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Toenail iron, genetic determinants of iron status and the risk of glioma

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Abstract

Purpose—Iron is essential for oxygen transport and oxidative metabolism; however, elevated iron stores can trigger overproduction of reactive oxygen species and induce DNA damage. Little is known about the association between body iron stores and glioma risk. This study examined associations of iron levels measured in toenails and genetic variants linked to body iron stores with risk of glioma in a clinic-based case-control study.

Methods—Samples were collected a median of 24 days following glioma diagnosis in the cases (10th-90th percentile range: 10-44 days). Nail iron levels were measured in 300 cases and 300 controls using neutron-activation analysis. A total of 24 genetic variants associated with iron status were genotyped in 622 cases and 628 controls. Logistic regression was used to estimate odds ratios (OR) and 95% confidence intervals (CI) for glioma risk according to toenail iron and the examined genotypes.

Results—No association was observed between toenail iron and glioma risk when restricting to cases with nails collected within ~3 weeks of diagnosis (OR=0.93; 95% CI: 0.46, 1.87 comparing those with high (> 14 µg/g) versus low (<6 µg/g) iron levels). In contrast, an inverse association with increasing iron was observed after restricting to cases with a delay of 3 weeks or greater (OR=0.42; 95% CI: 0.19, 0.95) reflecting potentially insidious effects of advancing disease on iron levels among the cases. No associations were observed for any of the examined genetic variants.

Conclusion—The results do not support a role for body iron stores as a determinant of glioma risk.

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Keywords

glioma; iron; single nucleotide polymorphism; case-control

INTRODUCTION

Gliomas encompass nearly 80% of all malignant adult brain tumors [1]. Although relatively uncommon, these tumors are associated with high morbidity and extremely poor prognosis [2]. High-dose ionizing irradiation [3, 4] and rare hereditary disorders [5] are the only established risk factors for glioma, and these factors only account for a small proportion of cases. A high level of iron in the brain has been linked to neurodegenerative disorders including Alzheimer's, Parkinson's, amyotrophic lateral sclerosis, and Huntington's disease [6], and a number of trace metals including iron have been associated with the risk of cancer [7]. Men tend to have higher levels of iron than premenopausal women who experience menstrual blood loss and pregnancy [8, 9]. Men are also more likely develop glioma, with the greatest difference in incidence across genders occurring during the reproductive years [10]. It is currently unknown whether body iron stores play a role in the onset of glioma.

Iron is a micronutrient essential for oxygen transport and cellular oxidative metabolism, however, free non-protein bound iron can be cytotoxic and potentially carcinogenic [11]. Redox cycling of free iron via the Haber-Weiss and Fenton reactions generates reactive oxygen species (ROS) and hydroxyl free radicals that can induce DNA damage, lipid peroxidation, and oncogene activation [12]. Overproduction of ROS due to elevated iron levels can cause oxidative stress which has been linked to carcinogenesis [13]. Iron may further promote cancer progression by serving as a nutrient that supports cell growth and proliferation [14]. Humans lack a mechanism to excrete excess iron from the body [15]; therefore the ability to maintain iron homeostasis and regulate the levels of free iron in the body is important to prevent excessive ROS formation. Interindividual differences in the ability to regulate the uptake and storage of iron is suggested by the identification in genome-wide association studies (GWAS) of common polymorphisms associated with serum iron concentrations and other measures of body iron stores [16-22].

Epidemiologic studies that have examined the association between iron levels and cancer risk have been inconclusive. Several studies have suggested that high dietary iron intake and/or high body iron stores are associated with an increased risk for cancer [23-27] whereas other studies observed an inverse association between markers of iron levels and cancer risk [28-33]. Apart from a small case-control study that measured serum iron levels in 22 glioma cases and 22 controls [34], no study has examined the association between iron levels and glioma risk. The aim of this study was to investigate the association between toenail iron concentrations, common genetic variants associated with body iron stores, and glioma risk in a clinic-based case-control study conducted at medical centers in the southeastern US.

MATERIAL AND METHODS

Study Population

A description of the study population has been published [35]. Briefly, cases were individuals aged 18 and older recently diagnosed (within 3 months) with a primary, non-recurrent glioma. Cases were identified at neurosurgery and neuro-oncology clinics at major medical and oncology centers in the Southeastern US including Vanderbilt University Medical Center in Nashville, Tennessee; Moffitt Cancer Center in Tampa, Florida; the University of Alabama at Birmingham; Emory University in Atlanta, Georgia; and the Kentuckiana Cancer Institute in Louisville, Kentucky. As eligibility in the case-control

study required a recent diagnosis of glioma, only primary glioblastoma multiforme (GBM) and *de novo* anaplastic astrocytoma were included in the case group. Controls included friends and other non-blood related associates of the cases as well as residents from the same communities as the cases identified in white page listings. Controls were excluded if they reported a personal history of a brain tumor. Eighty-seven percent of eligible glioma patients were enrolled in the study, a median of 1.0 month following the glioma diagnosis (interquartile range: 2 weeks – 1.7 months). An estimated 50% of contacted eligible households yielded a participating control. Interviewer-administered questionnaires were used to collect data on demographic characteristics and potential glioma risk factors. Study protocols were approved by the institutional review committees at each participating center and all study participants provided written informed consent.

DNA Collection and Genotyping

Genomic DNA samples were self-collected by oral rinse or the saliva method using Oragene kits (www.dnagenotek.com). A total of 25 single nucleotide polymorphism (SNPs) associated with body iron stores in GWAS [16-22] were genotyped, including SNPs in *ARSB* (rs2052550); *BTN1A1* (rs13194984); *C7ORF10* (rs29880); *FLJ43390* (rs973968); *GHR* (rs13188386); *GTSCR1* (rs2660917); *HFE* (rs1799945, rs1800562); *HIST1H2BJ* (rs13194491); *KRT18P33* (rs1457451); *LRRC16* (rs2274089); *PCSK7* (rs236918); *RSPO3* (rs972275); *SCGN* (rs932316); *SLC17A1* (rs17270561); *TF* (rs1799852, rs3811647, rs1049296, rs1830084); *TMPRSS6* (rs4820268); *TOPBP1* (rs2718812); *TOPBP1* (rs1867504); *TRIM38* (rs12216125); and *WTAP* (rs4516970). Genotyping was performed at the Center for Genome Technology at the Hussman Institute for Human Genomics, University of Miami using Illumina's GoldenGate technology (Illumina, San Diego, CA). Taqman OpenArray was used to genotype SNPs that failed on the Illumina array. Quality control samples (water, CEPH DNA, as well as blinded and unblinded DNA samples) were included in genotyping runs. Laboratory staff was blinded to the case-control status of the samples. Of the 655 glioma cases and 658 controls (all Caucasian) that were submitted for genotyping, 33 cases (5.0%) and 30 controls (4.6%) were excluded due to low call rates, leaving 622 cases and 628 controls in the final analysis. One SNP (*KLHL13* rs2430212) exhibited a departure from Hardy-Weinberg Equilibrium among the controls (p-value of <0.0001) and was excluded from analysis. Concordance of genotype calls in 94 blinded duplicate pairs ranged from 89% to 100% (mean, 99.6%).

Toenail Iron Measurement

Toenail samples harvested from the great toe were examined in 300 glioma cases and 300 controls. Nail clippings from cases were collected a median of 24 days, with a range of 0 days to 88 days, following glioma diagnosis (10th-90th percentile range: 10-44 days). Toenail iron concentrations were determined using neutron-activation analysis at the University of Missouri Research Reactor Facility in Columbia, Missouri. Samples were analyzed in 3 batches each containing 100 cases and 100 controls matched on age, gender and state of residence. There was adequate sample mass for neutron activation analysis in all samples. Matched case-control sets were handled identically in each analytical run with laboratory personnel blinded to case-control status. In preparation for analysis, a cleaning procedure was performed where nail samples were immersed in 10% (v/v) nitric acid and sonicated for 10 minutes. Following the acid wash the nail samples were decanted, immersed in 18M -cm water, sonicated for 10 minutes and rinsed with 18M -cm water. The cleaned toenail samples were freeze dried and weighed into pre-cleaned high purity quartz vials for analysis. Samples were irradiated for 40 hours, allowed to decay for 5-15 days and counted for 2 hours each using a high purity germanium detector system. For quality control, a total of 12 NIST SRM 1577 bovine liver samples and 12 NCS DC 73347 hair samples were co-analyzed with the toenail samples. The mean iron levels (mean ±

standard deviation) measured in the liver ($249 \pm 14 \mu\text{g/g}$) and hair ($188 \pm 5 \mu\text{g/g}$) samples agreed with the accepted values of ($268 \pm 8 \mu\text{g/g}$) and ($190 \pm 9 \mu\text{g/g}$), respectively.

Statistical Analysis

The association between toenail iron levels and glioma risk was estimated with odds ratios (OR) and 95% confidence intervals (CI) using conditional logistic regression that took account matching on age, gender, state of residence, and analytic batch, and with further adjustment for education. To test for linear trend, an ordinal term reflecting categories of toenail iron were included in regression models. Cox proportional hazards regression was used to estimate hazard ratios (HR) and 95% CIs for the association between toenail iron and glioma-related mortality, adjusting for age, gender, histologic subtype of glioma, Karnofsky Performance Score, and days from diagnosis to nail clipping. Unconditional logistic regression was used to estimate the association between individual genetic variants and glioma risk adjusting for age and gender. Additive, dominant, and recessive inheritance models were tested. To test for linear trend, each SNP was modeled as an ordinal term coded 0, 1, or 2, corresponding to the number of variant alleles. Multinomial logistic regression was used to examine associations between genotypes and histologic subtypes of glioma (GBM, lower grade astrocytomas, and oligodendrogliomas). A test for heterogeneity was performed in each multinomial model to evaluate whether associations for each SNP differed across histologic subtypes. The false discovery rate (FDR) was used to control for multiple testing according to the method of Storey et al [36]. ANOVA was used to evaluate whether toenail iron levels varied according to genotype for each evaluated SNP after adjustment for age and gender. Statistical analysis was performed using SAS Version 9.1 (SAS Institute, Inc., Cary, NC) and statistical significance was defined as a two-sided p-value <0.05 .

RESULTS

Among subjects included in the toenail analysis, the median age was 54 years in cases and controls and the majority in both groups was male (61%) (Table 1). The study population was predominately Caucasian. Approximately 64% of cases had a diagnosis of GBM, 18% had grade I, II, or III astrocytic tumors, 15% had oligodendroglial tumors (grades II-III), and 2% had another tumor subtype (ganglioglioma, subependymoma, etc.) or a glioma with no specific subtype specified in pathology reports. The median level of toenail iron was similar in cases ($8.8 \mu\text{g/g}$) and controls ($9.0 \mu\text{g/g}$) ($p = 0.308$). Among subjects included in the genotype analysis, cases and controls were similar on age ($p = 0.912$) and gender ($p = 0.060$); all subjects in the genetic analyses were Caucasian.

Table 2 presents results for toenail iron. We observed an inverse association with risk comparing the referent ($<6 \mu\text{g/g}$) to higher levels of iron with no evidence of a dose response (ORs: 0.57, 0.66, 0.61 for increasing category of nail iron). Unexpectedly, given rapid harvesting of toenails in the study, an inverse association was demonstrated among the cases between iron levels and time elapsed between glioma diagnosis and collection of nails (Spearman $r = -0.18$, $p\text{-value} = 0.0002$) (Figure 1). When associations were considered according to the time between tumor diagnosis and nail collection in the cases (above or below the median of ~ 3 weeks), the inverse association was restricted to cases in which toenail collection was delayed (median: 34 days, range: 25 – 88 days) (Table 2). In these analyses, an approximate 50% reduction in risk was suggested in those with high ($\geq 14 \mu\text{g/g}$) versus low ($<6 \mu\text{g/g}$) iron levels (OR = 0.42; 95% CI: 0.19, 0.95). In contrast, no association of nail iron with glioma risk was observed among cases with rapid nail collection (median: 15 days, range: 0 – 24 days) (OR = 0.93; 95% CI: 0.46, 1.87 comparing high ($\geq 14 \mu\text{g/g}$) and low ($<6 \mu\text{g/g}$) iron levels). No association was observed in the latter group regardless of histological subtype of glioma (Table 2) and subject gender (not shown). Among the 252

patients with high grade glioma tumors, there was no association with mortality for the highest ($\geq 14 \mu\text{g/g}$) versus lowest ($<6 \mu\text{g/g}$) levels of toenail iron (HR = 0.89; 95% CI: 0.54, 1.46). When results were stratified by time from diagnosis to nail collection, no significant associations with survival were observed for patients with rapid nail collection (HR = 1.56; 95% CI: 0.78, 3.14) or among patients that provided a nail sample more than 3 weeks after diagnosis (HR = 0.47; 95% CI: 0.18, 1.25).

None of the examined genetic variants were significantly associated with overall risk of glioma under any inheritance model (Supplemental Table 1). However, when examined according to histologic subtype, several of the variants were associated with lower grade pure astrocytomas or oligodendroglial lineage. The minor allele in rs236918 (*PCSK7*) conferred a reduced risk for lower grade astrocytic tumors under an additive (per allele OR = 0.50; 95% CI: 0.29, 0.88; $p = 0.015$) and dominant (OR = 0.52; 95% CI: 0.28, 0.93; $p = 0.029$) model, whereas the minor allele in rs1049296 (*TF*) conferred an increased risk under a recessive model (OR = 3.03; 95% CI: 1.11, 8.27; $p = 0.031$). For pure oligodendrogliomas or mixed oligoastrocytomas, the variant allele in rs4820268 (*TMPRSS6*) was associated with an increased risk in additive (per allele OR = 1.42; 95% CI: 1.03, 1.94; $p = 0.030$) and recessive (OR = 1.91; 95% CI: 1.14, 3.22; $p = 0.015$) models, as was the minor allele in rs12216125 (*TRIM38*) (OR = 1.67; 95% CI: 1.03, 2.72; $p = 0.039$) under a dominant model. We also examined iron related polymorphisms in association with survival among 441 high grade gliomas. Higher risk of mortality was observed for the variants rs13188386 *GHR* (recessive model: OR = 1.69; 95% CI: 1.04, 2.75; $p = 0.034$) and rs1799945 in *HFE* (dominant model: OR = 1.27; 95% CI: 1.02, 1.59; $p\text{-value} = 0.032$). When restricting to the 320 glioma patients that received the current standard of care, only rs972275 in *RSPO3* (dominant model: OR = 1.40; 95% CI: 1.06, 1.87; $p\text{-value} = 0.020$) was associated with survival. No associations with risk or survival remained significant after adjustment for FDR (not shown).

We considered whether genotypes were associated with toenail iron levels among the 119 controls in which both iron measures and genotype data were available. For rs1457451 (*KRT18P33*), toenail iron was significantly higher among subjects with 1 or 2 variant alleles (mean: $19.34 \mu\text{g/g}$) when compared to those with none (mean: $10.93 \mu\text{g/g}$) ($p = 0.0016$). No significant differences in nail iron were observed for any of the other variants studied.

DISCUSSION

In the present study, neither body stores of iron as measured in toenails nor genetic variants associated with body iron stores were associated with risk of glioma. The inverse association observed when restricting analyses to cases with a modest delay in nail collection after diagnosis (median 34 days; maximum 88 days) could be indicative of iron depletion in the cases associated with progression of their disease; no association was demonstrated among cases for whom toenails were collected with no more than a minimal delay (median 15 days; maximum 24 days following diagnosis). None of the examined germline variants linked to body iron stores in GWAS was associated with glioma risk after adjustment for multiple testing. Taken together, the current results do not offer support for the hypothesis that iron stores play a significant role in the onset of glioma. However, results based on toenail measures should be interpreted with caution given the time dependent changes noted in toenail iron measures among the cases.

To our knowledge ours is the first study to consider whether iron levels as measured in toenails provide a marker of glioma risk. One small study examined serum iron concentrations among 22 glioma cases and 22 controls [34] and found significantly higher iron levels among the cases. However, serum iron levels have been shown to be highly

variable over time [37] and the single measure may not have provided a reliable measure of long-term iron status. Furthermore, blood samples were collected after glioma diagnosis in the cases and results may have been subject to changes in diet, treatment or other effects of disease on body iron stores. By contrast, toenails measured in the present study were intended to reflect usual iron status preceding the diagnosis of glioma (6-12 months in the past) which is etiologically relevant to disease.

Evidence for declining iron levels with increasing delay to nail collection among the cases was an unexpected finding in our data. Other metals examined in this series of cases including chromium, selenium, mercury and zinc did not show a similar pattern. Iron levels measured from the great toe should reflect ingestion of iron in the diet a minimum of 6 months in the past [38], well before subjects were diagnosed and began treatment for the brain tumor. While drugs used to manage symptoms including seizures [39] may reduce iron stores in the body [40], the majority of cases in the present study reported a recent onset of symptoms (a median of approximately 2 months before diagnosis), and few if any would have taken anti-seizure medications during the period when nail formation was taking place. Thus, the observed pattern of declining iron stores is unlikely to have reflected treatment for glioma. A more likely explanation relates to the heavy metabolic demands of the evolving brain tumor which may have progressively depleted iron stores in the cases. Although we cannot address this hypothesis directly, it is notable that gliomas have been shown to overexpress transferrin receptors (TfR1 and TfR2) which mediate the cellular uptake of iron from the blood [41, 42]. Furthermore, silencing of TfR2 receptors has been shown to reduce GBM cellular proliferation [42] consistent with a role for iron in tumor progression. Altered expression of genes known to modulate iron levels has also been demonstrated in high grade tumors [43]. Taken together, these findings suggest that iron may be important in the expansion of gliomas reflected in the present data by the observable decline in iron levels over a narrow range of time between glioma diagnosis and nail collection (90% collected within ~ 6 weeks). Results also imply that case-control differences in toenail iron should be interpreted with caution given evidence that nail measures may have reflected at least in part tumor burden in the cases and hence were not a reliable biomarker for prediagnostic iron exposure. Finally, the lower than optimal response rate among the controls is a potential limitation; however, it is unlikely that nonparticipation is associated with iron status.

To the best of the author's knowledge, ours is one of only a few studies to consider whether genetic variants associated with iron status are associated with cancer risk and the first ever to address this question in glioma. An increased risk for cancer has been suggested in several [44-47] though not all [48, 49] studies of *HFE* genetic variants which have been linked to hemochromatosis, a hereditary disorder that causes elevated body iron stores [50]. Two variants in *HFE* (rs1800562 and rs1799945) were unrelated to glioma risk in the present data; both are relatively uncommon (minor allele frequencies: 0.068 and 0.154, respectively) and power to detect associations correspondingly limited in our study. One other study that examined variants in genes involved in the uptake, absorption, and metabolism of dietary iron observed no associations with risk of colon cancer [29]. We examined one of the SNPs from that report (rs1049296 in *Tf*) and observed no association with glioma risk overall although carriers of the variant allele were at increased risk of lower grade tumors (nonGBMs). In the current study, toenail iron levels varied according to genotype in the controls for a variant which has been associated with transferrin saturation (rs1457451 in *KRT18P33*) [20]; no association with glioma risk was demonstrated for this variant in the present data.

In summary, our results do not support a role for iron status in glioma risk. While none of the examined genetic variants were associated with overall glioma risk, suggestive findings for glioma subtypes warrant examination in larger studies. Furthermore, prospective studies

in which toenail or blood samples are collected long before glioma diagnosis will be needed for a more definitive examination of the role of dietary iron in glioma risk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Cubic spline graph illustrating the negative correlation between toenail iron levels and time from diagnosis until nail collection among glioma cases, excluding one outlier with 88 days between diagnosis and nail collection.

Table 1

Characteristics of the study population.

Characteristic	Toenail samples		p-value *	Genotype samples		p-value *
	Cases (n = 300)	Controls (n = 300)		Cases (n = 622)	Controls (n = 628)	
Age, median (range)	54 (18-87)	54 (20-85)	matched	54 (18-87)	54 (20-85)	0.912
Gender, N (%)						
Female	118 (39.3)	118 (39.3)		233 (37.5)	268 (42.7)	
Male	182 (60.7)	182 (60.7)	matched	389 (62.5)	360 (57.3)	0.060
Race, N (%)						
Caucasian	286 (95.3)	280 (93.3)		622 (100.0)	628 (100.0)	
Non-Caucasian	14 (4.7)	20 (6.6)	0.202	0 (0.0)	0 (0.0)	---
State of residence						
Florida	152 (50.7)	154 (51.3)		168 (27.0)	149 (23.7)	
Alabama	71 (23.7)	70 (23.3)		97 (15.6)	80 (12.7)	
Georgia	35 (11.7)	35 (11.7)		72 (11.6)	67 (10.7)	
Tennessee	26 (8.7)	27 (9.0)		163 (26.2)	207 (33.0)	
Other	16 (5.3)	14 (4.7)	matched	122 (19.6)	125 (19.9)	0.1377
Toenail iron (µg/g), median (range)	8.8 (2.2-21.2)	9.0 (2.1-89.9)	0.308	---	---	---
Histology type, N (%)						
Glioblastomas	193 (64.3)	---	---	341 (54.8)	---	---
Lower grade astrocytomas	54 (18.0)	---	---	146 (23.5)	---	---
Oligodendroglial tumors	46 (15.3)	---	---	94 (15.1)	---	---
Other gliomas	7 (2.3)	---	---	41 (6.6)	---	---

* Differences between cases and controls were tested using the Wilcoxon rank-sum test for continuous data and the chi-squared test for categorical data.

Table 2

Association between toenail iron levels and glioma risk.

Toenail iron (µg/g)	Cases (n = 300)	Controls (n = 300)	Total Sample OR (95% CI) *	Time between diagnosis and nail clipping **	
				24 days OR (95% CI) *	> 24 days OR (95% CI) *
<6	85 (28.3)	63 (21.0)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
6 - <10	88 (29.3)	105 (35.0)	0.57 (0.35, 0.91)	1.01 (0.49, 2.08)	0.31 (0.15, 0.62)
10 - <14	57 (19.0)	57 (19.0)	0.66 (0.38, 1.14)	1.05 (0.47, 2.36)	0.47 (0.21, 1.02)
14	70 (23.3)	75 (25.0)	0.61 (0.37, 1.02)	0.93 (0.46, 1.87)	0.42 (0.19, 0.95)
P for trend			0.130	0.816	0.044
All gliomas					
<6	85 (28.3)	63 (21.0)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
6	215 (71.7)	237 (79.0)	0.60 (0.40, 0.91)	0.98 (0.53, 1.84)	0.37 (0.21, 0.68)
P-value			0.017	0.956	0.001
GBM only					
<6	59 (30.6)	44 (22.8)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
6	134 (69.4)	149 (77.2)	0.59 (0.36, 0.97)	1.06 (0.53, 2.11)	0.25 (0.11, 0.61)
P-value			0.037	0.8737	0.002
Non-GBM glioma only					
<6	26 (24.3)	19 (17.8)	1.00 (Ref)	1.00 (Ref)	1.00 (Ref)
6	81 (75.7)	88 (82.2)	0.72 (0.16, 3.24)	0.97 (0.38, 2.48)	0.65 (0.26, 1.61)
P-value			0.666	0.957	0.351

OR = odds ratio, CI = confidence interval.

Bold values indicate p-value <0.05.

* Conditional logistic regression model matched on age, gender, state of residence and analytic batch, adjusting for education (high school or less, some college, college graduate, graduate school).

** Median time from diagnosis to nail clipping among cases was 24 days.