incorrect p values whenever allele frequencies are unequal. If we denote \( P_{\text{DOST}}(\xi) \) as the value of \( P_{\text{DOST}} \) associated with sample-configuration \( \xi \), we can guarantee that, under the null hypothesis of HWE, \( P_{\text{DOST}}(\xi) \leq P_{\text{PHWE}}(\xi) \) whenever allele frequencies are unequal. In contrast, using \( P_{\text{PHWE}} \), \( P_{\text{low}} \), \( P_{\text{high}} \) guarantees a properly calibrated test statistic so that, for example, \( P_{\text{PHWE}} \leq P_{\text{HWE}}(\xi) \) regardless of allele frequency.

A simple example is illustrative. Consider a sample of 100 individuals in whom two copies of the rare allele are present. Two configurations are possible, one with two heterozygotes and another with a single rare allele homozygote. The first configuration has probability of \( 198/199 \), and the second has a probability of \( 1/199 \). Suppose a single homozygote is observed. This gives a \( \chi^2 \) test p value of \( \frac{1}{200} \) (without continuity correction) or \( < 10^{-6} \) (with continuity correction). Both are clearly wrong. Using \( P_{\text{DOST}} \) is "better," giving \( p = 2/199 \), but still incorrect. In contrast, \( P_{\text{PHWE}} \) correctly specifies that a configuration such as this occurs with \( p = 1/199 \). For rare alleles and unlikely configurations, \( P_{\text{DOST}} \) and \( P_{2a} \) are effectively equal to \( 2P_{\text{PHWE}} \). For common alleles and large samples, the three statistics converge to more similar values, but, in those settings, \( \chi^2 \) test approximations can be conveniently used.

The fact that \( P_{\text{DOST}} \) and \( P_{2a} \) detect fewer departures from HWE is not a virtue. It simply reflects that they are poorly calibrated statistics. If an investigator wishes to discard fewer SNPs due to HWE departures, it is more appropriate to lower \( \alpha \), the threshold for rejection. Just as we found no reason to recommend \( P_{2a} \) originally, we find no reason to recommend \( P_{\text{DOST}} \) now.

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Tuba8 Is Expressed at Low Levels in the Developing Mouse and Human Brain

To the Editor: Sheridan and colleagues recently reported that mutations in the tubulin gene TUBA8 result in polymicrogyria with optic nerve hypoplasia (PMGOH [MIM 613180]).¹ This conclusion is based on the mapping of two consanguineous families of Pakistani origin to a 7.42 Mb region on chromosome 22q11.2 that contains ~230 genes including TUBA8. Drawing on our previous finding that mutations in TUBA1A cause lissencephaly² and that mutations in TUBB2B cause asymmetric polymicrogyria,³ Sheridan and colleagues sequenced TUBA8 and found a 14 bp deletion in intron 1 that affects splicing. They provide further evidence that TUBA8 is involved in the disease state by analyzing its expression in the developing mouse brain by in situ hybridization. They report that Tuba8 is widely expressed in developing neural structures, with strongest expression in the cortical plate at E15.5 and E18.5 and in the cortical plate, subplate, and hippocampus at P0.

A meaningful analysis of individual tubulin gene expression by in situ hybridization requires the use of probes that avoid cross-hybridization among the highly conserved coding regions, relying exclusively on either the variant 5' or 3' untranslated regions. The probe employed by Sheridan and colleagues was 443 nucleotides in length, of which 415 correspond to sequences contained within the conserved coding region. Consequently, this probe shares a very high sequence homology with other \( \alpha \)-tubulins.⁴ An Ensembl BLAST search with the Sheridan probe against total mouse cDNA results in six other hits, each being at least 300 nucleotides in length with at least 80% sequence identity. Each of these hits corresponds to one of the six other members of the \( \alpha \)-tubulin family and includes a 374 nucleotide stretch that shares 84.2% identity with the coding sequence of Tuba1a, a gene that is highly expressed in the developing CNS.⁵

To establish whether the results reported by Sheridan and colleagues are a consequence of cross-hybridization, we conducted in situ hybridization on the developing (E14.5, E16.5, and P0) and adult mouse brain employing their probe and two others that we designed. We first confirmed the sequence of Tuba8 mRNA by amplifying

The American Journal of Human Genetics 86, 813–823, May 14, 2010 819
and cloning it from a C57BL/6 mouse adult olfactory bulb cDNA library (AK032157, GU591980) (Figure S1 available online). Next we designed two probes, a short probe (224 base pairs in length [nt 1367–1911]) and a long probe (545 base pairs in length [nt 1367–1911]) targeted to the highly variant 3' UTR of Tuba8. High levels of staining are observed in the developing mouse brain with the Sheridan probe (particularly in the cortical plate [CP] and to a lesser extent in the intermediate zone [IZ] and ventricular zone [VZ]), but none is apparent with probes that exclusively target the 3' UTR. We observed no staining when employing control sense probes (data not shown). Scale bars represent 50 μm. MZ indicates the marginal zone.

We compared the expression of Tuba8 to those tubulin genes (Tuba1a [MIM 611603], Tubb2b [MIM 610031], Tubb3 [MIM 600638]) that have been shown to cause distinct neurodevelopmental diseases. We found that Tuba1a, Tubb2b, and Tubb3 are highly expressed in the developing mouse brain from E12.5 to P6, whereas Tuba8 is expressed at low levels (Figure 2A). For instance, at E14.5, a peak time for neuronal migration, Tuba1a is expressed at ~94 times the control gene level, whereas Tuba8 is barely detectable, expressed at just 0.03 times the control gene level. We next analyzed expression levels of Tuba8 in different regions of the adult mouse brain by qPCR, again comparing it to Tuba1a, Tubb2b, and Tubb3 (Figure 2B). We observed regional differences in the expression levels of these genes, but generally Tuba1a was most highly expressed, followed by Tubb3 and Tubb2b. Tuba8 was again expressed at very low levels, with the exception of the olfactory bulbs and the cerebellum, where its modest level of expression was concordant with the results from our in situ hybridization experiments. We extended our expression analysis beyond the adult brain to the major adult organs in the mouse (Figure S2).

Because there is evidence for an alternatively spliced variant of Tuba8 with a shorter UTR, we confirmed our in situ hybridization results by undertaking real-time quantitative PCR (qPCR) with primers targeted to the coding region (Table S1). When designing intron-spanning primers for this task, we took care to ensure that each primer pair was specific, undertaking BLAST searches with multiple search engines (Ensemble, UCSC, and NCBI). In addition, we only used primer pairs with an efficiency between 95% and 105% that produced a melt curve indicative of a single product. To prepare templates for amplification, we dissected and flash froze embryonic brains (E10.5 [head], E12.5, E14.5, E16.5, E18.5), postnatal brains (P0, P6), various regions of the adult brain (8 weeks) and adult organs (C57BL/6), prior to RNA extraction (QIAGEN, 74804, 74704). Three independent RNA samples from each developmental time point, brain region, or organ were then quantitated and pooled for reverse transcription (Invitrogen, 45-0640). Quantitative PCR followed on a BioRad Cycler with SYBR green, alongside reverse transcriptase (RT) and cDNA negative controls. All reactions were performed in triplicate and normalized to the geometric mean of three reference genes (Pgk1, Tfic, and Hprt).8,9

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Finally, we investigated the expression of TUBA1A, TUBB2B, TUBB3, and TUBA8 in human fetal brain at GW13 (Biochain, C1244051) and GW22 (Biochain, C1244035), again employing three reference genes for normalization (HPRT, PGK1, TBP). Consistent with our mouse data, we observed very high levels of expression of TUBA1A, TUBB2B, and TUBB3, but only low levels of TUBA8 (Figure 2C). For instance, in the frontal lobe at GW13, a peak time for neuronal migration in humans,11 expression of TUBA1A is ~224 times the control level, whereas TUBA8 is expressed at just 0.18 times the control gene level. We replicated our qPCR results with alternative sets of primers (in both mouse and human), confirming that our qPCR results are not due to alternative splicing (data not shown).

The data presented here show that Tuba8 is expressed at a low level in the developing mouse and human brain. This raises a puzzling question: How is it that a gene that is preferentially expressed in testis, heart, and skeletal muscle presents as a rare brain malformation? This question is particularly pertinent given that those tubulin genes known to cause neurodevelopmental disorders are highly expressed in the developing CNS, whereas Tuba8 is not. We suggest three possible explanations. First, it is plausible that an unidentified mutation lies in one of the 230 unsequenced genes in the 7.42 Mb candidate interval that contributes to, or is responsible for, the observed phenotype. Second, it is conceivable that despite its low expression level, Tuba8 serves some unique function that is essential to the developing brain and that is not vital in other tissues. Third, one might imagine a scenario in which the deletion described by Sheridan results in the expression of a truncated tubulin polypeptide and that this might confer some deleterious effect. The generation of appropriate transgenic mouse models or the identification of additional genetically unrelated patients harboring mutations in TUBA8 would help to resolve these issues.

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**Supplemental Data**

Supplemental Data include three figures and one table and can be found with this article online at http://www.cell.com/AJHG.
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Web Resources

The URL for data presented herein is as follows:


References


Response to Braun et al.

To the Editor: We note with interest the discordance between the TUBA8 (MIM 605742) expression data obtained by Keays et al. and those we previously presented.1 We accept that there is a possibility of cross-hybridization of the probes we used with other members of the a-tubulin family.

We suggest, however, that the tissue distribution of expression in the adult animal is of limited relevance when considering the pathogenesis of a neurodevelopmental phenotype. The high level of expression of TUBA8 in the adult testis has been previously noted. Although this may imply a specific role of TUBA8 in spermatogenesis, it does not imply the absence of an important role elsewhere. (We would also point out that the severe disability of the patients with homozygous TUBA8 mutations precludes any knowledge concerning their fertility or testicular histology.)

Keays et al. also draw a contrast between low brain expression of TUBA8 they observe and the much greater expression of TUBA1A (MIM 602529) and TUBB2B (MIM 612850), previously shown to be mutated in neurodevelopmental disorders. However, such a comparison implies very little, given that the disease-causing TUBA1A and TUBB2B mutations are de novo dominants, in contrast to the autosomal-recessive inheritance in our families.

There are many examples of apparent discordance between patterns of tissue-specific gene expression and the phenotype manifested when a gene is mutated. In some cases, these may reflect differential sensitivity of tissues to loss of gene product; neural tissues are notable in this regard. Mutations in the genes encoding the globally expressed pre-mRNA splicing factors PRPF8 (MIM 607300) and PRPF31 (MIM 606419), for example, result in a highly tissue-specific phenotype (retinitis pigmentosa) but no discernable phenotypic effects in other tissues in which they are highly expressed.2 Similarly, the distinctive neurodevelopmental phenotype, Rett syndrome, results from mutation of a ubiquitously expressed DNA binding protein, MeCP2 (MIM 300005).3 Other highly specific phenotypes associated with mutations of widely expressed genes include motor neuron disease (SOD1 [MIM 147450])4 and a-thalassaemia mental retardation (ATRX [MIM 300032]).5

In the patients we described, almost all TUBA8 transcripts are aberrantly spliced as a result of the 14 bp polypyrimidine tract mutation, in both lymphoblastoid cells and fibroblasts (unpublished data). The phenotype

822 The American Journal of Human Genetics 86, 813–823, May 14, 2010