Central Precocious Puberty due to Hypothalamic Hamartomas Correlates with Anatomic Features but Not with Expression of GnRH, TGFβ, or KISS1

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Results: Hypothalamic hamartomas associated with CPP were more likely to contact the infundibulum or tuber cinereum and were larger than hamartomas not associated with CPP. GnRH, TGFβ, and GnRHR were expressed by all hamartomas studied. Expression of KISS1, GPR54, and GRM1A did not differ significantly between hamartomas associated and not associated with CPP. Conclusion: Anatomic features rather than expression patterns of candidate molecules distinguish hypothalamic hamartomas that are associated with CPP from those that are not. Copyright © 2010 S. Karger AG, Basel

Introduction

Central precocious puberty (CPP) is due to premature activation of the hypothalamic-pituitary-gonadal (HPG) axis and can be caused by a variety of pathological condi-
Precocious Puberty and Hypothalamic Hamartomas

We have analyzed radiologic characteristics and examined the expression of KISS1, GnRH, TGFβ, and GRM1A in a sample of 18 hypothalamic hamartomas resected from patients with and without a history of CPP, the largest expression study of hamartoma samples reported to date. We further examined the expression of the GnRH receptor (GnRHR) and GPR54 to determine whether signaling by GnRH or kisspeptin within hypothalamic hamartomas might have a role in causing CPP.

Materials and Methods

Patients

Patients underwent resection of hypothalamic hamartomas at the Barrow Neurological Institute of St. Joseph’s Hospital and Medical Center due to intractable seizures [19, 20]. Patients were considered to have CPP if medical records noted sexual development and elevated gonadotropin levels before 8 (girls) or 9 (boys) years of age. Patients considered not to have CPP were at least 8 (girls) or 9 (boys) years old and had no history of CPP. All patients had medically refractory epilepsy, half had mental retardation, and patient HH2013 had features of oro-facial-digital syndrome type II (Online Mendelian Inheritance in Man 252100). All studies were approved by the Institutional Review Board of the Barrow Neurological Institute, informed consent was obtained for all subjects, and a waiver for use of de-identified tissue was granted by the Institutional Review Board of Massachusetts General Hospital.

Radiologic Characterization

All magnetic resonance imaging was evaluated by a neuroradiologist (E.C.P.) who was blind to the diagnosis of CPP. Hamartomas were typed using the classification of Delalande and Fohlen [21], with a type 4 (‘giant’) hamartoma defined as having a volume >8 cm³. Volumes of hypothalamic hamartomas were determined by summing manually measured areas, multiplied by slice thickness, on sequential, contiguous, coronal T2-weighted images using Voxar 3D imaging software (DR Systems) [20]. Maximal diameter was the largest of the three diameters measured in each orthogonal plane (X-Y-Z). Hamartomas were also scored for the presence or absence of contact with the tuber cinereum and/or infundibulum, and whether the site of attachment to the hypothalamus was unilateral or bilateral.

Sample Collection and Preparation

After surgical resection, pieces of hamartoma tissue were immediately immersed in RNAlater (Applied Biosystems) and frozen for expression profiling, or fixed in 10% formalin overnight, embedded in paraffin, and sectioned at 5 μm. Due to limited tissue quantity, not all hamartomas were used for all analyses.

Immunohistochemistry

Immunostaining was performed using a Ventana Benchmark XT automated immunostainer (Ventana Medical Systems, Tucson, Ariz., USA) per the manufacturer’s protocol with pro-
priental antibodies. Ventana antigen retrieval was performed for all sections. Each section was stained with a single primary antibody and counterstained with hematoxylin. Primary antibodies used were polyclonal GnRH (1:500; Abcam); GnRH-R (clone A9E4) mouse monoclonal antibody (1:50, Cell Sciences); and TGFα rabbit polyclonal antibody (RB-9241, Lab Vision Corp.).

To confirm the TGFα staining pattern, the TGFα mouse monoclonal antibody Ab-1 (Clone MF9, Lab Vision Corp.) was used on the same cohort. Sections were exposed to biotinylated secondary antibodies (anti-rabbit IgG or anti-mouse IgG; 1:200) and the reaction product was visualized with standard diaminobenzidine protocols (Vector Laboratories). Negative control groups included tissue that did not express the antigen and antigen-positive sections that were processed without primary antibodies. No significant labeling was detected under the negative control conditions. Cell types were readily distinguishable by their morphology [22], and neurons ≥20 μm in diameter were classified as ‘large’.

Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from 2–25 mg of frozen hamartoma tissue using the RNeasy Lipid kit (Qiagen), and first-strand cDNA synthesis was performed with an oligo-dT primer using the SuperScript® III First-Strand Synthesis System (Invitrogen). Total human hypothalamic RNA (Ambion) was used as a positive control. Quantitative real-time PCR was performed using the TaqMan PCR Master Mix (Applied Biosystems) with primers at 400 nM final concentration; primer sequences are listed in online supplementary table 1 (www.karger.com/doi/10.1159/000308162). Reactions were run on an Mx4000 Quantitative PCR System (Stratagene) with 40–50 cycles of denaturation at 94°C for 30 s and annealing and elongation at 58°C for 1 min, and the cycle threshold (Ct) was determined using the accompanying software. Amplification of KISS1, GPR54, GRM1 (using a probe specific for the α isoform), and GAPDH was performed in triplicate. Quantitative RT-PCR performed on reactions from which reverse transcriptase was omitted did not produce a detectable signal (not shown). To determine relative expression of these genes, control cDNA was serially diluted and used to produce standard curves, which were used to convert average Cts from hamartoma samples to a fold difference in mRNA abundance relative to the control RNA sample; these differences were in turn normalized to the fold difference for GAPDH relative to control.

Statistics

Fisher’s two-tailed exact test was used to compare presence or absence of attachment to the tuber cinereum/infundibulum, bilateral attachment, and expression of KISS1 and GPR54 between hypothalamic hamartomas associated and not associated with CPP. Two-tailed t tests were used to compare numerical radiologic parameters and GRM1A expression levels. Hamartoma volumes and diameters and GRM1A mRNA expression levels were log transformed for analysis, as these did not exhibit normal distributions by the kurtosis/skew normality test. Linear and rank-order correlation analyses were used to compare age of puberty onset with hamartoma volume. Statistics were calculated using GraphPad Prism 4 for Windows.

Results

Clinical and Radiologic Characteristics

Characteristics of patients from whom hypothalamic hamartoma tissue was resected are summarized in table 1. In this cohort of patients with refractory epilepsy, all types of hamartomas (using the classification of Deland and Fohlen [21]) were found in patients both with and without a history of CPP. All 7 hamartomas in patients with CPP were found to contact the tuber cinereum and/or infundibulum (fig. 1; table 1), hypothalamic regions that contain axonal projections from endogenous GnRH neurons. In contrast, only 2 of 11 hamartomas in patients without CPP contacted these structures (p = 0.002).

Hypothalamic hamartomas associated with CPP had significantly larger volumes (geometric mean 6.6 cm³, 95% confidence interval [CI] 2.7–15.9 cm³) than those not associated with CPP (geometric mean 1.1 cm³, 95% CI 0.5–2.2 cm³, p = 0.002). They also had significantly larger maximal diameters (geometric mean 95% CI 20.2 mm [14.8–27.6] in CPP group, 11.7 [8.9–15.3] in no CPP group, p = 0.008). Hamartomas associated with CPP were more likely to have bilateral attachment to the hypothalamus (p = 0.01); differences in the size of the base of attachment were not statistically significant (mean ± SEM 1.26 ± 0.25 vs. 0.74 ± 0.13 mm², p = 0.1). There was no correlation between hamartoma volume and age at diagnosis of precocious puberty (p ≥ 0.86 by linear and rank-order correlation analyses).

Expression of GnRH

GnRH expression was detected in all hypothalamic hamartomas, both those associated and those not associated with CPP (n = 13; table 2). Moderate immunostaining was present in a predominantly nuclear pattern in ~50% of small neurons (<20 μm diameter) and ~70% of large neurons (fig. 2a); large neurons also exhibited staining in cell bodies. Weak glial staining was observed in two samples (data not shown). The neuropil also showed weak to moderate immunoreactivity in all cases.

Expression of TGFα

TGFα expression was also observed in all hypothalamic hamartomas studied (n = 14; table 2). About half of neurons, both large and small, exhibited immunoreactivity primarily in cell bodies (fig. 2b). Nearly all glial cells exhibited strong immunoreactivity in both cell-body and nuclear patterns. The neuropil exhibited little
to no staining. Because the nuclear staining pattern was unexpected, immunostaining was repeated and confirmed with a second antibody against TGFβ1 (data not shown).

Expression of the GnRH Receptor
Like GnRH and TGFβ1, the GnRH receptor was expressed in all hypothalamic hamartoma tissues studied (n = 13; table 2; fig. 2c), largely in the cell bodies of both large and small neurons. No staining was seen in glial cells, and moderate neuropil or background staining was present in all samples.

Expression of KISS1 and GPR54
Due to the lack of availability of antibodies against human kisspeptin, we examined expression of KISS1 by reverse transcription-polymerase chain reaction (RT-PCR). Quantitative RT-PCR of cDNA prepared from hamartoma tissue revealed expression of KISS1 mRNA in 1 of 7 samples from children with CPP and 1 of 11 samples from patients with no history of CPP (table 2; p = 1 by Fisher’s exact test). We also examined expression of GPR54 in hypothalamic hamartomas using RT-PCR. Like KISS1, GPR54 message was detectable in only some hamartomas and was seen in hamartomas that were both associated and not associated with CPP (table 2; p = 0.32).

Expression of GRM1A
We examined GRM1A expression in our cohort by quantitative RT-PCR. GRM1A levels were higher in the CPP group, but this difference was not statistically significant (table 2; geometric mean [95% CI] of expression relative to control 0.61 [0.13–2.90] in CPP group, 0.19 [0.08–0.45] in no CPP group, p = 0.1).

### Table 1. Clinical and radiologic characteristics

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<th>Patient</th>
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<th>Age at puberty years</th>
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<th>Maximal diameter mm</th>
<th>Area of attachment base, mm²</th>
<th>Volume cm³</th>
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</table>

CPP = Central precocious puberty; N/A = not available; L = left; R = right; B = bilateral. Hamartoma type is according to the classification of Delalande and Fohlen [21]: type 1, subhypothalamic; type 2, intraventricular; type 3, both subhypothalamic and intraventricular; type 4, ‘giant’ hamartoma, here defined as volume >8 cm³.

a Voice change. b Breast development. c Menarche.
Discussion

To our knowledge this is the largest series of hypothalamic hamartomas studied for expression of potential stimulatory factors that may contribute to the pathogenesis of CPP, namely, GnRH, GnRHR, TGFβ, and KISS1, GPR54, and GRM1A. Our principal findings are: (1) association of CPP with larger hamartoma size and contact with critical hypothalamic structures (the infundibulum and tuber cinereum), consistent with previous studies of patients with hypothalamic hamartomas and seizures [23, 24]; (2) expression of GnRH and TGFβ in all hamar-
hamartomas analyzed, regardless of the patient’s history of CPP; and (3) limited expression of KISS1 and GPR54 that also did not correlate with CPP.

Our results confirm and extend the findings in a recent study by Parent and Matagne et al. [18] of five hypothalamic hamartomas, two from patients with CPP. Expression of GNRH1 and TGFA was seen in all five hamartomas, KISS1 expression in none, and GPR54 expression in one of the two hamartomas associated with CPP and two of the three hamartomas not associated with CPP. Our collective results indicate that, despite their potent stimulatory properties, expression of GnRH, TGFFs, or kispeptin alone is not sufficient for a hypothalamic hamartoma to cause CPP. Indeed, the limited expression of KISS1 and GPR54 in our series suggests that kispeptin signaling is unlikely to contribute to the pathogenesis of CPP in most cases.

Rather, we found that anatomic features better distinguished hamartomas that were associated with CPP from those that were not. Both larger size and contact with the infundibulum/tuber cinereum were associated with CPP in our series. However, large hamartoma size does not appear to be a prerequisite for causing CPP, as other series have reported small hypothalamic hamartomas in patients with CPP. In particular, Debeneix et al. [25] found that patients with CPP without seizures (a population not studied in our cohort) had smaller hamartomas than patients with seizures, whether with or without CPP. Together, these findings suggest that a hamartoma’s anatomic contacts may determine whether it causes CPP, and in patients with seizures larger hamartomas are more likely to have these critical contacts.

We identified two large hamartomas that contacted the infundibulum/tuber cinereum were associated with CPP in our series. However, large hamartoma size does not appear to be a prerequisite for causing CPP, as other series have reported small hypothalamic hamartomas in patients with CPP. In particular, Debeneix et al. [25] found that patients with CPP without seizures (a population not studied in our cohort) had smaller hamartomas than patients with seizures, whether with or without CPP. Together, these findings suggest that a hamartoma’s anatomic contacts may determine whether it causes CPP, and in patients with seizures larger hamartomas are more likely to have these critical contacts.

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nically, it is also possible that normal tissue may have been present in some hamartoma samples, resulting in inaccurate measurement of GRM1A expression. Further examination of GRM1A expression in hypothalamic hamartomas will help to elucidate its role in the pathogenesis of CPP.

As stated earlier, hypothalamic hamartomas have been hypothesized to cause CPP by stimulating the HPG axis or by interfering with inhibitory pathways that restrain the HPG axis in childhood. While our study focused on stimulatory factors, our results do not exclude the latter possibility. Indeed, patients have been described to develop CPP after resection of their hypothalamic hamartomas, even when the resection appeared to be complete [e.g., 18]. The emergence of CPP after surgery could be due to surgical disruption of inhibitory pathways. Alternatively, posttraumatic activation of glial cells could lead to production of stimulatory factors such as TGFα [14].

In summary, we have used a unique collection of hypothalamic hamartoma tissue to examine expression of molecules that activate the reproductive endocrine axis. Contrary to our initial hypothesis that these molecules would be expressed exclusively in hamartomas associated with CPP, we found no correlation between CPP and expression of these molecules. Instead, we found significant correlation between anatomic features of the hamartomas and CPP. As we enter the era of gene-expression profiling for the prognosis and treatment of tumors [26], our results serve as a reminder that anatomic and functional characteristics remain important determinants of the clinical consequences of masses, particularly in the brain. Based on histopathological [22], ultrastructural [27], electrophysiological [19, 28], and now endocrine characterization of hypothalamic hamartomas resected at the Barrow Neurological Institute, we believe that the clinical consequences of these lesions are not simply due to mass effects. Rather, hypothalamic hamartomas are intrinsically active masses, with their effects determined by functional connections with critical brain regions: the tuber cinereum/infundibulum to cause precocious puberty and other regions, possibly the mammillary bodies [24], to cause seizures.

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