Introduction

Glioblastoma is the most common tumor of the central nervous system, affecting patients of all ages, and being essentially refractory to treatment; the clinical outcome remains dismal regardless of age at diagnosis. The median survival of a patient with glioblastoma is 15 months, and this survival benefits in glioblastoma are largely restricted to the subset of patients lacking expression of the DNA repair enzyme O6-methylguanine-DNA-methyl-transferase (MGMT; ref. 4). Temozolomide induces cytotoxic O6-guanine methyl adducts that are removed directly by functional MGMT, thereby producing drug resistance. Downregulation of MGMT usually occurs in tumors by gene promoter hypermethylation, in which >50% methylation has been shown to silence gene expression (5).

Deficiencies in DNA mismatch repair (MMR) are also linked to resistance to alkylating agents such as temozolomide (6), as are elevated levels of Ape1/Ref-1, a major component of the base excision repair (BER; ref. 7) system, with attempts to enhance temozolomide-induced cytotoxicity by disrupting BER by means of inhibition of poly(ADP-ribose)-polymerase (PARP) proving effective in vitro and in vivo (8).

The vast majority of the above work has taken place in adult glioblastoma and preclinical models derived from adult patients. In the pediatric setting, MGMT promoter hypermethylation predicts for response to alkylating agents (9); however, the survival of children treated with adjuvant temozolomide does not seem to be improved when compared with historical controls (10–14). The mechanisms of drug resistance in

Abstract

Sensitivity to temozolomide is restricted to a subset of glioblastoma patients, with the major determinant of resistance being a lack of promoter methylation of the gene encoding the repair protein DNA methyltransferase MGMT, although other mechanisms are thought to be active. There are, however, limited preclinical data in model systems derived from pediatric glioma patients. We screened a series of cell lines for temozolomide efficacy in vitro, and investigated the differential mechanisms of resistance involved. In the majority of cell lines, a lack of MGMT promoter methylation and subsequent protein overexpression were linked to temozolomide resistance. An exception was the pediatric glioblastoma line KNS42. Expression profiling data revealed a coordinated upregulation of HOX gene expression in resistant lines, especially KNS42, which was reversed by phosphoinositide 3-kinase pathway inhibition. High levels of HOXA9/HOXA10 gene expression were associated with a shorter survival in pediatric high-grade glioma patient samples. Combination treatment in vitro of pathway inhibition and temozolomide resulted in a highly synergistic interaction in KNS42 cells. The resistance gene signature further included contiguous genes within the 12q13-q14 amplicon, including the Akt enhancer PIKE, significantly overexpressed in the KNS42 line. These cells were also highly enriched for CD133 and other stem cell markers. We have thus shown an in vitro link between phosphoinositide 3-kinase–mediated HOXA9/HOXA10 expression, and a drug-resistant, progenitor cell phenotype in MGMT-independent pediatric glioblastoma. Cancer Res; 70(22); OF1–10. ©2010 AACR.
Pediatric high-grade glioma are poorly understood, in part due to the lack of availability of suitable models of the disease. We screened a series of pediatric and adult glioma cell lines for temozolomide efficacy in vitro, and investigated the differential mechanisms of resistance involved, highlighting the involvement in pediatric cells of processes outside of the usual MGMT/MMR/BER axis.

**Materials and Methods**

**Cell culture**

Adult glioblastoma cell lines A172, LN229, SF268, U87MG, U118MG, and U138MG, and pediatric glioma cell lines SF188, KNS42, UW479, Res259, and Res186 were obtained and cultured as previously described (15). For the spheroid formation assay, cells were grown in neurosphere medium, which consisted of NDiff RHB-A medium (Stem Cell Sciences) supplemented with epidermal growth factor and fibroblast growth factor 2, each at 20 ng/mL.

**Growth inhibition studies**

Temozolomide was obtained from Apin Chemicals, O6-benzylguanine from Calbiochem, and PI-103 from Piramed Pharma or synthesized in-house. Growth inhibition was determined using the sulforhodamine B (16) or MTS (17) assay as previously described. To attempt reversion of resistance to temozolomide, O6-benzylguanine was added at the highest nontoxic concentration (10−15% of cell growth inhibition).

**Figure 1.** Sensitivity of pediatric and adult glioma cell lines to temozolomide and relationship to MGMT status. A, adult (LN229, A172, U118MG, U138MG, U87MG, SF268) and pediatric (SF188, KNS42, UW479, Res186, Res259) glioma cells were treated with temozolomide, and cytotoxicity was assessed by the sulforhodamine B assay. IC50 values are plotted on a log10 scale. B, Western blot for MGMT protein expression correlated with extent of promoter methylation as assessed by MS-PCR and MS-MLPA. In most cases expression correlates with temozolomide resistance, with the exception of U87MG and KNS42 cells, which are hypermethylated, do not express the protein, and are resistant to temozolomide. C, SF188 and KNS42 cells were treated with MGMT substrate analogue O6-benzylguanine, showing the MGMT-dependent nature of temozolomide (TMZ) resistance in SF188, but not KNS42 cells. Growth inhibition was determined by the sulforhodamine B assay. Concentration of temozolomide is on a log10 scale.
of 5-Aza-2′-deoxycytidine (5-aza) methylation was assessed by comparing expression profiles against those obtained from MRC-Holland; ref. 20. Analysis was performed according to the manufacturer's instructions (MRC-Holland; ref. 20). Supervised analysis was performed using an absolute signal-to-noise metric of >1.5 in GenePattern software (http://www.broad.mit.edu/cancer/software/genePattern/). Coordinate gene regulation was identified using gene set enrichment analysis (GSEA; www.broad.mit.edu/gsea/), with a nominal P value cutoff of 0.001. "Core enriched" genes are defined as belonging to the leading-edge subset within the gene set, and thus contribute the most to the enrichment result. Assessment of HOX gene expression after 24-hour treatment with PI-103 at 5 × IC_{50} was carried out using Illumina HT-12 BeadChips (ArrayExpress accession number E-TABM-890). Affymetrix U133 expression data from The Cancer Genome Atlas (TCGA) glioblastoma study (21) was assessed for cross-correlations of probesets corresponding to HOX on by calculating Pearson’s correlation coefficients in R. GSEA and clinical correlations were further carried out on a published dataset (22) of Affymetrix U133 expression array profiling of 78 pediatric high-grade gliomas (Gene Expression Omnibus accession number GSE19578; http://www.ncbi.nlm.nih.gov/geo/).

Immunofluorescence and flow cytometry

CD133 protein expression was measured by both flow cytometry using a BD fluorescence-activated cell sorting Vantage SEDiVa system (BD Biosciences) and immunofluorescence on cytopsin preparations, using anti-CD133 antibody (AC133/1, Miltenyi Biotec) at 1:50 and 1:100 dilution, respectively. Cells were stained with nestin (196908, R & D Systems) and visualized with 4′, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc.). Cell cycle analysis was also carried out by flow cytometry.

Results

Sensitivity of glioma cell lines to temozolomide in vitro is largely but not exclusively dependent on MGMT promoter methylation and lack of protein expression

We first determined the response to temozolomide in vitro of our panel of five pediatric (SF188, KNS42, UW479, Res259, Res186) and six adult (A172, LN229, SF268, U87MG, U118MG, U138MG) glioma cell lines. Four lines (A172, LN229, SF268, Res259) were classed as temozolomide sensitive, with IC_{50} values of between 10 and 20 μmol/L (Fig. 1A). The remaining cells were resistant to treatment with the alkylating agent, with IC_{50} values of >500 μmol/L.

We assessed MGMT promoter methylation by methylation-specific PCR and MLPA, and protein expression by Western

Promoter methylation analysis

Cell line DNA was treated with sodium bisulphite using the Epitect kit (Qiagen) according to the manufacturer’s instructions. Methylation-specific (MS) PCR for the MGMT promoter was performed as described previously (19). MS–multiplex ligation-dependent probe amplification (MLPA) was carried out as previously reported (15) according to the manufacturer’s instructions (MRC-Holland; ref. 20). HOX A9/HOX A10 methylation was assessed by comparing expression profiles of 5-Aza-2′-deoxycytidine–treated cells with vehicle-treated controls on Illumina Human-6 v2 Expression BeadChips (Illumina Inc.), ArrayExpress accession number E-TABM-858.

Western blot analysis

Immunodetection was performed as previously described (15) using antibodies against MGMT (1:500; Zymed), MLH1 (1:500; Pharmingen), MLH3 (1:500; Santa Cruz Biotechnolo-
Using expression microarrays, we identified 135 genes differentially expressed between sensitive and resistant glioma cell lines (Fig. 3A). Included in this list were MGMT and PARP2, despite these enzymes not explaining the resistance in all cell lines. Enzymes involved in DNA MMR (MLH1, MLH3, MSH2, MSH3, MSH6, PMS2) were evaluated by Western blot (Fig. 2A). Although we identified three temozolomide-resistant lines with abrogated expression of MSH3 (U138MG, UW479, and Res186), there was no deficiency in KNS42 or U87MG cells. These data correlated well with levels of promoter methylation assessed by MS-MLPA (15). We further investigated components of the BER pathway, including PARP1/2, XRCC1, and APE1. Although temozolomide-resistant UW479 and Res186 seemed to lack PARP1/2 and XRCC1 expression, there were no apparent alterations in other glioma lines, including KNS42 or U87MG (Fig. 2B).

Identification of a HOX/stem cell gene expression signature associated with temozolomide resistance in pediatric glioblastoma cells

Using expression microarrays, we identified 135 genes differentially expressed between sensitive and resistant glioma cell lines (Fig. 3A). Included in this list were MGMT and PARP2, despite these enzymes not explaining the resistance in all cell lines. Also included were several kinases, including MAPK9 and CDK6, which may prove suitable targets for pharmacologic modulation; PIK3C3 (Vps34), suggesting a possible link to the autophagic response (24); and genes encoding elements of the immune response such as IL10 and IL16.

When we applied GSEA to our data, we observed coordinated differential expression of the HOX_GENES set (MSigDB C2:curated gene sets) in resistant versus sensitive cell lines (Fig. 3B), with an enrichment score of 0.54 [nominal \( P = 0.01 \); false discovery rate (FDR) \( q = 0.403 \)]. Furthermore, the HOX_GENES list was also identified as significant using a GSEA “Preranked” analysis based on differentially expressed genes between KNS42 alone and temozolomide-sensitive cell lines (Fig. 3C). In these analyses, the genes in both core-enriched lists, which contribute to the leading-edge subset within the gene set (25), included HOXA9, HOXA10, HOXB13, HOXC4, HOXC10, HOXC11, HOXD13, HOXD1, and Gbx2.

As coordinated expression of HOX genes had recently been noted in glioblastoma clinical samples (26), and was reported as evidence of a “self-renewal” signature as it included the stem cell marker PROM1 (CD133), we sought further evidence for this in a published glioblastoma dataset and our cell line models. When we investigated TCGA expression profiles of 163 glioblastomas for genes that correlated with the top-ranking HOX gene in our KNS42 GSEA list, HOXA9, we noted a remarkable parallel expression of numerous other homeobox genes (Fig. 4A). Of the 47 genes by this analysis, 18 were homeobox genes found at 9 distinct genomic loci, and 7 were included in the self-renewal signature of Murat and colleagues (26). These latter genes included PROM1. Intriguingly, the vast majority of the non–homeobox genes identified by this analysis are contiguous genes found commonly amplified in glioblastoma at the genomic locus 12q13-1q4.

To determine whether an enrichment of the stem cell marker CD133 may be playing a role in the resistance of KNS42 cells to temozolomide, we assessed the levels of mRNA expression relative to the other cell lines (Fig. 4B), and noted considerably higher levels of PROM1 in KNS42 cells than in any other line in our panel. Of note, the only other two cell lines to express PROM1 at above background levels were the similarly temozolomide-resistant U87MG and SF188. This was visualized by immunofluorescent staining for CD133, colabeled with nestin (Fig. 4C). We had previously reported the relatively high levels of stem cell markers in SF188 and KNS42 by immunocytochemistry (15), and to more accurately quantify this, we used the more sensitive flow cytometry analysis to reveal an usually high degree of expression in KNS42, with 17.0% of cells positive for CD133, compared with 0.0% to 0.02% in other cell lines. KNS42 cells grown as monolayers also expressed by far the greatest levels of other stem cell markers such as nestin, SOX2, and musashi-1 (Affymetrix U133, data...
Consistent with the cancer stem/progenitor cell–associated gene expression profile, neurosphere formation assays showed that KNS42 cells form tight three-dimensional spheroids, which may be serially passaged and undergo self-renewal. In contrast, SF188 cells form smaller, more loosely packed spheres, whereas U87MG grow as cell aggregates rather than neurospheres per se. Neither UW479, Res259, nor Res186 formed spheres under these conditions. Taken together, KNS42 cells seem to have a significant cancer stem/progenitor cell–associated gene expression signature and biological phenotype.

**Expression of HOXA9/HOXA10 is a result of phosphoinositide 3-kinase–mediated demethylation, inhibition of which synergistically interacts with temozolomide in KNS42 cells**

Treatment with the demethylating agent 5-aza-2′-deoxycytidine resulted in highly differential levels of expression of HOXA9 and HOXA10 in all pediatric cell lines with the exception of KNS42, in which no changes were observed, indicative of a lack of methylation in the untreated cells (Fig. 5A). As a recent study has proposed a mechanism for this observation whereby transcriptional activation of the HOX4 cluster is reversible by a phosphoinositide 3-kinase (PI3K) inhibitor through an epigenetic mechanism involving histone H3K27 trimethylation (27), we sought to investigate whether this mechanism was active in our system. Treatment for 1, 8, and 24 hours with the dual PI3K/mTOR inhibitor PI-103 (28–30) at 5 × IC50 resulted in significantly reduced expression of both HOXA9 and HOXA10 in KNS42 cells in a time-dependent manner (Fig. 5B). These effects were not due to fluctuations in HOX gene expression with the cell cycle, as diminished HOXA9/HOXA10 was observed as early as one hour posttreatment, at which time there was no evidence of G1 arrest (Fig. 5C), despite inhibition of PI3K as seen by reduced phospho-Akt levels (Fig. 5D).

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**Figure 4.** Coordinated upregulation of HOX genes in primary glioblastomas and a striking degree of CD133 positivity on KNS42 cells. A, genes coordinately expressed with HOXA9 from the TCGA dataset (21) were determined by calculating Pearson's correlation coefficients. All genes with values >0.3 are listed, in rank order. Yellow highlight, homeobox genes; orange highlight, genes also present in the HOX gene/self-renewal signature of Murat and colleagues (26), including PROM1 (CD133; box); gray highlight, genes found within the 12q13-q14 amplicon. B, Affymetrix expression analysis of PROM1 (CD133) mRNA levels in the panel of glioma cell lines, plotted as relative log2 expression. C, immunofluorescence assay showing extensive expression of stem cell markers CD133 (green) and nestin (red) in KNS42 cells grown as a monolayer. D, neurosphere formation assay highlighting the tight three-dimensional spheroids formed by KNS42 cells in contrast to the other cell lines studied.
Next we sought to determine whether there was any specific dysregulation of the PI3K/PTEN system in KNS42 cells that may be responsible for the \textit{HOX} gene overexpression. Mutation screening for \textit{PTEN}, \textit{PIK3CA}, \textit{PIK3R1}, and \textit{PIK3R3} did not identify any sequence variations (data not shown), and Western blot analysis confirmed a lack of overexpression of PI3K regulatory and catalytic subunits (Fig. 5E). By contrast, there were significantly elevated levels of the enhancer proteins PIKE-A and especially PIKE-L in KNS42 cells in comparison with the other lines. Both PIKE proteins are encoded by the \textit{CENTG1} (AGAP2) gene found within the 12q13-q14 amplicon coordinately upregulated in association with the \textit{HOX} cluster, and likely represent a significant target for this genomic event in human glioblastoma.

Finally, we investigated the efficacy of targeting PI3K as a strategy for overcoming temozolomide resistance in our pediatric glioma cells. Combination treatment \textit{in vitro} of temozolomide with the dual PI3K/mTOR inhibitor PI-103 resulted in a highly synergistic interaction in KNS42 as measured by median effect analysis (combination index = 0.43; Fig. 5F). By contrast, in SF188 cells an antagonistic response was observed with the same combination (combination index = 1.40).

**HOXA9/HOXA10 expression is associated with shorter survival in pediatric high-grade glioma patients**

To assess the translational relevance of \textit{HOX} gene expression in pediatric high-grade glioma patient samples, we examined published data detailing expression profiles of 78 tumors arising in childhood (22). Although the number of long-term (>3 years) survivors is small, we identified 49 genes that were differentially expressed between patients with long and short (<1 year) overall survival (Fig. 6A). Included in this list were \textit{HOXA2}, \textit{HOXA5}, \textit{HOX7}, and \textit{HOXA9}. By applying GSEA to the dataset, we identified coordinated upregulation in the short-term survivors of genes at the chromosome 7p15 cytoband, with an enrichment score of 0.68 (nominal \(P < 0.001\), FDR \(q < 0.001\); Fig. 6B). There was considerable correlation between gene expression of all members of the \textit{HOX} family in the pediatric samples (Pearson's correlation coefficients 0.03–0.90). Taking the values of \textit{HOXA9} and \textit{HOXA10}, and segregating samples into "high" and "low" expressers (combined expression greater or less than the 75th percentile of all values, respectively), we showed a
significantly reduced overall survival of pediatric high-grade glioma patients with high HOXA9/HOXA10 expression (log-rank test, \( P = 0.0453 \); Fig. 6C) independent of the WHO grade of the tumor (\( P = 0.635 \), Fisher’s exact test).

### Discussion

Promoter methylation of the MGMT gene is generally accepted as the major determinant of sensitivity to the alkylating agent temozolomide in glioblastoma cells, and as such has major significance in the treatment of these patients. We identified the pediatric glioblastoma cell line KNS42 to be resistant to temozolomide in vitro despite an absence of MGMT expression, a competent MMR system, and an intact double-strand break repair pathway. Clues as to the mechanism of resistance in these cells may help in identifying factors that contribute to childhood glioblastoma patients who remain refractory to temozolomide treatment.

Gene expression profiling of a panel of pediatric and adult glioma cell lines highlighted coordinated expression of numerous HOX genes in the resistant cell lines, most especially KNS42, and provided in vitro model system evidence in support of data from temozolomide-treated adult glioblastoma patients (26). Using a similar expression profiling and GSEA approach, Murat and coworkers identified a HOX-dominated gene cluster as an independent predictive factor of resistance. Integrating the core gene lists of the present study to that dataset highlights HOX9 and HOX10 as the key effectors in both systems. This converges with a recent study identifying the HOX4 cluster, and HOX10 in particular, to be independent negative prognostic markers in adult glioblastoma (27). Herein we provide evidence for an additional prognostic role in pediatric high-grade glioma.

**Figure 6.** High levels of HOXA gene expression are associated with shorter survival in pediatric high-grade glioma patients. A, heatmap representing differentially expressed genes between short (<1 year; yellow highlight) and long-term (>3 years; gray highlight) survivors. WHO grade IV (black) and III (green) tumors are indicated. Light blue, HOXA genes. B, GSEA analysis showing significant enrichment of genes at the chromosome 7p15 locus (enrichment score = 0.68, nominal \( P < 0.001 \), FDR \( q < 0.030 \)), and of HOXA genes in particular (enrichment score = 0.90, nominal \( P < 0.001 \), FDR \( q < 0.001 \)), upregulated in short-term survivors. C, Kaplan-Meier plot showing a significantly shorter survival of patients with high levels of HOX9/HOXA10 gene expression (\( P = 0.0453 \), log-rank test).
alkylating agents. Costa and colleagues propose that HOXA9 exerts antiapoptotic and proliferative effects after upregulation via an epigenetic mechanism controlled by PI3K and independent of mTOR (27). Our findings confirm this observation, and add to previous evidence showing the synergistic interactions of temozolomide and PI3K pathway inhibitors in *in vitro* and *in vivo* models of adult glioblastoma (33); this combination may therefore also be beneficial in glioblastoma patients with a MGMT-independent HOX gene signature–associated mechanism of resistance to temozolomide.

Murat and colleagues suggested strong HOXA10 expression in glioblastoma-derived neurospheres to be in line with a role of HOX genes in the glioma stem-like cell compartment, and showed that the resistance signature as a whole is evocative of self-renewal (26). Of note is the presence of a high proportion (17.0%) of CD133-positive cells present in our KNS42 cell monolayer cultures, with additional expression of other stem cell markers in coordination with the HOX gene signature. Such a HOX/stem cell signature was also found to be tightly regulated in an analysis of TCGA glioblastoma expression data (21). The pediatric glioblastoma cell line KNS42 may be an excellent experimental model for investigating such interactions with MGMT-independent treatment failure.

We also found a remarkable link between the HOX/stem cell signature and coordinated overexpression of genes within the CDK4 amplicon at 12q13-q14 in glioblastoma patient samples. This association was also present, and correlated with poor response to temozolomide chemoradiotherapy, in the Murat and colleagues dataset (26). Here we provide a possible mechanism for this coexpression of HOX and 12q13-q14 genes in the form of overexpression of the Akt enhancer PIKE (*CENTG1, AGAP2*), also present at very high levels in the unamplified KNS42 cells, which may drive the changes in H3K27 methylation of the HOX cluster mediated via PI3K pathway signaling (27).

It is apparent that a variety of processes, not all involving repair or tolerance of alkyl lesions, may promote alkylator resistance (23). Along with providing therapeutic guidance for those patients whose tumors are intrinsically resistant to treatment, characterization of additional determinants of resistance is necessary to develop new targets for therapy in tumors that acquire resistance to temozolomide *in vivo* in the presence of hypermethylated MGMT.

### Disclosure of Potential Conflicts of Interest

N. Gaspar, L. Marshall, L. Perryman, D.A. Bas, S.E. Little, M. Viana-Pereira, S.Y. Sharp, A.D.J. Pearson, P. Workman, and C. Jones are or were employees of The Institute of Cancer Research, which has a commercial interest in the development of PI3K inhibitors and operates a rewards-to-inventors scheme. P. Workman and his team have been involved in a commercial collaboration with Yamanouchi (now Astellas Pharma) and with Piramed Pharma, and intellectual property arising from the program has been licensed to Genentech. P. Workman was a founder of, consultant to, Scientific Advisory Board member of, and stockholder in Piramed Pharma, which was acquired by Roche.

### Acknowledgments

We thank Dr. Daphne Haas-Kogan (University of California, San Francisco) and Dr. Michael Bobola (University of Washington) for provision of the pediatric glioma cell lines, and Dr. Michael Hubank (Institute of Child Health, University College London) for assistance with the expression profiling.

### Grant Support

Cancer Research UK (C1178/A10294, C309/A2187, C309/A8274), the Oak Foundation (L. Marshall), and La Fondation de France (N. Gaspar). We acknowledge NIH funding to the NIH Neuro-Oncology Program. P. Workman is a Cancer Research UK Life Fellow.

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Received 04/09/2010; revised 09/14/2010; accepted 09/17/2010; published OnlineFirst 10/08/2010.

### References


MGMT-Independent Temozolomide Resistance in Pediatric Glioblastoma Cells Associated with a PI3-Kinase–Mediated HOX/Stem Cell Gene Signature


Cancer Res  Published OnlineFirst October 8, 2010.

Updated version  Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-10-1250

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