N^6-Methyladenine DNA Modification in Human Genome

Chuan-Le Xiao[1, 2, #], Song Zhu[1, #], Ming-Hui He[3, #], Ying Chen[2], Guo-Liang Yu[4], De Chen[1], Shang-Qian Xie[2], Feng Luo[5], Zhe Liang[6], Xiao-Feng Gu[7, *], Kai Wang[8, *], Guang-Rong Yan[1, 9, *]

[1]Biomedicine Research Center, the Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, 510150, China

[2]State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou 510060, China

[3]CookGene Biosciences Center, Guangzhou 510320, China

[4]Grandomics Biosciences, Beijing 102206, China

[5]School of Computing, Clemson University, Clemson, SC 29634-0974, USA

[6]Department of Biological Sciences, National University of Singapore, 117543, Singapore

[7]Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, China

[8]Department of Biomedical Informatics, Columbia University, New York, NY 10032, USA

[9]Key Laboratory of Protein Modification and Degradation, Guangzhou Medical University, Guangzhou, China

[#]Equal contribution

*Corresponding author: Prof. Guang-Rong Yan, E-mail: jxygr007@yahoo.com, or Kai Wang, E-mail: kw2701@cumc.columbia.edu, or Prof. Xiao-Feng Gu, E-mail: guxiaofeng@caas.cn.

Summary

In human cells, DNA 5-methylcytosine (5mC) modification plays an important role as an epigenetic mark. However, DNA N6-methyladenine modification (6mA), which is predominantly present in prokaryotes and a limited number of eukaryotes, is considered to be absent in human genomic DNA. Here, we show that 6mA is present in human genome, and we identified 881,240 6mA sites whose density is about 0.051% of the total adenines in the human genome DNA, but more than 0.18% in the mitochondrion genome. [G/C]AGG[C/T] was the most significant motif associated with 6mA modification. 6mA sites are enriched in the exon coding regions (P=0.02) and associated with transcriptional activation ($P<0.001$). We further identify that DNA N6-methyladenine and N6-demethyladenine modification is mediated by 6mA methytransferase N6AMT1 and 6mA demethytransferase ALKBH1, respectively. The 6mA abundance is significantly lower in cancer tissues compared to adjacent normal tissues, always accompanying with lower N6AMT1 and higher ALKBH1 level. Collectively, we uncover a DNA modification in human and describe a potential role of the N6AMT1/ALKBH1-6mA regulatory axis in the progression of human disease, such as cancer.

Introduction

DNA methylation is a crucail component of epigenetic regulation and plays an important role in regulating genomic imprinting, X-chromosome inactivation, transposon suppression, gene expression, epigenetic memory maintenance, embryonic development and human tumorigenesis (Bergman and Cedar, 2013; Smith and Meissner, 2013; von Meyenn et al., 2016). The most predominant DNA methylation modification in eukaryotes is 5-methylcytosine (5mC). Research has focused on characterizing 5mC due to its abundance and significance in eukaryotes. In contrast, N6-methyladenine DNA (6mA) modification is the most prevalent DNA modification in prokaryotes and considered to be absent in eukaryotes including human due to its undetectable contents (Ratel et al., 2006). 6mA plays crucial roles in the regulation of restriction-modification (R-M) system, DNA replication and mismatch repair, nucleoid segregation, gene expression, and is essential for viability of some bacterial strains (Luo et al., 2015; Vasu and Nagaraja, 2013; Wion and Casadesus, 2006).

With the developments of deep-sequencing, 6mA was found to be present in a limited number of eukaryotes including *Chlamydomonas reinhardti* (Fu et al., 2015), *Caenorhabiditis elegans* (Greer et al., 2015), *Drosophila melanogaster* (Zhang et al., 2015), fungi (Mondo et al., 2017), mouse (Wu et al., 2016), zebrafish and pig (Liu et al., 2016b). Among of them, 6mA participated into the regulation of gene and transposon expression including activation and suppression (Fu et al., 2015; Wu et al., 2016; Zhang et al., 2015). 6mA has also been identified as an epigenetic mark of carrying heritable epigenetic information in C. elegans (Greer et al., 2015). Although

the mRNA 6mA modification is widely occurred in eukaryotes including human and plays crucial roles in RNA splicing, mRNA stability and gene expression (Adhikari et al., 2016; Lence et al., 2017; von Meyenn et al., 2016; Xiang et al., 2017), DNA 6mA modification was considered to absent in human genome DNA due to its low level or detectable technology (Ratel et al., 2006). Because the important function of 5mC modification in human has attracted much interest, the issue of whether 6mA modification occurs in general and its related roles in human genome DNA remains largely unresolved.

In this study, we used a new method of PacBio single-molecule real-time (SMRT) sequencing to decode and identify the presence of 6mA in human genome DNA, in especially mitochondria genome (Flusberg et al., 2010; Ye et al., 2017). 6mA was broadly distributed across the human genome DNA and [G/C]AGG[C/T] was the most prevalent motif sequence of the 6mA modification. 6mA density was significantly enriched in the exon regions and associated with gene transcriptional activation. N6AMT1 and ALKBH1 were identified as methyltransferase and demethylase for 6mA modification in human, respectively. 6mA level was down-regulated in human cancer tissues accompanying N6AMT1 down-regulation and ALKNH1 up-regulation compared to normal tissues. Our results indicate that DNA 6mA is broadly present in human and may participate into the regulation of human disease such as cancer.

Results

6mA occurs in human genome DNA

Using PacBio sequencing data from Huaxia1 (HX1) human genome (103X genome-wide coverage) (Shi et al., 2016), we identified 881,240 6mA modification sites whose density (6mA/A) is about 0.051% of the total adenines in the human genome (Supplementary Table S1). We showed that 6mA density in human was lower than that in fungi (0.048%-2.8%) (Mondo et al., 2017), Chlamydomonas (~0.4%) (Fu et al., 2015) and C. elegans (~0.7%) (Greer et al., 2015), while similar to that in Drosophila (~0.07% at early embryo stage) (Zhang et al., 2015).

To accurately quantify the level of 6mA in human genomic DNA, we applied an LC-MS/MS assay using the pure 6mA nucleoside (Berry& Associates PR 3740) as an external standard. The 6mA modification in blood genome DNA sample was identified only when both its retention time and fragmentation pattern matched with 6mA standard. Our results showed that the 6mA/A ratio was 0.056% in the blood genome DNA sample, consistent with the ratio resolved by SMRT sequencing (~0.051%). As SMRT sequencing approach does not discriminate between 6mA and 1mA, we performed LC-MS/MS assays using the pure 1mA nucleoside as an external standard (Berry& Associates PR 3032) to examine whether 1mA was present in our blood DNA sample. We found that 1mA was not detectable in blood genomic DNA sample used in this study (Fig. 1A), suggesting that SMRT sequencing was suitable for detecting 6mA in this study.

To further confirm the credibility of our identified DNA 6mA database in human

genome from SMRT sequencing, we used 6mA-IP-qPCR technology to investigate the 6mA modification of the 18 randomly selected 6mA-methylated loci from SMRT sequencing data. The results showed that the genes containing our identified 6mA sites had more obvious enrichment of 6mA modification compared with the negative control *actin* (Fig. 1B), suggesting that 6mA database in human genome from SMRT sequencing was accurate and credited. Collectively, our results indicated that DNA 6mA modification was present in human genome DNA, and we provided the genomic DNA 6mA profiling containing 6mA modification sites in human.

6mA is low in chromosome X and Y, but enriched in mitochondria genome

A broad distribution of the 6mA across autosomal chromosomes (0.050%-0.064%) was observed in the human genome, while about a half of the 6mA density in autosomal chromosomes was observed across sexual chromosomes X (0.023%) and Y (0.024%) (Fig. 2A), in which there are always a higher DNA 5mC modification. Previous study demonstrated that a strong negative relationship between the presence of 6mA epigenomic mark and the presence of 5mC epigenomic mark in fungi (Mondo et al., 2017). This may suggest that a shift of the epigenomic mark in human chromosome X and Y genome DNA. However, a higher 6mA density (0.184%) was observed in mitochondria genome DNA than that in autosomal chromosome genome DNA (Fig. 2A).

We divided the 6mA modification level into low (0-30%, green circle), middle (30%-70%, blue circle), high (70%-100%, orange cicle) three categories and present the data in circus plot format in which concentric rings represent the density

distribution of 6mA across all human chromosomes in the given category. We found the 6mA density was enriched in middle 6mA modification level group (Fig. 2B), indicating that the middle 6mA level was more dynamic across the genome. Moreover, the 6mA density in the sexual chromosomes was not different with that in autosomal chromosomes when 6mA modification level was high. We also found a lower 6mA modification density across the centrosome in which there are many repeat elements and many gaps that were not disposed in the reference genome (Fig. 2B).

6mA is significantly enriched in exon regions

We found that each a gene has the different 6mA density (Supplementary Table S2). Mapping genomic distributions of 6mA modification can be an effective first step for uncovering potential functions of 6mA DNA modification(Jones, 2012; Smith and Meissner, 2013). Therefore, the distributions of 6mA on the different functional regions in genome were further investigated. We found that most of 6mA modification sites located in the intron and intergenic regions of genome (42% and 53%, respectively) (Fig. 2C). This is because the intron and intergenic regions accounted for most of the human genome (>95%). We found that about 23,000 genes harbored 6mA modification sites in exon or/and intron regions, indicating DNA 6mA modification was common in the human genome. Surprisingly, we found the 6mA modification density was significantly enriched in the gene exon-coding regions ($p=0.02$ Wilcoxon test, Fig. 2D), but not in the gene upstream, 5'UTR, intronic, 3'UTR, downstream, intergenic and transposon regions, suggesting that 6mA modification possibly participates into the gene expression regulation (Fig. 2D).

It is well known that the DNA 5mC methylation medification in gene promoter region play an important role in epigenetic regulation of gene transcription (Bergman and Cedar, 2013). Therefore, the distribution of 6mA modification around the upstream -3kb region and downstream 3kb region of transcription start sites (TSS) was further investigated. However, no a significant enrichment of 6mA around TSS was observed in the human genome (Fig. 2E), different from in the C. reinhardtii genome (Fu et al., 2015).

Collectively, our results indicated that the 6mA is highly enriched in at exon regions and the distribution of 6mA in the human genome was different from those in other prokarypotes and eukaryotes. Reminiscent of the genome distribution of 5mC, 6mA distribution patterns are thus species-specific, likely reflecting different functions and mechanisms of the DNA 6mA modification.

6mA prevalently occurs in AGG sequence motif

DNA sequence motif is short nucleotides that is widespread and is conjectured to have a biological sense such as binding to specific region for gene transcription. Most of the 6mA sites are located within palindromic sequences in prokaryotes and *T. thermophila (Touzain et al., 2011)*. The 6mA sites are mainly located at ApT motif around TSS in *Chlamydomonas* (Fu et al., 2015), at AGAA and GAGG sequence motif in C. *elegans* (Greer et al., 2015). We further investigated the master sequences motif of the 6mA sites in human genome. We found that there was high probability (frequency higher than 50%) of guanine (G) in upstream or downstream 1bp of 6mA modification sites, which constitute a core sequence pattern (GAG) within the 6mA

site flank sequence (Fig. 3A). Using MEME to analyze the flanking region to find the prevalent motif sequence(Bailey et al., 2009), the [G/C]ApGG[C/T] motif was most prevalent in 6mA sites in the human genome (Fig. 3B), similar to the motif in the C. elegans genome (Greer et al., 2015).

Genes with higher 6mA density focus on GPCR gene family

To explore the possible functions of the 6mA modification, we performed GO enrichment analysis for the TOP 200 genes with the high 6mA modification density in transcribe region. Interestingly and surprisingly, we found the TOP200 genes were significantly enriched in G-protein coupled receptor (GPCR) category (Supplementary Fig. S1, Supplementary Table S3). Our 6mA-IP-qPCR assay also showed that the GPCR genes had high 6mA modification ratio (Fig. 1B). GPCRs are well known as receptors involving in several cell signal transduction to change the cell state. GPCRs are involved in many diseases such as cancers, and are the targets for approximately 40% of all drugs (Wacker et al., 2017). The 6mA modification of the GPCR genes may play an important role in GPCR gene expression. Collectively, our results indicated that GPCR genes had a most prevalent 6mA modification density.

Increasing 6mA density in exon is positively associated with gene transcription

Adenine methylation in eukaryotic mRNA was recently shown to have profound effects on gene expression (Adhikari et al., 2016; Lence et al., 2017). The potential roles of 6mA in shaping gene expression remain largely unknown. Previous study showed that the 6mA modification in TSS actives gene expression in Chlamydomonas

(Fu et al., 2015), while epigenetic-silences gene expression in mouse embryonic stem cells (Wu et al., 2016). Therefore, we further investigate the influences of 6mA modification on gene expression. RNA-seq data from Huaxia1 (HX1) human blood, which were also used for SMRT sequencing and 6mA identification, was down-loaded and analyzed (Shi et al., 2016). We selected the TOP 1000 high and TOP 1000 low expression genes as two groups and plotted their 6mA density. We found a general trend that genes with higher RNA expression tend to have high density of 6mA around the gene exon regions ($p<0.001$ Student T-test, Fig 4). Our results reveal that 6mA marks the exon regions of actively transcribed genes in human.

N6AMT1 is a methyltransferase for 6mA in human

Next, we focus on discovering the methyltransferase responsible for 6mA in human. N-6 adenine-specific DNA methyltransferase 1(putative) (N6AMT1) was originally named based on the presence of an adenine methyltransferase-characterized amino acid motif (D/N/S) PP (Y/F/W) which was a potential S-Adenosyl-l-methionine (AdoMet) dependent methytransferase, partly similar with M.TaqI-like 6mA DNA methyltransferases of bacterial restriction-modification systems(Timinskas et al., 1995). Therefore, we speculate that N6AMT1 is a methyltransferase for 6mA in human. To validate the hypothesis, we investigate the influences of N6AMT1 on the 6mA level in human genome DNA in vivo and in vitro. Dot blotting assay showed that silencing of N6AMT1 decreased the 6mA modification level in human cancer cells (Fig. 5A and 5B), while over-expression of N6AMT1 increased the 6mA modification level in a dose-dependent manner (Fig. 5C and 5D).

Furthermore, we performed an in vitro methylation assay to further determine whether N6AMT1 is a direct 6mA methyltransferase in human. The recombinant N6AMT1-Flag fushion proteins were purified from N6AMT1-Flag-transfected HEK293T cells. The recombinant N6AMT1-Flag directly efficiently increased the 6mA modification level from four synthetic oligonucleotide substrates (Fig. 5E), while not from the A mutation synthetic oligonucleotide substrates (only A was mutated to G in oligo#1 substrates which contained 23 A) (Fig. 5F). Taken together, our results indicated N6AMT1 is a methyltransferase responsible for 6mA in human genome.

ALKBH1 is a demethylase for 6mA in human

The ALKBH family proteins which contain the conserved $Fe2^{+}$ and 2-oxoglutarate-dependent, dioxygenase domain were promising candidates (Liu et al., 2016a; Westbye et al., 2008). Among them, ALKBH2 and ALKBH3 can efficiently remove 1mA or 3mC from DNA or RNA, but not 6mA (Aas et al., 2003; Duncan et al., 2002). We speculate ALKBH1 as a promising demethylase for 6mA in human given that Homo sapiens ALKBH1 amino acid sequences share the high humongous with Mus musculus ALKBH1 amino acid sequences (Wu et al., 2016). To validate the hypothesis, we investigated whether ALKBH1 could demethylate 6mA from human genome DNA. We found that silencing of ALKBH1 increased the 6mA modification level in human cancer cell genome DNA (Fig. 6A and 6B), while ectopic expression of ALKBH1 decreased the 6mA modification level in a dose-dependent manner (Fig. 6C and 6D).

Next, we performed an in vitro demethylation assay to further determine whether ALKBH1 is a direct 6mA demethylase in human. The recombinant ALKBH1-Flag fusion proteins were purified from ALKBH1-Flag-transfected HEK293T cells. We showed that the recombinant ALKBH1-Flag could directly efficiently decreased 6mA modification level from the synthetic oligonucleotide substrates in a dose-dependent manner (Fig. 6E). We further demonstrated the demethylation activities of ALKBH1 were dependent on $Fe2^{+}$ and 2-oxoglutarate (Fig. 6F), as expected for an active dioxygenase. Collectively, our results indicated ALKBH1 is a demethylase responsible for 6mA in human genome.

6mA levels down-regulated and are positively associated with N6AMT1, negatively with ALKBH1 in human tissues

To further investigate the functions and significance of the 6mA modification, the 6mA level was further analyzed in human diseases, such as cancers. We next investigated the 6mA levels in five pairs of matched fresh frozen primary human gastric cancer tissues (T) and their corresponding adjacent non-tumoral gastric tissues (N). The 6mA and its methyltransferase *N6AMT1* levels were frequently down-regulated in these primary cancer tissues compared with those in their corresponding non-tumoral (N) tissues, while the 6mA demethylase *ALKBH1* levels were up-regulated in these primary cancer tissues (Fig. 7A and 7B). The microarray gene expression data deposited in the Oncomine database also demonstrated that N6AMT1 mRNA level was down-regulalted and ALKBH1 was up-regulated in human cancer tissues compared to normal tissues (Cho et al., 2011; D'Errico et al.,

2009; Deng et al., 2012; Rhodes et al., 2007) (Fig. 7C and 7E). We further found that N6AMT1 gene copy number was decreased in cancer tissues (Fig. 7D), but ALKBH1 was not (Supplementary Fig. S2), compared to normal tissues, suggesting that N6AMT1 down-regulation was induced by N6AMT1 gene loss in cancer cells. The *N6AMT1* and *ALKBH1* mRNA levels were positively (R^2=0.6673, p=0.0039) and negatively (R^2=-0.7532, p=0.0011) correlated with 6mA levels in human tissues, respectively (Fig. 7F). Collectively, our results indicate that the 6mA modification may involve in the progression of disease such as carcinogenesis.

Discussion

In this study, we find that 6mA is extensively present in the human genome DNA, especially in mitochondria genome. [G/C]AGG[C/T] is the most prevalent motif for 6mA modification. 6mA density is enriched in the exon region and associated with transcriptional activation. We identify methyltransferase N6AMT1 and demethylase ALKBH1 responsible for the 6mA modification in human. Interestingly, a lower 6mA level is observed in human cancer tissues compared to normal tissues.

The first human genome DNA 6mA modification profiling containing 6mA modification sites was here provided in this study. Our conclusion that 6mA is extensively present in human genome DNA is supported by multiple lines of evidence. First, 6mA on human genome DNA was detected using SMRT sequencing (103X genome-wide coverage). Although SMRT sequencing method has caveats about distinguishing between 6mA and 1mA, our LC-MS/MS assay demonstrated that the

DNA sample subjected to SMRT sequencing had undetectable 1mA, indicating that our identified DNA methylation modifications likely represent 6mA. Second, the presence of 6mA was identified by LC-MS/MS assay. The 6mA density identified by SMRT sequencing (0.051%) and LC-MS/MS (0.056%) is similar. Third, the presence of 6mA was also identified by 6mA-specific antibody assay including (6mA-IP-qPCR and dot blotting). Fourth, we identified the methyltranferase N6AMT1 and demethylase ALKBH1 for addition and removal of 6mA in human genome DNA. Finally, the 6mA level was down-regulated, positively with N6AMT1 and negatively with ALKBH1 in human cancer tissues.

Our SMRT sequencing data identified a broad 6mA genomic distribution with the prevalent motif sequence [G/C]AGG[C/T] in human genome DNA, similar to one of two motifs in *C. elegans* (GAGG and AGAA) (Greer et al., 2015), different from that in *Chlamydomonas* (AT in TSS region) (Fu et al., 2015), mouse (GAA, G[A/G]AATA) (Wu et al., 2016), and significantly different from that in prokaryotes in which most of 6mA sites are located with the palindromic sequence (Touzain et al., 2011), indicating that the 6mA modification pattern was not conserved across the eukaryotes specie genome and species-specific, likely reflecting the potential diverse biological functions and mechanisms. Recent studies from a limited number of eukaryotes have exhibited the function differences. Our and other's studies indicated that DNA 6mA modification activates gene expression in human, C. *elegans (Greer et al., 2015)*, C. *reinhardtii* (Fu et al., 2015), and fungi (Mondo et al., 2017), and transposon expression in D. melanogaster (Zhang et al., 2015), while suppresses gene expression

in mouse (Wu et al., 2016). In addition, in the gene activation of 6mA, the 6mA-enriched regions in genome were also different in the different eukaryotes. 6mA was enriched in the exon regions in human, TSS in C. *reinhardtii (Fu et al., 2015)*, transposon in D. melanogaster (Zhang et al., 2015). The detailed regulatory mechanism of 6mA on gene and transposon expression is sophisticated and still unclear.

Searching for specific 6mA methyltranferase and demethylase is important for understanding the potential role of 6mA in human cells. In this study, we identify methyltranferase N6AMT1 and demethylase ALKBH1 responsible for addition and removal of 6mA in human genome DNA, respectively. The demethylase ALKBH1 for DNA 6mA demethylation in human was high homologous with the reported demethylase ALKBH1 in mouse, but was different with the demethylase DMAD in Drosophila (Zhang et al., 2015), NMAD-1 in C.*elegans (Greer et al., 2015)*. However, both NMAD-1 and ALKBH1 belongs to AlkB family proteins which contain the conserved Fe2+ ion and 2-oxo-glutarate-dependent, dioxygenase domain (Liu et al., 2016a; Westbye et al., 2008). And the AlkB family proteins, such as ALKBH5 and FTO in mammals, also catalyze the demethylation of 6mA in RNA (Jia et al., 2011; Zheng et al., 2013). Taken together, these data suggested that the demethylases for DNA 6mA demethylation may be evolutionary conserved in mammalians and belong to AlkB family.

The methyltranferase for 6mA addition in genomic DNA was not still discovered in reported eukaryotes fungi (Mondo et al., 2017), C. *reinhardtii* (Fu et al., 2015), D.

melanogaster (Zhang et al., 2015) and mouse, except from C. *elegans* (Greer et al., 2015). Here, we identify N6AMT1 as 6mA methyltranferase in human. However, our identified methyltranferase N6AMT1 was not homologous with the methyltranferase DAMT-1 in C.*elegans*. The 6mA methylase DAMT-1 in *C. elegans* is a member of MT-A70 domain family evolving from the m.MunI-like bacterial 6mA DNA methyltransferase. But, in human, METTL3, which belongs to MT-A70 family, is a 6mA RNA methyltransferase, not 6mA DNA methyltransferase (Liu et al., 2014). Interestingly and surprisingly, the amino acid sequence of our identified human 6mA DNA methyltransferase N6AMT1 has regions of similarity with M.TaqI-like 6mA DNA methyltransferases of bacterial restriction-modification systems(Timinskas et al., 1995). Both of them contain an adenine methyltransferase-characterized amino acid motif (D/N/S) PP (Y/F/W), suggesting that human DNA 6mA methyltransferase are evolving from bacterial.

Importantly and interestingly, the 6mA level was down-regulated in human cancer tissues compared to their corresponding adjacent non-tumoral tissues accompanying a N6AMT1 down-regulation and gene copy number loss and ALKBH1 up-regulation. The significantly positive and negative associations of the 6mA level with the N6AMT1 and ALKBH1 level were observed in human cancer tissues, respectively. These data indicated that DNA 6mA modification may play crucial role in human diseases such as cancer. The DNA 5mC methylation biomarkers including circulating tumor DNA (ctDNA) in liquid biopsy have been used in cancer diagnosis, detection, classification and prognosis (Costa-Pinheiro et al., 2015; Ma et al., 2015). The DNA

6mA methylation modification may be a novel biomarker bank for cancer diagnosis and therapy.

In summary, we identified that 6mA is extensively present in human genome DNA, and [G/C]AGG[C/T] is the most prevalent motif of the 6mA modification. The 6mA modification is associated with gene expression activation. N6AMT1 and ALKBH1 were identified as methyltransferase and demethylase for 6mA modification in human, respectively. The 6mA level is down-regulated in human cancer tissues accompanying N6AMT1 down-regulation and ALKNH1 up-regulation. The discovery of 6mA in human cells sheds new light on epigenetic regulation during human diseases such as cancer.

Materials and methods

Tissue and DNA samples

Matched primary cancer tissues and their corresponding adjacent non-tumoral tissues were collected from gastric cancer patients at the Third Affiliated Hospital of Guangzhou Medical University (Xu et al., 2015). These cancer patients had not received preoperative anticancer treatment. Informed consent was obtained from each patient, and the collection of tissue specimens was approved by the Internal Review and Ethics Boards at the Third Affiliated Hospital of Guangzhou Medical University.

Blood genomics DNA samples used in the validation of 6mA (Measurement of the 6mA/A ratio and 6mA-IP-qPCR) were from our group-preserved samples which were used in long-read DNA SMRT sequencing and short-read RNA sequencing

(SRX1424851 and SRX1423750 in NCBI project PRJNA301527)(Shi et al., 2016).

Identification of 6mA in human genome DNA

The raw h5 format files of PacBio sequencing reads were downloaded from the NCBI SRA database (SRX1424851 in PRJNA301527) (Shi et al., 2016). We used the PacBio SMRT analysis platform (version 2.3.0) for DNA 6mA modification detection (http://www.pacb.com/products-and-services/analytical-software/smrt-analysis/analysis-applications/epigenetics/). Briefly, DNA raw data were aligned to the hg38 reference genome by pbalign. The polymerase kinetics information was further loaded after alignment by loadChemistry.py and loadPulses scripts from raw h5 format files. The two post-aligned datasets were then merged and sorted by using cmph5tools. The 6mA was identified by using ipdSummary.py script. We further filtered 6mAs with less than 25X coverage in autosome chromosomes and 12X in sex chromosomes. High-quality 6mAs were annotated by ANNOVAR (Wang et al., 2010) based on RefGene annotation file.

Measurement of the 6mA/A ratio

Blood Genomic DNA was digested into single nucleosides in a digestion buffer containing phosphodiesterase I (0.01 U) and nuclease P1 (1 U) in 2mM zinc chloride and 30mM sodium acetate at pH 6.8 for 3h at 37°C, and dephosphorylated with bacterial alkaline phosphatase (10 U) for 1h at 37°C. Enzymes were removed by filtration (Amicon Ultra 10K MWCO). Individual nucleosides were resolved on a Hypersil GOLD aQ reverse phase column (Thermo Scientific), and samples were then subjected to liquid chromatography coupled with tandem mass spectrometry

(LC-MS/MS) analysis on an Agilent 6490 Triple Quadrupole mass spectrometer. Nucleosides were quantified using the nucleoside-to-base ion mass transitions of 266.1 to 150.0 for 6mA and 1mA and 252.1 to 136.0 for A.

6mA-IP-qPCR

6mA-IP-qPCR was performed using freshly drawn blood samples harvested independently of the blood samples for SMRT sequencing. Blood genomic DNA was extracted from freshly drawn blood samples and treated with RNase to remove RNA contamination. The DNA sample was sonicated to produce ~250bp fragments. And the fragmented blood DNA was incubated with specific anti-6mA antibody (Synaptic Systems) in immunoprecipitation buffer (2.7 mM KCl, 2 mM potassium phosphate, 137 mM NaCl, 10 mM sodium phosphate, 0.05% Triton X-100, pH 7.4) for 2h at 4 °C. The mixture was then immunoprecipitated by Protein A/G Plus-Agarose (Santa Cruz) that was pre-bound with bovine serum albumin (BSA) at 4°C for 2h. After extensive washing, the bound DNA was eluted from the beads in elution buffer (50 mM Tris, pH 8.0, 1% SDS, 10 mM EDTA) at 65°C, treated with proteinase K and purified using QIAquick PCR Purification Kit (Qiagen). Fold-enrichment of each fragment was determined by quantitative real-time PCR. The Primer used for 6mA-IP-qPCR assays were listed in Supplementary Table S4.

Bioinformatics analysis

The genome-wide 6mA profiles across all chromosomes were generated from Circos (Krzywinski et al., 2009).

For each 6mA modification site, we extracted 20bp from the upstream and

downstream sequences of the 6mA site. MEME program was then used to identify motifs in flank region (Bailey et al., 2009).

The short-read RNA sequencing raw data were down-loaded from the NCBI database (SRX1423750)(Shi et al., 2016). RNA-seq short-read were aligned to the hg38 reference genome by HISAT (Kim et al., 2015). The Reads per Kilobase of transcript per Million mapped reads (RPKM) measured by RNA-SeQC was used for quantification of gene expression (DeLuca et al., 2012).

Web Gestalt was used to test the statistical enrichment of genes in Gene Ontology (Wang et al., 2017). P-values were adjusted by the Benjamini and Hochberg's approach, the adjusted P-value (FDR) <0.01 was considered statistically significant.

Dot blotting

Dot blotting was performed as previously described with minor modification (Zhang et al., 2015). Briefly, the extracted DNA was denatured at 95℃ for 10min and cooled down on ice for 5min. DNA was then loaded on the nitrocellulose membrane (Protran BA 85, Whatman), air dry for 5min. The membrane was baked at 80°C for 2h and then blocked in the blocking buffer (1% BSA and 5% milk in PBST) for 1.5h at room temperature. The membranes were incubated with a specific anti-6mA antibody (Millipore, 1:1000) overnight at 4°C, followed by incubation with HRP-conjugated anti-rabbit IgG secondary antibody (Bioworld, 1:4000) at room temperature for 1.5h. The antibody-bound 6mA was detected by using the enhanced chemiluminescence Supersignal West Pico kit (Thermo, USA) followed by exposure to autoradiographic film in darkroom.

N6AMT1 methylase assays in vitro

See Supplementary Materials and Methods.

ALKBH1 demethylase assays in vitro

See Supplementary Materials and Methods.

Statistical analysis

Statistical analyses were performed using Prism5 software. Two-tailed unpaired Student's t-tests were applied for comparisons between two groups. The data are presented as the means ± SEM except where stated otherwise. The *$p<0.05$ or **$p<0.01$ values were considered statistically significant.

Author Contributions

GRY and CLX conceived the project and designed the experiments; CLX performed the identification of 6mA; SZ and GRY performed the experiments for identifying for 6mA modification and detecting 6mA in samples in vitro and in vivo; MHH, YC, SQX and FL performed the informatics analysis; XFG and ZL performed LC-MS/MS and 6mA-IP-qPCR assays; GRY wrote the paper; KW revised the paper and provided the suggestions; KW and GLY provided the blood sample; DC collected the cancer samples.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (81272285, 31600667), the R&D Plan of Guangzhou (201704020115), the Yangcheng

Scholars program from the Ministry of Education of Guangzhou (1201561583), Innovative Research Team of Ministry of Education of Guangzhou (1201610015), the National Funds of Developing Local Colleges and Universities (B16056001), the Fundamental Research Funds for the Central Universities (15ykjc23d)， and Guangdong Natural Science Foundation (2015A030313127), China Postdoctoral Science Foundation (2017M612798).

Competing financial interests

The authors declare no conflicts of interest.

References

Aas, P.A., Otterlei, M., Falnes, P.O., Vagbo, C.B., Skorpen, F., Akbari, M., Sundheim, O., Bjoras, M., Slupphaug, G., Seeberg, E.*, et al.* (2003). Human and bacterial oxidative demethylases repair alkylation damage in both RNA and DNA. Nature *421*, 859-863.

Adhikari, S., Xiao, W., Zhao, Y.L., and Yang, Y.G. (2016). m(6)A: Signaling for mRNA splicing. RNA Biol *13*, 756-759.

Bailey, T.L., Boden, M., Buske, F.A., Frith, M., Grant, C.E., Clementi, L., Ren, J., Li, W.W., and Noble, W.S. (2009). MEME SUITE: tools for motif discovery and searching. Nucleic Acids Res *37*, W202-208.

Banerjee, A., and Rao, D.N. (2011). Functional analysis of an acid adaptive DNA adenine methyltransferase from Helicobacter pylori 26695. PLoS One *6*, e16810.

Bergman, Y., and Cedar, H. (2013). DNA methylation dynamics in health and disease. Nat Struct Mol Biol *20*, 274-281.

Cho, J.Y., Lim, J.Y., Cheong, J.H., Park, Y.Y., Yoon, S.L., Kim, S.M., Kim, S.B., Kim, H., Hong, S.W., Park, Y.N.*, et al.* (2011). Gene expression signature-based prognostic risk score in gastric cancer. Clin Cancer Res *17*, 1850-1857.

Costa-Pinheiro, P., Montezuma, D., Henrique, R., and Jeronimo, C. (2015). Diagnostic and prognostic epigenetic biomarkers in cancer. Epigenomics *7*, 1003-1015.

D'Errico, M., de Rinaldis, E., Blasi, M.F., Viti, V., Falchetti, M., Calcagnile, A., Sera, F., Saieva, C., Ottini, L., Palli, D.*, et al.* (2009). Genome-wide expression profile of

sporadic gastric cancers with microsatellite instability. Eur J Cancer *45*, 461-469.

DeLuca, D.S., Levin, J.Z., Sivachenko, A., Fennell, T., Nazaire, M.D., Williams, C., Reich, M., Winckler, W., and Getz, G. (2012). RNA-SeQC: RNA-seq metrics for quality control and process optimization. Bioinformatics *28*, 1530-1532.

Deng, N., Goh, L.K., Wang, H., Das, K., Tao, J., Tan, I.B., Zhang, S., Lee, M., Wu, J., Lim, K.H., *et al.* (2012). A comprehensive survey of genomic alterations in gastric cancer reveals systematic patterns of molecular exclusivity and co-occurrence among distinct therapeutic targets. Gut *61*, 673-684.

Duncan, T., Trewick, S.C., Koivisto, P., Bates, P.A., Lindahl, T., and Sedgwick, B. (2002). Reversal of DNA alkylation damage by two human dioxygenases. Proc Natl Acad Sci U S A *99*, 16660-16665.

Flusberg, B.A., Webster, D.R., Lee, J.H., Travers, K.J., Olivares, E.C., Clark, T.A., Korlach, J., and Turner, S.W. (2010). Direct detection of DNA methylation during single-molecule, real-time sequencing. Nat Methods *7*, 461-465.

Fu, Y., Luo, G.Z., Chen, K., Deng, X., Yu, M., Han, D., Hao, Z., Liu, J., Lu, X., Dore, L.C., *et al.* (2015). N6-methyldeoxyadenosine marks active transcription start sites in Chlamydomonas. Cell *161*, 879-892.

Greer, E.L., Blanco, M.A., Gu, L., Sendinc, E., Liu, J., Aristizabal-Corrales, D., Hsu, C.H., Aravind, L., He, C., and Shi, Y. (2015). DNA Methylation on N6-Adenine in C. elegans. Cell *161*, 868-878.

Jia, G., Fu, Y., Zhao, X., Dai, Q., Zheng, G., Yang, Y., Yi, C., Lindahl, T., Pan, T., Yang, Y.G., *et al.* (2011). N6-methyladenosine in nuclear RNA is a major substrate of the

obesity-associated FTO. Nat Chem Biol *7*, 885-887.

Jones, P.A. (2012). Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nat Rev Genet *13*, 484-492.

Kim, D., Langmead, B., and Salzberg, S.L. (2015). HISAT: a fast spliced aligner with low memory requirements. Nat Methods *12*, 357-360.

Krzywinski, M., Schein, J., Birol, I., Connors, J., Gascoyne, R., Horsman, D., Jones, S.J., and Marra, M.A. (2009). Circos: an information aesthetic for comparative genomics. Genome Res *19*, 1639-1645.

Lence, T., Soller, M., and Roignant, J.Y. (2017). A fly view on the roles and mechanisms of the m6A mRNA modification and its players. RNA Biol, 1-9.

Liu, F., Clark, W., Luo, G., Wang, X., Fu, Y., Wei, J., Wang, X., Hao, Z., Dai, Q., Zheng, G.*, et al.* (2016a). ALKBH1-Mediated tRNA Demethylation Regulates Translation. Cell *167*, 816-828 e816.

Liu, J., Yue, Y., Han, D., Wang, X., Fu, Y., Zhang, L., Jia, G., Yu, M., Lu, Z., Deng, X.*, et al.* (2014). A METTL3-METTL14 complex mediates mammalian nuclear RNA N6-adenosine methylation. Nat Chem Biol *10*, 93-95.

Liu, J., Zhu, Y., Luo, G.Z., Wang, X., Yue, Y., Wang, X., Zong, X., Chen, K., Yin, H., Fu, Y.*, et al.* (2016b). Abundant DNA 6mA methylation during early embryogenesis of zebrafish and pig. Nat Commun *7*, 13052.

Luo, G.Z., Blanco, M.A., Greer, E.L., He, C., and Shi, Y. (2015). DNA N(6)-methyladenine: a new epigenetic mark in eukaryotes? Nat Rev Mol Cell Biol *16*, 705-710.

Ma, M., Zhu, H., Zhang, C., Sun, X., Gao, X., and Chen, G. (2015). "Liquid biopsy"-ctDNA detection with great potential and challenges. Ann Transl Med *3*, 235.

Mondo, S.J., Dannebaum, R.O., Kuo, R.C., Louie, K.B., Bewick, A.J., LaButti, K., Haridas, S., Kuo, A., Salamov, A., Ahrendt, S.R., *et al.* (2017). Widespread adenine N6-methylation of active genes in fungi. Nat Genet *49*, 964-968.

Ratel, D., Ravanat, J.L., Berger, F., and Wion, D. (2006). N6-methyladenine: the other methylated base of DNA. Bioessays *28*, 309-315.

Rhodes, D.R., Kalyana-Sundaram, S., Mahavisno, V., Varambally, R., Yu, J., Briggs, B.B., Barrette, T.R., Anstet, M.J., Kincead-Beal, C., Kulkarni, P., *et al.* (2007). Oncomine 3.0: genes, pathways, and networks in a collection of 18,000 cancer gene expression profiles. Neoplasia *9*, 166-180.

Shi, L., Guo, Y., Dong, C., Huddleston, J., Yang, H., Han, X., Fu, A., Li, Q., Li, N., Gong, S., *et al.* (2016). Long-read sequencing and de novo assembly of a Chinese genome. Nat Commun *7*, 12065.

Smith, Z.D., and Meissner, A. (2013). DNA methylation: roles in mammalian development. Nat Rev Genet *14*, 204-220.

Timinskas, A., Butkus, V., and Janulaitis, A. (1995). Sequence motifs characteristic for DNA [cytosine-N4] and DNA [adenine-N6] methyltransferases. Classification of all DNA methyltransferases. Gene *157*, 3-11.

Touzain, F., Petit, M.A., Schbath, S., and El Karoui, M. (2011). DNA motifs that sculpt the bacterial chromosome. Nat Rev Microbiol *9*, 15-26.

Vasu, K., and Nagaraja, V. (2013). Diverse functions of restriction-modification

systems in addition to cellular defense. Microbiol Mol Biol Rev *77*, 53-72.

von Meyenn, F., Iurlaro, M., Habibi, E., Liu, N.Q., Salehzadeh-Yazdi, A., Santos, F., Petrini, E., Milagre, I., Yu, M., Xie, Z.*, et al.* (2016). Impairment of DNA Methylation Maintenance Is the Main Cause of Global Demethylation in Naive Embryonic Stem Cells. Mol Cell *62*, 848-861.

Wacker, D., Stevens, R.C., and Roth, B.L. (2017). How Ligands Illuminate GPCR Molecular Pharmacology. Cell *170*, 414-427.

Wang, J., Vasaikar, S., Shi, Z., Greer, M., and Zhang, B. (2017). WebGestalt 2017: a more comprehensive, powerful, flexible and interactive gene set enrichment analysis toolkit. Nucleic Acids Res.

Wang, K., Li, M., and Hakonarson, H. (2010). ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. Nucleic Acids Res *38*, e164.

Westbye, M.P., Feyzi, E., Aas, P.A., Vagbo, C.B., Talstad, V.A., Kavli, B., Hagen, L., Sundheim, O., Akbari, M., Liabakk, N.B.*, et al.* (2008). Human AlkB homolog 1 is a mitochondrial protein that demethylates 3-methylcytosine in DNA and RNA. J Biol Chem *283*, 25046-25056.

Wion, D., and Casadesus, J. (2006). N6-methyl-adenine: an epigenetic signal for DNA-protein interactions. Nat Rev Microbiol *4*, 183-192.

Wu, T.P., Wang, T., Seetin, M.G., Lai, Y., Zhu, S., Lin, K., Liu, Y., Byrum, S.D., Mackintosh, S.G., Zhong, M.*, et al.* (2016). DNA methylation on N(6)-adenine in mammalian embryonic stem cells. Nature *532*, 329-333.

Xiang, Y., Laurent, B., Hsu, C.H., Nachtergaele, S., Lu, Z., Sheng, W., Xu, C., Chen,

H., Ouyang, J., Wang, S., *et al.* (2017). RNA m6A methylation regulates the ultraviolet-induced DNA damage response. Nature *543*, 573-576.

Xu, S.H., Huang, J.Z., Xu, M.L., Yu, G., Yin, X.F., Chen, D., and Yan, G.R. (2015). ACK1 promotes gastric cancer epithelial-mesenchymal transition and metastasis through AKT-POU2F1-ECD signalling. J Pathol *236*, 175-185.

Ye, P., Luan, Y., Chen, K., Liu, Y., Xiao, C., and Xie, Z. (2017). MethSMRT: an integrative database for DNA N6-methyladenine and N4-methylcytosine generated by single-molecular real-time sequencing. Nucleic Acids Res *45*, D85-D89.

Zhang, G., Huang, H., Liu, D., Cheng, Y., Liu, X., Zhang, W., Yin, R., Zhang, D., Zhang, P., Liu, J., *et al.* (2015). N6-methyladenine DNA modification in Drosophila. Cell *161*, 893-906.

Zheng, G., Dahl, J.A., Niu, Y., Fedorcsak, P., Huang, C.M., Li, C.J., Vagbo, C.B., Shi, Y., Wang, W.L., Song, S.H., *et al.* (2013). ALKBH5 is a mammalian RNA demethylase that impacts RNA metabolism and mouse fertility. Mol Cell *49*, 18-29.

Figure Legend

Figure 1. Validation of 6mA modification in human genome DNA. (A) LC-MS/MS chromatograms showing 1mA and 6mA levels in standard oligos and genomic DNA from blood sample. (B) Validation of the 18 identified 6mA-methylated GPCR family gene loci from SMRT sequencing by 6mA-IP-qPCR assay (n=3). Actin was as a negative control.

Figure 2. Distribution of 6mA in chromosomes and genome. (A) 6mA modification density across all chromosomes. (B) Circos plots of the density distribution of 6mA across all human chromosomes in the different 6mA modification level category. Green, blue, and orange circle represents lowly methylated (0-30%), middle methylated (30%-70%), and highly methylated (70%-100%) 6mA, respectively. (C) The 6mA modification distribution in the functional elements of human genome DNA. (D) Comparison of observed versus expected distributions of 6mA modification in each functional elements. (E) Distribution of 6mA density surrounding transcriptional start sites (TSS).

Figure 3. AGG is the prevalent motif sequences of 6mA modification. (A) Base constitution percentage in the upstream and downstream flanking region of the 6mA site ("0" position). (B) The motif sequence of 6mA identify by MEME assay.

Figure 4. The 6mA density in exon region is significantly correlated with active genes. Two groups of genes with TOP 1000 high and TOP 1000 low expression levels (RNA-seq data(Shi et al., 2016)) are ploted with its methylation density ($p<0.001$, Student T-test).

Figure 5. N6AMT1 is the methyltransferase for DNA 6mA methylation in human. (A, B) The indicated human cells were transfected with anti-N6AMT1, the N6AMT1 levels detected by RT-PCR, the 6mA levels of genomic DNA were subjected to dot blotting assays using a specific anti-6mA antibody. (C, D) The indicated cells were transfected with N6AMT1-Flag plasmids, the N6AMT1 levels were determined by western blotting, the 6mA levels of genomic DNA were subjected to dot blotting assays. (E) Recombinant N6AMT1 directly methylated the DNA oligos in vitro methylation reaction. Four oligo substractes were used for 6mA-methylating by recombinat N6AMT1-Flag. (F) Recombinant N6AMT1 did not methylate the mutated DNA oligos (A mutated to G) in vitro methylation reaction.

Figure 6. ALKBH1 is the demethylase for DNA 6mA demethylation in human. (A, B) The indicated human cells were transfected with anti-ALKBH1, the ALKBH1 levels detected by RT-PCR, the 6mA levels of genomic DNA were subjected to dot blotting assays using a specific anti-6mA antibody. (C, D) The indicated cells were transfected with ALKBH1-Flag plasmids, the ALKBH1 levels were determined by western blotting, the 6mA levels of genomic DNA were subjected to dot blotting assays. (E) Recombinant ALKBH1 directly demethylated at 6mA the DNA oligos in vitro demethylation reaction. (F) ALKBH1 demethylase activities were dependent on Fe^{2+} and α-KG in vitro demethylation reaction.

Figure 7. The 6mA level was downregulated in cancer tissues compared to their corresponding adjacent non-tumoral tissues, positively with *N6AMT1* level and gene copy number, while negatively with *ALKBH1* level in human tissues. (A) The 6mA

levels were detected by DNA dot blotting in five pairs of matched primary gastric cancer tissues (T) and corresponding adjacent non-tumoral gastric tissues (N). (A) The mRNA levels of *N6AMT1* and *ALKBH1* in human tissues as (A) were detected. (C, D) *N6AMT1* RNA levels (C) and gene copy number (D) between normal gastric tissues (N) and gastric cancer tissues (GC) were analyzed in the Oncomine database. (E) *ALKBH1* RNA levels between normal gastric tissues (N) and gastric cancer tissues (GC) were analyzed in the Oncomine database. (F) The *N6AMT1* and *ALKBH1* mRNA levels were positively and negatively correlated with the 6mA levels in human cancer tissues, respectively.

Figure 1

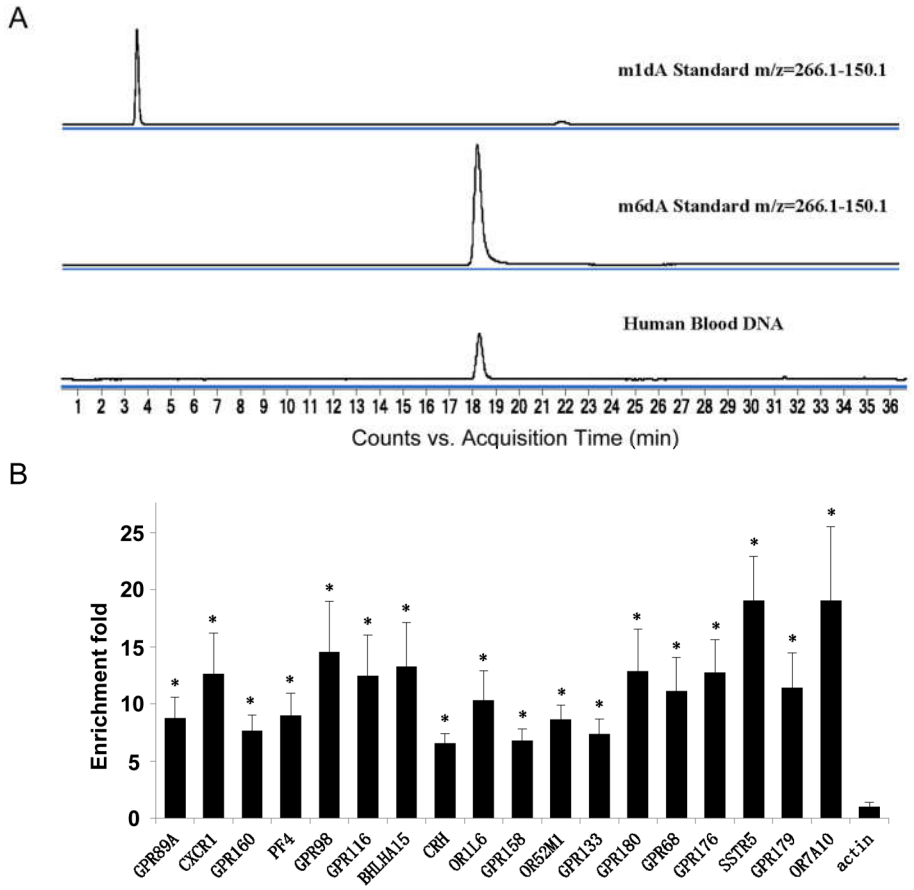

A
m1dA Standard m/z=266.1-150.1
m6dA Standard m/z=266.1-150.1
Human Blood DNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36
Counts vs. Acquisition Time (min)
B
Enrichment fold
0
5
10
15
20
25
GPR89A
CXCR1
GPR160
PF4
GPR98
GPR116
BHLHA15
CRH
OR1L6
GPR158
OR52M1
GPR133
GPR180
GPR68
GPR176
SSTR5
GPR179
OR7A10
actin

Figure 2

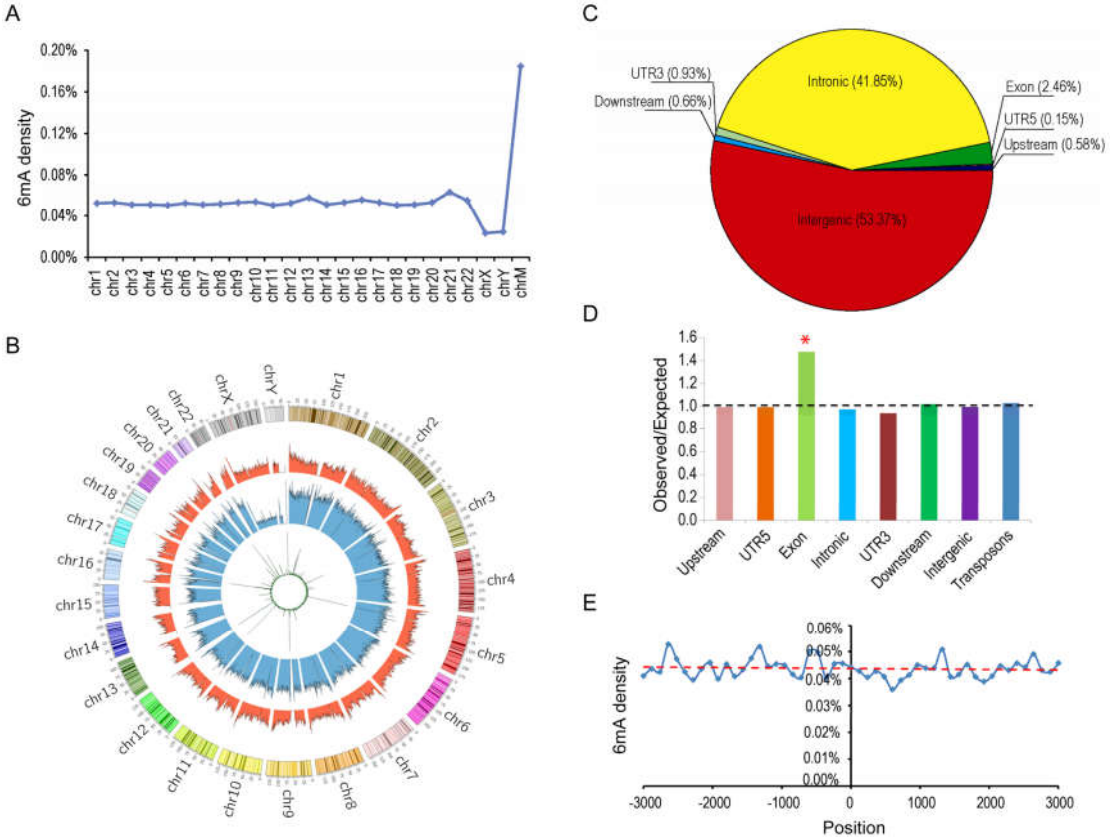

A
6mA density
0.20%
0.16%
0.12%
0.08%
0.04%
0.00%
chr1 chr2 chr3 chr4 chr5 chr6 chr7 chr8 chr9 chr10 chr11 chr12 chr13 chr14 chr15 chr16 chr17 chr18 chr19 chr20 chr21 chr22 chrX chrY chrM
B
chr1 chr2 chr3 chr4 chr5 chr6 chr7 chr8 chr9 chr10 chr11 chr12 chr13 chr14 chr15 chr16 chr17 chr18 chr19 chr20 chr21 chr22 chrX chrY
C
Intronic (41.85%)
UTR3 (0.93%)
Downstream (0.66%)
Exon (2.46%)
UTR5 (0.15%)
Upstream (0.58%)
Intergenic (53.37%)
D
Observed/Expected
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Upstream
UTR5
Exon
Intronic
UTR3
Downstream
Intergenic
Transposons
E
6mA density
0.06%
0.05%
0.04%
0.03%
0.02%
0.01%
0.00%
-3000
-2000
-1000
0
1000
2000
3000
Position

Figure 3

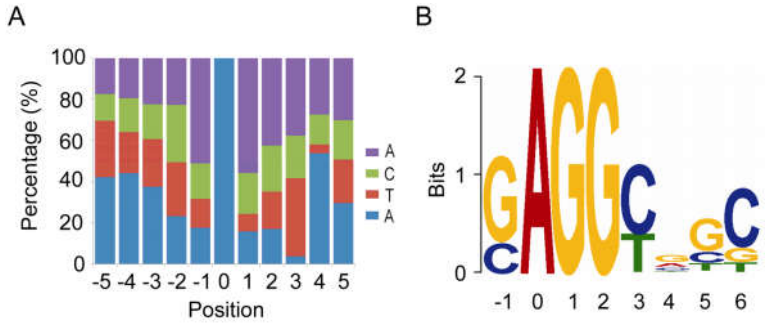


Figure 4

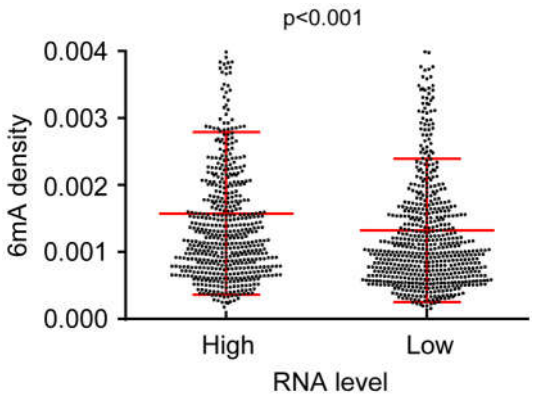


Figure 5

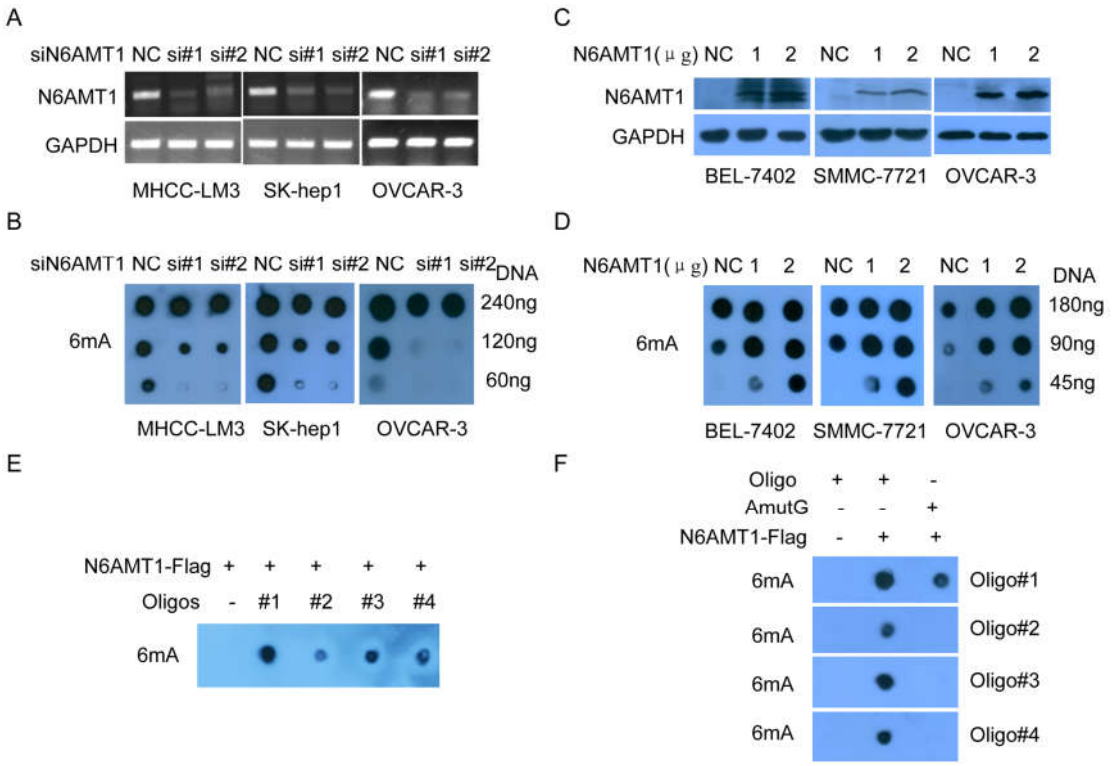

A
siN6AMT1 NC si#1 si#2 NC si#1 si#2 NC si#1 si#2
N6AMT1
GAPDH
MHCC-LM3 SK-hep1 OVCAR-3
B
siN6AMT1 NC si#1 si#2 NC si#1 si#2 NC si#1 si#2
DNA
240ng
120ng
60ng
6mA
MHCC-LM3 SK-hep1 OVCAR-3
C
N6AMT1(μg) NC 1 2 NC 1 2 NC 1 2
N6AMT1
GAPDH
BEL-7402 SMMC-7721 OVCAR-3
D
N6AMT1(μg) NC 1 2 NC 1 2 NC 1 2
DNA
180ng
90ng
45ng
6mA
BEL-7402 SMMC-7721 OVCAR-3
E
N6AMT1-Flag + + + + +
Oligos - #1 #2 #3 #4
6mA
F
Oligo + + -
AmutG - - +
N6AMT1-Flag - + +
6mA
Oligo#1
6mA
Oligo#2
6mA
Oligo#3
6mA
Oligo#4

Figure 6

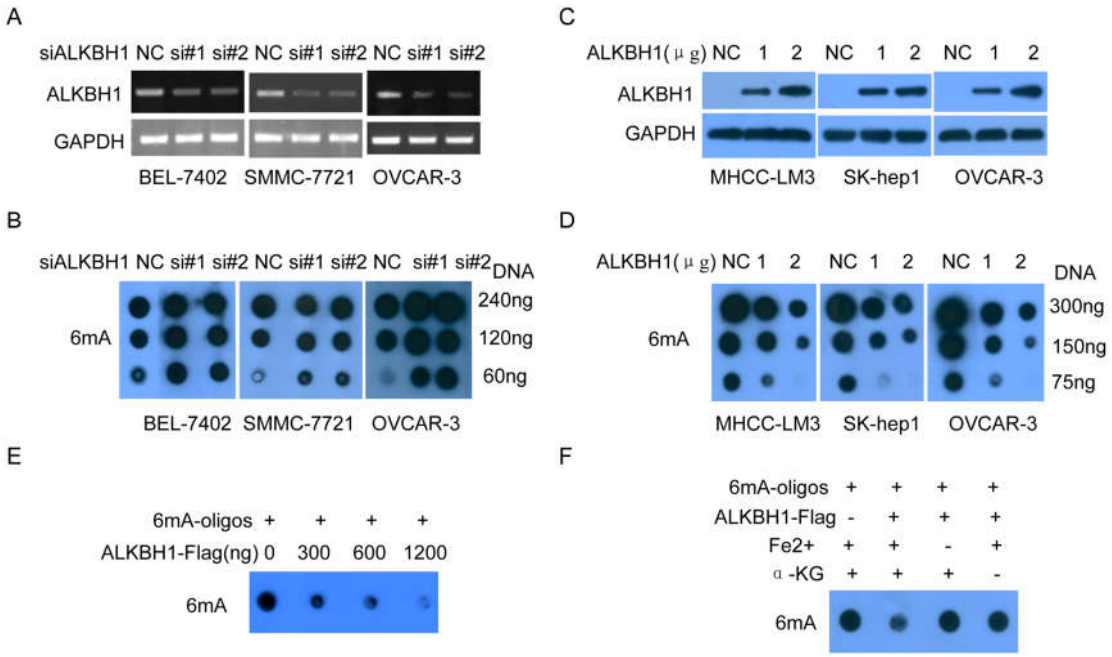
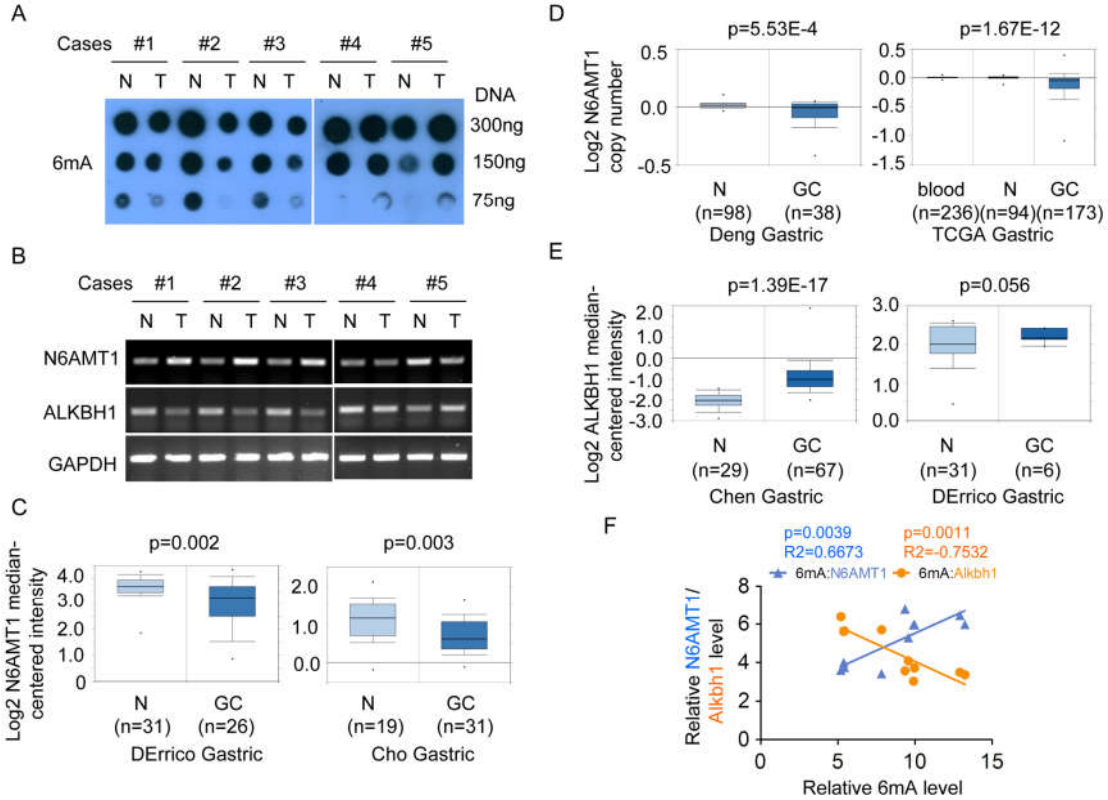

A
siALKBH1 NC si#1 si#2 NC si#1 si#2 NC si#1 si#2
ALKBH1
GAPDH
BEL-7402 SMMC-7721 OVCAR-3
B
siALKBH1 NC si#1 si#2 NC si#1 si#2 NC si#1 si#2
DNA
240ng
120ng
60ng
6mA
BEL-7402 SMMC-7721 OVCAR-3
C
ALKBH1(μg) NC 1 2 NC 1 2 NC 1 2
ALKBH1
GAPDH
MHCC-LM3 SK-hep1 OVCAR-3
D
ALKBH1(μg) NC 1 2 NC 1 2 NC 1 2
DNA
300ng
150ng
75ng
6mA
MHCC-LM3 SK-hep1 OVCAR-3
E
6mA-oligos + + + +
ALKBH1-Flag(ng) 0 300 600 1200
6mA
F
6mA-oligos + + + +
ALKBH1-Flag - + + +
Fe2+ + + - +
α-KG + + + -
6mA

Figure 7


A
Cases #1 #2 #3 #4 #5
N T N T N T N T N T
DNA
300ng
150ng
75ng
6mA
B
Cases #1 #2 #3 #4 #5
N T N T N T N T N T
N6AMT1
ALKBH1
GAPDH
C
Log2 N6AMT1 median-centered intensity
p=0.002
p=0.003
N (n=31) GC (n=26)
DErrico Gastric
N (n=19) GC (n=31)
Cho Gastric
D
Log2 N6AMT1 copy number
p=5.53E-4
p=1.67E-12
N (n=98) GC (n=38)
Deng Gastric
blood (n=236) N (n=94) GC (n=173)
TCGA Gastric
E
Log2 ALKBH1 median-centered intensity
p=1.39E-17
p=0.056
N (n=29) GC (n=67)
Chen Gastric
N (n=31) GC (n=6)
DErrico Gastric
F
p=0.0039
R2=0.6673
p=0.0011
R2=-0.7532
6mA:N6AMT1
6mA:Alkbh1
Relative N6AMT1/ Alkbh1 level
Relative 6mA level