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Functional variant of *MTOR* rs2536 and survival of Chinese gastric cancer patients

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We previously reported that some single nucleotide polymorphisms (SNPs) of candidate genes involved in the MTOR complex1 (MTORC1) were associated with risk of gastric cancer (GCa). In the present study, we further evaluated associations of eight potentially functional SNPs of *MTOR*, *MLST8* and *RPTOR* with survival of 1002 GCa patients and also investigated molecular mechanisms underlying such associations. Specifically, we found that the *MTOR* rs2536 C allele at the microRNA binding site was independently associated with a 26% reduction of death risk (HR = 0.74, 95% CI = 0.57–0.96, $p = 0.022$). The results remained noteworthy with a prior false positive probability of 0.1. Genotype–phenotype correlation analysis in 144 patients' adjacent normal gastric tissue samples revealed that the *MTOR* expression levels were lower in rs2536 TC/CC carriers than that in wild-type TT carriers ($p = 0.043$). Dual luciferase assays revealed that the rs2536 C allele had a higher binding affinity to microRNA-150, leading to a decreased transcriptional activity of *MTOR*, compared to the rs2536 T allele. Further functional analysis revealed that *MTOR* knockdown by small interference RNA impaired proliferation, migration, and invasion ability in GCa cell lines. In conclusion, The *MTOR* rs2536 T › C change may be a biomarker for survival of Chinese GCa patients, likely by modulating microRNA-induced gene expression silencing. Additional studies are needed to validate our findings.

Key words: gastric cancer, gene expression, genetic variants, MTORC1, survival
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Introduction

The incidence of gastric cancer (GCa) has been declining over time worldwide,[1] because of life-style change and the decreased prevalence of *Helicobacter pylori* infection.[2,3] However, GCa remains the second most common and also the second leading cause of cancer-related deaths in China, only next to lung cancer.[4] Because GCa is characterized by a long latency at onset but a rapid progression thereafter, more effective treatment strategies are needed to improve the currently poor 5-year survival. For example, to improve the prognosis of GCa patients, the D2 radical surgery has become a routine in China.[5] Besides, other novel anticancer therapies including chemotherapy,[6] radiotherapy,[7] molecular-targeted therapy[8] and their combination[7,9,10] have also improved the prognosis of the patients.[11] Additionally, lymph node metastasis, advanced pathological T stage, vascular and nerve infiltration, which are known to be correlated with a high recurrence risk and poor prognosis, have been used as clinical indicators for adjuvant chemotherapy. Furthermore, four molecular-based phenotypes of GCa have been identified by The Cancer Genome Atlas (TCGA) project, which are associated with prognosis and chemotherapy responses.[12]

It is known that MTOR, a key component of the PIK3-Akt-MTOR pathway, is over activated in GCa,[13] especially in the

What's new?

While MTORC1 is known to play an important role in the development of gastric cancer (GCa), its role in GCa progression remains unclear. This study of 1002 Chinese GCa patients demonstrated for the first time that the *MTOR* rs2536 T › C change predicted better overall survival. Functional studies showed that the 3′UTR of *MTOR* with rs2536 C had a better binding affinity to miRNA-150, leading to down-regulated *MTOR* expression and a less aggressive phenotype of GCa cells. The *MTOR* rs2536 T › C change may thus be a biomarker for survival of Chinese GCa patients, likely acting by modulating microRNA-induced gene expression silencing.

diffuse subtype.[14] In cells, there are two MTOR complexes: MTOR complex1 (MTORC1) and complex2 (MTORC2).[15] The MTORC1 is mainly formed by MTOR, RPTOR and MLST8,[15] playing an important role in autophagy,[16] in response to rapamycin and growth factor signals by protein–protein interaction.[15] For example, phosphorylation of MTOR has been reported as an indicator for poor prognosis of GCa patients,[14,17] because the activation of MTOR in the form of MTORC1 further phosphorylates two down-stream effectors, 4E-binding protein 1 (4EBP1) and S6 kinase 1 (S6K1), leading to an increased translation of spliced mRNA and protein synthesis needed by cell growth.[18] Moreover, MTOR has been an interesting therapeutic target of metformin to inhibit gastric cancer stem cells proliferation.[19]

It is well known that GCa patients, who suffer from the same disease stage and pathological type, may have a different response to treatment and prognosis, likely because of genetic variants responsible for variable survival, in addition to the heterogeneity of tumor mutation spectrum. Single nucleotide polymorphisms (SNPs), as a major form of genetic variation, have been consistently shown to be associated with susceptibility to different types of cancer as well as therapeutic response and survival in cancer patients. For example, genetic variants in the *NFKB1* gene were recently demonstrated to be associated with clinical outcome of patients with locoregional gastric cancer.[20] We also reported that genetic variants involved in DNA repair pathways were associated with chemotherapy-related outcomes.[21] However, few studies have investigated whether genetic variants of MTORC1 genes are associated with survival of GCa patients, despite some reported associations between MTORC1 variants and risk of GCa[22] and esophageal squamous cell carcinoma.[23] In the present study, we further investigated associations between genetic variants of the MTORC1 genes and survival of GCa patients and explored mechanistic basis of the observed associations.

Material and Methods

Study population

The present study included 1,002 patients with histopathologically confirmed diagnosis of GCa and available clinical information, who were recruited between 2009 and 2011 in previously published molecular epidemiologic studies.[21,22] Patients' peripheral blood and surgical samples were obtained from tissue bank of Fudan University Shanghai Cancer Center (FUSCC). These blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes and stored at −80°C until use.[24,25] In this cohort, there were 144 patients who also had formalin-fixed paraffin-embedded (FFPE) gastric cancer tissues as well as the adjacent normal tissues available for further analysis. All patients had signed an informed consent for donating their blood samples or tissues (if available) to tissue bank of FUSCC for the purpose of scientific research. The present study was approved by the FUSCC Institutional Ethics Review Board.

In silicon selection of potentially functional SNPs and genotyping

We used SNPinfo (https://snpinfo.niehs.nih.gov/) database to select potentially functional SNPs and HaploReg (http://archive.broadinstitute.org/mammals/haploreg/haploreg.php) and Regulome DB (http://www.snps3d.org/) databases to further confirm the functional relevance of the SNPs used for genotyping. We initially selected SNPs involved in MTORC1, including *MTOR*, *RPTOR* and *MLST8*, based on our defined criteria: (*i*) SNPs predicted to influence the transcriptional factor binding ability or microRNA (miRNA) binding ability to the gene region (e.g., regions of promoter or 3′UTR); (*ii*) with a minor allele frequency (MAF) higher than 5%; (*iii*) not included in the published genome-wide association studies (GWASs). The selected SNPs were further evaluated for functional prediction using HaploReg (http://archive.broadinstitute.org/mammals/haploreg/haploreg.php) and Regulome DB (http://www.snps3d.org/) database. Finally, the potentially functional SNPs predicted by all the three databases were included for genotyping by the TaqMan ABI7900HT PCR system. To control for the quality of genotyping results, two positive and four negative control wells were set up in each 384 plate. Moreover, 5% of the samples were repeatedly genotyped, and the results were 100% in accordance, ensuring the high quality for the genotyped data for analysis.

RNA extraction and real-time quantitative PCR analysis

From among the 1,002 patients, the total RNA from the paired GCa and adjacent normal tissues of 144 patients was also extracted by Trizol Regent (Invitrogen, The United States) according to the recommended protocol. The total RNA was then reversely transcribed to cDNA by the PrimeScript RT Master Mix system (Takara Bio, Japan). The cDNA was then mixed with the SYBR Premix Ex Taq II (Takara Bio,

Japan) and the specified primer for the targeted coding sequence of *MTOR* (Supporting Information Table S1), and the mixture was finally subjected to the real-time quantitative PCR (qPCR) analysis. The CT value is the cycle threshold during the real-time qPCR process. The expression levels of *MTOR* were normalized to that of *GAPDH* by a formula of $2^{-\bigtriangledown CT}$, in which $\bigtriangledown CT = CT_{MTOR} - CT_{GAPDH}$. All experiments were performed with triplicate measurements.

Expression quantitative trait loci analysis

Expression quantitative trait loci (eQTL) analyses were performed using SNP data and the corresponding mRNA expression from GTEx database, hapmap3 databse and the 1,000 Genome Project to assess the trend of association between rs2536 genotype and *MTOR* expression. The results of e-QTL from GTEx database (https://www.gtexportal.org/home/) were visually and directly provided as a box plot stratified by multiple normal tissues at the website. Sponsored by Geuvadis RNA sequencing project of the 1,000 Genomes samples,[26] the mRNA expression data from the 1,000 Genomes Project was obtained from RNA-sequencing data of 465 lymphoblastoid cell lines from European populations from CEPH (CEU), Finns (FIN), British (GBR) and Toscani (TSI) (n = 373) and African population from Yoruba (YRI) (n = 92) (https://www.ebi.ac.uk/Tools/geuvadis-das/). In the present study, rs2536 genotyping and mRNA expression data were available for 445 individuals from the 1,000 Genomes Project, including 358 Europeans descendants and 87 Africans (YRI), which we used for e-QTL analysis. We further used Hapmap3 datasets for additional eQTL analysis, which contained Asian [Japanese in Tokyo, Japan (JPT), n = 81; Han Chinese in Beijing, China (CHB), n = 79], European [Utah residents with Northrn and Western European ancestry from the CEPH collection (CEU), n = 107] and African [Yoruba in Ibadan, Nigeria (YRI), n = 108; Maasai in Kinyawa, Kenya (MKK), n = 135; Luhya in Webuye, Kenya (LWK), n = 83] populations.

Cell lines and culture

Human embryonic kidney 293T cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) as described in a recently published study.[27] The 293T cells have been authenticated by the ATCC-based assays for morphology and karyotyping as well as the PCR analysis.[27] The GCa cell lines, AGS and HGC-27, were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China), which were also used in previously published reports.[28,29] These cell lines have also been authenticated by the short tandem repeat (STR) DNA profiling analysis.[28] Cells were cultured in the DMEM medium added with 10% FBS (Gibco, The United States) at 37°C, supplemented with 5% CO_2.

Plasmid construction and luciferase reporter assay

The 3′UTR region sequence with a 950-bp full length covering the rs2536 locus was obtained from Ensemble genome browser (http://www.ensembl.org/index.html). By using a relative primer (Supporting Information Table S1), we amplified the 3′UTR region containing the *MTOR* rs2536T (wild type) and C (minor allele) allele from the control carriers detected and described in our previously published study.[22] Finally, by using In-Fusion Cloning System (TransGen Biotech, China), we cloned the amplified region into the psi-CHECK2 vector that was located between *hRluc* and *hluc+* sides of the vector. We seeded human embryonic kidney 293T cells and GCa AGS cells into the 96-well plates, respectively, at a density of 5×10^3 per well. Having been cultured for 24 hr, the cells were transfected with the Lipofectamine 3000 (lifetech, The United States) reagent according to the protocol, in which the psi-CHECK2 rs2536 T or psi-CHECK2 rs2536 C vector along with or without the miRNA negative control and mimics (Biotend, China) were co-transfected into the cells. About 48 hr after the transfection, the cells were washed and lysed and then subjected to the tests for the luciferase activity of both *Firefly* and *Renilla* using the Dual Luciferanse Reporter Assay system (Promega, The United States). Finally, the relative luciferase activity was calculated based on the *Firefly* activity divided by the *Renilla* activity. All experiments were performed in triplicate.

Site-directed mutagenesis

To investigate whether there is a restoration/loss of effect, we incorporated the psi-CHECK2 vector with rs2536 T to C and C to T mutagenesis into the luciferase analysis to further biologically validate our results and ensure a high quality control. In brief, the target fragment was cut off from the psi-CHECK2 vector using AsisI and XmaI restriction enzymes. Then, with a primer designed to target sequences around the site for mutagenesis (Supporting Information Table S1) and the PFU DNA polymerase, we made site-directed mutagenesis for the target fragment covering *mTOR* 3′UTR and rs2536 T or C using PCR methods. With a designed primer (Supporting Information Table S1), full length of the target fragment was amplified and was finally re-connected to the psi-CHECK2 vector that was sent for DNA sequencing to ensure the success of the mutagenesis.

Gene knockdown and validation

The knockdown of *MTOR* was made by using the small interference (siRNA)-based approach. Three different types of siRNAs with 20–25 base pairs in length (Biotend, China; see Supporting Information Table S1 for the detailed sequences), which may down-regulate the *MTOR* gene, were transfected into AGS and HGC-27 cells and confirmed by PCR and western blot analyses. Briefly, cells were lysed with an RIPA buffer (Sigma, The United States) in the presence of a protease inhibitor, and the total protein was extracted and then

separated with 8% SDS-PAGE and transferred to the PVDF membrane. After being blocked with milk, the membrane was probed with the rabbit anti-*MTOR* (Cell Sig Tech, The United States) or mouse anti-α-tubulin (Sigma, The United States) antibody overnight at 4°C and then was probed with goat anti-rabbit or -mouse IgG (H + L), HRP conjugated secondary antibody (Proteintech, The United States) for 1 hr, respectively. Finally, the membrane was subjected to further analysis by using Super Luminol Chemiluminescent Kit (Share-bio, China), respectively. Results were obtained by the Tanon-5200 Multifunction Imaging System (Tanon Science & Technology, China). All experiments were performed in triplicate.

Proliferation assay

HGC-27 and AGS cells were seeded into the 6-well plates, respectively, with a density of 2×10^3 cells per well. After 1-week incubation, cells were washed and stained with 0.5% crystal violet solution. The colony number was then counted for further analysis. To visualize the proliferation activity in cells, we detected the incorporation of 5-ethynyl-2′-deoxyuridine (EdU) (RiboBio, China) into *de novo* DNA (the EdU assay) 48 hr after culture of the cells by using a 4-well plate with a density of 5×10^4 cells per well. The cells were co-incubated with EdU for 6 hr before permeabilization, EdU staining and fixation as well as fluorescent staining by DAPI (4′,6-diamidino-2-phenylindole) (RiboBio, China) to visualize the nuclei of the cells. Finally, using the laser scanning confocal microscopy (Leica, Germany), the status of EdU staining was captured and the proportion of cells with a relatively high proliferation activity was calculated. All experiments were performed in triplicate.

Transwell invasion and migration assay

HGC-27 and AGS cells were seeded into 24-well transwell Boyden chamber wells (Costar, Bedford, MA), respectively, with a density of 5×10^4 cells per well. After 36-hr culture, cells that migrated into the lower transwell chamber were stained with 0.5% crystal violet solution and counted for further analysis. To assess both the migration and invasive ability of the cells, the upper transwell chamber was plated without and with matrigel (BD bioscience, The United States), respectively. All experiments were performed with triplicate measurements.

Statistical analysis

The Cox proportional hazards regression model was used to estimate the associations between the selected genetic variants and overall survival of the GCa patients. We adopted the additive model in Cox regression analysis to estimate the impact of an allelic change on survival of the patients. We defined the overall survival as the period between diagnosis and death, and the patients who are alive and lost by follow-up were censored. Hazards ratio (HR) with its 95% confidence interval (CI) was used to estimate the associations. To correct for multiple tests, we calculated the false positive rate probability (FPRP), which is the probability of false positive findings by chance, to test the strength of the results.[30] Three parameters: statistical power, p value and prior probability are needed to compute the FPRP. In the present study, we adopted a prior probability of 0.01 and 0.10, along with an HR of 1.50 to compute the FPRP. An FPRP of 0.20 was set as a threshold. Any significant association with FPRP < 0.20 were considered noteworthy findings. For the genotype–phenotype correlation analysis, the multivariate linear regression model was used to assess the correlation between genotypes and gene expression that was nominalized to the expression of *GAPDH*. For the subsequent functional analysis, Student t test was used to compare for the difference among groups. Two-tailed p values less than 0.05 considered significant. All the statistical analyses were performed with R (version 3.3.2, R Foundation, Vienna, Austria), SAS (version 9.4, Q5Chapel Hill, NC) or STATA (version 14, StataCrop, TX) software.

Results

Patient population

In the 1002 GCa patients included in the final analysis, the median follow-up time was 63.43 months, and there were more male patients (70.86%) than female patients (29.14%). Consistent with most previously published studies, disease stage, surgery and chemotherapy were independent and significant predictors for GCa patients' survival. Compared to those having I–II stage disease, GCa patients with stage III–IV had a 6.24-fold higher death risk (HR = 6.24, 95% CI = 4.69–8.31, $p < 0.001$). Chemotherapy and surgery were also significantly associated with death risk of GCa patients (HR = 0.58, 95% CI = 0.37–0.88, $p = 0.01$; and 0.62, 0.49–0.78, $p < 0.001$, respectively). Moreover, patients having combinational intravenous chemotherapy and radiotherapy tended to have a non-significantly lower death risk ($p = 0.47$ and 0.30, respectively), compared to those having oral fluorouracil alone. All the results were adjusted for age, sex, disease stage, tumor differentiation and treatment in the multivariate analysis (Table 1).

Genetic variants involved in MTOR complex 1 genes and survival of GCa patients

Eight SNPs were initially selected by the SNPinfo database based on our defined criteria that might potentially affect functions of the genes involved in MTORC1, including *MTOR* (rs2536 T > C and rs1883965 G > A), *MLST8* (rs3160 T > C, and rs26865 A > G) and *RPTOR* (rs3751934 C > A, rs1062935 T > C, rs3751932 T > C and rs12602885 G > A (Supporting Information Table S2). Using HaploReg and Regulome DB databases, the selected SNPs were further predicted to be 3′UTR (miRNA binding sites) functional annotation or have available predicted score for their involvement in transcription factor binding (Supporting Information Table S2). Among these SNPs, only rs2536 T > C was found by the single-locus

Table 1. Characteristics of the GCa patients and their associations with survival in a Chinese population

Variable	No. of patients (%)	No. of deaths (%)	Univariate analysis		Multivariate analysis[1]	
			HR (95% CI)	*p*	HR (95% CI)	*p*
Age (year)						
‹60	519 (51.80)	214 (41.23)	1.00		1.00	
60	483 (48.20)	251 (51.97)	1.37 (1.11–1.65)	‹0.001	1.07 (0.88–1.31)	0.494
Sex						
Male	710 (70.86)	336 (47.32)	1.07 (0.87–1.31)	0.509	1.03 (0.83–1.28)	0.786
Female	292 (29.14)	129 (44.18)	1.00		1.00	
TNM stage						
I–II	437 (43.61)	76 (17.39)	1.00		1.00	
III–IV	565 (56.39)	389 (68.85)	6.21 (4.85–7.95)	‹0.001	6.24 (4.69–8.31)	‹0.001
Tumor differentiation						
Poor	745 (74.35)	355 (47.65)	1.00		1.00	
Medium	177 (17.66)	70 (39.55)	0.78 (0.59–0.98)	0.033	0.84 (0.65–1.10)	0.201
Well	55 (5.49)	18 (32.73)	0.61 (0.38–0.98)	0.041	1.11 (0.67–1.85)	0.681
Chemotherapy						
No	308 (30.74)	115 (37.34)	1.00		1.00	
Yes	694 (69.26)	350 (50.43)	1.42 (1.15–1.75)	0.001	0.62 (0.49–0.78)	‹0.001
Neoadjuvant therapy[2]	26 (2.59)	18 (69.23)	2.14 (1.30–3.52)	0.003	0.52 (0.29–0.93)	0.03
Adjuvant therapy	558 (55.69)	235 (42.11)	1.09 (0.87–1.37)	0.442	0.67 (0.52–0.85)	‹0.001
Palliative therapy	136 (13.57)	115 (84.56)	3.54 (2.72–4.62)	‹0.001	0.37 (0.26–0.53)	‹0.001
Chemotherapy agents						
Oral Fu	146 (14.57)	57 (39.04)	1.00		1.00	
CI-Chemotherapy[3]	493 (49.20)	267 (54.16)	1.52 (1.14–2.02)	0.005	0.89 (0.66–1.22)	0.471
Chemoradiotherapy	55 (5.49)	26 (47.27)	1.20 (0.75–1.90)	0.454	0.76 (0.45–1.28)	0.300
Surgery[4]						
No	44 (4.39)	43 (97.73)	1.00		1.00	
Yes	958 (95.61)	422 (44.05)	0.17 (0.12–0.23)	‹0.001	0.58 (0.37–0.88)	0.012

[1]Adjusted for age, sex, TNM stage, tumor differentiation and treatment.
[2]Patients received neoadjuvant therapy were also all received adjuvant therapy after surgery.
[3]Platinum or Fu-based combinational intravenous chemotherapy.
[4]About 6 tumors of stage II and III patients, and 38 of stage IV patients were surgically unrespectable. Other patients who received surgery have resectable primary tumors or resectable metastasis (stage IV).
Abbreviations: GCa, gastric cancer; HR, hazards ratio; TNM, tumor, node and metastasis; p, p value; Fu, fluorouracil; CI-Chemotherapy, combinational intravenous chemotherapy.

analysis to be independently associated with decreased death risk by 26% after adjustment for age, sex, disease stage, tumor differentiation and treatment (HR = 0.74, 95% CI = 0.57–0.96, $p = 0.022$, Table 2). The association remained noteworthy after correction by an FPRP less than 0.20 under a prior probability of 0.10 (FPRP = 0.199). In the subgroup analysis, although the reduced death risk by rs2536 T > C remained significant in patients with stage III-IV diseases (HR = 0.75, 95% CI = 0.57–1.00, $p = 0.049$, Table 3), the FPRP reached as high as 0.854 and 0.347, under the assigned prior probability of 0.01 and 0.1, respectively, indicating a relatively high probability of false positive by chance for the finding in stage III-IV patients. The association of rs2536 T > C change with reduced death risk was more prominent in patients with poorly differentiated tumor (HR = 0.68, 95% CI = 0.50–0.91, $p = 0.009$) and among those who had undergone surgery (HR = 0.70, 95% CI = 0.53–0.91, $p = 0.008$, Table 3) after correction by FPRPs for both results when a prior probability of 0.1 was assigned (FPRP < 0.2 for all, Table 3).

Genotype–phenotype correlation analysis of MTOR rs2536

Because only *MTOR* rs2536 was found to be an independent predictor for survival of GCa patients in the initial analysis, we further explored genotype–phenotype correlation of this SNP and other molecular mechanisms. The RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) predicted that the substitution of *MTOR* rs2536 T to C or A to G change in the matched mRNA base paring may affect the centroid secondary structure of mRNA, with a minimal free energy (MFE) changed from −10.80 to −6.00 kcal/mol,

Table 2. SNPs of genes involved in the MTOR complex1 selected in the present study and their associations with survival of 1,002 GCa patients

Location	Gene	rs#	Allele change	WW No. of patients (deaths %)	WM No. of patients (deaths %)	MM No. of patients (deaths %)	MAF (%)	Univariate analysis HR (95% CI)	p	Multivariate analysis [1] HR (95% CI)	p	FPRP 0.01	FPRP 0.1
1p36.22	***MTOR***	**rs2536**	**T > C**	**836 (46.53)**	**159 (47.80)**	**7 (0)**	**8.63**	**0.88 (0.70–1.11)**	**0.283**	**0.74 (0.57–0.96)**	**0.022**	0.732	**0.199**
		rs1883965	G > A	821 (46.04)	172 (48.26)	9 (44.44)	9.48	1.02 (0.82–1.27)	0.838	1.16 (0.92–1.46)	0.199	–	–
16p13.3	MLST8	rs3160	T > C	255 (49.80)	489 (44.17)	258 (47.29)	50.15	1.01 (0.89–1.14)	0.916	1.02 (0.89–1.17)	0.783	–	–
		rs26865	A > G	358 (48.04)	471 (44.80)	173 (47.40)	40.77	1.03 (0.90–1.17)	0.698	1.07 (0.93–1.22)	0.334	–	–
17q25.3	RPTOR	rs3751934	C > A	177 (39.55)	456 (45.61)	369 (50.68)	59.58	1.14 (1.00–1.29)	0.055	1.01 (0.88–1.15)	0.938	–	–
		rs1062935	T > C	266 (50%)	501 (46.51)	235 (42.13)	48.45	0.91 (0.80–1.04)	0.171	0.98 (0.86–1.12)	0.776	–	–
		rs3751932	T > C	745 (45.91)	232 (46.55)	25 (60.00)	14.07	1.08 (0.90–1.29)	0.396	1.03 (0.85–1.23)	0.787	–	–
		rs12602885	G > A	558 (47.49)	383 (43.60)	61 (54.10)	25.20	0.99 (0.85–1.15)	0.879	0.97 (0.83–1.14)	0.717	–	–

[1]Adjust for age, sex, stage, tumor differentiation and treatment. The statistically significant results are marked in **bold**.

Abbreviations: SNP, single nucleotide polymorphisms; GCa, gastric cancer; HR, hazards ratio; rs#, reference SNP ID; p, p value; FPRP, false positive report probability; WW, homogenous wild genotype; WM, heterogeneous genotype; MM, homogenous genotype for minor allele; MAF, minor allele frequency

indicating the potential of this SNP in regulating *MTOR* expression (http://rna.tbi.univie.ac.at, Fig. 1*a* and 1*b*).

In addition, 144 patients with available gastric tissues were also included for the real-time qPCR analysis, whose characteristics are presented in Supporting Information Table S3. From the qPCR analysis of 144 paired adjacent normal and GCa tumor tissues, we found that patients with a genotype of rs2536 TC or CC had a lower mRNA expression level of *MTOR* than those with the rs2536 TT genotype in adjacent normal tissues (p = 0.043, Fig. 1*c*), suggesting an effect of *MTOR* rs2536 C allele on the regulating the gene expression. However, genotype-phenotype analysis seemed to be insignificant in tumor tissues (p = 0.095, Fig. 1*d*), which is likely confounded by possible somatic mutations presented in the tumors.

Using the GTEx database, further eQTL analysis indicated a significant trend toward lower expression of *MTOR* mRNA by rs2536 T > C change in transformed fibroblasts (p < 0.001, Supporting Information Fig. S1*a*) and ovarian tissues (p = 0.028, Supporting Information Fig. S1*b*). These results indicate that *MTOR* expression may be regulated by the rs2536 locus. However, the trend was undetectable in gastric tissues (p = 0.870, Supporting Information Fig. S1*c*) from the GTEx database and populations from the 1,000 Genomes Project (p > 0.05 for all, Supporting Information Figs. S1*d*–S1*f*), which may reflect racial differences in gene regulations. Because the 1,000 Genomes project do not contain available gene expression data for Asian populations, we also used Hapmap3 datasets for additional eQTL, which included Asian (Japanese and Chinese), European and African populations with both genotyping and gene expression data in 593 lymphoblastoid cell lines. As seen in the Supporting Information Figure S2, there was no rs2536 CC homozygotes in all populations, but the *MTOR* expression levels were significantly lower in rs2536 TC heterozygotes than in rs2536 TT homozygotes in both JPT and YRI (p = 0.037 for JPT, Supporting Information Fig. S2*a*; and p = 0.001 for YRI, Supporting Information Fig. S2*d*), but not in CHB (p = 0.567, Fig. 2*b*), LWK (p = 0.381, Supporting Information Fig. S2*e*) and MKK (p = 0.926, Supporting Information Fig. S2*f*), nor in the combined JPT and CHB populations (Asians) (p = 0.323, Supporting Information Fig. S2*g*). Because CEU did not have rs2536 TC carriers (Supporting Information Fig. S2*c*), no CEU-specific eQTL could be performed. By combing all the populations, except for CEU, we did find a significantly lower expression of *MTOR* in the 111 lymphoblastoid cell lines for rs2536 TC carriers in comparison with 375 rs2536 TT carriers (P < 0.001, Supporting Information Fig. S2*h*). Although we did not observe a genotype–phenotype correlation in Chinese population, which may be due to small sample size and selection bias, the result from the combined all populations is biologically consistent with our qPCR results, indicating the potential involvement of rs2536 in regulation of the *MTOR* expression.

Table 3. Subgroup analysis of the association between MTOR rs2536 and survival of GCa patients

Variable	No. of patients (TT, %)	No. of patients (TC, %)	No. of patients (CC, %)	Univariate analysis		Multivariate analysis[1]		FPRP	
				HR (95% CI)	p	HR (95% CI)	p	0.01	0.1
Age (year)									
59	438 (84.39)	74 (14.26)	7 (1.35)	0.85 (0.61–1.20)	0.355	0.71 (0.49–1.04)	0.078	–	–
>59	398 (82.40)	85 (17.60)	0 (0.00)	0.89 (0.64–1.24)	0.490	0.78 (0.55–1.11)	0.162	–	–
Sex								–	–
Female	247 (84.59)	43 (14.73)	2 (0.68)	0.84 (0.53–1.34)	0.456	0.68 (0.41–1.15)	0.149	–	–
Male	589 (82.96)	116 (16.34)	5 (0.70)	0.91 (0.69–1.19)	0.492	0.77 (0.58–1.04)	0.087	–	–
TNM Stage								–	–
I- II	371 (84.90)	60 (13.73)	6 (1.37)	0.78 (0.42–1.44)	0.420	0.73 (0.38–1.41)	0.348	–	–
III-IV	465 (82.30)	99 (17.52)	1 (0.18)	0.83 (0.64–1.09)	0.178	**0.75 (0.57–1.00)**	**0.049**	0.854	0.347
Differentiation									
Poor	624 (83.76)	115 (15.44)	6 (0.80)	0.83 (0.63–1.09)	0.173	**0.68 (0.50–0.91)**	**0.009**	0.560	**0.104**
Medium	144 (81.36)	32 (18.08)	1 (0.56)	1.13 (0.66–1.94)	0.657	1.20 (0.66–2.19)	0.543	–	–
Well	46 (83.64)	9 (16.36)	0 (0.00)	1.72 (0.57–5.26)	0.338	1.78 (0.26–12.08)	0.557	–	–
Unknown	22 (0.88)	3 (0.12)	0 (0.00)	0.49 (0.11–2.10)	0.333	0.18 (0.02–1.56)	0.119	–	–
Chemotherapy								–	–
No	254 (82.47)	52 (16.88)	2 (0.65)	0.81 (0.50–1.32)	0.396	0.67 (0.39–1.14)	0.139	–	–
Yes	582 (83.86)	107 (15.42)	5 (0.72)	0.91 (0.69–1.19)	0.472	0.77 (0.57–1.03)	0.075	–	–
Chemotherapy agents								–	–
Oral Fu	125 (85.62)	21 (14.38)	0 (0.00)	1.35 (0.68–2.68)	0.387	0.62 (0.29–1.31)	0.207	–	–
CI-Chemotherapy	412 (83.57)	77 (15.62)	4 (0.81)	0.85 (0.62–1.15)	0.287	0.79 (0.57–1.10)	0.163	–	–
Chemoradiotherapy	45 (81.82)	9 (16.36)	1 (1.82)	0.75 (0.29–1.97)	0.564	0.79 (0.30–2.14)	0.648	–	–
Surgery								–	–
No	37 (84.09)	7 (15.91)	0 (0.00)	1.33 (0.58–3.03)	0.500	2.88 (0.77–10.85)	0.118	–	–
Yes	799 (83.40)	152 (15.87)	7 (0.73)	0.87 (0.68–1.12)	0.275	**0.70 (0.53–0.91)**	**0.008**	0.507	**0.086**

[1]Adjusted for age, sex, stage, tumor differentiation and treatment.
Abbreviations: GCa, gastric cancer; HR, hazards ratio; TNM, tumor, node, and metastasis; p, p value; Fu, fluorouracil; CI-Chemotherapy, combinational intravenous chemotherapy; FPRP, false positive report probability.

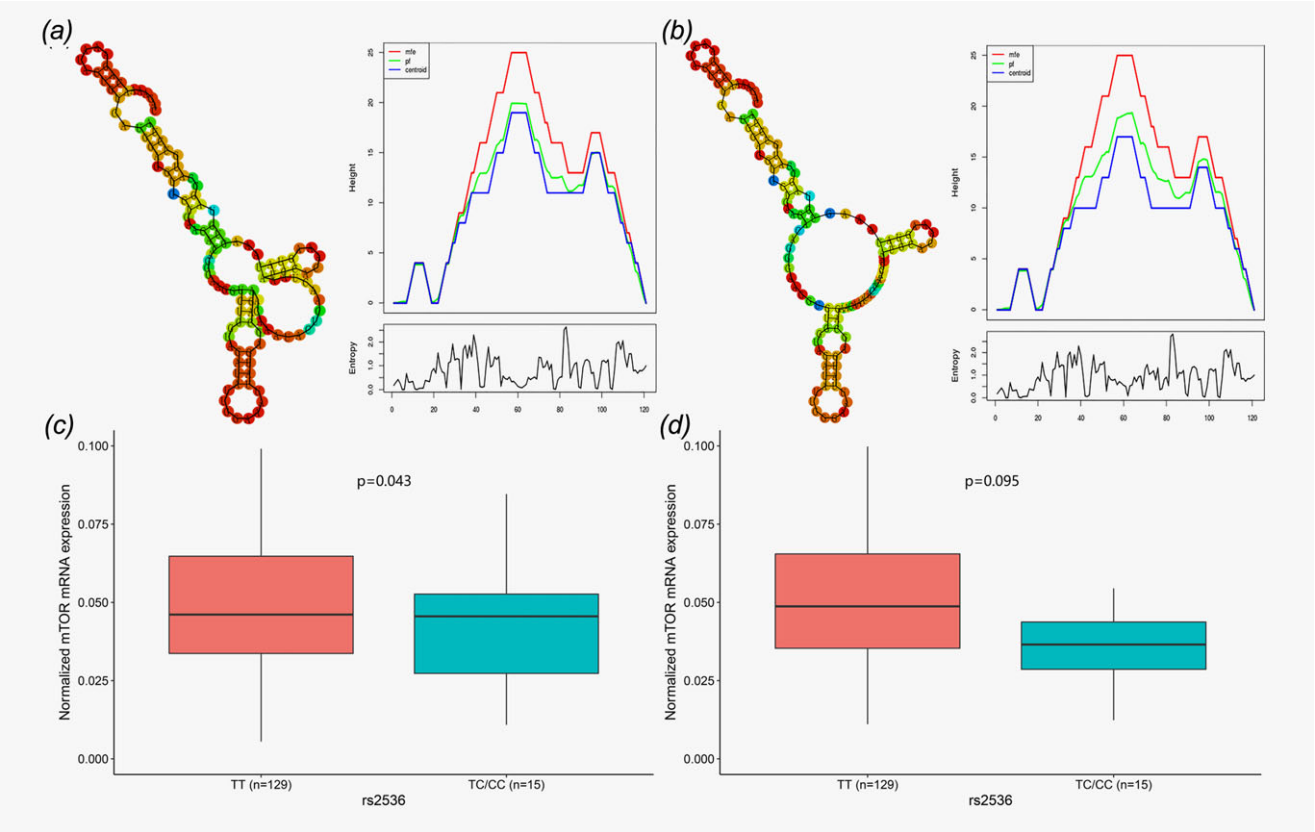

Figure 1. Potential modulation of *mTOR* expression by rs2536. The centroid secondary structure of mRNA and mountain plots predicted by RNA fold, (*a*) and rs2536 C, or G in the matched mRNA (*b*). The mountain plots described the MFE structure, the thermodynamic ensemble of RNA structures and the centroid structure of the mRNA; the real-time qPCR analysis to investigate the association of rs2536 with mRNA expression of *mTOR* in adjacent normal tissues (*c*) and cancer tissues (*d*).

MTOR rs2536-related transcriptional activity of the gene and binding affinity of miRNA-150

We next investigated the mechanism for potential regulation of *MTOR* expression by rs2536. As shown by Figure 2*a*, rs2536 is located at the 3′UTR of the *MTOR* mRNA, and miRNA-150 was predicted to bind to rs2536 C more preferably than to rs2536 T (Fig. 2*a*). To experimentally prove this prediction, we constructed a psi-CHECK2 vector carrying the 3′UTR of the *MTOR* gene, with the wild TT type and the minor CC type of rs2536, respectively, and used in the luciferase transfection analysis (Figs. 2*b* and 2*c*). A mutagenesis approach was also adopted to conversely construct the vector (rs2536 T to C and C to T mutagenesis) and evaluated by the luciferase analysis. The sequencing results of vectors processed by mutagenesis are presented in Figure 2*d*. In both 293T and AGS cells, we observed a down-regulated *Firefly* luciferase activity for the rs2536 C allele, compared to that for the T allele (p = 0.040 for 293T cells and 0.002 for AGS cells, Figs. 2*e* and 2*f*). With site-directed mutagenesis for rs2536, we observed an attenuated luciferase activity associated with the T-to-C mutagenesis in both 293T (p = 0.014, Fig. 2*e*) and AGS cells (p = 0.006, Fig. 2*f*). Interestingly, the luciferase activity was restored when C was changed to T by the same mutagenesis approach in these cell lines, (p = 0.008 for 293T cells and 0.040 for AGS cells, Figs. 2*e* and 2*f*), further indicating the potential role of rs2536 variant in regulating the gene transcriptional activity. Moreover, in 293T cells, miRNA-150 repressed the transcriptional activity of the *luciferase* gene with a downstream rs2536 C more significantly (p = 0.013) than that of a downstream rs2536 T (p = 0.103, Fig. 2*g*). This effect was not observed in AGS cells (p = 0.192, Fig. 2*h*). Interestingly, consistent with the 293T cells, miRNA-150 significantly inhibited the luciferase activity, when a reporter gene carrying rs2536 C was transfected into the AGS cells (p = 0.018, Fig. 2*h*). The impaired repression on luciferase activity was also associated with an rs2536 C to T mutagenesis, with only a borderline trend for 293T cells (p = 0.087, Fig. 2*g*) but not for AGS cells (p = 0.167, Fig. 2*h*), while this repression was restored after a site-directed change of rs2536 T to C by the same mutagenesis approach (p = 0.020 for 293T cells and 0.029 for AGS cells, Figs. 2*g* and 2*h*). These results provided additional support for the possible molecular mechanism underlying the observed genotype–phenotype correlation, in which *MTOR* expression was likely to be regulated by the rs2536 C, but not rs2536 T, possibly via an interaction with miRNA-150.

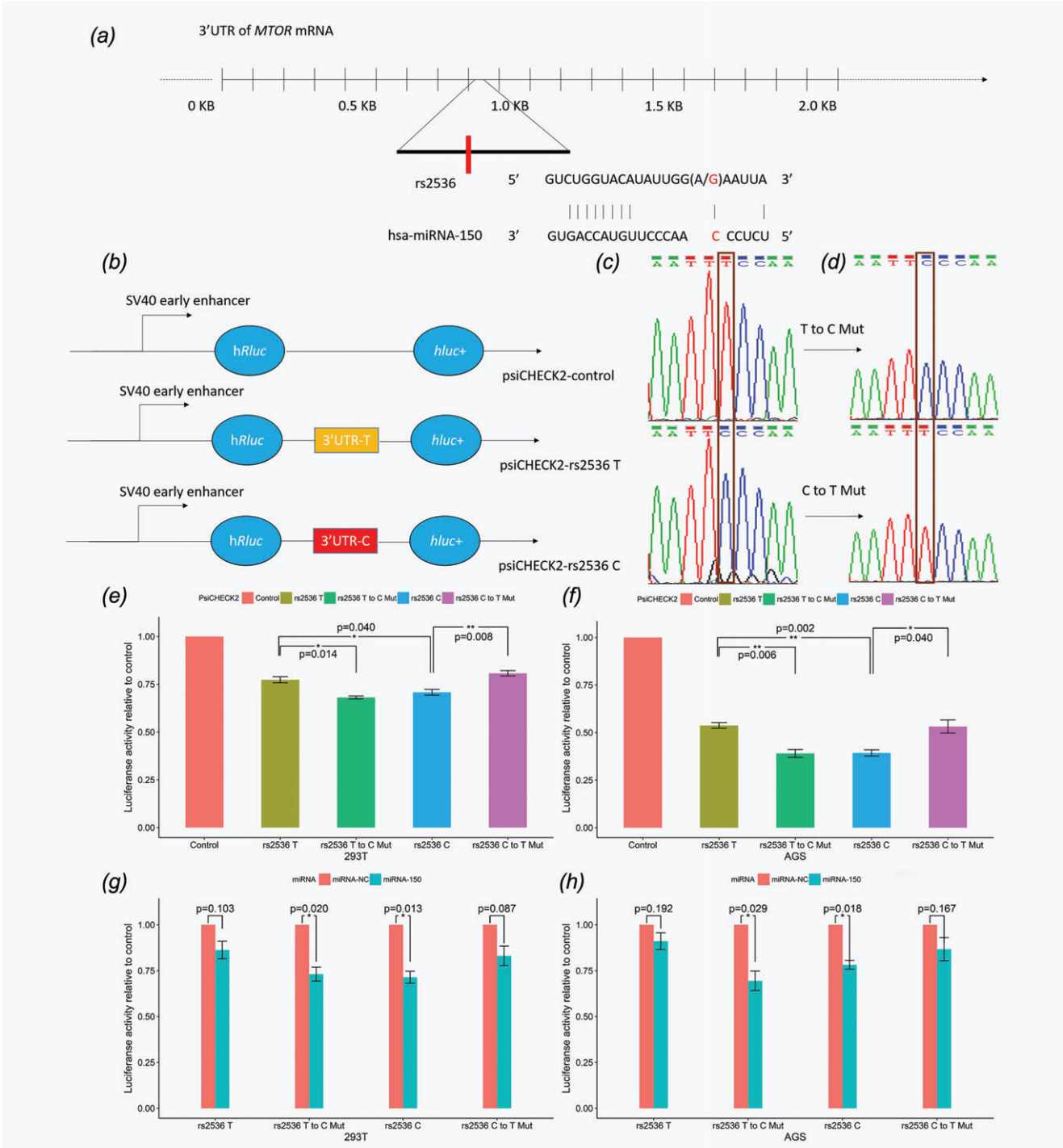

Figure 2. The luciferase reporter assay revealed a higher binding ability of rs2536 C to miRNA-150, compared to rs2536 T. Graphic representation of the detailed location of rs2536 in the 3′UTR of *mTOR* mRNA (Upper side in *a*), and the specified binding of miRNA-150 to the *mTOR* 3′UTR covering rs2536 C, or rs2536 G according to the principle of complementary base pairing (Lower side in *a*); Construction plot of the luciferase reporter system (*b*) and sequencing results of the Psi-CHECK2 vector containing rs2536 T or C allele (*c*); sequencing results of the luciferase vector with rs2536 mutagenesis **(*d*)**; luciferase activity of the rs2536 T, rs2536 C, along with the mutagenesis vectors in 293T cells (*e*) and AGS cells (*f*); luciferase activity in the presence of the miRNA-150 transfected into 293T cells (*g*) and AGS cells (*h*). Abbreviation: Mut, Mutagenesis.

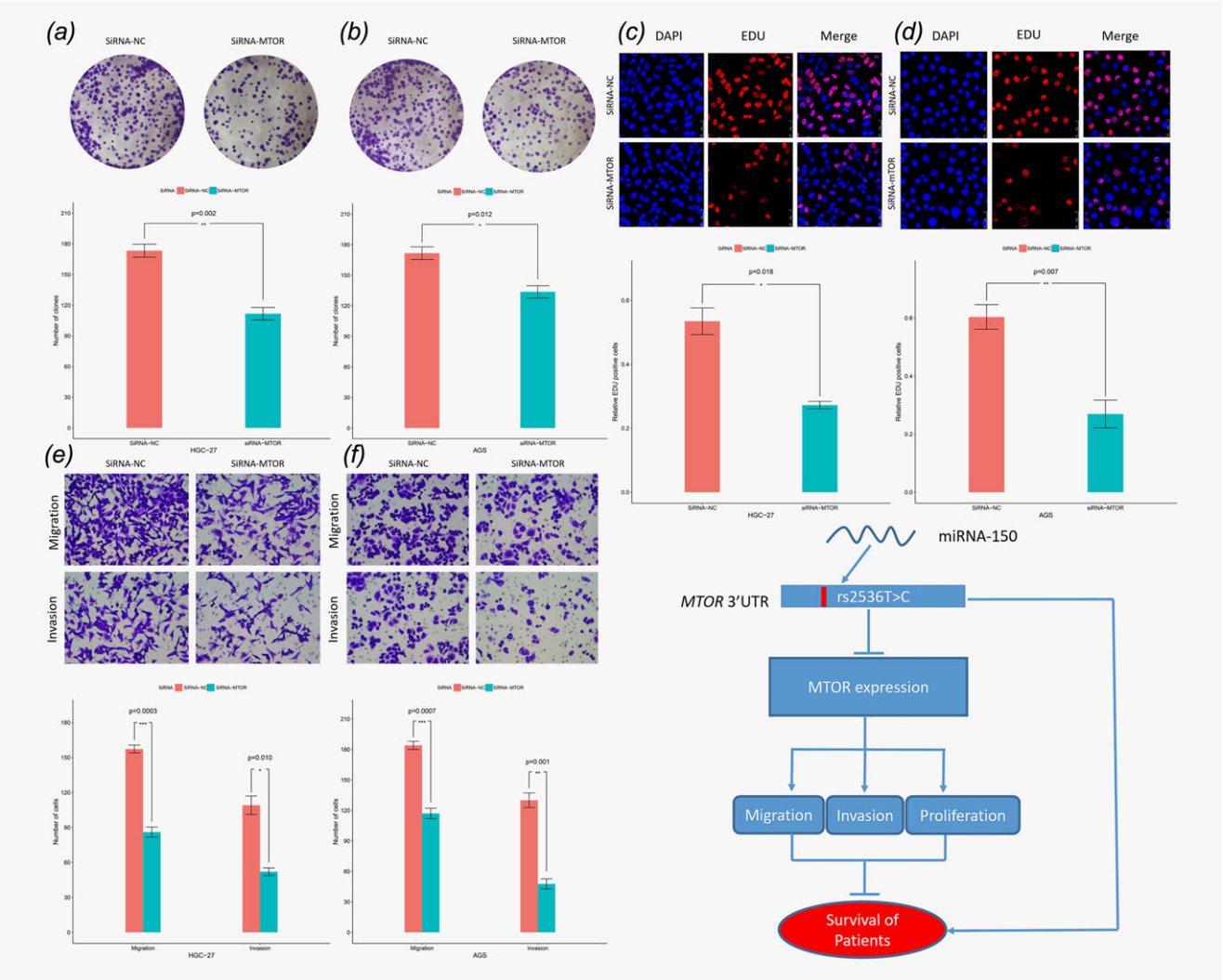

Figure 3. Knockdown of *mTOR* impaired the malignant phenotype of GCa cells. *mTOR* was knocked down in AGS and HGC-27 GCa cells by siRNA. The malignant performance of AGS and HGC-27 cells transfected with siRNA or the negative control were assessed by the colony formation assays (*a*: HGC-27 and *b*: AGS), EdU assay (*c*: HGC-27 and *d*: AGS) and the transwell assays with or without matrigel (*e*: HGC-27 and *f*: AGS). Overall findings of this study are presented as a schematic model (*g*).

The role of MTOR expression in malignant phenotype of GCa

We further investigated the potential role of *MTOR* down-regulation by rs2536 C in the malignant phenotype of GCa. Among the three siRNAs we designed, we selected siRNA-2 that presented a promising efficiency of *MTOR* knockdown at both protein and mRNA levels (Supporting Information Fig. S3) for functional analysis. The proliferation was significantly inhibited by down-regulation of *MTOR* as indicated by the colony formation assay in HGC-27 cells (p = 0.002) and AGS GCa cells (p = 0.012) (Figs. 3*a* and 3*b*). The EdU assay produced similar results that the proportion of GCa cells at the proliferation phase was reduced in the presence of *MTOR* knockdown (p = 0.018 for HGC-27 and 0.007 for AGS cells, Figs. 3*c* and 3*d*). Moreover, impaired ability of invasion and migration caused by siRNA-2 transfection was also observed in the transwell assay with matrigel (p = 0.010 for HGC-27 and 0.001 for AGS cells) and without matrigel (p = 0.0003 for HGC-27 and 0.0007 for AGS cells) (Figs. 3*e* and 3*f*). Overall findings of these experiments are summarized and presented as a schematic model in Figure 3*g*.

Discussion

In this relatively large survival study of 1002 GCa patients followed by functional analysis, we demonstrated for the first time that the *MTOR* rs2536 T > C change predicted a better overall survival of Chinese GCa patients. The possible molecular mechanism underlying the observed association is likely because of a higher binding affinity of miRNA-150 to rs2536 C that may down-regulate the expression of *MTOR*, compared to rs2536 T, leading to the inhibition of malignant phenotype of GCa cells.

In response to the upstream regulators, such as the PIK3-AKT signaling pathway, the TCS complex and the Rag

complex, MTOR is a central reactive element in MTORC1.[31] RPTOR and MLST8, a subunit regulator and a scaffolding protein of MTOR, respectively, are essential for the stability of MTORC1.[31] MTORC1 potentiates tumorigenesis and cancer progression from a series of events. First, the phosphorylation of downstream reactors by MTORC1, including HIF1a[32] and VEGF,[33] was associated with cancer progression. A recent report revealed that MTORC1 could stabilize SKP2 by phosphorylation, by which the function of the oncogene *SKP2* was sustained in GCa cells.[34] Second, the activated MTORC1 may drive transcriptional activities of genes involved in the metabolism. The metabolic reprogramming induced by MTORC1 activation may in turn provide sufficient energy by glycolysis to promote cell growth.[35] Third, MTORC1 is involved in the regulation of autophagy, a common biological process in cells that is associated with cancer progression.[16,36] Moreover, the therapy targeted against MTOR has been considered an important anticancer strategy.[37,38] Taken together, our results further suggest that the MTORC1 variants may be also involved in tumor progression, exerting an impact on cancer patients' survival.

There is limited evidence demonstrating that whether or not the *MTOR* gene expression can affect the progression of GCa. In the present study, we demonstrated a role of a *MTOR* variant in association with a malignant phenotype of GCa, including proliferation, migration, and invasion of the cancer cells. The genotype–phenotype analysis derived from qPCR analysis, GTEx and Hapmap3-based eQTL analysis provided consistent support for a potential role of *MTOR* rs2536 in regulating the gene expression. Therefore, the present study provides a new piece of evidence for the role both the *MTOR* gene and the related genetic variant may play in the progression of GCa.

Consistent with the present study, one recently published study also reported that *MTOR* rs2536 T > C was independently associated with a longer survival of 146 Chinese patients with small cell lung cancer.[39]. Another study addressed the remarkable involvement of genetic variants of *MTOR* in clinical outcome of 182 esophageal cancer patients who had undergone chemo-radiotherapy and surgery.[40] Moreover, genetic variants of *TCS1*, an upstream regulator of MTORC1, were also associated with survival of colorectal cancer in a study with 772 patients.[41] The results of the present study with a much larger sample size provided some new information on the involvement of genetic variants of *MTOR* in GCa prognosis. Interestingly, *MTOR* rs2536, a potential independent predictor for survival of GCa patients, seemed to have no association with risk of GCa according to our previous case–control studies.[22,42]

In the present study, we included common germline variants that have a minor allele frequency higher than 5% for analysis. It is not surprising that such variants have only moderate contribution to risk and progression of cancers, if any, particularly compared to germline mutations that are associated with some distinct disease phenotypes. This is particularly true for a single locus change such as the *MTOR* rs2536 T > C change, which is similar to other functional studies focused on single germline variant.[43–45] The effect of such germline variants on gene expression is also likely to be affected by other molecular events that govern gene expression regulation, such as long coding RNAs and circular RNAs that are competing endogenous RNAs (ceRNAs),[46,47] which may disrupt the binding of miRNA to 3′UTR of the genes. This might be the reason for the relatively less strong impact of miRNA-150 on modulating the *MTOR* expression associated with the *MTOR* rs2536 T > C change.

In the subgroup analysis, the impact of rs2536 T > C on survival of GCa patients was more prominent in patients with poorly differentiated tumors, and those who underwent surgery but not in other subgroups, likely due to the reduction in the number of observations in each of the strata that might have led to decreased statistical power. Another limitation for the present study is its retrospective design in nature. Clinical characteristics retrospectively reviewed by expert clinicians may have introduced some bias. Lastly, because the results were derived from a single hospital and therefore did not represent the general population of GCa patients in China, future Asian/Chinese population-based survival analysis and genotype–phenotype correlation studies are needed to validate our findings.

In conclusion, with 1,002 patients included in the present study, we found that *MTOR* rs2536 independently predicted the survival of GCa patients. The 3′UTR of *MTOR* with rs2536 C had a better binding affinity to miRNA-150, leading to the downregulation of *MTOR* expression and a less aggressive phenotype of GCa cells, which provides support for a potential mechanism underlying the observed association. To facilitate the translation of the present findings into clinical management of GCa patients, larger and prospective studies with additional mechanistic and follow-up studies are warranted to validate our findings.

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