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Analysis of 60 Reported Glioma Risk SNPs Replicates Published GWAS Findings but Fails to Replicate Associations From Published Candidate-Gene Studies

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Abstract

Genomewide association studies (GWAS) and candidate-gene studies have implicated single-nucleotide polymorphisms (SNPs) in at least 45 different genes as putative glioma risk factors. Attempts to validate these associations have yielded variable results and few genetic risk factors have been consistently replicated. We conducted a case-control study of Caucasian glioma cases and controls from the University of California San Francisco (810 cases, 512 controls) and the Mayo Clinic (852 cases, 789 controls) in an attempt to replicate previously reported genetic risk factors for glioma. Sixty SNPs selected from the literature (eight from GWAS and 52 from candidate-gene studies) were successfully genotyped on an Illumina custom genotyping panel. Eight SNPs in/near seven different genes (*TERT*, *EGFR*, *CCDC26*, *CDKN2A*, *PHLDB1*, *RTEL1*, *TP53*) were significantly associated with glioma risk in the combined dataset ($P < 0.05$), with all associations in the same direction as in previous reports. Several SNP associations showed considerable differences across histologic subtype. All eight successfully replicated associations were first identified by GWAS, although none of the putative risk SNPs from candidate-gene

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studies was associated in the full case-control sample (all *P* values > 0.05). Although several confirmed associations are located near genes long known to be involved in gliomagenesis (e.g., *EGFR*, *CDKN2A*, *TP53*), these associations were first discovered by the GWAS approach and are in noncoding regions. These results highlight that the deficiencies of the candidate-gene approach lay in selecting both appropriate genes and relevant SNPs within these genes.

Keywords

glioma; SNP; GWAS; candidate-gene

Introduction

Heritable susceptibility to glioma was originally suggested by the increased risk observed in patients with an affected first-degree relative, and also by the association of glioma with several well-defined Mendelian disorders (e.g., Neurofibromatosis Type 1, Lynch syndrome, Li-Fraumeni syndrome) [Malmer et al., 2007]. Gliomagenesis is, however, a complex and multifaceted process influenced by both heritable and somatic genetic variation. Studies of glioma tumor DNA have found that mutations in the genes *IDH1* and *IDH2* occur in approximately 50–80% of grades 2–3 glioma, but in < 10% of primary glioblastomas [Christensen et al., 2010; Yan et al., 2009]. These mutations are associated with younger age of onset and better survival among glioma patients, and are also associated with other somatic genetic and epigenetic alterations [Hartmann et al., 2009].

Studies of constitutional DNA from glioma patients have implicated at least 44 different genes in gliomagenesis. These investigations have mostly been candidate-gene studies, which frequently examine genes involved in a biological pathway of interest, such as DNA repair [Bethke et al., 2008c; Felini et al., 2007; Liu et al., 2007], apoptosis [Bethke et al., 2008a; Rajaraman et al., 2007], or folate metabolism [Bethke et al., 2008b]. However, robustly replicated risk genes have not emerged from these candidate studies and inconsistent associations are the norm.

Genomewide association studies (GWAS) of glioma have been conducted in recent years, identifying eight single-nucleotide polymorphisms (SNPs) in seven different genes which are independently associated with glioma risk [Sanson et al., 2011; Shete et al., 2009; Stacey et al., 2011; Wrensch et al., 2009]. The “hypothesis-free” GWAS approach has realized greater success than the candidate-gene approach, at least in part because it is not gene-centric. Indeed, across all the GWAS conducted to date for more than 500 diseases/traits, most significantly associated SNPs have been found in noncoding regions of the genome [Hindorff et al., 2009]. Furthermore, because of the lack of prior hypotheses, most GWAS are designed to include a replication phase to minimize false positives. Despite the shortcomings of the candidate-gene approach, such studies frequently evaluate well-considered a priori hypotheses. Because several of the glioma risk genes identified through GWAS had prior data indicating their potential involvement in gliomagenesis (i.e., *TP53, p15/CDKN2B, EGFR*), there is strong rationale to attempt replication of putative glioma risk loci appearing in the candidate-gene literature.

In order to assess the role of genetic variation at loci previously implicated in influencing glioma risk, we conducted a case-control study of Caucasian glioma patients and ancestry-matched controls. A total of 2,963 individuals recruited at The University of California, San Francisco and the Mayo Clinic were genotyped on an Illumina GoldenGate (Illumina, San Diego, CA) custom panel containing candidate SNPs selected from 28 previous publications. In total, 61 candidate SNPs were assayed, including eight previous GWAS hits

on chromosomes 5, 7 (two loci), 8, 9, 11, 17, and 20. We sought to replicate previously detected SNP associations using a larger sample size than any of the candidate-gene studies, and also to evaluate these associations within specific histologic subgroups.

Materials and Methods

Study Population

This study included European-ancestry glioma cases and controls from two collaborating institutions: The University of California, San Francisco (810 cases, 512 controls) and the Mayo Clinic (852 cases, 789 controls). All participating institutions received institutional review board approval and informed consent was obtained from subjects. Patient recruitment methods have been described in detail elsewhere [Jenkins et al., 2012]. Briefly, UCSF cases and controls were taken from the San Francisco Bay Area Adult Glioma Study. Cases aged 20 or older, diagnosed with histologically confirmed incident glioma were recruited from the local population-based registry, the Northern California Rapid Case Ascertainment program, and the UCSF Neuro-oncology clinic between 1997 and 2012. Controls aged 20 years or older from the same residential area as cases were ascertained through random digit dialing, had no history of brain tumor at time of recruitment, and were frequency matched to population-based cases on age, sex, and ethnicity.

Mayo Clinic cases consisted of patients 18 years of age and older that had surgical resection or biopsy of a glioma between 1989 and 2012. Cases were identified at diagnosis (for those initially seen at the Mayo Clinic) or at the time of pathologic confirmation (for those initially diagnosed elsewhere and subsequently treated at Mayo). The Mayo control group consisted of consented individuals, 18 years of age and older that underwent a general medical examination at the Mayo Clinic, and had no previous history of a brain tumor. Subject characteristics, including histopathologic classification of glioma cases, are outlined in Table 1. Pathology review was performed as previously described [Jenkins et al., 2012; Wrensch et al., 2009].

SNP Selection

A Medline search was performed on September 1, 2011 to retrieve association studies identifying at least one significantly associated glioma risk SNP using the following search expression (glioma[Title/Abstract] OR glioblastoma[Title/Abstract] OR astrocytoma[Title/Abstract] OR oligodendroglioma[Title/Abstract]) AND association[Title/Abstract] AND (SNP[Title/Abstract] OR single nucleotide polymorphism). Additionally, the bibliographies of selected articles were scanned to identify pertinent publications which the electronic search may have missed. Results were not filtered by language. Only studies which assayed SNPs were included because other variant types (e.g., microsatellites) are not amenable to the genotyping platform used in our study. Manuscripts were scanned and eliminated from further analysis as outlined in Supporting information Figure SI.

SNPs from 28 publications which investigated the role of inherited genetic variation in influencing glioma risk were included, of which 4 studies were GWAS [Sanson et al., 2011; Shete et al., 2009; Stacey et al., 2011; Wrensch et al., 2009] and 24 were candidate-gene studies [Bethke et al., 2008a,b,c; Brenner et al., 2007; Caggana et al., 2001; Chang et al., 2008; Chen et al., 2000; Dobbins et al., 2011; Dou et al., 2010; Felini et al., 2007; Liu et al., 2009, 2007, 2008; Rajaraman et al., 2007; Ruan et al., 2011; Schwartzbaum et al., 2005, 2007, 2010; Semmler et al., 2006; Wang et al., 2010; Wiemels et al., 2007; Wiencke et al., 2005; Wrensch et al., 2005; Yang et al., 2005]. Sample size, patient ethnicity, histological inclusion criteria, and the study hypothesis of included studies are outlined in Table 2.

From these studies, 61 SNPs, including eight identified in glioma GWAS, were selected for replication analysis in our study sample. The remaining 53 SNPs were reported to be associated with glioma risk in candidate-gene studies, with reported *P* values ranging from 1.45×10^{-4} to 0.042.

Genotyping

GoldenGate custom genotyping arrays were designed by Illumina (San Diego, CA). Genotyping was performed by the Mayo Clinic Genotyping and UCSF Genome Center core facilities as previously published [Jenkins et al., 2012]. Samples were submitted in 96-well plates containing intra- and inter-plate replicates to ensure genotype reproducibility. All cluster plots were visually inspected.

For both study sites, samples with genotyping call rate <95% were excluded from analysis. SNPs with genotyping call rates <95% in any site were excluded from all analyses. To exclude poorly genotyped SNPs, any SNP with a Hardy-Weinberg Equilibrium (HWE) *P*-value < 0.001 in controls, stratified by site, was removed from further analyses.

Statistical Analysis of SNP Associations

Single SNP association statistics were calculated using logistic regression in Plink v1.07, assuming a log-additive model [http://pngu.mgh.harvard.edu/purcell/plink/] [Purcell et al., 2007]. The effect of individual SNPs on glioma risk was calculated in the full case-control dataset using a logistic regression model adjusted for sex, age, and study site. Reported associations are for an allelic additive model, adjusted for these covariates, where odds ratios are for each additional copy of the minor allele. All *P* values are two-sided. SNP associations were assessed in the full case-control sample, and also stratified by tumor grade/histology (glioblastoma vs. controls, anaplastic astrocytoma vs. controls, grade 2 astrocytoma vs. controls, oligodendroglioma vs. controls, mixed oligoastrocytoma vs. controls) and *IDH* mutation status of patient tumors (cases with *IDH* mutant tumors vs. controls).

Of the 60 candidate SNPs for which replication was attempted, three were originally reported on in a glioma GWAS published by our group [Wrensch et al., 2009]. That GWAS case-control sample partially overlaps with the individuals reported on in this manuscript. We therefore removed 433 individuals from the analysis of these three SNPs in order to eliminate sample overlap with the previous GWAS report (i.e., $N = 2{,}963$ for 57 SNPs and $N = 2530$ for 3 SNPs).

Assessment of IDH Mutation Status

UCSF tumor specimens were sequenced to identify *IDH1* and *IDH2* mutations using previously described methods [Christensen et al., 2010]. Briefly, the region spanning the R132 codon of *IDH1* and the region spanning the R172 6 codon of *IDH2* were amplified by polymerase chain reaction with M13 tagged primers to facilitate amplification and sequencing. Products were run on a 1.5% agarose gel and subsequently sequenced in both directions at the UCSF Genomics Core Facility according to the manufacturer's protocol. Sequences were analyzed with Applied Biosystems Sequence Scanner Software v1.0. Mayo Clinic tumor specimens were assayed for *IDH1* mutations using pyrosequencing and *IDH2* mutations using both pyrosequencing and Sanger sequencing as previously described [Kipp et al., 2012].

Results

After excluding samples with call rates <95%, 2,959 participants remained for analysis (1,660 cases, 1,299 controls). After excluding SNPs that did not meet call-rate or HWE thresholds, 60 SNPs remained for analysis.

In the combined analysis of all glioma tumor histologies, seven SNPs were associated with case-control status at a *P*-value < 0.05. This included seven of the eight SNPs identified to be associated with glioma risk in previous GWAS. The eighth SNP identified via GWAS, rs498872 on chromosome 11, was associated only when analyses were restricted to the oligodendroglioma, mixed oligoastrocytoma, or *IDH* mutant subgroups, consistent with previous reports of the histologic specificity of this association (Table 3) [Jenkins, 2012]. For all eight SNPs, the direction of association in our data matches that in previous GWAS.

Many of the significantly associated SNPs in Table 3 show marked differences in association across histologic subtypes. As previously reported, rs4295627 on chromosome 8 and rs498872 on chromosome 11 are more strongly associated with tumors having an oligodendroglial component or an *IDH* mutation [Jenkins, 2012; Jenkins et al., 2011]. SNPs in *EGFR* and *CDKN2A*, on the other hand, show weak associations with oligodendroglial tumors but stronger associations with high-grade astrocytic tumors. SNPs in *TERT*, *TP53*, and *RTEL1* show only modest differences in effect size across histology strata and appear to be more general glioma risk factors.

Of the 52 remaining successfully genotyped SNPs, all selected from the candidate-gene literature, no significant associations were detected in analysis of the full case-control dataset (all *P* values > 0.05, Supporting information Table SI). Associations stratified by tumor histology and by *IDH*-mutation status can also be found in Supporting information Table SI. We were able to determine the previously reported direction of association and reference allele for 39 of these 52 SNPs. The direction of association reported in the literature matched that observed in our data for just 13/39 (33%) of the putative risk SNPs abstracted from the candidate-gene literature, compared with 50% expected by chance and 100% for SNPs identified by previous GWAS.

Discussion

The importance of performing robust SNP replication studies cannot be understated, as additional samples help to validate purported genotype-phenotype correlations and extend them to additional populations. We replicated associations at all eight glioma risk SNPs originally identified by the GWAS method, attesting to the efficacy of this approach and its ability to produce robust associations. Furthermore, the GWAS approach can identify associated genes acting in biological pathways not previously known to influence disease pathogenesis. Two such genes with relevance to glioma are involved in telomere elongation: *TERT* and *RTEL1* (rs2736100 and rs6010620, respectively). Before GWAS were conducted, telomere function was not linked to gliomagenesis and as a result, this important aspect of glioma biology remained unstudied.

In the case of glioma, *TP53* and $p16^{Ink4a}$ (containing *CDKN2A*, *CDKN2B*, and *ANRIL*) are obvious candidate loci based on the glioma-associated Mendelian syndromes resulting from deletion of these regions. Inherited mutations in *TP53* cause Li-Fraumeni syndrome (*Online Mendelian Inheritance in Man* (OMIM): 151623) and inherited deletions of *CDKN2A* cause familial melanoma-astrocytoma syndrome (OMIM: 155755). Additionally, the relevance of *EGFR* to gliomagenesis has long been apparent, as it is commonly amplified in glioma tumor samples [Wong et al., 1987]. Yet, significant and robustly replicated SNPs in *TP53*, *CDKN2B*, and *EGFR* were first identified by GWAS, despite being preceded by a plethora

of candidate-gene studies. Although the most obvious drawback of the candidate-gene approach is that selection of relevant genes is limited by the current state of biological knowledge, selecting the relevant SNPs within those genes can be a comparable challenge.

Of the eight significant SNPs identified through GWAS, none are located in coding regions (two intergenic, four intronic, one 3′-UTR, one 5′-UTR). This is in stark contrast to the 53 SNPs identified by candidate-gene studies, nearly half of which are located in exons but none of which were replicated. The impetus to identify coding variants of potential functional relevance is understandable. However, researchers performing candidate-gene studies in the future should recognize that genotyping such variants does not align with the genetic paradigm recently revealed by GWAS: Namely, that common variants are associated with common diseases, but such loci are often noncoding variants of a regulatory nature or are manifestations of “synthetic associations” [Dickson et al., 2010]. Selecting SNPs to genotype based on position within exons and predicted effect on protein function, as opposed to their ability to tag haplotype blocks, appears to be a poor strategy for identifying new associations given the results of the GWAS published to date.

The associations appearing in Supporting information Table SI can serve as a resource for researchers interested in these particular genes or in performing meta-analyses of SNPs potentially associated with glioma risk. The identification of constitutional genetic polymorphisms associated with the development of glioma has brought us closer to understanding the causal mechanisms underlying gliomagenesis. Additionally, excluding erroneous associations generated by studies with immoderate Type 1 error rates helps to focus research on more salient endeavors, such as fine-mapping of the confirmed risk loci.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Demographic and tumor histology characteristics of UCSF Adult Glioma Study and Mayo Clinic glioma cases and controls used in the genetic association analyses

	UCSF (*N* = 1322)		Mayo Clinic (*N* = 1,641)	
	Case[a]	Control	Case[b]	Control
Sample size	810	512	852	789
Mean ± SD				
Age	49.8 ± 14.4	57.9 ± 15.3	48.1 ± 14.4	49.8 ± 14.1
n (%)				
Male	501 (61.9)	289 (56.4)	488 (57.3)	450 (57.0)
Glioblastoma	390	NA	330	NA
Anaplastic astro	104	NA	188	NA
Grade 2 astro	84	NA	65	NA
Mixed oligoastro	59	NA	166	NA
Oligodendroglioma	168	NA	98	NA

[a] Numbers by histologic type add to 805 because three astrocytomas were of indeterminate grade and two gliomas had no histology information.

[b] Numbers by histologic type add to 847 because five astrocytomas were of indeterminate grade.

Table 2

Characteristics of 28 glioma case-control association studies from which 60 candidate SNPs were selected for replication

Publication	Cases	Controls	Patient ethnicity	Histologies included	Study hypothesis
Bethke et al., 2008c	1,012	1,016	Caucasian	All subtypes	Variants in DNA repair genes
Bethke et al., 2008a	1,005	1,011	Caucasian	All subtypes	*CASP8* and apoptosis
Bethke et al., 2008b	1,005	1,101	Caucasian	All subtypes	Folate metabolism genes
Brenner et al., 2007	756	1,190	Caucasian	All subtypes	Cytokine gene variation
Caggana et al., 2001	187	169	Caucasian	All subtypes	*ERCC2* polymorphisms
Chang et al., 2008	112	112	Caucasian	Glioblastoma	Pathways analysis with random forests
Chen et al., 2000	122	159	Caucasian	All subtypes	Polymorphisms in ERCC1
Dobbins et al., 2011	1,878	3,670	All	All subtypes	Atopy SNPs
Dou et al., 2010	643	656	Chinese	All subtypes	miRNA polymorphisms
Felini et al., 2007	441	487	Caucasian	All subtypes	Variants in DNA repair genes
Liu et al., 2007	771	752	Han Chinese	All subtypes	Nonhomologous end-joining genes
Liu et al., 2008	771	752	Han Chinese	All subtypes	Nonhomologous end-joining genes
Liu et al., 2009	373	365	Caucasian	All subtypes	Variants in DNA repair genes
Rajaraman et al., 2007	388	553	Caucasian	All subtypes	Apoptosis and cell-cycle genes
Ruan et al., 2011	677	698	Han Chinese	All subtypes	Genes related to allergy/immunity
Sanson et al., 2011	4,147	7435	Caucasian	All subtypes	GWAS
Schwartzbaum et al., 2005	111	422	Caucasian	Glioblastoma	Genes related to asthma
Schwartzbaum et al., 2007	217	1171	Caucasian	Glioblastoma	Genes related to allergy/immunity
Schwartzbaum et al., 2010	1,436	4,977	All	Glioblastoma	Immune function SNPs
Semmler et al., 2006	328	400	Caucasian	Glioblastoma	Methionine metabolism genes
Shete et al., 2009	1,878	3,670	All	All subtypes	GWAS
Stacey et al., 2011	207	45,081	Icelandic whites	All subtypes	GWAS of Li-Fraumeni associated tumors
Wang et al., 2010	677	698	Han Chinese	All subtypes	Epidermal growth factor polymorphisms
Wiemels et al., 2007	456	541	Caucasian	All subtypes	Genes related to allergy/immunity
Wiencke et al., 2005	461	541	All	Astrocytic tumors	*MGMT* functional polymorphisms
Wrensch et al., 2005	450	500	Caucasian	All subtypes	Polymorphisms in *ERCC1/ERCC2*
Wrensch et al., 2009	692	3,992	Caucasian	Grades 3–4 astrocytic tumors	GWAS
Yang et al., 2005	141	108	Caucasian	All subtypes	SNPs in/near 19q deletion region

Table 3

Glioma association results from UCSF Adult Glioma Study and Mayo Clinic cases and controls for eight SNPs significantly associated with glioma risk in a previous GWAS

					All glioma		GBM		AA		A2		MOA		Oligo		IDH mutant tumors[e]	
SNP	**Position**	**Nearest gene**	**Allele[b]**	**MAF[c]**	**OR[d]**	**P**	**OR[d]**	**P**	**OR[d]**	**P**	**OR[d]**	**P**	**OR[d]**	**P**	**OR[d]**	**P**	**OR[d]**	**P**
rs2736100[a]	Chr5:1286516	*TERT*	C/A	0.504	0.751	8.76E-07	0.756	1.94E-04	0.677	6.17E-04	0.781	0.073	0.698	1.78E-03	0.802	0.034	0.694	1.58E-04
rs2252586	Chr7:54978924	*EGFR*	G/A	0.275	1.145	0.023	1.145	0.066	1.230	0.039	1.074	0.611	1.160	0.221	1.108	0.362	1.086	0.426
rsll979158	Chr7:55159349	*EGFR*	A/G	0.173	0.819	6.04E-03	0.812	0.024	0.793	0.076	0.843	0.323	0.809	0.162	0.880	0.357	0.834	0.158
rs4295627	Chr8:130685457	*CCDC26*	A/C	0.168	1.515	1.14E-09	1.214	0.026	1.504	2.75E-04	1.673	4.95E-04	2.081	7.149E-09	2.247	8.665E-12	2.070	6.51E-11
rsl412829[a]	Chr9:22043926	*P15/ANRIL*	A/G	0.408	1.260	6.95E-05	1.433	1.496E-06	1.223	0.069	1.279	0.071	1.137	0.256	1.004	0.972	0.996	0.970
rs498872	Chrll:118477367	*PHLDB1*	G/A	0.319	1.107	0.067	1.016	0.820	1.165	0.109	1.080	0.562	1.334	8.95E-03	1.299	0.011	1.521	1.06E-05
rs78378222	Chrl7:7571752	*TP53*	A/C	0.014	2.655	8.26E-07	2.828	5.034E-06	3.131	3.893E-05	3.015	2.59E-03	2.696	5.10E-03	1.637	0.210	2.426	8.41E-03
rs6010620[a]	Chr20:62309839	*RTEL1*	G/A	0.244	0.702	6.43E-07	0.613	3.714E-07	0.748	0.036	0.765	0.117	0.810	0.121	0.766	0.035	0.836	0.112

[a] SNP was previously referenced in Wrensch et al., 2009. 433 individuals were removed from analysis of these SNPs to eliminate sample overlap with the previous GWAS report.

[b] Minor allele listed second.

[c] Minor allele frequency calculated among controls.

[d] OR for each additional copy of the minor allele, estimated in a logistic regression model adjusted for: age, sex, and study site.

[e] Includes 367 patients whose gliomas were found to carry mutations in IDH1 or IDH2 (48 GBMs, 85 AA, 47 A2, 81 MOAs, 106 Oligos).

Abbreviations: GBM, glioblastoma; AA, anaplastic astrocytoma; A2, grade 2 astrocytoma; MOA, mixed oligoastrocytoma; Oligo, Oligodendroglioma.