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Molecular characterization and analysis of the porcine *betaine homocysteine methyltransferase* and *betaine homocysteine methyltransferase -2* genes

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Abstract

Betaine homocysteine methyltransferase (BHMT) and BHMT-2 enzymes methylate homocysteine to form methionine using betaine and S-methylmethionine, respectively. These activities are observed only in the liver of adult rodents, but in adult humans and pigs these activities are detected in both the liver and kidney, indicating the pig is a more appropriate model for studying the biochemical and physiological roles of these enzymes in human biology. Porcine *BHMT* and *BHMT-2* cDNAs were cloned and sequenced, and their 5' and 3' UTR were amplified using RLM-RACE. The *BHMT* transcript had significantly longer 5' and 3' UTRs than *BHMT-2*. The pig *BHMT* and *BHMT-2* genes span approximately 26kb and 16kb, respectively, and both genes have 8 exons. The deduced amino acid sequences of BHMT and BHMT-2 contain 407 and 363 amino acids, respectively, and shared 78% amino acid identity. No promoter element (TATA or CAAT box) was observed for either *BHMT* or *BHMT-2*, although a CpG island surrounding the promoter and transcriptional start site were observed in both genes implying that methylation could regulate their expression. Using qPCR, it was determined that *BHMT* and *BHMT-2* transcripts are very abundant in liver and kidney cortex, whereas the expression is significantly less in other tissues. These findings confirm that the expression pattern of *BHMT* and *BHMT-2* genes in pigs is similar to humans, supporting the use of the pig as an animal model to study the genetics and regulation of *BHMT* and *BHMT-2* expression.

Keywords

BHMT; BHMT-2; homocysteine; pig

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1. Introduction

Hyperhomocysteinemia has been associated with increased risk for vascular diseases, psoriasis, renal insufficiency and Alzheimer's disease (Ueland and Refsum, 1989; Pajares and Perez-Sala, 2006; Mizrahi et al., 2002), as well as adverse pregnancy outcomes, including spina bifida and placental abruptions (Steegers-Theunissen et al., 1991; Hague 2003). Due to its association with human diseases, the genetic and nutritional factors influencing plasma homocysteine (Hcy) levels have been the subject of intense study over the past 20 years, including the role of betaine-homocysteine methyltransferase (BHMT) in Hcy metabolism. Recently, using a specific and high affinity inhibitor of BHMT, it has been shown that inhibition of this enzyme *in vivo* (mice) results in severe hyperhomocysteinemia (Collinsova et al., 2006). The recent discovery that *BHMT-2* encodes an enzyme that does not use betaine, but rather the plant metabolite, S-methylmethionine, has sparked new interest in the role BHMT enzymes have in sulfur amino acid metabolism across the spectrum of mammalian development, as well as how animals adapt to securing adequate amounts of methionine (Met) from diverse nutritional sources.

Hcy lies at a metabolic branch point; it can participate in the transsulfuration pathway and thereby contribute a sulfur atom for cysteine biosynthesis, or it can be methylated to reform Met, the amino acid from which it was originally derived. The conversion of Hcy to Met is referred to as remethylation, a process that is critical for cell function since the need for Met in downstream S-adenosylmethionine (SAM)-dependent methyltransferase reactions is robust and must continue during postprandial and fasting conditions. Three enzymes can methylate Hcy; Met synthase, BHMT and BHMT-2. Quantitatively, the majority of the SAM-derived methyl groups are used in phosphatidylcholine and creatine biosynthesis, but it has been estimated that the human genome contains ~200 genes encoding SAM-dependent methyltransferases (Petrossian and Clarke, 2010).

Met synthase is ubiquitously expressed (Chen et al., 1997) but the distribution of BHMT is more restricted and has been the subject of numerous studies using adult animals (Delgado-Reyes et al., 2001 and references therein). In mammalian liver, BHMT activity has been shown to dramatically increase during Met deficiency (Park and Garrow, 1999) and reportedly represents 1% or more of the total soluble protein (Garrow 1996). In addition to being present in adult liver, BHMT activity also is present at significant levels in the pancreas of ruminants and guinea pigs, and in the kidney cortex of guinea pigs, pigs and primates (Delgado-Reyes et al., 2001; Delgado-Reyes and Garrow, 2005). In humans, pigs and guinea pigs, BHMT has been specifically localized to the proximal tubules of kidney. *BHMT-2* mRNA has been shown to be expressed at significant levels in the liver and kidney with much lower levels of expression detected in the brain, heart and skeletal muscle of human tissues (Chadwick et al., 2000). Despite the detection of *BHMT-2* transcripts in extrahepatic tissues, BHMT-2 activity has only been observed at significant levels in the livers of mice and rats (Szegedi et al., 2008). It is unknown whether the diet influences *BHMT-2* gene expression in any organ, and further study is required to determine the impact of diet on *BHMT* expression in kidney and pancreas. Besides being important for the methylation of Hcy, BHMT is also required in the degradation of betaine to glycine. This is important since betaine functions as a renal osmolyte whose concentration is regulated by cell tonicity (Burg et al., 2007). For example, studies in guinea pigs have shown that high salt intake results in a dramatic decrease in *BHMT* mRNA and protein abundance in both liver and kidney cortex (Delgado-Reyes and Garrow, 2005).

This study is the first to characterize the pig *BHMT* and *BHMT-2* genes and their complete cDNA sequences. Since pigs and humans are omnivores and both express BHMT enzymes in the same adult organs, we believe the pig is the best animal model to explore the spatial

and temporal expression of the BHMT throughout development, as well as the physiological and genetic aspects of BHMT in human health and disease.

2. Material and Methods

2.1. Porcine RNA isolation and cDNA synthesis

Liver, kidney cortex, kidney medulla, lungs, heart, and brain tissues were obtained from three different Yorkshire pigs. The pigs were fed a diet containing soybean meal (14%), dical (0.78%), lime (0.7%), Swine TM (0.3%), Vitamin ADEK (0.5%), Tylan-40 (0.025%), lysine (0.05%), corn (83.1%) and qualfat (1%). This diet contains Met at a level that is not limiting for growth. Total RNA was isolated from frozen tissues using a RNeasy Mini Kit (Qiagen, Valencia, CA) and treated with RNase-free DNase (Qiagen, Valencia, CA) to eliminate genomic DNA contamination. Reverse transcription was performed using random primers and a Biotool cDNA synthesis kit (Biotool, Taunton, MA).

2.2. Full-length porcine *BHMT* and *BHMT-2* cDNA cloning and sequencing

A porcine BAC clone (CU468550) encoding both *BHMT* and *BHMT-2* genes was identified by blasting human *BHMT* and *BHMT-2* nucleotide sequences against the porcine genome (http://www.sanger.ac.uk/Projects/S_scrofa/). The intron-exon junctions were located using the SPIDEY tool (<http://www.ncbi.nlm.nih.gov/spidey/>). Three primer sets (Table 1): 1-F/1-R, 2-F/2-R, and 3-F/3-R, which were designed from the exonic regions of the *BHMT* gene, were used to amplify three overlapping cDNAs that encoded the complete ORF of *BHMT*. The amplicons were cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and sequenced using ABI Prism® BigDye™. Similarly, two overlapping cDNAs that encoded the complete ORF of *BHMT-2* were obtained using the following primer sets: 4 -F/4-R, and 5-F/5-R (Table 1).

The 5' and 3' untranslated regions of three *BHMT* and *BHMT-2* clones were amplified using total RNA extracted from liver and the FirstChoice™ RNA ligase-mediated (RLM)-RACE kit (Ambion, Austin, TX) according to the instructions given in the manual. This procedure used primers supplied with the kit and the nested gene-specific primers listed in Table 1. Reverse transcription was performed followed by nested PCR. The products were then gel purified (Qiagen Valencia, CA), cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA), and three colonies were sequenced for each 3' and 5' gel-purified product. The full-length cDNA sequences of porcine *BHMT* (accession number: HQ130333) and *BHMT-2* (accession number: HQ130334) were submitted to NCBI Genbank. Comparative analysis of cDNA sequences and predicted protein sequences were carried out using Biology Workbench (<http://workbench.sdsc.edu/>). MFOLD program, version 3.2 developed by Zuker and Turner (2003); (<http://www.bioinfo.rpi.edu/applications/mfold>) was used to predict secondary structures and calculate free energy values. The promoter region was predicted using Proscan software (<http://www.bimas.cit.nih.gov/molbio/proscan/>) and TSSG software (<http://www.softberry.ru/berry.phtml>). The transcriptional binding sites were identified using TESS (<http://www.cbil.upenn.edu/cgi-bin/tess/tess>) and TFSearch engine (<http://www.cbrc.jp/research/db/TFSEARCH.html>). The repeat elements were detected using the repeatmasker software (<http://repeatmasker.org/cgi-bin/WEBRepeatMasker>). Microsatellite repeats were identified using Gramene software (<http://www.gramene.org/db/markers/ssrtool>). Poly A software (<http://www.softberry.ru/berry.phtml>) was used to detect 3' UTR poly A signal sites. SMART (<http://smart.embl-heidelberg.de/>), Scanprosite (<http://expasy.org/tools/scanprosite/>) and Pfam programs were used to predict the domain and motifs of BHMT and BHMT-2 proteins.

2.3. Real time qRT-PCR of porcine *BHMT* and *BHMT-2* transcripts

The *BHMT* and *BHMT-2* transcripts were quantified by qRT-PCR using the SYBR® GreenER™ PCR Mastermix (Invitrogen, Carlsbad, CA). Each tissue was collected from the same three pigs and for each sample three replicates were performed. RNA was extracted, followed by reverse transcription with the OmniscriptKit (Qiagen, Valencia, CA) using the protocol of Chen et al. (2006). Primers (Table 1) were designed using Primer Express software (Applied Biosystems, Foster city, CA). The total number of *BHMT* and *BHMT-2* transcripts in a given tissue was determined using the following primer sets; qBHMT-f and qBHMT-r, and qB2-f and qB2-r, respectively. Total 18s rRNA was used as an internal control (Chen et al. 2006) and each reaction contained 100ng of cDNA. Negative controls (minus template or reverse transcriptase) were also run in triplicate. Absolute copy number, as well as normalized values were determined individually for each transcript. PCR products were cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA), verified by sequencing, and then used for the construction of standard curve. RT-PCR assays were performed using the ABI 7900HT fast real time PCR system (Applied Biosystems, Foster city, CA). Absolute quantification was performed to determine if the high level of BHMT protein expression in liver (> 1% of total soluble protein in pigs: Garrow, JBC 1996) correlates to high level of mRNA expression.

3. Results

3.1. Analysis and genomic organization of porcine *BHMT* and *BHMT-2* genes

Complete genomic sequences of porcine *BHMT* and *BHMT-2* genes were determined from the BAC clone CU468550. A schematic representation of the *BHMT* and *BHMT-2* genes is shown in Fig. 1 and exonic region details are provided in Table 2. The *BHMT* gene spans 26,508 bp and includes 77 bp of 5' UTR, 1,142 bp of 3' UTR and 1,224 bp of coding region. The *BHMT-2* gene spans 15,997 bp and includes 17 bp of 5' UTR, 893 bp of 3' UTR and 1,092 bp of coding sequence (Ganu et al. 2009). The *BHMT* gene (Table 3) has 9,996 bp (37.7%) of interspersed repeats, including 5,088 bp short interspersed elements (SINEs-19.19%), 3,295 bp long interspersed repeats (LINEs-12.43%), 433 bp LTR (1.63%) and 1,180 bp DNA elements (4.45%). The *BHMT-2* gene (Table 3) has 5,514 bp (34.47%) of interspersed repeats that include 3,049 bp short interspersed elements (SINEs 19.06%), 1,631 bp long interspersed repeats (LINEs 10.2%), 615 bp LTR (3.84%) and 219 bp DNA elements (1.37%). Five microsatellite markers were identified within the *BHMT* gene; introns 1, 5 and 7 each have one, and intron 2 contains two microsatellites. Two microsatellite markers were found in the *BHMT-2* gene, one in intron 2 and one in intron 3. Each of these microsatellite markers are di-nucleotide repeats of varying numbers ranging from 5–25 (Supplementary Table 1). The position of microsatellite makers are depicted on Figure 1.

3.2. Analysis of porcine *BHMT* and *BHMT-2* cDNAs and deduced amino acid sequences

A RLM-RACE protocol was used to amplify cDNA sequences of *BHMT* gene using total RNA isolated from adult liver. The complete ORF of *BHMT* gene was 1,224 bp long and encoded a protein of 407 amino acids with a calculated M_r (molecular weight) of 45 and a theoretical pI (isoelectric point) of 7.22. The ORF of *BHMT-2* gene was 1,092 bp long and encoded a 363-residue protein with a calculated M_r of 40 and a theoretical pI of 6.86. Amino acid sequence analysis predicts that BHMT has seven N-myristoylation sites (PS000008), five protein kinase C phosphorylation sites (PS00005) and four casein kinase II phosphorylation sites (PS00006). BHMT-2 is predicted to contain seven consensus N-myristoylation sites (PS000008), six protein kinase C phosphorylation sites (PS00005) and five casein kinase II phosphorylation sites (PS00006) (Supplementary Fig. 1a and 1b).

3.3. Promoter region and 5'UTR

The tissue-specific expression of *BHMT* and *BHMT-2* in adult tissues could be the result of regulation at the promoter and/or 5' UTR region. The leader sequences were predicted to have secondary structures with free energies of -19.5 kcal/mol for the *BHMT* mRNA and -2.6 to -3.2 kcal/mol for the *BHMT-2* mRNA. A BLAST search using the NCBI porcine resources identified one EST (AK233096) that was homologous to the porcine *BHMT* gene and contained a 5' UTR that was 532 bases longer than the porcine *BHMT* transcript reported here. This suggested that there may be more than one mRNA splice variant, a possibility that warrants further investigation.

A putative TATA-less promoter was present in intron 1 of the porcine *BHMT* gene that is 541 bases downstream of the transcriptional start site. For the porcine *BHMT-2* gene, a predicted promoter region (250 bases) was found between -194 and $+56$ relative to the translational start site. The region from $1-56$ bp includes the entire 5' UTR and most of exon 1. For the porcine *BHMT-2* gene also, no TATA box was predicted. Potential transcriptional regulatory elements which included NF-1 (GCCA, CTTGC, TCCA), GATA-1 (CGGATGGTAC, ATCTCCAAC), CP1 (GATTTGGATTGG), IRF-1 (TTCCCTTTTCGAAG), p300 (GGGAGTGAT, GAGTTCCTG), GR (TGAAGCTC, TCTTCT, CAGAG), CdxA (CTTTATA, AATATG, AATT, GAAAGCTGG), LVc (CCTGCA, CTGCA), Sp1 (CGCC, CAGGCCCTTAA, CCCCTCCT), and LBP-1 (CCTGG) were detected upstream and downstream from the transcriptional start site for both the porcine *BHMT* and *BHMT-2* genes. Additionally GAL4 (GAGGA), H4TF-2 (GCCTC) and HSTF (CAGAAA, AGGAAA) were detected for *BHMT* gene.

3.4. Detection of CpG island in *BHMT* and *BHMT-2* gene

A CpG island (865 bp with 64.9% GC content) was detected in the porcine *BHMT* gene. The CpG island started 100 bp upstream of the translational start site and contained exon 1 and part of intron 1. One CpG island (627 bp in length; 72% GC content) was also detected in the 5' flanking region (359 bp upstream) of the porcine *BHMT-2* gene that overlapped with the predicted promoter region (Fig. 1). These islands may represent elements of epigenetic (methylation) control of *BHMT* and *BHMT-2* transcription.

3.5. 3'UTR analysis

The porcine *BHMT* cDNA contains a consensus polyadenylation signal (ATTAAG) in the 3'UTR resulting in a 3' UTR length of 1,142 bp. Also, two additional polyadenylation signals were detected at 530 and 1023 nucleotides downstream of the translational termination site in the 3'UTR. The transcripts resulting from the above transcription termination might represent two splice variants that could not be cloned due to their low expression levels.

An additional polyadenylation signal at 90 bp upstream of the 3'UTR of *BHMT-2* gene was also detected, suggesting the presence of splice variants. Additionally, a BLAST search of NCBI porcine resources identified a *BHMT-2* EST (AK232714.1) that had 43 more bases in the 3' UTR than the one reported here. Thus, there could be *BHMT-2* splice variants that were not cloned, presumably due to either a low expression level or because they are not expressed in liver.

3.6. Tissue specific expression analysis of porcine *BHMT* and *BHMT-2* transcripts

Quantitative real-time PCR analysis revealed that adult porcine liver has the highest expression of total *BHMT* transcripts, which is approximately 1.5-fold higher as compared to kidney cortex (Table 4). The kidney medulla and lungs expressed significantly lower (4- and 39-fold lower, respectively) *BHMT* transcripts compared to kidney cortex;

307,780/100ng and 31,249/100ng of total mRNA, respectively, and the heart and brain contained negligible levels of *BHMT* transcripts as compared to kidney cortex.

In the kidney cortex *BHMT-2* transcripts were abundant. Although in contrast to *BHMT*, the level of *BHMT-2* transcripts were 3-fold higher in the kidney cortex compared to liver (Table 4). The level of *BHMT-2* transcript expression in kidney medulla, lungs, heart and brain was negligible compared to kidney cortex.

4. Discussion

This study provides full-length porcine *BHMT* and *BHMT-2* cDNAs sequences, as well as the genomic organization of their respective genes. A single BAC clone (Accession number: CU468550) was identified that contained both of the porcine *BHMT* and *BHMT-2* genes. These genes are adjacent to each other in the human, mouse, rat and pig genomes. Similar to that observed in the human and mouse *BHMT* and *BHMT-2* genes, the porcine genes contained 8 exons and 7 introns (Park and Garrow, 1999). The exonic sequences of the porcine *BHMT* gene shared 90%, 85%, and 86% nucleic acid identity with the human, rat and mouse *BHMT* genes. The exonic sequence of the porcine *BHMT-2* gene shared 87%, 82%, and 83% nucleic acid identity with the human, rat and mouse *BHMT-2* genes. The first 957 nucleotides code for the N-terminal domain of *BHMT* and *BHMT-2* that contain the active site (Evans et al., 2002; Castro et al., 2004). Thus, *BHMT* and *BHMT-2* genes are conserved for the function of Hcy conversion to Met across mammals.

The deduced amino acid sequence of porcine *BHMT* is 95%, 96%, 94% and 96% identical to human (NM_001713), bovine (AY854632), rat (NM_030850) and mouse (AF033381) *BHMT*, respectively, whereas the deduced amino acid sequence of porcine *BHMT-2* is 88%, 87% and 87% identical to human (NP_060084), rat (NM_001014256) and mouse (AF257474) *BHMT-2*, respectively (Fig. 2). Over the sequences that porcine *BHMT* and *BHMT-2* align, they shared 78% amino acid identity, including the residues required for Hcy and zinc binding (Evans et al. 2002), suggesting a common ancestral protein. However, relative to *BHMT-2*, *BHMT* has two additional regions of amino acid sequence; a 9 amino acid insertion (residues 86–94) in the N-terminal region and a 34 amino acid sequence (residues 373–407) at the carboxy terminus. The 9 amino acid insertion in the N-terminal region of *BHMT*, which is missing in *BHMT-2*, is involved in conferring betaine specificity. When that insertion is removed from *BHMT*, the enzyme still binds zinc but has no betaine-homocysteine methyltransferase activity (Castro and Garrow, unpublished data). Interestingly, this region has not been resolved in any of the crystal structures of *BHMT* solved to date (Evans et al., 2002;González et al., 2004) and is sensitive to trypsin digestion (Szegedi and Garrow, 2004), suggesting that this region displays considerable flexibility. The additional 34 amino acids at the carboxy terminus of *BHMT* are critical for oligomerization. The crystal structure of the rat enzyme has revealed this region to be involved in important dimer-dimer contacts (González et al. 2002;González et al., 2004), and removing these 34 amino acids from the human enzyme resulted in a loss of activity, presumably because of its inability to oligomerize into a tetramer (Szegedi and Garrow, 2004).

The promoter region for porcine *BHMT* gene was detected in intron 1 and no TATA box was observed. For humans, the *BHMT* promoter region is positioned just upstream of the 5' UTR (Park and Garrow, 1999). A promoter region of 250 bases for the porcine *BHMT-2* gene was detected and no TATA box was observed; however, previous findings have indicated that more than 50% of all promoters may lack a TATA box (Lewin, 2004). Such TATA-less promoters often show multiple transcriptional sites resulting in differences in the UTR lengths. Thus, it is possible that there are splice variants of the porcine *BHMT* and

BHMT-2 genes. In fact, our preliminary studies indicate the presence of alternative 5' transcription start sites for the *BHMT* gene in other tissues.

The *BHMT* 3' UTR varies across vertebrate species ranging from 574 to 1142bps, with humans having the longest 3' UTR. The porcine *BHMT* cDNA contains several polyadenylation signals. The *BHMT*-2 3' UTR was also variable among these species and ranged from 803 to 893 bps with pigs having the longest 3' UTR (893 bps) (Table 2). For both *BHMT* and *BHMT*-2, 5' UTRs in human, pig, mouse and rat and 3' UTRs in human, pig, mouse and rat shared no significant similarity. Thus, the ORFs has been conserved but divergence exists in the untranslated regions during speciation similar to other mammalian genes (Zolfaghari and Ross, 2004).

In pigs, the lungs, heart and brain contained lower levels of *BHMT* mRNA compared to liver and kidney cortex, similar to the findings in other mammalian species (Delgado-Reyes et al., 2001). RT-qPCR revealed that liver has 1.5-fold more *BHMT* mRNA than the kidney cortex. Due to the high copy number, it is clear that the level of BHMT protein simply reflects the high level of *BHMT* transcripts in the liver cell. In contrast, the kidney cortex contained 3-fold more *BHMT*-2 mRNA than liver (Table. 4). Both porcine *BHMT* and *BHMT*-2 genes contain CpG islands surrounding the transcriptional start site and that overlap the predicted promoters. Several mammalian genes contain CpG islands around the promoter region (Hannenhalli and Levy, 2001). Previous studies have shown that BHMT and BHMT-2 activity is high in liver and kidney cortex, but negligible in lungs, heart and brain (Sunden et al., 1997; Chadwick et al., 2000). Methylation could be a factor associated with low levels of BHMT and BHMT-2 expression in most organs.

Previous studies have shown that BHMT protein represents 1 to 2% of total liver protein (Garrow, 1996), which is consistent with its high level of mRNA. However, the k_{cat} of BHMT is about 1000-fold lower than its B12- and folate-dependent counterpart, Met synthase, which is a very low abundance protein. This chasm between enzyme level and catalytic efficiency of BHMT vs. Met synthase suggests that BHMT might have other, currently unknown biological functions. Since the carbon skeleton of Hcy is both critical in regenerating the essential amino acid, Met, as well as being toxic if allowed to accumulate to high levels, it is possible that another critical function of BHMT, besides methylation, is to sequester Hcy, thus preventing its oxidation and/or ability to initiate pathological processes. This study shows that the expression pattern and organization of *BHMT* and *BHMT*-2 genes is similar in pigs and humans, indicating pigs can be used as a model to study regulation and diseases associated with *BHMT* and *BHMT*-2 genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Schematic depiction of genomic structure of porcine *BHMT* and *BHMT-2* genes. The vertically shaded boxes depict the predicted promoter region, the gray boxes represent the CpG islands, and black boxes represent exons (E). SC denotes stop codon. The black dots represent the location on microsatellite markers. Scale is approximate.

Figure 2.

Comparison of amino acid sequences of porcine, rat, mouse and human BHMT (B1) and BHMT-2 (B2). Identical amino acids are not shaded and non-identical amino acids are shaded dark grey.

Table 1Primers used for amplifying porcine *BHMT* (B1) and *BHMT-2* (B2) cDNA

Primer name	Primer sequence (5'-3')	Tm	Amplicon Size(bp)
1-F	TCCGTTTTCGAGTACCATCC	60	778
1-R	AAGGGCTGACTCATCAGGTG		
2-F	TTTCGGCAGCAGTTAGAGGT	60	1899
2-R	CCCAGATGGGCTTTTGAGTA		
3-F	ACTTCCCCTATGTTTCAGACCAC	60	1071
3-R	CAGCTACACCAACCCAAGAA		
3'RACE-outer	Supplied with kit	60	
B1-3r-outer	ACTTCCCCTATGTTTCAGACCA	60	1164
3'RACE-inner	Supplied with kit	60	
B1-3r-inner	ATCAGTTAAGACTGGGGAGCAG	60	326
5'RACE-outer	Supplied with kit	60	
B1-5r-outer	TCCGTCTCCAATCACAACCT	60	203
5'RACE-inner	Supplied with kit	60	
B1-5r-inner	ATATTCGAGGTGTCCGGATG	60	115
4-F	TTGGAGGACAAACCTAAGAAGC	60	748
4-R	AGAAGGGAATACTGGGAGAACC		
5-F	AGTGAAGGACAGAAAGGTCTGC	60	981
5-R	TAACTCTGGAGAAGAGGGGCTA		
B2-5r-outer	CCCGTCTCAAGAATCCAT	60	262
B2-5r-inner	TAGCCCTCTTCTCCAGAGTTA	60	166
B2-3r-outer	CCCTGACGTCTCCAGCTACT	60	846
B2-3r-inner	GCACTGAACCCAGTGATGAA	60	596
qBHMT-f	TGGTGGCAGGAGGTGTGA	79	71
qBHMT-r	ACACTTTTTTGA CTCCGTTTCG		
qB2-f	GCCGTGGTAGAGCATCCAA	77	68
qB2-r	GACATTCGATCCCGCTCTCA		

Table 2

Exon sizes of *BHMT* and *BHMT-2* gene from human, pig, rat and mouse

	<i>BHMT</i> gene						<i>BHMT-2</i> gene					
	Human		Pig		Rat		Mouse		Human		Pig	
	Size (bp)	AA	Size (bp)	AA	Size (bp)	AA	Size (bp)	AA	Size (bp)	AA	Size (bp)	AA
Exon 1	138	11	197	11	103	11	295	11	56	11	50	11
Exon 2	133	44	133	44	133	44	133	44	133	44	133	44
Exon 3	119	40	119	40	119	40	119	40	92	31	92	31
Exon 4	192	64	192	64	192	64	192	64	192	64	192	64
Exon 5	148	49	148	49	148	49	148	49	148	49	148	49
Exon 6	183	61	183	61	183	61	183	61	183	61	183	61
Exon 7	229	77	229	77	229	77	229	77	228	76	229	77
Exon 8	1358	60	1329	61	759	61	772	61	975	27	975	26
3' UTR length	1189	-	1142	-	590	-	595	-	892	-	893	-

AA denotes amino acids.

Table 3Elements detected in *BHMT* and *BHMT-2* gene

Elements	Number of elements		Length (bp)		Percentage of sequence (%)	
	BHMT	BHMT-2	BHMT	BHMT-2	BHMT	BHMT-2
SINEs	24	14	5088	3049	19.19	19.06
MIRs	4	4	628	735	2.37	4.59
LINEs	10	8	3295	1631	12.43	10.20
LINE1	5	4	2269	1116	8.56	6.98
LINE2	5	4	1026	515	3.87	3.22
LTR elements	1	2	433	615	1.63	3.84
ERV1-MaLRs	1	1	433	328	1.63	2.05
ERV class I	-	1	-	287	-	1.79
DNA elements	7	2	1180	219	4.45	1.37
hAT-Charlie	5	-	763	-	2.88	-
TcMar-Tigger	2	-	417	-	1.57	-
Total interspersed repeats	-	-	9996	5514	37.71	34.47
Simple repeats	1	2	50	71	0.19	0.44
Low complexity	4	1	119	35	0.45	0.22

Table 4

Absolute quantification of expression of *BHMT* and *BHMT-2 transcripts* using real time RT-PCR

Tissue	BHMT transcript copy number/100ng cDNA	BHMT-2 transcript copy number/100ng cDNA
Liver	1741064 ± 763294*	76606 ± 29481**
Kidney cortex	1231119 ± 615560*	249609 ± 151946**
Kidney medulla	307780 ± 73867*	ND
Lungs	31249 ± 12311*	ND

Three replicates were performed for each sample. Negative controls (non-template control and minus reverse transcriptase control) were also run in triplicates. Each transcript was cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA), and was verified by sequencing and used for construction of a standard curve. Each reaction contained 100ng of cDNA. Absolute copy number was determined for each transcript. ND denotes not detectable. The expression was significantly different ($p < 0.01$) in tissues for both *BHMT** and *BHMT-2*** expression using Kruskal Wallis* and Mann-Whitney U test** non parametric test.