The Cyclic AMP Pathway Is a Sex-Specific Modifier of Glioma Risk in Type I Neurofibromatosis Patients

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Abstract

Identifying modifiers of glioma risk in patients with type I 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 cyclase gene adenylate cyclase 8 (ADCY8) that 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. Cancer Res; 75(1); 16–21. ©2014 AACR.

Introduction

Neurofibromatosis type I (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 have shown that alterations in cAMP levels could vary the stereotypical pattern of OPG formation, and that pharmacologic elevation of cAMP levels could block OPG growth in an established genetically engineered mouse (GEM) model of NF1-associated OPG (5–7). 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

Animal studies

Animals were used in accordance with an Animal Studies Protocol (#20121074) 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 (NIH, Rockville, MD).

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

Chemicals, reagents, and antibodies
All chemicals were obtained from Sigma-Aldrich 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 (St. Louis, MO), the University of Toronto (Toronto, ON, Canada), University of Utah (Salt Lake City, UT), and New York University (New York, NY). 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, and fluid filled optic nerve sheaths did not qualify as OPGs (8). Patients without OPG had negative MRIs obtained after the age of 10 years. DNA was extracted from blood using Qiagen DNA Blood Mini Kits and from saliva using DNA Genotek Oragene DNA kits according to the manufacturers’ instructions. Following quality checks and concentration optimization, DNA was hybridized to Affymetrix 6.0 single-nucleotide polymorphism (SNP) microarrays at The Genome Institute, Washington University or ARUP, Salt Lake City, UT. 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 arraymrix 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 \( P \leq 1 \times 10^{-06} \) or missingness test \( P < 0.1 \) or with a major allele frequency (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 four 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 base pairs in the sliding window = 100k; 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 (OR) 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 that was obtained by comparing the full logistic regression model with 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 \( P \) values 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 permuted \( P \) value ≤ the original LR \( P \) value corresponding to the nonpermuted status. LD analysis was conducted using PLINK and the LD measure \( R^2 \) was reported. MAF for SNPs in the general population was determined using 1,093 total samples in the 1000 Genome phase 1 data released on May 2011 using ENGINEs (SPSmart version 5.1.1 and dbSNP build 132; ref. 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\(^{flx/flx}\), GFAP-Cre) mice at postnatal day 1 to 2 as described previously (5). The sex of the newborn mice was determined by Jarid 1C/Jarid 1D PCR. Astrocytes of the same sex were combined and cryopreserved. Wild-type (WT) astrocytes were similarly derived from neonatal Nf1\(^{flx/flx}\) mice. Western blot analysis for neurofibromin expression was performed by standard methods using rabbit anti-NF1 antibody (1:200; Santa Cruz Biotechnology), 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 four 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). 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) using primers as indicated in Supplementary Table S3. Triplicate measures were made for each sample and corresponding GAPDH control. PCR and data collection were done using the Bio-Rad MiniOpticon Real-Time PCR machine and Opticon Monitor 3 Software from Bio-Rad. 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 hours), and then treated with the ADCY activator, forskolin (FSK; 10 \( \mu \)mol/L), and the phosphodiesterase (PDE) inhibitor, IBMX (1 mmol/L), 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 \( \mu \)mol/L) or CXCL12 (0.1 mg/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)
according to the manufacturer's instructions and as previously described (5).

Statistical analysis
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 \( P < 0.05 \) was considered statistically significant.

Results
DNA samples were obtained from 243 individuals with NF1 and genotyped using Affymetrix whole-genome human SNP array 6.0. Two hundred and thirty six specimens, 123 from individuals and genotyped using Affymetrix whole-genome human SNP array were medium with both dSNP 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. cPermutation and FDR adjustment was separately conducted on the SNPs on the cAMP pathway.

bLR \( P \) was derived from LR test on the SNP

cMAF in the population was determined in 1,093 total samples from the 1000 Genome phase I data, including AFRICA (\( N = 246 \)), EUROPE (\( N = 380 \)), EAST ASIA (\( N = 286 \)), and AMERICA (\( N = 181 \)).

dThe 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 toward 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 postnatal day 1 forebrain astrocytes from WT and Nf1fl/fl/lox/lox, GFAP\(^{lox/lox}\) (\( Nf1^−/− \)) mice based on expression of X and Y chromosome–encoded paralogs \( Jarid 1C \) and \( Jarid 1D \) (Fig. 1A; ref. 14) and verified equivalent deletion of neurofibromin (Fig. 1B).

We first looked for sex differences in cAMP regulator expression (Fig. 1C) and in intracellular cAMP levels. Although 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 (Fig. 1D). Intracellular cAMP levels were consistently lower in male compared with 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, FSK, in the presence of complete inhibition of cAMP degradation by the pan-PDE inhibitor, IBMX (Fig. 2A). Under these conditions, sex differences in cAMP levels were no longer observed (Fig. 2B). Both male and female astrocytes showed equivalent maximal cAMP levels, indicating that sex differences in cAMP activity were due to sex differences in cAMP breakdown. A sex difference in cAMP activity occurs in the postnatal day 1 astrocytes (Fig. 2C). Relative to females, sex differences in cAMP activity were observed for the first time in NF1 astrocytes (Fig. 2D).

Our analysis focused on 2,761 unique SNPs in 22 key regulators of intracellular cAMP levels (Supplementary Table S2). Calculations for OR for glioma between genotypes within males and females as sex differences in glioma-associated symptoms have been reported (13).

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.

<table>
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<th>Table 1. SNPs with significant association with optic glioma risk in individuals with NF1</th>
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\( ^6 \)MAF in the population was determined in 1,093 total samples from the 1000 Genome phase I data, including AFRICA (\( N = 246 \)), EUROPE (\( N = 380 \)), EAST ASIA (\( N = 286 \)), and AMERICA (\( N = 181 \)).

\( ^7 \)LR \( P \) was derived from LR test on the SNP × Sex interaction term in logistic regression models.

\( ^8 \)Permutation and FDR adjustment was separately conducted on the SNPs on the cAMP pathway.

\( ^9 \)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.
conditions, differences in cAMP levels reflect differences in synthetic capacity and not differences in degradation. Cyclic AMP levels increased to 650 and 900 pmol/mg protein in male and female Nf1/C0/C0/C0 astrocytes, respectively, indicating greater cAMP synthetic capacity in female Nf1/C0/C0/C0 astrocytes.

To detect sex differences in degradative (PDE) capacity, we treated with FSK alone (Fig. 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 6-fold and 3-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 have shown that ADCY inhibition with 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 (Fig. 2C).

The effect of DDA on Nf1/C0/C0/C0 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/C0/C0/C0 astrocytes (Fig. 2D), but only the female astrocytes exhibited a growth response (Fig. 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 astrocytes.
Sexual dimorphism in cAMP regulation in Nf1−/− astrocytes. Cyclic AMP levels were measured by ELISA in male and female Nf1−/− astrocytes treated with the ADCY activator, FSK (10 μmol/L), and the pan-PDE inhibitor, IBMX (1 mmol/L; A), or FSK (10 μmol/L; B) 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 the two-way ANOVA. C, cell number was measured by Trypan blue exclusion in male and female Nf1−/− astrocytes treated with vehicle or DDA (100 μmol/L). 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 the two-way ANOVA. D, cyclic AMP levels were measured by ELISA in male and female Nf1−/− astrocytes treated with CXCL12 (0.1 μg/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 (0.1 μg/mL; 48 hours). 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 the two-tailed t test.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N.M. Warrington, T. Sun, J. Luo, R.C. McKinstry, S. Ganzhorn, T.E. Druley, U. Tabori, D.H. Gutmann, J.B. Rubin
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.B. Rubin
Study supervision: J.B. Rubin

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Disclosure of Potential Conflicts of Interest
T.E. Druley is a consultant/advisory board member for Alex’s Lemonade Stand Foundation Scientific Advisory Board. No potential conflicts of interest were disclosed by the other authors.

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Figure 2.
Sexual dimorphism in cAMP regulation in Nf1−/− astrocytes. Second, despite comparable suppression of cAMP levels, CXCL12 stimulated the growth of female but not male astrocytes. Although not demonstrated to 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.

Disclosure of Potential Conflicts of Interest
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Sexually Dimorphic cAMP Signaling in Gliomas

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