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The cyclic AMP pathway is a sex-specific modifier of glioma risk in type 1 neurofibromatosis patients

Nicole M. Warrington[1,*], **Tao Sun**[1,*], **Jingqin Luo**[2,*], **Robert C. McKinstry**[1,3], **Patricia C. Parkin**[4], **Sara Ganzhorn**[1], **Debra Spoljaric**[1], **Anne C. Albers**[5], **Amanda Merkelson**[6], **Douglas R. Stewart**[7], **David A. Stevenson**[8], **David Viskochil**[9], **Todd E. Druley**[1], **Jason T Forys**[1], **Karlyne M. Reilly**[10], **Michael J. Fisher**[11,12], **Uri Tabori**[4], **Jeffrey C. Allen**[6], **Joshua D. Schiffman**[8], **David H. Gutmann**[5], and **Joshua B. Rubin**[1,13]

[1]Division of Pediatric Hematology/Oncology, Department of Pediatrics, Washington University School of Medicine, St Louis, MO

[2]Division of Biostatistics, Washington University School of Medicine, St Louis, MO

[3]Mallinckrodt Institute of Radiology, Washington University School of Medicine, St Louis, MO

[4]Department of Pediatrics, The Hospital for Sick Children, University of Toronto, Toronto, Ontario

[5]Department of Neurology, Washington University School of Medicine, St Louis, MO

[6]Department of Pediatrics, New York University Langone Medical Center, New York, NY

[7]Clinical Genetics Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, Rockville, MD

[8]Division of Medical Genetics, University of Utah, Salt Lake City, UT

[9]Huntsman Cancer Institute, University of Utah, Salt Lake City, UT

[10]Rare Tumors Initiative, Office of the Director, Center for Cancer Research, National Cancer Institute, NCI, Bethesda, MD

[11]Division of Oncology, The Children's Hospital of Philadelphia, PA

[12]Department of Pediatrics, The Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA

[13]Department of Anatomy and Neurobiology, Washington University School of Medicine, St Louis, MO

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Abstract

Identifying modifiers of glioma risk in patients with type 1 neurofibromatosis (NF1) could help support personalized tumor surveillance, advance understanding of gliomagenesis and potentially identify novel therapeutic targets. Here we report genetic polymorphisms in the human adenylate

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Corresponding author: Joshua B. Rubin, MD, PhD, Campus Box 8208, 660 South Euclid Ave, St Louis, MO 63110, (Tel): 314-286-2790, (Fax): 314-286-2892, rubin_j@kids.wustl.edu.

*These authors contributed equally.

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cyclase gene ADCY8 which correlate with glioma risk in NF1 in a sex-specific manner, elevating risk in females while reducing risk in males. This finding extends earlier evidence of a role for cAMP in gliomagenesis based on results in a genetically engineered mouse model (Nf1 GEM). Thus, sexually dimorphic cAMP signaling might render males and females differentially sensitive to variation in cAMP levels. Using male and female Nf1 GEM, we found significant sex differences exist in cAMP regulation and in the growth promoting effects of cAMP suppression. Overall, our results establish a sex-specific role for cAMP regulation in human gliomagenesis, specifically identifying ADCY8 as a modifier of glioma risk in NF1.

Introduction

Neurofibromatosis type 1 (NF1) is a common autosomal dominant cancer predisposition syndrome that affects males and females of all races and ethnicities, and variably results in multiple developmental abnormalities and neoplasias (1). Currently, the severity with which multiple body systems will be affected by complications of NF1 remains largely unpredictable, which significantly hinders the delivery of care (2). Controversies surrounding the management of optic pathway gliomas (OPG) in these patients illustrate this point. These NF1-associated brain tumors occur in approximately 20% of affected individuals, and in up to 50% of NF1 OPG cases, chemotherapy is initiated, usually prompted by vision loss (3). The unpredictable growth of OPGs has impeded the adoption of consensus guidelines for care and confounds assessments of treatment efficacy (4). Identifying biomarkers for OPG risk would transform our management of NF1 patients.

The majority of NF1-associated gliomas occur in the anterior optic pathway of young children (< 7 years old). Previously, we showed that alterations in cAMP levels could vary the stereotypical pattern of OPG formation, and that pharmacological elevation of cAMP levels could block OPG growth in an established genetically engineered mouse (*GEM*) model of NF1-associated OPG (5),(6, 7). These studies established the cAMP pathway as a candidate modifier of glioma risk in NF1. Here, we provide a measure of validation for these studies by showing that polymorphisms in adenylate cyclase 8 (*ADCY8*) modify NF1 glioma risk in a sex-specific fashion. Moreover, we found that sexual dimorphism in cAMP signaling and sex differences in cAMP-dependent growth regulation are well modeled in murine *Nf1-/-* astrocytes.

Materials and Methods

Ethics Statement

Animal studies—Animals were used in accordance with an Animal Studies Protocol (# 20120174) approved by the Animal Studies Committee of the Washington University School of Medicine per the recommendations of the Guide for the Care and Use of Laboratory Animals (National Institutes of Health).

Human Studies—DNA specimens acquired from individuals with NF1 were processed and are being reported in accordance Institutional Review Board (IRB)-approved Human Studies Protocols at each of the participating institutions.

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Chemicals, Reagents, and Antibodies—All chemicals were obtained from Sigma-Aldrich (St Louis, MO) unless otherwise indicated.

Human DNA sample collection—Individuals with NF1 were recruited for this study from NF1 Clinical Programs at Washington University in St. Louis (WU), the University of Toronto (TORONTO), University of Utah (UTAH) and New York University (NYU). Those with and without OPG were identified from MRI scans. Criteria for OPG included clear optic nerve or chiasm enlargement or enhancement. Other optic nerve abnormalities such as tortuosity or dilated, fluid filled optic nerve sheaths did not qualify as OPGs (8). Patients without OPG had negative MRIs obtained after the age of ten. DNA was extracted from blood using Qiagen DNA Blood mini-kits (Valencia, CA) and from saliva using DNA Genotek Oragene DNA kits (Kanata, ON, CA) according to the manufacturers' instructions. Following quality checks and concentration optimization, DNA was hybridized to Affymetrix 6.0 single nucleotide polymorphism microarrays at The Genome Institute, Washington University or ARUP, Salt Lake City. Intensity scanning was performed in the same laboratories in which hybridization occurred. All data are accessible through the geo database, accession number GSE62215 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62215).

High-density Affymetrix Genome-wide SNP Array Analysis—The Birdseed-v2 was used to make initial genotype calls. Samples with a genotyping call rate <95% and contrast QC<0.4 according to the Affymetrix genotyping console analysis were removed and genotypes were regenerated using the remaining samples. PLINK (9) was used for SNP QC to exclude those failing Hardy-Weinberg test (P 1e-06) or missingness test (P<0.1) or with a MAF <0.05. A total of 680,187 SNPs were analyzed. The logistic regression model for glioma risk was modeled with a SNP, Sex, SNP × Sex interaction, biospecimen (saliva/blood) and cohort (WU/UTAH/TORONTO/NYU), as well as the first 4 principal components from principal component analysis (PCA) using linkage disequilibrium (LD) pruned SNPs to control for population stratification. The bioConductor package “SNPRelate” (10) was used for LD pruning (the maximum basepairs in the sliding window=10e06, LD threshold=0.2 and the “composite” method was adopted for LD metrics) and PCA analysis and “GWASTools” (11) was used for genome wide association analysis using logistic regression modeling under the dominant genetic model. The odds ratios (ORs) of male, female, ratio of the ORs for glioma risk between males and females (the SNP × Sex interaction) and the likelihood ratio (LR) P-values on the ratio which was obtained by comparing the full logistic regression model to the model leaving the interaction out were reported. To account for multiple comparisons, the permutation-adjusted P-values and the false discovery rate (FDR) adjusted P-value were calculated. Specifically, the case/control status was permuted (for 500 times) and the LR P-values of SNP × Sex corresponding to each permuted phenotype were calculated under the same full logistic regression model. The permutation-adjusted P-value was finally computed as the proportion of permutations with at least one SNP's permutated P-value the original LR P-value corresponding to the non-permuted status. Linkage disequilibrium analysis was conducted using PLINK and the LD measure R^2 was reported. Major Allele Frequency (MAF) for SNPs in the general population was determined using 1093 total samples in the 1000

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Genome phase I data released on May 2011 using ENGINES (SPSmart version 5.1.1 and dbSNP build 132) (12).

Primary Astrocyte Cultures—Animals were maintained on a C57Bl/6 background. Primary *Nf1*-/- astrocytes were isolated from the cortices of individual neonatal *Nf1*-CKO (*Nf1$^{flox/flox}$*; *GFAP-Cre*) mice at postnatal day 1-2 as described (5). The sex of the newborn mice was determined by *Jarid 1C/Jarid 1D* PCR. Astrocytes of the same sex were combined and cryo-preserved. Wildtype (*WT*) astrocytes were similarly derived from neonatal *Nf1$^{flox/flox}$* mice. Western blot analysis for neurofibromin expression was performed by standard methods utilizing rabbit anti-Nf1 antibody (1:200, Santa Cruz), mouse anti-β-actin antibody (1:30,000, Sigma), and IRdye680 or 800-conjugated donkey anti-mouse or rabbit IgG (1: 30,000, LI-COR). Only cells at low passage numbers (<6) were used. Each experiment included at least 4 separate cultures derived from at least two litters/sex.

Real-time PCR—RNA was extracted from *WT* and *Nf1-/-* astrocytes using the Qiagen RNeasy kit (Qiagen, Valenica, CA). cDNA was generated using the Superscript first-strand cDNA synthesis system (Invitrogen). Real-time quantitative PCR reactions were performed using Power Sybr Green PCR master mix (Applied Biosystems (Carlsbad, CA)) using primers as indicated in Supplemental Table 3. Triplicate measures were made for each sample and corresponding GAPDH control. PCR and data collection were done using the BioRad MiniOpticon Real Time PCR machine and Opticon Monitor 3 Software from BioRad (Hercules, CA). Relative transcript copy number was calculated using the delta-delta-C(t) method. The relative expression values of cAMP modulators in cells derived from female *Nf1-/-* astrocytes were normalized to those from male expression levels (n = 3-5 separate litters/genotype).

Drug Treatments—For cAMP measurements, astrocytes were cultured in serum free DMEM/F12 media (24 hrs), and then treated with the ADCY activator, Forskolin (FSK, 10 μM) and the phosphodiesterase inhibitor, IBMX (1mM), FSK alone, or DMSO control as indicated. For cell number experiments, 75,000 cells per well were plated in 6-well plates. Twenty-four hours after plating, cells were serum starved for 24 hours, and then treated with dideoxyadenosine (DDA, 100 μM) or CXCL12 (0.1μg/ml (Peprotech)) in serum free DMEM/F12 as indicated. Cells cultured in DMEM/F12 + vehicle served as control. Cell number was determined by trypan blue exclusion.

cAMP ELISA—cAMP was measured by competitive immunoassay using a Correlated Enzyme Immunoassay Kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's instructions and as previously described (5).

Statistics—Baseline Data were analyzed using GraphPad Prism version 4.00 (GraphPad Software) or Stata10 (Stata). Specific statistical tests are as indicated in the text and figure legends. All tests were two-sided and a *p*-value < 0.05 was considered statistically significant.

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Results

DNA samples were obtained from 243 individuals with NF1 and genotyped using Affymetrix whole genome human SNP array 6.0. Two hundred thirty-six specimens, 123 from individuals with OPG and 113 from individuals without OPG, passed quality control filtering (Supplemental Table 1). Both the tumor and non-tumor groups had equivalent numbers of males and females (P=0.90, Fisher's exact test). The average genotyping rate in the 236 individuals was 98.43%.

Our analysis focused on 2,761 unique SNPs in 22 key regulators of intracellular cAMP levels (Supplemental Table 2). Calculations for odds ratio (OR) for glioma between genotypes within males and females, the ratio of the male OR to female OR, and corrections for multiple comparisons were calculated as described in Materials and Methods. At the 5% statistical significance level on the FDR-adjusted P-values, we identified three SNPs in adenylate cyclase 8 (*ADCY8:* rs724365, FDR P=0.014; rs4736688, FDR P=0.014; rs1435446, FDR P=0.043) (Table 1). Linkage disequilibrium (LD) analysis indicated that recombination rarely occurred between rs724365 and rs1435446 in the population (R^2=0.92) while the LDs between each of the loci with rs4736688 were medium with both R^2 slightly above 0.5. Additionally, SNPs in *CXCR7* (rs2568554) and *ADCYAP1* (rs16952813) were nearly significant at the 10% significance level (Table 1).

Unexpectedly, associations between *ADCY8* SNPs and glioma risk were sex-dependent. The minor alleles of each *ADCY8* SNP elevated glioma risk in females and decreased risk in males (Table 1). The resulting SNP × Sex interaction effects were highly significant, indicating that sequence variants in *ADCY8* are potential sex-specific modifiers of glioma risk in NF1.

As the SNPs had sex-specific effects, we reviewed NF1 OPG case series for evidence of sex disparity. We found 543 OPG cases diagnosed from both, routine surveillance scans of asymptomatic individuals and scans obtained to evaluate symptoms. Six series reported higher frequency of OPG in females, four reported higher frequency in males, and three reported equal incidence. Overall, 297 or 55% of cases occurred in females (Table 2), suggesting a slight female predominance. However, not all cited studies were population-based, and in those series that include scans for symptoms the results may be skewed towards increased rates in females as sex differences in glioma-associated symptoms have been reported (13).

Prior murine studies suggested that spatiotemporal regulation of CXCL12 and intracellular cAMP during development could influence the pattern of tumorigenesis in NF1 (5, 7). Identification of *ADCY8* as a sex-specific modifier of glioma risk in NF1 potentially provides important human validation for these studies. To examine whether cAMP exerts a sex-specific effect on tumorigenesis, we established primary cultures of male and female post-natal day one forebrain astrocytes from wildtype (*WT*) and $Nf1^{flox/flox}$;$GFAP^{cre}$ (*Nf1-/-*) mice based on expression of X and Y chromosome encoded paralogs *Jarid 1C* and *Jarid 1D* (14) (Figure 1A) and verified equivalent deletion of neurofibromin (Figure 1B).

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We first looked for sex differences in cAMP regulator expression (Figure 1C) and in intracellular cAMP levels. While there were no sex differences in *Adcy8* expression, there were clear effects of sex and neurofibromin loss on the expression of multiple other components of the cAMP pathway (Figure 1D). Intracellular cAMP levels were consistently lower in male compared to female *Nf1-/-* astrocytes (Male: 6.97 +/- 1.5, Female: 10 +/- 0.89 pmol/mg protein, (P=0.03, t test, n=3 independent litters)), indicating that cell intrinsic sexual dimorphism in cAMP regulation exists in *Nf1-/-* astrocytes.

Next we looked for functional differences in cAMP synthesis and degradation. We assessed differences in synthesis (ADCY activity) by treating male and female with the pan-ADCY activator, forskolin (FSK), in the presence of complete inhibition of cAMP degradation by the pan-phosphodiesterase (PDE) inhibitor, IBMX (Figure 2A). Under these conditions, differences in cAMP levels reflect differences in synthetic capacity and not differences in degradation. Cyclic AMP levels rose to 650 and 900 pmol/mg protein in male and female *Nf1-/-* astrocytes, respectively, indicating greater cAMP synthetic capacity in female *Nf1-/-* astrocytes.

To detect sex differences in degradative (PDE) capacity, we treated with FSK alone (Figure 2B). Under these conditions cAMP levels are determined by total ADCY capacity and counter-regulatory increases in PDE activity. Cyclic AMP levels reached a plateau at approximately six-fold and three-fold above baseline in female and male astrocytes, respectively, indicating male astrocytes have greater capacity to upregulate PDE activity.

The SNP array data suggested that variation in ADCY activity has a sexually dimorphic effect on glioma risk. Previously, we showed that ADCY inhibition with dideoxyadenosine (DDA) promotes astrocyte growth (5). Here, we looked for sex differences in DDA effects. We found that, paralleling the human data, inhibition of ADCY activity promoted growth of female astrocytes but suppressed the growth of male astrocytes (Figure 2C).

The effect of DDA on *Nf1-/-* astrocytes was previously shown to phenocopy the growth-promoting effects of CXCL12 (5). Here, CXCL12 treatment suppressed cAMP levels in both male and female *Nf1-/-* astrocytes (Figure 2D), but only the female astrocytes exhibited a growth response (Figure 2E). Together these observations identify sex differences in the growth promoting effects of ADCY inhibition and cAMP suppression.

Discussion

Sex is a significant determinant of many human diseases (15) and has been shown to interact with genetic modifier loci to determine risk in a mouse model of high–grade glioma associated with combined loss of *Nf1* and *p53* (16, 17). This however, is the first study to confirm a role for cAMP regulation in human gliomagenesis and to report that cAMP's effect is modified by sex.

Two lines of evidence suggest that sexually dimorphic growth responses to ADCY activity are relevant. First, inhibition of ADCY by DDA had opposing effects on the growth of female and male *Nf1-/-* astrocytes. Second, despite comparable suppression of cAMP levels, CXCL12 promoted the growth of female but not male astrocytes. While not demonstrated to

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specifically involve ADCY8, the close parallel between these results and the effect of polymorphisms in *ADCY8* on glioma risk in males and females with NF1 suggest that these mechanisms are relevant to human disease and that this *Nf1 GEM* will be an important model for studying sexual dimorphism in the cAMP pathway.

Despite dramatically opposing effects of *ADCY8* SNPs on OPG risk in males and females, there is little sex disparity in OPG incidence. Thus, we hypothesize that OPGs that arise through variation in ADCY may represent a subset of disease that is more common in females. Sex disparities limited to molecular subsets of brain tumors are established in other childhood brain tumors like medulloblastoma (18).

Finally, these observations provide a rationale for clinical evaluation of personalized glioma surveillance in NF1 using SNP-based tools to identify those at the greatest risk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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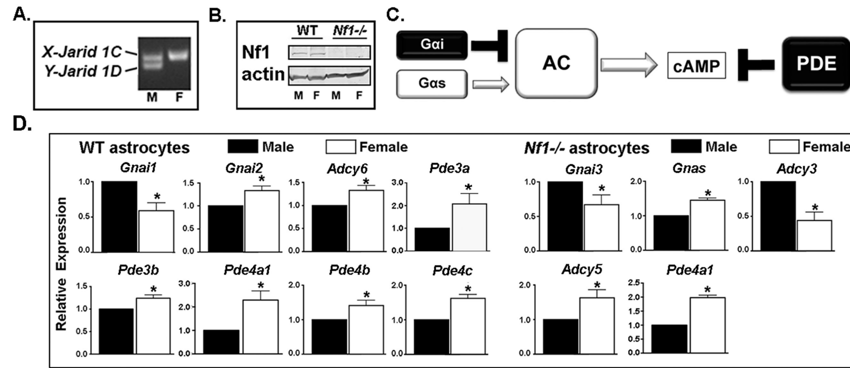

Figure 1. Sex differences in cAMP regulator expression in *WT* and *Nf1-/-* astrocytes

(**A**) Neonatal mice were sexed by *Jarid 1C/Jarid 1D* PCR. “M” indicates male and “F” indicates female derived samples. (**B**) Western blot analysis confirmed loss of neurofibromin expression in *Nf1-/-* astrocytes. Actin serves as loading control. “M” indicates male and “F” indicates female derived samples. (**C**) Schematic of cAMP regulation indicating the different families of regulators whose expression was evaluated. (**D**) Significant differences in expression were detected for cAMP regulators in *WT* and *Nf1-/-* astrocytes as indicated. Shown are the mean and SEM of expression in female cells relative to expression in male cells from 3-5 separate litters per genotype. *p 0.05, as detailed in the text.

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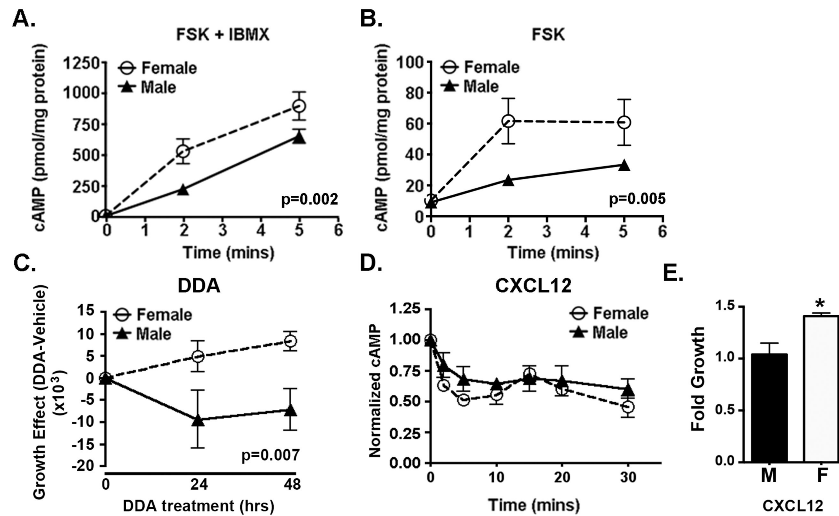

Figure 2. Sexual dimorphism in cAMP regulation in *Nf1-/-* astrocytes

Cyclic AMP levels were measured by ELISA in male and female *Nf1-/-* astrocytes treated with (**A**) the ADCY activator, FSK (10 μM) and the pan-PDE inhibitor, IBMX (1 mM), or (**B**) FSK (10 μM) alone for the times indicated. Shown are the means and SEM of cAMP levels from four independent experiments. P values are as indicated and were determined by two way ANOVA. (**C**) Cell number was measured by Trypan Blue exclusion in male and female *Nf1-/-* astrocytes treated with vehicle or dideoxyadenosine (DDA, 100 μM). Shown are the means and SEM of the differences between DDA and vehicle treated male and female cultures derived from three independent experiments. P value is as indicated and was determined by two way ANOVA. (**D**) Cyclic AMP levels were measured by ELISA in male and female *Nf1-/-* astrocytes treated with CXCL12 (0.1μg/ml) for the times indicated. Shown are the means and SEM of three independent experiments measuring the responses of male and female *Nf1-/-* astrocytes normalized to their basal values. (**E**) Cell number was measured by Trypan Blue exclusion in male and female *Nf1-/-* astrocytes treated with vehicle or CXCL12 (0.1μg/ml, 48 hrs). Shown are the means and SEM of the ratio of cell numbers in CXCL12-treated/vehicle-treated cultures derived from three independent experiments. *=$p<0.05$ as determined by two tailed t-test.

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Table 1

SNPs with significant association with optic glioma risk in individuals with NF1

	SNP	rs724365	rs4736688	rs1435446	rs2568554	rs16952813
SNP identifiers	**Gene Symbol**	*ADCY8*	*ADCY8*	*ADCY8*	*CXCR7*	*ADCYAP1*
	Chr	8	8	8	2	18
Major Allele frequency (MAF)	**Population**[*]	0.263	0.39	0.241	0.2	0.14
	NF1 dataset	0.242	0.361	0.239	0.11	0.102
Odds Ratio (OR)	**Female**	4.09	2.59	3.13	4.79	1.88
	Male	0.31	0.19	0.31	0.31	0.1
Male/Female OR	**Estimate**	0.0746	0.07474	0.0982	0.0638	0.0524
	95% CI	0.0233~0.239	0.0227~0.2457	0.0313~0.3076	0.0138~0.296	0.0088~0.3128
	LR P[+]	6.35E-06	9.31E-06	4.33E-05	0.0002	0.0002
	Permutated LR P[&]	0.018	0.03	0.138	0.446	0.472
	FDR LR P[&]	0.014	0.014	0.043	0.102	0.102
SNP ranking	**LR P**	5	9	40	177	185
	FDR LR P	4	4	26	99	99

[*] MAF in the population was determined in 1093 total samples from the 1000 Genome phase I data, including AFRICA (N=246), EUROPE (N=380), EAST ASIA (N=286), AMERICA (N=181).

[+] LR P was derived from likelihood ratio (LR) test on the SNPxSex interaction term in logistic regression models.

[&] Permutation and FDR adjustment was separately conducted on the SNPs on the cAMP pathway.

[#] SNP ranking based on raw LR P and FDR-adjusted LR P (on all SNPs) compared the performance of each SNP in the cAMP pathway to all SNPs on the array.

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Table 2

Frequency of Optic Pathway Gliomas in Males and Females with NF1

Study	Location	Total	M	F
Listernick *et al.* (1995)	Chicago, IL	17	5	12
Chateil *et al.* (2001	Cedex, FRA	14	7	7
Czyzyk *et al.* (2003)	Warsaw, POL	51	19	32
Thiagalingam *et al.* (2004)	Sydney, AUS	54	27	27
Blazo et al. (2004)	TX	24	10	14
Pascual-Castroviejo I *et al.* (2008)	Madrid, SPN	80	22	58
Nicolin *et al.* (2009)	Toronto, CA	78	48	30
Segal *et al.* (2010)	Montreal, CN	44	24	20
de Blank *et al.* (2013)	Philadelphia, PA	50	25	25
Incecik *et al.* (2013)	Turkey	9	5	4
Gooden *et al.* (2014)	Liverpool, UK	19	11	8
Kalin-Hajdu *et al.* (2014)	Montreal, CA	7	3	4
Diggs-Andrews *et al.* (2014)	St Louis, MO	96	40	56
Total %		**543**	**246 (45%)**	**297 55%)**