Table 3. Changes in concentration of the intracellular metabolites involved in glycolysis, PPP, TCA cycle and glutaminolysis in the *GLO1*-knockdown gastric cancer cells, NUGC3-sh*GLO1* and MKN28-sh*GLO1*

Pathway cluster	*ID*	*Compound name*	*Comparative analysis*			
			NUGC3-shGLO1/NUGC3-scr		*MKN28-shGLO1/MKN28-scr*	
			Ratio	*P-value*[a]	*Ratio*	*P-value*[a]
Glycolysis	A_0088	Glucose 6-phosphate	0.4	0.064	0.3	0.022*
Glycolysis	A_0087	Fructose 6-phosphate	0.7	0.000***	0.4	0.018*
Glycolysis	A_0111	Fructose 1,6-diphosphate	0.9	0.238	0.6	0.001***
Glycolysis	A_0094	2,3-Diphosphoglyceric acid	1.0	0.686	0.8	0.093
Glycolysis	A_0059	3-Phosphoglyceric acid	0.7	0.011*	0.4	0.001**
Glycolysis	A_0058	2-Phosphoglyceric acid	0.7	0.142	0.4	0.004**
Glycolysis	A_0045	Phosphoenolpyruvic acid	0.7	0.023*	0.4	0.001**
Glycolysis	A_0004	Pyruvic acid	0.6	0.267	1 <	NA
Glycolysis	A_0006	Lactic acid	0.6	0.004**	0.4	0.002**
Glycolysis	A_0089	Glucose 1-phosphate	0.6	0.009**	0.3	0.000***
Glycolysis	A_0048	Dihydroxyacetone phosphate	0.3	0.012*	0.5	0.009**
Pentose phosphate pathway	A_0088	Glucose 6-phosphate	0.4	0.064	0.3	0.022*
Pentose phosphate pathway	A_0095	6-Phosphogluconic acid	0.4	0.004**	0.5	0.004**
Pentose phosphate pathway	A_0082	Ribulose 5-phosphate	0.6	0.028*	0.5	0.002**
Pentose phosphate pathway	A_0083	Ribose 5-phosphate	0.6	0.175	0.4	0.001**
Pentose phosphate pathway	A_0087	Fructose 6-phosphate	0.7	0.000***	0.4	0.018*
Pentose phosphate pathway	A_0097	Sedoheptulose 7-phosphate	0.9	0.135	0.4	0.005**
Pentose phosphate pathway	A_0073	Erythrose 4-phosphate	NA	NA	NA	NA
TCA cycle	A_0066	Citric acid	0.6	0.017*	0.5	0.004**
TCA cycle	A_0051	*cis*-Aconitic acid	0.6	0.036*	0.5	0.002**
TCA cycle	A_0067	Isocitric acid	0.8	0.177	0.6	0.030*
TCA cycle	A_0031	2-Oxoglutaric acid	0.4	0.012*	1 <	NA
TCA cycle	A_0016	Succinic acid	1.4	0.020*	0.4	0.001**
TCA cycle	A_0012	Fumaric acid	0.8	0.082	0.6	0.004**
TCA cycle	A_0024	Malic acid	0.8	0.186	0.6	0.001***
Glutaminolysis	C_0061	Glutamine	0.4	0.025*	0.3	0.029*
Glutaminolysis	C_0064	Glutamic acid	0.8	0.014*	0.5	0.013*
Glutaminolysis	A_0031	2-Oxoglutaric acid	0.4	0.012*	1 <	NA

Abbreviations: NA, not available; PPP, pentose phosphate pathway; TCA, tricarboxylic acid. [a]Welch *t*-test: * < 0.05, ** < 0.01, *** < 0.001.

gastric cancer cells. These include several cancer-related genes such as *PPIL1*,[27] *PIM1*,[28] *GLO1*,[21,22] *TFEB*,[29] *CCND3*,[11,12] *CUL7*,[30] *C6orf108*,[31] *VEGFA*[14] and *CDC5L*.[13] To focus generally on growth-promoting genes, we examined activities for *in vitro* colony formation and *in vivo* tumor formation using HEK293 and NIH3T3 cells, because no immortalized gastric epithelial cells were available. More than two-fold enhanced colony-forming activities were observed in six genes by enforced overexpression (Figure 2b). *In vivo* tumor-formation efficiencies were: *GLO1*, 83%; *C6orf64*, 67%; *GNMT*, 38%; *GTPBP2*, 33%; *C6orf130*, 17%; and *TBCC*, 17% (Table 2). These results demonstrated that *GLO1* is the most likely oncogene in the 6p21 amplicon. Furthermore, these genes and some others such as *CCND3*, *C6orf108* and *VEGFA* showed marked enhancement of growth-promoting activity in the presence of *GLO1* overexpression (Figure 2c). Therefore, we concluded that the 6p21 amplicon includes at least six potential oncogenes and several cell growth-supporting genes and that all of these genes might function cooperatively in cancer formation and/or progression. Recent reports have indicated that *GNMT* encodes an enzyme glycine *N*-methyltransferase, involved in methionine metabolism, which has a role in liver regeneration,[32] and that *C6orf108* encodes an enzyme, deoxynucleoside 5′-monophosphate *N*-glycosidase, which is implicated in purine or pyrimidine salvage and stimulation of glycolysis by supplying deoxyribose 5-phosphate (P) as an energy source.[33] Chromosomal amplification may be the most efficient mechanism for activating a subset of 6p21 genes favorable for cancer cell growth. Although a recent search has identified *GLO1* to be the commonly amplified gene in various cancer cells,[22] our study is the first to provide experimental evidence that *GLO1* exhibited tumorigenic potential.

The glyoxalase system comprises two enzymes, GLO1 and GLO2, and sequentially metabolizes cytotoxic MG to D-lactate via the intermediate *S*-D-lactoylglutathione, using reduced glutathione as a cofactor (Figure 4f). MG is mainly produced from dihydroxyacetone-P or glyceraldehyde 3-P during glycolysis. Accumulation of MG causes oxidative stress and damage to cells, then leads to apoptosis.[34] Therefore, overexpression of *GLO1* in a rapidly proliferating cancer cell may confer a growth advantage by reducing MG stress, in which aerobic glycolysis (the Warburg effect) is predominantly active.[35–37] Metabolome analysis has demonstrated an enhanced glycolysis and glutaminolysis, which is an alternative energy-producing pathway involving a glucose-independent tricarboxylic acid cycle, in gastric cancer tissues.[18] In this study, we observed that the concentration of many metabolic intermediates involved in central carbon metabolism was significantly lowered in both gastric cancer cells NUGC3 and MKN28 when *GLO1* is stably downregulated (Table 3 and Supplementary Figure S5). We also showed that the expression ratio of *GLO1/GLO2* mRNA tends to be high in BBGC-insensitive relative to BBGC-sensitive cells (Figure 3c, right).

Furthermore, *GLO1* was found to function as a modulator of the transcriptional activities of NF-κB and AP-1 (Figures 4a–d). The mechanism as to how *GLO1* influences on the activities of NF-κB and AP-1 is not clear, but two recent reports have demonstrated that *GLO1* silencing or MG administration in prostate cancer cell induces mitochondrial apoptotic pathway through inactivation of NF-κB by the decrease in serine 32-phosphorylated IκBα and the increase in total IκBα levels.[38,39] A previous promoter sequence analysis of *GLO1* gene has indicated a possible positive regulation by NF-κB and AP-1 under oxidative stress.[40] Also, *GLO1* has been

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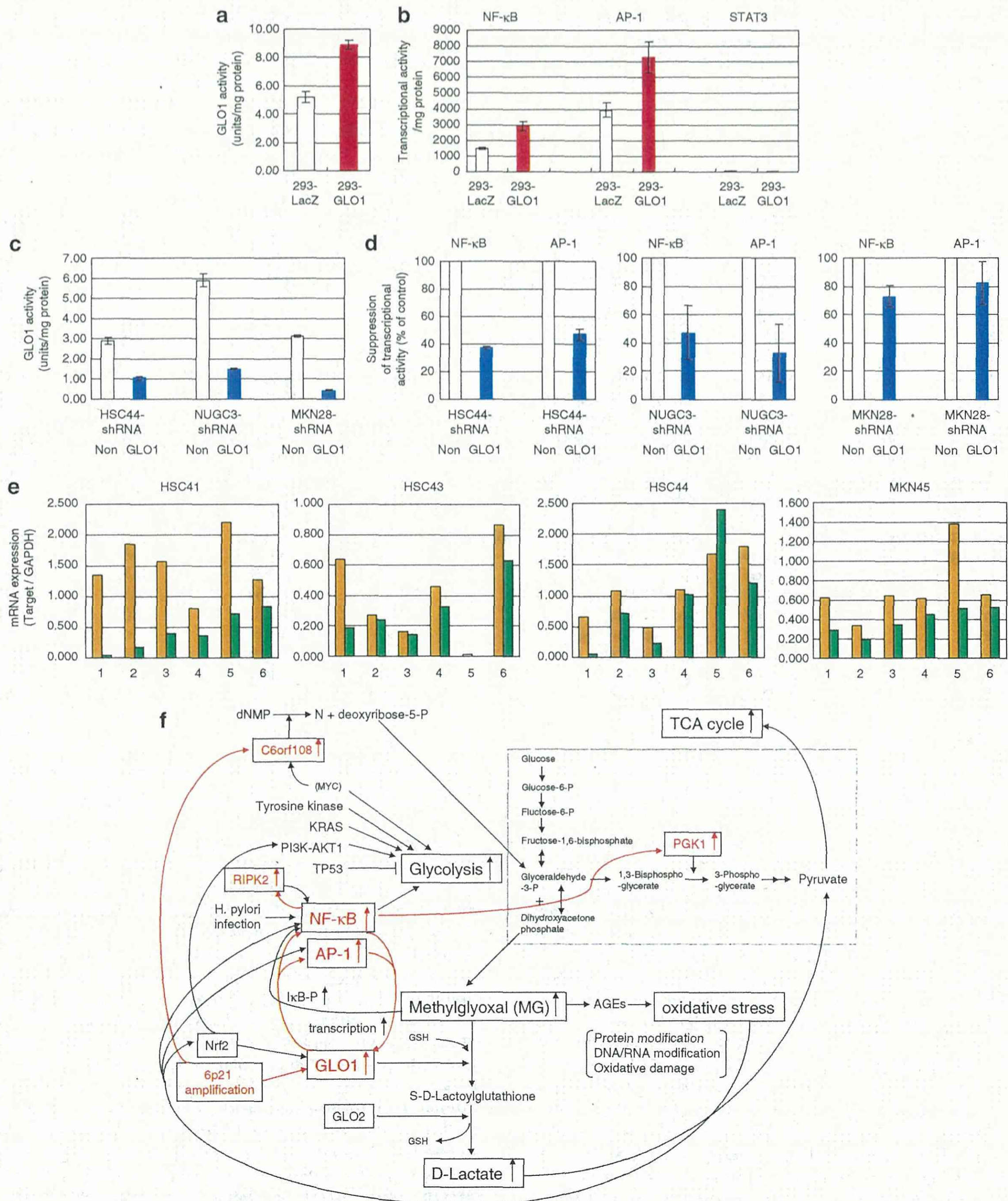

Figure 4. Function of *GLO1* as a modulator of the transcriptional activities of NF-κB and AP-1. (**a**) GLO1 activity is upregulated in a *GLO1*-overexpressing HEK293 clone (red) in comparison with a *LacZ*-overexpressing HEK293 clone (white). (**b**) Enhancement of transcription factor-dependent reporter gene expression in the *GLO1*-overexpressing clone. The transcription factor-dependent luciferase activity was measured using a cell lysate prepared at 24 h after transfection. Data represent means ± s.e.m. (**c**) GLO1 activity is decreased in a *GLO1* shRNA-expressing gastric cancer cells (blue) in comparison with a non-targeting shRNA-expressing cells (white). (**d**) Suppression of NF-κB- and AP-1-dependent reporter gene expression levels in the *GLO1* shRNA-expressing gastric cancer cells. Data represent means ± s.e.m. (**e**) NF-κB target genes upregulated in the tumors expressing high level of *GLO1* are downregulated in a *GLO1* shRNA-expressing gastric cancer cells (green) in comparison with a non-targeting shRNA-expressing cells (orange). Messenger RNA expression was quantitatively measured about *GLO1* and five NF-κB target genes. Lanes: 1. *GLO1*, 2. *BIRC2*, 3. *RIPK2*, 4. *TNFRSF10B* (variant 1), 5. *S100A6*, 6. *PGK1*. (**f**) Schematic representation of cancer-specific activated glycolysis and *GLO1* function. Upregulation of GLO1 via chromosome 6p21 amplification or transcriptional regulation by NF-κB, AP-1 and Nrf2 leads to an activated glycolysis through relieving MG/oxidative stress. Furthermore, *GLO1* can activate the transcriptional activities of NF-κB and AP-1.

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shown to be a target of another stress-responsive transcription factor Nrf2 that functions for the sustained activation of phosphatidylinositol 3′-kinase-AKT signaling and the metabolic reprogramming to contribute maintenance of malignant phenotype of cancer.[41] We identified 21 upregulated NF-κB target genes in a class of *GLO1* highly expressing gastric tumors, including an NF-κB activator *RIPK2* and a glycolytic enzyme *PGK1* (Supplementary Table S8).

Based on these observations, *GLO1* would belong to a novel type of metabolic oncogene that regulates both cancer-specific metabolism and gene regulation, and *IDH1* is a prototype of this category.[42] Figure 4f schematically shows metabolomic reprogramming, especially in glycolysis, by cancer-related genes,[35,36] including our findings: that chromosome 6p21 amplification causes multiple gene overexpression, including several metabolic oncogenes such as *GLO1* and *C6orf108*; that GLO1 activates glycolysis through controlling MG/oxidative stress; that *GLO1* overexpression upregulates the transcriptional activities of NF-κB and AP-1; and that the NF-κB target genes, such as an NF-κB activator *RIPK2* and a glycolytic enzyme *PGK1*, are differentially upregulated in *GLO1*-overexpressing gastric tumors.

In conclusion, the present data indicate that 6p21 genomic amplification has considerable clinical significance and that the locus includes a number of possible therapeutic targets for gastric cancer. In particular, several novel enzymatic activities would be feasible molecular targets for the development of new drugs. GLO1 is one of such attractive targets for gastric cancer, and development of an appropriate indicator of its sensitivity to inhibitors is warranted to ensure effective treatment.

MATERIALS AND METHODS

Clinical samples and cell lines

Gastric tissue samples were collected from patients who underwent gastric surgery at the National Cancer Center Hospital, Tokyo, Japan between 1998 and 2001 as described previously.[43] Clinicopathological features of the 163 cases of gastric cancer are summarized (Supplementary Table S1). This study was approved by the Ethics Committee of the National Cancer Center and written informed consent was obtained from all patients. Human gastric cancer cell lines, human HEK293 and mouse NIH3T3 cell lines were obtained from the Riken Cell Bank (Ibaraki, Japan) or the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). The HSC series of human gastric cancer cell lines were established at our institution and have been characterized previously.[44] Identification of cancer cell line was performed using Cell ID System (Promega, Madison, WI, USA; Supplementary Table S2).

aCGH and expression microarray analyses

DNA sample preparation from gastric tumor cells and matched normal gastric epithelial cells isolated by laser capture microdissection and array hybridization were carried out as described previously,[43] using our custom-made bacterial artificial chromosome arrays, MCG Whole Genome Array-4500 and MCG Cancer Array-800.[5,43] Total RNA was extracted from frozen gastric tissue samples by homogenizing in TRIZOL Reagent (Invitrogen, San Diego, CA, USA) and purified using RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany). Comparative expression analysis was performed using a GeneChip Human Genome U133 Plus 2.0 Array in accordance with the manufacturer's protocols (Affymetrix, Santa Clara, CA, USA).[45]

Gene knockdown analysis with siRNA or lentiviral shRNA

Pre-designed siRNA (Dharmacon siGenome SMART pool) targeting the 6p21 individual genes and a non-targeting siRNA were purchased from Thermo Scientific (Lafayette, CO, USA). The shRNA sequences targeted for *GLO1* (5′-GTTCTTGGAATGACGCTAA-3′) and for a non-targeting negative control (5′-TAATATCGAGTATGCTCGG-3′, designed by B-Bridge International at Cupertino, CA, USA) were synthesized together with the 5′- and 3′-flanking sequences and cloned into the pSIF1-H1-Puro vector (System Biosciences, Mountain View, CA, USA). Sequences are as follows: *GLO1*-sense: 5′-GATCCGTTCTTGGAATGACGCTAACTTCCTGTCAGATTAGCGTCATTCCAAGAACTTTTTG-3′; *GLO1*-antisense: 5′-AATTCAAAAAGTTCTTGGAATGACGCTAATCTGACAGGAAGTTAGCGTCATTCCAAGAACG-3′; non-targeting-sense: 5′-GATCCGTAATATCGAGTATGCTCGGCTTCCTGTCAGACCGAGCATACTCGATATTATTTTTG-3′; and non-targeting-antisense: 5′-AATTCAAAAATAATATCGAGTATGCTCGGTCTGACAGGAAGCCGAGCATACTCGATATTACG-3′. Human gastric cancer cells were transfected with each siRNA using Lipofectamine 2000 (Invitrogen) or infected with lentivirus at a multiplicity of infection of 5. The infected cells were selected by incubation with 2 μg/ml puromycin (Sigma-Aldrich, St Louis, MO, USA), and the cell clones stably expressing a decreased amount of *GLO1* mRNA were isolated. For transient assay, reverse transcription (RT) was performed on cell lysates 1 day after transfection using a FastLane Cell RT-PCR kit (Qiagen). Real-time PCR was carried out using the universal probe library (Roche, Indianapolis, IN, USA) and gene-specific PCR primers or TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) (Supplementary Table S3). Cell growth inhibition was evaluated by CellTiter-Glo Luminescent Cell Viability Assay (Promega).

Construction of gene expression vectors, plasmid transfection and colony-formation assay

Individual full-length cDNAs of the 6p21 genes were synthesized from a commercial human stomach cDNA (Clontech, Mountain View, CA, USA) or gastric cancer cell line cDNA by nested PCR amplification using gene-specific primers (Supplementary Table S4) and cloned into the pcDNA3.1D/V5-His-TOPO vector (Invitrogen). For double transfection experiments, *GLO1* cDNA (+V5-His tag) was transferred to the pcDNA3.1/Zeo (+) vector. Introduction of recombinant plasmid DNA into HEK293 or NIH3T3 cells was performed using an Amaxa Nucleofector electroporator (Lonza, Basel, Switzerland). Efficiency of colony formation was evaluated by counting Giemsa-stained colonies after 12 days of growth under drug selection.

Tumor-formation assay in nude mice

In all, 1×10^6 HEK293- or NIH3T3-derived polyclonal cells expressing the 6p21 genes in 0.2 ml of phosphate-buffered saline were injected subcutaneously on the flanks of 8-week-old female BALB/c nude mice (CLEA Japan, Tokyo, Japan) on day 0. Tumor sizes were measured in two dimensions at once a week for 3 months. The protocol of the experiment was approved by the Committee for Ethics in Animal Experimentation and conducted in accordance with the Guidelines for Animal Experiments of the National Cancer Center.

Promoter reporters and dual luciferase assays

One hundred thousand cells in a 24-well format were transfected with 0.4 μg each of pNFκB-Luc, pAP1-Luc or pSTAT3-TA-Luc (Clontech) using Lipofectamine 2000. Transfection efficiency was normalized by cotransfection with 0.04 μg of pRL-TK (Promega) containing a full-length renilla luciferase gene under control of the herpes simplex virus thymidine kinase promoter. Twenty-four hours after transfection, the cells were lysed in passive lysis buffer. Firefly luciferase and renilla luciferase activities were quantified using the Dual-Luciferase Reporter Assay System and a GloMax 96 Microplate Luminometer (Promega).

GLO1 enzyme assay and cytotoxicity assay

GLO1 enzyme activity was analyzed according to a standard spectrophotometric method monitoring the rate of formation of *S*-D-lactoylglutathione.[46] The drug sensitivity of human gastric cancer cell lines was evaluated by cell growth inhibition. Five thousand cells in a 96-well format were incubated with various concentrations of BBGC for 24 h,[20] and then the cell viability was estimated by CellTiter-Glo Luminescent Cell Viability Assay.

Metabolome analysis

Intracellular metabolites in the *GLO1*-knockdown gastric cancer cell and its reference cell grown in a standard medium were extracted by ice-cold methanol according to the manufacturer's instructions. All charged metabolic intermediates were quantitatively analyzed using a CE-TOFMS method by HMT (Human Metabolome Technologies, Inc., Tsuruoka, Japan).[18]

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Statistical analyses

Statistical analyses were performed using the JMP5.1 (SAS Institute Japan, Tokyo, Japan) and Statview 5.0 (Abacus Concepts, Piscataway, NJ, USA) statistical software packages. The overall survival curve was calculated by JMP5.1 using the Kaplan–Meier method and log-rank test. The Cox proportional hazards model was used for multivariate analysis to determine independent factors related to survival and recurrence, based on the variables selected by univariate analysis in Statview 5.0.

ABBREVIATIONS

aCGH, array-based comparative genomic hybridization; AP-1, activator protein-1; BAC, bacterial artificial chromosome; BBGC, S-p-bromobenzyl glutathione cyclopentyl diester; CE-TOFMS, capillary electrophoresis-time-of-flight mass spectrometry; CNA, copy number alteration; GLO1, glyoxalase I; GLO2, glyoxalase II; MG, methylglyoxal; NF-κB, nuclear factor kappa B; PPP, pentose phosphate pathway; P, phosphate; shRNA, short hairpin RNA; siRNA, small interfering RNA; TCA, tricarboxylic acid.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

Study concept and design: FH, MO and TS. Provision of study materials or patients: HT, HK, JI, II and KY. Acquisition of data: FH, YA, NO, HS and MM. Data analysis and interpretations: FH, YA, NK and TS. Manuscript writing: FH and TS.

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