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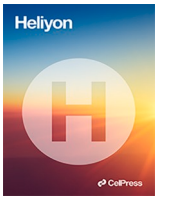

Research article

rs2736098, a synonymous polymorphism, is associated with carcinogenesis and cell count in multiple tissue types by regulating *TERT* expression

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ABSTRACT

rs2736098 is a synonymous polymorphism in TERT (telomerase reverse transcriptase), an enzyme involved in tumor onset of multiple tissues, and should play no roles in carcinogenesis. However, a search in cancer somatic mutation database indicated that the mutation frequency at rs2736098 is much higher than the average one for *TERT*. Moreover, there are significant H3K4me1 and H3K27Ac signals, two universal histone modifications for active enhancers, surrounding rs2736098. Therefore, we hypothesized that rs2736098 might be within an enhancer region, regulate *TERT* expression and influence cancer risk. Through luciferase assay, it was verified that the enhancer activity of rs2736098C allele is significantly higher than that of T in multiple tissues. Transfection of plasmids containing *TERT* coding region with two different alleles indicated that rs2736098C allele can induce a significantly higher *TERT* expression than T. By chromatin immunoprecipitation, it was observed that the fragment spanning rs2736098 can interact with USF1 (upstream transcription factor 1). The two alleles of rs2736098 present evidently different binding affinity with nuclear proteins. Database and literature search indicated that rs2736098 is significantly associated with carcinogenesis in multiple tissues and count of multiple cell types. All these facts indicated that rs2736098 is also an oncogenic polymorphism and plays important role in cell proliferation.

1. Introduction

Telomere is a special region of repetitive DNA at the both ends of chromosome and can protect chromosome ends [1]. In cell division, the telomere is shortened slightly [1]. When telomere length is reaching a specific threshold, cell division can not be initiated and apoptosis will be induced [2]. Therefore, telomere lengthening after each cell division plays an important role in cancer and self-renewing cells. This process is conducted by telomerase, a nucleoprotein complex [3]. Among this complex, TERT (telomerase reverse transcriptase) is the most important and rate-limiting catalytic subunit [4]. TERT overexpression has been frequently observed in numerous tumor tissues [5]. Moreover, multiple genetic variations which can increase *TERT* expression have been suggested to be

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involved in carcinogenesis [6,7]. Based on these results, *TERT* has been proposed to be an oncogene for multiple cancer types [8].

Breast cancer is the most common cancer type in woman [9]. Recent genome-wide association studies (GWAS) have suggested that C allele of rs2736108 is significantly associated with breast cancer risk (see GWAS Catalog at https://www.ebi.ac.uk/gwas/for detail) [10–14]. This association has been verified by multiple association studies (see PubMed at https://pubmed.ncbi.nlm.nih.gov for detail). Linkage disequilibrium (LD) analysis on 1000 genomes project data indicated that there are another four SNPs, rs2736098 (in coding region), rs2853669 (−245 relative to *TERT* translation start in genome; the same for following mutations), rs2736109 (−1655) and rs2736107 (−2750), are in strong LD ($r^2 \geq 0.80$) with rs2736108 (−2384; see Table S1) in three representative populations, CEU (Utah Residents with Northern and Western European Ancestry), CHB (Han Chinese in Beijing) and YRI (Yoruba in Ibadan, Nigeria). Consequently, two distinct haplotypes can be observed (see Table 1). Functional genomics work observed that the allele in common haplotype, i.e., C of rs2736107, rs2736108 and rs2736109 and A of rs2853669, can increase *TERT* promoter activity [15–17]. Further analysis verified that rs2736108 is significantly associated with telomere length [17–21]. All these efforts illuminated the connection between genetic variations in this locus and breast cancer risk.

Within these two haplotypes, rs2736098 (915 relative to *TERT* translation start in mRNA) is a synonymous polymorphism and located at the second exon. Therefore, it seems that this polymorphism is not likely to influence carcinogenesis. However, a search in COSMIC (https://cancer.sanger.ac.uk), a database for cancer somatic mutations database, indicated that the mutation probability at rs2736098 is much higher than nearby positions (see Fig. S1). Indeed, *TERT* coding region is with a length 3399 bases and only 1773 somatic mutation events occur in all cancer types (If we limit the search in breast cancer, the mutation events are too small to draw a conclusion.). In contrast, 17 somatic mutation events are observed at rs2736098 position, which is ~31.6-fold higher than the average frequency. If this synonymous polymorphism play no roles in carcinogenesis, it is difficult to image such a phenomenon. Moreover, a search in ENCODE project (https://www.encodeproject.org/) indicated that there are strong H3K4me1 and H3K27ac signals, two common histone modification in active enhancers [22], appearing in multiple cell lines, including breast ones (see Fig. S2). Therefore, we hypothesized that rs2736098 might be within an enhancer region for *TERT* and further influence breast cancer risk.

In this study, we compared the enhancer activity and *TERT* expression difference induced by rs2736098 through plasmids construction and transfection. Further mechanism was also investigated.

2. Materials and methods

2.1. Reporter gene assay

Genomic DNA was isolated from MCF-7 cell line by starndard phenol-chloroform method. rs2736098 nearby region (~1.5 kb; chr5: 1293195–1294669; corresponding to partial exon 2 and intron 2 of *TERT*) was amplified by using primers in Table S2 from MCF-7 DNA. After digestion by restriction enzymes *Kpn*I and *Xho*I (NEB, Ipswich, MA), the PCR product and pGL3-promoter plasmid (Promega, Madison, WI) were ligated by utilizing T4 DNA ligase (NEB). PCR was carried out with Q5 High-Fidelity DNA Polymerase (NEB) to exclude artificial mutations. The plasmid containing the corresponding allele for rs2736098 was constructed using Q5 Site-Directed Mutagenesis Kit (NEB) and the primers listed in Table S2. All recombinant plasmids were sequenced to avoid the possibilities of any artificial mutations and confirm the haplotypes orientation of mutated and wild-type DNA segments. Besides rs2736098, there were no other SNPs in the cloned region between the two plasmids.

Breast cancer cell line MCF-7 was maintained in Dulbecco's modified Eagle's medium (high glucose; HyClone, Logan, UT) with 10% fetal bovine serum (Biological Industries, Cromwell, CT) in 5% CO_2 at 37 °C. Before transfection, MCF-7 cells (~10^5) were grown in 24-well plates. After 24 h of cultivation, 475 ng constructed plasmid and 25 ng pRL-TK (Promega) were transiently co-transfected into MCF-7 cell utilizing Lipofectamine 2000 (Thermo Fisher Scientific, Grand Island, NY) according to the manufacturer's guidance. After 48 h of transfection, cells were lysed and luciferase activity was assessed by utilizing Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Six independently replicates were carried out for this experiment.

2.2. Overexpression plasmid construction and TERT expression measurement

TERT coding region was amplified by nested PCR with primers in Table S3 and Q5 High-Fidelity DNA Polymerase (NEB) from MCF-7 cDNA. After *Nhe*I and *EcoR*I (NEB) digestion, PCR product was inserted into overexpression vector pEGFP-N1 (Clontech, Mountain View, CA). The plasmid containing another allele of rs2736098 was obtained by mutagenesis as abovementioned. The two overexpression plasmids (500 ng) were transfected into MCF-7 cell line as abovementioned. After 48 h culture, total RNA was isolated by

Table 1
SNPs in core haplotypes.

rs ID	position[a]	Common haplotype	Rare haplotype
rs2736098	1294086	C	T
rs2853669	1295349	A	G
rs2736109	1296759	C	T
rs2736108	1297488	C	T
rs2736107	1297854	C	T

[a] Based on human genome build 37.

TRIzol (Thermo Fisher Scientific) and cDNA library was prepared by RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). *TERT* expression was assessed by quantitative PCR (qPCR) with iQ SYBR green (Bio-Rad, Hercules, CA) and primer pair AGAGGGGAAAGGGTGTCCATGG and GTGAAAGGCAAGGAGCAGACGG. *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) expression was also measured as a control as abovementioned with published primer [23]. Three independently replicates were carried out for this experiment.

2.3. Chromatin immunoprecipitation (ChIP)

Potential transcription factors (TFs) USF1 (upstream transcription factor 1) and MYC (MYC proto-oncogene, bHLH transcription factor) were predicted by TRANSFAC (http://www.gene-regulation.com/) to bind rs2736098 surrounding. ChIP assay was conducted in MCF-7 cell line by using ChIP Assay Kit (Beyotime Biotechnology, Shanghai, China) following the manufacturer's guidance. In brief, approximately 1×10^7 MCF-7 cells were fixed by adding formaldehyde (1% final concentration) and incubated for 10 min at 37 °C. To end cross-linking, glycine was added for 5 min at 25 °C. After harvesting, cells were lysed for 10 min on ice and sonicated by Ultrasonic Cell Disruptor (Scientz Biotechnology, Ningbo, China) to obtain 200-800 bp DNA fragments. Chromatin samples were immunoprecipitated utilizing anti-mouse USF1 antibody, anti-mouse MYC antibody or IgG (Santa Cruz Biotechnology, Santa Cruz, CA). After immunoprecipitating, DNA was purified by GeneJET Gel Extraction Kit (Thermo Fisher Scientific) and subjected to qPCR to assess the enrichment with the following primer air GGCGTACACCGGGGGACAAG and CAGGACGCGTGGACCGAGTGACC. The *APLN* (apelin) promoter region, which was verified to be USF1 binding site in breast tissues [24], was amplified by primer pair GCTGCAGAGTGCGTGCCTGGAG and GAGCGGCAGCGGCGAGCTCTTTCTTAG as a positive control. In contrast, one random selected region, which was without USF1 binding prediction, was amplified by primer pair AGCAGCTCAGGCACACTTCTT and AGTCTCTGAACCCTTGGCTCC as a negative control.

2.4. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts (5 μg) were prepared from MCF-7 cells utilizing the Nuclear Protein Extraction Kit (Beyotime). The probe consisted of 27 bp sequences centered on the rs2736098 SNP alleles were shown in Table S4 and labeled by 3′-Biotin labeling kit (Beyotime, Shanghai, China). After annealing, the duplex probes (10 fmol) were incubated with the nuclear extracts at 37 °C for 20 min. The biotin-labeled probes without nuclear protein as controls and unlabeled probes (200-fold molar excess) were added as competition reactions. The reaction samples were electrophoresed on a 4.9% polyacrylamide gel for 60 min at 100 V and then transferred to a positively charged nylon membrane (Beyotime). After transferring, the membrane was incubated with Streptavidin-HRP (horseradish peroxidase) conjugate and image was gained on Luminescent Imaging Workstation system (Tanon, Shanghai, China).

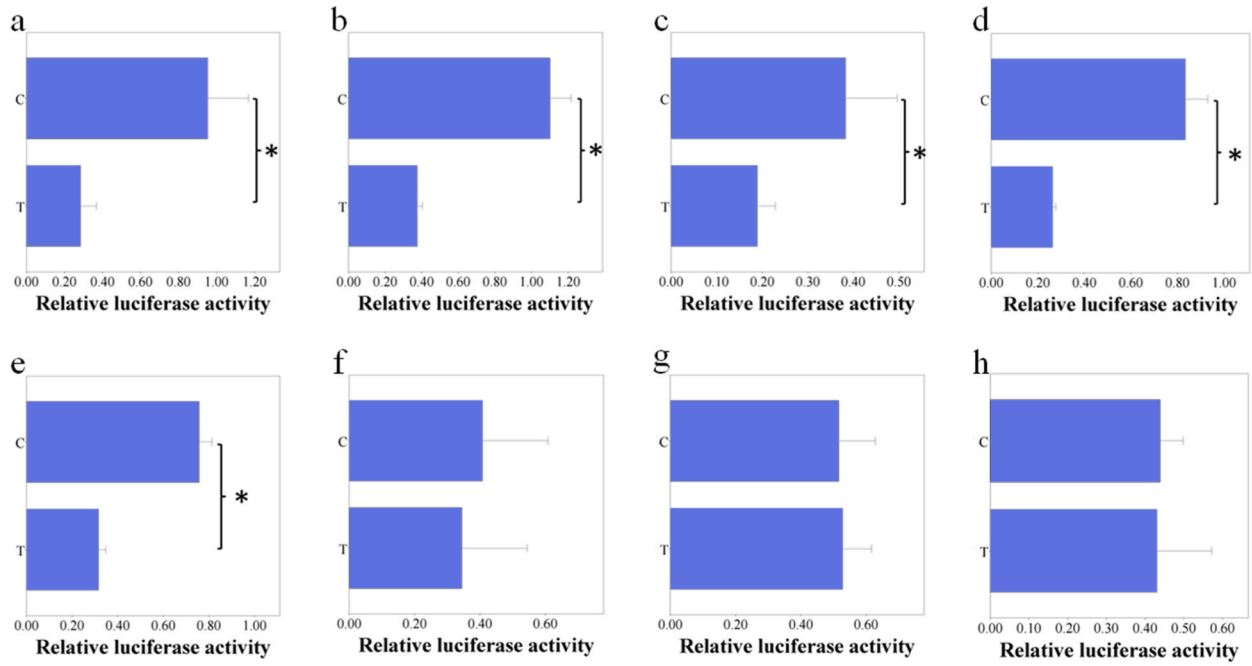

Fig. 1. The different enhancer activities between rs2736098 alleles in MCF-7 (a), HCT116 (b), HeLa (c), SK-OV-3 (d), SGC-7901 (e), HepG2 (f), Beas-2B (g) and PA-TU-8988T (h) cells. The *x* axis represents the relative luciferase expression amount. All data are displayed as mean ± standard deviation (SD). $^*P < 0.0001$.

3. Statistics

Independent student's *t*-test was used to compare relative luciferase activity, *TERT* expression and ChIP enrichment. All statistics analysis was performed in SPSS 20.0 (IBM, Armonk, NY). When $P < 0.05$, the difference was supposed to be significant.

4. Result

4.1. Function of rs2736098

To disclose the potential enhancer activity difference induced by rs2736098, we generated the plasmids with different rs2736098 alleles and transfected them into MCF-7 cell line. As shown in Fig. 1a, the relative luciferase activity of C allele is approximately 2.36 fold higher than that of the T ($P = 6.03 \times 10-^{8}$), thus verifying that rs2736098 should be functional in breast tissue.

To verify whether the function of rs2736098 is limited in breast cell, we further cultured colon cancer cell line HCT116, cervical cancer cell line HeLa, ovarian cancer cell line SK-OV-3, gastric cancer cell line SGC-7901, hepatocellularcarcinoma cell line HepG2, lung/bronchus epithelial cell line Beas-2B and pancreatic cancer cell line PA-TU-8988T in the same condition with MCF-7 and transfected the two plasmids. As shown in Fig. 1, C allele of rs2736098 displays a significantly higher luciferase expression than T in HCT116 (Fig. 1b), HeLa (Fig. 1c), SK-OV-3 (Fig. 1d) and SGC-7901 (Fig. 1e; all $P < 0.0001$). In contrast, no significant difference was observed in HepG2 (Fig. 1f), Beas-2B (Fig. 1g) and PA-TU-8988T (Fig. 1h; all $P > 0.60$). These results indicated that rs2736098 can influence enhancer activity in multiple tissues.

4.2. Effect of rs2736098 in TERT expression

Considering the function and location of rs2736098, it is highly possible that rs2736098 can influence *TERT* expression. However, due to the close distance between rs2736098 and *TERT* promoter (1018 bp), the classic loop model between enhancer and promoter might not apply to this case and chromosome conformation capture might not disclose the interaction between them. To substantiate the effect of rs2736098 on *TERT* expression, we further generated overexpression plasmids containing two alleles of rs2736098, transfected them into MCF-7 and measured *TERT* expression. Compared with the cell without transfection, *TERT* expression in transfected cell increases ~8000 fold (result not shown), which indicated that intrinsic *TERT* expression is negligible in our experiment. As shown in Fig. 2, *TERT* expression of the plasmid containing C allele is ~6.06 fold higher than that of T ($P = 0.02$), which was consistent with our luciferase result. All these facts indicate that rs2736098 indeed can influence *TERT* expression.

4.3. TF interacting with rs2736098

Our above results identified that rs2736098 is a functional SNP. Since rs2736098 is located in enhancer, it seems reasonable to

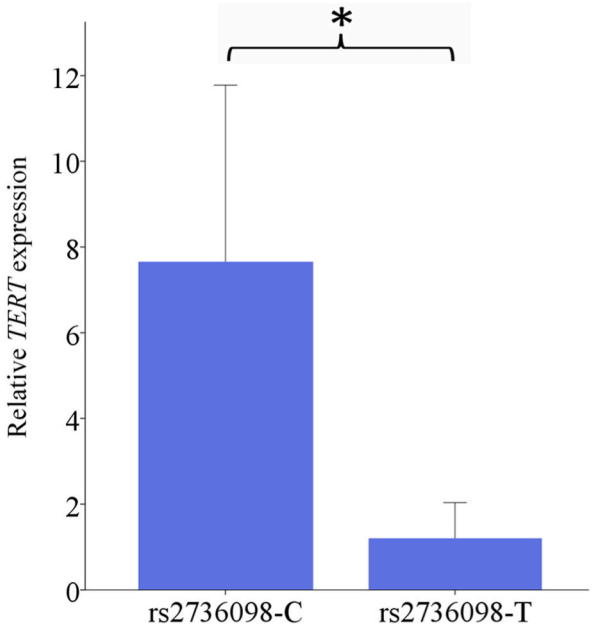

Fig. 2. Differential *TERT* expression between rs2736098 alleles. The *x* axis denotes different alleles of rs2736098 while *y* axis represents *TERT* expression. All data are displayed as mean ± SD. *$P < 0.05$.

assume that it could interact with TF and influence TF binding affinity. Bioinformatics prediction suggested that the substitution from T to C at rs2736098 may influence the binding affinity of MYC and USF1. To verify this prediction, ChIP assay was carried out using MCF-7 cell line with related antibodies and qPCR was used to assess the relative chromatin enrichment. For both antibodies, we included positive and negative controls. The result indicated that positive control region can be immunoprecipitated while the negative one not (results not shown), thus suggesting that our assay is effective to detect TF for rs2736098 surrounding region. As shown in Fig. S3, the MYC antibody failed to enrich chromatin compared with IgG ($P = 0.66$). In contrast, compared with IgG, the region containing rs2736098 was significantly enriched by USF1 antibody ($P = 0.042$; see Fig. 3a), thus verifying that USF1 could bind the rs2736098 surrounding region in MCF-7 cell.

4.4. *Different TF binding affinity between rs2736098 alleles*

We next performed EMSA to verify the possibility that rs2736098 interacts with USF1 in an allele specific pattern. It can be observed that there is a specific protein-DNA complex band composed of the core sequence containing rs2736098C allele and nuclear proteins, which verified that the rs2736098C allele could interact with protein factors (see Fig. 3b and Fig. S4). In contrast, this band is almost disappearing for T allele of rs2736098 (see Fig. 3b and Fig. S4), thus verifying the low affinity between rs2736098 T allele and protein factors. This pattern was consistent with our luciferase and *TERT* expression result.

5. Discussion

In current study, we used functional genomics approaches to investigate the role of rs2736098 in *TERT* expression. The result indicated that the common allele of rs2736098, C, is with the ability to enhance *TERT* expression. Interestingly, the common allele at other four positions (see Table 1) can also increase *TERT* promoter activity [15–17], thus constituting a haplotype with high expression and leading to a significant signal at rs2736108 in GWAS [10–14].

In current stage, most identified enhancers are located in non-coding region. However, enhancers in coding region have also been reported [25–27]. Most enhancers in coding region can regulate nearby gene expression [26]. Therefore, it is not surprising to observe that rs2736098 is a novel *cis*-regulatory element for *TERT*.

Our luciferase result indicated that rs2736098 effects through a tissue-specific manner. Indeed, the *cis*-regulatory function of rs2736098 is lost in some tissues. This phenomenon has also been reported [28] and can be explained by the *trans*-regulatory environment in different tissues. Indeed, rs2736098 will lose its function if the supposed TF, i.e., USF1, is absent or rs2736098 surrounding region is competitively occupied by other proteins.

rs2736098 has been suggested to be significantly associated with carcinogenesis in multiple tissues besides breast, including lung, basal cell, bladder, prostate, cervix, liver, pancreas, colon, ovary, B cell, head and neck [29–41]. Further search in GWAS Catalog, UK BioBank (https://www.ukbiobank.ac.uk) and FinnGen (https://www.finngen.fi/fi) indicate that rs2736098 is also significantly

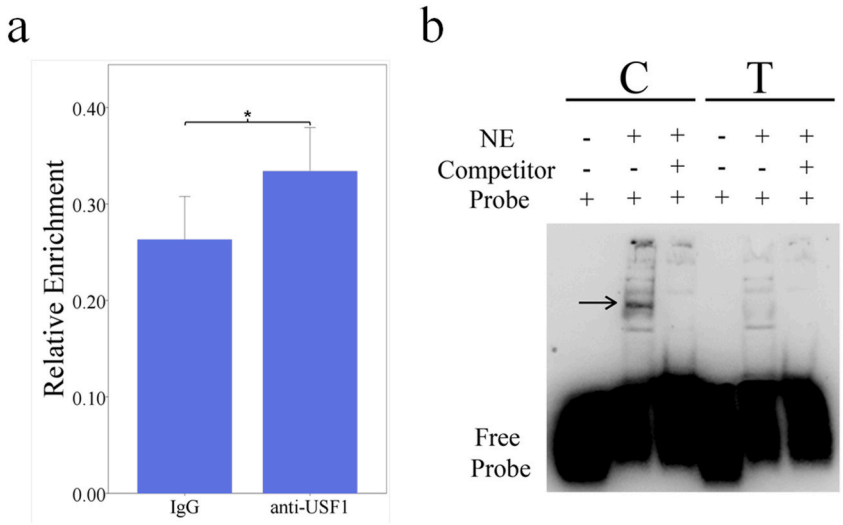

Fig. 3. Interaction between USF1 and rs2736098. Part a displays the relative enrichment of chromatin surrounding rs2736098 by USF1 antibody. *$P < 0.05$. Part b indicates binding affinity difference between rs2736098 alleles. The top line indicates different alleles. NE denotes nuclear extracts, and the arrow points out the position of protein-probe complex. The original image for part b is Fig. S4

chromatin immunoprecipitation, ChIP; electrophoretic mobility shift assay, EMSA; genome-wide association study, GWAS; Han Chinese in Beijing, CHB; Linkage disequilibrium, LD; MYC proto-oncogene, bHLH transcription factor, MYC; telomerase reverse transcriptase, TERT; transcription factor, TF; upstream transcription factor 1, USF1; Utah Residents with Northern and Western European Ancestry, CEU; Yoruba in Ibadan, Nigeria, YRI.

associated with count of platelet, red and white blood cell, neutrophil, basophil neutrophil and monocyte (all $P < 5 \times 10^{-8}$; results not shown) [42–44]. Considering the role of rs2736098 and the LD pattern in this locus, it can be concluded that the association is resulting from, at least partially, the *cis*-regulation of *TERT* by rs2736098.

Our and previous functional genomics work indicated that the allele in common haplotype, i.e., C of rs2736098, rs2736107, rs2736108 and rs2736109 and A of rs2853669, is the high expression one for *TERT* [15–17]. Therefore, the common haplotype should induce a high *TERT* expression. Moreover, there might be some synergistic effects among these five SNPs. However, due to the relatively long distance between rs2736098 and other four SNPs (>1264bp), we can not clone them into one segment and evaluate the synergistic effect by mutagenesis. Further gene expression analysis in individuals with recombination at this locus might better illuminate this issue.

The frequency of rs2736098 varies significantly in human populations. As shown in Fig. S5, the frequency of rs2736098C allele is ~54%, ~62%, ~73%, ~78% and ~92% in South Asian, East Asian, European, American and African from 1000 genomes project, respectively. Considering the function of rs2736098C allele, it can be concluded that *TERT* expression distribution might be different among populations. However, since cancer is a group of complex diseases due to multiple loci in genome, it is difficult to deduce the effect in cancer morbidity.

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Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Xin-Xin Zhang: Writing – original draft, Visualization, Validation, Investigation. **Xin-Yi Yu:** Investigation. **Shuang-Jia Xu:** Investigation. **Xiao-Qian Shi:** Investigation. **Ying Chen:** Investigation. **Qiang Shi:** Writing – original draft, Funding acquisition, Conceptualization. **Chang Sun:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27802.

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