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The role of transcription factor Sp1 in the regulation of gamma-glutamyl hydrolase gene expression by the rs3758149 polymorphism in CEM/C1 cells

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Gamma-Glutamyl hydrolase (GGH) plays an important role in the disposition of anti-folate analogs. Several studies noted the pharmacological relevance of rs3758149 C/T polymorphism located in the human *GGH* promoter. The present study aimed to investigate the role of rs3758149 C/T polymorphism and transcription factors in the regulation of *GGH* expression in human acute lymphoblastic leukemia (ALL) CEM/C1 cells. Compared with the rs3758149 T allele, the C allele showed significantly higher transcriptional activity in luciferase reporter assays, as well as a stronger binding affinity for the nuclear protein extracts in an electrophoretic mobility shift assay. Sp1 was identified as the target transcription factor that exhibited allele-specific binding to the location of rs3758149 C/T polymorphism in the chromatin immunoprecipitation assay. Overexpression of Sp1 led to enhanced *GGH* promoter activity and *GGH* mRNA expression in allele-specific manners. These findings suggested that Sp1 acted as a positive regulator of human *GGH* transcription through the rs3758149 polymorphism in CEM/C1 cells. This study contributed to the present understanding of the mechanisms underlying variable responses of ALL to anti-folates.

1. Introduction

Gamma-Glutamyl hydrolase (GGH) is a lysosomal peptidase involved in the disposition of anti-folate analogs such as methotrexate (MTX) (Chave et al. 2003; Schneider and Ryan 2006). It catalyzes the hydrolysis of glutamyl residues from γ-linked polyglutamates that are attached to anti-folate drugs by folypolyglutamate synthetase (FPGS) (Galivan et al. 2000). Polyglutamates of anti-folates act as stronger inhibitors of enzymes involved in one-carbon metabolism and are thus retained longer within cells than their monoglutamyl forms (Cheng et al. 2004). The efficacy of anti-folates depends on the concentration and persistence of intracellular polyglutamates (Galivan et al. 1999). An inverse relationship between GGH activity and the accumulation of MTX polyglutamates was previously shown in acute lymphoblastic leukemia (ALL) cells (Cheng et al. 2004). Increased MTX polyglutamates were observed in acute non-lymphoblastic leukemia cells incubated with a GGH inhibitor (Göker et al. 1993). GGH was one of the important factors that affected sensitivity to anti-folates (Schneider and Ryan 2006). Increased GGH activity was therefore found in human acute myeloid leukemia cells, human soft tissue sarcoma cells, and in rat hepatoma cells resistant to MTX (Rots et al. 1999; Li et al. 1993a; Rhee et al. 1993b).

There is marked inter-individual variability in human GGH activity (Cheng et al. 2004). GGH activity in human leukemia cells is regulated by genetic and epigenetic changes. The gene encoding human GGH is located on chromosome 8q12.23-13.1 (Chave et al. 2003). Higher GGH activity and less accumulation of MTX polyglutamates were demonstrated in hyperdiploid ALL cells with chromosome 8 trisomy (Cheng et al. 2005). Cheng et al. (2006) found that the methylation of two CpG islands (CpG1 and CpG2) in the GGH promoter resulted in reduced GGH mRNA expression and catalytic activity, with subsequent increased accumulation of MTX polyglutamates in ALL cells. Li et al. (2017) confirmed that the methylation of CpG1 or hypermethylation of CpG2 could reduce GGH mRNA expression in leukemia cells.

Several single nucleotide polymorphisms (SNP) were identified in the coding and promoter regions of the human *GGH* gene (Chave et al. 2003). A well-documented functional SNP is the rs11545078 C/T polymorphism (T127I) located on exon 5, which was associated with reduced GGH activity and greater accumulation of long-chain MTX polyglutamates in hyperdiploid ALL cells (Chave et al. 2003). The underlying mechanism involved a modification of the molecular surface structure of human GGH at its binding domain, which effectively decreased binding affinity to its substrates (Chave et al. 2003). Another widely investigated SNP is the rs3758149 C/T located in the promoter of *GGH*. Organista-Nava et al. (2010) found a significant association between the rs3758149 polymorphism and the relapse risk of ALL in a Mexican population. In our previous work, we also found that the rs3758149 polymorphism was associated with the proportion of MTX above the therapeutic threshold in Chinese children with ALL (Wang et al. 2014). SNPs in the promoter region may affect gene expression due to the gain/loss of transcription factor binding sites (Chave et al. 2003). However, functional studies on the rs3758149 polymorphism remained limited in ALL cells. The aim of the present study was to investigate the role of rs3758149 C/T polymorphism and transcription factors in the regulation of *GGH* expression in human ALL CEM/C1 cells.

2. Investigations and results

2.1. Rs3758149 C/T polymorphism affected GGH promoter activity

To determine whether the rs3758149 C/T polymorphism influenced *GGH* promoter activity *in vitro*, we performed luciferase reporter assays with two luciferase reporter constructs containing a C or T allele at rs3758149 in the *GGH* promoter. As shown in Fig. 1, promoters with a C allele showed significantly higher transcriptional activity than that of the T allele in CEM/C1 cells (4.8-fold, $P < 0.05$).

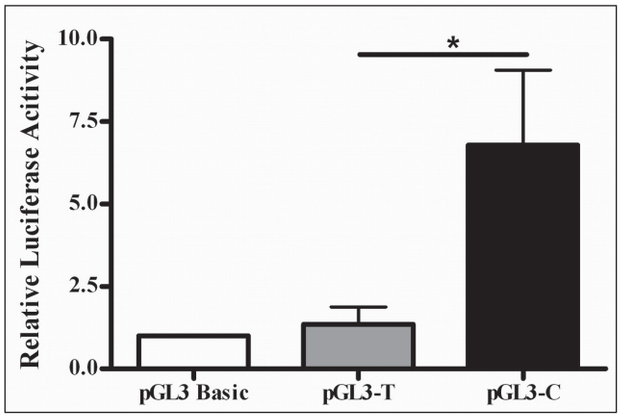

Fig. 1: Effects of rs3758149 C/T polymorphism on *GGH* promoter activity in human ALL CEM/C1 cells. Luciferase expression assays were performed with constructs containing a C or T allele at rs3758149 in the GGH promoter. Data are presented as mean±SD. *$P < 0.05$. Abbreviations: GGH, gamma-glutamyl hydrolase; ALL, acute lymphoblastic leukemia.

2.2. GGH rs3758149 C/T polymorphism modulated transcription factor affinity to the promoter

To verify whether the rs3758149 C/T polymorphism altered the binding affinity of transcription factors to the *GGH* promoter, we performed electrophoretic mobility shift assay (EMSA) with nuclear extracts from CEM/C1 cells and radiolabeled duplex oligonucleotide probes harboring either rs3758149 C or T allele. As shown in Fig. 2, the C allele had a stronger binding affinity for the protein–DNA complex as compared to the T allele. The formation of the DNA-protein complex was dramatically inhibited by unlabeled oligonucleotide probes containing a C or T allele. These results indicated that the T-allele probe had less affinity for the nuclear proteins of CEM/C1 cells than the C-allele probe.

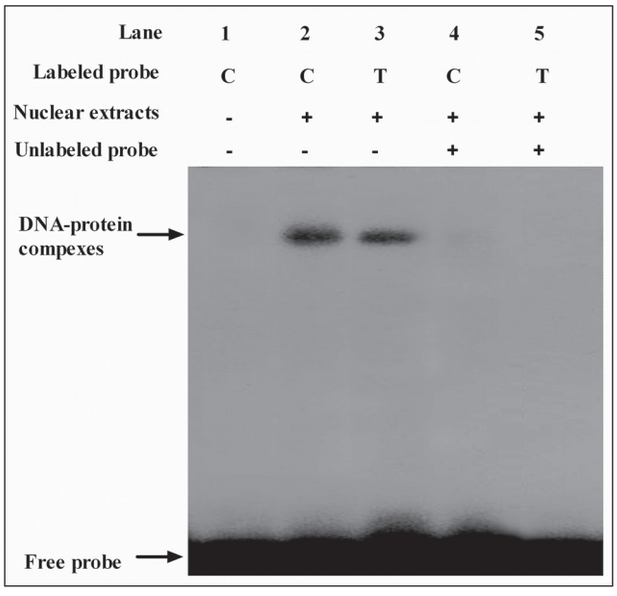

Fig. 2: Effects of rs3758149 C/T polymorphism on the binding affinity between nuclear extracts and the promoter of *GGH* in human ALL CEM/C1 cells. EMSAs were performed with probes containing rs3758149 C or T allele and nuclear extracts from CEM/C1 cells. Abbreviations: GGH, gamma-glutamyl hydrolase; ALL, acute lymphoblastic leukemia.

2.3. Sp1 was identified as the target transcription factor that exhibited allele-specific binding to the location of rs3758149 C/T polymorphism

According to bioinformatic analyses, a Sp1 binding site was predicted when the rs3758149 C allele was present, but not with a T allele. In order to test this hypothesis, lysates from CEM/C1 cells were subjected to chromatin immunoprecipitation (ChIP) assays.

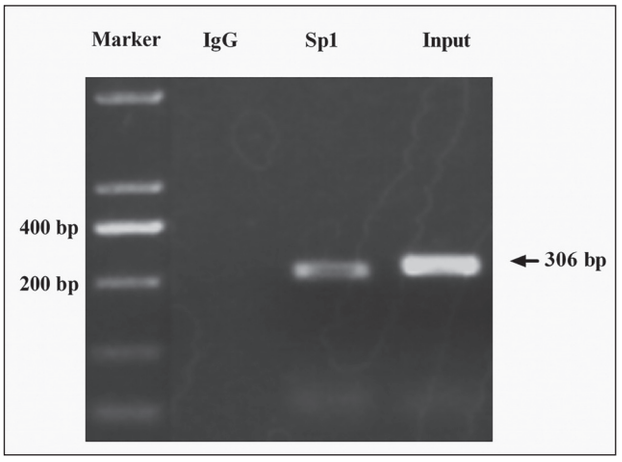

Fig. 3: Chromatin immunoprecipitation assays in human ALL CEM/C1 cells using Sp1 and IgG antibodies. Abbreviations: ALL, acute lymphoblastic leukemia.

As shown in Fig. 3, the -401 position of the *GGH* promoter could be precipitated with specific Sp1 antibody. However, no amplification products were observed for the immunoprecipitation with nonspecific rabbit IgG, serving as the negative control. These results indicated that Sp1 could bind to the promoter of *GGH*, and the difference in *GGH* promoter activities between the rs3758149 C and T alleles was presumably due to their distinct binding affinity to Sp1.

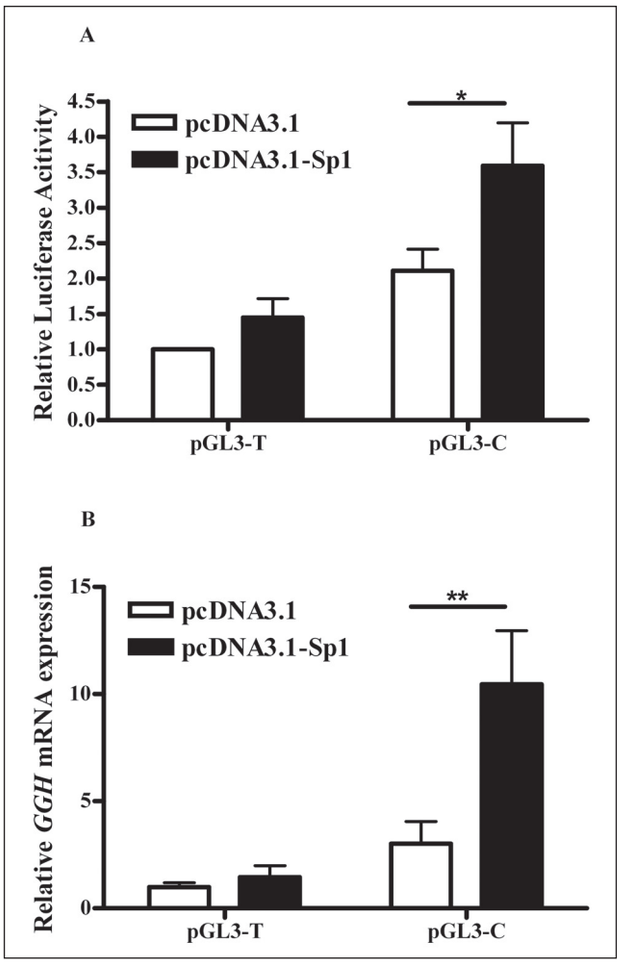

Fig. 4: Analysis of *GGH* promoter activity (A) and mRNA expression (B) in CEM/C1 cells cotransfected with luciferase reporter plasmids containing rs3758149 C or T allele and pcDNA3.1 (control) or pcDNA3.1-Sp1 expression plasmid. Data are presented as mean±SD. *$P < 0.05$, **$P < 0.01$. Abbreviations: GGH, gamma-glutamyl hydrolase; ALL, acute lymphoblastic leukemia.

2.4. Sp1 regulated GGH expression in allele-specific manners

To investigate the role for Sp1 in the regulation of *GGH* expression and to determine whether Sp1 was responsible for the rs3758149 polymorphism-related changes of *GGH* promoter activity, CEM/C1 cells were co-transfected with an Sp1 expression vector (pcDNA3.1-Sp1) along with the luciferase reporter plasmid driven by the *GGH* promoter with rs3758149 C/T alleles. As shown in Fig. 4A, overexpression of Sp1 led to significantly increased luciferase expression driven by the *GGH* promoter with a rs3758149 C allele in CEM/C1 cells (1.7-fold, $P < 0.05$). However, the increased luciferase expression was not significant in cells co-transfected with the T-allele-containing plasmid, even when Sp1 was overexpressed. To further confirm these results, we examined *GGH* mRNA levels from CEM/C1 cells co-transfected with the two constructs. As shown in Fig. 4B, overexpression of Sp1 also led to significantly higher *GGH* mRNA expression in CEM/C1 cells co-transfected with a construct harboring the rs3758149 C allele (3.5-fold, $P < 0.01$). In contrast, the increased *GGH* mRNA expression was not significant in cells co-transfected with the construct harboring a rs3758149 T allele, even when Sp1 was overexpressed. Therefore, these data supported the hypothesis that Sp1 could have allele-specific effects on *GGH* expression by increasing the transcriptional activity of the C-allele promoter, due to a higher binding affinity to this region.

3. Discussion

GGH plays an important role in the metabolism of folate and anti-folates (Chave et al. 2003; Schneider and Ryan 2006). Indeed, GGH activity was associated with the response to anti-folates (Schneider and Ryan 2006). Increased GGH activity might lead to MTX resistance in ALL, osteosarcoma, and rheumatoid arthritis (RA) (Schneider and Ryan 2006). GGH activity was also directly related to its mRNA expression in ALL cells (Cheng et al. 2006). Thus, factors that regulate GGH expression are likely to be important determinants of antifolate sensitivity. In the present study, we showed that the transcription factor Sp1 was involved in the regulation of *GGH* expression by the rs3758149 polymorphism in human ALL CEM/C1 cells.

Several studies noted the pharmacological relevance of rs3758149 C/T polymorphism in different diseases. Koomdee et al. (2012) found that GGH rs3758149 CT and TT genotypes increased the risk of severe leukopenia and thrombocytopenia in ALL children treated with high dose MTX. Dervieux et al. (2004) reported that the rs3758149 C/T polymorphism was predictive of MTX polyglutamate levels in patients with rheumatoid arthritis. Oppeneer et al. (2012) showed that a rs3758149 C/T polymorphism was associated with plasma homocysteine levels in a healthy Singaporean population. Hegyi et al. (2017) demonstrated that the rs3758149 C/T polymorphism affected the area under the curve of MTX in children with osteosarcoma. However, some studies did not find an association between the investigated polymorphism with intracellular MTX polyglutamates and the response to MTX in patients with RA. This inconsistency might be due to ethnicities, sample sizes, MTX dosage, and genetic variations of other enzymes involved in MTX metabolism.

Chave et al. (2003) reported higher transcriptional activity of the rs3758149 T variant in HepG2 and MCF-7 cells. However, opposite results were observed in our study. Reporter constructs with a rs3758149 C variant showed significantly higher promoter activity in CEM/C1 cells. This might be due to the tissue-specific expression patterns of *GGH*. Dervieux et al. (2004) found a significantly higher risk of MTX toxicities in RA patients with a rs3758149 CC genotype. Hashiguchi et al. (2016) demonstrated higher mRNA expressions in healthy Japanese adults with a rs3758149 CC genotype. These results supported our observation, indicating that the rs3758149 C variant might be associated with high GGH activity.

The molecular mechanisms by which the rs3758149 C/T polymorphism regulated GGH expression remain poorly understood. Chave et al. (2003) performed a search in the TRANSFAC 4.0 database and found that the rs3758149 T allele led to the loss of binding sites for MZF1 and IK2. However, they did not provide the experimental evidence for this hypothesis. It was found that the rs3758149 C/T polymorphism was within an Sp1 Binding Site with AliBaba 2.1. We performed an EMSA assay and noted that the rs3758149 C allele had a higher affinity for nuclear proteins. Sp1 also exhibited allele-specific binding to the location of rs3758149 C/T polymorphism in ChIP assays. Overexpression of Sp1 enhanced *GGH* promoter activity and mRNA expression in allele-specific manners. These observations indicated that Sp1 might have a role in the regulation of GGH expression. Given the association between GGH activity and the responses to anti-folates, Sp1 might alter the sensitivity to anti-folates through the regulation of GGH expression. However, further research is needed to verify this claim. Gazzoli and Kolodner (2003) also observed that Sp1 regulated the human MSH6 Gene through polymorphisms in its promoter. This meant that Sp1 could regulate gene expression in an allele-specific manner.

There were several limitations to our study. Firstly, there was no concentration gradient in our EMSA assay. Secondly, we did not investigate whether Sp1 knockdown influenced *GGH* expression. Thirdly, studies on the role of Sp1 in the regulation of *GGH* expression in other leukemic cell lines and human samples are required to support our findings.

In summary, we demonstrated that the transcription factor Sp1 was involved in the regulation of GGH expression by the rs3758149 polymorphism in human ALL CEM/C1 cells. The study contributed to our present understanding of the mechanisms underlying variable responses of ALL to anti-folates.

4. Experimental

4.1. Cells and plasmids

Human ALL CEM/C1 cells were grown and maintained as previously described (Wang et al. 2018). The promoter regions of *GGH* containing the rs3758149 C or T allele (Invitrogen, Carlsbad, CA, USA) were cloned into pGL3 Basic vectors (Promega, Madison, WI, USA) to generate two SNP constructs (PGL3-C and PGL3-T). The primers utilized were sense 5'-TCCCCCGGGttttttgtgtgtaacgccggg-3'; antisense 5'- CCCAAGCTTgaaacgcctggggcgggtac-3'. The Sp1 cDNA was cloned into the mammalian expression vector pcDNA3.1 to construct pcDNA3.1-Sp1 expression plasmid. The primers utilized were sense 5'-CTAGCTAGCtttttgtgtgtaacgccggg-3'; antisense 5'-ATAAGAATGCGGCCGCgaaacgcctggggcgggtac-3'. All constructs were verified by DNA sequencing.

4.2. Transfection and luciferase reporter assay

CEM/C1 cells with a density of 1×10^5 cells/ml were seeded in 96-well plates 48 h before transfection. The pGL3-C or pGL3-T reporter plasmids were transfected into CEM/C1 cells, or co-transfected with a pcDNA3.1-Sp1 plasmid or empty pcDNA3.1 vector by electroporation using an Amaxa Nucleofector II system. After 48 h, luciferase activities were measured with the Luciferase Reporter Assay Kit (Beyotime, Haimen, China) according to the manufacturer's instructions. The luciferase signals from the *GGH* promoter reporter constructs were calculated and normalized to the Renilla luciferase activity. Relative luciferase activities were calculated with the luciferase signals from the *GGH* promoter reporter constructs normalized to the transfection control plasmids. Each sample was tested in triplicate.

4.3. Electrophoretic mobility shift assay (EMSA)

The 26 bp oligonucleotides bearing the rs3758149 C or T allele were used as probes. Complementary strands were radiolabeled with γ^{32}P-ATP using a T4 Polynucleotide Kinase (Beyotime, Haimen, China). The sequences of the sense probes were 5'-ggacacccaacctcccctctcgagga-3' and 5'-ggacacccaacctccctctcgagga-3'. The sequences of the antisense probes were 5'-tcctcgagagggaggttgggtgtcc-3' and 5'-tcctcgagagggaggttgggtgtcc-3' (the polymorphic sites were underlined). Nuclear protein extracts were prepared from CEM/C1 cells using the Nuclear Extraction Kit (Beyotime, Haimen, China). *In vitro* binding reactions between probes and nuclear extracts were conducted according to the manufacturer's protocols. The reaction products were separated on a 4 % polyacrylamide gel and visualized by autoradiography.

4.4. Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed in CEM/C1 cells using the ChIP Assay Kit (Beyotime, Haimen, China). Chromatin was crosslinked with 1 % formaldehyde for 10 – 15 min and subsequently quenched by a 125 mM glycine solution. CEM/C1 cells were lysed and sonicated to shear DNA to a length between 200-1000 bp. The sheared chromatin was subjected to immunoprecipitation with antibodies against Sp1 or nonspecific rabbit IgG (Beyotime, Haimen, China). The immunoprecipitated target of the *GGH* promoter region was identified by PCR analysis using the following primers (which generated a 306-bp fragment): sense 5'-TCCCGTCTTCACTTCCTAC-3'; antisense 5'- CCTTCCCTTTCAACTGTTAC -3'.

4.5. Quantitative real-time PCR (qPCR)

RNA from CEM/C1 cell were isolated using a TRIzol reagent (Tiangen, Beijing, China) according to the manufacturer's instructions. All RNA samples were subjected to reverse transcription using a PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Dalian, China). qPCR analysis was performed to measure *GGH* mRNA levels on an ABI7500 PCR system (Applied Biosystems, Foster City, CA, USA), with β-actin as an internal reference gene. The relative expression levels of *GGH* mRNAs were calculated using the ΔΔCt method (Livak and Schmittgen 2001). Each reaction was done in triplicate. The primers used for *GGH* were sense 5'-CCAAGAAGC-CCATCATCGGAA-3'; antisense 5'-ACTGGTACAACTCTCGCACC-3', and for β-actin were sense 5'-CGCGGCTACAGCTT CACCAC-3'; antisense 5'-GGAAG-CAGCCGTGGCCAT -3'.

4.6. Statistical analysis

Graphpad Prism Software version 4.0 was used for all statistical analyses. Data were expressed as mean±SD. Comparisons of luciferase reporter gene expression or mRNA levels from cells transfected with different plasmids between two groups, were performed using unpaired two-tailed Student's t test. $P < 0.05$ was considered statistically significant.

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Conflicts of interest: All authors declare no competing interests.

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