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Malformations of the brain in two fetuses with a compound heterozygosity for two PAX6 mutations

Bogna Schmidt-Sidor[1], Krystyna Szymańska[2,3], Kathleen Williamson[4], Veronica van Heyningen[4], Tomasz Roszkowski[5], Teresa Wierzba-Bobrowicz[1], Jacek Zaremba[6]

[1]Department of Neuropathology, Institute of Psychiatry and Neurology, Warsaw, Poland; [2]Department of Clinical and Experimental Neuropathology, Mossakowski Medical Research Center, Polish Academy of Sciences, Warsaw, Poland; [3]Department of Child Psychiatry Medical University of Warsaw, Warsaw, Poland; [4]MRC Human Genetics Unit, Western General Hospital, Edinburgh, UK; [5]Department of Gynaecology and Obstetrics, The Medical Centre of Postgraduate Education, Warsaw, Poland; [6]Department of Genetics, Institute of Psychiatry and Neurology, Warsaw, Poland

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Abstract

PPAX6 is an important transcription factor which plays an essential role in brain morphogenesis and eye development. It is related to migration of neuroblasts to the cerebral cortex and deep telencephalic nuclei, and the specification of cellular and regional identity. Disturbances of brain development in two sib fetuses whose parents were aniridic (both sporadic cases) are reported. Molecular analysis in both parents has shown different mutations in PAX6 gene and a compound heterozygosity for two PAX6 mutations in both fetuses. Neuropathologically both cases showed severe brain malformations with increased germinal proliferation, gross disturbances of migration and organization of the CNS.

Key words: *PAX6 mutation (compound heterozygosity), fetal development, brain malformations, cell proliferation, migration, neuropathology.*

Introduction

The *PAX6* gene located on 11p13 chromosome cods one of PAX family proteins which are transcription factors. It plays a key role during eye and olfactory system development and in brain morphogenesis [13]. *PAX6* is also required for the development and maintenance of islet cells of pancreas [13].

PAX6 mice mutants provide a good model for morphological and molecular studies of the *PAX6* role during brain development. During development *PAX6* is expressed in the telencephalon and diencephalon and also in the hindbrain and spinal cord. There is a clean boundary at midbrain where expression of *PAX6* stops [13]. The results of Estivill-Torrus et al. investigation suggests that *PAX6* may be an essential in developing cortical progenitors to influence cell cycle times and rate of progression from symmetrical to asymmetrical division [3].

Communicating author:

Teresa Wierzba-Bobrowicz, Department of Neuropathology, Institute of Psychiatry and Neurology, 9 Sobieskiego, 02-957 Warsaw, Poland. Phone number: +48 22 458 26 46, Fax number: +48 22 458 25 19, Email: bobrow@ipin.edu.pl

PAX6 takes part in forebrain patterning and cerebral cortical arealization [1,5,7]. *PAX6* plays an important role in thalamo-cortical axonal navigation [8]. At the time of development of cerebellum *PAX6* regulates granule cells polarization during parallel fiber formation [14].

The experience and knowledge about outcome of *PAX6* mutation is, for the most part, based on investigations of mouse pathology. In the mouse heterozygosity *PAX6* (*PAX6*$^{+/Sey}$) results in the *Small eyes* (*Sey*), homozygotes *Pax6* (*Pax6*$^{Sey/Sey}$) result in many brain and cerebellum abnormalities as well as lack of eyes [9,11]. Homozygous loss of *Pax6* function is neonatally lethal.

The *Pax6*$^{Sey/Sey}$ causes many defects of the molecular regionalization and boundary formation in telencephalon [7,8]. Radial glia differentiation and also cortical progenitor cells proliferation and migration are disturbed [12]. The axonal pathfinding is abolished [8].

Van Heyningen and Willamson in 2008 reviewed the literature describing anomalies associated with heterozygous *PAX6* mutations in human; there were malformations of eye: aniridia and related eye anomalies; in some cases there were behavioral problems, increased incidence of epilepsy, anosmia with olfactory bulbs hypoplasia and structural brain abnormalities such as absence of anterior commissure as well as absence or hypoplasia of the pineal gland [13].

The *PAX6* null homozygotic human was reported for the first time in 1994 by Glaser et al. [4]. They have found in a human newborn (43 weeks of gestation) the disturbances of brain development very similar to those described in homozygotic mice fetuses *PAX6*$^{Sey/Sey}$ [4]. Glaser et al. stated a compound heterozygosity for two *PAX6* nonsense mutations in the malformed newborn. The aniridic mother carried a nonsense mutation in codon 103 truncating *PAX6* within the N-terminal paired domain. The father with milder fenotype of congenital cataract and late onset corneal dystrophy carried another nonsense mutation in codon 353 in the C-terminal PST domain.

We report neuropathological findings in two other cases of malformed sib fetuses whose parents were aniridic. Molecular analysis in each parent has shown heterozygous mutations in *PAX6* gene and a compound heterozygosity for two *PAX6* mutations in one of the sib fetuses in whom molecular analysis has been performed.

Clinical data

Family history and clinical data of aniridic parents

The parents were aniridic (both sporadic cases). They were not consanguineous, and neither had family history of poor vision or any other ocular conditions. CT brain scan of the parents showed in each of them aplasia of pineal gland, hypoplasia of corpus callosum and anterior commissure. They have one child with aniridia – from the second pregnancy. The first and the third pregnancy were terminated at 24th and 21st week of gestation respectively because of the severe fetal brain malformations detected by ultrasonography (US).

PAX6 mutations analysis

Molecular analysis (V.van H. and K.W.) has shown that the father has the novel *PAX6* mutation c.117_128del12. The deletion of 12 bp does not cause a frameshift and, if translated, may generate protein that lacks part of the paired domain. Since this will almost certainly disrupt DNA binding of *PAX6* protein it may be predicted to cause loss of function. The mother has the *PAX6* mutation c.112delC. This is a 1 bp frameshift deletion that, if translated, will generate truncated protein that will almost certainly be non-functional. This is therefore a loss of function mutation. The analysis has revealed both of these mutations, i.e. compound heterozygosity, in the fetus (case 1).

Phenotypes of malformed fetuses

Case 1

First pregnancy. Fetal ultrasonography (US) at 18th week of gestation revealed ventriculomegaly and malformations of the brain described as "possible schizencephaly" and anophthalmia. Gestation period according to US measurements of the fetus corresponded to 24th week of gestation. The stillborn female fetus weighted 495 g (after fixation in formalin), its length was 36 cm, head circumference 23 cm. Postmortem X-ray showed normal for age shape and size of the skull, but the brain was very small, filling only 1/3 of the cranial cavity; above the brain there was a large volume of fluid (Fig. 1). General autopsy showed:

fusion of palpebral fissures and absence of eyes and orbits; the nose was rudimentary with very narrow anterior nostrils; the palate was high arched; there was palmar contraction of both wrists. The inner organs of the thorax and abdominal cavity were normal.

Case 2

Third pregnancy. Fetal US at 21st week of gestation showed severe malformations of the brain described as "alobar holoprosencephaly" and anophthalmos or microphthalmos and there was a very small nose. The pregnancy was terminated. The stillborn male fetus weighted 370 g, its length was 29 cm, head circumference measured 20 cm. General autopsy showed: antimongoloid, fused palpebral fissures and anophthalmos; the nose was very small and flat with closed anterior nostrils; the ears were small and low-set; there was micrognathia and enlarged lips. The inner organs of thorax and abdominal cavity were normal.

The post mortem X-ray of the skull showed its normal shape and size.

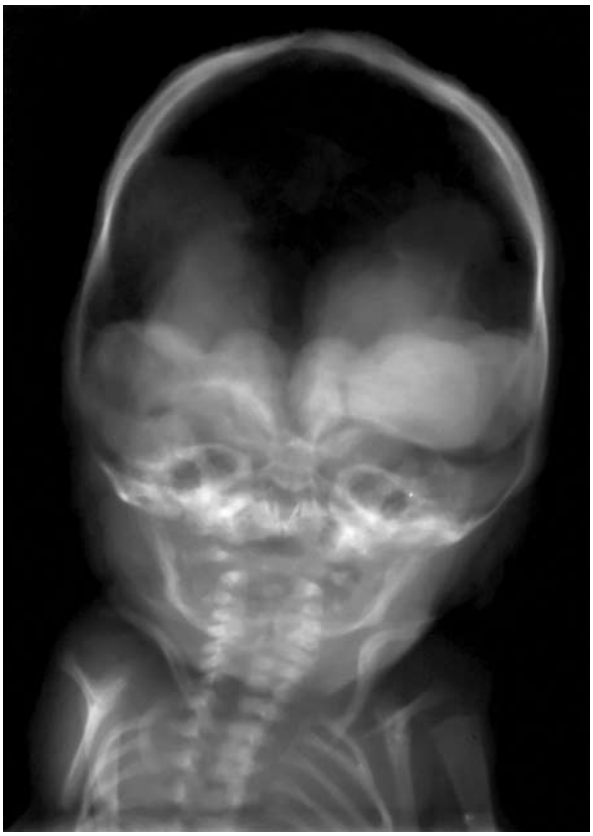

Fig. 1. Clinical case 1: Postmortem X-ray of the head: normal shape and size of the skull. The brain very small, filling 1/3 of the cranial cavity. Frontal view.

Material and Methods

The brains were fixed in formalin. Then specimens from the cerebral hemispheres, the brain stem and the cerebellum were taken and embedded in paraffin. The sections were stained with hematoxylin-eosin and Klüver-Barrera methods.

Neuropathology

Case 1 – fetus aged 24 weeks of gestation

Gross neuropathological evaluation showed very small brain hemispheres of polyglobal structure; corpus callosum was absent and the midline fissure of the brain was very wide (Fig. 2A). The surface of the brain hemispheres seemed to be constructed of loosely joined global structures of the brain tissue. The olfactory bulbs, optic nerves, chiasm and tracts were absent. The brain stem and the cerebellum were small. The spinal cord was normal in shape and size (Fig. 2B). The sections of the brain showed irregular structure of the hemispheres with better preserved frontal part of the brain hemispheres. Lateral ventricles were small and ill-shaped, in the posterior part of the brain hemispheres there were bilateral, schizencephalic fissures connecting lateral ventricles with submeningeal space. The third ventricle was normal in shape. Microscopic evaluation: the most striking feature of the microscopic structure of the brain hemispheres was enormous amount of germinal matrix. Masses of germinal matrix were situated mainly in the inner parts of the hemispheres, but there was also a great amount of germinal cells on the surface of the hemispheres and in the meningeal space, narrow cerebral cortex, areas with axon bundles (Fig. 3). The structure of the cerebral cortex was disturbed (Fig. 4): on the surface of the cerebral cortex there was wide and dense layer of germinal matrix. The marginal zone was poorly formed with irregular wideness and dispersion of cells. The deeper part of the cortex was narrow also with irregular dispersion of undifferen-

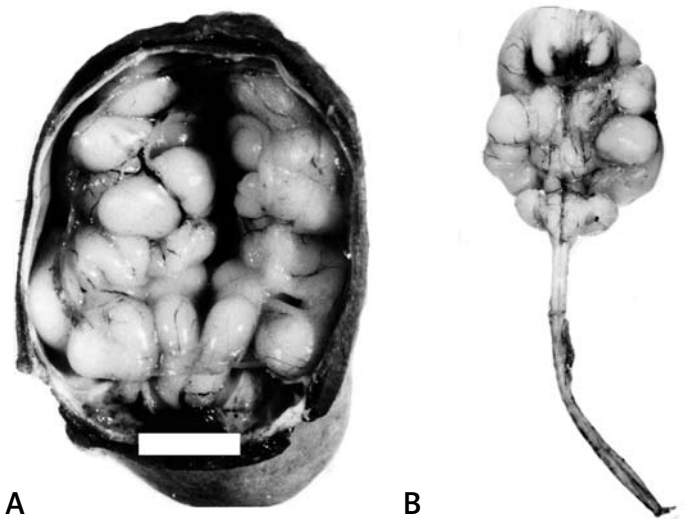

Fig. 2A-B. Case 1. Macroscopic appearance of the brain. A. Upper view of the brain hemispheres: polyglobal structure of the brain surface, wide midline fissure due to agenesis of corpus callosum. B. Basal view of the brain: polyglobal structure of the brain, small brain stem and cerebellum. Spinal cord of normal shape and size.

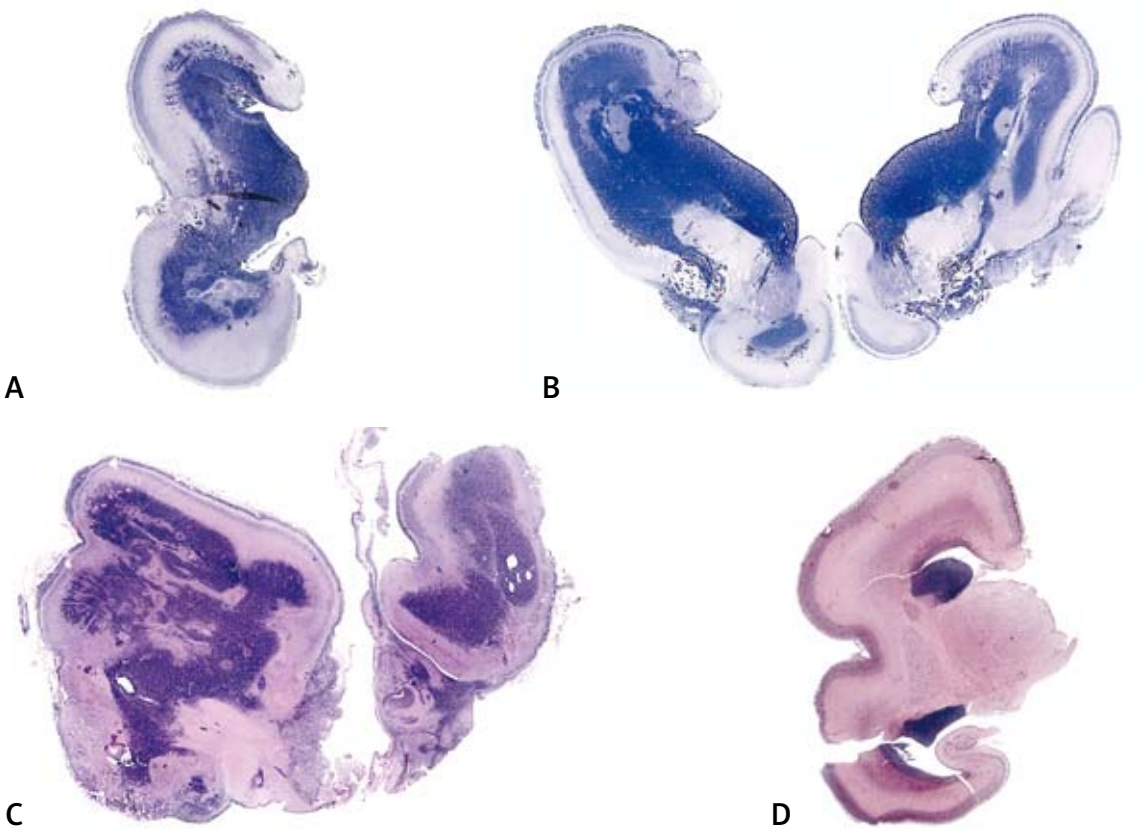

Fig. 3A-D. Case 1. Coronal section of the brain hemispheres: Frontal (A), middle (B) and posterior part (C) of the brain hemisphere: haphazard shape of the middle and posterior part of the hemispheres, enormous amount of germinal matrix. D. Control age-matched case. A, B, C Klüver-Barrera method. Lupe. D. H-E. Lupe.

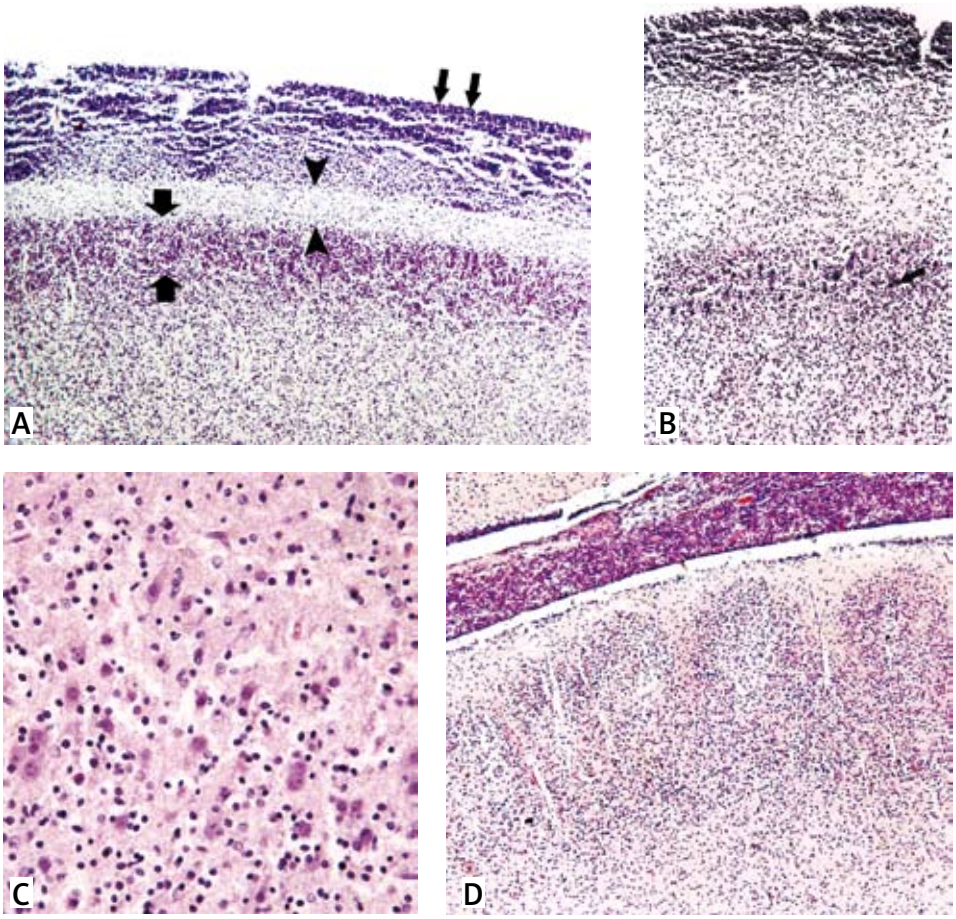

Fig. 4A-D. Case 1. Development disturbances of the brain cortex: A. Layer of germinal cells on the surface of the cortex (arrows); marginal zone poorly formed (head arrows); deeper part of the cortex was narrow, without stratification, with clusters of undifferentiated cells (fat arrows). B. Place with irregular distribution of cells with large dysplastic neurons surrounded by undifferentiated cells (arrow). C. Cortex with paucity of cells with irregular distribution of large neurons. D. Focal polymicrogyria. Klüver-Barrera. Original magn. ×100.

tiated cells stratification; there was not normal for age stratification of the cortex. In some places there were dense clusters of cortical cells. The cortex was narrow with paucity of cells. In some parts of the cortical plate there were mainly small undifferentiated cells; in others there were large, dysplastic cells resembling neurons. There was focal polymicrogyria. In the entire cerebral cortex the cells were mainly in clusters. The white matter was difficult to discern with regions showing hypercellularity or paucity of cells with many heterotopia of germinal cells and also with clusters of undifferentiated cells (Fig. 5). The microscopic structure of basal part of cerebral hemisphers showed agglomeration of neural cells resembling normal neurons of the thalamic nuclei separated by bundles of neuronal fibers, but there were no normal structures of the thalamic nuclei (Fig. 6). On the surface of the cerebral hemispheres there were several small cysts lined with ependymal cells with small patches of choroid plexus. The cerebellum showed marked dysplastic changes: paucity of convolutions which were small with irregular cortical layers with paucity of Purkinje and inner granular cells. Some parts of the cerebellum revealed normal structure, especially in the cerebellar vermis. The structure of the brain stem and spinal cord was normal, except the absence of the pyramidal tracts and the pontine nuclei (Fig. 7). The spinal cord on all levels showed normal structure with exception of lack of pyramidal tracts. The signs of myelination in spinal cord were corresponding to the gestational age of the fetus.

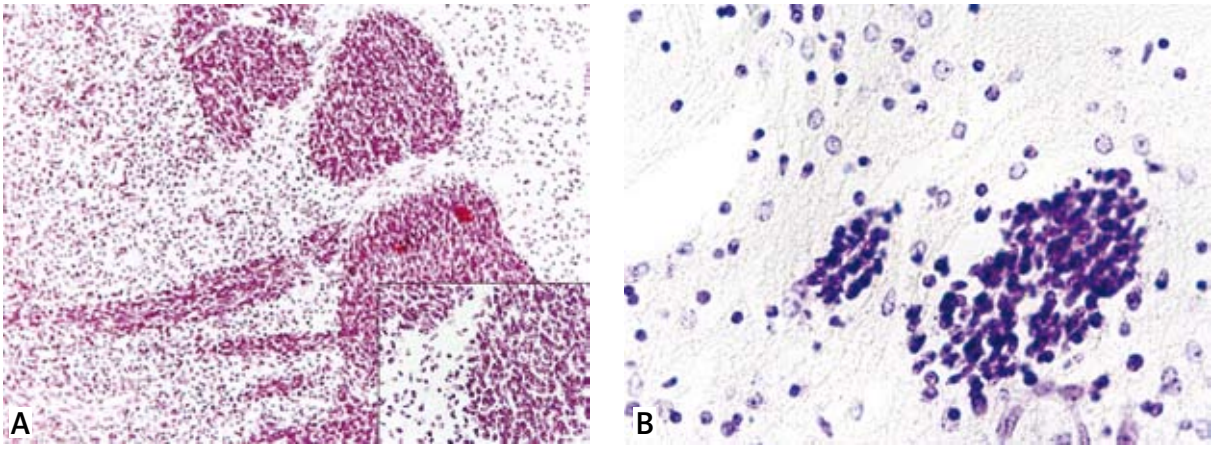

Fig. 5A-B. Case 1. A. White matter with many heterotopia. B. Dense clusters of germinal cells. H-E. Original magn. ×200.

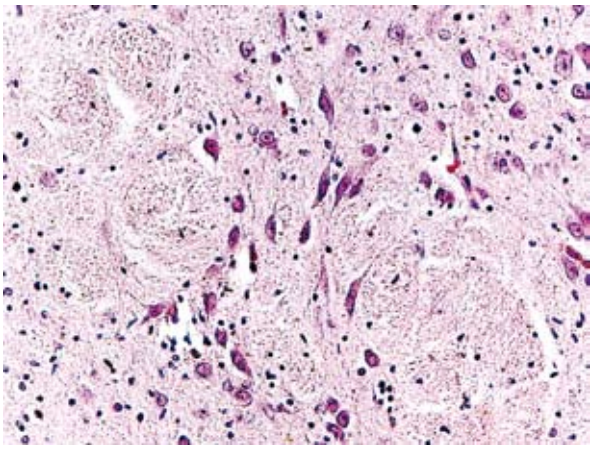

Fig. 6. Case 1. Basal ganglia with agglomeration of large neurons separated by bands of axons. H-E. Original magn. ×200.

Case 2 – fetus aged 21 weeks of gestation

Gross neuropathological evaluation showed a very small brain filling only 1/3 of the cranial cavity, above the brain there was a collection of fluid. Brain hemispheres had polyglobal structure with deep sulci between each other of them. The corpus callosum was absent (Fig. 8). The olfactory bulbs, optic nerves and tracts were absent. In the left brain hemisphere in the place of a lateral ventricle there were several narrow fissures not connected with each other. The lateral ventricle in the right hemisphere was widely opened on the middle side of the hemisphere. The brain hemispheres were connected with brain stem only by the crus cerebri. Brain stem and cerebellum were smaller than normal for age with poor foliation of the cerebellum. Spinal cord was of normal structure. Microscopic evaluation showed haphazard structure of the brain hemispheres with enormous amount of germinal matrix, very scanty cortical mantel with thick layer of matrix cells on the surface of the cortex; the white matter was poorly defined; there were areas of bands of fibers (Fig. 9). The ventricular zone didn't show any columnar arrangement of the cells, enlarged undifferentiated germinal cells were extending on the cortical surface and there were ectopia within arachnoid. On the surface of the brain there was a layer of germinal cells and heterotopia in the cortex (Fig. 10). The pattern of the cerebral cortex was disturbed; there was poorly formed marginal zone, in some places with finger like collections of germinal cells expanding from the superficial layer toward deeper parts of the cortex. The cerebral cortex was very narrow and there was focal polymicrogyria (Fig. 11). The cerebral white matter was no well defined; there were plenty of immature cells within it and many heterotopia of dense cluster of germinal cells. There were scanty bundles of fibers in brain hemispheres: the axon bundles separated in some places the germinal matrix from the intermediary zone, they were also seen in the cerebral cortex, within germinal matrix, and though in some places they formed considerable bunch they did not form any tracts. Sometimes between the bundles of fibers there were dense clusters of the matrix cells (Fig. 12). The basal ganglia were difficult to the identification within huge amo-

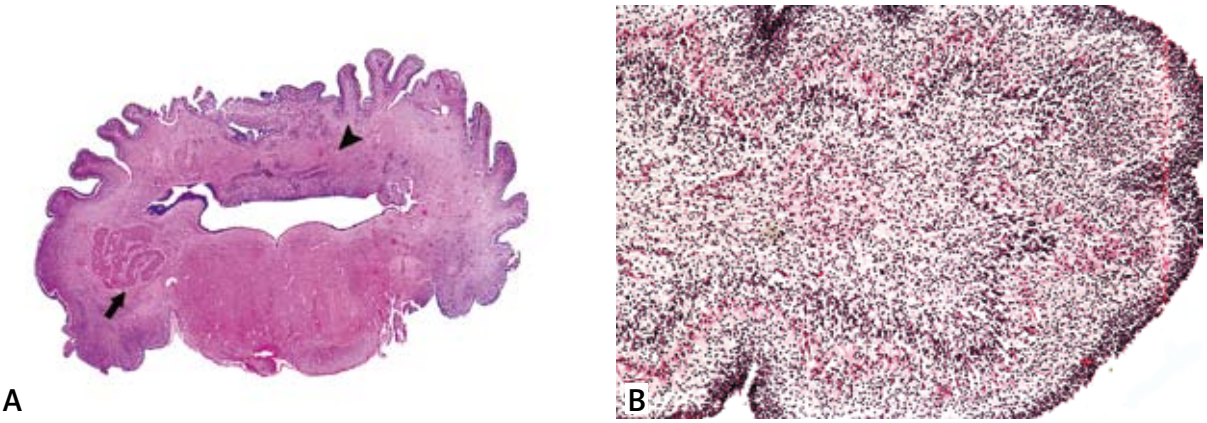

Fig. 7A-B. Case 1. Cerebellum and pons: A. Paucity and small convolutions of cerebellum; Irregular, fragmented dentate nucleus (arrow); and asymmetrical nuclei fastigi (head arrows). Lack of cortico-spinal tracts and nuclei pontis. H-E. Lupe. B. Dysplasia of all cerebellar cortex layers, wide external granular layer. H-E. Original magn. ×100.

unt of germinal matrix. On the surface of one brain hemisphere there was ependymal cyst with small patches of choroidal plexus (Fig. 13). The cerebellum was poorly foliated with few flat folia without normal for age cortical layers and the white matter (Fig. 14), but some small parts of vermis showed normal for age cortical layers. The nucleus dentatus was wider than normal. In the brain stem there was lack of pyramidal tracts and nuclei pontis; the olivary nuclei were wider than normal and fragmented (Fig. 15). The spinal cord oat all levels was of normal structure with exception of lack of pyramidal tracts.

Discussion

Schmahl et al. in 1993 [11] have performed the first neuropathological study on homozygous *Pax6*$^{sey/sey}$ and heterozygous *Pax*$^{6+/sey}$ mutant mice during fetal development. The *Pax6*$^{sey/sey}$ mutants were affected with anophthalmia, lack of the nasal cavities and olfactory bulbs and dysgenesis of telencephalon, basal ganglia, diencephalon and metencephalon. The increased volume of germinal epithelium within those structures of the CNS was observed. There were heterotopias of germinal cells in the grey matter and ectopias of germinal cells within subarachnoid space. The putative neurons accumulated within intermediate zone after leaving the subventricular zone formed the irregular layer. Authors also described drastically reduced cell content in the cerebral plate and absence of definite marginal zone. The basal ganglia were severely hypoplastic with loss of structural compartmentation. There were no abnormalities of the thalamus, hypothalamus and pituitary. In the cerebellum there was marked enlargement of the external granular layer. In some mice schizencephaly and microgyria were observed. Agenesis or hypoplasia of corpus callosum was present in the most of the *Pax6*$^{sey/sey}$ mutant mice [11].

The first neuropathological observations in human *PAX6* compound heterozygote was reported by Glaser et al. [4]. Authors described developmental disturbances throughout the brain, cerebellum and brain stem similar to those observed by Schmahl and coworkers in mice: there were absence of olfactory bulbs and corpus callosum, reduced size of brain hemispheres and brain stem, disturbed stratification of the cerebral cortex with heterotopic islands of germinal and ependymal cells, as well as focal polymicrogyria of the cerebral cortex. In the leptomeninges there were ectopic foci of glial cells and choroid plexus. The hypothalamus was abnormal truncated and there was enlarged ventricular system. There were also dysplastic changes in all elements of the cerebellum. In medulla there were prominent olivary nuclei and reduction of pyramidal tracts [4]. Glaser et coworkers compared neuropathological findings in their human *PAX6* compound heterozygote with developmental disturbances of the brain in homozygous *Pax6*$^{Sey/Sey}$ mice and they found that anatomical and histopathological findings in mice and human were correlated well with each other [4].

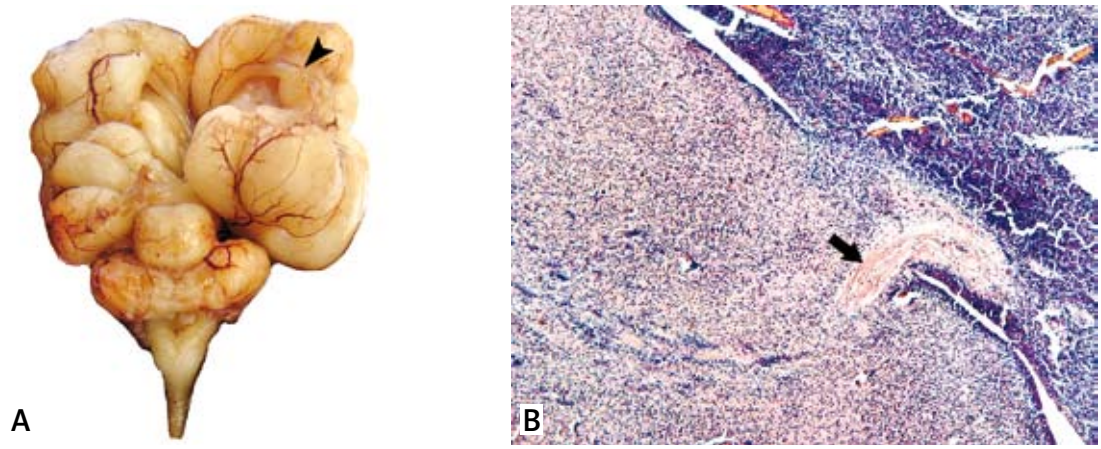

Fig. 8A-B. Case 2. A. Upper view of the brain. Surface of the brain hemispheres with polyglobal structures. Agenesia of corpus callosum except of Probs bundles (head arrow). B. The place of junction of two global structure with bundle of fibers joining each other (arrow). H-E. Original magn. ×100.

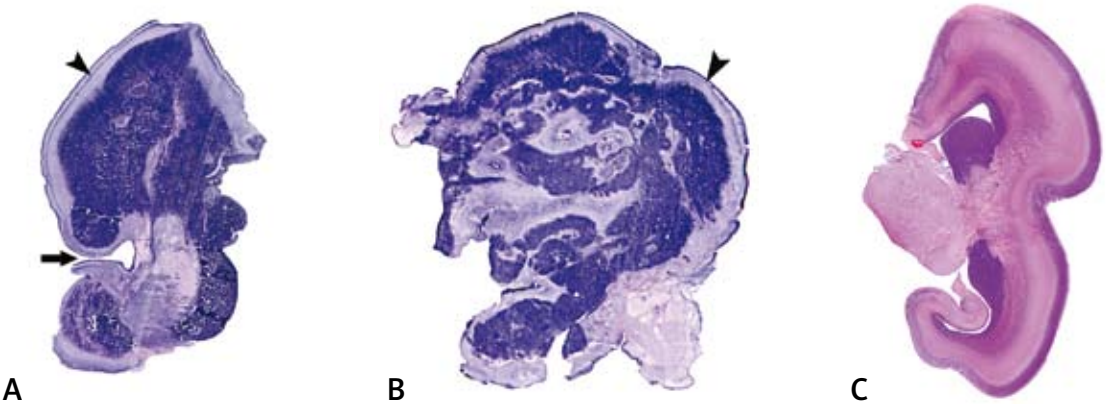

Fig. 9A-C. Case 2. A. Frontal section of brain hemisphere: A. Frontal part of hemisphere with lateral fissure (arrow). B. Posterior part of the hemisphere with haphazard structure. In both sections enormous amount of germinal matrix and scanty cortical mantel (head arrows) H-E. Lupe. C. Control aged-matched case. H-E. Lupe.

In two fetuses described by us with a compound heterozygosity for two *PAX6* mutations (confirmed in one fetus) we found completely disorganized structures of the brain hemispheres and cerebellum. Similar as in the case of Glaser et al. [4] the pattern of CNS defects in our cases suggests an abnormality of the migration of neural precursor cells into the cortex manifested by macroscopic structure of the brain hemispheres, microscopic dysgenetic changes in the cerebral cortex, the heterotopic foci of germinal cells on the surface of the brain hemispheres, as well as in the leptomeninges. The severe disturbance of the white matter development was also observed. The intermediate zone was absent. In several places of the cortex and also between clusters of neuroblasts inside the brain hemispheres there were fascicles of axons which didn't form normal tracts. It is a known fact, that *PAX6* is required for normal development of the forebrain connections [5] and normal thalamocortical development requires the action of *PAX6* within the dorsal thalamus itself [1,8]. In two cases reported by us there are dense clusters of cells characteristic of the germinal matrix, within undifferentiated cells of the cerebral cortex, between the

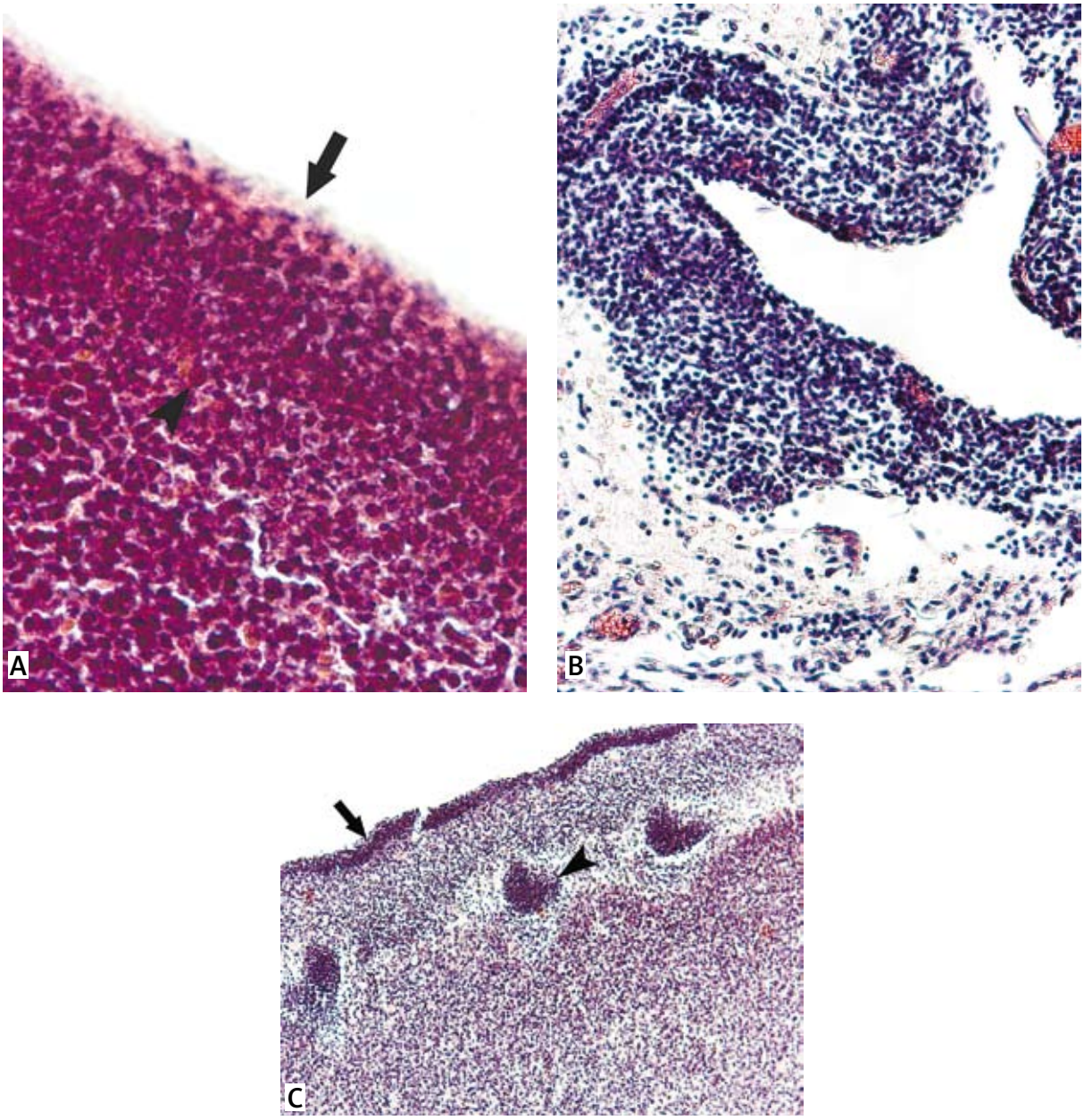

Fig. 10A-C. Case 2. A. Ventricular zone showed cells without columnar arrangement (arrow), subventricular zone with amorphous appearance (head of arrow). H-E. Original magn. ×400. B. Ectopia of germinal cells in meningeal space. H-E. Original magn. ×200. C. Layer of germinal cells on the surface of the cerebral cortex and heterotopia of germinal cells within the cortex (head arrow). H-E. Original magn. ×100.

bands of axons, in heterotopias and in ectopia. In the experiments of Tyas et al. mutant cells *in vitro* and *in vivo* migrated together and formed clusters. They postulated that cortical cells lacking *Pax6* have altered adhesive properties and increased tendency to aggregate [12]. It is possible that this property of neuroblasts is responsible for dense clusters observed in all cerebral structures in the cases presented by us. The most striking alteration of cerebellar development observed in *Pax6*$^{Sey/Sey}$ mice is the lack of foliation [2]. In cerebellum of the fetuses described here there was paucity of convolutions but some,

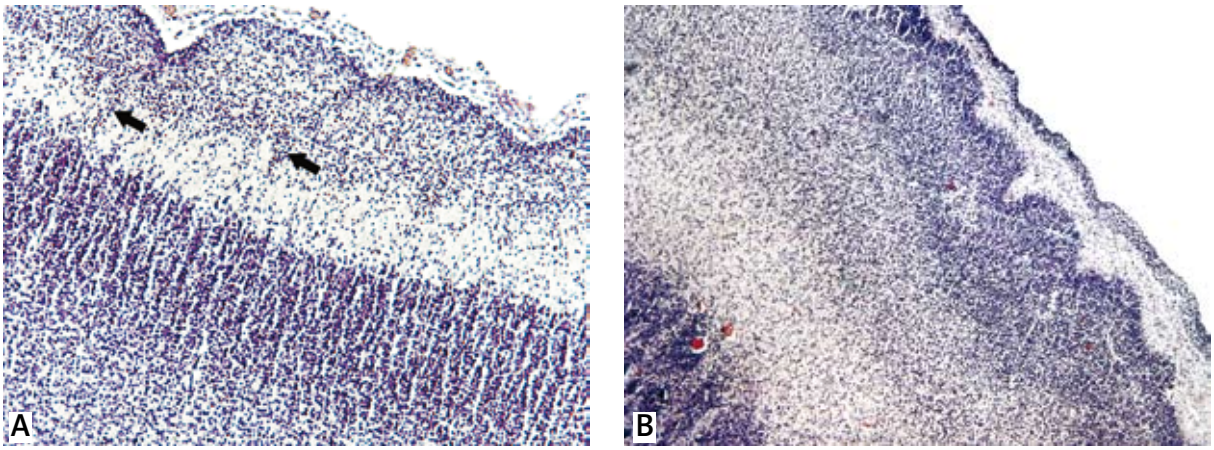

Fig. 11A-B. Case 2. Dysgenesis of cerebral cortex: A. Poorly formed marginal zone with finger like collections of germinal cells expanding toward deeper parts of the cortex (arrows). B. Focal polymicrogyria. Klüver-Barrera method. Origin magn. ×100.

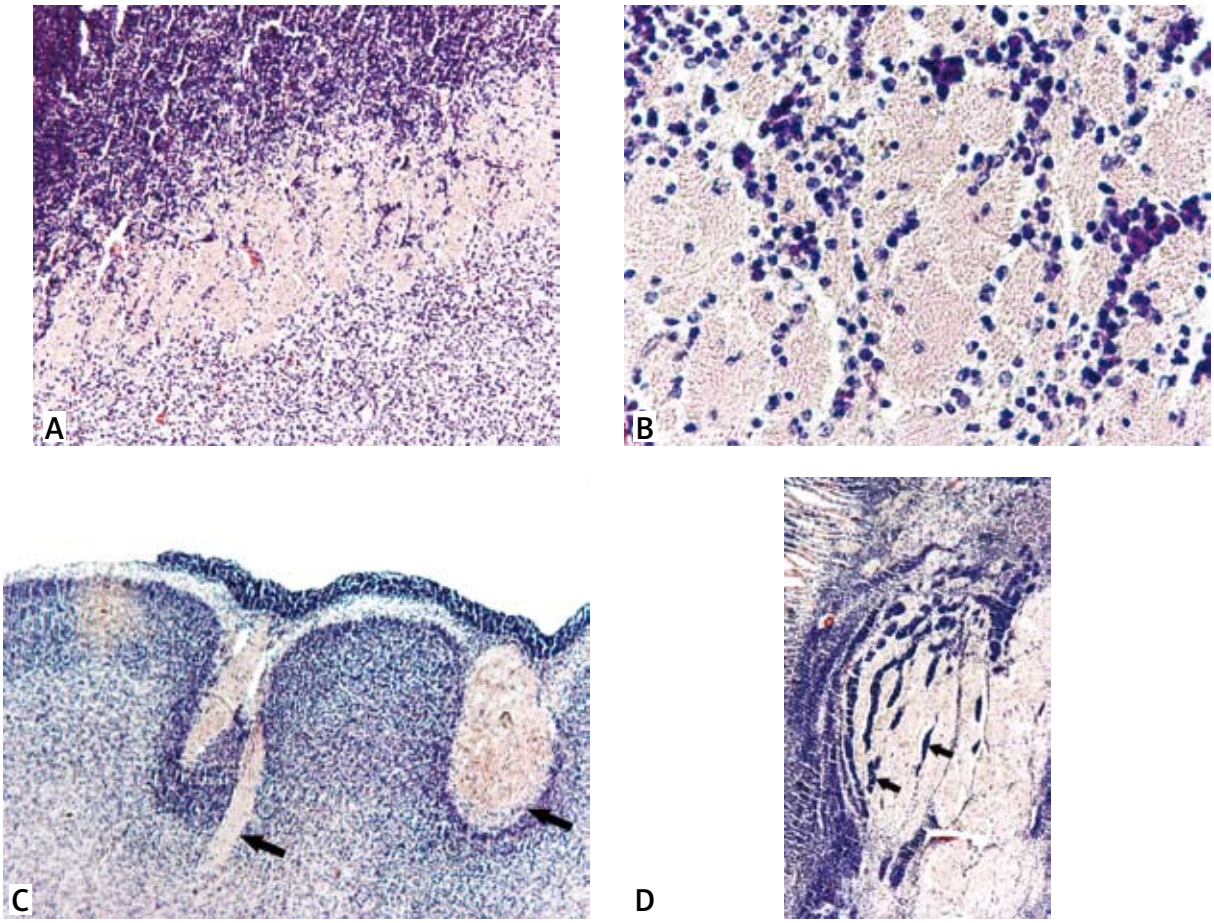

Fig. 12A-D. Case 2. Bundles of the axons: A. Demarcation by axon bundles between subventricular zone and intermediate zone. H-E. Magn. ×100. B. High magnification from A. Magn. ×400. C. Bundles of axons in the cerebral cortex (arrows). H-E. Magn. ×100. D. Bundles of axons in the region of lateral fissure with many dens clusters of germinal cells (arrows). H-E. Original magn. ×100.

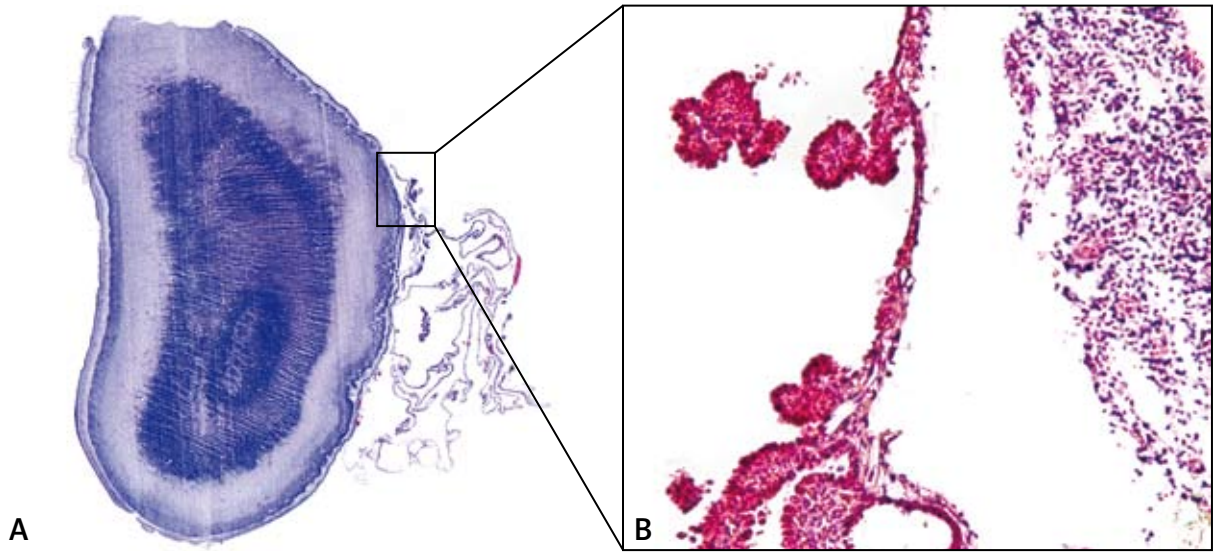

Fig. 13A-B. Case 2. A. Section of frontal part of brain hemisphere with ependymal cyst on the surface with patches of choroid plexus B.. Kluver-Barrera method. Lupe. B. Klüver-Barrera. Original magn. ×200.

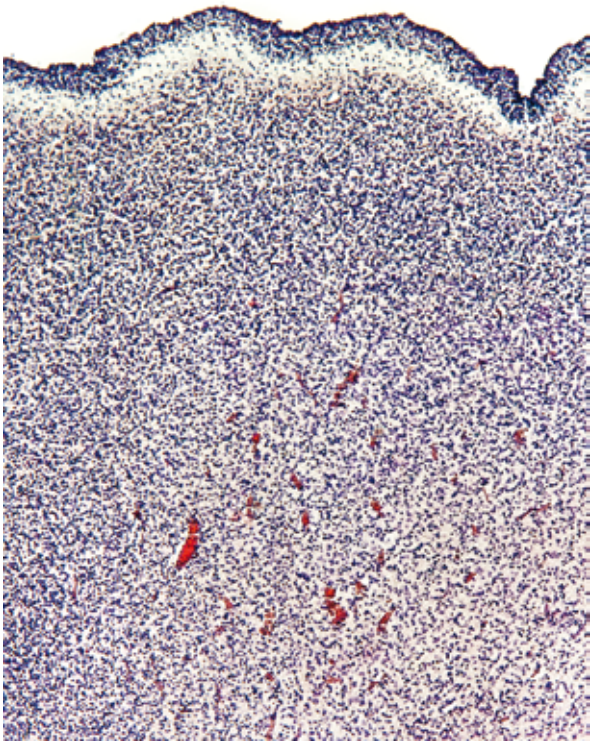

Fig. 14. Case 2. Cerebellum: few flat folia without normal cortex and white matter, wide external granular layer. H-E. Original magn. ×100.

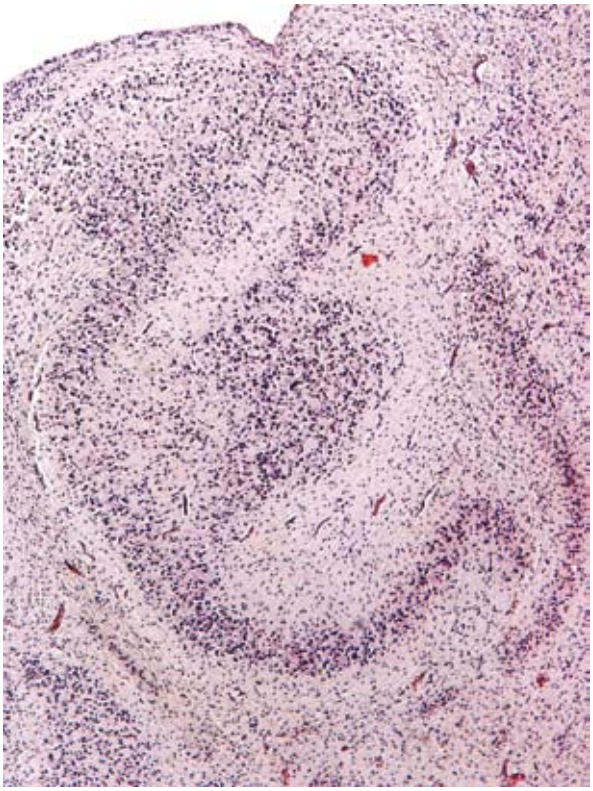

Fig. 15. Case 2. Dysplastic development of inferior olive: partially wider than normal and fragmented. H-E. Magn. ×100.

small parts of vermis showed normal for age cortical layers. Contrary to our observations the cerebellar folia in the case of Glaser et al. were excessively convoluted [4]. The different morphological pictures may be to some extent the reflection of the failure of neural and glia circuit organization due to migration disturbances [6]. Other differences between our and Glaser's cases consisted in larger amount of germinal cells and more pronounced disturbances of migration in each of our cases. To some degree those differences may be due to different gestation age of the case reported by Glaser et al. – 43 weeks and of the two cases presented here – 21 and 24 weeks respectively. More probable, however, are phenotype variations due to different mutations in the compound heterozygosity cases. This makes the analysis of single mutated gene expressions and the interpretation of programmed malformations of the brain more difficult [10].

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