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ORIGINAL ARTICLE

Genetic variants in m⁶A modification genes are associated with colorectal cancer risk

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Abstract

The N6-methyladenosine (m⁶A) modification plays important regulatory roles in gene expression, cancer occurrence and metastasis. Herein, we aimed to explore the association between genetic variants in m⁶A modification genes and susceptibility to colorectal cancer. We used logistic regression models to investigate the associations between candidate single-nucleotide polymorphisms (SNPs) in 20 m⁶A modification genes and colorectal cancer risk. The false discovery rate (FDR) method was used for multiple comparisons. Dual luciferase assays and RNA m⁶A quantifications were applied to assess transcriptional activity and measure m⁶A levels, respectively. We found that *SND1* rs118049207 was significantly associated with colorectal cancer risk in a Nanjing population (odds ratio (OR) = 1.69, 95% confidence interval (95% CI) = 1.31–2.18, $P = 6.51 \times 10^{-6}$). This finding was further replicated in an independent Beijing population (OR = 1.36, 95% CI = 1.04–1.79, $P = 2.41 \times 10^{-2}$) and in a combined analysis (OR = 1.52, 95% CI = 1.27–1.84, $P = 8.75 \times 10^{-6}$). Stratification and interaction analyses showed that *SND1* rs118049207 multiplicatively interacted with the sex and drinking status of the patients to enhance their colorectal cancer risk ($P = 1.56 \times 10^{-3}$ and 1.41×10^{-2}, respectively). Furthermore, rs118049207 served as an intronic enhancer on *SND1* driven by *DMRT3*. *SND1* mRNA expression was markedly increased in colorectal tumour tissues compared with adjacent normal tissues. The colorimetric m⁶A quantification strategy revealed that *SND1* could alter m⁶A levels in colorectal cancer cell lines. Our findings indicated that genetic variants in m⁶A modification genes might be promising predictors of colorectal cancer risk.

Introduction

Globally, colorectal cancer is the third most common cancer and the third leading cause of cancer death in men and women (1). Colorectal cancer incidence and mortality rates vary by up to 10 times worldwide (2). The number of new colorectal cancer cases is expected to increase to more than 2.2 million, while the number of deaths will increase by 1.1 million by 2030 (3). Unlike other highly developed countries, China has experienced an increasing trend in colorectal incidence in the past

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Abbreviations

CI	confidence interval;
FDR	false discovery rate;
GTEx	Genotype-Tissue Expression;
HWE	Hardy-Weinberg equilibrium;
LD	linkage disequilibrium;
MAF	minor allele frequency;
OR	odds ratio;
SMD	standardized mean difference;
SNPs	single-nucleotide polymorphisms

few decades (4,5). Colorectal cancer is a heterogeneous tumour that arises due to both genetic and environmental risk factors, and accumulating evidence indicates that genetic variants play a pivotal role in colorectal cancer development (6). Single-nucleotide polymorphisms (SNPs) contribute to the progression of diseases (7). Studies on cancer chronology support the idea that genetic and epigenetic alterations define malignant tumour behaviour (8).

The N6-methyladenosine (m^{6}A) modification, which is considered the most abundant mRNA post-transcriptional modification (9,10) is the most prevalent internal modification in eukaryotic mRNAs (11). m^{6}A was first detected in poly (A) RNA fractions in 1974 (12,13), and shares the same characteristics as epigenetic DNA and histone modifications (14,15). Studies have recently investigated RNA modifications, revealing the instability of m^{6}A (16). However, all of the modifications remain incompletely understood (17,18). RNA is methylated by writers and demethylated by erasers, while a third group of readers bind to m^{6}A and decide the fate of the modified mRNA (19,20). The m^{6}A modification is widely observed in prokaryotes and eukaryotes, and it plays a central regulatory role in gene expression, RNA cleavage and mRNA translation (14). Recent studies have revealed that m^{6}A is closely related to cancer occurrence and metastasis (21), embryonic development (22), lipid metabolism (23), circular RNA translation (24) and DNA damage repair (25). RNA m^{6}A methylation has multiple functions in a variety of cells and cancers (19), including glioblastoma stem cells (26), haematopoietic stem cells (27), acute myeloid leukaemia (28), hepatocellular carcinoma (29) and colorectal cancer (30). Moreover, a few studies have examined the relationship between SNPs involving the m^{6}A enzyme and major depressive disorders (31).

Given the evidence that cells modulated by m^{6}A contribute to tumourigenesis, we hypothesize that genetic variants of m^{6}A modification genes are associated with colorectal cancer risk. In this study, we conducted two case–control studies to investigate the association of SNPs with susceptibility to colorectal cancer. Further functional validations in luciferase activity experiments and RNA m^{6}A quantification tests were applied to confirm our hypothesis.

Materials and Methods

Study populations

Our discovery study included 1150 colorectal cancer cases and 1342 control subjects, all of which were of the Han Chinese population (32). The patients, confirmed to have colorectal cancers histopathologically, were recruited from the First Affiliated Hospital of Nanjing Medical University and the Affiliated Nanjing First Hospital since September 2010, with no age or gender restrictions utilized. The controls were randomly recruited from more than 25 000 cancer-free individuals in the same geographical region and subjected to physical examinations. The controls were frequency-matched to the patients based on age (±5 years) and sex. Individuals who consumed one or more alcoholic drinks per week for > 1 year were deemed drinkers, and those who smoked daily for > 1 year were deemed smokers. Upon recruitment, peripheral venous blood samples (5 ml) were collected from each subject after written informed consent was obtained. In the interview, a questionnaire on the lifestyles and demographic factors of the subjects was administered via trained interviewers in face-to-face interviews. The Institutional Review Board of Nanjing Medical University gave official approval for this research.

The replication study includes 932 cases and 966 controls from a genome-wide association study (GWAS) dataset in a Beijing population. The cases were recruited in local hospitals and had pathologically proven disease. Cancer-free control subjects were recruited in local hospitals for individuals receiving routine physical examinations or in the communities for those participating in screenings for non-communicable diseases. More details of the characteristics and participant recruitment have been described previously (33).

Selection of genes

The keywords 'RNA methylation', 'N6-methyladenosine', 'm^{6}A', 'cancer', 'tumour', and 'carcinoma' were searched in PubMed, EMBASE, Web of Science and other online databases. The last search update was conducted on 1 March 2018. Non-English-language papers were excluded. After excluding genes located on the X chromosome, the resultant 20 related autosomal chromosome genes involved in the m^{6}A modification of methylated RNA were obtained. Briefly, we included seven literatures (14,19,34–38), which described the YTH N6-methyladenosine RNA-binding protein 1 (*YTHDF1*), YTH N6-methyladenosine RNA-binding protein 3 (*YTHDF3*), YTH N6-methyladenosine RNA-binding protein 2 (*YTHDF2*), YTH domain-containing 1 (*YTHDC1*), heterogeneous nuclear ribonucleoprotein C (*HNRNPC*), eukaryotic translation initiation factor 3 subunit A (*EIF3A*) and eukaryotic translation initiation factor 4E (*EIF4E*) genes as readers; the alkB homologue 5 (*ALKBH5*) and alpha-ketoglutarate-dependent dioxygenase (*FTO*) genes as erasers; the WT1-associated protein (*WTAP*), methyltransferase-like 3 (*METTL3*), methyltransferase-like 14 (*METTL14*), RNA-binding motif protein 15 (*RBM15*) and RNA-binding motif protein 15B (*RBM15B*) genes as writers; and the RNA-specific (*ADAR1*), adenosine deaminase, RNA-specific B1 (*ADARB1*), *DICER1*, staphylococcal nuclease and tudor domain containing 1 (*SND1*), *DGCR8* and drosha ribonuclease III (*DROSHA*) as binding proteins.

Selection of SNPs

SNPs genotyped within these 20 genes and 5-kb regions upstream were selected from the 1000 Genomes Project based on data from Japanese individuals in Tokyo, Japan (JPT), and Han Chinese individuals in Beijing, China (CHB) (39). The quality control criteria for deriving these SNPs were as follows: (a) call rate ≥95%, (b) minor allele frequency (MAF) ≥0.05 and (c) Hardy–Weinberg equilibrium (HWE) ≥0.05. Pairwise linkage disequilibrium (LD) analysis ($r^2 \geq 0.8$) was applied using HaploView 4.2 software to confirm the SNPs analysed in this study. HaploReg (40) (http://compbio.mit.edu/HaploReg), SNPinfo (41) (http://snpinfo.niehs.nih.gov/), PancanQTL (42) (http://bioinfo.life.hust.edu.cn/PancanQTL/), RegulomeDB (43) (http://regulomedb.org/) and GTEx (44) (https://www.gtexportal.org/) were used to predict the potential functions of the significant SNPs.

Genotyping and expression correlation analysis

Genomic DNA was isolated from EDTA-treated venous blood using a Qiagen Blood Kit (Qiagen). The methods utilized for extracting genomic DNA were described in previous studies (45). Genotyping for the discovery stage was conducted using Illumina Human Omni ZhongHua Bead Chips. We imputed the non-genotyped SNPs based on the 1000 Genomes Project (Phase I, version 3, 1092 individuals) using IMPUTE2 (46). A series of filtering criteria for the imputed SNPs were implemented. Imputed SNPs were excluded if they had (i) MAF < 0.05; (ii) call rate < 95% or (iii) HWE < 0.001; (iv) imputation confidence score, INFO < 0.3. The association between genotype data for imputed SNPs and colorectal cancer risk were analyzed by the SNPTEST 2.5 program.

Genotyping for the replication stage was conducted using Affymetrix Axiom Genome-Wide CHB1 and CHB2 arrays. The following

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criteria were used to exclude SNPs: (i) MAF < 0.02; (ii) call rate < 95%; (iii) HWE < 1.0×10^{-5}; (iv) lack of mapping to autosomal chromosomes and (v) a significantly different miss rate between the cases and controls ($P < 1.0 \times 10^{-5}$). After the quality control process, a total of 932 cases and 966 controls comprising 1 129 636 SNPs were included for further analyses.

We downloaded a total of 676 samples from The Cancer Genome Atlas (TCGA) datasets (June 2016) (http://cancergenome.nih.gov/), including 625 colorectal tumour tissues and 51 normal colorectal tissues. RNA-Seq by expectation maximization (RSEM)-normalized counts were used to calculate changes in the expression levels of the selected genes.

Cell lines and quantitative RT-PCR

HCT116 and DLD-1 cells were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum with no mycoplasma contamination. The siRNAs targeting *SND1* and *DMRT3* were purchased from Ribo (RiboBio, China) was co-transfected into colorectal cell lines with DharmaFECT (Dharmacon, USA). The *DMRT3* overexpression vector was purchased from Generay Biotechnology (Shanghai, China).

Total RNA was isolated from cultured cells using TRIzol (Invitrogen, USA) and quantified by ultraviolet spectrometry. Total RNA was reversed transcribed into complementary DNA (cDNA) using SYBR Green Master Mix (Roche, Germany) according to the manufacturer's instructions. The primer sequences are listed in Supplementary Table 1, available at *Carcinogenesis* Online. The relative mRNA expression levels of *SND1* and the internal control genes were detected using a LightCycler 480 II Real-Time PCR system (Roche, Germany).

Luciferase activity

The 1000-bp containing the rs118049207 A or G alleles of the enhancer sequence (chr7: 127 890 317–127 891 317) and *SND1* promoter region (chr7: 127 650 989–127 651 989) were synthesized and cloned into the pGL3-basic vector (Promega, USA) using the *Nhe*I and *Xho*I restriction sites. All constructs were confirmed by DNA sequencing.

For luciferase assays, HCT116 and DLD-1 cells were plated onto 24-well plates (3×10^5 cells per well) and transfected with reporter plasmids using Lipofectamine 3000 (Lifetech, USA). As an internal standard, all plasmids were co-transfected with 10 ng pRL-SV40, which contained the Renilla luciferase gene. Twenty-four hours after transfection, the cells were harvested and then assayed for luciferase activity with the Dual-Luciferase Reporter Assay System (Promega, USA). Relative luciferase activity was normalized to Renilla luciferase.

RNA m^{6}A quantification

An m^{6}A RNA methylation quantification kit (Abcam, UK) was used to measure the m^{6}A content in the total RNA (29). Briefly, 200 ng of RNA was coated on assay wells. Capture antibody solution and detection antibody solution were then added to assay wells separately in a suitable diluted concentration following the manufacturer's instructions. The m^{6}A levels were quantified colourimetrically by reading the absorbance of each well at a wavelength of 450 nm. We then performed statistical calculations based on the standard curve.

Statistical analysis

To evaluate the correlations between genetic variants and colorectal cancer risk, unconditional univariate and multivariate logistic regression models were used to calculate the crude and adjusted odds ratios (ORs) and their 95% confidence intervals (CIs). The χ^2 test and Student's *t*-test were used to evaluate the differences between colorectal cancer cases and controls based on their demographic distributions involving categorical and continuous variables, respectively. For multiple testing corrections, the false discovery rate (FDR) approach was used to reduce the probability of false-positive findings. The goodness-of-fit χ^2 test was used to compute the HWE in the control groups. The interaction effects between SNPs and colorectal cancer risk with or without environmental factors were estimated using multivariate logistic regression models. The measure of heterogeneity was tested using Cochran's Q statistics and I^2. If the *P* value of Cochran's Q-test was > 0.1 and the heterogeneity statistic (I^2) was <50%, a fixed-effects model was employed. Statistical analyses were performed using SAS (version 9.4), PLINK (version 1.90) or R (version 3.5.1) unless otherwise specified. *P* values < 0.05 were considered statistically significant.

Results

Demographic and geographic characteristics of the study populations

The frequency distributions of the selected variables in colorectal cancer patients and cancer-free controls on our discovery study are summarized in Supplementary Table 2, available at *Carcinogenesis* Online. Cases and controls were favourably matched by their age and sex. There were no significant differences in the age, sex, smoking status or drinking status of the patients and cancer-free controls (*P* = 0.994, 0.738, 0.334 and 0.077, respectively). In addition, there were more drinkers in the case group (30.3%) than in the control group (27.0%). In total, there were 586 (51.0%) colon cancer patients and 564 (49.0%) rectal cancer patients. Among the patients, 15.4% had poor tumour grades, while 84.6% had well or moderate grades. In addition, 44.3% of the colorectal cancer patients were in the Dukes A/B stage, and 55.7% were in the C/D stage.

Selection of m^{6}A modification genes and SNPs

We searched, filtered and summarized five writers, two erasers, six readers and six other binding proteins from seven published studies. A total of 20 genes related to m^{6}A modification in tumourigenesis and cancer development were selected from the published studies (Supplementary Table 3 and Supplementary Figure 1, available at *Carcinogenesis* Online). The cellular pathways of m^{6}A in nuclear and cytoplasmic RNAs from the literature reviews are referenced and summarized (19,34,47).

The process for filtering the SNPs of these genes is shown in Figure 1. After performing quality control analysis, a total of 1783 SNPs remained. As shown in Supplementary Table 4, available at *Carcinogenesis* Online, a total of 240 independent SNPs were retained for genotyping after LD analysis ($r^2 \geq 0.8$) and functional prediction.

Associations between SNPs in m^{6}A modification genes and colorectal cancer risk

We estimated the associations between common SNPs and colorectal cancer risk using unconditional univariate and multivariate logistic regression analyses. In an additive model, 14 SNPs were significantly associated with susceptibility to colorectal cancer ($P < 0.05$, Table 1). However, only rs118049207 in *SND1* met the FDR multiple testing correction criteria (OR = 1.69, 95% CI = 1.31–2.18, $P = 6.51 \times 10^{-5}$, $P_{FDR} = 1.56 \times 10^{-2}$, Supplementary Table 5, available at *Carcinogenesis* Online).

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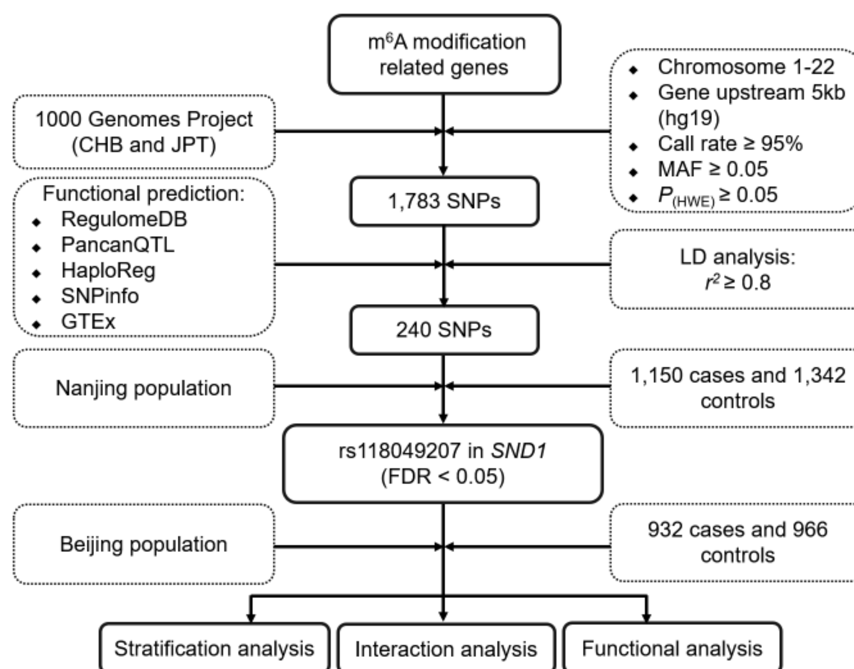

Figure 1. Flow diagram of SNP selection. The workflow of the analysis includes the screening criteria and the methods. MAF, minor allele frequency; HWE, Hardy–Weinberg equilibrium; LD, linkage disequilibrium.

To confirm the significance of this association, we genotyped rs118049207 in an additional Han Chinese population from Beijing. Similarly, rs118049207 showed a significant association consistent in direction with the previous results (OR = 1.36, 95% CI = 1.04–1.79, P = 2.41 × 10^{-2}, Table 2). We further conducted a combined analysis of our study in the Nanjing population and Beijing population. We found that the rs118049207 G allele had an increased risk of colorectal cancer without heterogeneity between the two sets (OR = 1.52, 95% CI = 1.27–1.84, P = 8.75×10^{-6}, P_{het} = 0.262, I^2 = 20.37, Table 2).

We further focused on *SND1* variants in high LD with rs118049207. We found that four SNPs had r^2 values of 0.8 or higher with rs118049207 (Supplementary Table 6, available at *Carcinogenesis* Online). Functional data for these five SNPs were not available in PancanQTL and SNPinfo. The expression quantitative trait loci (eQTLs) in rs11761621 and rs3757769 with other genes had been reported based on GTEx. RegulomeDB suggested the presence of a transcription factor binding motif, any motif, DNase footprint and DNase sensitivity peak for rs118049207. In HaploReg and RegulomeDB, rs118049207, rs3823996 and rs11761621 had a potential effect on histone marks in colorectal tissues and on motifs. Because the purpose of the present study was to identify potentially functional SNPs, the stringent criterion of a DNase sensitivity peak and motif were used to select rs118049207.

Stratification analysis of rs118049207 with colorectal cancer risk

We performed a stratified analysis of lifestyle-related characteristics. As shown in Table 3 and Supplementary Figure 2, available at *Carcinogenesis* Online, rs118049207 was associated with colorectal cancer patients of different age groups and different smoking statuses. It showed similar associations in age ≤ 60 (OR = 1.58, 95% CI = 1.09–2.29, P = 1.47 × 10^{-2}), age > 60 (OR = 1.80, 95% CI = 1.26–2.58, P = 1.27 × 10^{-3}), non-smokers (OR = 1.63, 95% CI = 1.16–2.23, P = 2.08 × 10^{-3}) and smokers (OR = 1.77, 95% CI = 1.12–2.79, P = 1.47 × 10^{-2}). With stratification based on sex and drinking status, increased risks of colorectal cancer were observed for males (OR = 2.49, 95% CI = 1.73–3.58, P = 7.72 × 10^{-7}) and drinkers (OR = 3.51, 95% CI = 1.96–6.28, P = 2.30 × 10^{-5}).

Next, we investigated the clinicopathological feature relationships between rs118049207 and colorectal cancer risk. As shown in Supplementary Table 7 and Supplementary Figure 2, available at *Carcinogenesis* Online, increased risks were associated with the following subgroups: poor tumour grade (OR = 1.71, 95% CI = 1.32–2.23, P = 6.81 × 10^{-5}), colon tumour site (OR = 1.71, 95% CI = 1.26–2.32, P = 6.36 × 10^{-4}), rectal tumour site (OR = 1.68, 95% CI = 1.24–2.28, P = 8.44 × 10^{-4}), Dukes A/B stage (OR = 1.63, 95% CI = 1.19–2.24, P = 2.52 × 10^{-3}) and Dukes C/D stage (OR = 1.75, 95% CI = 1.30–2.36, P = 2.44 × 10^{-4}). No significant association between rs118049207 and colorectal cancer risk was observed in the poor tumour grade subgroup under the additive model.

Moreover, we analysed the association between *SND1* rs118049207 and the clinicopathologic characteristics of colorectal cancer. However, no statistically significant association was observed for the tumour grade (well, moderate or poor), tumour site (colon or rectum) or Dukes stage (A/B or C/D) (P = 0.648, 0.990 and 0.800, respectively, Supplementary Table 7, available at *Carcinogenesis* Online).

Interactions between the characteristics and *SND1* rs118049207

To further explore gene–environment interactions, *SND1* rs118049207 and basic characteristics were included in the interaction analysis. Significant multiplicative interactions were observed between rs118049207 and the sex and drinking status risk factors ($P_{interaction}$ = 1.56 × 10^{-3} and 1.41 × 10^{-2}, respectively; Table 3). Compared with non-drinkers or females with the A allele, a significantly increased risk was observed in drinkers or males with the rs118049207 G allele according to logistic regression analysis (P = 2.30 × 10^{-5} and 7.72 × 10^{-7}, respectively; Table 3). These differences reflected the different gene–environment interactions occurring when the samples were collected.

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Table 1. Association between the selected SNPs and colorectal cancer risk

SNPs	Gene	Chr	Position	Allele[a]	MAF		Call rate	$P_{(HWE)}$[b]	OR (95% CI)[c]	P[c]	Adjusted OR (95% CI)[d]	P[d]
					Cases	Controls						
rs2304512	*RBM15B*	3	51425481	T/C	0.31	0.34	1.00	0.715	0.89 (0.79–1.00)	4.53×10^{-2}	0.89 (0.79–1.00)	4.56×10^{-2}
rs115267066	*METTL14*	4	119610266	C/T	0.11	0.10	0.99	0.113	1.21 (1.01–1.46)	4.35×10^{-2}	1.21 (1.01–1.46)	4.45×10^{-2}
rs118049207	*SND1*	7	127530870	A/G	0.07	0.04	0.98	0.723	1.68 (1.30–2.17)	7.27×10^{-5}	1.69 (1.31–2.18)	6.51×10^{-5}
rs7477908	*SND1*	7	127732268	G/A	0.05	0.06	0.97	0.650	0.71 (0.55–0.92)	9.03×10^{-3}	0.71 (0.55–0.92)	8.88×10^{-3}
rs1243370	*HNRNPC*	14	21674214	T/C	0.07	0.05	1.00	0.590	1.27 (1.01–1.60)	4.30×10^{-2}	1.27 (1.01–1.60)	4.31×10^{-2}
rs13078	*DICER1*	14	9555747	T/A	0.06	0.04	0.99	0.177	1.30 (1.02–1.67)	3.62×10^{-2}	1.30 (1.02–1.67)	3.74×10^{-2}
rs74019730	*FTO*	16	54134100	C/T	0.11	0.09	0.98	0.604	1.27 (1.05–1.53)	1.47×10^{-2}	1.28 (1.06–1.54)	1.19×10^{-2}
rs4386132	*FTO*	16	53759123	C/T	0.27	0.24	1.00	0.061	1.17 (1.03–1.33)	1.95×10^{-2}	1.17 (1.03–1.33)	1.93×10^{-2}
rs74558124	*FTO*	16	53884664	C/T	0.40	0.36	0.98	0.952	1.14 (1.02–1.28)	2.40×10^{-2}	1.14 (1.02–1.28)	2.50×10^{-2}
rs12324955	*FTO*	16	54019686	G/A	0.16	0.19	1.00	0.530	0.85 (0.74–0.99)	3.28×10^{-2}	0.85 (0.73–0.99)	3.18×10^{-2}
rs8056666	*FTO*	16	53794830	C/A	0.31	0.28	0.99	0.946	1.14 (1.01–1.29)	3.49×10^{-2}	1.14 (1.01–1.29)	3.39×10^{-2}
rs2124370	*ALKBH5*	17	18109328	G/A	0.26	0.23	0.98	0.875	1.15 (1.01–1.31)	3.97×10^{-2}	1.15 (1.01–1.31)	3.83×10^{-2}
rs2253763	*ADARB1*	21	46642764	G/C	0.37	0.34	0.98	0.525	1.13 (1.01–1.28)	4.14×10^{-2}	1.13 (1.00–1.28)	4.21×10^{-2}
rs2838770	*ADARB1*	21	46492897	G/C	0.26	0.23	0.98	0.534	1.18 (1.04–1.35)	1.31×10^{-2}	1.18 (1.04–1.35)	1.33×10^{-2}

OR, odds ratio; CI, confidence interval; MAF, minor allele frequency.
[a]Reference/effect allele.
[b]HWE: Hardy Weinberg Equilibrium in control subjects.
[c]P for additive model not adjusted in logistic regression model.
[d]P for additive model adjusted for age and sex in logistic regression model.

Potential regulatory role of rs118049207 on *SND1*

The SNP rs118049207 is located in the intron (between exons 13 and 14) of the *SND1* gene. It was considered to have enhancer histone markers in colorectal cancer tissue (Supplementary Table 6, available at *Carcinogenesis* Online). *DMRT3*, *DMRT4*, *DMRT7* are predicted to bind in this region according to RegulomeDB (Supplementary Figure 3, available at *Carcinogenesis* Online). Therefore, putatively functional r118049207-centred regions may affect the binding efficiency.

To determine the function of the rs118049207-containing enhancer in *SND1* regulation, we constructed enhancer luciferase reporter vectors containing the rs118049207-centred region or the *SND1* promoter. From the JASPAR database, we further confirmed that rs118049207 altered the binding motif of *DMRT3*. We then knocked down and overexpressed *DMRT3* in HCT116 and DLD-1 cells (Supplementary Figure 4, available at *Carcinogenesis* Online). We selected siRNA1 and siRNA2, which exhibited promising knockdown efficiencies at the mRNA level, for functional analysis. The rs118049207 A allele showed significantly higher enhancer activity than the G allele (Supplementary Figure 5A-B, available at *Carcinogenesis* Online), suggesting that the switching of the enhancer function is, at least partially, genotype-dependent.

In addition, to further demonstrate the role of the transcription factor *DMRT3* in the enhancer assay, we mediated the knockdown of *DMRT3* by siRNA. The differences between the activity levels of the rs118049207 A allele and G allele were abrogated, and the activity levels of both were lower than that of the *SND1* promoter. In contrast, the activity levels of rs118049207 A allele and G allele were significantly higher than that of the *SND1* promoter after *DMRT3* overexpressing in two different cell lines (Supplementary Figure 5C, available at *Carcinogenesis* Online). The putative enhancer activity of the A allele intronic segment is dependent on the presence of *DMRT3*, and presumably its ability to bind its motif. The TCGA database indicates that *DMRT3* is highly expressed in colorectal cancer (Supplementary Figure 6, available at *Carcinogenesis* Online). This high *DMRT3* expression could alter the transcription of the rs118049207 risk allele and reference allele, which may partially affect the high expression of *SND1* in colorectal cancer.

Functional relevance of *SND1* mRNA expression

As shown in Figure 2, *SND1* mRNA expression was significantly higher in tumours than in normal samples found in the TCGA database, TCGA paired data, GSE32323, GSE21510, GSE5261, GSE15781 and in-house RNA-Seq data ($P = 2.20 \times 10^{-16}$, 2.21×10^{-15}, 3.02×10^{-4}, 4.05×10^{-15}, 3.87×10^{-3}, 2.11×10^{-2} and 7.70×10^{-7}, respectively). Overall, the expression level of *SND1* in colorectal tumour tissues was significantly higher than that in adjacent normal tissues (standardized mean difference (SMD) = 0.77, 95% CI = 0.69–0.85).

We demonstrated that age, sex and tumour type subgroups between *SND1* and colorectal cancer patients in TCGA carried no significant risk effects ($P = 0.103$, 0.549 and 0.198, respectively). Relative *SND1* mRNA expression was higher in stages 3 and 4 than in stages 1 and 2 ($P = 0.008$, Supplementary Figure 6, available at *Carcinogenesis* Online).

We next investigated the associations between the mRNA expression of *SND1* with writers and erasers in the GSE106535, GSE21510, GSE32323, GSE5261, GSE96528 and GSE101896 datasets using colorectal cancer tissues. We found that *METTL3* exhibited a significantly positive correlation with *SND1* in mRNA expression (Supplementary Table 8, available at *Carcinogenesis* Online).

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Table 2. Statistical comparisons of the *SND1* rs118049207 genotypes between two independent populations of colorectal cancer cases and their matched groups

Genotypes	Cases, n (%)	Controls, n (%)	Adjusted OR (95% CI)[a]	P^a	P_{het}	I^2
Nanjing population						
AA	998 (96.8)	1185 (91.7)	1.00			
AG	149 (13.0)	106 (8.2)	1.68 (1.29–2.19)	1.00×10^{-4}		
GG	3 (0.3)	1 (0.1)	3.55 (0.37–34.25)	2.73×10^{-1}		
Additive model			1.69 (1.31–2.18)	6.51×10^{-5}		
Beijing population						
AA	799 (85.8)	866 (89.6)				
AG	131 (14.1)	96 (9.9)	1.48 (1.12–1.96)	6.46×10^{-3}		
GG	1 (0.1)	4 (0.4)	0.28 (0.03–2.53)	2.58×10^{-1}		
Additive model			1.36 (1.04–1.79)	2.41×10^{-2}		
Combined-analysis						
Additive model			1.52 (1.27–1.84)	8.75×10^{-6}	0.262	20.37

OR, odds ratio; CI, confidence interval;
[a]P for additive model adjusted for age and sex in logistic regression model.

Table 3. Stratification analyses of rs118049207 genotypes and colorectal cancer risk in the additive model

	AA		AG		GG				
Variables	Cases, n (%)	Controls, n (%)	Cases, n (%)	Controls, n (%)	Cases, n (%)	Controls, n (%)	Adjusted OR (95% CI) [a]	P^a	$P_{interaction}$
Age									8.57×10^{-1}
≤ 60	521 (48.6)	552 (51.4)	76 (49.4)	52 (40.6)	1 (100.0)	0 (0.0)	1.58 (1.09–2.29)	1.47×10^{-2}	
> 60	477 (43.0)	633 (57.0)	73 (57.5)	54 (42.5)	2 (66.7)	1 (33.3)	1.80 (1.26–2.58)	1.27×10^{-3}	
Sex									1.56×10^{-3}
Male	590 (45.7)	699 (54.3)	96 (68.6)	44 (31.4)	2 (66.7)	1 (33.3)	2.49 (1.73–3.58)	7.72×10^{-7}	
Female	408 (45.6)	486 (54.4)	53 (46.1)	62 (53.9)	1 (100.0)	0 (0.0)	1.06 (0.72–1.55)	7.78×10^{-1}	
Smoking status									8.10×10^{-1}
Never	651 (44.9)	800 (55.1)	98 (56.3)	76 (43.7)	2 (100.0)	0 (0.0)	1.63 (1.16–2.23)	2.08×10^{-3}	
Ever	347 (47.4)	385 (52.6)	51 (63.0)	30 (37.0)	1 (50.0)	1 (50.0)	1.77 (1.12–2.79)	1.47×10^{-2}	
Drinking status									1.41×10^{-2}
Never	701 (45.1)	854 (54.9)	99 (52.4)	90 (47.6)	2 (66.7)	1 (33.3)	1.35 (1.01–1.81)	4.49×10^{-2}	
Ever	297 (47.3)	331 (52.7)	50 (75.8)	16 (24.2)	1 (100.0)	0 (0.0)	3.51 (1.96–6.28)	2.30×10^{-5}	

OR, odds ratio; CI, confidence interval;
[a]P for additive model adjusted for age and sex in logistic regression model.

M6A modification of *SND1* down-regulation in colorectal cancer

Furthermore, we conducted siRNA-mediated knockdown of *SND1* expression in HCT116 and DLD-1 cells (Supplementary Figure 4, available at *Carcinogenesis* Online). We selected all three siRNAs, which presented promising knockdown efficiencies at mRNA level, for functional analysis. Using the colorimetric m6A quantification strategy, we found that m6A levels were increased when *SND1* expression was suppressed in colorectal cancer cell lines (Figure 3). Our findings suggest that *SND1* has an effect on altering m6A levels.

Discussion

In the present study, we performed the analysis for associations between SNPs in m6A modification genes and colorectal cancer risk in two case–control studies. We identified and validated *SND1* rs118049207, which was significantly associated with colorectal cancer risk in Chinese populations. m6A modifications have gradually been found to play important roles in tumourigenesis and cancer development. However, no previous studies have explored the association between genetic variants in m6A modification genes and the risk of colorectal cancer. In this study, we identified *SND1* rs118049207 as a potential susceptibility locus for colorectal cancer. SNP rs118049207 also showed a potential regulatory role on *SND1*. *SND1* mRNA expression was markedly increased in colorectal tumour tissues compared with that in adjacent normal tissues obtained from public and in-house databases. Furthermore, our findings suggest that *SND1* performs an inhibitory function by altering m6A levels.

Colorectal cancer is affected by environmental factors. Clinical and preclinical studies have led to the discovery of gender-associated differences in the development of colorectal cancer (48). Some studies have demonstrated that female colorectal cancer patients had significantly better survival outcomes than male patients (49). However, these results were inconsistent. In this study, we found that males with the G allele of rs118049207 were more likely to be susceptible to colorectal cancer than females with the A allele. Despite the gender-specific differences associated with cancer risk involving different factors, the data needed to produce specific sex summary estimates are limited. Drinking has been identified as a causative factor for colorectal cancer in a considerable number of studies (50–55). In this study, the association of rs118049207 in *SND1* was lifestyle-dependent, with more pronounced risk enhancements being observed in males and drinkers. Most interestingly, the G allele

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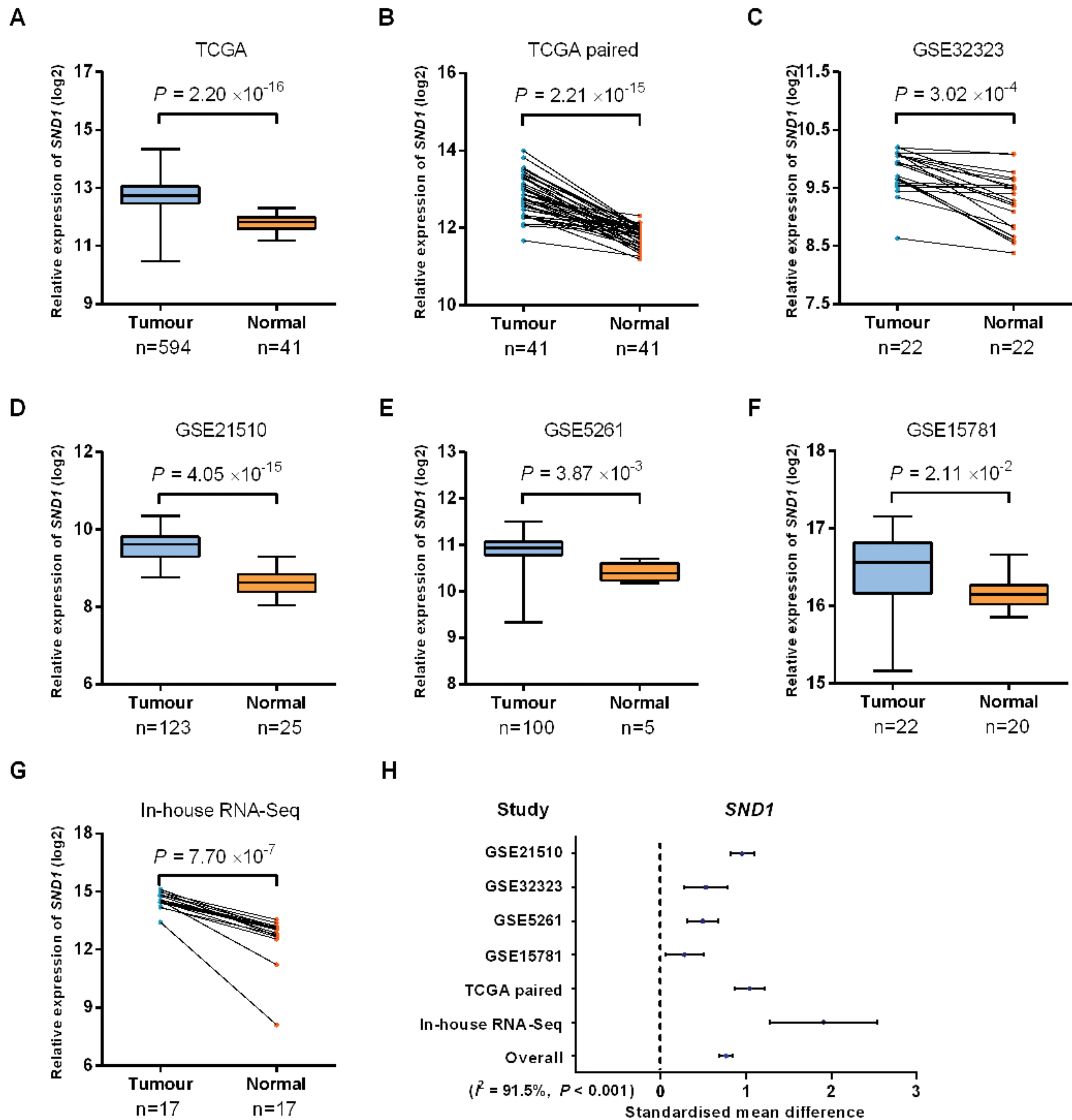

Figure 2. mRNA expression levels of *SND1* in cancer and normal adjacent tissues. The mRNA expression of *SND1* in the TCGA database (A-B), GSE32322 (C), GSE21510 (D), GSE5261 (E), GSE15781 (F) and in-house RNA-Seq database (G-H) are shown in a forest plot of the meta-analyses of the associations between *SND1* and colorectal cancer risk in the six studies. The horizontal axis depicts the SMD and 95% CI. A total of 676 samples were downloaded from the TCGA database. However, no mRNA expression of *SND1* was found in the 31 tumour tissues and 10 normal colorectal tissues.

of rs118049207 remained significant in the final combined analysis including the drinker subgroup. We also provided evidence that rs118049207 and drinking status were the strongest two risk factors and may advance the aetiology of colorectal cancer. Interactive effects on the risk of the G allele were observed between individuals with *SND1* rs118049207 and drinking.

SND1 has been suggested to act as a novel gene transcription activator in breast cancer, prostate cancer and hepatic carcinoma (56–58). Thus far, the mechanisms underlying SNPs in intronic regions and their relationships with disease development have not been elucidated. Functional annotations have predicted that *SND1* rs118049207 can recognize the motif domains of *DMRT3*, *DMRT4* and *DMRT7*. Although the rs118049207 A allele exhibited greater enhancer activity than the *SND1* promoter, the implications of this finding are not clear. Interestingly, the rs118049207 A allele was regulated by the *DMRT3* transcription factor and abrogated the enhancer activity in luciferase assays, an effect mimicked by transfection of the *DMRT3* overexpression vector. Therefore, the contribution of rs118049207 to the development of colorectal cancer may result from the preferential binding

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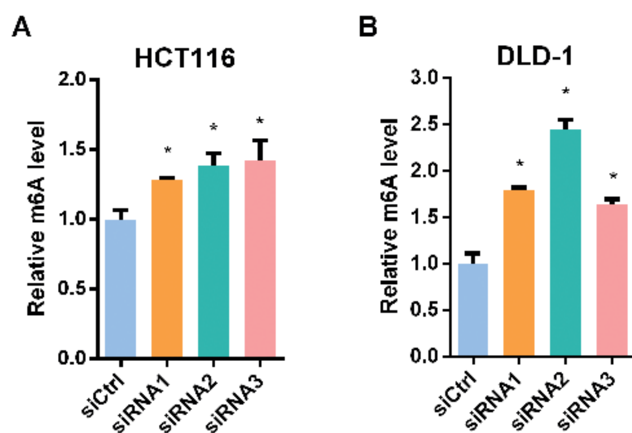

Figure 3. M⁶A modification of *SND1* in colorectal cancer. Colorectal cancer cells were transfected with *SND1* knockdown siRNA (with siCtrl as the control). The m⁶A content in total RNA from *SND1* knockdown (A) HCT116 and (B) DLD1 cells is shown. *t*-test, mean ± SD, n = 3, *P < 0.05.

ability of the rs118049207 A allele to the *DMRT3* transcription factor-binding site. However, the lack of reliable functional data and eQTLs for this SNP can be explained by the observation that the entire *SND1* region is under a very complex gene regulation. Previous studies emphasize the importance interpreting the results of dual-luciferase reporter assays with caution (59). The most recent research reveals a SNP-mediated promoter-enhancer conversion mechanism (60). Notably, although the non-risk allele favours enhancer activity, both alleles are bifunctional and regulated, suggesting that other factors such as the local chromatin structure and abundance/occupancy of transcription factors may also play a role in the modulation of bifunctionality and should be considered in future studies.

SND1 is a multifunctional protein that regulates transcription, mRNA splicing, RNA editing, and miRNA-mediated mRNA degradation as a nuclease in the RNA-induced silencing complex (35,61–63). *SND1* is upregulated in numerous human cancers, such as hepatocellular carcinoma, breast cancer and prostate cancer, and has been assigned multiple functional roles as an oncogene (56,64,65). Previous research demonstrates that *SND1* is both upregulated in human colon cancers and implicated in early-stage colon carcinogenesis (66). Furthermore, *SND1* mRNA is expressed at higher levels in late tumour development stages than in early-onset colorectal cancer, consistent with our results obtained herein (67). We observed that the *SND1* mRNA expression levels were significantly increased in tumour tissues, compared with those in normal tissues harvested from public and in-house databases. These results implied that *SND1* may act as a tumour enhancer in colorectal cancer.

During the past a few years, m⁶A modification in mRNAs or non-coding RNAs has been reported to play a critical role in virtually all major normal bioprocesses including self-renewal and differentiation of embryonic stem cells, primary microRNA processing, and RNA-protein interactions (10,68). Several articles explained the distinct roles of *SND1* in transcription and splicing (69). Previous research demonstrates that *SND1* binds methylated ligands (70,71). In addition, the capacity of *SND1* to interact with proteins of the spliceosome can be altered by methyltransferase inhibitors (72). In this study, we evaluated the relationship between the change in *SND1* mRNA expression levels and m⁶A modification capacity using functional prediction and a colorimetric m⁶A quantification strategy. Using knockdown experiments, we found that *SND1* preferentially binds to m⁶A-containing RNA in colorectal cancer cells. Strong correlations between *SND1* and the writer enzyme (*METTL3*) confirmed the reliability of our experiments. Considering that *SND1* could specifically recognized and degraded both edited pri-miRNAs and edited pre-miRNAs, inhibition of *SND1* can hinder the editing process, leading to enrichment of m⁶A, such an alternative reading process. To our knowledge, this is the first study to examine the association between genetic variants in m⁶A modification genes and colorectal cancer risk.

There are some limitations in the present study. First, the two available datasets we used were from Chinese populations. Therefore, our findings may not be generalized to the general population. Despite the fact that we used the FDR to control false-positive results and provided *in silico* and *in vitro* functional evidence, multistage designs are required to further replicate our findings. Next, *SND1* rs118049207 will require additional molecular biology experiments to fully elucidate the function and mechanism. The lack of reliable functional data and eQTLs for this SNP can be explained by the observation that rs118049207 does not influence the *SND1* expression level. The entire *SND1* region is under a complex gene regulation; thus, the leading SNPs and/or other SNPs in strong LD with rs118049207 may multiplicatively affect *SND1* expression. Further biochemical studies and functional experiments are required to validate our results. The results of different luciferase reporter assays suggested that other factors such as the local chromatin structure and abundance/occupancy of transcription factors may also be involved; thus, additional molecular biology experiments are needed to further confirm our hypothesis. Third, there is currently insufficient evidence to suggest that the identified SNPs can affect the m⁶A modification. The extra identification of genetic variants that target m⁶A modification sites is costly and time-consuming. Finally, due to the relatively limited clinicopathological factors and life factors analysed, information on the nutritional status and additional details were not applicable in this study.

In summary, this study investigated the associations between genetic variants in m⁶A modification genes and colorectal cancer risk. Using gene expression analyses, we report for the first time that rs118049207 in *SND1* may be involved in the genetic mechanisms underlying colorectal carcinogenesis. Our results also suggest that the rs118049207-centred region regulated by *DMRT3* is involved in *SND1* regulation. The change in *SND1* leads to alteration of m⁶A in colorectal cancer. This study clarified the vital role of genetic variants and genes in m⁶A methylation modification in cancer tissues and provided new ideas for the prevention of colorectal cancer.

Supplementary material

Supplementary data are available at *Carcinogenesis* online.

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Beijing population. The authors would also like to acknowledge Shuai Ben and Qiuyuan Zhu (Nanjing Medical University) for their assistance in experiments.

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