

Identification of a novel human cancer/testis gene MAEL that is regulated by DNA methylation

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Received: 27 July 2009 / Accepted: 5 August 2009 / Published online: 20 August 2009
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Abstract The mouse maelstrom (MAEL) gene has been found to be expressed in male germ cells and to play a role in spermatogenesis. Here, we cloned the human MAEL gene by digital differential display and found that, among human tissues, MAEL is only expressed in the testis, but it is also expressed in various cancer cell lines. The transcription start site of the MAEL gene is 74-bp upstream of the start codon. The region from -216 to $+150$ is the basal promoter of the MAEL gene, and a CpG island (-295 to $+148$) is located in this region. Treatment with the demethylating agent 5'-Aza-2-Deoxycytidine significantly upregulated MAEL expression. These results suggest that MAEL is a novel cancer/testis-associated gene and is regulated by DNA methylation.

Keywords Maelstrom · Cancer/testis gene · Promoter · CpG island · DNA methylation

Introduction

Cancer/testis (CT) genes (also termed Cancer/testis associated genes [1]) are a group of genes that are normally expressed only in germ cells of the testis, and yet are aberrantly expressed in a range of human cancers [1, 2]. To date, 44 CT genes have been identified; among these, 19 CT genes have been demonstrated to encode antigens that are immunogenic and elicit humoral and cellular immune responses in cancer patients [3, 4]. Because of their restricted expression profile, CT antigens are becoming useful biomarkers for the diagnosis and treatment of human cancers [5]. Currently, the CT genes provide a model to better understand complex gene regulation and aberrant gene activation during cancer [2].

The Maelstrom gene was originally identified in *Drosophila*, and functions in oocyte polarity [6]. Subsequently, Maelstrom was identified as a component of nuage and was found to be required for repression of selfish genetic gene expression in *Drosophila* [7, 8]. In mouse models, the maelstrom homolog (MAEL) is only expressed in the male germ line and also is a component of nuage [9]. Knockout of MAEL in mouse leads to the failure of spermatogenesis via meiotic arrest and derepression of transposable elements [10]. Recently, MAEL was found to play a crucial role in the piRNA pathway [11].

Here, we cloned the human MAEL gene by Digital Differential Display, and found that the human MAEL is only expressed in testis, among normal tissues. However, MAEL is ubiquitously expressed in many human cancer cells, similar to other CT genes in human. Furthermore, we found that the expression of human MAEL is controlled by DNA methylation of a CpG island in its basal promoter.

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Materials and methods

Electronic cloning of testis-specific genes

A data-mining tool called Digital Differential Display (DDD) (http://www.ncbi.nlm.nih.gov/UniGene/info_ddd.shtml) was used to search for testis-specific genes [12, 13]. Four human testis-derived EST libraries (pool A) were compared with other human organ-derived EST libraries (pool B, $N = 270$). The hits showing >10 fold difference between pool A and pool B were selected and further analyzed. ORF Finder and BLAST, at <http://www.ncbi.nlm.nih.gov/Tools/>, were used for identifying ORFs and comparing gene and protein sequences against others in public databases, respectively.

RNA ligase mediated rapid amplification of cDNA (RLM-RACE)

The 5'- and 3'- ends of human MAEL were obtained by RLM-RACE methods using FirstChoice RACE-Ready Testes cDNA kit (Ambion, USA) according to the manufacturer's instructions. By this method, only intact mRNAs with a 5'-cap structure are reverse transcribed. For 5'-RACE, 0.5 ng of RACE-ready testes cDNA was used as a template for PCR amplification with a gene-specific outer primer (5'-CCTCCTCAGTGGTGTGAAAAC-3') and the 5'RACE outer primer provided in the kit. The products were further amplified by a subsequent nested PCR reaction using a gene-specific inner primer (5'-CAGTCTGAGGAG CAGTAAGGGA-3') and the 5'RACE inner primer provided in the kit. The 3'-RACE was performed using the same protocol as the 5'-RACE, and the gene-specific outer primer and inner primer were as follows: 5'-AAGCATA TGGCAAAGGCATC-3', 5'-AGCAACACAAGGTGCAA GTG-3', respectively. The final PCR products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining. Gel-purified products were cloned into the pMD18-T vector (TaKaRa) and sequenced.

Northern blotting

A multiple human tissue northern blot membrane (Inno-gent, China) was sequentially hybridized with MAEL and β -Actin cDNA probes according to the manufacturer's user manual.

RNA in situ hybridization

The single-strand DNA probe (5'-GCT TCG AAT CCA AGT CTT AGA GGG CTC C-3' complementary to the human MAEL mRNA) was synthesized and labeled with digoxigenin by Sangon Co. Paraffin sections of testis (from

a 68-year-old man) were purchased from Chaoying Biomedicine R&D Incorporated (CBRDI). In situ hybridization was performed according to CBRDI's instructions. Detection was carried out with an alkaline phosphatase-conjugated anti-DIG antibody (BCIP/NBT). Counterstaining was performed with nuclear fast red [9]. The signal was visualized with a fluorescence microscope (Axioskop 2 plus, Zeiss).

Computer analysis of 5'-flanking sequence of the human MAEL gene

The transcription factor binding sites were predicted by the MatInspector program (<http://www.genomatix.de>). The presence of CpG islands was determined by the EMBOSS program CpGplot (www.ebi.ac.uk).

Promoter constructs and luciferase assays

The 5'-flanking sequence (nucleotides -1510 to +150) of the MAEL gene was amplified from human DNA and inserted upstream of the promoterless firefly luciferase gene in the *Bgl*III/*Hind*III sites of pTAL-Luc vector (Clontech), denoted as pTAL-p (-1510 to +150). The primers used were: MAEL-p-F (5'- GAGATCTGACGCACCCTC-CAAAGATGTA-3') and MAEL-p-R (5'- AAGCTTCTCGTCGCCGTAGTTCG-3'). A series of 5'- and 3'-deletion constructs were generated through different restriction enzyme digestion and ligation of pTAL-p(-1510 to +150): pTAL-p(-1149 to +150) (*Xho*I), pTAL-p(-606 to +150) (*Nsp*I + *Hind*III), pTAL-p(-216 to +150) (*Bsr*GI + *Hind*III) and pTAL-p(-1510 to -235) (*Bsr*GI + *Bgl*III). All the above constructs were confirmed by DNA sequencing. GC-1 and MCF-7 cells were seeded in 35 mm dishes and transfected at 70% confluence using Lipofectamine 2000 (Invitrogen) according to the manufacturer's manual. Luciferase activity was measured as previously described [14].

5'-Aza-2-Deoxycytidine (5'-aza-CdR) Treatment

Cells were grown at low density in six-well plates and treated with the indicated dose of 5-aza-CdR (Sigma, USA). RNA was isolated from each of the samples after 72 h.

Isolation of DNA and RNA, reverse transcription and RT-PCR

The E.Z.N.A total DNA/RNA isolation kit (Omega, USA) was used to isolate DNA and RNA simultaneously from cells. The first-strand cDNA was synthesized using AMV reverse transcriptase (Invitrogen, CA), and used as the

template for PCR. For semi-quantitative RT-PCR, the PCR cycles for linear amplification were selected in preliminary experiments. The final PCR products were separated by electrophoresis in a 2% agarose gel and visualized by ethidium bromide. The primers used were MAEL (forward: 5'-AAGCATATGGCAAAGGCATC-3', reverse: 5'-CAC-TTGACCTTGTGTTGCT-3', β -actin (forward: 5'-CGTG CGTGACATTAAGGAGA-3', reverse: 5'-CACCTTCAC CGTTCCAGTTT-3'). Real-time PCR reactions were performed in the Prism 7500 Sequence Detection System (ABI) using SYBR-Green PCR master mix. The real-time PCR results were analyzed with the SDS 7300/7500 software (ABI).

Results and discussion

Cloning of the human MAEL gene

To search for novel testis-specific genes, we compared human testis EST libraries with EST libraries from all other tissues using the Digital Differential Display (DDD) program. The DDD results revealed that 126 EST contigs are upregulated by greater than 10 fold in testis in comparison to other tissues or cell lines. Hs. 180191 (hypothetical protein FLJ14904) is one of contigs that showed significantly upregulated expression in testis. The full-length cDNA of hypothetical protein FLJ14904 was amplified from human testes RACE-Ready cDNA (Ambion) by the RLM-RACE method. Sequencing of the obtained PCR products revealed that the full-length cDNA sequence consists of 1751 bases, and its transcription start site is A, corresponding to the position 74-bp upstream of the translation start codon. The deduced amino acid sequence predicts a protein of 434 amino acids with a calculated molecular mass of 49.2 kDa. PSI-BLAST search revealed that FLJ14904 is similar to *Drosophila melanogaster* maelstrom, so we termed it human maelstrom homolog (MAEL) after acquiring the consent of HUGO Gene Nomenclature Committee. The sequence data of human MAEL gene was deposited in GenBank with accession number DQ076156.

Cancer/Testis-specific expression of the MAEL gene

To detect the tissue distribution of MAEL expression, Northern blot analysis was performed on a blot membrane containing mRNA isolated from eight different normal human tissues. As shown in Fig. 1A, a unique transcript of approximately 1.7 kb was detected exclusively in testis, but not in other tissues. Further, RNA in situ hybridization was performed to determine the specific cell types within testis tissue where MAEL is expressed. We found that

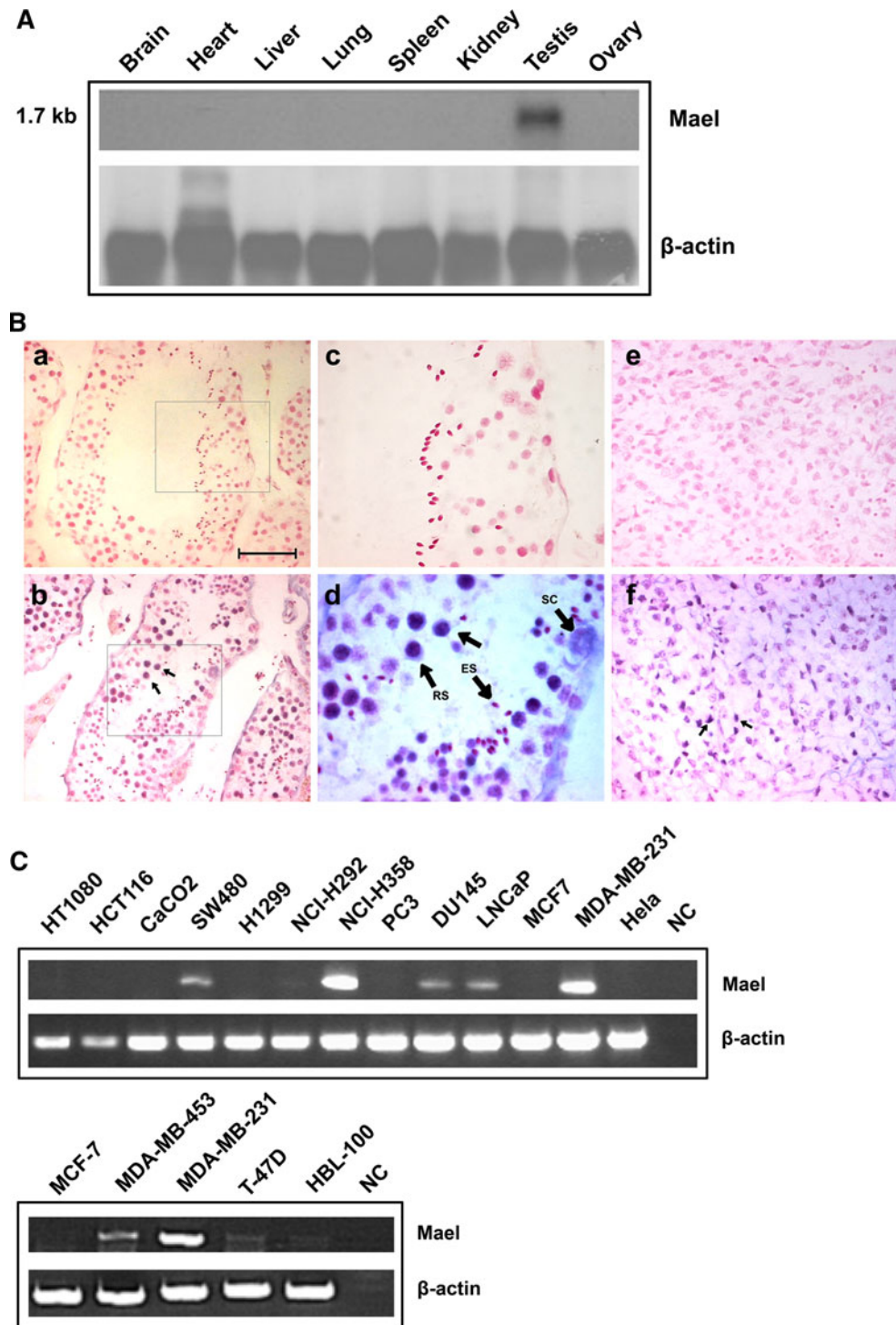
MAEL mRNA was present in spermatocytes, round and early elongating spermatids (Fig. 1B b and d). This result is in accordance with that obtained in mouse testis tissue by Costa et al. [9]. However, interestingly, MAEL was highly expressed in the testis tumor cells (Fig. 1B f) as well as in the normal testis. To determine whether MAEL is also expressed in other human cancer cells, we examined the expression of MAEL mRNA by RT-PCR in five types of human cancer cell lines from different origins, including lung cancer (H1299, NCI-H292, NCI-H358), breast cancer (MCF-7, MDA-MB-231, MDA-MB-453, HBL-100 and T-47D), colon cancer (HCT116, SW480, CaCO2 and HT1080), prostate cancer (PC3, DU145 and LNCaP) and cervical cancer (Hela). We found that MAEL was ubiquitously expressed in 4 of 5 types of human cancer cell lines. In these cancer cells, MAEL was highly expressed in NCI-H358 (lung cancer), MDA-MB-231 (breast cancer) cells and relatively lowly expressed SW480 (colon cancer), MDA-MB-453 (breast cancer), DU145 and LNCaP (prostate cancer) cells, but not detected in the other cancer cells or the normal cell line HBL-100 (breast) (Fig. 1C). The specific expression in testes and cancer cells suggests that MAEL is a novel member of the cancer/testis gene class.

Identification of transcription regulatory region of MAEL gene

To explore what transcription factors contribute to the regulation of the MAEL gene, we used several online software programs to discover potential transcriptional *cis*-acting elements in the 5'-flanking sequence. We found that there are three conserved CACCC-boxes (−288 to −284, −185 to −181 and −133 to −129), one conserved CCAAT-box (−178 to −174), one inverted CCAAT-box (−47 to −43) and a GC-box (+5 to +10) upstream of the translation start codon (Fig. 2a). A CpG island was detected surrounding the transcription start site (TSS) from −325 to +148 (Fig. 2a).

To examine the effects of the above six *cis*-acting elements on transcription of MAEL, we cloned the 5'-flanking sequence (−1510 to +150) upstream of luciferase into the promoterless luciferase reporter vector pTAL-Luc, and generated a series of constructs with deletions in the 5' flanking sequence (Fig. 2a). As shown in Fig. 2b, in MCF-7 cells, the largest construct pTAL-p (−1510 to +150) displayed the highest transcriptional activity, approximately 9-fold of that of the control (pTAL-Luc). However, the truncated construct pTAL-p (−1510 to −235), in which five of the above 6 *cis*-acting elements were deleted, had no transcriptional activity, while pTAL-p (−216 to +150), containing five *cis*-acting elements, showed the minimal transcriptional activity, approximately 4-fold of that of the

Fig. 1 Cancer/testis-specific expression of human MAEL gene. **A** Northern blot. A multiple human tissue Northern blot membrane was sequentially hybridized with MAEL and β -actin cDNA probe. **B** RNA in situ hybridization. The presence of MAEL was revealed by purple staining (as *black arrows* indicated) in human normal testis (b, d) and in human testis tumor (f). MAEL was present in spermatocytes (SC) and round (RS) and early elongating spermatids (ES) (d). No positive signal was detected in negative controls of human normal testis (a, c) and of human testis tumor (e). Scale bar in (a) is 10 μ m, as well as (b, e, f). (c) and (d) are the 2.5-folds magnification of the rectangular region in (a) and (b), respectively. **C** Expression of MAEL mRNA in human cell lines was detected by RT-PCR



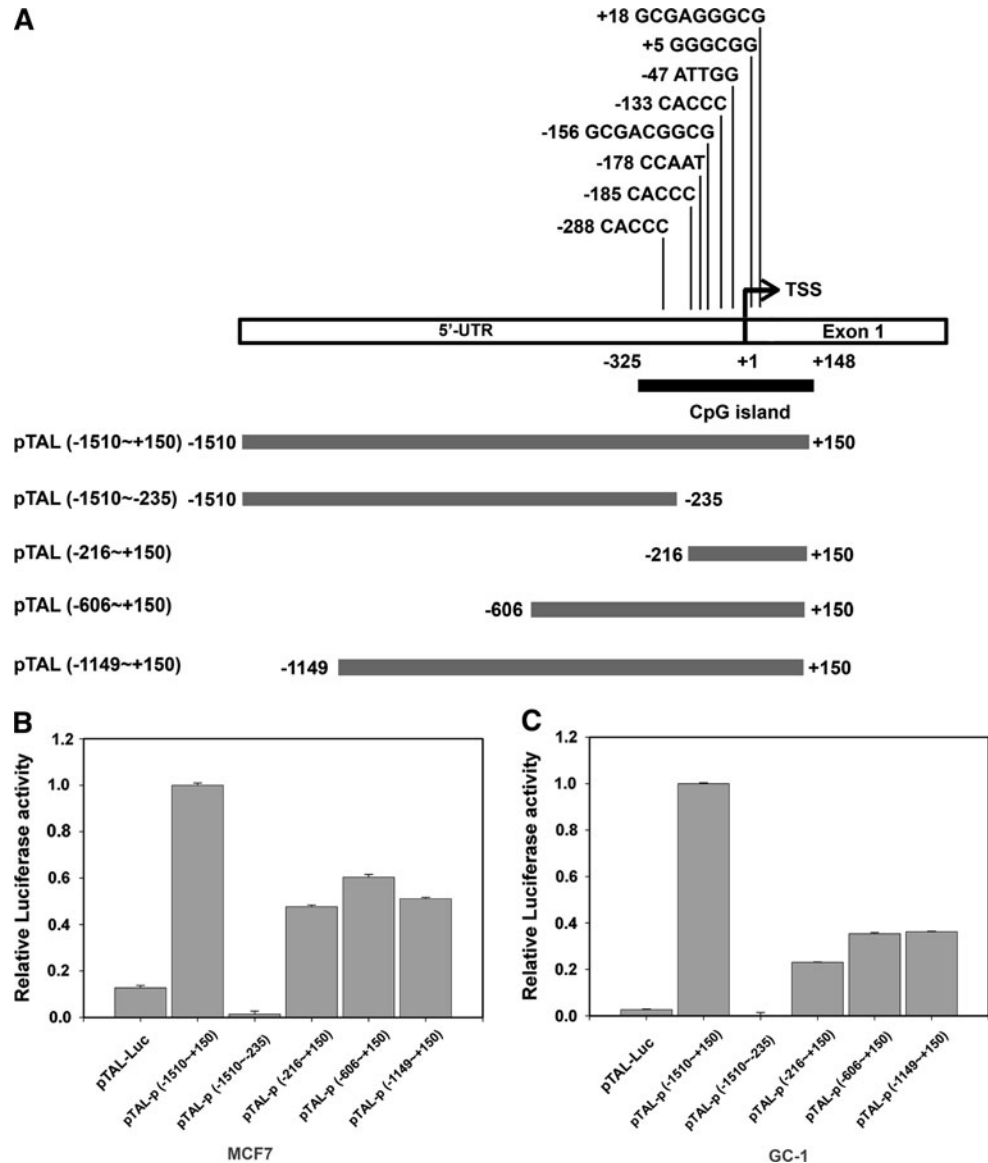
control. Both pTAL-p (−606 to +150) and pTAL-p (−1149 to +150) contained one more CACCC-box than pTAL-p (−216 to +150), but their transcription activities were only slightly higher than that of pTAL (−216 to +150). This suggests that these five *cis*-acting elements are indispensable for maintaining basal transcription activity. Similar results were obtained in GC-1 cells (Fig. 2c). Taken together, this suggests that the region from −216 to +150 is the

basal promoter of the MAEL gene. A CpG island (−295 to +148) is also located in this region.

The demethylating agent 5'-aza-CdR upregulates MAEL expression

Previous studies have shown that the methylation status of CpG islands in promoters of several CT genes is involved

Fig. 2 a Schematic diagrams show the potential cis-acting elements in human MAEL promoter and promoter constructs used in transient transfection experiments. Numbers indicate the nucleotide position relative to the transcription start site (TSS, +1). Promoter constructs were co-transfected with β -gal expression vector pCMV β which was used as an internal control to normalize luciferase activity. Luciferase activities in MCF-7 (b) and GC-1 (c) were measured at 36 h post-transfection. Data (means \pm S.D.) were represented as the percent activity relative to luciferase activity of pTAL-p (-1510 to +150)



in the regulation of their transcription [15–17]. The CpG island in MAEL is possibly associated with the regulation of its basal transcription.

To confirm whether the DNA methylation of the CpG island in the 5'-UTR contributes to transcriptional repression of MAEL, we examined MAEL expression in both normal breast cells and breast cancer cells with or without 5'-aza-CdR (an inhibitor of DNA methyltransferase) treatment. As shown in Fig. 3a, MAEL expression was remarkably upregulated by 5'-AZA treatment in MCF-7, MDA-MB-453, T-47D and HBL-100 cells. Moreover, the upregulation of MAEL expression by 5'-AZA is dose-dependent. However, the expression of MAEL in MDA-MB-231 cells showed little change after 5'-AZA treatment, which may be because of the high level of expression of MAEL in non-treated MDA-MB-231 cells. Furthermore,

we performed quantitative real-time PCR to confirm this. In comparison with non-treated cells, MAEL expression was upregulated by 10 μ M 5'-AZA 2.5-, 1.3-, 1.4-, 2.6- and 3.2-fold in MCF-7, MDA-MB-231, MDA-MB-453, T-47D and HBL-100 cells, respectively (Fig. 3b). Together, these results showed that demethylating agent, 5'-AZA can upregulate the expression of the MAEL gene. These data also confirmed that methylation of the CpG island in the 5'-UTR of MAEL represses its expression.

In the present study, we have identified a novel human cancer/testis gene and characterized its promoter and transcriptional control by DNA methylation. The mouse maelstrom (MAEL) gene has been found to be expressed in male germ cells and to play a role in spermatogenesis. Our finding of its expression in cancer cells indicates that MAEL possibly plays a role in carcinogenesis.

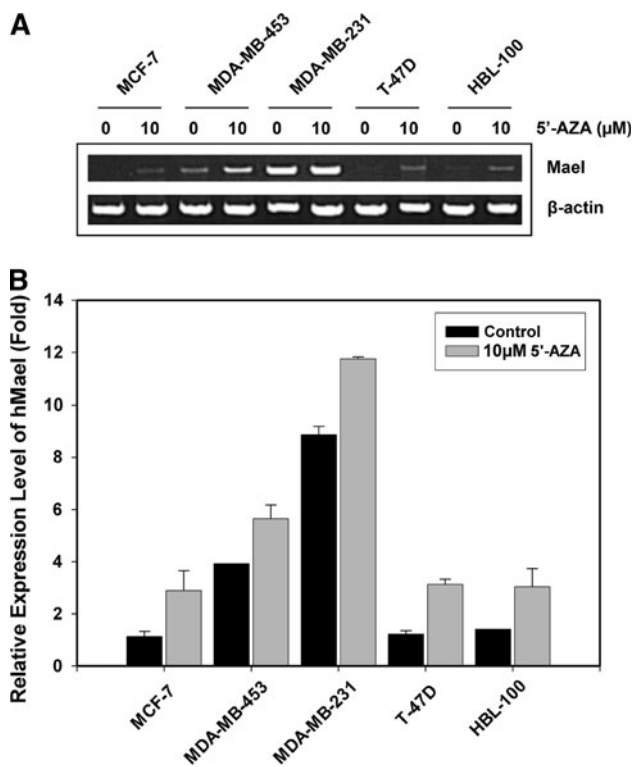


Fig. 3 Upregulation of MAEL expression by 5'-aza-2'-CdR treatment. Cells were harvested after treatment with 0 or 10 μM 5'-aza-CdR for 72 h, and then RNA was isolated. **a** MAEL expression detected by RT-PCR. β -actin was used as internal control to assess equal loading of template. **b** MAEL expression detected by quantitative real-time PCR. The MAEL expression level was normalized to β -actin mRNA level of the same sample

Acknowledgements This work was supported by National Basic Research Program of China (No. 2008CB517306), Hunan Provincial Department of Science & Technology (No. 2007CK3052) and Scientific Research Fund of Hunan Provincial Education Department.

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