immunohistochemistry decreased expression was observed for MLH1, MSH2 and MSH6 in a subset of initial low- and high-grade astrocytomas, but DNA copy number status and paired recurrences were not assessed [46]. A larger cohort of paired samples will be needed to determine if loss of heterozygosity of *MGMT* and/or MMR genes in initial tumors has predictive or prognostic value. Contrary to primary GBM, where copy number loss of the entire chromosome 10 is a frequent event [4], we observed variability in the size of the region lost in initial LGG and secondary GBM in our cohorts [55]. *MGMT* hypermethylation and corresponding impaired MGMT activity prior to TMZ treatment could also be a predisposing factor, but we did not detect statistically significant differences when analyzing *MGMT* methylation level alone between the initial tumors of the TMZ-HM and TMZ-non-HM subgroups. An alternative hypothesis is that, because TMZ-HM tumors appear to derive from a very limited number of cells, *MGMT* methylation in a small number of cells in the initial tumor may allow positive selection and hypermutation. Other studies with variable designs, and predominantly examining HGGs, were also unable to identify a correlation between MGMT methylation and MMR status [18, 36]. Similar to GBM [15, 21, 23], variation in *MGMT* methylation levels among multiple regions of the initial LGG of our patients was negligible, suggesting that single samples of the initial and recurrent tumor may be sufficient to elucidate temporal patterns. However, because the TMZ-HM group had an increased level of *MGMT* methylation relative to more variable patterns in the other groups, recurrences in the TMZ-HM group may exhibit greater intratumoral heterogeneity if the initial tumor resection was incomplete and sampling at recurrence included hypermutated and non-hypermutated regions.

The results presented here and in prior studies [1, 5], along with the well-established mechanisms of DNA repair by MMR and MGMT, further suggest that compromised DNA repair contributes to the onset of hypermutation and subsequent malignant transformation. Taken together, the data suggest a working model in which a hypermutated tumor arises through clonal expansion of cells with high levels of *MGMT* methylation, pre-existing loss of heterozygosity of a key MMR gene and/or *MGMT*, and TMZ-associated mutation in MMR genes. Tumor tissue and clinical data from LGG patients participating in clinical trials with TMZ treatment will be required to follow-up these initial findings [59, 60] and to assess the clinical relevance of the TMZ-associated hypermutator phenotype.

Data availability

Whole exome sequence data are uploaded to the European Genome-phenome Archive (EGA) for patients 1–23 (accession number EGAS00001000579), and shallow whole-genome sequencing data of patients 90–296 (EGAS00001000643). Data of patient 24 was deposited to the Japanese Genotype–phenotype Archive under accession number JGAS00000000004.

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References

1. Cancer Genome Atlas Research Network (2008) Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature 455:1061–1068
2. Bady P, Sciuscio D, Diserens AC, Bloch J, van den Bent MJ, Marosi C, Dietrich PY, Weller M, Mariani L, Heppner FL, Mcdonald DR, Lacombe D, Stupp R, Delorenzi M, Hegi ME (2012) MGMT methylation analysis of glioblastoma on the Infinium methylation BeadChip identifies two distinct CpG regions associated with gene silencing and outcome, yielding a prediction model for comparisons across datasets, tumor grades, and CIMP-status. Acta Neuropathol 124:547–560
3. Baumert B, Mason WP, Ryan G, Bromberg JE, van den Bent M, Hoang-Xuan K, Brandes AA, Kantor G, Taphoorn MJ, Ben Hassel M, Rees J, Wick W, von DA, Hartmann C, Kros JM, Hegi ME, Dif N, Lacombe D, Gorlia T, Stupp R (2013) The international EORTC/NCIC-CTG/TROG/MRC-CTU low grade trial: temozolomide chemotherapy versus radiotherapy in molecularly characterized (1p loss) LGG. 4th Quadrennial Meeting of the World Federation of Neuro-Oncology. San Francisco, CA, USA
4. Beroukhim R, Getz G, Nghiemphu L, Barretina J, Hsueh T, Linhart D, Vivanco I, Lee JC, Huang JH, Alexander S, Du J, Kau T, Thomas RK, Shah K, Soto H, Perner S, Prensner J, Debiasi

RM, Demichelis F, Hatton C, Rubin MA, Garraway LA, Nelson SF, Liau L, Mischel PS, Cloughesy TF, Meyerson M, Golub TA, Lander ES, Mellinghoff IK, Sellers WR (2007) Assessing the significance of chromosomal aberrations in cancer: methodology and application to glioma. Proc Natl Acad Sci 104:20007–20012

5. Bodell WJ, Gaikwad NW, Miller D, Berger MS (2003) Formation of DNA adducts and induction of lacI mutations in Big Blue Rat-2 cells treated with temozolomide: implications for the treatment of low-grade adult and pediatric brain tumors. Cancer Epidemiol Biomarkers Prev 12:545–551
6. Brada M, Viviers L, Abson C, Hines F, Britton J, Ashley S, Sardell S, Traish D, Gonsalves A, Wilkins P, Westbury C (2003) Phase II study of primary temozolomide chemotherapy in patients with WHO grade II gliomas. Ann Oncol 14:1715–1721
7. Brandes AA, Franceschi E, Tosoni A, Bartolini S, Bacci A, Agati R, Ghimenton C, Turazzi S, Talacchi A, Skrap M, Marucci G, Volpin L, Morandi L, Pizzolitto S, Gardiman M, Andreoli A, Calbucci F, Ermani M (2010) *O*(6)-methylguanine DNA-methyltransferase methylation status can change between first surgery for newly diagnosed glioblastoma and second surgery for recurrence: clinical implications. Neuro Oncol 12:283–288
8. Cahill DP, Levine KK, Betensky RA, Codd PJ, Romany CA, Reavie LB, Batchelor TT, Futreal PA, Stratton MR, Curry WT, Iafrate AJ, Louis DN (2007) Loss of the mismatch repair protein MSH6 in human glioblastomas is associated with tumor progression during temozolomide treatment. Clin Cancer Res 13:2038–2045
9. Cankovic M, Mikkelsen T, Rosenblum ML, Zarbo RJ (2007) A simplified laboratory validated assay for MGMT promoter hypermethylation analysis of glioma specimens from formalin-fixed paraffin-embedded tissue. Lab Invest 87:392–397
10. Chao EC, Velasquez JL, Witherspoon MS, Rozek LS, Peel D, Ng P, Gruber SB, Watson P, Rennert G, Anton-Culver H, Lynch H, Lipkin SM (2008) Accurate classification of MLH1/MSH2 missense variants with multivariate analysis of protein polymorphisms-mismatch repair (MAPP-MMR). Hum Mutat 29:852–860
11. Cibulskis K, Lawrence MS, Carter SL, Sivachenko A, Jaffe D, Sougnez C, Gabriel S, Meyerson M, Lander ES, Getz G (2013) Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. Nat Biotechnol 31:213–219
12. Costello JF, Futscher BW, Kroes RA, Pieper RO (1994) Methylation-related chromatin structure is associated with exclusion of transcription factors from and suppressed expression of the *O*-6-methylguanine DNA methyltransferase gene in human glioma cell lines. Mol Cell Biol 14:6515–6521
13. Costello JF, Futscher BW, Tano K, Graunke DM, Pieper RO (1994) Graded methylation in the promoter and body of the *O*6-methylguanine DNA methyltransferase (MGMT) gene correlates with MGMT expression in human glioma cells. J Biol Chem 269:17228–17237
14. Douw L, Klein M, Fagel SS, van den Heuvel J, Taphoorn MJ, Aaronson NK, Postma TJ, Vandertop WP, Mooij JJ, Boerman RH, Beute GN, Sluimer JD, Slotman BJ, Reijneveld JC, Heimans JJ (2009) Cognitive and radiological effects of radiotherapy in patients with low-grade glioma: long-term follow-up. Lancet Neurol 8:810–818
15. Dunn J, Baborie A, Alam F, Joyce K, Moxham M, Sibson R, Crooks D, Husband D, Shenoy A, Brodbelt A, Wong H, Liloglou T, Haylock B, Walker C (2009) Extent of MGMT promoter methylation correlates with outcome in glioblastomas given temozolomide and radiotherapy. Br J Cancer 101:124–131
16. Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG (1999) Inactivation of the DNA repair gene *O*6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. Cancer Res 59:793–797
17. Everhard S, Tost J, El AH, Criniere E, Busato F, Marie Y, Gut IG, Sanson M, Mokhtari K, Laigle-Donadey F, Hoang-Xuan K, Delattre JY, Thillet J (2009) Identification of regions correlating MGMT promoter methylation and gene expression in glioblastomas. Neuro Oncol 11:348–356
18. Felsberg J, Thon N, Eigenbrod S, Hentschel B, Sabel MC, Westphal M, Schackert G, Kreth FW, Pietsch T, Loffler M, Weller M, Reifenberger G, Tonn JC (2011) Promoter methylation and expression of MGMT and the DNA mismatch repair genes MLH1, MSH2, MSH6 and PMS2 in paired primary and recurrent glioblastomas. Int J Cancer 129:659–670
19. Fritz G, Kaina B (1992) Genomic differences between O6-methylguanine-DNA methyltransferase proficient (Mex+) and deficient (Mex−) cell lines: possible role of genetic and epigenetic changes in conversion of Mex+ into Mex−. Biochem Biophys Res Commun 183:1184–1190
20. Giraldo A, Gomez A, Salguero G, Garcia H, Aristizabal F, Gutierrez O, Angel LA, Padron J, Martinez C, Martinez H, Malaver O, Florez L, Barvo R (2005) MLH1 and MSH2 mutations in Colombian families with hereditary nonpolyposis colorectal cancer (Lynch syndrome)–description of four novel mutations. Fam Cancer 4:285–290
21. Grasbon-Frodl EM, Kreth FW, Ruiter M, Schnell O, Bise K, Felsberg J, Reifenberger G, Tonn JC, Kretzschmar HA (2007) Intratumoral homogeneity of MGMT promoter hypermethylation as demonstrated in serial stereotactic specimens from anaplastic astrocytomas and glioblastomas. Int J Cancer 121:2458–2464
22. Groenendijk FH, Taal W, Dubbink HJ, Haarloo CR, Kouwenhoven MC, van den Bent MJ, Kros JM, Dinjens WN (2011) MGMT promoter hypermethylation is a frequent, early, and consistent event in astrocytoma progression, and not correlated with TP53 mutation. J Neurooncol 101:405–417
23. Hamilton MG, Roldan G, Magliocco A, McIntyre JB, Parney I, Easaw JC (2011) Determination of the methylation status of MGMT in different regions within glioblastoma multiforme. J Neurooncol 102:255–260
24. Harris LC, Remack JS, Brent TP (1994) Identification of a 59 bp enhancer located at the first exon/intron boundary of the human *O*6-methylguanine DNA methyltransferase gene. Nucleic Acids Res 22:4614–4619
25. Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, Kros JM, Hainfellner JA, Mason W, Mariani L, Bromberg JE, Hau P, Mirimanoff RO, Cairncross JG, Janzer RC, Stupp R (2005) MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med 352:997–1003
26. Hunter C, Smith R, Cahill DP, Stephens P, Stevens C, Teague J, Greenman C, Edkins S, Bignell G, Davies H, O'Meara S, Parker A, Avis T, Barthorpe S, Brackenbury L, Buck G, Butler A, Clements J, Cole J, Dicks E, Forbes S, Gorton M, Gray K, Halliday K, Harrison R, Hills K, Hinton J, Jenkinson A, Jones D, Kosmidou V, Laman R, Lugg R, Menzies A, Perry J, Petty R, Raine K, Richardson D, Shepherd R, Small A, Solomon H, Tofts C, Varian J, West S, Widaa S, Yates A, Easton DF, Riggins G, Roy JE, Levine KK, Mueller W, Batchelor TT, Louis DN, Stratton MR, Futreal PA, Wooster R (2006) A hypermutation phenotype and somatic MSH6 mutations in recurrent human malignant gliomas after alkylator chemotherapy. Cancer Res 66:3987–3991
27. Jiricny J (2006) The multifaceted mismatch-repair system. Nat Rev Mol Cell Biol 7:335–346
28. Johnson BE, Mazor T, Hong C, Barnes M, Aihara K, McLean CY, Fouse SD, Yamamoto S, Ueda H, Tatsuno K, Asthana S, Jalbert LE, Nelson SJ, Bollen AW, Gustafson WC, Charron E, Weiss WA, Smirnov IV, Song JS, Olshen AB, Cha S, Zhao Y, Moore RA, Mungall AJ, Jones SJ, Hirst M, Marra MA, Saito N, Aburatani H, Mukasa A, Berger MS, Chang SM, Taylor BS, Costello JF

(2014) Mutational analysis reveals the origin and therapy-driven evolution of recurrent glioma. Science 343:189–193
29. Koekkoek JA, Dirven L, Heimans JJ, Postma TJ, Vos MJ, Reijneveld JC, Taphoorn MJ (2014) Seizure reduction in a low-grade glioma: more than a beneficial side effect of temozolomide. J Neurol Neurosurg Psychiatry. doi:10.1136/jnnp-2014-308136
30. Komine C, Watanabe T, Katayama Y, Yoshino A, Yokoyama T, Fukushima T (2003) Promoter hypermethylation of the DNA repair gene *O6*-methylguanine-DNA methyltransferase is an independent predictor of shortened progression free survival in patients with low-grade diffuse astrocytomas. Brain Pathol 13:176–184
31. Li H, Durbin R (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25:1754–1760
32. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, Scheithauer BW, Kleihues P (2007) The 2007 WHO classification of tumours of the central nervous system. Acta Neuropathol 114:97–109
33. Malley DS, Hamoudi RA, Kocialkowski S, Pearson DM, Collins VP, Ichimura K (2011) A distinct region of the MGMT CpG island critical for transcriptional regulation is preferentially methylated in glioblastoma cells and xenografts. Acta Neuropathol 121:651–661
34. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo MA (2010) The genome analysis toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 20:1297–1303
35. Nakamura M, Watanabe T, Yonekawa Y, Kleihues P, Ohgaki H (2001) Promoter methylation of the DNA repair gene MGMT in astrocytomas is frequently associated with G: C–>A: T mutations of the TP53 tumor suppressor gene. Carcinogenesis 22:1715–1719
36. Nguyen SA, Stechishin OD, Luchman HA, Lun XQ, Senger DL, Robbins SM, Cairncross G, Weiss S (2014) Novel MSH6 mutations in treatment naive glioblastoma and anaplastic oligodendroglioma influence temozolomide resistance independently of MGMT methylation. Clin Cancer Res
37. Pace A, Vidiri A, Galie E, Carosi M, Telera S, Cianciulli AM, Canalini P, Giannarelli D, Jandolo B, Carapella CM (2003) Temozolomide chemotherapy for progressive low-grade glioma: clinical benefits and radiological response. Ann Oncol 14:1722–1726
38. Parkinson JF, Wheeler HR, Clarkson A, McKenzie CA, Biggs MT, Little NS, Cook RJ, Messina M, Robinson BG, McDonald KL (2008) Variation of *O*(6)-methylguanine-DNA methyltransferase (MGMT) promoter methylation in serial samples in glioblastoma. J Neurooncol 87:71–78
39. Peltomaki P (2003) Role of DNA mismatch repair defects in the pathogenesis of human cancer. J Clin Oncol 21:1174–1179
40. Pieper RO, Costello JF, Kroes RA, Futscher BW, Marathi U, Erickson LC (1991) Direct correlation between methylation status and expression of the human *O*-6-methylguanine DNA methyltransferase gene. Cancer Commun 3:241–253
41. Pignatti F, van den Bent M, Curran D, Debruyne C, Sylvester R, Therasse P, Afra D, Cornu P, Bolla M, Vecht C, Karim AB (2002) Prognostic factors for survival in adult patients with cerebral low-grade glioma. J Clin Oncol 20:2076–2084
42. Quinn JA, Reardon DA, Friedman AH, Rich JN, Sampson JH, Provenzale JM, McLendon RE, Gururangan S, Bigner DD, Herndon JE, Avgeropoulos N, Finlay J, Tourt-Uhlig S, Affronti ML, Evans B, Stafford-Fox V, Zaknoen S, Friedman HS (2003) Phase II trial of temozolomide in patients with progressive low-grade glioma. J Clin Oncol 21:646–651
43. Raevaara TE, Korhonen MK, Lohi H, Hampel H, Lynch E, Lonnqvist KE, Holinski-Feder E, Sutter C, McKinnon W, Duraisamy S, Gerdes AM, Peltomaki P, Kohonen-Ccorish M, Mangold E, Macrae F, Greenblatt M, de la Chapelle A, Nystrom M (2005) Functional significance and clinical phenotype of nontruncating mismatch repair variants of MLH1. Gastroenterology 129:537–549
44. Ramalho-Carvalho J, Pires M, Lisboa S, Graca I, Rocha P, Barros-Silva JD, Savva-Bordalo J, Mauricio J, Resende M, Teixeira MR, Honavar M, Henrique R, Jeronimo C (2013) Altered expression of MGMT in high-grade gliomas results from the combined effect of epigenetic and genetic aberrations. PLoS One 8:e58206
45. Reijneveld JC, Bottomley A, Taphoorn MJ, Coens C, Bromberg JE, Mason, Hoang-Xuan K, Brandes AA, Stupp R, Kantor G, Ben Hassel M, Ryan G, Wick W, Theissen B, Lacombe D, Gorlia T, Baumert B. (2013) Health related Quality of Life results from temozolomide chemotherapy vs. radiotherapy in molecularly characterized (1p loss) low-grade glioma. A randomized phase III Intergroup study by the EORTC/NCIC-CTG/TROG/MRC-CTU (EORTC 22033-26033). Neuro Oncol 15. 4th Quadrennial Meeting of the World Federation of Neuro-Oncology. San Francisco, CA, USA
46. Rodriguez-Hernandez I, Garcia JL, Santos-Briz A, Hernandez-Lain A, Gonzalez-Valero JM, Gomez-Moreta JA, Toldos-Gonzalez O, Cruz JJ, Martin-Vallejo J, Gonzalez-Sarmiento R (2013) Integrated analysis of mismatch repair system in malignant astrocytomas. PLoS One 8:e76401
47. Rohde C, Zhang Y, Reinhardt R, Jeltsch A (2010) BISMA–fast and accurate bisulfite sequencing data analysis of individual clones from unique and repetitive sequences. BMC Bioinform 11:230
48. Ruda R, Magliola U, Bertero L, Trevisan E, Bosa C, Mantovani C, Ricardi U, Castiglione A, Monagheddu C, Soffietti R (2013) Seizure control following radiotherapy in patients with diffuse gliomas: a retrospective study. Neuro Oncol 15:1739–1749
49. Scheinin I, Sie D, Bengtsson H, van de Wiel MA, Olshen AB, van Thuijl HF, van Essen HF, Eijk PP, Rustenburg F, Meijer GA, Reijneveld JC, Wesseling P, Pinkel D, Albertson DG, Ylstra B (2014) DNA copy number analysis of fresh and formalin-fixed specimens by shallow whole-genome sequencing with identification and exclusion of problematic regions in the genome assembly. Genome Res 24:2022–2032
50. Shaw EG, Wang M, Coons SW, Brachman DG, Buckner JC, Stelzer KJ, Barger GR, Brown PD, Gilbert MR, Mehta MP (2012) Randomized trial of radiation therapy plus procarbazine, lomustine, and vincristine chemotherapy for supratentorial adult low-grade glioma: initial results of RTOG 9802. J Clin Oncol 30:3065–3070
51. Shin YK, Heo SC, Shin JH, Hong SH, Ku JL, Yoo BC, Kim IJ, Park JG (2004) Germline mutations in MLH1, MSH2 and MSH6 in Korean hereditary non-polyposis colorectal cancer families. Hum Mutat 24:351
52. Silber JR, Blank A, Bobola MS, Ghatan S, Kolstoe DD, Berger MS (1999) *O6*-methylguanine-DNA methyltransferase-deficient phenotype in human gliomas: frequency and time to tumor progression after alkylating agent-based chemotherapy. Clin Cancer Res 5:807–814
53. Taal W, Dubbink HJ, Zonnenberg CB, Zonnenberg BA, Postma TJ, Gijtenbeek JM, Boogerd W, Groenendijk FH, Kros JM, Kouwenhoven MC, van Marion R, van Heuvel I, van der Holt B, Bromberg JE, Sillevis Smitt PA, Dinjens WN, van den Bent MJ (2011) First-line temozolomide chemotherapy in progressive low-grade astrocytomas after radiotherapy: molecular characteristics in relation to response. Neuro Oncol 13:235–241
54. van de Wiel MA, Kim KI, Vosse SJ, van Wieringen WN, Wilting SM, Ylstra B (2007) CGHcall: calling aberrations for array CGH tumor profiles. Bioinformatics 23:892–894
55. van Thuijl HF, Scheinin I, Sie D, Alentorn A, van Essen HF, Cordes M, Fleischeuer R, Gijtenbeek AM, Beute G, van den

Brink WA, Meijer GA, Havenith M, Idbaih A, Hoang-Xuan K, Mokhtari K, Verhaak R, van der Valk P, van de Wiel MA, Heimans JJ, Aronica E, Reijneveld JC, Wesseling P, Ylstra B (2014) Spatial and temporal evolution of distal 10q deletion, a prognostically unfavorable event in diffuse low-grade gliomas. Genome Biol 15:471

56. Venkatraman ES, Olshen AB (2007) A faster circular binary segmentation algorithm for the analysis of array CGH data. Bioinformatics 23:657–663
57. Weber RG, Sabel M, Reifenberger J, Sommer C, Oberstrass J, Reifenberger G, Kiessling M, Cremer T (1996) Characterization of genomic alterations associated with glioma progression by comparative genomic hybridization. Oncogene 13:983–994
58. Wick W, Meisner C, Hentschel B, Platten M, Schilling A, Wiestler B, Sabel MC, Koeppen S, Ketter R, Weiler M, Tabatabai G, von DA, Gramatzki D, Westphal M, Schackert G, Loeffler M, Simon M, Reifenberger G, Weller M (2013) Prognostic or predictive value of MGMT promoter methylation in gliomas depends on IDH1 mutation. Neurology 81:1515–1522
59. www.clinicaltrials.gov (2013) NCT00003375, observation or radiation therapy with or without combination chemotherapy in treating patients with low-grade glioma
60. www.clinicaltrialsregister.eu (2013) EORTC 22022-26033, evaluating primary chemotherapy with temozolomide continuative low dose in patients with low grade gliomas grade II WHO
61. Ye K, Schulz MH, Long Q, Apweiler R, Ning Z (2009) Pindel: a pattern growth approach to detect break points of large deletions and medium sized insertions from paired-end short reads. Bioinformatics 25:2865–2871
62. Yip S, Miao J, Cahill DP, Iafrate AJ, Aldape K, Nutt CL, Louis DN (2009) MSH6 mutations arise in glioblastomas during temozolomide therapy and mediate temozolomide resistance. Clin Cancer Res 15:4622–4629

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ONCOGENOMICS

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Integrated genomic and functional analyses reveal glyoxalase I as a novel metabolic oncogene in human gastric cancer

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Chromosomal abnormalities are good guideposts when hunting for cancer-related genes. We analyzed copy number alterations of 163 primary gastric cancers using array-based comparative genomic hybridization and simultaneously performed a genome-wide integrated analysis of copy number and gene expression using microarray data for 58 tumors. We showed that chromosome 6p21 amplification frequently occurred secondary to *ERBB2* amplification, was associated with poorer prognosis and caused overexpression of half of the genes mapped. A comprehensive small interfering RNA knockdown of 58 genes overexpressed in tumors identified 32 genes that reduced gastric cancer cell growth. Enforced expression of 16 of these genes promoted cell growth *in vitro*, and six genes showing more than two-fold activity conferred tumor-forming ability *in vivo*. Among these six candidates, *GLO1*, encoding a detoxifying enzyme glyoxalase I (GLO1), exhibited the strongest tumor-forming activity. Coexpression of other genes with *GLO1* enhanced growth-stimulating activity. A GLO1 inhibitor, *S*-p-bromobenzyl glutathione cyclopentyl diester, inhibited the growth of two-thirds of 24 gastric cancer cell lines examined. The efficacy was found to be associated with the mRNA expression ratio of *GLO1* to *GLO2*, encoding glyoxalase II (GLO2), another constituent of the glyoxalase system. *GLO1* downregulation affected cell growth through inactivating central carbon metabolism and reduced the transcriptional activities of nuclear factor kappa B and activator protein-1. Our study demonstrates that *GLO1* is a novel metabolic oncogene of the 6p21 amplicon, which promotes tumor growth and aberrant transcriptional signals via regulating cellular metabolic activities for energy production and could be a potential therapeutic target in gastric cancer.

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INTRODUCTION

Gastric cancer is the second most common cause of cancer-related death worldwide and shows the highest incidence in Eastern Asia.[1] Previous investigations have identified multiple genetic/epigenetic alterations that result in activation of the proto-oncogenes *MET*, *FGFR2*, *ERBB2* and *KRAS* and inactivation or silencing of the tumor-suppressor genes *p53*, *p73*, *APC*, *p16*, *RUNX3* and *CDH1* in gastric cancer.[2,3] In primary tumors and gastric cancer cell lines, many copy number alterations (CNAs) have been identified using array-based comparative genomic hybridization (aCGH) techniques.[4–6] However, only a few studies have analyzed the genome-wide correlations between CNA and transcriptional changes.[7–9] Molecular characterization of CNA that is directly related to changes in gene expression or gene structure is essential for understanding the genetic basis of gastric cancer, which may eventually facilitate in identification of critical genes in cancer development.

In the present study, to comprehensively identify novel candidate oncogenes, we performed a genome-wide integrated analysis of a total of 163 CNA profiles consisting of 79 differentiated and 84 undifferentiated adenocarcinomas and an oligonucleotide expression microarray analysis of 58 tumors and 16 non-cancerous tissues. The analysis revealed many target genes whose expression was significantly associated with changes in copy number, including candidate genes of potential clinicopathological value in gastric cancer.

Among them, we focused on the chromosome 6p21 amplicon and identified multiple candidate oncogenes with the ability to enhance cell growth *in vitro* and *in vivo* by systematic functional screening using RNA interference and enforced gene expression. We find that *GLO1* gene encoding glyoxalase I (GLO1) exibits the strongest oncogenic activity in the amplicon and also other six genes cooperatively function with *GLO1* to stimulate cell growth. Inhibition of the enzymatic activity and knockdown of mRNA expression resulted in defect in gastric cancer cell growth. Metabolome analysis indicated that *GLO1* knockdown affected key pathways for energy production, such as glycolysis, the pentose phosphate pathway and the tricarboxylic acid cycle. Furthermore, we find that *GLO1* downregulation caused suppression of the transcriptional activities of nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1).

RESULTS

Genomic CNAs and correlation between DNA copy number and mRNA expression in gastric cancer

To identify a comprehensive pattern of genomic CNA in gastric cancer, we performed aCGH analysis of 163 primary gastric

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adenocarcinomas, consisting of 79 well and moderately differentiated tubular adenocarcinomas and 84 poorly differentiated adenocarcinomas, using bacterial artificial chromosome arrays covering the whole human genome with 0.6-megabase resolution (Supplementary Figure S1). The regions with recurrent genomic amplification (signal ratio $\geqslant 2.5$) included 133 loci, and recurrent homozygous deletions (signal ratio < 0.4) were detected at 30 loci within the hemizygously deleted regions (signal ratio < 0.75) (Supplementary Tables S5 and S6). Frequent amplification was observed at the *ERBB2* locus at 17q12 (12.9% of cases), the *VEGFA* locus at 6p21.1 (8.6%), the *CCND1* locus at 11q13.3 (7.4%), the *CCNE1* locus at 19q12 (7.4%), the *BCAS1* locus at 20q13.2 (7.4%) and the *MYC* locus at 8q24.21 (5.5%).

To determine the correlation between gene copy number and mRNA expression in tumors, we performed genome-wide gene expression analysis of 58 tumors (tub 1, 24; tub 2, 18; por 1, 16) that had been analyzed by aCGH. By calculating the Pearson correlation coefficient (r) between the aCGH signal ratio detected using the nearest neighbor bacterial artificial chromosome clone and the expression signal value for each gene in the same samples, we identified genes whose expression was correlated with CNA. All the genes showing $r>0.5$ are listed (Supplementary Table S5) and well-known oncogenes, such as *EGFR* ($r=0.96$), *FGFR2* ($r=0.94$), *CCNE1* ($r=0.86$), *CCND1* ($r=0.76$) and *ERBB2* ($r=0.76$), exhibited significant concordance with increased gene copy number. These analyses help us to identify bona fide pathogenetic oncogene in gastric cancer.

Chromosome 6p21 genomic amplification in gastric cancer

Chromosomal amplification at the 6p21 locus has been detected in gastric cancer[4,7–9] and in other tumors.[10–14] In this study, we also detected frequent gains (signal ratio $\geqslant 1.3$, up to 32.5% of tumors) and high-level amplifications (signal ratio $\geqslant 2.5$, up to 8.6% of tumors) in the chromosomal band. Most of the 6p21 copy number gains/amplifications in primary cases were spread over a rather broad region, and high-level amplifications occurred intensively in the region of 6p21.2-p21.1 (Figure 1a). Multivariate analysis using the Cox proportional hazards model revealed that copy number gain detected by RPCI11–89L17 at 6p21.1 was an independent factor related to overall survival of gastric cancer, in addition to the depth of invasion and the status of lymph node and extranodal metastasis (Table 1). This observation strongly suggested that chromosome 6p21 gain/amplification has an important role in the pathogenesis of gastric cancer.

In the 6p21 region, most genes showing significant correlations between expression patterns and copy number gains were located within the more proximal region of 6p21.2-p21.1 (Figure 1b). Fifty-nine (49.2%) out of the 120 genes in this region showed significant correlation with increased copy number. The list included candidate genes that have been reported as targets for 6p21 amplification or as cancer-related genes in various tumors (Supplementary Table S7).

Loss-of-function assay shows that multiple genes at the 6p21 locus are associated with gastric cancer cell growth

To isolate potential oncogenes at this locus, we used functional assays. We first tested endogenous growth-promoting activity using comprehensive small interfering RNA (siRNA) knockdown on two gastric cancer cell lines HSC58 and HSC60 with moderate copy number increase (signal ratio ≈1.3) at the 6p21 locus (Supplementary Figure S2). Downregulation of 58 out of the 59 candidate genes in the 6p21 locus was successful using specific siRNAs. This screening identified 45 and 40 siRNAs, which inhibited cell growth at $< 70\%$ compared with the control siRNA

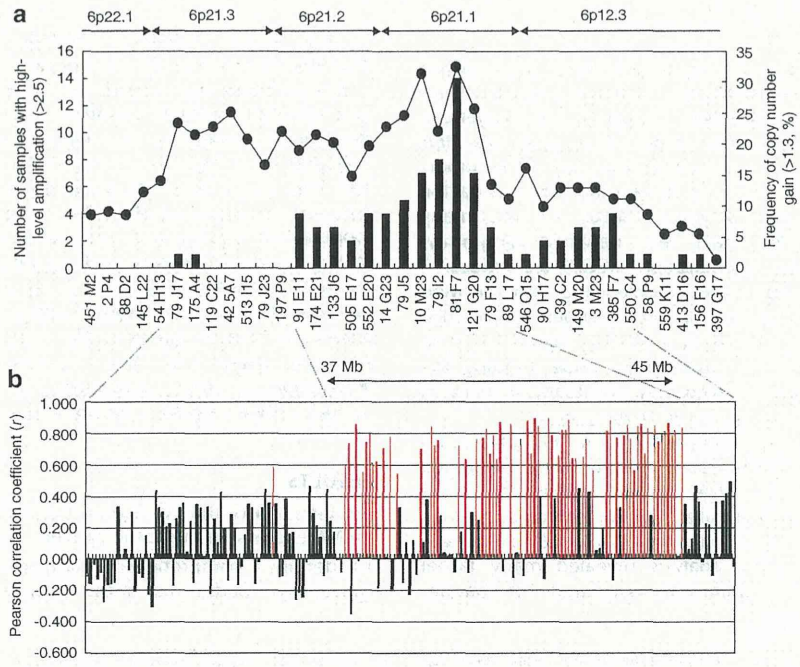

Figure 1. Chromosome 6p21 amplification in gastric cancer. (**a**) Chromosome 6p21 shows frequent copy number gains and high-level genomic amplifications. Horizontal line: 37 bacterial artificial chromosome clones mapped around chromosome 6p21. Vertical line (left): number of tumor samples with high-level amplification (signal ratio $\geqslant 2.5$) shown as a black bar. Vertical line (right): frequency of copy number gain (signal ratio $\geqslant 1.3$) shown as a line graph. (**b**) Tight correlations are observed between CNA and gene expression at the 6p21 locus. Horizontal line: 189 genes mapped at the 6p21 locus. Vertical line: the Pearson correlation coefficient (r) of each gene calculated using the gene copy number and gene expression value. Gene showing $r \geqslant 0.5$ is shown as a red bar.

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Table 1. Multivariate analysis of influencing factors for overall survival and recurrence

Variable	*Hazard ratio*	*95% Confidence interval*	P-*value*
For overall survival (n = 141)			
Copy number changes detected by RPCI11-89L17			
Neutral	1		
Gain	2.506	1.230–5.102	0.0114
Depth of invasion			
T1-T2	1		
T3-T4	2.481	1.342–4.587	0.0037
Lymph node metastasis			
(−)	1		
(+)	5.076	1.157–22.222	0.0313
Extranodal metastasis			
(−)	1		
(+)	1.742	1.055–2.882	0.0301
For recurrence (n = 115)			
Lymph node metastasis			
(−)	1		
(+)	2.967	0.952–9.259	0.0606
Extranodal metastasis			
(−)	1		
(+)	1.976	1.138–3.436	0.0157

in HSC58 and HSC60 cells, respectively. In total, downregulation of 36 genes caused concordant reduction of cell growth in both cell lines (Figure 2a).

Multiple genes at the 6p21 locus confer colony-forming activity *in vitro* and tumorigenicity *in vivo*

To further evaluate the oncogenic activities attributable to their overexpression, we introduced the candidate genes on the 6p21 amplicon into HEK293 epithelial cells and tested whether their overexpression enhanced colony-forming activity *in vitro*. Sixteen of the 51 genes examined enhanced the colony-forming activity: (1) *PPIL1*, (3) *MTCH1*, (5) *TBC1D22B*, (10) *GLO1*, (11) *C6orf64*, (13) *C6orf130*, (20) *CCND3*, (25) *TBCC*, (30) *GNMT*, (32) *MEA1*, (38) *PTK7*, (40) *C6orf108*, (45) *POLR1C*, (48) *GTPBP2*, (51) *VEGFA* and (58) *AARS2* (Figure 2b). Reproducible colony-forming activities of NIH3T3 fibroblast cells were observed at similar stimulation rates in almost all of them (Table 2).

We then attempted to examine whether the six genes showing more than two-fold enhancement of *in vitro* cell growth could confer *in vivo* tumorigenicity by transplanting polyclonal cells expressing them into mice. Overexpression of the six genes led to tumor development *in vivo* with frequencies ranging from 17% to 83% within 12 weeks (Supplementary Figure S3). The results indicated that at least these six genes upregulated in the 6p21 amplicon had the individual potential to stimulate cell growth and form tumors *in vivo* (Table 2). Among them, *GLO1* showed the strongest tumor-forming ability, with the tumors developing at the earliest time (4–5 weeks after injection), growing more quickly and attaining a larger size than the others.

GLO1 exhibits a stimulated oncogenic activity in cooperation with other genes on 6p21

Integration of copy number analysis, gene expression analysis and three different kinds of functional analyses identified *GLO1* as the most likely oncogene. However, each 6p21 amplicon in primary cases usually lies within a wide range and is accompanied by coamplification of multiple potential oncogenes (Supplementary Figure S1b), we hypothesized that there might be cooperative tumorigenic activity between *GLO1* and other genes within the 6p21 amplicon. We double-transfected seven genes into a *GLO1*-expressing clone (HEK293-*GLO1*-Zeo) and tested their growth-stimulatory activities. Significant enhancement of colony formation was detected for all the genes examined, except for *PPIL1* (Figures 2c and d), indicating that *GLO1* confers a broad synergistic effect on tumor formation with other genes on 6p21.

In addition to the high-level amplification of the *GLO1* gene, we searched for somatic mutation of the *GLO1* gene in our gastric cancer cohort (72 tumors) and 23 cell lines. However, we detected germline variations only in the 5′-untranslated region of exon 1, in intron 1 and in the coding exon 4 (Supplementary Figure S4). GLO1 is a ubiquitous detoxifying enzyme of methylglyoxal (MG) that is a potent glycating agent and induces oxidative stress and apoptosis.[15] A recent report has demonstrated that upregulation of *GLO1* expression occurs in response to oxidative stress, by binding a stress-responsive transcription factor Nrf2 to the ARE (antioxidant-response element) in 5′-untranslated region of exon 1 (from − 19 to − 10, numbered from the start codon).[16] Whether the variations found in this study relate to the regulation by Nrf2 remains unknown.

GLO1 is a novel metabolic oncogene affecting gastric cancer cell growth by regulating energy producing pathways

We tried to decrease the expression of *GLO1* stably in eight gastric cancer cell lines (HSC41, HSC43, HSC44, HSC45, HSC58, HSC60, MKN28 and NUGC3) using a lentiviral short hairpin RNA (shRNA) and were able to isolate seven of them (with the exception of HSC58) exhibiting reductions of expression ranging from 11% to 69% relative to the negative control. Remarkable reduction of cell growth was detected in HSC43, HSC44, HSC45, HSC60, MKN28 and NUGC3 (Figure 3a) but not in HSC41 (data not shown). *GLO1* stably knockdown HSC60 clones exhibited the most severe growth retardation and ceased proliferating within a month after isolation. Together with the failure to establish HSC58-*GLO1*-knockdown clones, the data indicated that the growth of some gastric cancer cells is strongly dependent on *GLO1* function, whereas the dependency is less marked in others.

Because GLO1 is an enzyme that detoxifies MG generated during glycolysis, we assumed that downregulation of *GLO1* would reduce glycolytic activity by accumulation of MG.[17] The intracellular metabolites of key pathways for energy production were analyzed by capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS), which enables comprehensive and quantitative analysis of charged metabolites.[18] The CE-TOFMS system identified and quantified 239 and 228 candidate compounds comparable in NUGC3-sh*GLO1* vs NUGC3-scr and MKN28-sh*GLO1* vs MKN28-scr, respectively. Unexpectedly, lower concentration of almost all the intermediates that are included in glycolysis, the pentose phosphate pathway and tricarboxylic acid cycle were detected in both NUGC3-sh*GLO1* and MKN28-sh*GLO1* cells compared with their respective reference cells (Table 3 and Supplementary Figure S5). In addition, we found relative lower levels of glutamine/glutamic acid than that of the other amino acids. It might indicate glutaminolysis activation to compensate for reduced central carbon metabolism as a secondary effect, in which glutamine converts to lactic acid via tricarboxylic acid cycle.[19]

Suppression of gastric cancer cell growth by *S*-p-bromobenzyl glutathione cyclopentyl diester (BBGC), a glyoxalase I inhibitor

We further tested whether a GLO1 inhibitor, BBGC, was able to exert an anti-growth effect on gastric cancer. We treated 24 gastric cancer cell lines with a range of BBGC concentrations and found that 16 cell lines showed reproducible reduction of growth (the IC50 value = 4.4–13.1 μM, Figure 3b). Previous studies have demonstrated that sensitivity to BBGC is correlated with *GLO1* overexpression/amplification in cell lines.[20–22] Because of the relatively low-level copy number gain at the *GLO1* locus in the cell

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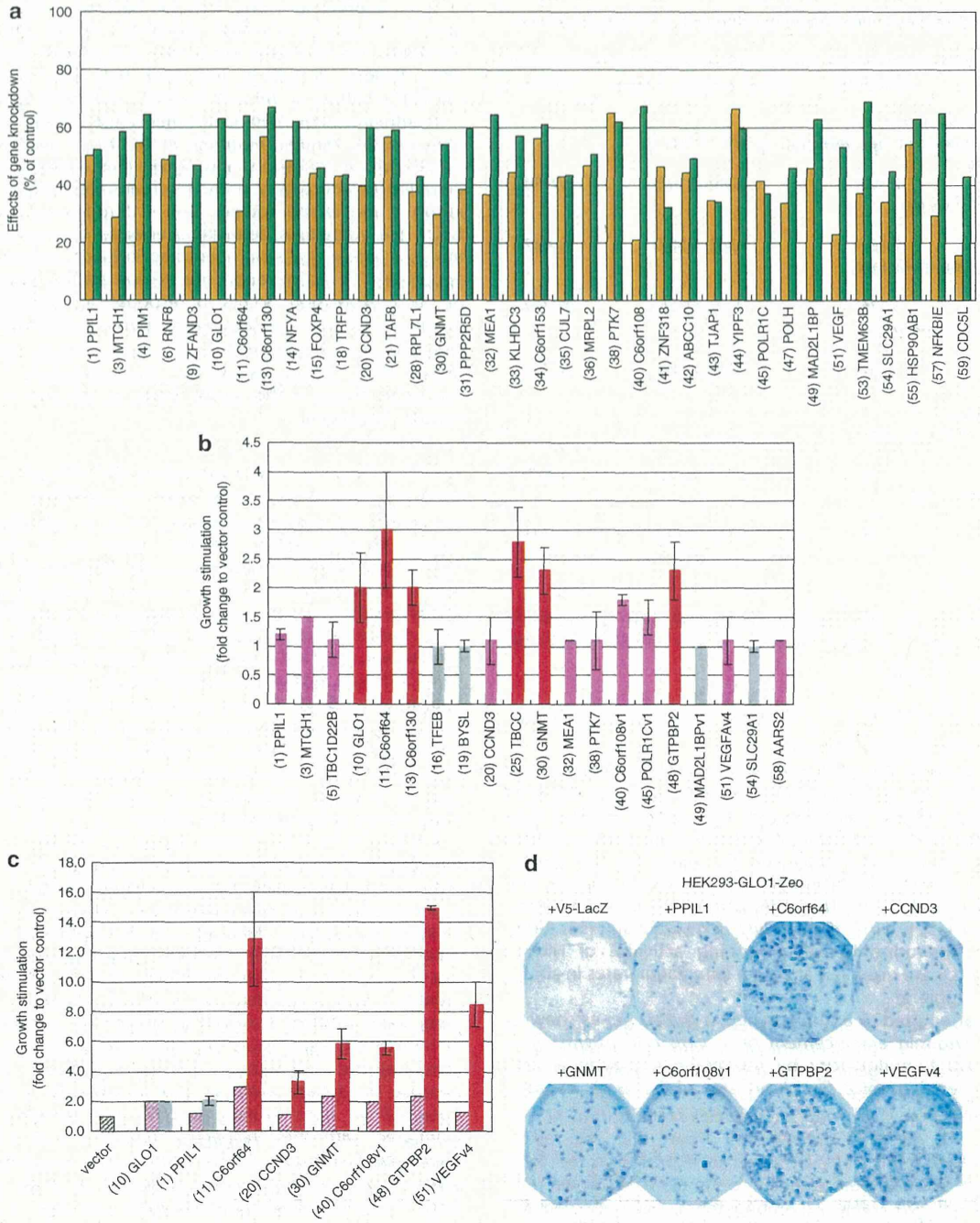

Figure 2. Effects of 6p21 genes in cell growth *in vitro*. (**a**) Multiple 6p21 genes affect gastric cancer cell growth by knockdown of expression. Horizontal lines: target genes in the 6p21 amplicon. Vertical lines: effects of gene knockdown on cell growth. Growth retardation with $<70\%$ growth in comparison with that elicited with a non-targeting control is shown. Experiments were carried out in triplicate and repeated at least twice. Downregulation of 36 genes caused concordant reduction of cell growth in both cell lines HSC58 (orange) and HSC60 (green). (**b**) Multiple 6p21 genes promote cell growth by enforced expression. The individual gene cloned into the pcDNA3.1D/V5-His-TOPO expression vector was transiently transfected into HEK293 cells, and after 12 days of incubation, the number of colonies was counted. Horizontal lines: candidate genes in the 6p21 amplicon showing growth-promoting activities. Vertical lines: efficiency of colony formation relative to the pcDNA3.1D/V5-His-*LacZ* plasmid used as a negative control. Growth promotion of more than 2.0-fold is shown as a red bar, that between 1.1 and 2.0 as a dark pink bar and that between 1.0 and 1.1 as a grey bar. Three independent experiments were carried out in triplicate. Data represent means $\pm$ s.e.m. (**c**) Synergistic effects on growth stimulation in a HEK293 clone stably expressing *GLO1*. HEK293-*GLO1*-Zeo clone was transfected with the respective gene and cultured for 12 days. The relative efficiencies of colony formation in double transfectants were significantly enhanced (solid red bar) in comparison with the single transfectants (shaded bar), except for *PPIL1* (solid gray bar). Data represent means $\pm$ s.e.m. All recombinant expression plasmids carried cDNA with V5-His tag fused to its carboxyl terminus. Expression of the fusion protein was confirmed by western blotting analysis using part of the cell culture at 24 h after transfection (data not shown). (**d**) Colony formation of HEK293-*GLO1*-Zeo cells after transfection of the indicated gene. Three independent experiments were carried out in triplicate and summarized in panel (**c**).

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lines (signal ratios 1.20 ~ 1.34), we were unable to identify any correlations between higher sensitivity to BBGC and increased *GLO1* gene dosage (Figure 3c, left). We measured GLO1 activity and mRNA expression levels of *GLO1* and *GLO2*, encoding glyoxalase II (GLO2), another constituent of the glyoxalase system.[15] No correlation between the BBGC sensitivity and GLO1 activity (Figure 3c, middle) or *GLO1* mRNA quantity (data not shown) was evident. We found that the expression ratio of *GLO1*/*GLO2* mRNA is high in BBGC-insensitive compared with BBGC-sensitive cells (1.238 vs 0.478). The difference was statistically significant at $P=0.0162$ (Figure 3c, right).

GLO1 regulates the transcriptional activities of NF-κB and AP-1 in gastric cancer cells

A recent study has demonstrated that overexpression of *GLO1* suppresses basal and tumor necrosis factor-induced NF-κB activity in HEK293 cells.[23] As activation of NF-κB has been shown to have critical oncogenic roles in a variety of solid tumors including gastric cancer,[24–26] we examined the transcriptional activities of NF-κB, AP-1 and signal transducer and activator of transcription factor 3 (STAT3) using a stably *GLO1*-overexpressing HEK293 clone. The GLO1 activity of HEK293-*GLO1* cells was increased by 1.8-fold, and the transcriptional activities of NF-κB and AP-1 were increased by 2.0-fold and 1.9-fold relative to the basal levels, respectively (Figures 4a and b). In contrast, no increase of STAT3 transcriptional activity was seen. On the other hand, cells stably expressing *GLO1*-shRNA showed decreased GLO1 activity (14–36% of the negative control) (Figure 4c), and the basal activities of NF-κB and AP-1 were significantly suppressed in *GLO1*-downregulated HSC44, NUGC3 and MKN28 cells (Figure 4d). These results are consistent with the reverse observations in HEK293 *GLO1*-overexpressing cells, indicating that *GLO1* expression and/or GLO1 activity is positively linked to the transcriptional activities of NF-κB and AP-1.

To confirm NF-κB activation by *GLO1* overexpression, we searched for NF-κB target genes upregulated in the *GLO1*-overexpressing tumors. This was carried out by comparing the expression level for each gene among the three tumor groups divided by the expression level of *GLO1*: *GLO1*-high, averaged expression level = 2139 ($n=18$); *GLO1*-medium, averaged expression level = 1080 ($n=26$, $P=1\times10^{-8}$); and *GLO1*-low, averaged expression level = 785 ($n=14$, $P=4\times10^{-10}$). Twenty-one NF-κB target genes showed differential gene expression between *GLO1* highly expressing tumors and others among the 463 NF-κB target genes (Supplementary Table S8). The upregulated gene list includes apoptosis/anti-apoptosis-related genes, tumor invasion/metastasis-related genes, oncogenes *E2F3* and *MCTS1*, an NF-κB activator *RIPK2* and a glycolytic enzyme *PGK1*. We confirmed that some of these NF-κB target genes were almost downregulated in gastric cancer cells stably expressing *GLO1* shRNA compared with those expressing non-targeting shRNA (Figure 4e).

DISCUSSION

In this study, we identified a set of 808 genes whose overexpression correlated with copy number gain from 107 gain/amplification loci (Supplementary Table S5) and a set of 83 genes whose underexpression correlated with copy number loss from 41 hemizygous/homozygous deletion loci (Supplementary Table S6) in gastric cancer. In particular, genes showing strong correlation ($r\geqslant0.7$) appeared to be potential targets for frequent gene amplification, for example, *FAM84B* and *PVT1* at the 8q24.2 *MYC* locus and *C20orf43* and *RAB22A* at the 20q13.2 *BCAS1* locus, by analogy with the patterns of well-known oncogenes.

The frequency of high-level 6p21 amplification encompassing a stretch of 8 megabases was second only to the *ERBB2* locus, and the copy number gains at chromosome band 6p21.1 was an independent prognostic factor of overall survival of gastric cancer patients (Table 1). We expected that there would be multiple pathogenetic genes for gastric cancer in this region, because a tight and strong correlation between CNAs and gene expression was observed in almost half of the genes mapped (Figure 1b). Gene knockdown analysis using siRNA revealed that downregulation of 36 genes inhibit the growth in both HSC58 and HSC60 cells (Figure 2a). The results clearly demonstrated that many of the upregulated 6p21 genes have a role in the viability of

Table 2. *In vitro* growth-promoting activities and *in vivo* tumor-forming activities of the six candidate target genes for 6p21 amplification

Gene no.	*Gene symbol*	*Full name*	In vitro *growth-promoting activity*		In vivo *tumor-forming activity*		
			Colony-forming activity in HEK293 (folds of negative control)	*Colony-forming activity in NIH3T3 (folds of negative control)*	*Tumors developed/sites injected*	*Protein expression confirmed*	*Tumor-forming efficiency (%)*[a]
10	GLO1	Glyoxalase I	2.0	2.0	5/6	5/5	83
11	C6orf64	Chromosome 6 open reading frame 64	3.0	3.7	4/6	4/4	67
13	C6orf130	Chromosome 6 open reading frame 130	2.0	5.1	1/6	1/1	17
25	TBCC	Tublin folding cofactor C	2.8	2.0	1/6	1/1	17
30	GNMT	Glycine-N-methyltransferase	2.3	2.5	3/8	3/3	38
48	GTPBP2	GTP binding protein 2	2.3	2.3	3/6	2/3	33

[a]Tumor-forming efficiency was calculated by dividing the number of tumors expressing the exogenous protein by the number of sites injected.

Figure 3. Stable knockdown of *GLO1* using shRNA and inhibition of enzymatic activity by a GLO1 inhibitor, BBGC, result in growth inhibition of gastric cancer cells. (**a**) (Left) Growth curves of HSC43, HSC44, HSC45, HSC60, MKN28 and NUGC3 cell clones expressing *GLO1* shRNA (blue) and expressing non-targeting shRNA (black). (Right) Expression of *GLO1* mRNA in *GLO1* shRNA-expressing cells (blue) and in non-targeting shRNA-expressing cells (white). Data represent means ± s.e.m. A representative growth curve of HSC58 expressing *GLO1* siRNA by a transient transfection is also shown. (**b**) Assessment of cell viability after 24 h incubation with various concentration of BBGC. Dose-dependent cytotoxicity curves for 16 gastric cancer cell lines sensitive to BBGC (left) and for eight gastric cancer cell lines insensitive to BBGC (right). Experiments were performed in triplicate and were repeated more than three times. (**c**) *GLO1*/*GLO2* expression ratio, but not *GLO1* copy number and/or activity, is associated with sensitivity to BBGC in gastric cancer cells. Statistical analysis was performed using the Student's *t*-test. (Left) Differences of genomic copy number at *GLO1* locus detected by RPCI11-174E21 in BBGC-insensitive cells ($n=8$) and BBGC-sensitive cells ($n=13$) are not significant. (Middle) Differences of GLO1 activity in BBGC-insensitive cells ($n=8$) and BBGC-sensitive cells ($n=16$) are not significant. (Right) Differences of *GLO1*/*GLO2* mRNA expression ratio in BBGC-insensitive cells ($n=8$) and BBGC-sensitive cells ($n=14$) are significant.

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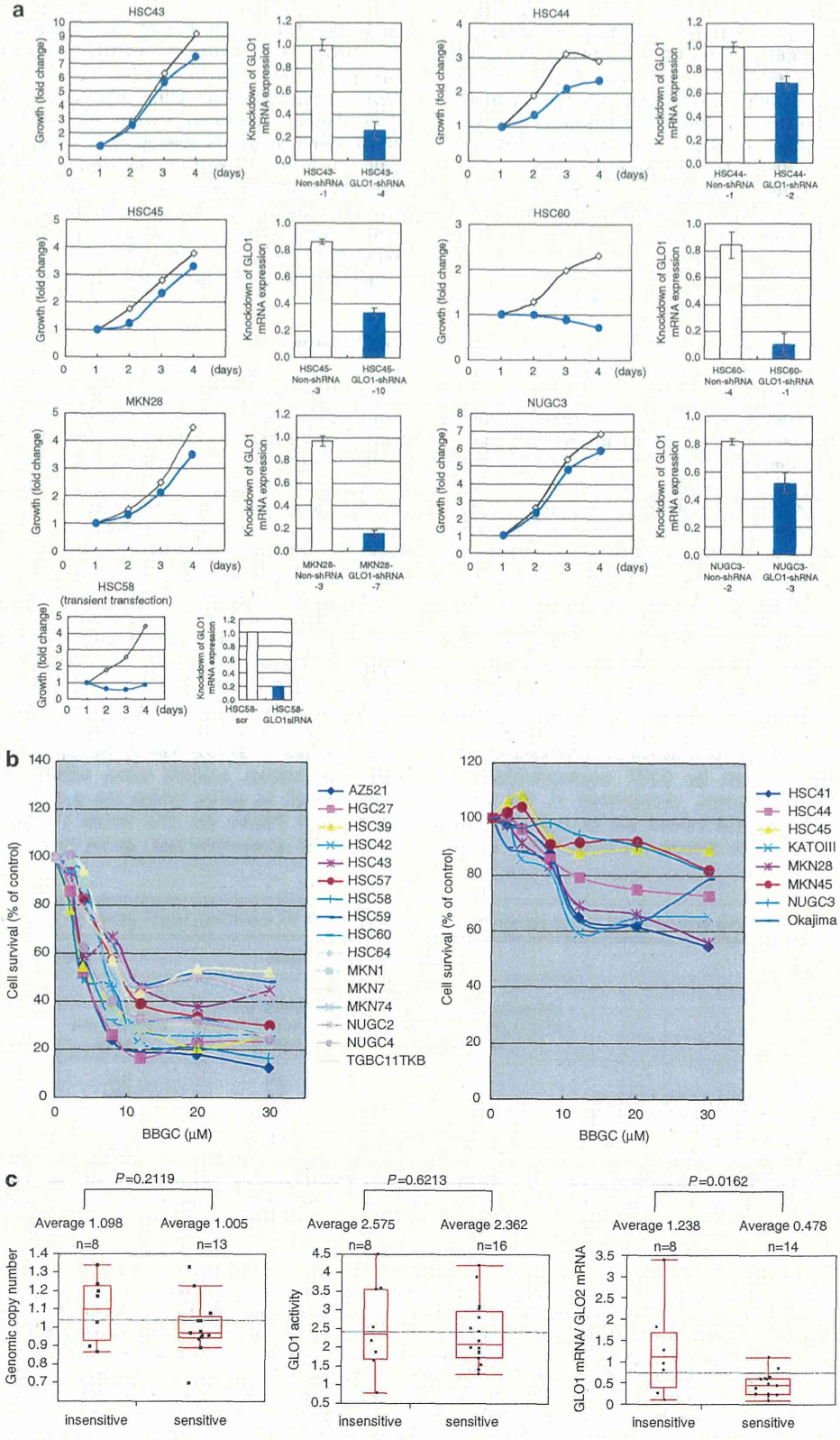

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