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KRT15, INHBA, MATN3, and AGT are aberrantly methylated and differentially expressed in gastric cancer and associated with prognosis

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ABSTRACT

Aim: The present study aims to identify aberrantly methylated and differentially expressed genes (DEGs) in gastric cancer (GC) and explore their potential role in the carcinogenesis and development of GC.

Methods: The original RNA-Seq, clinical information and Illumina Human Methylation 27 Chip data associated with GC were downloaded from The Cancer Genome Atlas (TCGA) database using the gdc-client tool. The DEGs and aberrantly methylated genes (AMGs) were screened with *edgeR* and *limma* package in R, respectively. The cut-off criteria for DEG identification were $P < 0.05$ and fold change (FC) > 2.0, and for AMG identification were $P < 0.05$ and $|t| > 2.0$. Genes which were both DEGs and AMGs were considered to be regulated by aberrant DNA methylation in GC. The common genes were used for further functional enrichment analysis in the categories of cellular component, molecular function, biological process and biological pathway.

Results: In total 465 genes including 336 down-regulated genes with hyper-methylation (DGs-Hyper) and 129 up-regulated genes with hypo-methylation (UGs-Hypo) were identified. Cellular component analysis showed that these genes were mainly expressed in the cytoplasm and plasma membrane. Molecular function and biological process analysis indicated that the genes primarily participate in cell communication, signal transduction, cell growth/maintenance and function as transcription factors, receptor, cell adhesion molecules, and transmembrane receptor protein tyrosine kinases. Biological pathway analysis revealed that the genes are involved in some crucial pathways including epithelial-to-mesenchymal transition, IL3-mediated signaling, mTOR signaling, VEGF/VEGFR and c-Met signaling. KRT15, INHBA, MATN3, and AGT are significantly associated with the prognosis of GC patients.

Conclusion: Our study identified several DEGs regulated by aberrant DNA methylation in GC. The mechanism of DNA methylation in the carcinogenesis and development of GC could be further explored in these genes, especially KRT15, INHBA, MATN3, and AGT.

1. Introduction

Gastric cancer (GC) is a malignant gastrointestinal tumor which has a high morbidity and mortality [1]. Most patients are in an advanced stage of GC when diagnosed and have a poor prognosis with a median overall survival of less than 1 year [2]. GC is known to be caused by various risk factors, including those associated with genetics, epigenetics, *Helicobacter pylori* infection and diet. Understanding the molecular mechanisms and pathways of GC could contribute to the diagnosis, treatment and prognostic management of GC patients.

Epigenetics is considered a mitotically or meiotically heritable change in gene expression that is not accompanied by changes in the DNA sequence [3]. The major epigenetic regulation of gene expression includes DNA methylation and histone modifications [4]. DNA methylation, a reversible chemical alteration in DNA sequence, frequently happens at CpG islands (DNA length > 200 bp and guanine/cytosine content > 0.5) and has been reported to be associated with the silencing of tumor suppressor genes and activation of oncogenes [5–7]. Nakamura et al. reported that aberrantly methylated genes (AMGs) could act as biomarkers for early detection and tumor prognosis assessment [8]. Therefore, identifying key genes mediated by aberrant methylation will facilitate the further investigation of GC development and progression.

In the present study, we downloaded, integrated and analyzed RNA-Seq, clinical information, and Illumina Human Methylation 27 Chip data associated with GC from The Cancer Genome Atlas (TCGA)

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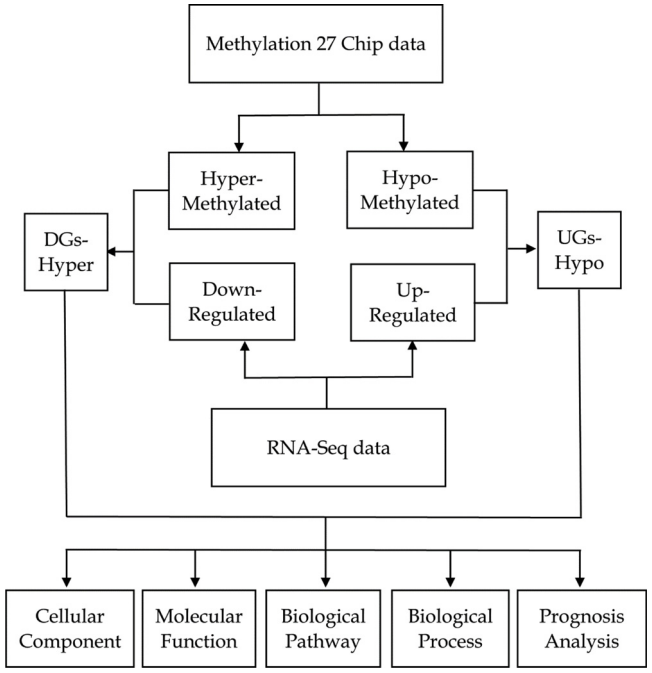

Fig. 1. The flowchart of data process. DGs-Hyper: down-regulated genes with hyper-methylation; UGs-Hypo: up-regulated genes with hypo-methylation.

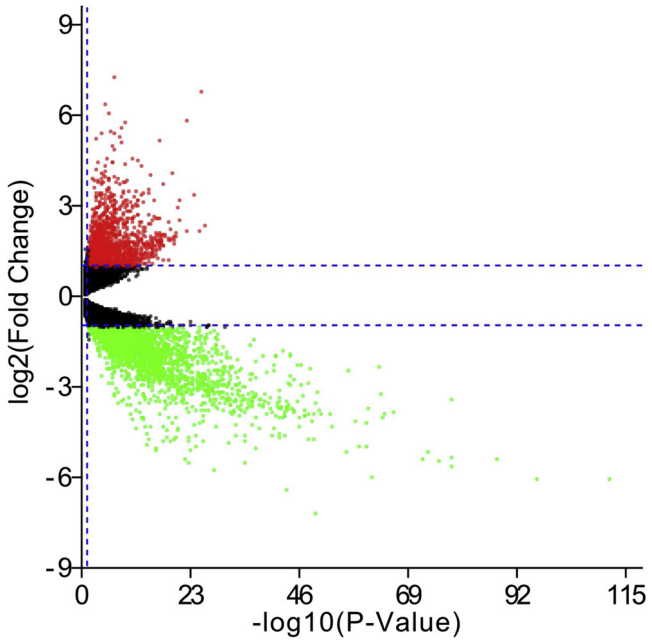

Fig. 2. The volcano plot of RNA-Seq genes. The difference in expression of all genes between 375 GC tissues and 32 normal tissues as presented in coordinate graphs. The cut-off criteria are P < 0.05 and fold change > 2.0. The red and green spots represent up-regulated and down-regulated genes, respectively. GC: Gastric cancer.

database. The results indicated that KRT15 was down-regulated and hyper-methylated in GC tissues, however, INHBA, MATN3, and AGT were up-regulated and hypo-methylated. Interestingly, the expression of these four genes is associated with the prognosis of GC patients.

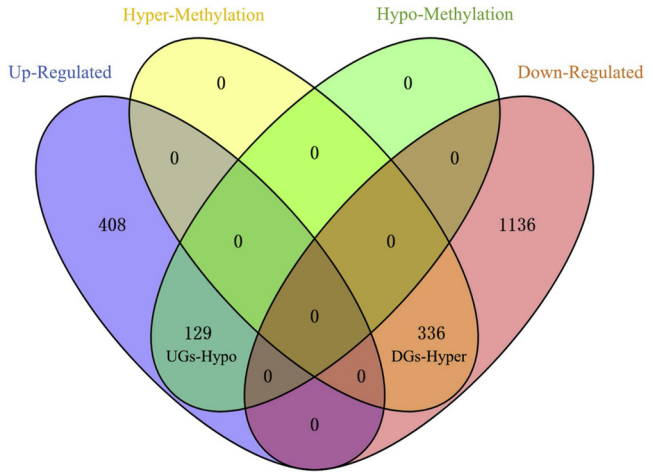

Fig. 3. The venn plot of DEGs and AMGs. UGs-Hypo are the common genes (129) of both Up-Regulated and Hypo-Methylation; DGs-Hyper are the common genes (336) of both Down-Regulated and Hyper-Methylation. DEGs: Differentially expressed genes; AMGs: Aberrantly methylated genes; DGs-Hyper: down-regulated genes with hyper-methylation; UGs-Hypo: up-regulated genes with hypo-methylation.

2. Materials and methods

2.1. DEG and AMG identification

RNA-Seq, clinical information, and Illumina Human Methylation 27 Chip data associated with GC were downloaded from TCGA database with the gdc-client tool. RNA-Seq expression data were used for differentially expressed gene (DEG) identification and the data processing criteria were as follow: (1) protein-coding genes were reserved expect for microRNAs, long non-coding RNAs, and similar; (2) genes were more than a half the samples' expression value was zero were eliminated; (3) the cut-off criterion were $P < 0.05$ and $|\log2FC| > 1.0$; (4) the edgeR package of R software was applied for processing. Illumina Human Methylation 27 Chip data were utilized for AMGs screening. The limma package was used for data processing and the cut-off criterion was $|t| > 2.0$. Then, the common down-regulated and hyper-methylated genes were defined as down-regulated genes with hyper-methylation (DGs-Hyper). Similarly, the common up-regulated and hypo-methylated genes were defined as up-regulated genes with hypo-methylation (UGs-Hypo).

2.2. Functional enrichment analyses of DEGs regulated by aberrant methylation

FunRich is a useful tool for the functional enrichment and interaction network analysis of gene or proteins [9]. In order to seek the detailed molecular function of these DEGs, we performed gene enrichment analyses in the modules of cellular component, molecular function, biological process and biological pathways. P values converted by $-\log10$ and the percentage of genes in each term were calculated. A value of $P < 0.05$ was considered statistically significant.

2.3. Association of DEGs and GC patients' prognosis

GC samples that had both tissue RNA-Seq data and follow-up associated data were selected for prognosis analysis. Overall, 354 samples were divided into two groups according to the median expression value of DEGs.

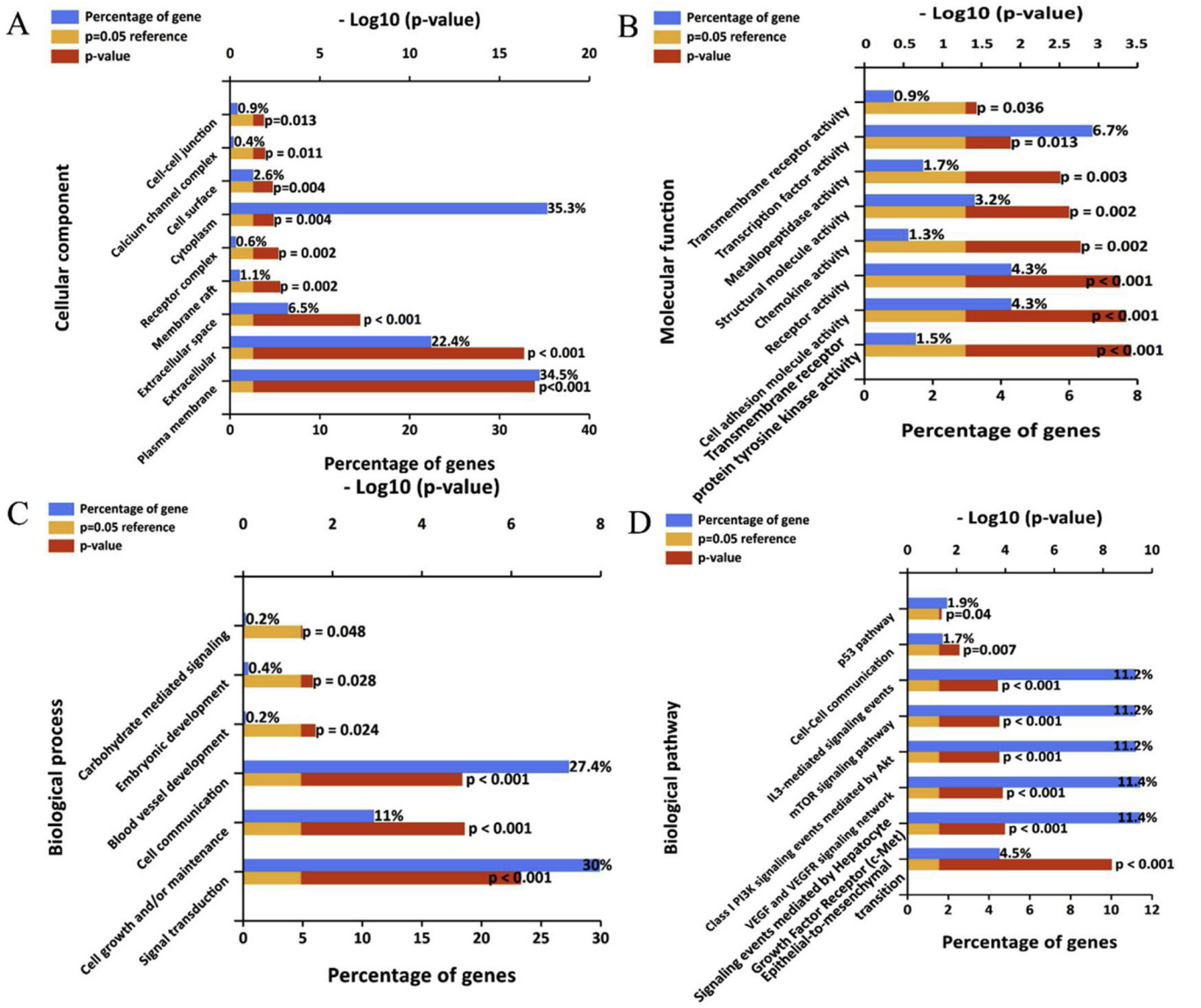

Fig. 4. Gene functional enrichment analysis. Up-regulated genes with hypo-methylation (UGs-Hypo) and down-regulated genes with hyper-methylation (DGs-Hyper) were selected for functional enrichment analysis with *FunRich* software. A: Cellular component analysis showed that these genes were mainly expressed in cytoplasm and plasma membrane. B: Molecular function analysis indicated that the genes primarily participate in the activity of transcription factor, receptor, cell adhesion molecule, and transmembrane receptor protein tyrosine kinase. C: Biological process analysis indicated that the genes primarily participate in cell communication, signal transduction, and cell growth/maintenance. D: Biological pathway analysis revealed that the genes are involved in some crucial pathways including epithelial-to-mesenchymal transition (EMT), IL3-mediated signaling, mTOR signaling, VEGF/VEGFR and c-Met signaling.

2.4. Statistical analysis

RNA-Seq and Illumina Human Methylation 27 Chip data were processed by the edgeR [10]and limma [11] packages in R software, respectively. The difference of gene methylation level between GC and adjacent normal tissues was analyzed by an unpaired *t*-test Kaplan–Meier and the Log-rank test were used to perform survival analysis. In all the statistical analysis, $P < 0.05$ was considered to be significantly different.

3. Results

3.1. Identification of DEGs and AMGs in GC

Fig. 1 displays a flow chart of our study. The RNA-Seq data contains 375 GC tissue samples and 32 normal tissue samples. In total, 2009 DEGs were identified according to the threshold of $P < 0.05$ and $|\log2FC| > 1.0$. The difference of whole genes in RNA-Seq was displayed in a volcano plot (Fig. 2). The methylation of 27 chip data contains 48 GC and 25 normal samples. Under the criterion of $|t| > 2.0$, numerous cg positions that showed a significant difference in methylation level were identified. According to the annotation of the chip, AMGs were obtained. Then, a Venn plot was generated to achieve the common genes between DEGs and AMGs. As a result, 336 DGs-Hyper and 129 UGs-Hypo were identified (Fig. 3).

3.2. Functional enrichment analyses of DGs-Hyper and UGs-Hypo

In order to further explore the function of DGs-Hyper and UGs-Hypo, the *FunRich* tool was used for gene functional enrichment analyses in the modules of cellular component, molecular function, biological process and biological pathways. Cellular component analysis showed that these genes were mainly expressed in cytoplasm and plasma membrane. Molecular function and biological process analysis

Table 1
Clinical characters of GC patients.

Variables	Case; n (%)
Age at diagnosis (yr)	
< 60	112 (29.9)
≥60	259 (69.1)
NA*	4 (1.0)
Gender	
Male	241 (64.3)
Female	134 (35.7)
T stage	
T1 + T2	99 (26.4)
T3 + T4 + Tx	276 (73.6)
Histologic grade	
g1 + g2	147 (39.2)
g3 + g4	228 (60.8)
Race	
White	238 (63.5)
Asian	74 (19.7)
Black or African American	11 (2.9)
NA*	52 (13.9)
Pathologic stage	
Stage I	53 (14.1)
Stage II	111 (29.6)
Stage III	150 (40.0)
Stage IV	38 (10.1)
NA*	23 (6.2)
Node status	
N0	111 (29.6)
N1-3	246 (65.6)
NA*	18 (4.8)
Metastasis	
M0	330 (88.0)
M1	25 (6.7)
Mx	20 (5.3)

*GC: Gastric cancer; NA: Not available.

indicated that the genes primarily participate in cell communication, signal transduction, cell growth/maintenance and function as transcription factors, receptors, cell adhesion molecules and transmembrane receptor protein tyrosine kinases. Biological pathway analysis revealed that the genes are involved in crucial pathways including epithelial-to-mesenchymal transition (EMT), IL3-mediated signaling, mTOR signaling, VEGF/VEGFR and c-Met signaling pathway. The results indicate that DGs-Hyper and UGs-Hypo may provide a vital role in investigating the DNA methylation regulation mechanism of GC (Fig. 4).

3.3. Identification of prognosis-associated DGs-Hyper and UGs-Hypo

To find prognosis-associated DGs-Hyper and UGs-Hypo, the clinical information of 375 GC patients was downloaded and used for survival analyses. The detailed clinical characteristics are enumerated in Table 1. The genes were ranked according to the values of fold changes, thus, the 100 top DGs-Hyper and UGs-Hypo were selected for survival analyses (Table 2). Finally, we found that the expression of KRT15 (DGs-Hyper) and INHBA, MATN3, AGT (UGs-Hypo) were significantly associated with the overall survival of GC patients (Fig. 5). The association analysis between the expression of four genes and clinical features was also performed. After excluding the samples which lack of detailed information, such as gender, age at diagnosis, TNM stage and so on, a total of 337 samples were included for analysis. The results indicated that KRT15, INHBA and MATN3 expression were significantly associated with T stage, M stage and histologic grade (Table 3). However, the association between AGT expression and clinical features has no difference in statistics. Moreover, differences in the methylation level of the four genes were also observed (Fig. 6). The two cg positions of each gene were all differentiated significantly between 48 GC and 25 normal samples except for cg 22647018.

Table 2
The top 100 DGs-Hyper and UGs-Hypo in GC.

Genes	DGs-Hyper*		Genes	UGs-Hypo*	
	logFC*	*P* value		logFC*	*P* value
APOA4	−6.40	5.27E-44	APOA2	7.28	1.74E-07
MAL	−5.00	2.39E-37	CST1	6.83	1.03E-25
ENDOU	−4.59	5.09E-33	MAGEA4	5.48	9.16E-07
KRT13	−4.35	5.99E-12	HOXC10	5.21	3.60E-17
CAPN14	−4.18	1.25E-19	CSAG1	5.12	6.20E-09
NCCRP1	−4.13	3.80E-26	LEFTY1	4.47	5.08E-07
DPT	−3.88	1.21E-64	PRAME	4.40	9.41E-10
BNIPL	−3.86	8.81E-40	NOX1	3.81	3.04E-09
ENPP3	−3.86	1.23E-49	NPSR1	3.81	7.75E-08
FAM19A4	−3.83	9.66E-33	CST4	3.78	1.16E-08
CWH43	−3.75	1.18E-26	ESM1	3.74	1.00E-17
C2orf40	−3.74	2.87E-30	CEMIP	3.60	1.29E-14
SCNN1B	−3.63	8.36E-29	HOXA11	3.50	5.75E-12
CALML3	−3.60	5.36E-10	STRA6	3.43	1.53E-09
KRT15	−3.58	1.19E-25	MMP7	3.36	3.75E-09
PGA5	−3.50	2.40E-08	INHBA	3.22	2.31E-21
PKP1	−3.50	8.73E-15	CST2	3.15	1.07E-10
RAET1E	−3.41	4.54E-31	MSLN	3.11	5.15E-10
SLC5A5	−3.41	7.19E-20	CXCL9	3.08	3.57E-12
CERS3	−3.39	6.21E-14	MMP10	3.00	5.31E-09
DSG1	−3.33	4.93E-14	HMGA2	2.91	6.63E-08
PAX9	−3.25	1.58E-27	ZIC2	2.91	1.75E-07
HPSE2	−3.17	7.21E-27	BAMBI	2.74	2.45E-09
MAMDC2	−3.09	2.53E-33	TREM2	2.65	7.88E-16
PRIMA1	−3.08	9.09E-28	CXCL1	2.63	2.48E-11
CIDEA	−3.07	1.58E-21	GDF15	2.63	3.37E-16
SLC6A4	−3.06	4.75E-28	LGR5	2.61	1.84E-08
ZNF365	−2.95	1.16E-21	ETV4	2.60	1.63E-16
PNCK	−2.95	1.92E-22	ASCL2	2.55	2.88E-08
CARTPT	−2.94	1.04E-11	LAMC2	2.43	8.74E-15
PYGM	−2.92	1.67E-30	MAPK15	2.39	1.44E-09
KCNA5	−2.90	1.42E-30	FOXS1	2.37	6.45E-13
RDH12	−2.90	1.66E-25	SDS	2.33	1.49E-13
FBP2	−2.88	8.49E-29	MMP12	2.33	2.03E-07
SOSTDC1	−2.86	1.94E-19	MATN3	2.32	4.64E-08

*DGs-Hyper: down-regulated genes with hyper-methylation; UGs-Hypo: up-regulated genes with hypo-methylation; FC: fold change.

4. Discussion

In recent decades, increasing evidence has proved that epigenetic modifications play an important role in the process of human disease, including GC [12]. Aberrant DNA methylation, a frequent event of epigenetic modifications, could regulate the expression of tumor suppressor genes and oncogenes [13,14]. Thus, understanding the mechanism of DNA methylation and identifying DEGs regulated by aberrant DNA methylation could contribute to the diagnosis, treatment and prognosis management of GC patients.

In the present study, we identified numerous DEGs regulated by aberrant DNA methylation in GC. For a further comprehension of these key genes, we performed gene functional enrichment analyses. The results showed that these genes were mainly expressed in the cytoplasm and plasma membrane and participated in the activities of cell adhesion molecules and transcription factors. Furthermore, they were involved in several vital tumor-associated pathways, such as EMT, IL3-mediated signaling, mTOR signaling, VEGF/VEGFR and c-Met signaling pathway. EMT, a process whereby epithelial cells are converted into mesenchymal cells, is well known in the carcinogenesis and progression of GC [15,16]. Moreover, mounting evidence has indicated that mTOR [17,18] and VEGF signaling [19,20] activation would promote invasion and metastasis of GC. This evidence supports our finding that these genes may have a vital role in the molecular mechanisms of GC.

In addition, we found that KRT15, INHBA, MATN3, and AGT were differentially expressed and aberrantly methylated in GC. Furthermore, the expression of KRT15 was positively associated with the overall survival time of GC patients whereas INHBA, MATN3, and AGT were

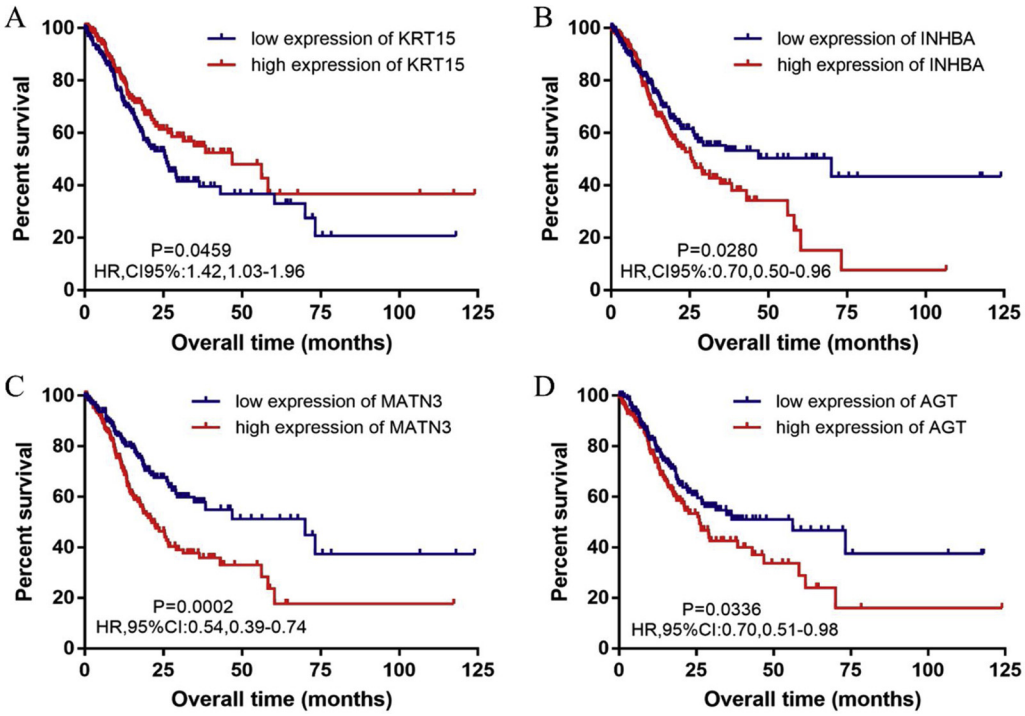

Fig. 5. The genes expression were associated with overall survival of GC patients. A: The expression of KRT15 is positively associated with overall survival time of GC patients. B: The expression of INHBA is negatively associated with overall survival time of GC patients. C: The expression of MATN3 is negatively associated with overall survival time of GC patients; D: The expression of AGT is negatively associated with overall survival time of GC patients. GC: Gastric cancer; KRT15: Keratin 15; INHBA: Inhibin subunit beta A; MATN3: Matrilin 3; AGT: Angiotensinogen.

Table 3
Association between the genes and clinical features.

Variable	KRT15 expression		*P*-value	INHBA expression		*P*-value	MATN3 expression		*P*-value	AGT expression		*P*-value
	Low	High		Low	High		Low	High		Low	High	
Age at diagnosis (yr)												
< 60	58	46	0.147	51	53	0.842	47	57	0.253	42	62	0.02[a]
≥60	110	123		117	116		121	112		126	107	
gender												
male	99	117	0.049[a]	109	107	0.764	105	111	0.543	100	116	0.081
female	69	52		59	62		63	58		68	53	
T stage												
T1	6	10	0.624	14	2	0.019[a]	13	3	0.029[a]	9	7	0.993
T2	37	32		35	34		39	30		35	34	
T3	77	83		77	83		74	86		80	80	
T4	48	44		42	50		42	50		44	48	
N stage												
N0	47	56	0.58	47	56	0.59	55	48	0.274	56	47	0.571
N1	43	47		48	42		49	41		40	50	
N2	38	32		38	32		34	36		36	34	
N3 + Nx	40	34		35	39		30	44		36	38	
M stage												
M0	156	144	0.025[a]	150	150	0.877	152	148	0.394	146	154	0.215
M1	12	25		18	19		16	21		22	15	
Histologic grade												
G1	3	5	0.037[a]	5	3	0.139	5	3	0.801	5	3	0.288
G2	47	71		65	53		59	59		66	52	
G3	113	90		92	111		101	102		94	109	
G4	5	3		6	2		3	5		3	5	
Pathologic stage												
I	20	27	0.354	29	18	0.195	32	15	0.049[a]	27	20	0.557
II	54	55		47	62		48	61		52	57	
III	80	67		75	72		71	76		70	77	
IV	14	20		17	17		17	17		19	15	

[a] $P < 0.05$, statistically significant.

negatively associated with the overall survival time. Studies have reported that INHBA is up-regulated in GC and associated with poor survival [21–23]. Moreover, the expression of INHBA is up-regulated after treatment with 5-aza-2′ deoxycytidine in lung adenocarcinoma, which indicates that INHBA may be regulated by DNA methylation in lung adenocarcinoma [24]. Zhou et al. found a six-gene signature, which included MATN3, could be used for predicting the recurrence of patients in stage III and IV after curative surgery plus chemoradiotherapy [25]. Research demonstrated that IL-6 treatment induced the increased expression of AGT by reducing DNA methylation activity in human adrenocortical cells [26]. However, whether these four genes are regulated by aberrant DNA methylation in GC has not yet been

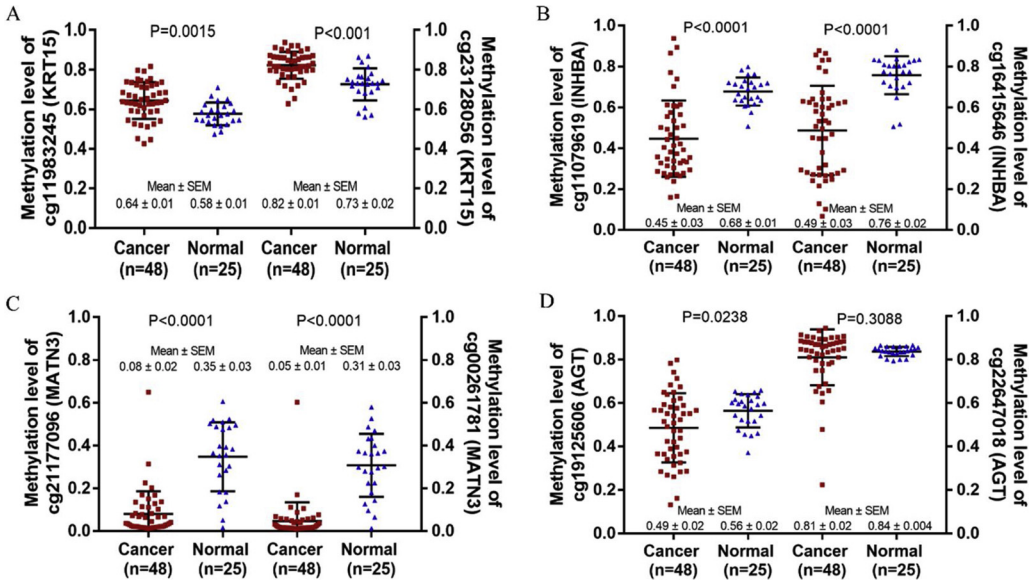

Fig. 6. The methylation level of genes cg positions. A: The methylation level of KRT15 cg positions in GC (n = 48) and normal tissues (n = 25); B: The methylation level of INHBA cg positions in GC (n = 48) and normal tissues (n = 25); C: The methylation level of MATN3 cg positions in GC (n = 48) and normal tissues (n = 25); D: The methylation level of AGT cg positions in GC (n = 48) and normal tissues (n = 25). GC: Gastric cancer; KRT15: Keratin 15; INHBA: Inhibin subunit beta A; MATN3: Matrilin 3; AGT: Angiotensinogen.

reported. Thus, the four genes should receive more attention in studying the DNA methylation mechanism of GC, which will be our aim in a future study.

Gene methylation could be used as a marker for the detection and diagnosis of GC. E-cadherin and p16 in the serum of GC patients were found to be hyper-methylated at the promoter region and may be a new serum biomarker for GC [27]. Therefore, our findings have indicated that KRT15, INHBA, MATN3, and AGT are likely to be useful gene methylation markers for the detection and diagnosis of GC. However, further clinical trials are needed to prove the reliability of these markers.

Furthermore, there are also some limitations in present study. In TCGA database, it is not all GC patients have RNA-Seq data of normal tissues. So the scale of cancer and normal tissues used for RNA-Seq analysis is not perfect enough. Besides, Illumina Human Methylation 27 Chip, which only cover 27 thousand cg locus in human genome, is an earlier technique for methylation detection. In recent years, more advanced methylation chips have been applied in scientific research, such as Illumina Human Methylation 850 Chip and Whole-genome bisulfite sequencing. Nevertheless, we will focus on the update of TCGA database and improve the present study in future.

In conclusion, we identified that KRT15, INHBA, MATN3, and AGT were DEGs and AMGs. Moreover, the four genes were all associated with the prognosis of GC patients. The results indicate that the four genes may play a crucial role in the DNA methylation mechanism of GC, and could be a promising signature in predicting the prognosis of GC patients.

Author contributions

Dong-Qiu Dai and Cheng Zhang conceived of this study; Cheng Zhang, Yu Liang, Kun-Zhe Wu and Ming-Hui Ma processed the data analysis; Cheng Zhang wrote the manuscript.

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Conflict-of-interest statement

The authors declare that there is no conflict of interest related to this study.

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