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ORIGINAL REPORT

Stem Cell–Related "Self-Renewal" Signature and High Epidermal Growth Factor Receptor Expression Associated With Resistance to Concomitant Chemoradiotherapy in Glioblastoma

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Terms in blue are defined in the glossary, found at the end of this article and online at www.jco.org.

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ABSTRACT

Purpose
Glioblastomas are notorious for resistance to therapy, which has been attributed to DNA-repair proficiency, a multitude of deregulated molecular pathways, and, more recently, to the particular biologic behavior of tumor stem-like cells. Here, we aimed to identify molecular profiles specific for treatment resistance to the current standard of care of concomitant chemoradiotherapy with the alkylating agent temozolomide.

Patients and Methods
Gene expression profiles of 80 glioblastomas were interrogated for associations with resistance to therapy. Patients were treated within clinical trials testing the addition of concomitant and adjuvant temozolomide to radiotherapy.

Results
An expression signature dominated by *HOX* genes, which comprises *Prominin-1* (CD133), emerged as a predictor for poor survival in patients treated with concomitant chemoradiotherapy (n = 42; hazard ratio = 2.69; 95% CI, 1.38 to 5.26; $P = .004$). This association could be validated in an independent data set. Provocatively, the *HOX* cluster was reminiscent of a "self-renewal" signature ($P = .008$; Gene Set Enrichment Analysis) recently characterized in a mouse leukemia model. The *HOX* signature and *EGFR* expression were independent prognostic factors in multivariate analysis, adjusted for the *O*-6-methylguanine-DNA methyltransferase (*MGMT*) methylation status, a known predictive factor for benefit from temozolomide, and age. Better outcome was associated with gene clusters characterizing features of tumor-host interaction including tumor vascularization and cell adhesion, and innate immune response.

Conclusion
This study provides first clinical evidence for the implication of a "glioma stem cell" or "self-renewal" phenotype in treatment resistance of glioblastoma. Biologic mechanisms identified here to be relevant for resistance will guide future targeted therapies and respective marker development for individualized treatment and patient selection.

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INTRODUCTION

In glioblastoma, introduction of combined chemoradiotherapy of concomitant and adjuvant temozolomide (TMZ) and radiotherapy (TMZ/RT→TMZ) has allowed significant prolongation of survival,[1,2] in particular in patients with an epigenetically silenced *O*-6-methylguanine-DNA methyltransferase (*MGMT*) DNA repair gene.[3,4] However, outcome remains unsatisfactory, and ongoing clinical trials explore modulation of MGMT or the addition of targeted agents.[1,5] Recognizing molecular tumor signatures of underlying biologic processes associated with resistance in patients treated with this new standard therapy will allow the identification of potential targets for improvement of therapy.

Recent concepts for cancer development suggest that a minority population of cancer stem-like cells may determine the biologic behavior of tumors, including response to therapy. Failure to cure cancer has been attributed to the fact that therapies are aimed at the tumor bulk without significantly harming tumor stem-like cells,[6] supported by experimental evidence in a respective mouse model showing that this glioblastoma subpopulation of cells is more

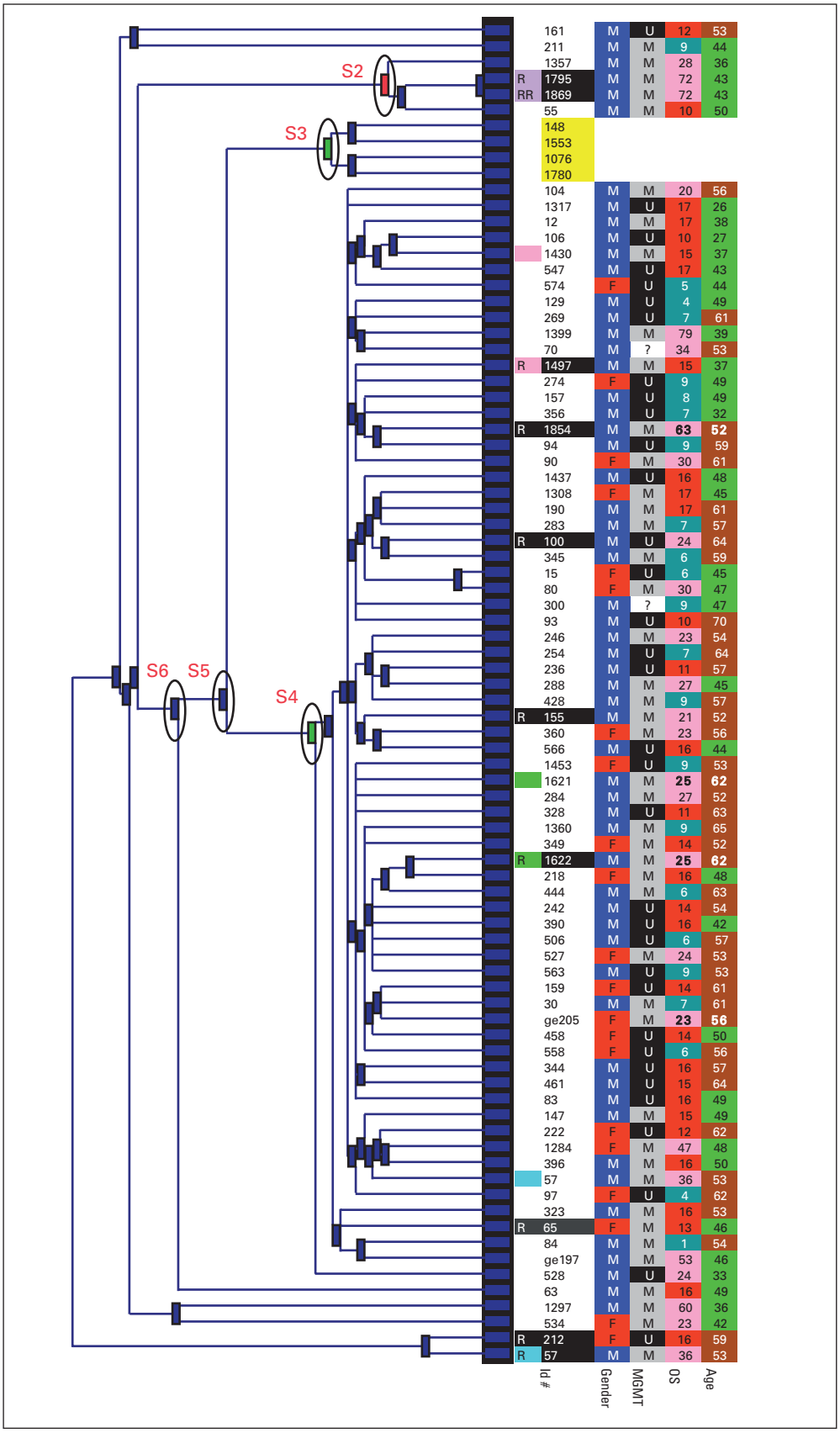

Fig 1. Sample dendrogram S1(G1) by Coupled Two-Way Clustering (CTWC). Sample dendrogram S1(G1) emerging from clustering all 84 samples S1 with all genes G1. Stable sample clusters S2 to S6 emerge. Clinical information: age at diagnosis (green, < 50 years; brown, > 50 years), overall survival in months (green, < 9; red, 9-18; pink > 18); *MGMT* methylation status (gray, methylated; black, unmethylated; white, unknown); sex (red, female); and sample Id# (sample identification number, nontumoral brain tissue, yellow; recurrent glioblastoma, black; color code tumor pairs from same patient, black only recurrent glioblastoma).

Table 1. Cox Analysis Using Stable Gene Clusters Identified in G1(S1) in the Cohort of Treated Patients (n = 42)

From G1(S1)							
Main Clusters	Subclusters	Description	No. of Probes in Cluster	*P**	False-Discovery Rate†	Hazard Ratio‡	95% CI
G2		Migration-related	19	.21	0.32	0.73	0.45 to 1.19
G7		Tumor blood vessel markers	77	**.04**	0.11	**0.73**	0.54 to 0.99
G9		Hypoxia-induced	32	.70	0.78	0.89	0.49 to 1.61
G11		SOX genes	15	.25	0.34	1.46	0.77 to 2.77
G12		Interferon-induced genes	80	.78	0.83	1.06	0.72 to 1.55
G13		Chromosome 12	39	**.02**	0.09	**1.13**	1.02 to 1.24
G14		Myeloid lineage/adhesion	26	**.03**	0.09	**0.61**	0.39 to 0.95
G16		Glial lineage	49	.07	0.16	1.64	0.96 to 2.81
G18		Brain physiology	25	**.03**	0.09	**1.94**	1.07 to 3.51
G20		??	24	.20	0.32	1.36	0.85 to 2.19
G21	> G17 > G10 > G3	Neural genes	213	.99	0.99	1.00	0.69 to 1.46
G22		Imprinted genes	34	.20	0.32	0.65	0.34 to 1.25
G23	> G19 > G4	Oligodendrocyte markers	110	.66	0.78	1.07	0.78 to 1.47
G24	> G15 > G8	Innate immune response	134	**.03**	0.09	**0.65**	0.44 to 0.96
G25		*EGFR*	18	**.002**	**0.03**	**2.78**	1.44 to 5.36
G27	> G5	Spermatogenesis	79	.10	0.20	1.38	0.94 to 2.03
G28		*HOX* genes	20	**.004**	**0.03**	**2.69**	1.38 to 5.26
G29	> G26 > G6	Proliferation	101	.43	0.55	1.27	0.70 to 2.32

NOTE. Bold text indicates statistical significance, $P < .05$.
*P value for the coefficient relative to the mean of the cluster in a multivariate Cox proportional hazards model adjusted for *MGMT* methylation status and age as additive independent risk factors ($y \sim \beta_1 \text{meanCluster} + \beta_2 MGMT + \beta_3 \text{age}$).
†False discovery rate to correct for multiple testing (Benjamini-Hochberg procedure).[17]
‡Each continuous variable was scaled to have the interquartile range equal to 1 and median equal to 0.

resistant to radiotherapy.[7] Facilitated by markers differentiating stem-cells and progenitors of the different lineages, the origin of leukemic stem cells has been traced back to hematopoietic stem cells as well as progenitor populations that have acquired self-renewal properties.[8,9] In contrast, the origin and concept of glioma stem-like cells remains to be fully elucidated. CD133 positivity has been postulated to be a glioma stem-cell marker, considering that this subpopulation of glioma-derived cells seems to have a higher potential to generate and maintain tumors in vivo.[7,10]

With the goal of identifying molecular mechanisms of treatment resistance, we report here on the analysis of glioblastoma-derived gene expression profiles from patients treated within two prospective clinical trials.[1,2] We have identified several biologic processes associated with resistance or responsiveness to combined chemoradiotherapy that provide important information guiding novel treatment strategies and aiming at individualized therapy. Intriguingly, an expression signature associated with resistance shows high similarity with a stem cell–related self-renewal signature.[9] Our data provide the first clinical evidence to our knowledge for the involvement of a tumor stem-cell phenotype in the escape of glioblastoma from chemoradiotherapy.

PATIENTS AND METHODS

Tumor Samples and Patient Characteristics

Gene expression profiles were established from 80 frozen glioblastoma samples obtained from 76 patients, comprising 70 tumors from initial surgery and 10 samples resected at recurrence, and from four non-neoplastic brain tissue samples. All patients were treated within a phase II or a randomized phase III trial[1,2] and provided written informed consent for molecular studies of their tumor. The protocol was approved by the ethics committee at each center. Sixty-eight patients with complete molecular and clinical information were included in survival analysis, with a median age of 51 years (range, 26 to 70 years) at enrollment. Thereof, 42 received TMZ/RT→TMZ, and 26 were randomly assigned to RT only. Second-line therapy frequently involved alkylating agents including TMZ. Patient characteristics are summarized in Table A1(online only). The validation set comprised 76 independent patients of the European Organisation for Research and Treatment of Cancer (EORTC)/National Cancer Institute of Canada (NCIC) study,[1] 39 randomly assigned to TMZ/RT→TMZ, and 37 to RT (median age, 54 years; range, 25-69 years), whose glioblastomas were available on a tissue microarray (TMA). There was no difference in survival compared with the general trial population, neither in the test population nor the validation set ($P > .2$).

Gene Expression Profiling

Total RNA was extracted from frozen tumor sections (10 to 15 mg; Qiagen RNeasy-Lipid Tissue Kit; Hilden, Germany). The first section served as reference for diagnosis and tumor content (> 70%). Probes were prepared with the Enzo BioArray-High Yield Kit (Enzo Life Sciences, Farmingdale, NY) for double amplification and were hybridized to Affymetrix HG-133Plus2.0 GeneChips (Affymetrix, Santa Clara, CA). The microarray data is deposited in the Gene Expression Omnibus (GEO) database at http://www.ncbi.nlm.nih.gov/geo/ (accession-number GSE7696). Quantitative reverse transcription polymerase-chain reaction (qRT-PCR) was performed on the ABI Prism7900 with SYBR Green (Applied Biosystems, Foster City, CA). Primers are listed in Table A2 (online only). Results were normalized to the expression of the *EIF2C3*, *DNAJA4*, and *B2M* genes that exhibit little variation in this data set.

Data Analysis and Statistical Methods

Analyses were carried out in R, a free software environment available at http://www.r-project.org/, SAS (V9.1.3; SAS Institute, Cary, NC), or Coupled Two Way Clustering (CTWC),[11-13] available at http://ctwc.weizmann.ac.il. The expression intensities for all probe sets from Affymetrix CEL-files were estimated using robust multiarray average with probe-level quantile

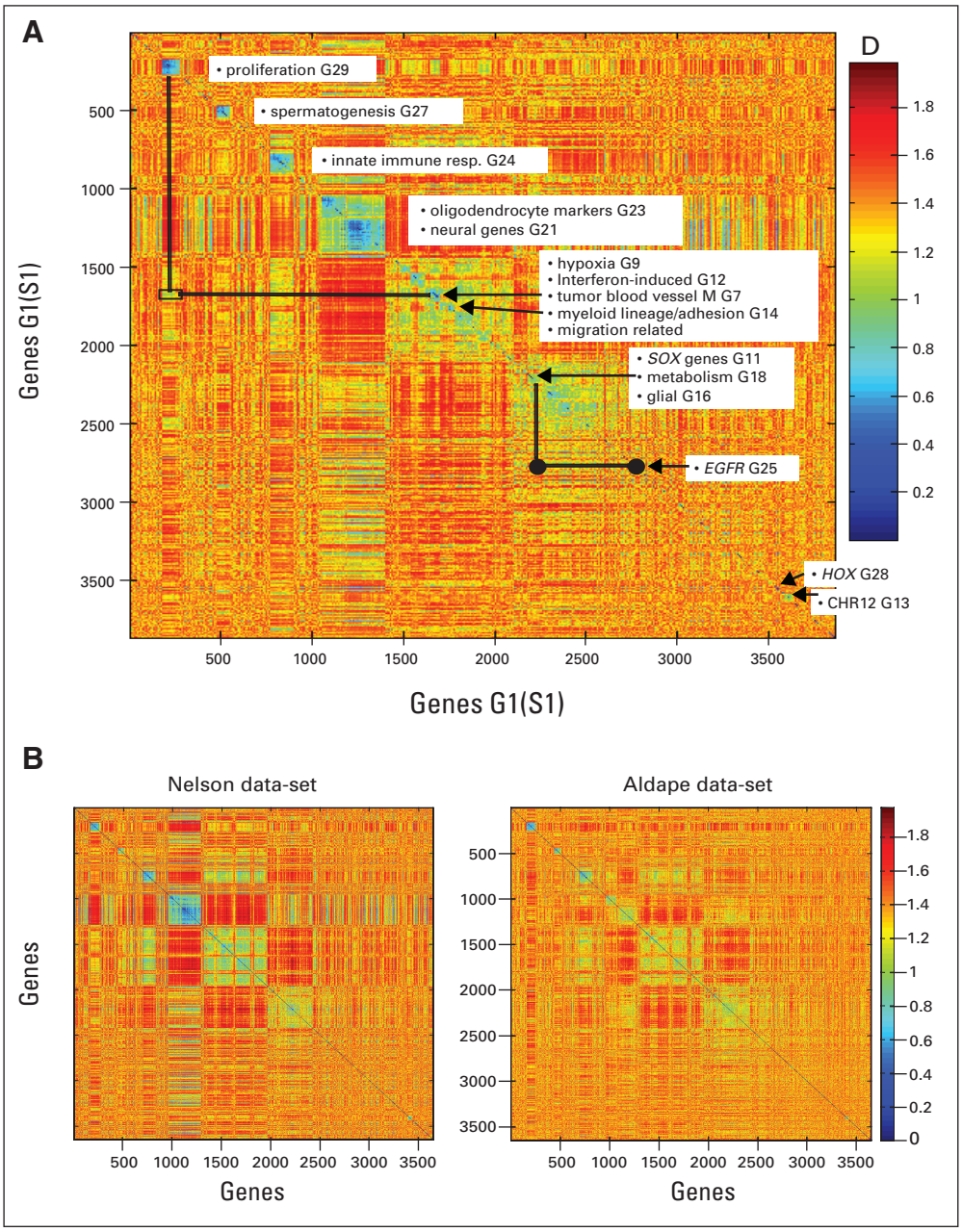

Fig 2. (A): The distance matrix of all filtered genes G1 (Euclidian distance) heatmap; blue indicates short distance (more similar), red indicates large distance. The distance ranges from 0 (corresponding to Pearson correlation = 1; ie, full similarity of the two expression profiles) to 2 (corresponding to anticorrelated profiles, Pearson = −1). Zero correlation indicated by orange. Annotation is based on gene dendrogram G1(S1), not shown. The distance matrix visualizes relationships between clusters as indicated by lines and arrows (eg, G7 is in the center of a supercluster, reflecting close relationship with G9, G12, G14 and G2). In addition G7 is related to G29, but not G23 or G21. (B) Respective distance matrices for two published data sets with 48 and 54 glioblastomas[14,15] comprising the common probe sets (3,649) and ordered according to the matrix in Figure 2A. All three data sets appear similar, particularly visible for the largest clusters annotated in Figure 2A.

normalization followed by median polish summarization as implemented in the BioConductor software (http://www.bioconductor.org/).

The expression matrix of 84 samples and 3,860 most variable probe sets (standard deviation, > 0.75) was input into CTWC using default parameters and two levels of clustering. CTWC analysis can be viewed at: http://bcf.isb-sib.ch/projects/cancer/glio/. Probe sets comprising stable gene clusters emerging from CTWC served as input for supervised analyses.

Details on the Cox and partial least square models, and published data sets used[9,14-16] are described in the Appendix and Table A3 (online only). The Benjamini-Hochberg procedure was applied for multiple testing correction (false-discovery rates).[17]

TMA and Immunohistochemistry

The TMA was constructed with glioblastoma from the EORTC/NCIC-trial.[1] HOXA10 (Santa Cruz Biotechnology, Santa Cruz, CA; sc-17159; dilution, 1:200) expression was determined by immunohistochemistry (citrate buffer: pH 6.0, 15 minutes in pressure cooker) and scored without knowledge of clinical information on a scale of 0 to 6 (0, no expression; 1, weak nuclear expression in < 20% cells; 2, strong in < 20% cells; 3, weak in 20% to 50% cells; 4, strong in 20% to 50% cells; 5, weak in > 50% cells; 6, strong in > 50% cells). Dichotomization for survival analysis was no to low score (1, 2) *v* intermediate to high expression (scores 2-6). Frozen sections of neurospheres were fixed with acetone and stained for HOXA10 and CD133 (Santa Cruz; sc-17159, dilution 1:100; Miltenyi AC133-2, 293C3, dilution 1:50).

Glioblastoma-Derived Neurospheres

Fresh glioblastoma tissue was dissociated in presence of papain and DNase I basically as described previously.[18] Cells were cultured under stem-cell conditions to form spheres using Dulbecco's modified Eagle medium/F12 medium containing B27 supplement and 20 ng/mL of both epidermal growth factor and fibroblast growth factor 2. Neurospheres from six glioblastomas propagated between 4 weeks and 14 months were used for immunostaining.

Table 2. Overall Cox Proportional Hazards Models for the Cohort of 42 Patients Treated With Concomitant and Adjuvant Temozolomide and Radiotherapy

Variable	Univariate Model				Multivariate Model			
	Hazard Ratio	95% CI	*P*	R^2	Hazard Ratio	95% CI	*P*	R^2
HOX cluster (G98), mean expression	1.89	1.03 to 3.46	.04	0.10	3.32	1.61 to 6.82	.001	0.67*
EGFR expression	1.87	1.09 to 3.22	.02	0.12	3.13	1.62 to 6.04	< .001	
MGMT methylation	0.15	0.06 to 0.37	< .001	0.38	0.06	0.0 to 0.20	< .001	
Age > 50 years	1.89	0.93 to 3.83	.08	0.07	2.61	1.22 to 5.57	.01	

*R^2 for the overall multivariate Cox proportional hazards model.

RESULTS

Gene Expression Signatures Associated With Tumor Resistance

There was no obvious association of patient characteristics or survival with genetic subtypes evident from the sample dendrogram S1(G1), clustering all samples (S1) and all genes (G1) passing a variation filter (Fig 1). The methylation status of the *MGMT* gene promoter appears to be randomly distributed. All stable gene clusters emerging from this analysis are listed in Table 1, named by the predominant biologic function suggested by the genes they comprise, whereas their inter-relationship is visualized in Fig 2A. Similar gene clusters are obtained when using only the subset of glioblastoma clustered in S4 [G1(S4); Appendix Table A4, online only]. Cluster S4 comprises most glioblastoma (69 of 80), but not the nontumoral tissues that form their own stable cluster, S3 (Fig 1). Similar gene clusters are present in other glioblastoma data sets as visualized in Fig 2B for data sets published by the groups of Nelson and Aldape, respectively.[14,15] All 18 nonoverlapping, stable gene clusters [G1(S1)] were interrogated for association with survival using Cox proportional hazards, adjusted for age (> 50 years) and *MGMT* methylation status.[3,4] Seven gene clusters were most influential for explaining survival in patients assigned to TMZ/RT→TMZ (Table 1; gene lists, Appendix Tables A5-A10, online only). A respective partial least square model yielded comparable results (Appendix Fig A1, online only). However, the *MGMT* methylation status was yet the most influential predictor of survival (Table 2; Appendix Fig A1, online only). For this study, we focused on the two most significant clusters characterized by *HOX* (homeobox) gene (G28/G98) and *EGFR* (epidermal growth factor receptor) gene expression (G25), respectively, and here we briefly comment on the biologic and clinical implications denoted by the other relevant clusters.

Self-Renewal Signature Associated With Resistance to Chemoradiotherapy in Glioblastoma

Increased expression of cluster G28, dominated by *HOX* genes and comprising the cell-cycle checkpoint gene *GADD45G*,[19] was found to be associated with worse outcome ($P = .004$; hazard ratio [HR] = 2.69; 95% CI, 1.38 to 5.26; Table 1). *HOX* genes are essential in axis determination during embryonic development and are known to be involved in cancer including glioblastomas.[20,21] The interaction term between this *HOX* cluster and chemoradiotherapy was significant ($P = .001$) in the Cox model when evaluating all 68 patients from the two treatment arms, implying that high expression of *HOX* genes may be predictive for resistance to TMZ/RT→TMZ therapy.

The *HOX* gene clusters G28 and G98 emerging from clustering all genes (G1) either with all samples (S1) or only with glioblastoma clustered in S4 are almost identical (Pearson correlation, 0.98; 19 of 21 probe-sets; Appendix Table A11, online only). Intriguingly, G98 in addition comprises *Prominin 1*, encoding the putative glioma stem–cell marker CD133 (Fig 3A), suggesting that in a subpopulation of glioblastoma, concerted upregulation of *HOX* genes might be associated with a tumor stem-like cell phenotype. In accordance, we find high HOXA10 protein expression in glioblastoma-derived neurospheres, cultured under stem-cell conditions, as displayed in Fig 3B together with CD133 expression. To include information on *Prominin 1*, we show results on G98 for all following analyses. Fig 3C visualizes the association of short survival with enhanced expression of G98.

Validation in Independent Data Sets

The association of the *HOX* signature with resistance to treatment was subsequently validated in a sample set of the trial not available for initial discovery, arrayed on TMA. HOXA10 was evaluated by immunohistochemistry (Fig 4) as a representative of the correlated set of *HOX* genes. Strong nuclear expression was often observed in patches of tumor cells situated in the vicinity of blood vessels. High HOXA10 expression was associated with worse outcome in patients randomly assigned to TMZ/RT→TMZ therapy (n = 39; HR = 2.57; 95% CI, 1.21 to 5.47; $P = .014$; Fig 5). The patients randomly assigned to RT only did not show such a relationship, suggesting that high HOXA10 expression may be predictive for resistance to a synergistic effect of concomitant chemoradiotherapy, in concordance with the significant interaction term between treatment and expression of G28 or G98 (Appendix Table A12, online only). Similar *HOX* clusters can be identified in the Nelson and Aldape glioblastoma data sets.[14,15] Correlating G98 gene expression with outcome in these data sets totaling 102 glioblastoma revealed a trend for worse outcome ($P = .09$; HR = 1.29; 95% CI, 0.97 to 1.72; Appendix Fig A2, online only). Of note, in contrast to our data, these patients were treated before the TMZ/RT→TMZ regimen was established. In accordance with better survival, anaplastic glioma (WHO grade 3) profiled in these publications revealed significantly lower expression of G98 genes compared with glioblastoma (WHO grade 4, $P < .001$ for the Aldape data set[14]; $P = .002$ for the Nelson data set[15]; Wilcoxon rank sum test with continuity correction; Appendix Fig A3, online only). However, within grade 3 glioma of the two data sets increased expression of G98 genes was associated with worse outcome (n = 44; $P = .007$; HR = 3.35; 95% CI, 1.39 to 8.08; Appendix Fig A2).

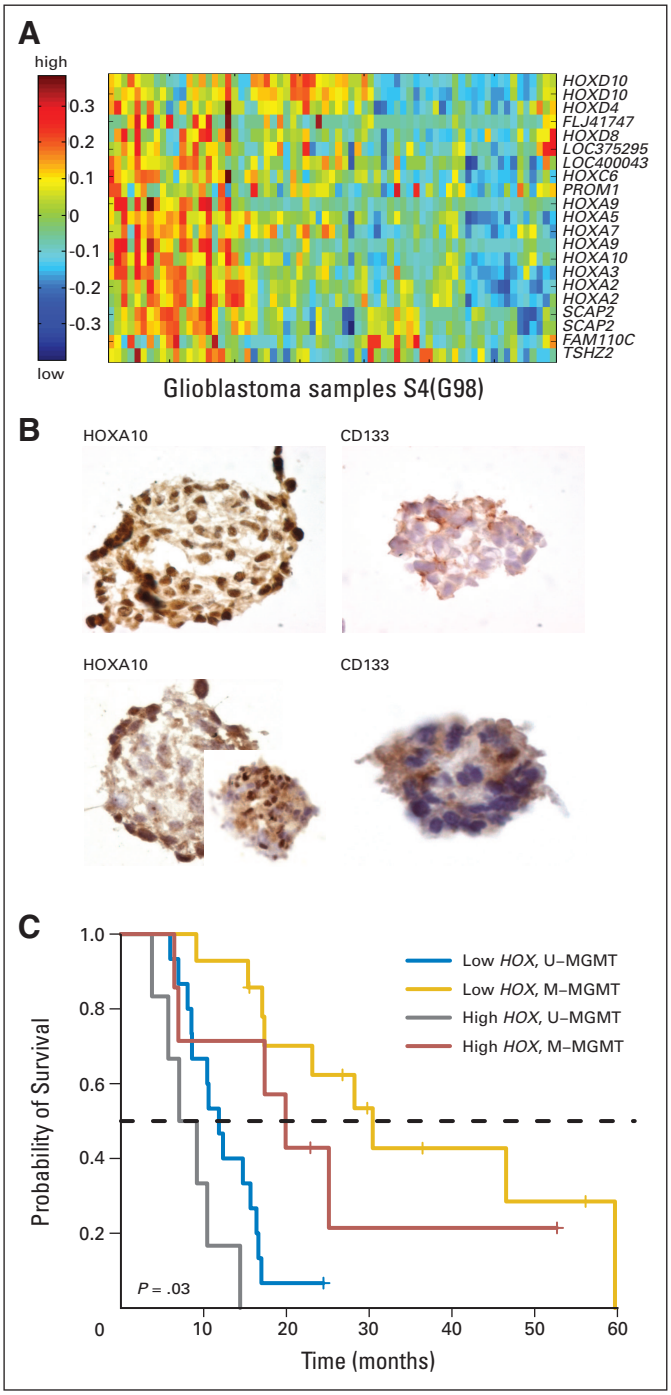

Fig 3. Relation of glioblastoma derived *HOX* gene signature with survival. (A) Stable cluster G98 was found by clustering all genes G1 with samples clustered in S4 (69 glioblastomas). Red indicates relative overexpression and blue relative underexpression. (B) Glioblastoma-derived neurospheres (40× magnification) cultured under stem-cell conditions exhibit strong nuclear staining for HOXA10 and express CD133 as determined by immunohistochemistry. Representative neurospheres from two glioblastoma are shown. (C) Kaplan-Meier survival estimates of the 42 patients treated with concomitant and adjuvant temozolomide (TMZ) and radiotherapy (TMZ/RT→TMZ) separated into high (High *HOX*) versus low (Low *HOX*) expressors of G98 genes (dichotomized according to Coupled Two-Way Clustering sample dendrogram). The *P* value of the log-rank test is shown for the two groups, stratified by the *MGMT* methylation status. M-MGMT, methylated *MGMT*; U-MGMT, unmethylated *MGMT*.

HOX *Signature Reminiscent of Self-Renewal*

The core of the *HOX* cluster was found to be part of the top 20 genes of a self-renewal signature identified by Krivtsov et al[9] in murine MLL-AF9-induced leukemic stem cells derived from committed progenitors. Our G98-derived signature was significantly enriched in genes discriminating self-renewal versus non–self-renewal in this expression data set according to Gene Set Enrichment Analysis (GSEA; $P = .008$; Appendix Fig A4 and Table A13, online only) and the Wilcoxon two-sample test (G98; $P < .001$).[22] The relevance of our signature was further demonstrated in a human data set[16] in which G98, similar to the original murine self-renewal signature,[9] was able to significantly differentiate MLL-rearranged–acute myeloid leukemia from acute myeloid leukemia (GSEA, $P < .001$; Wilcoxon two-sample test $P = .01$; Appendix Table A14, online only).

Interestingly, a significant, although low, correlation between the mean DNA copy number of the two BAC (bacterial artificial chromosome) clones (GS1-213H12 and CTB-23D20) bordering the *HOXA* gene locus on chromosome 7 and the mean expression of the *HOXA* genes was observed in a set of 60 glioblastoma (Pearson correlation coefficient $r = 0.27$; $P = .03$; manuscript in preparation). These flanking BACs were more amplified than their neighbors (Appendix Fig A4, online only). Hence, the herein-proposed *HOX*-dominated self-renewal signature for glioblastoma may in part be acquired by increased gene dosage.

High EGFR *Expression Is Associated With Tumor Resistance*

Two gene clusters representing amplification-mediated overexpression of proto-oncogenes were associated with tumor resistance: G13 ($P = .02$; HR = 1.1; 95% CI, 1.0 to 1.2) characterized by coordinated upregulation of contiguous genes on chromosome 12q13-15, comprising the proto-oncogenes *CDK4* and *MDM2*, and G25, dominated by *EGFR* probe sets ($P = .002$; HR = 2.8; 95% CI, 1.4 to 5.4; Table 1; Fig 6). The array-derived *EGFR* measurement (probe set 201983_s_at) was confirmed by qRT-PCR (Pearson correlation, 0.89). Expression of the constitutively activated *EGFRvIII* mutant (18 of 70; 26%), as measured by qRT-PCR, did not further influence outcome prediction ($P = .94$).

Cluster G18, associated with tumor resistance ($P = .03$; HR = 1.94; 95% CI, 1.07 to 3.51), displayed some correlation with *EGFR* expression ($r = 0.57$; Fig 2A) in particular with *Aquaporin 4* (*AQP4*). *AQP4* has been associated with brain tumor related edema.[23] Aquaporins require activation of mitogen-activated protein kinase signaling that may be mediated by EGFR activation.[24] Another family member, AQP1, has been linked with tumor angiogenesis and cell migration[25] and was associated with worse outcome in this study ($P = .003$; HR = 2.44, 95% CI, 1.36 to 4.04) and in the two external data sets (combined $P = .009$; HR = 1.51; 95% CI, 1.11 to 2.06).

Blood Vessels Markers Associated With Better Outcome

G7 is characterized by genes associated with endothelial cells, basement membranes, signaling pathways of vascular development and angiogenesis, and tumor-derived endothelial markers[26,27] (Table A5). Hence, G7 may differentiate tumors according to their angiogenic pattern that may be indicative for drug perfusion, and therefore show association with benefit from treatment (Table 1). G7 is in the center of a "super cluster" (Fig 2A) constituted of several stable gene

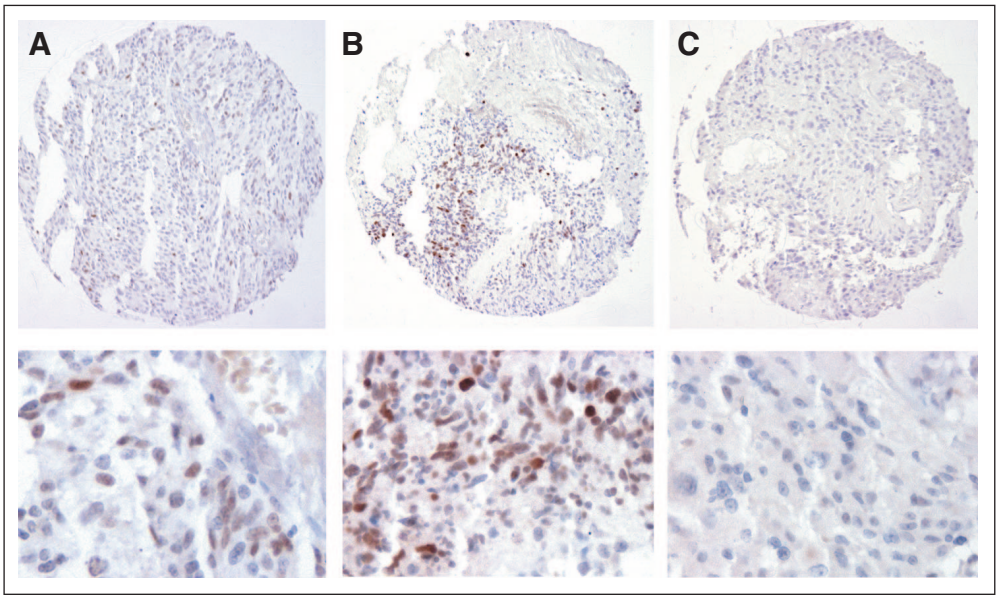

Fig 4. HOXA10 expression determined by immunohistochemistry on tumor microarray. Diameter of tissues in upper panel is 0.6 mm. (A) High nuclear expression; (B) focal high nuclear expression; and (C) no expression.

clusters with biologically related features, such as hypoxia-regulated genes G9, and the myeloid progenitor/adhesion cluster G14, which is also correlated with better outcome (Table 1). Beside genes related to cell adhesion, mesenchymal stem cells (*PRR16*), and homeobox genes (*MEOX1*, *MKX*), G14 includes aldehyde dehydrogenase[28] and bone morphogenetic protein 5 gene (*BMP5*)[29] both associated with differentiation of stem cells. The cluster comprises positive (*MEOX1*) and negative regulators (*SOSTDC1*) of BMPs, which recently have been shown to inhibit tumorigenic potential of human brain tumor stem cells by promoting their differentiation.[29] This cluster may reflect the perivascular microenvironment proposed recently to serve as niche for brain tumor stem cells.[30]

Innate Immune Response Associated With Better Survival

Alongside markers of innate immunity and macrophages (CD11b), G24 comprises numerous cell-surface receptors known as markers for M2-polarized macrophages,[31] such as CD163 (Appendix Table A9, online only). M2 polarization mediates tolerance and downregulates inflammation, alleviating immune surveillance.[31,32] A wide range of CD163-positive cells can be observed in glioblastoma (Appendix Fig A5, online only). The cluster also contains probes encoding major histocompatibility complex class II surface molecules, but lacks expression of costimulatory molecules critical for T-cell activation.[33] This immune signature may be relevant for strategies of tumor vaccination.

HOX *Signature and* EGFR *Expression Are Independent Prognostic Factors*

Multivariate analysis suggests that the *HOX* signature and *EGFR* expression, respectively, are independent prognostic factors for poor outcome in TMZ/RT→TMZ–treated glioblastoma patients, explaining 67% of the survival outcome variations together with the *MGMT* status and age (Table 2).

DISCUSSION

Gene expression signatures relevant for treatment resistance to TMZ/RT→TMZ have been identified in a prospectively treated population

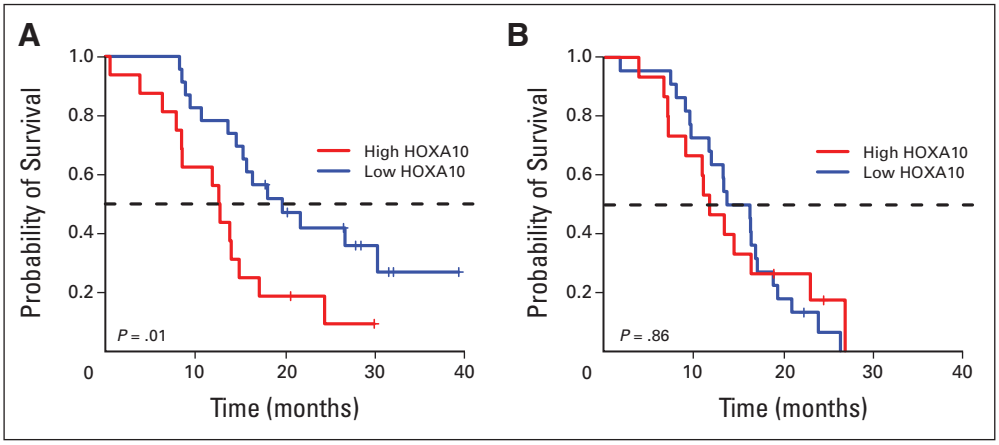

Fig 5. HOXA10 expression and overall survival in independent patient cohort. Kaplan-Meier survival estimates of (A) 39 patients randomly assigned to concomitant and adjuvant temozolomide (TMZ) and radiotherapy (TMZ/RT→TMZ) and (B) 37 patients randomly assigned to RT, separated into high versus low expressors of HOXA10 as determined by immunohistochemistry on tumor microarray.

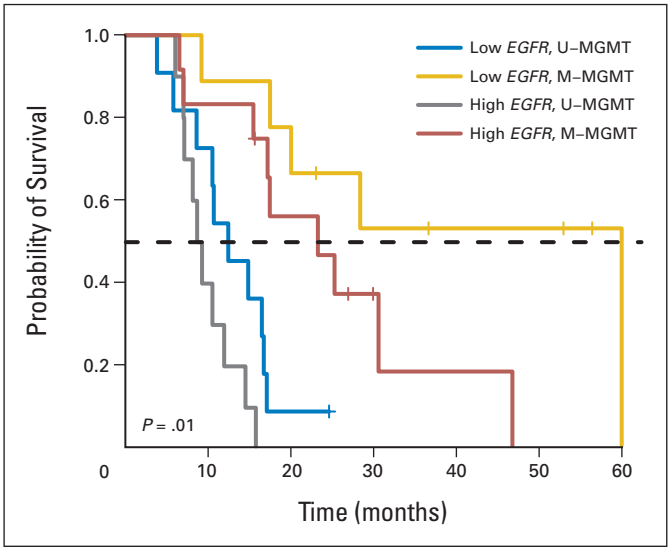

Fig 6. Association of *EGFR* expression with survival. Kaplan-Meier curves of the 42 patients in the concomitant and adjuvant temozolomide (TMZ) and radiotherapy (TMZ/RT→TMZ) cohort divided between low and high expression of *EGFR* (probe set 201983_s_at, dichotomized according to a Gaussian mixture model). The *P* value from log-rank test is shown for two groups defined by *EGFR* expression and stratified according to the *MGMT* methylation status. M-MGMT, methylated *MGMT*; U-MGMT, unmethylated *MGMT*.

of glioblastoma patients. Although epigenetic inactivation of the *MGMT* gene promoter remained the most prominent predictive factor, expression signatures allowed identification of patient subgroups that may benefit from specific additional therapies targeting particular mechanisms of resistance.

As an independent predictive factor of resistance, we have identified a *HOX*-dominated gene cluster, evocative of a self-renewal signature recently described for leukemia.[9] Strong HOXA10 expression of glioblastoma-derived neurospheres is in line with a role of *HOX* genes in the glioma stem-like cell compartment. These findings provide the first clinical evidence to our knowledge for the relevance of a stem-like cell phenotype in treatment resistance of glioblastoma.

In leukemia, expression of translocation-related fusion proteins led to MLL-mediated chromatin remodeling associated with re-expression of *HOX* genes.[9,34,35] In glioblastoma, however, such fusion proteins have not been described, and no indications from the present data set link MLL expression with the self-renewal signature. We provide evidence that the *HOX*-dominated gene signature emerges with malignant progression to glioblastoma, and may be acquired in some glioblastomas by low-level amplification, the latter supporting the notion that gliomas may also arise from progenitors, in agreement with mouse models.[36] The identification of *GADD45G* as part of the *HOX* signature may provide further evidence for an enhanced DNA repair potential that has recently been associated with radiation resistance of glioma stem cells.[7]

These molecular and clinical data underscore the importance of the self-renewal phenotype that could be explored as a potential treatment target.[37] First efforts blunting glioma stem cell–related self-renewal properties of tumors suggest that strategies forcing differentiation (eg, mediated by cytokines such as BMP4) may be promising.[29]

Targeting resistance to TMZ/RT→TMZ associated with overexpression of the *EGFR* gene is of particular clinical interest because this alteration affects a large proportion of patients, and inhibitors of several key targets in downstream signaling pathways are already available or are in advanced clinical development.[38] Similarly, inhibitors for *CDK4* and *MDM2* are under investigation, and both proto-oncogenes showed amplification-mediated overexpression, identified here as associated with worse outcome.

Surprisingly, good prognosis was associated with increased expression of a signature for tumor endothelium markers. This signature may predict improved cytotoxic activity by means of better perfusion of the tumor with the chemotherapy agent TMZ. This is in accordance with the concept suggesting that antiangiogenic agents may temporarily lead to "normalization" of aberrant tumor vasculature, resulting in more efficient delivery of drugs and oxygen to the tumor.[39,40] In a recent trial, addition of the antiangiogenic integrin-inhibitor cilengitide appears to confer increased antitumor activity in conjunction with TMZ/RT→TMZ in patients with a methylated *MGMT* gene promoter.[5]

Another interesting insight of our study suggests infiltration of M2-polarized macrophages into the tumors. The altered capacity of these glioma-infiltrating macrophages to induce effective antitumor T-cell response may obstruct therapeutic strategies aimed at boosting adaptive immunity against the tumor. M2 polarization is driven by tumor- and T-cell–derived cytokines,[31] consistent with the well-known expression of the immunosuppressive cytokines transforming growth factor-β and interleukin-10 in malignant glioma.[41] Thus, for effective immunotherapy/vaccination, full resection of the tumor may be required to remove the microenvironment conferring immunosuppression and tolerance.

The molecular signatures identified in this study associated with outcome underline the need for development of multimodality treatments targeting not only the tumor cells, but including strategies aimed at the glioma stem-like cell compartment, and interfering with tumor-host interaction that provides the specialized microenvironment relevant for the maintenance of tumor stem-like cells (the stem-cell niche), angiogenesis, and immune response. The hypotheses generated in this study need to be tested in prospective trials. Our findings may guide a rational choice of agents, targets, trial design, and appropriate patient selection, incorporating biomarkers defining mechanisms of response and resistance.

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Appendix

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

Glossary Terms

HOX (homeobox) genes: The proteins encoded by these genes, the homeodomain proteins, play important roles in the developmental processes.

MGMT: The DNA repair enzyme, O6-methylguanine DNA methyltransferase, which confers resistance to alkylating agents. Thus, cells are protected from the toxicity of alkylating agents, which frequently target the O6 position of guanine in DNA.

Clustering: Organization of data consisting of many variables (multivariate data) into classes with similar patterns. Hierarchical clustering creates a dendrogram based on pairwise similarities in gene expression within a set of samples. Samples within a cluster are more similar to one another than to samples outside the cluster. The vertical length of branches in the tree represents the extent of similarity between the samples. Thus, shorter the branch length, the fewer the differences between the samples.

Tissue microarray: Used to analyze the expression of genes of interest simultaneously in multiple tissue samples, tissue microarrays consist of hundreds of individual tissue samples placed on slides ranging from 2 to 3 mm in diameter. Using conventional histochemical and molecular detection techniques, tissue microarrays are powerful tools to evaluate the expression of genes of interest in tissue samples. In cancer research, tissue microarrays are used to analyze the frequency of a molecular alteration in different tumor type, to evaluate prognostic markers, and to test potential diagnostic markers.

EGFR (epidermal growth factor receptor): Also known as HER-1, EGFR belongs to a family of receptors (HER-2, HER-3, HER-4 are other members of the family) and binds to the EGF, TGF-α, and other related proteins, leading to the generation of proliferative and survival signals within the cell. It also belongs to the larger family of tyrosine kinase receptors and is generally overexpressed in several solid tumors of epithelial origin.

CD133: CD133, encoded by the *Prominin-1* gene, is used as a marker for stem cells of normal tissues such as neural and hematopoietic stem cells, but also as a marker for tumor stem-like cells of distinct origin such as brain tumors and breast and colon cancer.

Cancer stem-like cells: A cancer cell that has the potential to transfer disease or to form tumors after transplantation. Cancer stem-like cells have the potential to self-renew (see self-renewal), forming additional tumorigenic cancer cells of similar phenotype, and to give rise to phenotypically diverse cancer cells with more limited potential.

Self-renewal: Process by which a progenitor gives rise to daughter progenitors of equivalent developmental potential (ie, multipotent stem cells self-renew by dividing to generate one or two multipotent daughter cells).

Tumor-host interaction: The evolving cross talk between different cell types within the tumor and its host environment. Cancer cells can subvert the normal tissue architecture and reprogram the activity of stromal cells to their advantage, such as promoting the formation of new blood vessels or the recruitment of inflammatory cells, which are key events promoting cancer cell survival, invasion, and metastasis.

