Genome-wide high-density SNP linkage search for glioma susceptibility loci: results from the Gliogene Consortium


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ABSTRACT

Gliomas, which generally have a poor prognosis, are the most common primary malignant brain tumors in adults. Recent genome-wide association studies have demonstrated that inherited susceptibility plays a role in the development of glioma. Although first-degree relatives of patients exhibit a two-fold increased risk of glioma, the search for susceptibility loci in familial forms of the disease has been challenging because the disease is relatively rare, fatal, and heterogeneous, making it difficult to collect sufficient biosamples from families for statistical power. To address this challenge, the Genetic Epidemiology of Glioma International Consortium (Gliogene) was formed to collect DNA samples from families with two or more cases of histologically confirmed glioma. In this study, we present results obtained from 46 U.S. families in which multipoint linkage analyses were undertaken using nonparametric (model-free) methods. After removal of high linkage disequilibrium SNPs, we obtained a maximum nonparametric linkage score (NPL) of 3.39 (P=0.0005) at 17q12-21.32 and the Z-score of 4.20 (P=0.000007). To replicate our findings, we genotyped 29 independent U.S. families and obtained a maximum NPL score of 1.26 (P=0.008) and the Z-score of 1.47 (P=0.035). Accounting for the genetic heterogeneity using the ordered subset analysis approach, the combined analyses of 75 families resulted in a maximum NPL score of 3.81 (P=0.00001). The genomic regions we have implicated in this study may offer novel insights into glioma susceptibility, focusing future work to identify genes that cause familial glioma.
INTRODUCTION

Gliomas account for ~40% of all primary malignant brain tumors (PBT) and are responsible for ~13,000 cancer related deaths in the US each year. Irrespective of treatment, most gliomas are associated with a poor prognosis with the most common type of glioma, glioblastoma (GBM), having a median overall survival of only 10-15 months (1).

Evidence strongly suggests that inherited susceptibility plays a role in the development of glioma, as first-degree relatives of patients with glioma have a two-fold increased risk of glioma (2-4). Hereditary genetic disorders such as neurofibromatosis type I and II, and Li-Fraumeni and Turcot’s syndromes are known to predispose to glioma (5;6). These syndromes are, however, rare and collectively make only a minor contribution to the familial risk of glioma.

Families segregating glioma outside the context of these syndromes provide a strong rationale for seeking to identify moderate-high risk susceptibility loci for glioma through genome-wide linkage scans. To date only one genome-wide linkage scan of glioma has been conducted, which was based on the analysis of four Finnish families. Evidence of linkage of glioma to 15q23-26 was provided; however, no genetic mutation was identified in this region (7).
To facilitate the collection of glioma families informative for linkage analysis, we established the “Genetic Epidemiology of Glioma International Consortium” (GLIOGENE-Linkage) in 2006 (8). GLIOGENE now includes 15 institutions in the United States, United Kingdom, Sweden, Denmark, and Israel. We have undertaken a genome-wide scan of 75 glioma families in the U.S. ascertained through GLIOGENE. This search was conducted using high-density single-nucleotide polymorphism (SNP) arrays, thereby allowing us to maximize the power to identify a disease-causing locus. Here we report evidence for Mendelian predisposition to glioma and strong evidence for a disease locus at 17q12-21.32.
MATERIALS AND METHODS

Ascertainment and collection of families
Forty six glioma families with at least two biologically related family members diagnosed with a histologically-confirmed glioma (International Classification of Disease – Oncology codes: low grade glioma (WHO grade I, II): juvenile pilocytic astrocytoma (9421/3), fibrillary astrocytoma (9420/3), protoplasmic astrocytoma (9410/3), gemistocytic astrocytoma (9411/3), diffuse astrocytoma (9400/3), oligodendroglioma (9450/3), oligoastrocytoma (9382/3); ependymoma (9391/3), high grade glioma (WHO grade III and IV): anaplastic astrocytoma (9401/3), anaplastic oligodendroglioma (9451/3), anaplastic oligoastrocytoma (9382/3), anaplastic ependymoma (9392/3), gliosarcoma (9442/3), gliomatosis cerebri (9381/3), and glioblastoma multiforme (9440/3)) were ascertained through the GLIOGENE consortium. We excluded all families with reported or confirmed diagnosis of neurofibromatosis 1, neurofibromatosis 2, Turcot’s syndrome or tuberous sclerosis. The scheme for recruitment, and data collection of families has been previously described (8). These forty-nine families were ascertained and genotyped in years 2004-2009, and are referred as the first stage families. An additional twenty-nine families were collected using the same sampling criterion in 2010 and genotyped in 2011. These twenty-nine families are referred to in this manuscript as the second stage or the replication families. All members of the included families were self-reported to be non-Hispanic white. Blood or saliva samples were obtained from both the offspring and spouse of deceased
affected family members wherever possible to facilitate the genotype
reconstruction of deceased family members. DNA was extracted from EDTA-
venous blood samples and saliva samples that were collected using the Oragene
kits (DNA Genotek). Biosamples and clinico-pathological information from
patients and family members was collected with informed consent according to
protocols approved by each center’s Institutional Review Board in accordance
with the tenets of the Declaration of Helsinki.

**Genotyping**

Prior to genotyping all DNA samples were quantified by PicoGreen (Invitrogen,
Carlsbad, CA). Genotyping was conducted using Illumina Hap370K BeadChip
arrays according to manufacturer’s protocol. DNA with GenCall scores <0.25 at
any locus were considered ‘no-calls’. A SNP was considered to have failed if
<95% of DNA samples generated a genotype at the locus. Cluster plots were
manually inspected to resolve any ambiguities. We excluded SNPs from the
analyses if the Hardy-Weinberg proportion $P$-value was <0.0001. We also
removed SNPs for which the minor allele frequency was <5%.

**Data processing and error checking**

The pedigree relationship testing program *Pedcheck* software, version 1.1
(November 24, 1998) (9) was implemented to check for pedigree errors. Non-
Mendelian error checking of genotypes and generation of linkage format files from Illumina array files was performed using in-house generated scripts. The genotyping calls were processed using the BeadStudio Genotyping (BSGT) software, version 3.2.32. The map order and distances between SNP markers was based on the NCBI GenomeBuild version 36.

**Linkage analysis**

We conducted multipoint non-parametric linkage analysis using Allegro software, version 2.0f (10). We used an equal family weighting scheme in the exponential model with a scoring function $S_{pairs}$ (11), which is optimal over a range of disease models (12).

Multipoint linkage assumes that markers are in linkage equilibrium. However, for closely spaced markers, this is not always the case thus increasing false-positive evidence for linkage (13). Pairwise LD (as measured by $r^2$ value) between SNPs was calculated using Plink (v1.06) (14). Then, we obtained LD blocks based on the LD value criteria of $r^2 \geq 0.01$ and $r^2 \geq 0.004$. For each block, we retained the SNP from each set with the highest information content, defined by entropy information measure $I_E$ (15). Then, we performed linkage analysis using three different criteria: retaining all SNPs, excluding all SNPs with $r^2 > 0.01$, and excluding all SNPs with $r^2$ value $> 0.004$. For some pedigrees, individual
members not informative for linkage were removed to accommodate Allegro pedigree size limitations.

We performed haplotype analyses using SimWalk2 (version 2.83) (16-18). SimWalk2 uses Markov chain Monte Carlo methods and simulated annealing algorithms to perform multipoint haplotype analysis, which estimates the most likely set of fully-typed maternal and paternal haplotypes of the marker loci for each individual in the pedigree. The input files to SimWalk2 were automatically generated from the linkage analysis files using a utility program Mega2 (Manipulation Engine for Genetic Analysis), version 4.3.1 (19). The options for running Mega2 were set with “SimWalk2” for analysis option and “Haplotype analysis” for analysis sub-option.

Genetic heterogeneity is an important feature of complex disease etiology such as glioma. We performed the ordered subset analysis (OSA) using trait related covariates that provide maximal evidence for linkage (20;21). In the OSA method, the family members' covariate information is used to assign a covariate score to each family. Subsequently, families are ordered by family-specific covariate score and linkage analyses are performed on all subsets ranked by covariate scores. We used a computer program, FLOSS (version 1.4.1 for Windows), that implemented the OSA method (21). We used age of onset (minimum, maximum, mean, and range) and number of affected individuals per family as covariates in the FLOSS analyses.
RESULTS

Details of the 75 families: 46 from the first stage of analyses and 29 from the replication study are summarized in Table 1. For the first stage families, the average family size was 24.6 individuals, with the smallest family having eight individuals and the largest having 44 individuals. A total of 127 individuals (11.2%) were affected with a diagnosis of glioma, 835 (73.8%) were unaffected, and 169 (15.0%) were coded as “unknown affection status.” Of the 127 affected individuals, 61 (48%) were male compared to 50.1% of the total individuals. We genotyped 415 individuals (36.7%), with a range of 4 to 20 individuals genotyped per family. Twenty-eight pedigrees had two affected relatives per pedigree, twelve pedigrees had three affected relatives per pedigree, one pedigree had four affected relatives, three pedigrees had five affected relatives per pedigree, and two pedigrees had six affected relatives per pedigree. The average age of diagnosis for the probands was 46.9 years (standard deviation (sd)=13.4) and the average age of diagnosis for the other affected members in the pedigrees (excluding proband) was 46.9 years (sd=19.6) (Table 1).

Data quality

A number of parameters were used throughout the study to determine data quality. The average SNP call rate per array was >96%. Three pedigrees from
the stage 1 samples had excessive Mendelian inheritance errors, which were likely due to misspecified relationships or sample switching. These three pedigrees were removed from the analyses. On average, Mendelian inconsistency was found in < 0.4% of the SNPs. These error SNPs were randomly distributed across the genome and were removed from further analyses.

**Linkage analysis**

The linkage analyses based on the SNPs retained using the three LD criterions (see the methods section) resulted in LOD score peaks at identical locations (data not shown). The first two criterions based on retaining all the SNPs and excluding all SNPs with \( r^2 > 0.01 \) gave higher LOD scores than the criterion based on excluding all SNPs with \( r^2 > 0.004 \). Conservatively, we report linkage analysis results based on the last criterion (i.e. excluding all SNPs with \( r^2 \) value >0.004) which is based on a total of 23,476 SNPs across the genome.

For the initial 46 families, the most significant genome-wide linkage (NPL) score of 3.39 was obtained at physical location 42,504,408 on chromosome 17q12-21.32 (P=0.0005) (Table 2 and Figure 1). The corresponding Z-score in this region was 4.20 (P=0.000007). In addition, three other chromosomal regions had maximum NPL scores exceeding 2.0. These regions were chromosome 6 (max NPL=2.10, P=0.02 at physical location 22,393,991, 6p22.3), chromosome 12
(max NPL=2.07, P=0.02 at physical location 24,344,720, 12p13.33-12.1), and chromosome 18 (max NPL=2.45, P=0.008 at physical location 75,238,190, 18q23).

We further investigated the contribution of each family to the LOD scores on chromosome 17. We investigated the age of onset of the affected members of these families linked to the region on chromosome 17. Eleven families showed maximal evidence of linkage at chromosome 17. Each of these families had individual NPL score greater than 0.59 (equivalent to a point-wise significance of 0.05). The age of onset for all individuals affected with glioma in the families linked to the chromosome 17 region was lower than the age of onset for all individuals affected with glioma coming from unlinked families (36.8 versus 43.9 years); however, the difference was not statistically significant (p-value=0.17). Most of the families linked to chromosome 17 have affected siblings with glioma (8 out of 11 families). No significant glioma histological differences were noted between families.

To better understand the 17q region and to identify risk haplotypes shared by affected individuals from the eleven families linked to this region, we genotyped five highly polymorphic microsatellite markers in 17q region (D17S932, D17S950, D17S791, D17S943, and D17S1869). The average information content value for all the markers was 0.56 to 0.70. Haplotype analyses revealed that the risk haplotype segregated among affected relatives. However, we also observed that
a number of unaffected individuals were carriers of the disease haplotype implying decreased penetrance. The highest NPL score of 4.02 (P=0.000009) was obtained at the marker D17S950 using microsatellite-based multipoint linkage analyses.

**Replication and Joint Analyses**

To replicate the 17q12-21.32 region, we genotyped 29 independent glioma families ascertained using the same criteria as families in the first stage. The average family size was 19 individuals, with the smallest family having nine individuals and the largest had 32 individuals. A total of 70 individuals (12.6%) were affected with a confirmed diagnosis of glioma. Of the 70 affected individuals, 41 (58.6%) were male which is a higher percentage than the percentage of affected males in the first stage families; however, the difference is statistically not significant. We genotyped a total 309 individuals (56.0%) which is a higher percentage than the number of individuals genotyped in the first stage families. We were able to get DNA samples on more individuals in the second stage which could be due to the fact that stage 2 families were more recently ascertained and recruitment staff was more experienced. The majority of the families had only two affected relatives (n=21), and the other eight families had three to six affected relatives. The average age of diagnosis for the probands was 47.6 years (sd=14.8), and the average age of diagnosis for the affected members (excluding proband) was 46.6 years (sd=20.5) (Table 1) which was
very similar to the average age of diagnosis for the affected individuals in the first stage families.

Using Allegro, we obtained NPL score of 1.26 (P=0.008) at 17q12-21.32 region for the Stage 2 families. The corresponding Z-score in this region was 1.47 (P=0.035).

To account for hypothesized genetic heterogeneity, using the FLOSS program, we obtained an ordered subset analyses-based NPL score of 2.16 from the 29 replication families (P=0.0008) with the number of affected individuals per family as significant family-specific covariate. Even though, the NPL score based on stage 2 families did not reach the LOD score threshold of 3.0, it should be noted that we are not performing genomewide analyses with these replication families. Therefore, the point-wise p-value of 0.0008 should be considered as a replication of the genetic region identified using the first stage families.

We further performed joint OSA on 75 families (46 from stage 1 and 29 from stage 2). Using the FLOSS, the combined analyses gave us an NPL score of 3.81 (P=0.00001) with the number of affected individuals per family as significant family-specific covariate. When we re-analysed the data excluding those families deemed unlinked from the FLOSS analysis, the LOD score increased to 4.24 (P=0.000005), further supporting linkage to this region.
We also performed joint OSA on 75 families at the three chromosomal regions 6p22.3, 12p13.33-12.1, and 18q23 that were suggestive of linkage based on the first stage families. Using the FLOSS, the combined analyses gave respective NPL scores 2.34, 1.75, and 2.04 with the number of affected individuals per family as significant family-specific covariate.

DISCUSSION

Here we provide evidence for a moderate-high penetrance susceptibility to glioma mapping to 17q12-21.32. We searched the National Center for Biotechnology Information (NCBI) Hapmap database and identified potentially important target genes related to cancers in this area. WNT9B (17q21), mapping at 44,941,702 bps, belongs to the WNT gene family, is associated with basal cell carcinoma. The WNT gene family consists of several structurally related genes that encode signalling proteins that have been implicated in oncogenesis and in several developmental processes, including the regulation of cell fate and patterning during embryogenesis (22;23). Another gene that resides in this region is CDC27, at location 17q12-23.2. The protein encoded by this gene is a component of the anaphase-promoting complex (APC), an E3 ubiquitin ligase that controls the cell cycle. The DNA damage response mediator MDC1 directly interacts with this protein (24). A third gene in this region is NPEPPS, at location 17q21. This protein-coding gene encodes an enzyme that degrades enkaphalins in the brain, and mouse studies suggest that it is also involved in regulating the
cell cycle. Another candidate gene mapping to this region is NSF, located at 17q21, which has been shown to be associated with schizophrenia (25) and neurotransmission and neurodevelopment (26). Finally, the ITGB3 gene located at 17q21.32 has been reported to be is involved in brain ischemia (27).

We also identified three additional regions, on chromosomes 6p, 12p, and 18q, which are suggestive for linkage using data from first stage families. The associated allele-sharing LOD scores also exceeded threshold for suggestive evidence of linkage. The exact p-values for NPL scores in these regions were between 0.01 and 0.02. However, using the joint OSA on 75 families, only 6p22.3 and 18q23 regions remained suggestive for linkage.

The contribution of individual families to the genetic region implicated in this study varied, suggesting that gliomas have strong genetic heterogeneity. This provides justification for the further study of larger number of families at high risk for this relatively rare cancer.

Recently, our group conducted three independent genome-wide association studies (28-30) which identified seven risk loci for glioma at 5p15.33 (TERT rs2736100), 7p11.2 (EGFR rs11979158 and rs2252586), 8q24.21 (CCDC26 rs4295627), 9p21.3 (CDKN2A-CDKN2B rs4977756), 11q23.3 (PHLDB1 rs498872), and 20q13.33 (RTEL1 rs6010620). Collectively these loci accounted for only 7-14% of the excess familial risk of glioma (28). The linkage peak for the
GWAS hits was not significant in the families analyzed herein, underscoring that the genes associated with sporadic glioma have little contribution to familial glioma. Thus, additional studies are needed to discover additional risk loci for familial glioma.

In summary, our analysis provides evidence for disease locus for glioma susceptibility at 17q12-21.3. The next step for this study is to sequence this region in the affected and unaffected members of families that showed high linkage and determine if there is a pathogenic mutation in the germline. We will look for deleterious mutations (frame shift truncating or nonsense mutations, missense variants, insertions or deletions), that could be cancer promoting genes. If we identify specific genes from the sequencing analysis, we will then screen for these mutations in the linking relatives. To date, this is the largest genetic linkage study of glioma. The regions implicated in this study underscore glioma heterogeneity especially since they are different from those identified in previous GWAS. Further studies could potentially provide novel insights into the biological mechanisms and may ultimately lead to the identification of a causal gene or genes in these regions and to guidelines for genetic counseling.
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Conflict of Interest

The authors declare no competing financial interests.

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FIGURE LEGENDS

Figure 1. NPL scores for US glioma pedigrees across each chromosome.

Each plot shows NPL scores obtained after excluding all SNPs with r² value >0.004. The y-axis is NPL score and x-axis is physical location.
Table 1. Descriptive statistics and characteristics of glioma pedigrees from US used for linkage analyses

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<th>No. of affected individuals in a pedigree</th>
<th>No. of affected individuals in generation(s)</th>
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<td>3</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>6</td>
<td>1,2</td>
<td>--</td>
<td>--</td>
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<tr>
<td></td>
<td>3</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>No. of affected male (%)</td>
<td>61 (48.0)</td>
<td>41 (58.6)</td>
<td>102 (51.8)</td>
<td></td>
</tr>
<tr>
<td>No. of affected female (%)</td>
<td>66 (52.0)</td>
<td>29 (41.4)</td>
<td>95 (48.2)</td>
<td></td>
</tr>
<tr>
<td>Avg. age (yrs) at dx, proband (SD)</td>
<td>46.9 (13.4)</td>
<td>47.6 (14.8)</td>
<td>45.0 (14.4)</td>
<td></td>
</tr>
<tr>
<td>Age range at dx, proband</td>
<td>6 - 69</td>
<td>22 - 75</td>
<td>6 - 75</td>
<td></td>
</tr>
<tr>
<td>Avg. age (yrs) at dx, family (SD)</td>
<td>46.9 (19.6)</td>
<td>46.6 (20.5)</td>
<td>45.3 (19.1)</td>
<td></td>
</tr>
<tr>
<td>Age range at dx, all affected individuals</td>
<td>1 - 86</td>
<td>1 - 85</td>
<td>1 - 86</td>
<td></td>
</tr>
<tr>
<td>Total No. Individuals</td>
<td>1131</td>
<td>552</td>
<td>1683</td>
<td></td>
</tr>
<tr>
<td>Avg. family size</td>
<td>24.6</td>
<td>19.0</td>
<td>22.4</td>
<td></td>
</tr>
<tr>
<td>No. genotyped (%)</td>
<td>415 (36.7)</td>
<td>309 (56.0)</td>
<td>724 (43.0)</td>
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</tr>
<tr>
<td>chr</td>
<td>physical location</td>
<td>SNP</td>
<td>max NPL</td>
<td>NPL exact p-value</td>
</tr>
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<td>------------------</td>
<td>--------------</td>
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<tr>
<td>1</td>
<td>158,235,632</td>
<td>rs6676682</td>
<td>1.6120</td>
<td>0.0556</td>
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<tr>
<td>2</td>
<td>1,657,741</td>
<td>rs7560004</td>
<td>1.9369</td>
<td>0.0283</td>
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<tr>
<td>3</td>
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<tr>
<td>4</td>
<td>7,375,903</td>
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<td>1.1218</td>
<td>0.1320</td>
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<tr>
<td>5</td>
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<td>1.8552</td>
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<td>0.1111</td>
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<td>11</td>
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<td>1.7056</td>
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<tr>
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<tr>
<td>16</td>
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**NOTE:** chr, chromosome; physical location, the physical location at which maximum NPL score was observed; SNP, rs number of the SNP at which maximum NPL score was observed; max NPL, maximum NPL score obtained; NPL exact p-value, the exact p-value associated with maximum NPL score from Allegro; left physical location and right physical location, boundaries based on 1-LOD drop (approximating 95% confidence interval for the location of peak.)
Genome-wide high-density SNP linkage search for glioma susceptibility loci: results from the Gliogene Consortium


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