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Sexually dimorphic impact of the iron-regulating gene, *HFE*, on survival in glioblastoma

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Abstract

Background. The median survival for patients with glioblastoma (GBM), the most common primary malignant brain tumor in adults, has remained approximately 1 year for more than 2 decades. Recent advances in the field have identified GBM as a sexually dimorphic disease. It is less prevalent in females and they have better survival compared to males. The molecular mechanism of this difference has not yet been established. Iron is essential for many biological processes supporting tumor growth and its regulation is impacted by sex. Therefore, we interrogated the expression of a key component of cellular iron regulation, the *HFE* (homeostatic iron regulatory) gene, on sexually dimorphic survival in GBM.

Methods. We analyzed TCGA microarray gene expression and clinical data of all primary GBM patients (*IDH*-wild type) to compare tumor mRNA expression of *HFE* with overall survival, stratified by sex.

Results. In low *HFE* expressing tumors (below median expression, $n = 220$), survival is modulated by both sex and MGMT status, with the combination of female sex and MGMT methylation resulting in over a 10-month survival advantage ($P < .0001$) over the other groups. Alternatively, expression of *HFE* above the median (high *HFE*, $n = 240$) is associated with significantly worse overall survival in GBM, regardless of MGMT methylation status or patient sex. Gene expression analysis uncovered a correlation between high *HFE* expression and expression of genes associated with immune function.

Conclusions. The level of *HFE* expression in GBM has a sexually dimorphic impact on survival. Whereas *HFE* expression below the median imparts a survival benefit to females, high *HFE* expression is associated with significantly worse overall survival regardless of established prognostic factors such as sex or MGMT methylation.

Key Points

- There is a sex-specific effect of HFE mRNA expression in GBMs on survival.
- High *HFE* results in poor survival in GBM regardless of patient sex or MGMT status.
- Survival differences may be due to *HFE* related immunosuppression in the tumor microenvironment.

Importance of Study

Expression of the homeostatic iron-regulatory gene, *HFE*, is a novel prognostic indicator of survival for brain tumors. These data further support the sexually dimorphic nature of glioblastoma and, given the importance of HFE in regulating cellular iron uptake, may provide molecular indicators to focus interrogation of the biological basis for the sex-dependent outcomes. Moreover, the gene expression profiles support the role of *HFE* as both influencing the immune system and iron biology of the tumor microenvironment.

Glioblastoma (GBM) is the most common type of primary malignant brain tumor in adults. Despite aggressive therapy, the median survival for GBM patients has remained at about 1 year, necessitating the need for a new paradigm to GBM treatment.[1,2] Recent evidence suggests that GBM is a sexually dimorphic disease.[3,4] GBMs are less common in females, yet females have better survival.[5,6] Prognostic indicators like O[6]-methylguanine-DNA methyltransferase (MGMT) and Isocitrate Dehydrogenase (IDH) status also modify survival based on sex. Females with MGMT methylated GBMs have better survival rates than males and males have improved survival with *IDH*-mutant GBMs.[7,8] The molecular mechanism that underlies this sex-based difference has yet to be established.

Iron homeostasis is essential for cellular energy production and DNA synthesis, particularly in rapidly dividing cells, and thus plays a critical role in cancer development and maintenance.[9–11] Elevated expression of iron-regulating genes is correlated with worse survival in multiple cancers including breast, prostate, and colon.[10–14] In breast cancer, a molecular signature representing decreased iron acquisition is associated with better outcome whereas a signature indicating decreased iron export is associated with worse outcome.[15] In GBMs, both glioma and cancer stem cells can upregulate the iron transporter transferrin, conferring improved iron extraction from the tumor microenvironment to promote proliferation.[16,17]

A key regulator of cellular iron uptake is the homeostatic iron-regulator protein (HFE), which binds to transferrin receptors (TFRs) decreasing TFR affinity to transferrin, consequently reducing iron uptake. Mutations of the *HFE* gene result in cellular iron overload, and one of the mutations C282Y was first discovered as the genetic cause of an iron overload disorder found predominantly in men; hemochromatosis.[18] To date, the role of *HFE* in tumor biology has focused primarily on the impact of *HFE* mutations.[19–23] For example, we have previously shown female GBM patients who carry C282Y HFE polymorphism have shorter survival than both wild-type (WT) patients and male metastatic brain tumor patients with C282Y23.[23]

Despite the interest in the incidence and effect of common *HFE* gene mutations on the disease, including cancer, very little data have been published on the impact of expression of WT *HFE* gene levels in cancer. Reuben et al.[24] previously described higher levels of *HFE* expression in tumor cell lines from melanoma, lung, and kidney cancers, yet few studies have looked at *HFE* expression in patient tumor samples. We previously identified several iron metabolism genes associated with changes in survival for patients with low-grade glioma (LGG) and found high *HFE* gene expression resulted in a 5-year decrease in patient survival compared to low *HFE* expression.[25]

Here we present data revealing that there are significant sex differences in overall survival associated with *HFE* gene expression and that a synergistic interplay exists between MGMT methylation status, sex, and *HFE* expression that impact patient survival in GBM.

Methods

HFE mRNA Expression and Survival Data

Gene expression, tumor phenotype, and de-identified patient data were acquired from The Cancer Genome Atlas (TCGA) GBM (n = 525). This dataset was downloaded from a publicly available data visualization software program, GlioVis.[26] Microarray data were chosen for its large sample size (n = 525) and availability of data including sex, survival status, and tumor MGMT methylation status. Updated survival data for TCGA GBM were contributed by Dr Joshua B. Rubin's group. To identify and remove outliers, Cook's distance was calculated based on a linear regression of survival times with respect to *HFE* mRNA expression. Any observation with Cook's distance greater than 98th percentile was noted as an outlier and removed from data analysis (n = 10). We excluded nonprimary tumors (n = 21) and *IDH*-mutant samples (n = 34) from the analysis group resulting in 460 total samples for analysis.

We first determined the median *HFE* mRNA expression of all GBM samples to establish the high and low *HFE* groups. We then focused our analysis on primary, *IDH*-WT tumors (n = 460, low n = 220, high n = 240). To determine whether the impact of *HFE* on survival may be therapy dependent, we compared individuals who had received temozolomide (TMZ) (n = 274) versus those who did not (n = 170). Positive TMZ therapy was defined by any patient who had TMZ therapy, in combination with any other therapy, for example, concomitant radiation therapy. See Table 1 for demographics summary.

We attempted to replicate our main results in other datasets but to appropriately assess our model, we needed to identify datasets that contained all of the variables used in the original model, which includes survival time, sex, MGMT methylation status, and recurrence status, in addition to the gene expression profiles of the tumors. At least one of these main variables was missing from each of the available datasets except for the Murat dataset (although this did not include IDH status).

Table 1. Demographics of GBM Patients Included in the Study

		Overall ($n = 460$)	High *HFE* ($n = 240$)	Low *HFE* ($n = 220$)	*P*
Sex	Female, % (*n*)	61.52 (283)	58.75 (141)	64.55 (142)	.24
	Male, % (*n*)	38.48 (177)	41.25 (99)	35.45 (78)	
MGMT ($n = 301$)	Methylated, % (*n*)	42.52 (128)	41.33 (62)	43.71 (66)	.76
	Unmethylated, % (*n*)	57.48 (173)	58.67 (88)	56.29 (85)	
Age	Median (range)	61 (10–89)	60 (14–86)	61 (10–89)	.62
KPS	Median (range)	80 (20–100)	80 (20–100)	80 (20–100)	.83
Resection ($n = 457$)	Biopsy, % (*n*)	12.25 (56)	11.76 (28)	12.79 (28)	.85
	Tumor resection, % (*n*)	87.75 (401)	88.24 (210)	87.21 (191)	
TMZ ($n = 444$)	No, % (*n*)	38.29 (170)	39.47 (90)	37.04 (80)	.67
	Yes, % (*n*)	61.71 (274)	60.53 (138)	62.96 (136)	

TMZ, temozolomide; GBM, glioblastoma.
Two-sample test for equality of proportions was used for sex, MGMT, resection, and TMZ and Welch two-sample *t*-test was used for age and KPS. Median *HFE* mRNA expression was based on all GBM patients provided by TCGA GBM, including *IDH*-mutant, nonprimary, and outlier samples (determined by Cook's distance). Removal of those samples results in an $n = 20$ difference in *HFE* groups, as opposed to $n = 230$ equal split as would otherwise be expected. Not all phenotype data are available for each sample, thus the number of available samples are included next to category name.

Statistical Analysis

Sample test for equality of proportions was used to detect baseline differences for sex, MGMT, resection, and TMZ and Welch two-sample *t*-test was used for comparison of age and Karnofsky Performance Score (KPS). Data analysis was performed in R (v 3.5.1). Kaplan–Meier survival curves were plotted to characterize survival differences and *P*-values were generated using log-rank test. From R package "survival," Cox Proportional Hazard (CoxPH) models were used to control for sex, age, MGMT methylation status, KPS, and resection. For overall model fit, *P*-values were based on robustified variance estimates (known as Huber sandwich estimator).[27] We further confirmed results using Weibull Accelerated Failure Time model, to ensure the results hold true even in the absence of non-proportionality of hazards.[28] For all analyses, two-tailed *P*-values less than .05 were considered statistically significant.

Microarray Data Processing by GlioVis

HFE mRNA expression was reported by microarray data collected from the Affymetrix expression array HG-U133 platform. GlioVis downloaded available raw.CEL files from TCGA and processed in R using the Bioconductor suite. The "affy" package was used for robust multi-array average normalization followed by quantile normalization. The median of all probes was used for genes with several probe sets.

GlioVis provided mRNA data on approximately 12 000 genes analyzed in each GBM tumor sample. These data were downloaded and analyzed as described in Gene Expression Analysis section. To elucidate the meaning of differential gene expression, gene sets were analyzed by GO Enrichment Analysis.

Gene Expression Analysis

The aim of our gene expression analysis was to identify gene sets which were significantly different between low and high *HFE* groups and subsequently, the expression of which also correlated with *HFE* mRNA expression. Herein, we only focused on 2 subsets of gene expressions that were shown to have the greatest survival differences between low and high *HFE* while also presenting comparative information on sex differences: males with MGMT methylated tumors and females with MGMT methylated tumors. Genes found to be most different between the 2 groups were then analyzed through PANTHER GO Enrichment Analysis of Biological mechanisms.[29]

We followed a similar strategy described below for each of the subsets.

(1) First, we conducted nearly 12 700 univariate robust linear regressions (R package MASS) where the response was high versus low *HFE* group and the gene expression values were the predictors. False discovery rate (FDR) adjusted *P*-values were calculated to correct for multiple testing errors. We chose those genes which had FDR adjusted *P*-values less than .05, therefore this set of genes had expressions that differ significantly between high versus low *HFE*.
(2) Next, we calculated the simple Pearson correlation coefficient between *HFE* expression and the gene expressions obtained from step (1). We choose only those genes whose correlations are statistically significant after controlling the *P*-values by FDR adjustment. The level of significance for these adjusted *P*-values is set at .05.
(3) In a more realistic scenario, one needs to find the association between *HFE* expression and all the other significant gene expressions from step (1) put together as multivariate covariates. This mimics how one gene is

associated with *HFE* in the presence of several other genes. We conducted penalized elastic net regression (R package glmnet) with *HFE* expression as response and the gene expressions from step (1) as covariates. The elastic net model had a considerable higher weight for ridge regression than LASSO so that the number of resulting genes were not over-regularized. This regression controls the problem of multicollinearity by shrinking the coefficients of highly correlated gene expressions. We chose only those genes whose coefficients are non-zero, implying an association with *HFE*.

The final set of genes are both common to genes obtained from step (2) and step (3). Note that this multilevel strategy was stricter and controlled for spuriously correlated gene expressions. Please see Figure 3 for schematic and results of this analysis strategy.

Results

High *HFE* Expression Is Associated With Significantly Worse Patient Survival in Brain Tumors

In the TCGA GBM LGG microarray dataset, tumors expressing *HFE* above the median are associated with shortened survival for all brain tumor populations (114 months vs 21.3 months, log-rank $P < .0001$, Figure 1A). Restricting the analysis to the TCGA GBM data also revealed a similar and significant difference (Figure 1B; $P = .0088$) between the high and low *HFE* GBM expressing groups. *HFE* expression increases with increasing grade of the tumor (all adjusted $P < .05$, Figure 1C) and is the highest in GBM ($P < .0001$, Figure 1D). Furthermore, within GBM subtypes, *HFE* expression is highest in the mesenchymal subtype, associated with worse patient prognosis, and lowest in the proneural subtype ($P < .0001$, Figure 1E).

A CoxPH regression model was then run with *HFE* as a continuous variable. After controlling for known prognostic factors (sex, MGMT status, KPS, resection, and age), the impact of *HFE* on survival was not significant, yet neither was KPS, resection, sex, nor MGMT methylation status (Table 2 and Supplementary Table S1). Because MGMT and sex have been established as significant prognostic indicators, this was an unexpected finding. Thus, we considered whether *HFE*, MGMT status, and sex may interact, suggesting that *HFE* expression might be negatively influencing survival based on the patient's sex and MGMT methylation status. To nullify the possibility of confounding of *HFE* expression by sex and MGMT methylation status, a simple linear regression was performed, verifying *HFE* expression did

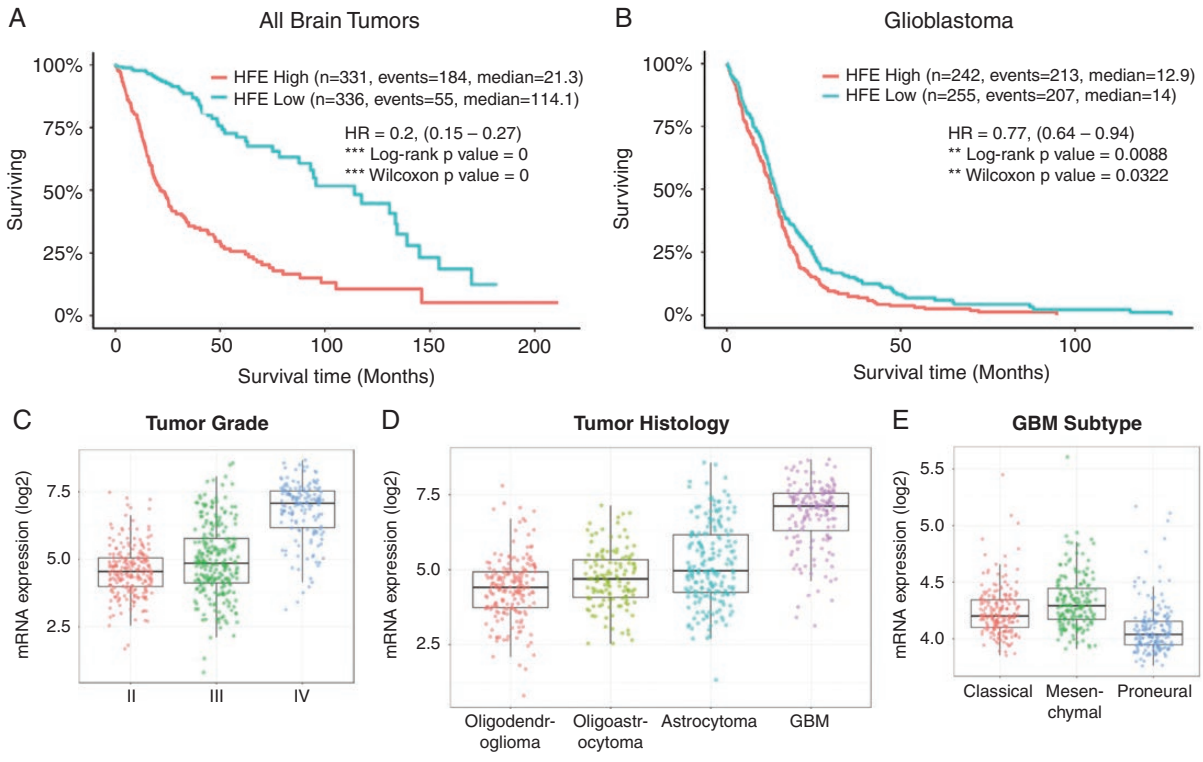

Figure 1. (A–E) Survival and expression differences of *HFE* in low-grade gliomas and glioblastoma. Survival in low *HFE* (blue) versus high *HFE* (red) in all brain tumors (A) and in glioblastoma (B). *HFE* mRNA expression based on tumor grade (C), tumor histology (D), and glioblastoma subtype (E). These figures were directly rendered by and downloaded from GlioVis.

Table 2. Survival Differences Based on *HFE* Group, MGMT Status, Sex, and Temozolomide Therapy

		All Data				Low *HFE* mRNA (<Median)				High *HFE* mRNA (≥Median)			
		N	Deaths	Median (CI)	*P*	*N*	Deaths	Median (CI)	*P*	*N*	Deaths	Median (CI)	*P*
Sex	Male	283	242	12.7 (11.9–14.0)		142	120	12.6 (10.9–13.9)		141	122	12.9 (11.9–14.9)	
	Female	177	142	12.5 (10.5–15.3)		78	62	14.7 (11.5–20.1)		99	80	10.7 (8.6–14.9)	
	Log rank				.8				.07				.1
	CoxPH[a]				.049*				.003**				.7
MGMT	Methylated	128	100	15.3 (12.9–17.8)		66	54	16.9 (13.8–21.2)		62	46	13.6 (10.7–18.1)	
	Unmethylated	173	132	12.2 (10.7–14.0)		85	66	11.5 (10.1–13.3)		88	66	12.9 (10.8–14.9)	
	Log rank				.002**				.002**				.4
	CoxPH[a]				.12				.014*				.6
Sex and MGMT (All therapy)	Male: Methylated	66	55	13.3 (10.7–17.6)		40	35	13.3 (10.2–19.5)		26	20	12.9 (7.6–20.4)	
	Female: Methylated	62	45	17.8 (13.8–21.3)		26	19	24.9 (17.8–33.2)		36	26	13.6 (7.0–20.7)	
	Male: Unmethylated	112	87	12.6 (10.6–14.0)		57	45	10.9 (9.5–14.0)		55	42	12.9 (11.9–15.0)	
	Female: Unmethylated	61	45	11.7 (9.9–17.5)		28	21	11.7 (9.9–22.2)		33	24	12.2 (8.8–17.9)	
	Log rank				.001**				<.0001****				.7
	CoxPH[b]				.002**				<.0001****				.3
Temozolomide Therapy	Male: Methylated	47	38	16.5 (13.3–20.8)		31	26	16.1 (12.7–21.1)		16	12	18.1 (12.9-NA)	
	Female: Methylated	43	28	20.7 (15.6–26.9)		19	14	25.7 (19.9-NA)		24	14	15.6 (12.4-NA)	
	Male: Unmethylated	81	60	12.9 (12.2–14.9)		40	31	12.7 (11.3–15.4)		41	29	13.9 (12.6–16.6)	
	Female: Unmethylated	38	27	14.5 (10.2–21.9)		18	13	11.7 (9.9-NA)		19	14	14.9 (12.2-NA)	
	Log rank				.0002***				<.0001****				.2
	CoxPH[b]				.0008***				.0002***				.6
No Temozolomide Therapy	Male: Methylated	18	17	3.1 (2.0–7.6)		9	9	2.0 (1.1-NA)		9	8	5.9 (2.8-NA)	
	Female: Methylated	15	14	6.6 (3.6–25.4)		5	4	15.1 (3.6-NA)		10	10	5.5 (2.3-NA)	
	Male: Unmethylated	27	23	5.8 (1.6–10.6)		16	13	7.6 (1.1-NA)		11	10	5.3 (1.6-NA)	
	Female: Unmethylated	20	16	6.6 (4.6–17.5)		8	7	6.1 (4.1-NA)		12	9	9.1 (2.9-NA)	
	Log rank				.06				.05				.7
	CoxPH[b]				.06				.03*				.8

NA, upper confidence interval could not be calculated/infinity.
[a]CoxPH *P*-value based on hazard coefficients.
[b]Robustified CoxPH *P*-value for the overall model fit.
**P*-value < .05, ***P*-value < .01, ****P*-value < .001, *****P*-value < .0001.

not differ significantly based on sex or MGMT status (Table 1).

High *HFE* Expression Abrogates Survival Benefit of Both Female Sex and MGMT Methylation

The dataset was then divided into 2 separate groups, low *HFE* and high *HFE* based on median *HFE* expression. CoxPH analysis of these groups, controlling for the same factors as mentioned above, revealed 2 distinct and sexually dimorphic survival patterns. A low *HFE* tumor expression level is associated with better survival for females (Figure 2A) compared to males but there is no sex difference in survival in the high *HFE* tumor expressing group (Figure 2D). The established MGMT tumor methylation survival benefit was seen in the low *HFE* group, but not in the high expressing *HFE* group (Figure 2B and E). There was a clear effect of sex and *HFE* status on the MGMT effect; females with low *HFE* and MGMT methylated tumors survive significantly longer than males regardless of their MGMT status. High *HFE* expression negated the MGMT and sex benefits to survival (Figure 2C and F).

We determined to use the median *HFE* expression to help maintain equal sample sizes for the analysis groups (low *HFE* vs high *HFE*) to prevent imbalance in the groups. To confirm that impact of *HFE* on survival does not depend on the chosen median cutoff, we reproduced our main result using 40th and 60th percentile cutoffs (Supplementary Table S2). Furthermore, interrogation of the Murat data using the median *HFE* expression generated similar results (Supplementary Table 3).

High *HFE* Negates the Impact of MGMT Methylation in TMZ Treatment

We confirmed that irrespective of *HFE* status, sex, and methylation status, receiving TMZ therapy versus treatments without TMZ (but did receive radiation) was beneficial for survival. Supporting our previous results, for patients taking TMZ, the survival advantage of being female is amplified by positive methylation status in the low *HFE* group (25.7 months vs 16.1 months in methylated males, Table 2). Regardless of MGMT status or sex, high *HFE* continued to abrogate survival advantages seen at low *HFE* (Table 2).

In patients who did not receive TMZ, we observed the same pattern of sex-based survival differences, with females having a survival advantage over males at low *HFE* and similar survival to males at high *HFE* (Table 2). At low *HFE*, the median survival of females in the methylated group was 15 months versus 2 months for males in the same group. Similarly, to the group treated with TMZ, high *HFE* expression negated the survival benefit of the combination of female sex and methylation status in the group that was not treated with TMZ (Table 2).

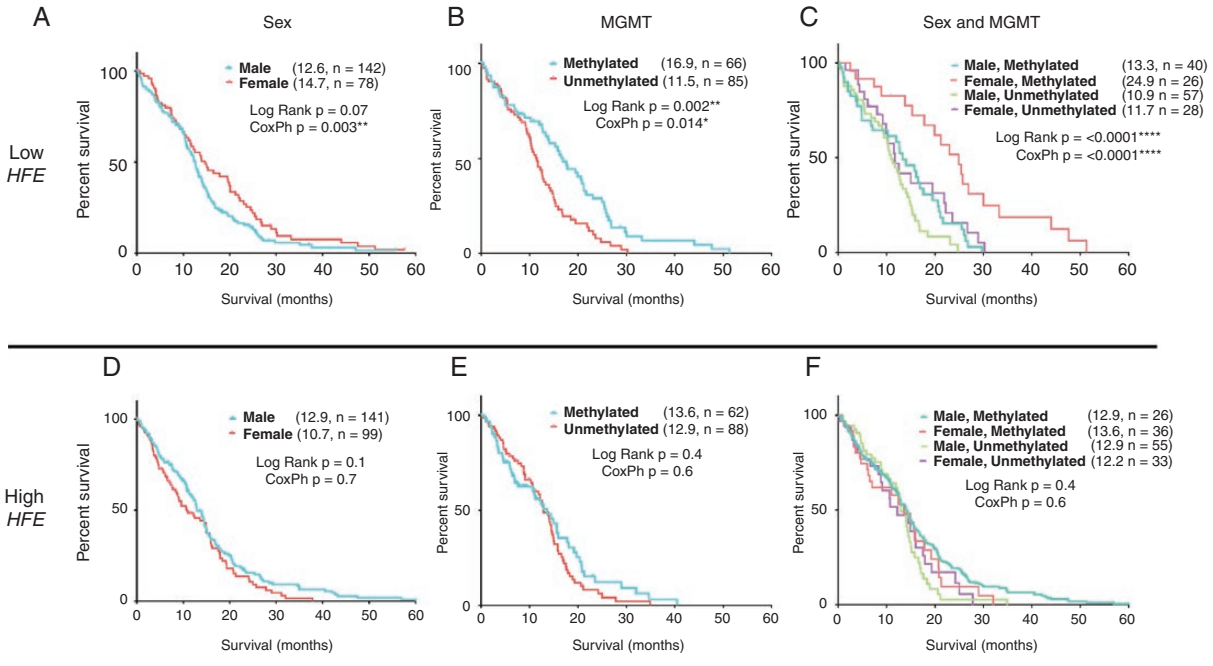

Figure 2. (A–F) Impact of *HFE* expression, sex, and MGMT status on survival in glioblastoma. The top row represents all samples with low *HFE* expression (below median) and is separated into 3 columns based on differences in survival in males versus females (A), MGMT status (B), or both sex and MGMT status (C). Similarly, the row below is also separated by the same comparisons as above yet representing individuals with high *HFE* expression (D–F).

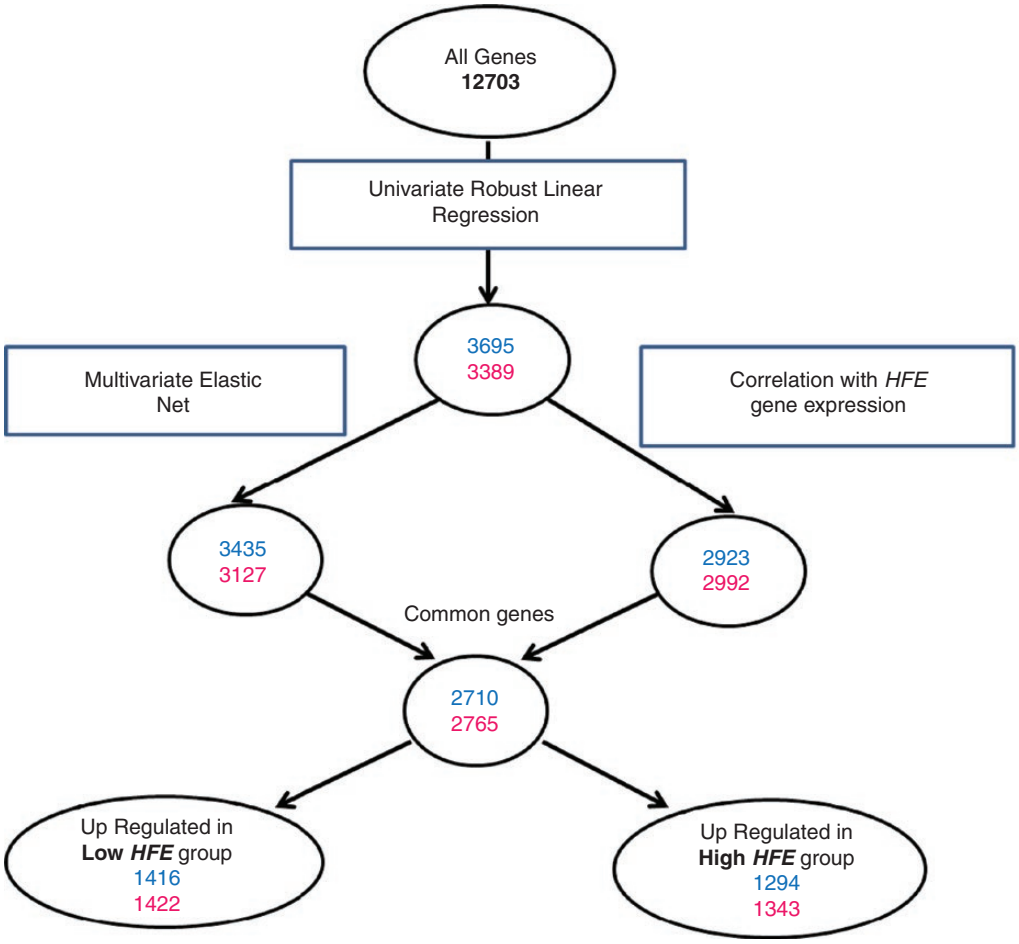

Figure 3. Gene expression analysis of low versus high *HFE* gene expression with the number of genes found at each stage of analysis. The number of genes upregulated in low *HFE* and in high *HFE* states, separated by sex. The number of genes from the analysis of low versus high *HFE* expression genes in females is depicted in magenta, while those of males are depicted in blue. Gene analysis focused on individuals with positive MGMT methylation since the greatest sex-based survival difference was seen between males and females with MGMT positive methylation.

Gene Expression Profiles

To begin to interrogate the relationship between *HFE* expression and the tumor microenvironment, over 3000 genes were found to be significantly different in the low and high *HFE* expressing groups (Figure 3) including sex-based differences. When comparing upregulated genes, males and females shared 884 genes in the low *HFE* group and 694 genes in the high *HFE* group (data not shown). Gene enrichment analysis in low *HFE* groups shows upregulation of genes responsible for DNA topological change, synaptic vesicle assembly, and microtubule development (Figure 4A and B) in both sexes. A unique pathway upregulated in males with low *HFE* includes negative regulation of histone H3-K9 methylation (Figure 4A). Females in the low *HFE* group show unique upregulation in nucleosome disassembly and response to hydroxyurea (Figure 4B).

High *HFE* expression, regardless of sex, is associated with upregulation of pathways involving T-cell activation, chemokine production, and cell apoptosis, among others (Figure 4C and D). Specifically, the most highly upregulated genes in both males and females with high *HFE* were those associated with the regulation of CD4-positive, T-cell proliferation (Figure 4C and D). Males with high *HFE* showed enrichment for genes associated with positive regulation of antigen presentation and processing (Figure 4C), while females uniquely showed upregulation of pathways associated with ganglioside catabolic processes and AMP biosynthesis (Figure 4D).

Discussion

We present data showing that the level of gene expression of a key iron-regulating protein, *HFE*, is a prognostic indicator of survival in patients with brain tumors. Furthermore, in GBMs, the prognostic value of *HFE* is impacted by sex and MGMT methylation status. Not only

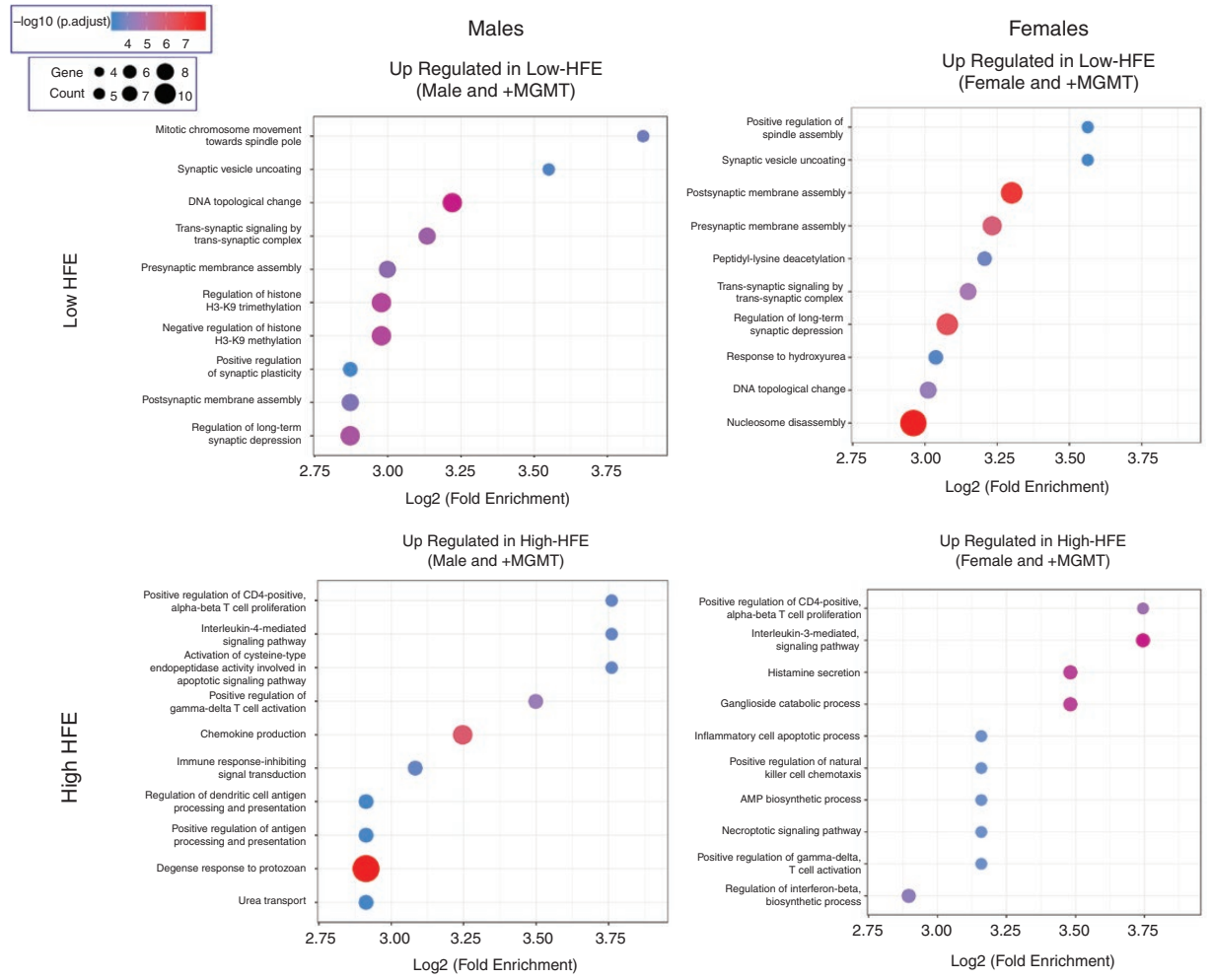

Figure 4. (A–D) GO Enrichment results of genes differentially upregulated in low versus high *HFE*, as observed separately in both males and females. The dot plots show the top 10 biological processes based on fold enrichment value (converted to log 2 scale). The sizes of the dots indicate the number of genes upregulated in that group that are also present in that biological process. The colors of the dots represent FDR adjusted *P*-values (converted to negative log 10 scale). The top row represents pathways enriched based on genes which were most significantly upregulated at low *HFE*, while the bottom row represents pathways of genes upregulated in high *HFE*. The columns represent sex of individuals in analysis (males on the left, females on the right). Thus, pathways upregulated in low *HFE* in males (A) and females (B) and those upregulated in high *HFE* in males (C) and females (D) are represented for comparison.

does *HFE* expression in tumors impact survival, but high expression completely abrogates previously published positive prognostic indicators, specifically female sex and positive MGMT methylation.

Furthermore, these data support recently published studies on the impact of sex differences in GBM, while also providing a critical new lens of analysis further informing those studies. For example, we show that female sex is an advantage only when *HFE* expression levels are low.[6,30] Moreover, this finding extends to MGMT methylated tumors where only females with low *HFE* expression and methylated tumors experience a significant survival advantage. When *HFE* tumor expression is high, methylation no longer conferred a survival advantage to females. In contrast, we did not find significant differences in male survival based on *HFE* expression status. Had we not separated these *HFE* analyses by sex, the significant differences for the expression data would have been washed out by the male survival data. There was is no additional benefit of *HFE* expression level to the MGMT methylation status and survival for males. These data are consistent with reports that females have a more significant survival benefit from MGMT methylation.[7]

Sex may also influence treatment response, with females responding better to the standard of care therapy, including TMZ, and our analysis found similar trends.[6] We confirmed that in patients treated with TMZ, methylation is advantageous to females, yet only when *HFE* levels are low. In the high *HFE* group, MGMT methylation no longer conferred a survival advantage to females who were

treated with TMZ. Because *HFE* status was determined by the median mRNA expression of all GBM patient tumor samples, our data predict that half of all females with GBM would not have the anticipated better survival due to MGMT methylation. In the TMZ group, survival differences widened slightly. Females at low *HFE* and MGMT methylated tumors have a median survival of 25.7 months, which is close to twice the median survival of patients with GBM overall. These data support results by Yang et al.,[30] yet, with the caveat that females had improved response to TMZ only if their *HFE* expression was lower than the median, otherwise, it was similar to males. From these data, it can be postulated that *HFE* is either actively modulating response to TMZ or is a marker of response to TMZ therapy. However, even those females who do not receive TMZ therapy still experience the greatest survival advantage when their tumors have the combination of MGMT methylation and low *HFE* expression. The effects of *HFE* in the tumor microenvironment do not simply reflect sex-based levels of *HFE* expression in the tumors (Table 2). Thus, the *HFE* survival effect appears to be intrinsic to the tumor microenvironment. The underlying mechanism is currently being explored but could be related to epigenetic influences. For example, iron is a required co-factor for demethylases,[31] thus its availability directly impacts on histone and DNA methylation, and consequently on gene expression. Iron overload dysregulates global methylation and mice with *HFE* mutations have lower rates of global methylation than WT mice.[32] Likewise, iron chelation also results in epigenetic alterations in breast cancer cells making them more susceptible to doxorubicin and cisplatin.[33] These data link *HFE* expression, which regulates cellular iron status, to methylation state. This information could have clinical value. For example, the gene expression data showing the upregulation of topoisomerase genes in both male and female low *HFE* groups suggests that these tumors could be more susceptible to topoisomerase inhibitors.

HFE and Immunity

Gene expression data acquired from tumor samples provide information not only on the neoplastic cells but also on the infiltrating immune cells which are critical to tumor proliferation. Macrophages make up the bulk of infiltrating immune cells in GBMs and play a critical role in regulating the tumor microenvironment.[34–38] *HFE* is highly expressed in macrophages and these cells are key regulators of iron.[39–41] Thus, *HFE* mRNA expression may be reflective of the volume of these cells in the tumor.

HFE expression may also be directly influencing the immune cell function in the tumor microenvironment. Expression of *HFE* interferes with MHC class I antigen presentation and impairs activation and differentiation of CD8+ T lymphocytes.[24,42–44] *HFE*'s role as a negative regulator of CD8+ T cells may allow for the predominance of other lymphocytes consistent with the significant upregulation of CD4+ T-cell genes identified in the high *HFE* groups.

Finally, an obvious goal of identifying the sex-based differences in GBMs prevalence and outcomes is to help develop models to guide therapeutic strategies. Although our studies and others in this field are in their infancy, insights can begin to be discussed. For example, a developing therapeutic area for GBM has been immunotherapy, specifically with regard to the use of PD-L1 inhibitors. Binding of PD-1 to PD-L1 delivers a signal to encourage the suppression of T-cell proliferation. Upregulation of PD-L1 in circulating monocytes and tumor-associated macrophages is associated with cytotoxicity to T cells, while also inducing and maintaining T regulatory cells (CD4 T cells) in glioma with increased expansion, perhaps to maintain immunosuppressive environment through T regulatory cells in the tumor, which is ultimately associated with worse GBM prognosis.[45–47] Interestingly, this finding is consistent with observations in the high *HFE* groups, both in males and females; an upregulation of genes involved in T-cell proliferation and activation.[48] PD-L1 expression is higher in *IDH*-WT glioma and higher in higher grade tumors, which seems to follow the same pattern as *HFE* expression (higher in *IDH*-WT and GBM). Both mechanisms may be related, and if so, may help explain the discrepancies seen between studies in PD-L1 inhibitor efficacy, as only half of all GBM patients would potentially benefit from PD-L1 inhibitors.[49] This is an example of how the use of *HFE* status of GBMs in clinical settings may prove to be useful in identifying patients who are likely to benefit from PD-L1 inhibitors.

Lastly, in contrast to our findings in GBMs, high *HFE* expression in breast cancer tumors was associated with better survival in an all-female study. Despite the differences in our findings, these studies underscore the key role of *HFE* gene expression in predicting survival but indicate that the results are sex- and cancer specific.

Conclusions

Expression of the iron-regulatory gene, *HFE*, is associated with sexually dimorphic survival in GBM patients. Furthermore, at low expression, the survival effect is impacted by sex and MGMT methylation status. Conversely, high *HFE* expression completely abrogates previously published positive prognostic indicators, specifically female sex and positive MGMT methylation. The data support further investigation of characterizing the sex-dependent iron signature of GBMs and suggest a relationship between the immune system (including tumor-associated macrophages) and iron biology in the tumor microenvironment.

Supplementary Data

Supplementary data are available at *Neuro-Oncology* online.

Keywords

glioblastoma | HFE | iron | sex | tumor

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