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Absence of allelic loss in cytomegalic neurons of cortical tuber in the Eker rat model of tuberous sclerosis

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Abstract The Eker rat is an animal model of tuberous sclerosis caused by a mutation in the *Tsc2* gene encoding a tumor suppressor protein, tuberin. According to Knudson's two-hit theory, renal carcinomas and other tumors develop in various organs. Although the incidence of brain lesions is lower in the Eker rat than in human tuberous sclerosis, a cortical tuber was recently found in the cerebrum of an Eker carrier. In this study, we examined whether neuronal cytomegaly in the Eker rat tuber is caused by deletion of the normal *Tsc2* allele and resultant loss of tuberin, as is the case with the majority of renal carcinomas. A combination of laser capture microdissection and semi-nested polymerase chain reaction demonstrated the presence of the wild-type *Tsc2* allele in the cytomegalic neurons isolated individually. Immunohistochemistry also detected positive tuberin immunoreactivity in many of these giant neurons. These findings were in sharp contrast to those of renal carcinoma cells deriving from allelic loss. Our results provide evidence that many if not all cytomegalic neurons of a cortical tuber occur in the absence of allelic loss.

Keywords Cortical tuber · Immunohistochemistry · Laser capture microdissection · Loss of heterozygosity · Tuberous sclerosis

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Introduction

Tuberous sclerosis complex (TSC) is caused by a loss-of-function mutation in either of two tumor suppressor genes, *TSC1* (chromosome 9q34) or *TSC2* (chromosome 16p13.3) [6, 28]. TSC is characterized pathologically by the occurrence of multiple hamartomas, such as renal angiomyolipomas and cardiac rhabdomyomas, in various organs [9]. Malignant tumors, such as renal cell carcinomas, may also develop in a small number of TSC patients [1]. The development of these TSC-associated hamartomas and tumors is explained on the basis of Knudson's two-hit theory. Using DNA markers on chromosome 9q34 and 16p13.3, allelic loss, or loss of heterozygosity (LOH) involving either of the *TSC* gene loci, is demonstrated in many of the renal angiomyolipomas, cardiac rhabdomyomas and renal cell carcinomas [5, 10, 11, 12, 26]. By contrast, the incidence of LOH is very low in brain lesions, notably cortical tubers [12, 25]. Although there are a small number of tubers with positive evidence of LOH [4], the mechanism of the "second hit" by which most tubers develop may be different from that of cardiac and renal lesions. A technical issue concerning the LOH studies of cortical tubers is that allelic loss, if present only in the cytomegalic neurons and/or balloon cells, could be concealed due to contamination by a large number of normal-sized neurons and glial cells in the same tissue specimen. A method to overcome this problem is the DNA analysis of individual cells by laser capture microdissection, which has previously been applied for only a few human patients with TSC [4].

The Eker rat is a naturally occurring animal model of TSC caused by an insertion mutation in the *Tsc2* gene [18]. This rat has a much larger propensity to develop malignant tumors, such as renal cell carcinoma, than human TSC patients do [14]. Most of these tumors show LOH at the *Tsc2* gene locus [21, 32]. Despite the absence of neurological symptoms, the Eker rat occasionally has cerebral lesions, such as subcortical and subependymal hamartomas [33], some of which show evidence of LOH [13].

Recently, we found a cortical tuber in a carrier of the Eker mutation. Although rare, this lesion provides useful material for investigating the histogenesis of cortical tubers in human TSC [24]. Using laser capture microdissection, we conducted a DNA analysis of individual cytomegalic neurons to examine whether LOH is present in these cells. Taking advantage of the fact that the germline mutation ("first hit") in this animal model is already known, we could directly test for the presence of the wild- and mutant-type alleles by polymerase chain reaction (PCR).

Material and methods

Histology

Twenty-one Eker carriers ($Tsc2^{Ek/+}$) underwent necropsy at the age of 17–24 months. Eight non-carriers ($Tsc2^{+/+}$) served as controls. The brains of 19 carriers and 6 non-carriers, as well as the kidneys of 2 carriers and 2 non-carriers, were dissected out, cut coronally, fixed in 10% neutral formalin, and embedded in paraffin. Sections of 6-μm thickness were prepared, deparaffinized, and stained with routine histological stainings. A cortical tuber was found in the cerebrum of a 19-month-old carrier. Histologically, the tuber showed loss of normal cortical lamination and the presence of many cytomegalic neurons [24]. Renal carcinomas were found in all the carriers' kidneys examined, but in none of the non-carriers' kidneys.

Laser capture microdissection and DNA extraction

Sections of the cortical tuber were deparaffinized and stained with hematoxylin. Individual cells were collected using a LM200 laser capture microdissection system (Olympus, Tokyo, Japan). From each section of the tuber, about 10 cytomegalic neurons, as well as about 50 normal-sized neurons, were removed and transferred onto a thermoplastic film with a laser beam of 7.5 μm in diameter. Brain cells of a non-carrier and renal carcinoma cells of a carrier (about 50 cells each) were also isolated by the same methods, and served as controls.

DNA was extracted from each transfer film by successive addition of 5 μl of an alkaline solution (20 mM KOH/50 mM dithiothreitol; 65°C for 10 min), 5 μl of a neutralizing buffer (900 mM TRIS-HCl, pH 8.3, 300 mM KCl, 200 mM HCl) and 20 μl of a 1x PCR buffer containing 0.5 mg/ml proteinase K. The samples were subjected to heat treatment (50°C for 1 h, followed by 95°C for 10 min) immediately before use to inactivate proteinase K.

PCR

The *Tsc2* allele in the DNA samples was amplified by the semi-nested PCR method. For the first PCR, the following primer sequences were used: 5MFJ (5'-ACCATCAGGATGCTGCTGAA-3') as the forward primer; 3MFJ (5'-GAGCACACAAGCAGGCAAG-3') and TSR27 (5'-GCGCCAGATTCACCTCATTA-3') as the reverse primers [7]. The reaction mixture (50 μl) contained each sample (30 μl), the primers (40 pmol each), 1.25 mM dTNP, 1.5 mM $MgCl_2$, 10 mM TRIS-HCl pH 8.3, 50 mM KCl, and 1.25 U Taq DNA polymerase (Takara, Tokyo, Japan). The cycling conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The product of the first PCR was then concentrated and subjected to the second PCR with the following primer sequences: 5MFJ as the forward primer; Nest-wild-R (5'-AAGCAGGCAAGCTCAGGCATC-3') and Nest-mut-R (5'-CATACAGCTGTCGCACCGCT-3') as the reverse primers. The reaction conditions were the same as that of the first PCR. Amplified products were then electrophoresed on a 3% agarose gel, stained with ethidium bromide, and visualized using ultraviolet light.

Immunohistochemistry

Tuberin expression in the cortical tuber and renal carcinoma was immunohistochemically examined using two rabbit polyclonal antibodies, anti-Tub-NT and anti-Tub-CT, which were raised against subsequences of human tuberin [22, 23] and demonstrated to cross-react with rat tuberin [24]. Following microwave treatment at 90°C for 10 min, brain and kidney sections of carriers and non-carriers were immunostained by the biotin-streptavidin-peroxidase method, as described previously [23].

Results

LOH assay

From DNA of normal-sized neurons of an Eker carrier, two products of 151 bp (wild-type allele-specific) and 129 bp (mutant-type allele-specific) were amplified by the first PCR, as previously shown in the Eker kidney tissues [7]. Only the 151-bp product was obtained from DNA of a non-carrier neurons. The second PCR provided 141-bp and 119-bp products derived from the wild-type and mutant-type allele, respectively. From DNA extracted from cytomegalic neurons of a cortical tuber, amplification products of both 141 bp and 119 bp were obtained, as from DNA of normal-sized neurons. By contrast, PCR amplification of DNA extracted from renal carcinoma cells yielded only the 119-bp product, providing evidence for LOH (Fig. 1).

Immunohistochemistry

Immunostaining demonstrated that tuberin expression was low in the cerebrum but high in the kidney, in both the carriers and non-carriers. The two antibodies used, anti-Tub-NT and anti-Tub-CT, yielded essentially similar results, although the brain tended to be labeled more intensely by the latter, and the kidney by the former.

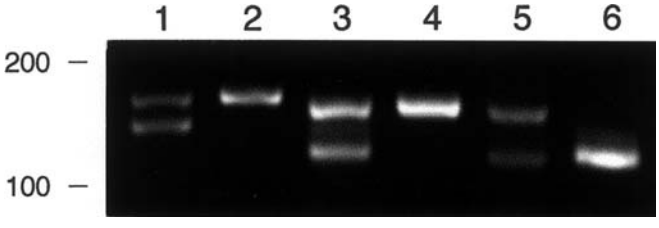

Fig. 1 Allele-specific PCR. After the first PCR of DNA from normal-sized neurons of an Eker carrier (*lane 1*) and non-carrier (*lane 2*), a band derived from the wild-type allele (151 bp) is detected in both the specimens, and a band from the mutant-type allele (129 bp) in the former (*lane 1*). After the second PCR of DNA from normal-sized neurons of a carrier (*lane 3*) and non-carrier (*lane 4*), cytomegalic neurons (*lane 5*) and renal carcinoma cells (*lane 6*) of a carrier, a band derived from the wild-type allele (141 bp) is detected in *lanes 3*, *4* and *5*, and a band from the mutant-type allele (119 bp) in *lanes 3*, *5* and *6*

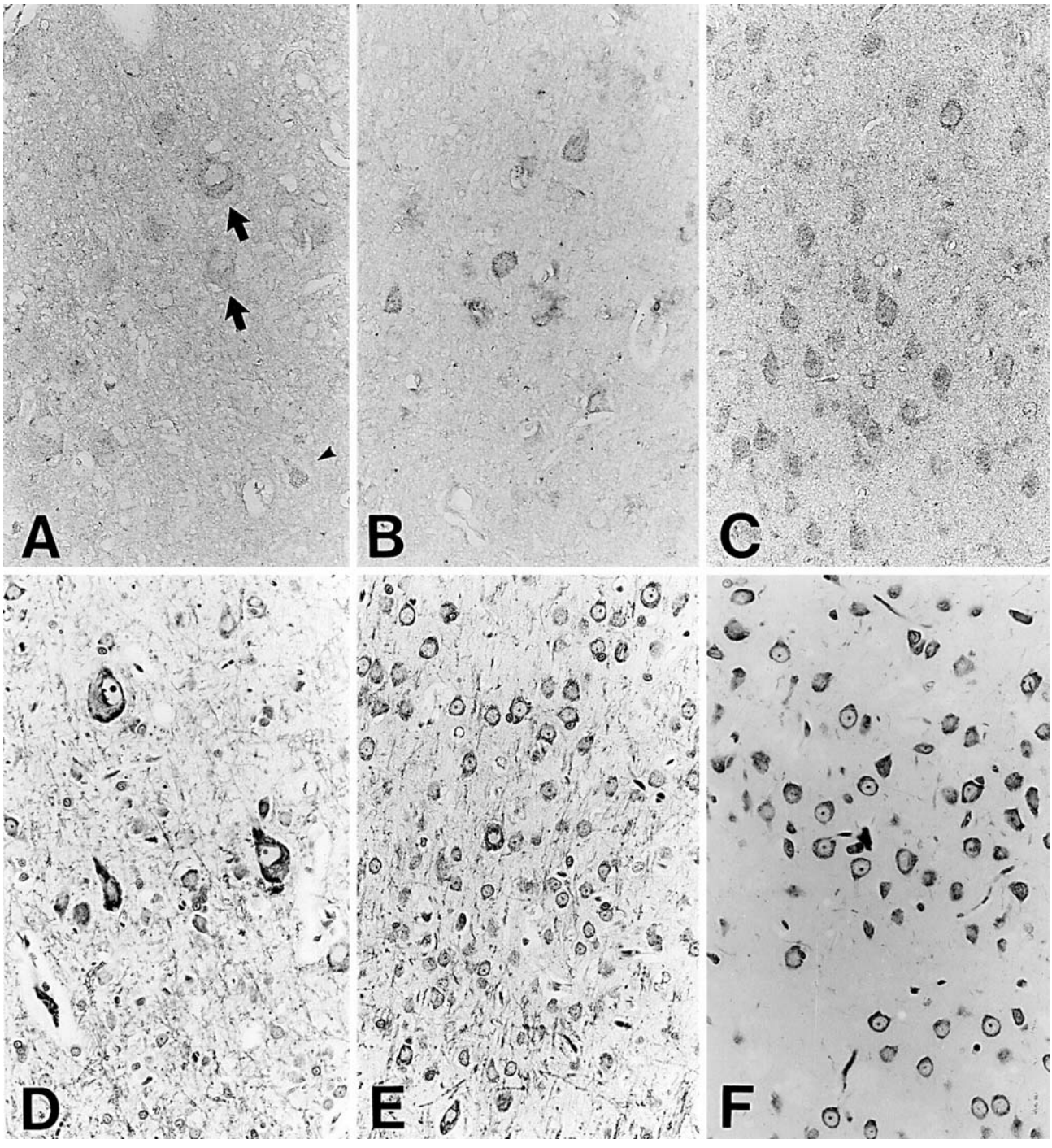

Fig. 2 A–F Immunoperoxidase staining with the anti-Tub-CT antibody (**A**–**C**) and Klüver-Barrera staining (**D**–**F**) of a cortical tuber (**A**, **D**) and contralateral cortex (**B**, **E**) of an Eker carrier, and a corresponding cortical region of a non-carrier (**C**, **F**). In the tuber (**A**), cytomegalic neurons (*arrows*) are weakly immunopositive for tuberin. Immunoreactivity is absent from all neurons and glial cells with normal morphology except for one neuron that is positively stained (*arrowhead*). In the contralateral (**B**) and control (**C**) cortex, there are tuberin-immunoreactive neurons, the number of which is somewhat larger in the control tissue. **A**–**F** ×230

In the Eker rat cortical tuber, many of the cytomegalic neurons showed weak tuberin immunoreactivity, whereas the vast majority of normal-sized neurons and glial cells were negative for tuberin (Fig. 2A, D). In the contralateral cerebral cortex without a tuber, there were no cytomegalic neurons. Scattered neurons were weakly stained for tuberin (Fig. 2B, E). The number of positively stained cells appeared smaller than that in control tissues of non-carriers (Fig. 2C, F), although the difference was slight and of equivocal significance.

In the kidneys of Eker carriers, positive tuberin immunoreactivity was localized in the epithelium of urinif-

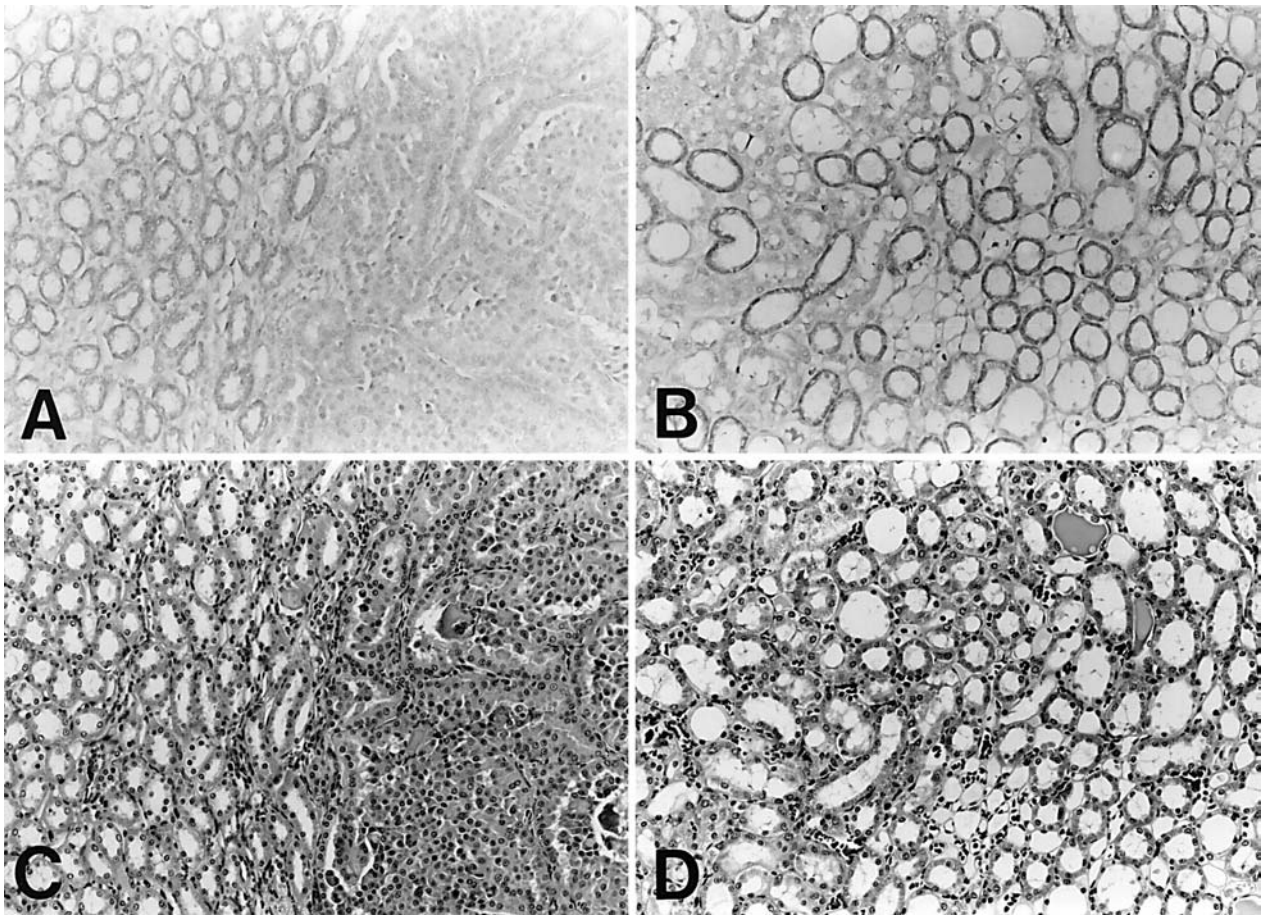

Fig. 3 A–D Immunoperoxidase staining with the anti-Tub-NT antibody (**A**, **B**) and hematoxylin and eosin staining (**C**, **D**) of renal tissues of an Eker carrier (**A**, **C**) and a non-carrier (**B**, **D**). A renal carcinoma lacks tuberin immunoreactivity (**A**, *right*). The surrounding collecting tubules show positive staining for tuberin (**A**, *left*), which is less intense than that of a control kidney (**B**). **A–D** ×120

erous and collecting tubules. By contrast, tuberin was absent from all cancer cells of the LOH-positive renal carcinoma (Fig. 3A, C). Renal tubules of the non-carriers were also immunopositive for tuberin. Many of them were stained more intensely than the carriers' tubules (Fig. 3B, D).

Discussion

In this study, we first isolated individual cytomegalic neurons of an Eker rat cortical tuber by laser capture microdissection, and compared their *Tsc2* gene status to that of renal carcinoma cells by semi-nested PCR. The results demonstrated the presence of both the wild-type and mutant-type *Tsc2* alleles in cytomegalic neurons, and the loss of the former in renal carcinoma cells (Fig. 1). Since we analyzed DNA extracted from about ten cytomegalic neurons, we cannot conclude that all these cells retained the wild-type allele. Nevertheless, our results clearly demonstrate that at least some of the cytomegalic neurons arose by a mechanism different from LOH, which accounts for the majority of renal carcinomas occurring spontaneously in the Eker carriers [21].

We next studied immunohistochemically the expression of tuberin protein in the cytomegalic neurons of the same tuber, and again compared the findings with those of an LOH-positive renal carcinoma. In the tuber, tuberin immunoreactivity was detectable in many of the cytomegalic neurons, and absent from most of the normal-sized neurons (Fig. 2). The staining pattern in the Eker rat brain was in sharp contrast to that in the kidney, where normal epithelial cells of renal tubules were positive and cancer cells of a renal carcinoma were negative (Fig. 3). Since both the Eker germline mutation and LOH reduce the amount of tuberin protein [13], the results of our immunohistochemical study were in good agreement with those of the LOH assay.

Our genetic and immunohistochemical findings on Eker rats are comparable in many respects to those previously obtained for human TSC patients. In the kidney of TSC patients, renal carcinomas occur only rarely, but benign tumors, named angiomyolipoma, often arise spontaneously. Genetic studies have demonstrated a high incidence of LOH, either for the *TSC1* or *TSC2* locus, in TSC-associated renal angiomyolipomas [10, 11, 12, 13, 25, 26]. Tuberin immunohistochemistry positively labels normal renal tubules but not the angiomyolipomas [13, 23], producing a staining pattern strikingly similar to that of the Eker

rat kidney. In the human TSC brain, cortical tubers occur much more frequently than in Eker rats. Previous studies have demonstrated that LOH is very rare in human tubers [12, 25]. Immunohistochemically, tuberin immunoreactivity is detectable in many of the human cytomegalic neurons and balloon cells, and is by no means weaker (and often even stronger) than in the normal-sized neurons and glial cells [16, 17, 23, 29]. These similarities suggest that the molecular mechanisms by which cerebral and renal lesions occur are similar between human TSC and Eker rats.

On the other hand, cortical tubers of human TSC patients and Eker rats show some important differences. First, the Eker rat tuber consists predominantly of cytomegalic neurons, and does not contain balloon cells, an essential component of human tubers [24]. Using laser capture microdissection, a recent study demonstrated LOH at the *TSC1* gene locus in balloon cells of several human TSC patients [4]. Clarifying whether cytomegalic neurons and balloon cells differ in their pathogenetic mechanism should be the aim of future studies. Second, tuberin immunoreactivity of the cerebral cortex is generally lower in rats than humans. In the Eker rat cortical tuber, the staining of cytomegalic neurons was weaker than that in human tubers [23]. In the cortex of the contralateral hemisphere and control cerebra, the number of immunoreactive cells was smaller than that in human cortex [23]. It is unlikely that the difference resulted from weak interspecies cross-reactivity of the antibodies used, since they labeled rat renal tubules as intensely as human ones.

It has recently been shown that cells comprising human cortical tubers often show clear clonality [25]. Since tuberin regulates cell growth via a target of the rapamycin (TOR)-mediated pathway [8, 20], the abnormal size of neurons probably results from dysfunction of tuberin intrinsic to their precursors. Given the fact that a minority of human tubers undergo LOH [4, 26], the most plausible cause of neuronal cytomegaly in LOH-negative tubers appears to be an intragenic somatic mutation of the *TSC2*/*Tsc2* gene, as previously demonstrated in LOH-negative renal carcinomas of Eker rats [19]. If most cortical tubers are caused by such a mutation, it is expected that intrauterine exposure to radiation and chemical mutagens would increase the incidence of cortical tubers. Recently, preliminary evidence supporting this hypothesis was provided [15, 31]. Although the small amount of DNA prohibited us from analyzing the base sequence of the *Tsc2* gene, the positive tuberin staining of cytomegalic neurons observed in this study is compatible with a second hit of this type, since point mutations and small deletions may not reduce the amount of immunoreactive tuberin protein.

However, some of our results cannot be fully explained on the basis of the *Tsc2* gene status alone. For example, the present immunohistochemical data on an Eker rat tuber and previous data on human tubers indicate that the level of tuberin expression in normal-sized neurons and glial cells is lower compared to that in normal cerebral tissues of control subjects and gliotic tissues of aberrant neurological conditions other than TSC [23, 29]. Why tuberin is down-regulated in these "bystander" cells remains unclear. Various factors may affect tuberin expression, since its level is secondarily altered in many neurological conditions other than TSC, such as hypoxic-ischemic encephalopathy [23], focal cortical dysplasia [27], hemimegalencephaly [2], astrocytoma, ependymoma [30] and ganglioglioma [3]. Even in the absence of a second hit affecting the *TSC2*/*Tsc2* locus, other genetic or epigenetic changes that interfere with either the expression or function of tuberin could initiate the pathological process of neuronal cytomegaly, if neuronal precursors are particularly susceptible to haploinsufficiency of the *TSC2*/*Tsc2* gene [31].

In conclusion, this study determined the *Tsc2* gene status in isolated cytomegalic neurons of an Eker rat cortical tuber, and correlated the data with immunohistochemical findings. We clearly demonstrated that cytomegalic neurons can arise in the absence of LOH. Further analyses of the second hit in these cells will require more sensitive methods of gene sequencing using a tiny amount of DNA from single cells on histological slides.

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References

1. Al-Saleem T, Wessner LL, Scheithauer BW, Patterson K, Roach ES, Dreyer SJ, Fujikawa K, Bjornsson J, Bernstein J, Henske EP (1998) Malignant tumors of the kidney, brain, and soft tissues in children and young adults with the tuberous sclerosis complex. Cancer 83:2208–2216
2. Arai Y, Edwards V, Becker LE (1999) A comparison of cell phenotypes in hemimegalencephaly and tuberous sclerosis. Acta Neuropathol 98:407–413
3. Arai Y, Ackerley CA, Becker LE (2000) Tuberin expression in ganglioglioma. Ultrastruct Pathol 24:93–98
4. Becker AJ, Urbach H, Scheffler B, Baden T, Normann S, Lahl R, Pannek HW, Tuxhorn I, Elger CE, Schramm J, Wiestler OD, Blumcke I (2002) Focal cortical dysplasia of Taylor's balloon cell type: mutational analysis of the *TSC1* gene indicates a pathogenic relationship to tuberous sclerosis. Ann Neurol 52: 29–37
5. Carbonara C, Longa L, Grosso E, Borrone C, Garre MG, Brisigotti M, Migone N (1994) 9q34 loss of heterozygosity in a tuberous sclerosis astrocytoma suggests a growth suppressor-like activity also for the TSC1 gene. Hum Mol Genet 3:1829–1832
6. European Chromosome 16 Tuberous Sclerosis Consortium (1993) Identification and characterization of the tuberous sclerosis gene on chromosome 16. Cell 75:1305–1315
7. Fukuda T, Hirayama Y, Mitani H, Maeda H, Tsutsumi M, Konishi Y, Hino O (1998) Generation of metastatic variants of Eker renal carcinoma cell lines for experimental investigation of renal cancer metastasis. Jpn J Cancer Res 89:1104–1108
8. Gao X, Zhang Y, Arrazola P, Hino O, Kobayashi T, Yeung RS, Ru B, Pan D (2002) Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling. Nat Cell Biol 4:699–704
9. Gomez MR (1991) Phenotypes of the tuberous sclerosis complex with a revision of diagnostic criteria. Ann N Y Acad Sci 615:1–7

10. Green AJ, Johnson PH, Yates JR (1994) The tuberous sclerosis gene on chromosome 9q34 acts as a growth suppressor. Hum Mol Genet 3:1833–1834
11. Green AJ, Smith M, Yates JR (1994) Loss of heterozygosity on chromosome 16p13.3 in hamartomas from tuberous sclerosis patients. Nat Genet 6:193–196
12. Henske EP, Scheithauer BW, Short MP, Wollmann R, Nahmias J, Hornigold N, van Slegtenhorst M, Welsh CT, Kwiatkowski DJ (1996) Allelic loss is frequent in tuberous sclerosis kidney lesions but rare in brain lesions. Am J Hum Genet 59: 400–406
13. Henske EP, Wessner LL, Golden J, Scheithauer BW, Vortmeyer AO, Zhuang Z, Klein-Szanto AJP, Kwiatkowski DJ, Yeung RS (1997) Loss of tuberin in both subependymal giant cell astrocytomas and angiomyolipomas supports a two-hit model for the pathogensis of tuberous sclerosis tumors. Am J Pathol 151:1639–1647
14. Hino O, Mitani H, Katsuyama H, Kubo Y (1994) A novel cancer predisposition syndrome in the Eker rat model. Cancer Lett 83:117–121
15. Hino O, Mitani H, Sakurai J (2002) "2nd hit" of *Tsc2* gene in radiation induced renal tumors of Eker rat model. Int Congress Ser 1236:163–174
16. Johnson MW, Emelin JK, Park S-H, Vinters HV (1999) Co-localization of TSC1 and TSC2 gene products in tubers of patients with tuberous sclerosis. Brain Pathol 9:45–54
17. Kerfoot C, Wienecke R, Menchine M, Emelin J, Maize JC Jr, Welsh CT, Norman MG, DeClue JE, Vinters HV (1996) Localization of tuberous sclerosis 2 mRNA and its protein product tuberin in normal human brain and in cerebral lesions of patients with tuberous sclerosis. Brain Pathol 6:366–377
18. Kobayashi T, Hirayama Y, Kobayashi E, Kubo Y, Hino O (1995) A germline insertion in the tuberous sclerosis (*Tsc2*) gene gives rise to the Eker rat model of dominantly inherited cancer. Nat Genet 9:70–74
19. Kobayashi T, Urakami S, Hirayama Y, Yamamoto T, Nishizawa M, Takahara T, Kubo Y, Hino O (1997) Intragenic *Tsc2* somatic mutations as Knudson's second hit in spontaneous and chemically induced renal carcinomas in the Eker rat model. Jpn J Cancer Res 88:254–261
20. Kobayashi T, Adachi H, Mitani H, Hirayama Y, Hino O (2003) Toward chemotherapy for *Tsc2*-mutant renal tumor. Proc Jpn Acad 79B:22–25
21. Kubo Y, Mitani H, Hino O (1994) Allelic loss at the predisposing gene locus in spontaneous and chemically induced renal cell carcinoma in the Eker rat. Cancer Res 54:2633–2635
22. Mizuguchi M, Kato M, Yamanouchi H, Ikeda K, Takashima S (1996) Loss of tuberin from cerebral tissues with tuberous sclerosis and astrocytoma. Ann Neurol 40:941–944
23. Mizuguchi M, Kato, M, Yamanouchi H, Ikeda K, Takashima S (1997) Tuberin immunohistochemistry in brain, kidneys and heart with or without tuberous sclerosis. Acta Neuropathol 94:525–531
24. Mizuguchi M, Takashima S, Yamanouchi H, Nakazato Y, Mitani H, Hino O (2000) Novel cerebral lesions in the Eker rat model of tuberous sclerosis: cortical tuber and anaplastic gangliogioma. J Neuropathol Exp Neurol 59:188–196
25. Niida Y, Stemmer-Rachamimov AO, Logrip M, Tapon D, Perez R, Kwiatkowski DJ, Sims K, MacCollin M, Louis DN, Ramesh V (2001) Survey of somatic mutations in tuberous sclerosis complex (TSC) hamartomas suggests different genetic mechanisms for pathogenesis of TSC lesions. Am J Hum Genet 69:493–503
26. Sepp T, Yates JR, Green AJ (1996) Loss of heterozygosity in tuberous sclerosis hamartomas. J Med Genet 33:962–964
27. Takaishi Y, Hashimoto K, Fujino O, Arai N, Mizuguchi M, Maehara T, Shimizu H (2002) A case of intractable epilepsy: diagnosis of tuberous sclerosis based on histopathological findings and immunohistochemical expression of hamartin and tuberin. No To Hattatsu 34:49–54
28. Van Slegtenhorst M, Hoogt R de, Hermans C, et al (1997) Identification of the tuberous sclerosis gene *TSC1* on chromosome 9q34. Science 277:805–808
29. Vinters HV, Kerfoot C, Catania M, Emelin J, Roper SN, DeClue JE (1998) Tuberous sclerosis-related gene expression in normal and dysplastic brain. Epilepsy Res 32:12–23
30. Wienecke R, Guha A, Maixe JC Jr, Heideman RL, DeClue JE, Gutmann DH (1997) Reduced *TSC2* mRNA and protein in sporadic astrocytomas and ependymomas. Ann Neurol 42:230–235
31. Yeung RS (2002) Tuberous sclerosis as an underlying basis for infantile spasm. Int Rev Neurobiol 49:315–332
32. Yeung RS, Xiao GH, Everitt JI, Jin F, Walker CL (1995) Allelic loss at the tuberous sclerosis 2 locus in spontaneous tumors in the Eker rat. Mol Carcinog 14:28–36
33. Yeung RS, Katsetos CD, Klein-Szanto A (1997) Subependymal astrocytic hamartoma in the Eker rat model of tuberous sclerosis. Am J Pathol 151:1477–1486