# Copy Number Variations in Structural Brain Malformations

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# I. Introduction:

The development of the human brain is a complex process involving the proliferation and migration of primordial cells which leads to the formation of the various layers and neuronal components of the mature brain. The proper spatio-temporal expression of structural and regulatory genes is essential to brain development. It has been a matter of great interest in the past years to identify these genes and their functions. Understanding brain development is a prerequisite for studying the molecular mechanisms that are involved in this process.

## 1. Development of the brain

The early steps of Central Nervous System (CNS) patterning is highly conserved and all vertebrate brains share a common developmental pathway. Much of what we know about the development of the brain is based on animal models.

The nervous system develops from the ectoderm of the embryo, influenced greatly by the interaction of a number of molecules secreted by the underlying notochord that will decide the fate of these cells. Neural cells are of two main categories: the neurons that are responsible for the signaling and the glial cells that provide the supporting structures. Differentiation and division of the cells of the primary ectoderm destined to become neural cells will give rise to the spinal cord and brain.

During the first 3 weeks of gestation, the embryonic ectoderm forms a thickened strip called the neural plate which then folds and closes to form the neural tube (Saladin, 2012). Following the anterior and posterior closure of the neural tube, lack of which will lead to anencephaly or to spina bifida, respectively, the cells at the anterior end with a neural fate will form the initial three vesicles that will later constitute the forebrain, midbrain and the hindbrain [Figure I.1].

The next stage of development involves the splitting of the forebrain into two vesicles: the telencephalon containing the cerebral cortex, basal ganglia and related structures, and the diencephalon containing the thalamus and hypothalamus. Simultaneously, the hindbrain splits to form the metencephalon containing the cerebellum and the pons, and the

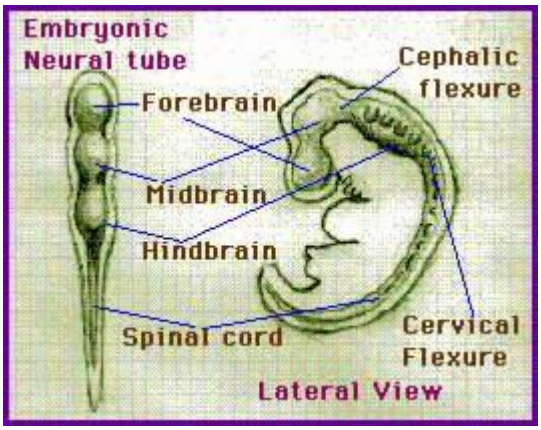



Figure I.1 By 3-4 weeks the anterior portion of the neural tube is divided into three distinct round cavities. These cavities will become the encephalon, whereas the middle and caudal portions remain tubular and will give rise to the spinal cord. At this stage, the embryonic neural tube has formed. http://www.ifc.unam.mx/Brain/devel.htm

myelencephalon containing the medulla oblongata. Within each region, multitudes of neurons and glial cells are generated [Figure I.2]. The fated neural cells on the sides of the midline of the initial neural tube called the neural crest cells will migrate to different parts of the body and form the peripheral nerves, roots and ganglion cells of the peripheral nervous system. The destiny of neural tissue depends upon their exposure to environmental signals that are specific to their location and cellular position. The sonic hedgehog (SHH) protein is secreted by the mesodermal tissue lying beneath the developing spinal cord and destines the adjacent neural cells to become a specialized class of glial cells (Poncet, 1996; Pringle, 1996).

Throughout the process of proliferation of the primary cells, regulatory genes and their products will decide the fate of the cells and conduct their differentiation. Migration of the rapidly proliferating cells is essential to the formation of the spinal cord, brain stem, cerebellum and cerebral hemispheres. This is a complicated process whereby sequential expression of genes and the presence of regulatory proteins are critical to its proper execution. For example, in the cortex, the cells must move outward to the external border or the telencephalon and form the six layers of the cortex from the inside out, each with its distinctive pattern of organization and connections. The proper migration of cells through the layers to their final destination determines the structure of the normal brain.

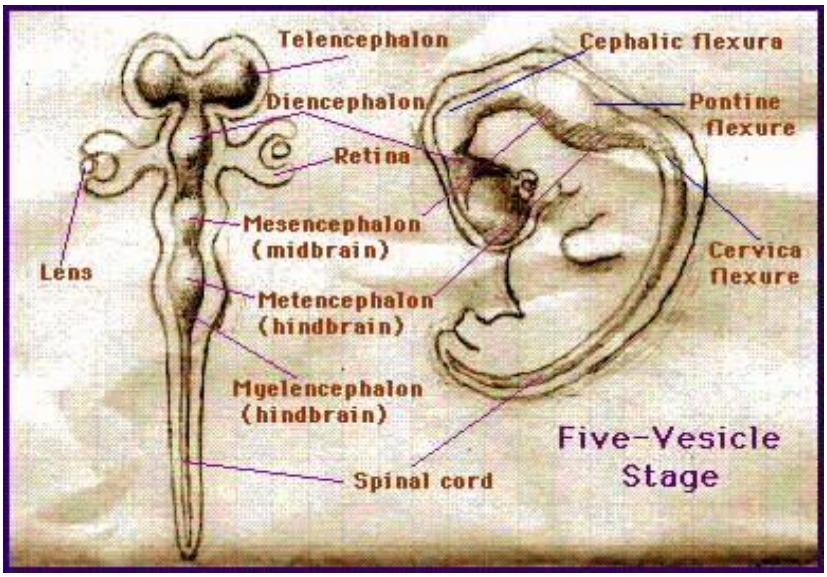



Figure I.2: The forebrain, now divided has an anterior portion called the telencephalon, which is divided laterally to form the cerebral hemispheres enfolding the cavities that become the lateral ventricles. The second division of the forebrain is the diencephalon, which will give rise to the central structures of the brain, and its cavity will become the third ventricle. From the lateral sides of this section will eventually grow the retina and the optic nerve. The division of the hindbrain is not as evident, and is only divided thru the middle. The anterior portion is the metencephalon and the posterior the myelencephalon. The midbrain and caudal portions (spinal cord) remain undivided, but continue to grow and differentiate, and in the case of the midbrain its cavity will derive in the cerebellar (Sylvian) aqueduct. http://www.ifc.unam.mx/Brain/devel.htm

Anomalies in this migration are thought to be the cause of abnormal behavior in the *reeler* and *staggerer* mutant mice (Scheibel, 2011).

As the cortical layers are developing, the growing fibers of nerve cells from the thalamus will arrive but are unable to reach their yet undeveloped destination. A series of cells will locate between the borders of the cortical layers (gray matter) and the underlying layer (white matter). Along with a second group of similar cells at the outermost edge of the neural tube wall, at the most superficial layer of the cortex, these cells will provide an interim connection for these fibers. Once the proliferative process has completed and the layers of the cortex have formed, these interim cells will disappear allowing the fibers to fill the juncture and make synaptic connections with the underlying cortical layers. Any errors in this transfer or disappearance of interim cells will lead to major and minor cognitive and emotional disorders; dyslexia is considered to be one such disorder (Scheibel, 2011).

The placement of the cells in their correct position is followed by their maturation whereby they develop dendritic branches providing increased surface area for synaptic terminals and

develop axons adjacent to the destination cells. The maturation of nerve cells is completed by the formation of the myelin sheaths which serve as the insulating covering of nerve cells; a process that is considered to occur in various specific sets of nerve cells at specific times of postnatal development.

## 1.1. Development of the anterior neural plate

During the early stages of neural plate formation the embryo consists of three layers: the ectoderm, to become the skin and neural tissues, the mesoderm that forms the muscles and the bones, and the endoderm, to constitute the cellular lining of digestive and respiratory tracts. Based upon chick models, fibroblast growth factors (FGFs) are the initial signals that induce a "prospective" neural state to the ectodermal cells prior to gastrulation (Stern, 2002). However, FGF signaling is not singularly sufficient for neural development. The bone morphogenetic proteins (BMPs) are currently believed to be the major components of neural specification in the anterior neural plate (Stern, 2002; Kuroda et al., 2004). Based upon the "neural default" model, it is postulated that ectodermal cells initiated by FGF signaling will develop into neural cells by default and their exposure to BMPs will deter this process (Munoz-Sanjuan and Brivanlou, 2002). BMP antagonists will protect cells from exposure to BMPs. Observations suggest that high BMP signaling promotes non neural fates while low BMP signaling allows for more medial neural plate fates (Munoz-Sanjuan and Brivanlou, 2002). At this point, the neural plate border co-expresses both neural and non-neural markers differentiating the ectodermal layer from the neural plate (Gestri et al., 2005).

Further development of the neural plate will encompass the differentiation of the cells and the formation of the anterior neural plate which will encase the brain structures. The patterning of the neural plate and orientation of the proliferating cells is of great significance and involves dorsal-ventral and anterior-posterior determination. It is believed that anterior posterior (AP) positioning patterning signals may have already aligned the growing embryo or may act simultaneously with the formation of the neural plate. WNTs are a family of proteins, the first of which, encoded by the *Wg* gene, was recognized in the wingless Drosophila and later identified to function in a signaling pathway that is of profound importance in establishing the development of all multicellular organisms. WNTs and WNT antagonists may contribute to the initial AP positioning of the growing embryo (Yamaguchi,

2001) and are thereafter active in the regionalization of the differentiating anterior neural plate. It is believed that the interaction between WNTs and SHH determines the dorsal-ventral patterning of the anterior neural plate (Wurst and Bally-Cuif, 2001). *WNTs* are expressed in high concentration at the most dorsal region of the neural plate conferring upon the region the characteristics of the future roof plate, while expression of *SHH* is concentrated at the ventral floor plate, promoting the notochord. An expression gradient is thus established and maintained by mutually inhibitory effects.

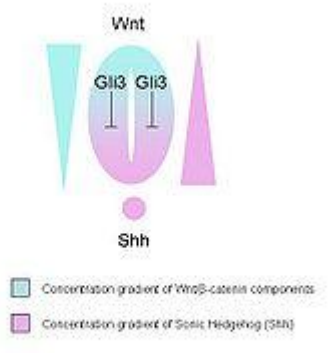



Figure I.3 The gradient created by Wnt and Shh will determine the caudalization of the anterior neural plate. http://en.wikipedia.org/wiki/Wnt_signaling_pathway.

The favored interpretation of the development of the anterior neural plate is that anterior neural plate identity is established in the absence of caudalizing factors. This is achieved by localized expression of the caudalization factors in caudal regions, their inhibition by localized antagonists in non-caudal regions and the morphogenetic movement of the anterior neural tissue away from the caudal expressing locale (Wilson and Houart, 2004).

To date FGFs, WNTs, BMPs and retinoic acid have been proposed as caudalizing factors (Villaneuva et al., 2002). In this model, the initial regionalization of the neural plate is again established by the gradient of secreted WNTs and other caudalizing signals expressed by the nascent neural plate (Dorsky et al., 2003). Thereafter, gradients of *WNT* activity are locally controlled and established by regional and spatial expression of agonists and antagonists of *WNT* signaling. This localized expression pattern further refines regional development (Houart et al., 2002).

One consequence of the initial dorsal ventral (DV) -AP patterning is the regionalization of the neural plate that entails the establishment of cell populations such as the floorplate and isthmic organizer, that are local sources of signaling within the neuroectoderm (Wilson and Houart, 2004). These local organizers mediate and regulate initial regional patterning such that, by the end of gastrulation, the expression domains of many newly induced genes begin to subdivide the neural plate into discrete territories that preshape the various structures of the mature CNS (Wilson and Houart, 2004).

## 1.2. Development of the forebrain

The cerebral cortex of the forebrain is the major component distinguishing the human brain from that of other vertebrates and mammals. Most human attributes derive from this region of the brain which is larger and more elaborate compared to other vertebrates and mammals, while it maintains the relatively conserved general organization.

The forebrain arises from a simple sheet of neuroepithelial cells of the anterior neuroectoderm protected from the caudalization factors (Wilson and Houart, 2004). The forebrain will later give rise to the telencephalon and diencephalon. Once the specification of the anterior neural plate is complete, the eyes will develop from the vesicles forming from the ventral forebrain (Wilson and Houart, 2004).

Suppression of *BMP* activity is an important step in the establishment of the prospective forebrain (Bachiller et al., 2000). Within the forming neural plate, lower levels of graded *BMP* activity contribute to the initial establishment of lateral (marginal) to medial pattern (Wilson and Houart, 2004). The induction, movements, patterning and maintenance of rostral CNS structures is influenced by the underlying mesendoderm (Wilson and Houart, 2004). Additionally, *WNT* and *WNT* antagonists mediate the patterning of forebrain structures (Kiecker and Niehrs, 2001b). The repression of *WNT* by *WNT* antagonists is essential for vertebrate telencephalic development, while *WNT* signaling promotes the development of the caudal region of the forebrain including the diencephalon. Two families of transcription factors that affect the *WNT* signaling pathway have been identified. The Sine-oculis (SIX) proteins promote anterior, and the Iroquois (IRX) proteins, the posterior fates within the prospective forebrain and midbrain regions of neural plate (Lagutin et al.,

2003). They are expressed in mutually exclusive regions of the brain (Wilson and Houart, 2004). In mice, the absence of Six3 protein, a *Wnt* antagonist, leads to an excess of Wnt in prospective forebrain tissue and subsequent loss of telencephalic tissue (Lagutin et al., 2003). Prospective caudal diencephalon and midbrain express various *IROQUOIX* (*IRX)* genes that are potential effectors of the WNT pathway (Wilson and Houart, 2004). The domain of *IRX* expression will subdivide into the diencephalon and the midbrain, most probably by further interaction of the IRX with *PAIRED BOX GENE 6 (PAX6, MIM#607108)* of the forebrain and Engrailed proteins of the prospective midbrain (Scholpp et al., 2003).

The zona limitans intrathalamica (zli) is localized by the interaction between *SIX* and *IRX* gene expression (Wilson and Houart, 2004) and secretes SHH and possibly other regulating signals that control the growth of the dorsal forebrain tissue (Kiecker and Lumsden, 2004) and defines the cell boundary of the telencephalon. The development of the hypothalamus is induced by the expression of *HH* genes in the prechordal mesendodermal tissue. Mice lacking Shh activity do not develop hypothalamic tissue (Chiang et al., 1996; Ohkubo et al., 2002). The expansion of posterior ventral hypothalamus resulting from abrogation of *HH* genes (Mathieu et al., 2002) implies that they also regulate DV patterning of the forebrain structures.

In addition to *WNT* antagonists, members of the FGF family of proteins with a significant role in a wide variety of developmental events, *FGF3* (MIM#164950) and *FGF8 (MIM#600483)*, are expressed at the anterior neural border (ANB). The *FGF* signaling pathways have profound roles in the regional patterning and polarization of the telencephalic neuroepithelium (Wilson and Houart, 2004). It has been shown that *WNT* antagonists locally induce *FGF8* expression in the anterior neural plates (Houart et al., 2002) suggesting that the ANB is established as a source of *FGF* signals consequent to local repression of *WNT* activity. Reduced FGF activity leads to proliferation and apoptosis defects in the telencephalon (Storm et al., 2003), midline defects (Walshe and Mason, 2003) and defects in morphogenesis of olfactory bulb (Herbert et al., 2003). High levels of FGF activity promote anterior cortical fates (Grove and Fukuchi-Shimogori, 2003) and polarize the neocortex. In the telencephalon, *FGF* activity represses (Storm et al., 2003) and is reciprocally modulated by *EMPTY SPIRACLES HOMOLOGUE 2 (EMX2, MIM#6000354)* transcription factor expression (Fukuchi-Shimogori and Grove, 2003). Polarization of the entire telencephalic neuroepithelium could conceivably be initiated by ANB-derived *FGF* signals at this stage.

Expression of *FGFs* and cellular response to it seem to be regulated by SIX3 and IRX proteins (Lagutin et al., 2003).

# 1.3. Development of the cortex

Cortical development entails the generation of stem cells, their differentiation into neurons and glia, migration to the cortex, and organization into functional layers. The cerebral cortex is formed by the migration of the neurons and glial cells originating from the ventricles of the brain (Boulder Committee, 1970). A thick layer, the proliferative neuroepithelium, forms around the ventricles as the multipotential precursor cells migrate. The pre-plate of the provisional cortex is the result of the first wave of migration, which is later replaced by the permanent cortical plate (Nadarajah et al., 2003). The permanent cortex is formed by neuronal migration in an "inside out, outside last" pattern. Neuronal migration occurs in two patterns: radial and tangential (Nadarajah et al., 2003).

Early glutamatergic neuronal migration to the cortex is radial and guided by radial glia which are neuronal and glial precursors. The nuclei of radial glial cells are located in the walls of the ventricles and their processes stretch vertically to the pial surface (Agamanolis, 2010) forming a scaffold along which the neurons migrate. In addition to adhesion molecules present on their membranes and on the radial glial fibers, neuronal migration is guided by chemical signals, some of which are produced by the pre-plate (Agamanolis, 2010).

The later neuronal migration of GABAergic (inhibitory) neurons occurs in a tangential direction. The GABAergic neurons are generated in the primordia of the basal ganglia. It appears that they are guided by signals that will determine their cortical layers (Nadarajah et al., 2003).

The proliferative neuroepithelium produces a surplus of neurons; those that do not establish functional synapses die, while others are eliminated by genetically programmed apoptosis (Agamanolis, 2010).

It is known that neurogenesis in fish, amphibians, birds, and rodents continues after birth, and until recently, it was believed that neurogenesis and migration in primates, except for the hippocampus and the granular layer of the cerebellum, is completed by mid-gestation.

Recent research shows that neurogenesis also occurs in the adult brain and that new neurons are generated in the periventricular area and migrate to the neocortex.

The cortex is organized in six layers with distinct morphological, projectile and neuronal characteristics. The layer restricted expression of a multitude of genes orchestrates the sequential and localized development of the layers of the cortex. *PAX6* and *EMX2* are expressed regionally in the neuroepithelium of the dorsal telencephalon and initiate morphogenesis of the cerebral cortex (Molyneaux et al., 2007). It is believed that these gene products confer regional organization by producing a regional expression gradient.

A multitude of other genes are involved, some of which have been identified by their expression at developmental stages. Elimination of *NEUROGENIN1 (NGN1, MIM#601726*) and *NEUROGENIN2 (NGN2, MIM#606624)* function appears to affect mostly the determination of neurons born early in corticogenesis which will migrate into the lower cortical layers (Schuurmans et al., 2004). *PAX6* is required for the specification of neurons born in the later stages of corticogenesis which migrate into the upper cortical layers (Schuurmans et al., 2004). The *T-CELL LEUKEMIA HOMEOBOX 1*(*TLX1, MIM#186770)* genes encoding a nuclear orphan receptor is also involved in the upper layering of the cortex. *CADHERIN-6 (MIM#603007)* and *EPHRIN-A5(MIM#601535)* are predominantly expressed in the parietal cortex, while *LIM DOMAIN ONLY 4 (LMO4, MIM#603129)* and *C-TERMINAL LIM DOMAIN 1A (CLIM1A)* are confined to the upper layers of the frontal and occipital cortex, respectively (Molyneaux et al 2007; Guillemot et al., 2006). Some genes that have been identified in cortical layering are presented in Table I.1.

# 1.4. Development of the cerebellum

The cerebellum is the region of the brain involved in motor control. The cerebellum arises from the anterior hindbrain and is positioned along the anterior/posterior axis of the neural tube by *FGF* and *WNT* signals from the isthmic organizer located at the mid hindbrain junction (Sato et al., 2004). The secretion of BMP, WNT and retinoic acid from the adjacent fourth ventricle roof plate influences the development (Chizhov et al., 2006). The progenitor

cells of the cerebellar ventricular zone express the bHLH transcription factor, Pancreas specific transcription factor 1A (PTF1A, MIM#607194) and migrate into the

Table I.1 Genes identified in the layering of the cortex (adapted from Molecular Mechanisms of Cortical Differentiation, Guillemot et al. 2006)

| **Gene** | **Layer 1** | **Layer2/3** | **Layer 4** | **Layer 5** | **Layer 6** | **Layer 6b** |
|---|---|---|---|---|---|---|
| *BHLHB5* | | + | + | | | |
| *BRN2* | | + | | | | |
| *CLIM1A* | | | | + | | |
| *CTIP2* | | + | + | + | | |
| *CUX2* | | + | + | | | |
| *DELTEX LIKE* | | + | + | | | |
| *ER81* | | | | + | | |
| *FAX01* | | + | | | | |
| *KlF6* | | | + | | | |
| *KORA1* | | | | + | | |
| *LATEXIN* | | | | | + | |
| *LMO3* | | + | + | | + | |
| *LMO4* | | + | + | | + | |
| *ME-2C* | | + | + | | | |
| *NR4A3* | | | | + | + | |
| *OTX1* | | | | + | + | |
| *REELIN* | + | | | | | |
| *ROR-B* | | | + | | | |
| *SATB 1* | + | | | + | + | + |
| *SATB 2* | | + | + | | | |
| *SCF* | | + | + | | | |
| *SCIP* | | + | | | | |
| *SVET1* | | + | + | | | |
| *TBR1* | + | | | | + | + |
| *UNC5H4* | | | + | | | |
| *ZFP312* | | | | + | + | |

cerebellar structure giving rise to all of the GABAergic cerebellar cell types: the glutamatergic deep cerebellar nuclei and cerebellar internuclei. Progenitor cells of the

rhombic lip express a different bHLH transcription factor, MATH1 and form the pontine nuclei and the external granular layer (Chizhikov et al., 2003). Proliferation of the granular layer is then stimulated by *SHH* signaling from the underlying Purkinje cells (Millen and Gleeson, 2008).

In murine and chick models, the diencephalic-mesencephalic boundary appears to form at least in part from interactions between the *PAX6, PAX2 (MIM#167409), ENGRAILED-1* and *ENGRAILED-2 (EN1, MIM#131290* and *EN2, MIM#131310)* genes and an established *PAX6* gradient (Barkovich et al., 2009). Expression of *PAX6* represses *PAX2* and *EN1* in the region of high *PAX6* expression conferring diencephalic fate. Expression of *PAX6* is repressed by *EN2* resulting in a low *PAX6* expression region conferring mesencephalic fate (Barkovich et al., 2009). The interaction between *ORTHODENTICAL HOMEOBOX 2 (OTX2, MIM#600037)* and *GASTRULATION BRAIN HOMEOBOX 2 (GBX2, MIM#601135)* specifies the location of the isthmus organizer which is essential to cerebellar and normal brainstem development (Nakamura et al., 2005).

## 1.5. Development of corpus callosum

Among the pathways that are developing at this stage is the bundle of nerves that will connect the two brain hemispheres and form the corpus callosum. The axons are guided along the glial wedge at the central component where the corpus callosum will form into the contralateral hemisphere and away from the wedge. This is accomplished by the individual and concerted role of both attractive- guidance, and negative- repellant, secretions. The pioneer axons that cross the midline secrete the guidance receptor neurophilin 1 and provide a path for later arriving neocortical axons. Other similar, as of yet unrecognized, secretions must exist that will provide the axons of the more caudal regions of the cortex with a pathway. Two signaling pathways have been identified: the SLIT-ROBO and the WNT-RYK pathways (Paul et al., 2007). SLIT2 is secreted by the glia dorsal to the corpus callosum which may help guide the axons to and across the wedge. The conditional fibroblast growth factor receptor/glial fibrillary acidic protein (FGFR1/GFAP) appears to be involved in this formation, as the elimination of FGFR1 from glia prevents the formation of the corpus callosum (Paul et al., 2007). *FGFR1* (MIM#136350) knock out in mice prevents the

migration of cells to, and the formation of the midline glial structures in earlier development suggesting that the formation of the midline glial structures at the cortico-septal boundary is among other roles of *FGFR1* (Tole et al., 2006). In *NUCLEAR FACTOR I/A (MIM#600727) and I/B(MIM#600728) (Nfia-*) and (*Nfib-)* knockout mice, the glial wedge is absent and the corpus callosum does not form (Shu et al., 2003; Steele-Perkins et al., 2005). However, in *Fgfr1* heterozygotes and *growth associated protein 43 (Gap43)* negative mice glial midline structures expressing *Slit2* form, but the axons fail to cross suggesting the involvement of other mechanisms (Shen et al., 2002). Severing the subcallosal sling in mice fetuses leads to the failure of corpus callosum formation. Recognition of target cells by axons, probably requires various other mechanisms that control the pruning and insertion of the axons (Paul et al., 2007).

# 2. Brain malformations

Brain malformations result from flawed development and are considered to be caused by chromosomal aberrations, single gene defects, imbalance of factors that control gene expression, or multifactorial patterns of inheritance. Distinguishing malformations from disruptions, that may result from environmental or extrinsic factors such as fetal infection during the developmental period, is often difficult. One major distinction that can often be referred to is that malformations tend to be either midline, or bilateral and symmetric.

Among common environmental factors that can mimic malformations in the brain are viral infections such as Cytomegalovirus (CMV), especially when contracted before midgestation, can cause microcephaly and polymicrogyria (Agamanolis 2010).

## 2.1 Classification of brain malformations

Classically, brain malformations are classified according to the morphological and structural criteria. With recent advances in the understanding of underlying molecular mechanisms involved in the development of the brain, attempts are being made to categorize these malformations according to the underlying genetic factors. To complete the new

classification and for its acceptance into clinical applications, further studies regarding the molecular mechanisms are required. The morphological structural classification is initially presented and thereafter the more modern approach of classification by underlying molecular mechanism is discussed.

## 2.1.1. Midline defects

Midline defects are the group of malformations that affect the midline structures of the brain. The two main malformations include the holoprosencephalies and various degrees of agenesis of the corpus callosum.

### 2.1.1.1. Holoprosencephaly

Between the fourth to sixth weeks of gestation the forebrain begins its cleavage into two hemispheres. Holoprosencephaly (HPE) includes a range of defects of incomplete cleavage of the two hemispheres that are distinguished by the degree of this cleavage. Complete absence of cleavage is called alobar and the brain consists of a single spherical forebrain structure with a single ventricle. Partial separation of posterior region of hemispheres is called semilobar, and separation of right and left hemispheres with various degrees of fusion is called lobar. In cases where there is fusion in the basal ganglia, thalami and absence of the body of the corpus callosum, the term middle interhemispheric fusion variant is used.

In 80% of cases, a visible midline defect is also present in the facial features and may vary from the most severe case where there is only one eye (cyclopia), one nostril, to hypotelorism, cleft lip and/or palate.

The pathogenesis of HPE is felt to involve multiple interacting genetic and environmental factors (Ming and Muenke, 2002). Most cases are sporadic, 25-50% have a numeric or structural chromosomal abnormality. Copy number variants have been detected in 10-25% of cases (Bendavid, 2009). Several candidate genes have been identified. One of the earliest genes identified is the *SHH* (MIM#600725) gene on 7q36 (MIM#142945). It has become obvious that various other genes involved in the *SHH* pathway are implicated in the etiology of HPE. Other genes are the *ZINC FINGER IN CEREBELLUM* 2 *(ZIC2, MIM#603073), SIX3, TALE FAMILY HOMEOBOX (TGIF, MIM#602360), GLI FAMILY ZINC FINGER 2 (GLI2, MIM#165230),* and *PATCHED-1 (PTCH1, MIM#601309)* (Dubourg et al., 2004). In

addition to a role in mediating the response to Sonic hedgehog protein signaling, *ZIC2* may have a role in axial midline establishment in early development and later, in the development of the dorsal telencephalon (Warr et al., 2008). *SIX3* (MIM#603714) is considered to be responsible for the activation of lens specification during eye formation, and transcriptional repression of *NODAL* (MIM#601265), *WNT*, and *BMP* signaling targets; it appears to influence cellular fate in the developing forebrain and to regulate *SHH* in the ventral forebrain (Geng et al., 2008). *TGIF1* (MIM# 602630) modulates the TGF beta pathway, components of which have been shown to be involved in HPE in animal models (Dubourg et al., 2004). Three *GLI* genes have been implicated in vertebrate Shh signal mediation, with *GLI2* acting as the central transcriptional activator in animal models (Roessler et al., 2005). *PTCH1*, the receptor for *SHH*, normally represses SHH signaling, which is relieved when *SHH* binds to *PTCH1* (Roessler et al., 2010).

## 2.1.1.2. Corpus callosum agenesis

The corpus callosum is the largest connective structure in the brain consisting of over 190 million axons that transfer information between the two hemispheres (Tomasch, 1954). Agenesis of corpus callosum (AgCC) occurs in 1/4000 individuals and encompasses the agenesis or hypogenesis of the corpus callosum. It can result from a disruption in cellular proliferation and migration, or in axonal growth or glial patterning at the midline. It can occur as an isolated defect, or as is most often the case, with other CNS or non CNS malformations.

There is evidence that different molecular mechanisms, including single gene mutations, either sporadic or familial and polygenic/multifactorial inheritance have a role in the etiology of corpus callosum agenesis. 30-45% of AgCC have recognizable causes: 10% have chromosomal aberrations, 20-35% have recognizable genetic syndromes (Bedeschi et al., 2006). Examples of Mendelian disorders are X-linked lissencephaly with AgCC and ambiguous genitalia (XLAG, MIM#300004) resulting from mutation in *ARISTALESS-RELATED HOMEOBOX* gene *(ARX*, MIM#300382) and corpus callosum agenesis, retardation, adducted thumbs, spastic paraplegia and hydrocephalus syndrome (CRASH, MIM#303350) resulting from a mutation in *L1 CELL ADHESION MOLECULE* (*L1CAM*, MIM#308840) gene that encodes a transmembrane cell adhesion protein. *L1CAM* disruption leads to hydrocephalus and causes agenesis of corpus callosum through interaction with other proteins (Itoh et al., 2004).

The Mowat-Wilson syndrome (MIM#235730) which manifests as Hirschsprung disease, congenital heart disease, genitourinary anomalies, microcephaly, epilepsy and severe cognitive impairment in addition to AgCC is an example of the complex genetics of corpus callosum agenesis. A heterozygous mutation in the gene *ZINC FINGER HOMEOBOX 1B* (*ZFHX1B,* MIM#605802) on chromosome 2q22 which codes SMAD interacting protein 1 (SIP1) causes the Mowat-Wilson syndrome. However, not all mutation carriers have AgCC. It appears that gene dosage of *SIP1* (MIM#602595) and its interaction with other genetic factors determines the callosal development (Mowat et al., 2003). Another example of complex genetics is the Sotos syndrome (MIM#117550), which is caused by the haploinsufficiency of the *NUCLEAR RECEPTOR-BINDING su-var (NSD1*, MIM#606681*)* gene. In some patients the haploinsufficiency can lead to corpus callosum agenesis, indicating that other genetic factors will influence the callosal formation (Schaeffer, 1997).

## 2.1.2 Neuronal migration defects

The process of neuronal migration and cortical organization is tied to the process of cortical folding. As there is extensive overlap between the generation and differentiation of stem cells into neurons and glia, and their migration and organization into the functional layers of the cortex, impairments in any of these processes can lead to a variety of malformations. These malformations are classified together as neuronal migrational defects. The major types consist of abnormal neuronal-glial proliferation or apoptosis which will manifest as microcephaly or megalencephaly, abnormal neuronal migration presenting as subependymal (periventricular) heterotopias, lissencephaly, subcortical band heterotopias, and cobblestone cortex. Abnormal cortical organization that involves the final stages of cortical migration, and most prominently, folding is manifested in polymicrogyria and cortical microdysgenesis.

Most neuronal migration disorders have a genetic basis, but genotypes and phenotypes overlap. Same gene mutations can cause different phenotypes by affecting protein functions differently. The presence of somatic mosaicism, or skewed X-inactivation can alter the severity of the malformation and hence, the phenotype.

### 2.1.2.1. Abnormal neuronal migration

The absence of neuronal migration from the ventricles results in periventricular heterotopias and half way migration, in subcortical band heterotopias. In cases where a small proportion

of neurons reach the cortex, there is a consecutive absence or decrease of cortical layers. They present as the "smooth brain" and are called lissencephaly and pachygyria. In some instances, neurons overshoot the cortex and reach the subarachnoid space, and are called marginal-leptomeningeal glioneuronal heterotopias, or cobblestone cortex.

The best recognized gene in the etiology of neuronal migration defects is the *reelin* gene expressed by the Cajal-Retzius cells of the preplate, the protein product of which acts as a signaling factor for migrating neurons.

### 2.1.2.1.1 Gray matter heterotopia

Gray matter heterotopias are common malformations of cortical development. Affected patients show presence of cortical cells in heterotopic positions, suggesting migration abnormalities. It appears that they result from a severe neuronal migration defect: a failure to migrate and subsequent differentiation at their original position. Based on the location of their formation, heterotopias are generally divided into three groups: periventricular, subcortical, and band heterotopia. They can be bilateral or unilateral, unique or numerous.

**Periventricular heterotopias (PVH)** line both ventricles. They are presumed to be situated close to their site of generation. In the mildest form, they are predominantly associated with seizures in individuals with normal intelligence. In more severe forms, individuals may have intellectual disability, dyslexia and difficulty with reading and writing. Less commonly, individuals with this brain malformation may have other severe brain malformations, developmental delays, and other problems.

Certain forms of bilateral periventricular heterotopias (MIM#300049) arise from loss of function mutations in *FILAMIN A (FLNA*, MIM#300017*)* (Masruha et al., 2006). *FLNA* is found on the X chromosome and mutations are most often lethal in males. *FLNA* is an actin binding protein linking beta proteins at plasma membrane to the cytoskeleton by interacting with integrins and transmembrane receptor complexes. Thus *FLNA* anchors the membrane proteins to actin cytoskeleton and is important for the integrity of the cytoskeleton and cellular movement.

A distinct form of PVH, associated with microcephaly (MIM#608097), is caused by mutations in *ADP-RIBOSYLATION FACTOR GUANINE NUCLEOTIDE-EXCHANGE FACTOR 2 (ARFGEF2*, MIM#605371*)*, encoding a vesicle and membrane trafficking

protein. The gene is expressed in progenitor cells and in migrating neurons. Mutations disrupt expression of these transmembrane proteins or cell adhesion molecules that are important for proliferation and migration. Variations on short arm of chromosome 5 have also been associated with this malformation (Sheen et al., 2003).

**Subcortical heterotopia** is characterized by the formation of distinct, most often focal nodes in the white matter. In general, patients present fixed neurologic deficits and develop partial epilepsy between the ages of 6 and 10. Patients with focal subcortical heterotopia have a variable motor and intellectual disturbance depending on the size and site of the heterotopion. The more extensive the subcortical heterotopia, the greater the deficit, bilateral heterotopias are almost invariably associated with severe developmental or intellectual disability. The cortex itself often suffers from an absence of gray matter and may be unusually thin or lack deep sulci.

**Subcortical band heterotopias (SBH)** refer to a band of heterotopic gray matter located just beneath the cortex and separated from it by a thin zone of normal white matter. Band heterotopia may be complete, surrounded by simple white matter, or partial. The frontal lobes seem to be more frequently involved when it is partial. Patients with band heterotopia may present at any age with variable developmental delay and seizure disorder, which vary widely in severity. Many cases of SBH are associated with lissencephaly or part of the spectrum of defects caused by the same genes that will be discussed presently.

### 2.1.2.1.2 Lissencephaly-Agyria (type 1 lissencephaly)

Lissencephaly means smooth brain, a term derived from the appearance of the brain in this disorder. The cortex becomes thick as a result of the lack of formation of the normal folds (gyri) and grooves (sulci) of the normal brain. It results from the migration of neurons from the ventricles but their failure to reach their final destination during the $12^{th}$ to $24^{th}$ weeks of gestation. Other terms, agyria, describing the lack of gyri and pachygyria, describing the broad gyri, are variations within the same malformation. Pachygyria is a milder variant of lissencephaly, characterized by broad gyri and a thick cortex with abnormal cytoarchitecture.

Children with various forms of lissencephaly are severely neurologically impaired and often die within several months of birth. Most lissencephaly cases are caused by *PLATELET-ACTIVATING FACTOR ACETYLHYDROLASE, ISOFORM 1B ALPHA SUBUNIT*

*(PAFAH1B1, MIM#601545)* mutations or heterozygotic loss of the gene (MIM#607432). The PAFAH1B1 protein is localized in microtubules. Various possible regulatory roles have been proposed for the protein, most of which are associated with cell division, migration, and intracellular transport. It has been postulated that PAFAH1B1 forms complexes with other proteins and is crucial in regulating the microtubule motor protein cytoplasmic dynein. This connection supports a role for PAFAH1B1 in cell motility, and suggests that defects in dynein mediated movement are responsible for lissencephaly.

*DOUBLECORTIN (DCX,* MIM#300121*)* gene on Xq22.3-q23 is the cause of X-linked lissencephaly (MIM#300067) and encodes the doublecortin protein that is also a microtubule (MT) associated protein. *DCX* is expressed by neuronal precursor cells and immature neurons in embryonic and adult cortical structures. Neuronal precursor cells begin to express *DCX* while actively dividing, and their neuronal daughter cells continue to express *DCX* for 2–3 weeks as the cells mature into neurons. *DCX* is not expressed in proliferating cells of the ventricular zone. It is expressed in postmigratory neurons of the cortical plate but not in mature neurons of the adult brain. Downregulation of *DCX* begins after 2 weeks, and occurs at the same time that these cells begin to express Neuronal specific antigen (NeuN), a marker for mature neurons. The regulation of MT cytoskeleton is critical for correct neuronal migration. It may be involved in vesicle trafficking, regulation of cell adhesion and protein stability and microtubule actin crosstalk.

As the brain malformation is caused by the degree of defect in underlying mechanism affected by the gene mutations, the same gene can lead to various phenotypic manifestations. Mutations in *DCX* and *PAFAH1B1* can also lead to subcortical bands. It appears that those restricted to the frontal lobes are more typically associated with mutations of *DCX*, while subcortical bands restricted to the posterior lobes are more typically associated with *PAFAH1B1* mutations (Dobyns et al., 1999).

Among other genes identified in relation to lissencephaly are *ARX* and *REEELIN (RELN,* MIM#600514) genes. Mutations in *ARX* are the cause of lissencephaly and agenesis of corpus callosum. *ARX* is a member of the group-II aristaless-related protein family whose members are expressed primarily in the central and/or peripheral nervous system. This gene is involved in CNS and pancreas development.

Mutations in *TUBULIN A1A (TUBA1A*, MIM#602529) gene have been shown to cause pachygyria in patients with microcephaly, mental retardation and additional cortical malformations (Poirier et al., 2007).

#### 2.1.2.1.3 Reelin-Type Pachygyria

Mutations in *RELN* are responsible for a form of lissencephaly, pachygyria, and cerebellar hypoplasia (MIM#257320). Reelin is a protein that promotes the differentiation of progenitor cells into radial glia. Reelin-secreting cells create a gradient that direct the fibers in the direction of its higher concentration. By affecting the orientation of these glial fibers, which in turn guide the migrating neuroblasts, it regulates the processes of neuronal migration and positioning in the developing brain.

In the mutant *reeler* mouse, where reelin is absent, the order of cortical layering becomes roughly inverted. Subplate neurons fail to stop and invade the upper most layers and consequently mix with Cajal-Retzius cells and other second layer cells. The marginal zone is displaced by a superplate while *PAFAH1B1* and *DCX* molecular layer is preserved. There is controversial evidence supporting the fact that the protein is a stop signal for the migrating cells. Other mechanism of action being considered is that it makes the cells more susceptible to some yet undescribed positional signaling cascade.

Reelin regulates neuronal migration and lamination via the RELN-DAB1 signaling pathway. It binds to very low density lipoprotein receptor (VLDLR, MIM#192977) and apolipoprotein E receptor type 2 expressed by migrating cells thus triggering a signaling cascade leading to tri phosphorylation of the cytoplasmic adapter Disabled-1 (DAB1). Mutations of *RELN*, *DAB1* (MIM#603448) and combined lipoprotein receptors in mice give rise to a reeler phenotype where cortical layers are not formed in a classical inside out fashion but instead neurons accumulate beneath the preplate in the order in which they are formed.

The RELN-DAB-1 pathway acts at the end stages of radial migration based on *REELIN* expression in the marginal zone. It is possible that *PAFAH1B1* and *DCX* act during earlier stages of migration, directly affecting the motility of migrating neurons and hence producing a more severe disruption of the cortical architecture.

Mutations in *VLDLR* gene cause cerebellar hypoplasia with cerebral gyral simplification, thickening of cerebral cortex, moderate to severe MR and ataxia (MIM#224050). The brain malformation in this instance is more cerebellar than cortical.

#### 2.1.2.1.4 Cobblestone cortex (type 2 lissencephaly)

This brain malformation is characterized by irregular grooves. The normally organized cortical layering is replaced by an irregular layering of neurons and molecules resulting from the inability of neurons to integrate into the matrix. Sometimes referred to as Type II lissencephaly, cobblestone lissencephaly is also caused by abnormalities in neuronal migration. Unlike classical lissencephaly where neurons do not migrate to the destination, in cobblestone lissencephaly they often move beyond the destination because of their inability to integrate. The result is the appearance of a bumpy cobblestone surface.

The cobblestone lissencephalies fall into three categories: (1) Fukuyama Congenital Muscular Dystrophy (MIM#253800); (2) Finnish muscle eye brain disorder (MIM#253280); and (3) Walker Warburg syndrome (MIM#236670, #613150). Symptoms of each disorder are unique, except that most individuals present with muscular and eye abnormalities as well as the characteristic cobblestone cortex. Four genes have been implicated in these syndromes: Walker Warburg syndrome is caused by mutations in *PROTEIN O-MANNOSYLTRANSFERASES 1* and *2* (*POMT1* MIM#607423 and *POMT2*, MIM#607439), Finnish muscle eye brain disorder by mutations in *O-MANNOSE BETA-1,2-N-GLUCOSYLAMINYLTRANSFERASE 1 (POMGNT1,* MIM#606822), and Fukuyama Congenital Muscular Dystrophy by mutations in *FUKUTIN* (MIM#607440).

They are caused by mutations in proteins that affect glycosylation. *POMT1*, and *POMT2* are members of an evolutionarily conserved family of proteins O-mannosyltransferases, and *POMGNT1* participates in O-mannosyl glycan synthesis. O-mannosylation is an important protein modification in eukaryotes. In yeast, these enzymes are located in the endoplasmic reticulum (ER) and are required for cell integrity and cell wall rigidity.

### 2.1.2.2 Disorders of neuronal organization

This classification is an extension and overlap of the neuronal migration disorders and involves the later stages of cortical organization. There are two major types of neuronal

migration disorders that can be grouped together in this category: the cortical dysplasias, and the polymicrogyria.

#### 2.1.2.2.1 Focal cortical dysplasia-microdysgenesis

The failure of neurons in an area of the brain to migrate or differentiate in the proper formation results in a focally thickened cortex with a disordered cytoarchitecture. The neurons are large, and abnormally oriented with hypertrophic astrocytes. Focal cortical dysplasia is a common cause of intractable epilepsy in children and is a frequent cause of epilepsy in adults. All forms of focal cortical dysplasia lead to disorganization of the normal structure of the cerebral cortex. Focal cortical dysplasia associated with enlarged cells is known as FCDIIB. The enlarged cells have large elliptical shape, displaced nucleus, and lack dendrites or axons; they are believed to be derived from neuronal or glial progenitor cells.

#### 2.1.2.2.2 Polymicrogyria

The association of polymicrogyria and schizencephaly is so close that the two entities are considered as part of a spectrum. Polymicrogyria is a disorder resulting in structurally abnormal cerebral hemispheres. As the name implies, the brain has excessive number of small gyri and consequently shallow sulci resulting from overfolding of cerebral hemispheres. The cortex is thickened with neuronal heterotopia and enlarged ventricles. Polymicrogyria can be diffuse or focal, bilateral or unilateral, symmetric or asymmetric. It is diverse in its etiology and pathogenesis. In some cases, where the disruption occurs in the four layered cortex, a post migration effect is indicated, and where there is an absence of layers, the disruption may have occurred earlier in development or may be some continuum of early and post migration effects. Patients with polymicrogyria have severe psychomotor retardation and seizures.

Some forms of polymicrogyria have a genetic basis. Bilateral frontal polymicrogyria (MIM#606854) is associated with mutations of *G-PROTEIN COUPLED RECEPTOR 56 (GPR56,* MIM#604110*).* The protein encoded by this gene is widely expressed throughout the body. It appears to be essential during human cerebral cortical development and patterning. However, individuals with a variety of other polymicrogyria syndromes including bilateral frontal polymicrogyria, bilateral perisylvian polymicrogyria, and bilateral generalized polymicrogyria have not been found to have mutations in *GPR56*.

Polymicrogyria and other developmental abnormalities have been observed in patients with deletion 22q11.21 (MIM#188400) (Sztriha et al., 2004; Robin et al., 2006). A potential locus for Aicardi syndrome associated with polymicrogyria and other cerebral malformations (Barkovich et al., 2001) for this condition at Xp22 has not been confirmed.

Other forms of polymicrogyria are acquired and are caused by disruptions. Polymicrogyria often occurs with fetal cytomegalovirus infection and prenatal hypoxic-ischemic encephalopathy, including vascular problems related to twinning.

## 2.1.2.3 Abnormal glial or neural proliferation/apoptosis

The size of the brain is the result of a delicate interplay between the proliferating and apoptotic factors and related to equilibrium between mitosis and apoptosis. Lack of proliferation and/or increased apoptosis will lead to a subsequent decrease in brain size or microcephaly and increased proliferation without sufficient apoptosis will result in megalencephaly.

### 2.1.2.3.1 Microcephaly

Microcephaly comprises a heterogeneous group of conditions that are characterized by failure of normal brain growth and is defined as a head circumference that is two standard deviations smaller than the age- and sex-related average. It encompasses acquired microcephaly due to abnormal neuronal maturation and postnatal brain development which can be caused by many injurious or degenerative conditions such as intrauterine exposure to alcohol, drugs, viral infection with CMV and rubella.

Otherwise, microcephaly can be congenital, most probably due to perturbed neuronal proliferation during development which can result from defects in pattern formation, cell proliferation, cell survival, cell differentiation, or cell growth. The architecture of the cortex is intact. Congenital microcephaly can be present in a variety of chromosomal abnormalities, metabolic disorders or associated with syndromes.

Congenital microcephaly can also be accompanied by other structural brain malformations including migrational brain malformations, organizational brain malformations, midline defects or malformations of the posterior fossa. Characterized instances of these malformations include microlissencephaly (MIM#257320) or the Norman Roberts Syndrome

in which microcephaly is associated with lissencephaly or microcephaly with simplified gyral pattern (MIM#603802) characterized by simplification of the gyri. *TUBA1A* mutations have been reported in a family with microcephaly, pachygyria, and hypoplasia of corpus callosum, brain stem and inferior vermis (MIM#611603) (Poirier et al., 2007). In periventricular heterotopias caused by mutations in *ARFGEF2* and *FLNA*, microcephaly is a consistent finding. Cerebellar hypoplasia/agenesis accompanies microcephaly in mutations of *EOMES* (MIM#604615), *CASK* (MIM#300549) and *SLC15A19* (MIM #606521) in the Amish Lethal Microcephaly (MIM #607196).

Microcephalia vera, is an autosomal recessive condition associated with mental retardation, and rarely, seizures and is characterized by head circumference of -3 SD of normal. Currently seven loci have been identified to date *MCPH1,2,3,4,5,6,7*. Mutations in the *MICROCEPHALIN* (MIM#607117) *MCPH1* gene, which is responsible for DNA damage response and regulator of chromosome condensation lead to premature chromosome condensation (Jackson et al., 2002). *MCPH2* (MIM#604317) and *MCPH4* have been mapped to chromosome 19q13.1-2 and 15q15-21, respectively. Recently *WDR62* (MIM#613583) on chromosome 19 was proposed as the gene involved in causing MCPH2. The protein functions as a molecular link between proliferation and migration in neurogenesis (Wollnik, 2010). Mutations in the *CEP152* (MIM#613529) gene on 15q21 were identified by linkage analysis followed by genome sequencing in three unrelated individuals with microcephaly (Guernsey et al. 2010). The gene encodes a centrosomal protein with crucial role in cell motility. The cause of MCPH3 (OMIM#604804), *CDK5RAP2* (MIM#608201), is a neuronal CDC2-like kinase which is involved in the regulation of neuronal differentiation (Bond et al., 2005). The protein encoded by this gene may be involved in neuronal differentiation. The encoded protein may also be a substrate of neuronal CDC2-like kinase. MCPH6 (MIM#608393) is caused by defective Centromere protein J (CDNPJ), encoded by the *CENPJ* (MIM#609279) gene belongs to the centromere protein family (Bond et al., 2005). During cell division, this protein plays a structural role in the maintenance of centrosome integrity and normal spindle morphology, and it is involved in microtubule disassembly at the centrosome. The most common form of microcephaly MCPH5 (MIM#608716) is caused by mutations in the *ABNORMAL SPINDLE LIKE MICROCEPHALY (ASPM*, MIM#605481*)* gene which expresses a protein involved in normal mitotic spindle function in embryonic neuroblasts and neurogenesis (Bond et al.,

2002). Mutations in the *STIL* (MIM#181590) gene on 1p32 are known to cause MCPH7 (MIM#612703) (Darvish et al., 2010).

Many of these genes are involved in the mitotic spindle formation and the centrosome. The centrosome plays a major role in regulating cell division, functioning as a microtubule organizing centre (MTOC). The loss of the interphase astral microtubule array radiating from the centrosome to the cell cortex is one of the most dramatic intracellular changes occurring in mitosis. The centrosome functions in nucleating microtubule polymerization from free tubulin subunits and organizing the microtubule arrays.

## 2.1.2.3 Malformations of the posterior fossa

A common finding in malformations of posterior fossa is hydrocephalus which is dilatation of the cerebral ventricles resulting from any obstruction of the cerebrospinal fluid (CSF) circulation. The common obstructive causes of this malformation/disruption are hypersecretion, defective filtration or increase in volume of cerebrospinal fluid. Other causes include obstruction of structures of the posterior fossa, fibrosis of the subarachnoid space, and loss of brain tissue.

The cases of hydrocephalus, most often considered to be genetic, are aqueduct stenosis, Chiari malformation and the Dandy Walker malformations which are usually accompanied by other CNS malformations. Mutations in the *L1CAM* gene are usually associated with hydrocephalus due to congenital stenosis of Aqueduct of Sylvius. The L1 protein is a nerve cell membrane receptor with a role in the development and organization of neurons, myelin and synapse formation.

### 2.1.2.3.1 Dandy Walker syndrome (DWS)

This is the most common congenital malformation of the human cerebellum characterized by a severely hypoplastic cerebellar vermis and a significantly enlarged fourth ventricle in an enlarged posterior skull. They are divided into three closely associated forms: DWS malformation, DWS mega cisterna magna and DWS variant. The DWS malformation is the most severe presentation of the syndrome with an enlarged posterior fossa due to cystic dilatation of the fourth ventricle. The tentorium is malpositioned and partial or complete agenesis of the cerebellar vermis is present.

In Mega cisterna magna, the enlargement of the posterior fossa is secondary to enlargement of the cisterna by a large accumulation of CSF. The cerebellar vermis and the fourth ventricle are normal.

The third type is the variant, which is less common and represents the most wide-ranging set of symptoms and outcomes of DWS.

*ZINC FINGER IN CEREBELLUM* genes *ZIC1* (MIM#600470) and *ZIC4* (MIM#603073) are transcription factor genes postulated to be causative in human DWS. They are regulators of *SHH* signaling and may be involved in the development of cerebellar vermian hypoplasia (Grinberg, 2004).

## 2.2 Molecular classification of brain malformations

The classification that has been presented thus far is based on the structural and morphological changes of the brain. With the elucidation of the molecular mechanisms underlying the brain malformations, the classification is being consistently revised to incorporate these concepts. Some distinctions have been made and many malformations are being grouped together based on the molecular mechanism. This approach has introduced some molecular classifications that we will present here.

### 2.2.1 Peroxisome biogenesis disorders

These disorders are caused by defects in peroxisome function. Most commonly there is a mutation in the *PEX* genes that encode peroxin, proteins responsible for normal peroxisome assembly and biogenesis. A distinct group of Peroxisome biogenesis disorders (PBDs) including infantile Refsum disease (MIM#266510), neonatal adrenoleukodystrophy (MIM#202370), and Zellweger syndrome (ZSD) (MIM#214100) are autosomal recessive developmental brain disorders that also result in skeletal and craniofacial dysmorphism, liver dysfunction, progressive sensorineural hearing loss, and retinopathy.

Peroxisome biogenesis disorders- Zellweger syndrome spectrum (PBD-ZSS) are a continuum of the three phenotypes that were recognized long before the underlying mechanism had been identified. The ZSD phenotype is the most severe with earlier onset and death within the first year of life. The other two phenotypes are less severe with later onset and variable severity of phenotypic manifestations.

Mutations in twelve PEX genes have been identified in these patients (Gould et al. 2001; Matsumot et al., 2003; Shimozawa et al. 2004). Mutations in the two most commonly involved genes *PEX1* (MIM#602136) and *PEX6* (MIM#170993) are present in the range of clinical phenotypes. Mutations in the less frequently involved genes *PEX10 (MIM#602859)*, *PEX12 (MIM#601758)*, and *PEX26 (MIM#608666)* also show variable expressivity. Data and information regarding other genes are not sufficient to determine the involvement in the continuum of phenotypes. All mutations result in the over-accumulation of very long chain fatty acids and branched chain fatty acids, such as phytanic acid. In addition, PBD-ZSD patients show deficient levels of plasmalogens necessary for normal brain and lung function.

## 2.2.2 Glycosylation abnormalities

Congenital disorder of glycosylation (CDG) is a group of inherited disorders affecting the processing of glycoconjugates. Many disorders are known to arise from the hypoglycosylation of proteins as both normal N- and O- glycosylation is necessary for the proper function of many proteins. Presently 16 different subtypes of CDG-type I which involve N-glycosylation are recognized with highly heterogeneous phenotypic manifestations. In the most common form, PMM2-CDG, the genetic defect leads to loss of the phosphomannomutase 2 enzyme. An enlarged cistern magna and superior cerebellar cistern are observed in infancy to early childhood of affected patients. Other reported brain malformations include DW malformation, myelination abnormalities and various cerebellar findings. Ocular disorders such as coloboma, cataract and glaucoma are common findings.

Brain development is affected by disorders of O-glycosylation. Lack or deficient O-glycosylation of the alpha dystroglycan component of the dystrophin associated glycoprotein complex results in the inefficient attachment of cells to the extracellular matrix. Best recognized types of this disorder include the Walker Warburg, Finnish muscle eye brain, and Fukuyama muscular dystrophy syndromes. These disorders are characterized by cobblestone lissencephaly and caused by gene mutations in *POMT1*, *POMT2*, *POMGnT1* and *FUKUTIN*, respectively. In the morphological classification of brain structural malformations, these conditions are grouped together according to the cobblestone brain phenotype. However, in the molecular classification, two other conditions resulting from mutations in O-glycosylation enzyme encoding genes, fukutin-related protein (FKRP, MIM#606596), and LARGE are included within one classification. In addition to muscular dystrophy, patients with

homozygous mutations of *FKRP* have been reported to have cerebellar hypoplasia, lissencephaly, and cobblestone lissencephaly (MIM#613153) (Beltran-Valero de Bernabe et al., 2004). Similarly patients with LARGE homozygous mutations have been reported to have hydrocephalus, DWS, cerebellar malformations/ hypoplasia/agenesis (van Reeuwijk et al., 2007; Mercuri et al., 2009; Clement et al., 2008).

### 2.2.3 Ciliopathies

The ciliopathies include all disorders resulting from defects in genes regulating the structure and function of cilia, ciliated bodies and related structures. They include a variety of disorders many of which include malformations of the brain and manifest as abnormalities in brain development. As cilia are involved in the regulation of *WNT* and *HH* signaling pathways, they can disrupt the initial morphogenesis of the brain as well as other organs.

A number of human congenital cerebellar malformations are associated with deficiencies in genes required for cilia function. Joubert syndrome is a heterogeneous group of disorders characterized by hypoplasia of cerebellar vermis and a range of neurological involvement such as developmental delay. Of all the genes identified in the etiology of Joubert syndrome: *TMEM216* (MIM#613277), *AHI1* (MIM#608899), *NPHP1 (*MIM#256100), *NPHP4* (MIM#607215), *CEP290* (MIM#610142), *TMEM67* (MIM#609884), *RPGRIP1L* (MIM#610937), *ARL13B* (MIM#608922), *CC2D2A* (MIM#612013), *KIF7* (MIM#611254), *TCTN1* (MIM#609863), *TMEM237* (MIM#614423), *CEP41* (MIM#610523), *TMEM138* (MIM#614459), only *CXORF5* (MIM#300170) is not a ciliary gene or involved in ciliary function. Mutations in some of these genes are also cause of Meckel Gruber syndrome. As cilia line the ependydymal lining of the cerebral ventricles and conduct CSF flow, a form of hydrocephalus in mice is caused by defects in the axonemal dynein heavy chain gene *MDHNA5* (MIM#603335) (Ibanez-Tallon et al., 2004).

Many of the genes identified in other brain malformations such as *PAFAH1B1*, *DCX*, *14-3-3e* (MIM#605066) and in lissencephaly though not ciliary genes are involved and interact with microtubules, tubulin, and are involved in the proper function of cellular motility.

# 3. Identification of genes

A common ground for the identification of genes involved in brain development and their roles in this process has been the study and molecular analysis of the instances of specifically malformed brain. A proof in case is the identification of the roles of *PAFAH1B1*, *14-3-3e*, *DCX*, *RELN*, and *ARX* genes in neuronal migration through patients with lissencephaly, the *SHH* gene in morphogenesis by studying patients with holoprosencephaly, *MCPH1*, *ASPM*, *CDK5RAP*, *CENPJ* in the regulation of brain size by researching patients with microcephaly. The examples are numerous and the search is ongoing and continuous.

# 3.1 Historical overview

One of the first diagnostic tests for detection and analysis of the genotype was the routine karyotype. As early as 1957 when the extra chromosome 21 was identified in patients with Down syndrome (Lejeune et al., 1958), this test was implemented for the detection of chromosomal imbalances. Whole chromosome aneuploidies, or more specifically trisomy 21, account for the most frequent cause of mental retardation. Shortly thereafter, and with the development of banding techniques (Yunis et al., 1978), the detection of microscopically visible partial imbalances became feasible and led to the identification of many more chromosomal disorders. Among the first to have been characterized, is the cri du chat syndrome or 5p-. A number of other partial deletions were identified and categorically classified such as the 9p, 13q, 18q,… deletion syndromes. Better banding techniques and more knowledge of the structure of the chromosomes brought about the identification of smaller deletions such as the 22q11.21 deletion syndrome. Many genes such as familial adenomatous polyposis (Herrera et al., 1986), retinoblastoma (Lele et al., 1963), Wilms tumor, aniridia, genitourinary anomalies and mental retardation syndrome (WAGR, MIM#194072) (Riccardi et al., 1978) have been cloned through the identification of small deletions.

However, it soon became evident that not all cases of these deletion syndromes could be identified on the routine karyotype and techniques with more specificity and objectivity such as fluorescent in situ hybridization (FISH) became an adjunct to the routine karyotype. These deletions have been instrumental in the determination of a number of other contiguous gene syndromes (Schmickel, 1986). For example, successful application of systematic deletion

analysis led to the identification of *SHH, ZIC2, SIX3* and *TGIF* genes for holoprosencephaly (Munke,; Roessler et al., 1996; Brown et al., 1998; Wallis et al., 1999; Gripp et al., 2000).

Table I.2 Landmarks in the history of conventional cytogenetic and molecular cytogenetics (adapted from Andrieux and Sheth, 2009)

| Year | Discovery | Reference |
|---|---|---|
| 1959 | First chromosomal anomaly trisomy 21 | Lejeune |
| 1969 | Molecular hybridization of radioactive DNA | Pardue & Gall |
| 1976 | High resolution banding | Yunis |
| 1981 | Isotopic in situ hybridization | Harper & Saunders |
| 1984 | Direct FISH | Landegent |
| 1986 | Interphase FISH | Cremer et al., Pinkel et al. |
| 1989 | Combinatorial labeling | Nederlof et al |
| 1989 | Primed in situ hybridization | Koch et al. |
| 1990 | Identification of translocation in interphase cell by FISH | Tkachuk et al |
| 1990 | Technique of ratio labeling | Nederlof et al. |
| 1992 | Comparative Genomic hybridization | Kallioniemi et al. |
| 1993 | Fiber FISH | Parra & Windle |
| 1996 | Multiplex FISH | Speicher et al. |
| 1996 | SKY | Schrock et al |
| 1997 | Cross species color banding | Muller et al |
| 1997 | DNA array (matrix) CGH | Solinas-Toldo |
| 1997 | Padlock probe FISH | Nilsson et al |
| 1998 | Array CGH using BAC clones | Pinkel et al. |

After the application of FISH for specific microdeletion syndromes, subtelomeric FISH promised a universal technique that would simultaneously analyze a larger portion of the genome for those cryptic otherwise undetected imbalances. The use of FISH for identification of subtelomeric imbalances made possible the identification of various range of cytogenetically invisible cryptic imbalances (Ravnan et al., 2006). Many duplications and deletions of these regions were thus detected. But the test was limited by the fact that similar to all FISH based tests detected was limited and specific to the probe [Table I.2].

It probably came as a surprise when the first cases with apparently microscopically balanced chromosomal rearrangements would paradoxically express a phenotypic defect. One of the first cases of this paradox to have been exploited for gene identification and mapping was the balanced Xp21 translocation in a female with Duchenne muscular dystrophy (Jacobs et al., 1998). The analysis of this case led to the mapping of the dystrophin gene to this locus. It became evident that visibly detectable chromosomal rearrangements or small deletions could lead to the identification of genes. The association of some of these structural chromosome

anomalies, balanced or unbalanced, with single gene disorder, alone or in contiguous gene syndromes has led to the concept of Mendelian cytogenetics (Tommerup, 1993). Based upon this association, *de novo* translocations in phenotypically manifesting individuals have been widely used for the mapping and identification of disease genes.

However, this approach is limited by the resolution of the karyotype and its ability to detect deletions and/or duplications which would be in the range of 5–10 Mb in the better technical preparations. In addition, the contribution of individual genes to disease may not always be apparent in patients with complex phenotypes due to cytogenetically visible alterations.

## 3.2 Array Comparative Genomic Hybridization

As it became increasingly clear that many so-called microdeletion syndromes are largely or completely due to the phenotypic effects of haploinsufficiency for single genes, detection of microdeletions and microduplications gained significance in the quest for genes. Pertinent examples are the *RETINOIC ACID-INDUCED GENE-1* (*RAI1*, MIM#607642) gene in Smith–Magenis syndrome (MIM#182290) (Slager et al., 2003), the *UBE3A* (MIM#601623) gene in Angelman syndrome (MIM#105830) (Kishino et al., 1997), and the *TBX1* (MIM#602054) gene in deletion 22q11 syndrome (Lindsay et al., 2001). For the *PAFAH1B1* gene in Miller-Dieker syndrome, however, the situation is more complex. Although the deletion of this gene is responsible for lissencephaly (Dobyns et al., 1993), the concomitant deletion of the *14-3-3epsilon* gene also contributes to this brain phenotype (Cardoso et al., 2003; Toyo-Oka et al., 2003).

A promising technology that was developed and would enable objective detection of deletions and duplications was comparative genomic hybridization (du Manoir et al., 1993). This technique is based upon the comparison of two genomes, differentially labeled, and their ability to hybridize to a third fixed genome. Metaphase spreads fixed on slides were initially used as the third genome. The technique also requires a software analysis system that measures and compares the differential signal intensities of the two genomes, and positions the signals on the chromosomes, thus identification imbalances of the test genome in relation to the control. CGH was initially applied to tumor samples and later to the study of other patients.

Based upon the ability to measure the differential intensity of signals after hybridization to the fixed platform genome, the concept was further developed into the array based comparative genomic hybridization. The availability of human genome libraries and databases developed in the human genome project provided the groundwork for developing a higher resolution platform genome. Array comparative genomic hybridization takes advantage of the basic concepts of CGH and replaces the metaphase, as the platform genome, by arrays of genomic DNA from human libraries. The analysis tools were also provided for in the databases generated by the project. The test was first applied to the study of tumor cells by Pinkel et al. (1998) and soon became an attractive means of analyzing the genome. It is presently being used extensively in the research of patients with developmental/intellectual delay, multiple congenital anomalies, epilepsy, autism, and a range of other disorders. The efficiency of the test for diagnostic purposes and its applicability for various disorders will hopefully transpire in the process.

Array CGH has already been successfully used in determining genotype/phenotype relationships in contiguous genes syndromes which is a critical step in localizing the genes involved in different aspects of the phenotype and in understanding the functional basis of the conditions. Array CGH has provided the ability to map aberrations with high resolution and is particularly advantageous when large numbers of loci need to be analyzed. For example, the tiling path array of chromosome 4p was used to map deletions in Wolf-Hirschorn syndrome. They were thus able to determine that the facial characteristics of this phenotype were attributed to a single gene and that other characteristics resulted from the interaction of various other genes (Van Buggenhout et al., 2004). The gene for CHARGE syndrome a pleiotropic disorder comprising of coloboma, heart defects, choanal atresia, retarded growth and development, genital hypoplasia, ear anomalies and deafness (MIM#214800) was identified by array CGH on a 1 Mb resolution genome-wide Bacterial Artificial Chromosome (BAC) array (Vissers et al., 2004).

The initial experimental microarray platforms used have been of lower resolutions, 0.5 to 1.0 Megabase, with or without full genome coverage. It is to be expected that higher resolution array platforms will have increased detection power and will yield more informative results that can be applied to the purpose of single gene identification.

We are investigating the role of CNVs in the etiology of brain malformations. Towards this end we have prospectively studied genomic variations in 100 patients with various brain

malformations by array CGH on a 200 kb overlapping BAC array platform. The high density array gives multiple fold coverage of the genome providing a high resolution analysis that will detect imbalances with a resolution that would be not be conceivable by standard karyotyping. For each of the potentially causative genomic imbalances detected we also discuss the role of individual genes in the pathogenesis of the relevant brain malformation.

# II. Materials and Methods

# 1. Materials

## 1.1 Equipment

The tests were performed using standard cytogenetics and molecular laboratory equipments. The following additional equipment was used for sample preparations, hybridization and image scanning in the procedures for array- and oligo array-CGH and FISH.

Sonicator

Thermocycler with heated lid

Waterbath (Memmert)

Slide Booster (Advalytix; distributed by Implen, Germany)

Incubator (Memmert)

Hybridization oven (Agilent)

Hybridization oven rotator for Agilent Microarray Hybridization Chambers

Hybridizatoin gasket slides (Agilent)

Hybridization Chamber (Agilent)

Laser microarray scanner (Agilent)

Leica photomicroscope (fluorescent and light)

Nanodrop

Zeiss photomicroscope (fluorescent)

## 1.2 Chemicals

All chemicals are listed in Table II.1

Table II.1 List of chemicals and manufacturers

| | | |
|---|---|---|
| Acetic Acid Glacial | Merck | Cat no. 100056 |
| Agarose | Invitrogen | Cat no. 16500100 |
| Bovine Serum Albumin | Sigma | Cat no. A-2153 |
| Cy3 dUTP | Amersham | Cat no. PA53022 |
| Cy5 dUTP | Amersham | Cat no. PA55022 |
| DAPI | Serva | Cat no. 18860.01 |
| Dextran sulfate | USB | Cat no. US70796 |
| Ethanol | MERCK | Cat no. 1085430250 |
| Ethidium Bromide | Serva | Cat no. 21251.01 |
| Green fluorophore | Vysis/Abbott | Cat no. 02N32-050 |
| Formamide | Merck | Cat no. 344206 |
| Herring sperm DNA (10mg/ml) | Invitrogen | Cat no. 15634-017 |
| Human Cot1-DNA | Roche | Cat no. 1581074 |
| Size ladder | Invitrogen | Cat no. 10594018 |
| Sodium Dodecyl Sulfate (10%) | MERCK | Cat no. 106022 |
| Sodium acetate | MERCK | Cat no. 567422 |
| Orange fluorophore | Vysis/Abbott | Cat no. 02N33-050 |
| Trypsin 1:250 | Invitrogen | Cat no. 17270018 |
| Tween 20 | Sigma | Cat no. P9416 |

## 1.3 Solutions

All solutions are prepared using autoclaved Millipore water with Merck or Sigma products unless otherwise specified. Table II.2

Table II.2 List of solutions

| | |
|---|---|
| 1X PBS, pH 7.3 | 137 mMNaCl,<br>2.7 mM KCl,<br>10.1 mM Na2HPO4,<br>1.8 mM KH2PO4 |
| 20XSSC, pH 7.0 | 300mM sodium citrate<br>3mM NaCl, |
| BAC array Wash solution1 | 50% formamide,<br>2xSSC,<br>0.1%SDS |
| BAC array post hybridization wash PN solution pH 8.0 | 100mM sodium phosphate<br>0,05% NP40 |
| BAC array slide pre-hybridization solution | 25% formamide,<br>4xSSC<br>0.1%SDS |
| Carnoy Fixative Solution | 2 part methanol<br>1 part acetic acid glacial |
| Ethidium Bromide (EtBr) | 10mg/mL EtBr |
| FDST, pH7<br>Heat to 70°C and mix vigorously to dissolve | 0.8g dextran sulfate<br>5ml formamide,<br>1ml 20xSSC<br>H2O to 7ml. |
| FISH wash solution I | 1x SSC at 75°C |
| FISH wash solution II | 4xSSC,<br>0.05% Tween 20, pH 7-7.5 |
| FISH pre-hybridization solution | 2x SSC,<br>70% Formamide |
| KCL hypotonic solution (0.075M) | 56 grams KCL in 1 liter water |
| 50X TAE buffer (pH 8.0) | 242g Tris base<br>57.1 ml glacial acetic acid<br>100 ml 500mM EDTA in 1 liter water |

# 1.4 DNA microarrays

## 1.4.1 BAC array

We used a whole genome tiling path BAC array of approximately 32400 BAC clones for the human Genome on a 24 X 60 mm platform. The array used is based on the human 32k Re-Array set (http://bacpac.chori.org/pHumanMinSet.htm; DNA kindly provided by Pieter de Jong (Krzywinski, et al., 2004; Osoegawa, et al., 2001; Ishkanian, et al., 2004), the 1Mb Sanger set (clones kindly provided by Nigel Carter, Wellcome Trust Sanger Centre)(Fiegler, et al., 2003) and a set of 390 subtelomeric clones (assembled by members of the COST B19 initiative: Molecular Cytogenetics of solid tumors).

## 1.4.2 Oligoarray

We used the Agilent 4X44 oligoarray per manufacturer's instructions.

# 1.5 Cell culture media and reagents

The following cell culture reagents and media were used for the preparation of cell cultures from peripheral blood lymphocytes for karyotyping and interphase/metaphase FISH.

| Product | Manufacturer | Catalogue number |
|---|---|---|
| Colcemid | Invitrogen | 15212012 |
| Foetal Bovine serum | Invitrogen | 10439016 |
| Ham F10 Liquid Media | Invitrogen | 1150043 |
| Penicillin Streptomycin | Invitrogen | 15070063 |
| Phytohemagglutinin M | Invitrogen | 10576015 |

# 1.6 DNA Purification and labeling kits

The following kits were used for purification of DNA and labelled samples, for CGH random primer labelling, and for human genome BAC probes nick translation labeling for FISH according to manufacturer and in-house protocols.

| Product | Manufacturer | Catalogue number |
|---|---|---|
| Bioprime Array CGH Kit | Invitrogen | 18095-011 (purification module included) |
| Bioprime purification module with Purelink | Invitrogen | Part no. 46 6335 |
| Nick translation kit<br>CGH Nick Translation Kit | Vysis,<br>Vysis has been changed to Abbott Molecular Inc. | 32-801024 (old)<br>32-801300 (2008) |
| PCR purification kits | Qiaquick | 28104-28106 |

# 1.7 Samples

## 1.7.1 Reference samples

Male reference 4981 and female reference 291 produced by whole genome amplification were used in all arrays as sex matched references.

## 1.7.2 Test samples

Whole genomic DNA from 100 Iranian cases with brain malformations and their parents was used. (See patient selection)

# 1.8 Information management and resources

## 1.8.1 Softwares

Chromosomal study, microphotography and karyotyping were performed using Leica Cytovision software. Microphotography and analysis of FISH were done using same or Zeiss Metasystems software. Processing and feature extraction of BAC array images were done individually using the Genepix software. Processed images were computed and analyzed using the CGH pro software. Processing, feature extraction and analysis of Oligoarray images was done using the Agilent Feature extraction and analysis software.

## 1.8.2 Databases

The following databases were used for the characterization of the CNVs, their gene contents, disease associations, and Bac probe identities. The inhouse database which consisted of healthy individuals and patients with phenotypes other than brain malformations studied on the same BAC array platform by our group was also used for screening for recurrent CNVs that could be considered artefacts of the array.

| | |
|---|---|
| UCSC genome browser | http://Genome.ucsc.edu |
| Database of genomic variants | http://projects.tcag.ca/variation/ |
| ENSEMBLE | http://www.ensembl.org |
| Online Mendelian Inheritance in Man | http://www.ncbi.nlm.nih.org/omim |
| Bacpac CHORI database | http://bacpac.chori.com |

# 1.9 Human genomic clones

The following human genomic clones were labeled for FISH analysis

| Patient | Chromosomal rearrangement | Clones | Chromosomal position |
|---|---|---|---|
| 9q rearrangement | 9q21.11 deletion | N0415N07 | 69382199-69553929 |
| | | N0419K08 | 71599849-71802575 |
| | 9q31.1 duplication | C23235 | 105362383-105529387 |
| | | N0703I18 | 106059921-106243510 |
| 1p deletion | 1p36 deletion | M2318 | 121039388-121078455 |
| | | K142280 | 6076167-6210159 |
| 16p duplication | 16p13.13 | RP11228K09 | 11067102-11239578 |
| | 16p13.13 | RP11547D14 | 11500108-11703585 |

The following commercially available FISH probes were used solely or in conjunction with labeled probes for confirmation purposes:

| FISH probes | Manufacturer | Catalogue number |
|---|---|---|
| 12pter | CYTOCELL | LPT12PG |
| 12cen | CYTOCELL | LPE012R |
| LIS 1, | CYTOCELL | LPU 019 |
| 19pter | CYTOCELL | LPT19PG |
| 19qter | CYTOCELL | LPT19QR |

# 2. Methods

# 2.1 Selection of cases

Cases were selected prospectively from among patients referred to Kariminejad Najmabadi Pathology and Genetics Center for genetic tests or counseling. All cases were initially

karyotyped at a resolution of 500 or higher, and those with normal cytogenetic results were candidate for inclusion in the study. During following sessions a complete clinical history and pedigree was taken. As there is a high endemic rate of consanguinity in the Iranian population, only sporadic cases were chosen in order to exclude cases with possible unidentified autosomal recessive disorders.

## 2.2 Documentation

Family history including couple's former pregnancies, relationship of couple, other sibs and relations with similar history are recorded. Family pedigree is drawn. The relationship of the parents is calculated in cases of consanguineous marriages

History of pregnancy and neonatal development including development during pregnancy, gestational age and delivery, head circumference, height, length, and weight at birth were noted. Clinical history included prenatal, perinatal and postnatal events. All cases where there was the indication of traumatic events are considered as probably causative perinatal factors and were not included in the study.

Complete physical examination including head circumference, height, weight, cranial, facial features, limb and digital characteristics, appearance of skin, hair, torso and other physical features are closely examined and noted.

Biochemical tests were conducted to eliminate possible metabolic involvement. Documents relevant to any other abnormality, defect, disorder, or malformation were registered and requested, so that the case could be clinically characterized. Perinatal infections were ruled out by TORCH study where applicable otherwise toxoplasmosis was tested. Brain malformation was documented by CT scan and/or MRI of the brain. The MRI or CT scan was later interpreted by a pediatric neuroradiologist to create uniform identification of malformations. An informed consent form was acquired from the families and when compliant, the patients were photographed for future reference. Blood was collected from patients and both parents.

# 2.3 Chromosomal study

Heparinized whole blood lymphocytes were seeded at approximately 1 million cells/ml. and cultured in two flasks containing 5 mls of Ham F10 culture media supplemented with 15% FBS, 1% penicillin streptomycin, and were induced by Phytohaemmaglutinin M (final concentration 5 ug/ml). Following 3 day incubation at 37 degrees centigrade, and one hour after addition of colcemid (final concentration 0.2 ug/ml) the cells were spun down and supernatant discarded. Ten mls of hypotonic solution (0.075M KCL) were added and samples were incubated at 37 degrees for 30 minutes. After centrifugation the supernatant was discarded and the cells fixed with Carnoys solution. Fixation step was repeated until pellet was clean and debris free. The cells were suspended on methanol washed glass slides and a minimum of 4 slides prepared from each suspension. All slides were banded and stained according to Giemsa-Trypsin-Giemsa banding technique. Metaphase spreads were analyzed microscopically at an average resolution of 500 bands. Five metaphase spreads were photographed, and 2 karyotypes prepared for future reference.

# 2.4 Sample preparation

## 2.4.1 DNA extraction

All DNA samples were extracted from peripheral blood in EDTA by salt extraction technique, simultaneously DNA samples from parents were obtained. DNA measurements were taken using Nanodrop prior to and after labeling.

## 2.4.2 DNA preparation for BAC array

For each sample and reference, 10ug of DNA in 200 ul of water was sonicated at a constant cycle with output set at 4 for 4 seconds. Quality and quantity of DNA was confirmed and determined by Gel electrophoresis and Nanodrop measurements. DNA was purified using the Bioprime purification module with Purelink per manufacturer's protocols.

# 2.5 BAC ArrayCGH

## 2.5.1 Labelling

For each patient 4 tubes were set up: 2 with input of 1ug genomic patient DNA and 2 with 1 ug genomic reference DNA to 4.4 ul 5.5 mM EDTA. All tubes were brought to 22 ul total volume by adding the necessary amount of water. Labelling was performed using Bioprime array CGH labeling kit with modifications to the manufacturer protocol. To each tube 20µl of 2.5x Random Primer Solution (Kit) was added. After spinning down, the DNA and primers were denatured in 95 degrees centigrade thermocycler. Tubes were taken out and put immediately on ice for a minimum of 5 minutes. To each tube 5 ul of 10x dUTP Nucleotide Mix (Kit), 3 ul of Cy3-dUTP for patient DNA or Cy5-dUTP for reference DNA and 1 ul Exo-Klenow Fragment (Kit) were added. The samples were then vortexed and spun down, before being placed in a 37 degree heat block for amplification for a minimum of 2 hours. Thereafter, 5 ul of 0.5mM EDTA was added to each tube to quench the reaction before they were purified using the Purification Module provided in the kit and eluted with elution buffer resulting in approximately 50 ul flow through. Nanodrop measurements of label incorporation were taken and recorded for Cy3 and Cy5 samples. Labelled DNA of each patient (2 tubes) was pooled together with DNA of sex matched reference (2 tubes).

## 2.5.2 DNA Precipitation

Labelled DNA samples were precipitated by ethanol (825 ul) sodium acetate (30 ul) with 100 ul of Cot-1 Human DNA (5ug/ul). DNA was allowed to precipitate for a minimum of 2 hours and maximum of 2 days at -20 degrees. After which ethanol was removed by centrifuge in 4 degree centrifuge at 14000 rpms for 30 minutes, and rehydrated by adding 100-200 ul of 70% ethanol before centrifuging another 15 minutes. The alcohol was removed and the pellet was allowed to dry.

## 2.5.3 Pre-Hybridization

Immediately prior to hybridization the array slides were primed in a pre-warmed pre-hybridization buffer solution supplemented with 0.3 BSA and 200 ul herring sperm DNA for 1h at 42°C. Slides were then rinsed with water and immediately dried by centrifugation at 150g room temperature.

The DNA pellet was resuspended and completely dissolved in 28 ul FDST supplemented by 4 ul of yeast tRNA and 8 ul of 10% SDS. Once dissolved, the sample was denatured in 70 degree water bath for 15 minutes. It was transferred to a 42 degree water bath for 2 hours before hybridization.

## 2.5.4 Hybridization

The slides were hybridized to the sample by placing the slide over the sample pipetted onto a prewarmed coverslip (on the surface of a 42 degree hot plate). Hybridization was done in Adalytix slide booster heated to 42 degrees for a minimum of 20 hours. Each slide pad was moistened with 30 ul of aquavison and the chambers were filled with humidifying buffer (FDST). The program was set for maximum hours at 3:7 pulse/mix settings.

## 2.5.5 Post hybridization Wash

Coverslips were removed by placing the slides in 2X SSC solution. The slides were immediately transferred to preheated Wash solution I and placed in water bath for additional 15 minutes. The slides were washed in PN solution and immersed in second PN solution and placed on moving table for 10 minutes. The slides were passed through PBS solution and water with tween solution and immediately placed in centrifuge.

## 2.5.6 Scanning

Slides were immediately scanned using Agilent laser scanner. Two tiff images were obtained for each slide, one for each fluorophore.

## 2.5.7 Analysis

The two tiff images corresponding to each slide were loaded into the Genepix software for feature extraction and processing. The resulting image was uploaded into the CGHpro software for genomic profiling.

# 2.6 Oligo-arrayCGH

All oligo arrays were performed on Agilent 4X44 formats using manufacturer's protocols. They were analyzed using Agilent Analysis software.

# 2.7 Interphase/metaphase FISH

FISH was performed as confirmation of detected CNVs in all cases where the *de novo* CNVs showed gain and/or the position of the CNV would be inducive to the interpretation and identification of the disease causing mechanism. Commercially available centromeric and telomeric probes were used and were hybridized according to manufacturer's instructions. In instances of specific CNVs with no compatible commercial probe, two relevant BAC human genome clones were labeled with FITC or spectrum orange by nick translation using VYSIS nick translation kits per manufacturer's instructions.

## 2.7.1 Probe Preparation by Nick translation

For nick translation 500ng - 1ug genomic DNA was used and diluted in 17.5 ul of water. To this was added the cocktail mix of 2.5 ul 0.2mM Spectrum orange or 0.2mM FITC, and (supplied in the kit) 2.5 ul of 0.1mM dTTP, 5 ul of 0.1mM dNTP mix (0µl 0.3M dATP + 10µl 0.3M dCTP + 10µl 0.3M dGTP ), 10 ul 10x nick translations buffer, and 5ul Nick Translation Enzymes. After vortexing they were placed in cycler preset at 15°C for 2 hours, then, the probes were transferred to 70-75 degree centrigrade water bath for 10 minutes.

## 2.7.2 EtOH precipitation

After combining 25µl (500ng) of the red and green labeled probes, to be used simultaneously, with 10µl human Cot-1 DNA (1mg/ml) and 1µl Herring Sperm -DNA (10mg/ml), it was then purified by precipitation Ethanol-Sodium Acetate, at –20°C overnight.

## 2.7.3 Pre-Hybridization

Slides were prepared by dehydrating in 70%, 90% and 100% EtOH and were dried by placing in 60C incubator for 2 hours and/or baked at 65 for 15 minutes. Then 130µl pre hybridization solution was applied to a 24 X 60 cover slip and brought together with the slide at room temperature for 10 minutes. The slides were denatured on a hot plate at 78°C between 1 min 15 sec and 1 min 45 sec or longer depending on condition of slides. The coverslips were shaken off and slides were transferred immediately to cold (-20°C) 70% EtOH for 2 minutes and processed through 90% EtOH and 100% EtOH for 2 minutes each. Slides were dried at room temperature, or with compressed air.

## 2.7.4 FISH hybridization

Commercially available probes were hybridized according to manufacturer's instructions using Thermobrite hybridization chambers. The probes labeled by nick translation were hybridized by resolving the pellet in 7 µl 100% Formamide and adding 7 µl 4x SSC, 20% Dextran Sulfate at 37°C for 20 minutes. The probes were then denatured on hot plate at 85°C for 5 minutes, and placed on ice for 2 minutes. They were then incubated for at least 30 minutes at 37°C (protected from light).

The prepared probe was transferred to an 18x18 mm cover slip and merged with the slide and sealed up with Fixogum Rubber Cement and placed in chamber in water bath at 37°C overnight.

## 2.7.5 Post hybridization Wash

After careful removal of the Fixogum, the cover slip was removed in 2x SSC and the slides were washed in 1x SSC at 75°C for 5 minutes. The slides were then washed in 4x SSC at room temperature for another 5 minutes followed by a wash in 1x PBS at room temperature for 3 minutes. The slides were dried with compressed air and then stained by spreading 35ul DAPI/ antifade (500ng/ul) to a cover slip (24x60mm) and merging with slide.

# III. Results

# 1. Clinical findings in patients with CNVs

## 1.1 Brain malformations

As previously outlined, the presence of a brain malformation was confirmed by CT/ MRI scans and all images were reviewed by a pediatric neuroradiologist. Many of the patients have more than one brain malformation and considering the extreme overlap between the various categories, an attempt was made to classify them according to the most likely possible underlying defect. [Table III.1]

Microcephaly was considered as head circumference of -2 SD for age and sex. Overall 42 patients had microcephaly. All those cases with microcephaly accompanied by pachygyria (4), lissencephaly (2) were considered to be a result of neuronal migrational disorders and classified therein. All cases of microcephaly and complete agenesis of corpus callosum, with (3) or without cerebellar hypoplasia (3), were classified as a midline defect. And the one case where microcephaly was associated with a frontal cortical dysplasia was considered to be a neuronal organization defect.

The cases where cerebellar hypoplasia (3) was not accompanied by other malformations were placed in the neuronal proliferation/ apoptosis category. As already indicated, where cerebellar hypoplasia (4) was accompanied by a structural defect in midline structures they were placed in the midline defect category.

Midline defects were considered to comprise total agenesis of corpus callosum with or without other malformation, and all cases of cerebellar vermian hypoplasia and holoprosencephaly.

The category of abnormal neuronal organization consisted of 10 cases with focal cortical dysplasia, one accompanied by microcephaly, 5 cases of schizencephaly and 1 case of polymicrogyria.

The category of abnormal neuronal migration consisted of pachygyria (13), lissencephaly (6) and periventricular heterotopias (2) and colpocephaly with hypogenesis of corpus callosum (2).

All malformations of the posterior fossa including Dandy Walker syndrome, mega cistern magna and variants have been put together and apart from other malformations of the cerebellar vermis and corpus callosum.

# 1.2 Other clinical findings

The cohort consisted of 56 males and 44 females ranging in age from 6 months to 16 years, with a median age of 3 years. All patients have variable range of intellectual disability. Many have additional clinical findings including 52 with seizures, 50 with craniofacial dysmorphism, 4 with autism spectrum disorders, 6 with a form of renal defects, 10 with congenital heart defects, 7 have limb defects, 4 have genital abnormalities and 24 of them have various sensory visual and hearing problems. These patient findings are presented in table III.2.

Table III.2 Additional findings in the patients

| **Clinical finding** | **Total** | **Case number of patients with CNVS** | |
|---|---|---|---|
| | | ***De novo*** | **Inherited** |
| Seizures | 52 | 17652, 20375, 22392, 23510 | 17807, 19958, 21003, 22245, 22748, 22943 |
| Craniofacial dysmorphisms | 50 | 17926, 19673, 20196, 21573, 22392, 23510, | 17500, 17671, 18986, 20673, 23779 |
| Sensory defects | 24 | 20196, 23510 | 17671, 18986, 20732, 22748, |
| Cardiac defects | 10 | 19673 | 17500 |
| Limb defects | 7 | - | - |
| Renal defects | 6 | 17926, 21573 | |
| Autism | 4 | 21835 | 22748 |
| Genital abnormalities | 4 | 19673, 20375 | |

Table III.1 The brain malformations of the patients according to the most prominent finding

| Brain classification | Brain malformation | Total | | *De novo* CNV | | Inherited CNV | |
|---|---|---|---|---|---|---|---|
| Abnormal neuronal glial proliferation or apoptosis | Microcephaly | 37 | 22 | 2 | 0 | 4 | 3 |
| | Microcephaly & cerebellar hypoplasia | | 4 | | 0 | | 1 |
| | Microcephaly & corpus callosum hypogenesis | | 3 | | 0 | | 0 |
| | Cerebellar hypoplasia | | 3 | | 0 | | 0 |
| | Megalencepaly | | 4 | | 2 | | 0 |
| | Hemimegalencephaly | | 1 | | 0 | | 0 |
| Abnormal neuronal organization | Focal cortical dysplasia | 16 | 7 | 2 | 1 | 3 | 2 |
| | Focal cortical dysplasia & microcephaly | | 1 | | 0 | | 1 |
| | Focal cortical dysplasia and hypogenesis of corpus callosum | | 2 | | 0 | | 0 |
| | Polymicrogyria | | 1 | | 1 | | 0 |
| | Schizencephaly | | 4 | | 0 | | 0 |
| | Schizencephaly with thinning of corpus callosum | | 1 | | 0 | | 0 |
| Abnormal neuronal migration | Pachygyria | 23 | 7 | 2 | 0 | 2 | 1 |
| | With corpus callosum hypogenesis | | 2 | | 0 | | 0 |
| | With microcephaly | | 4 | | 1 | | 1 |
| | Lissencephaly | | 4 | | 1 | | 0 |
| | With microcephaly | | 2 | | 0 | | 0 |
| | Periventricular heterotopias | | 2 | | 0 | | 0 |
| | Colpocephaly With corpus callosum hypogenesis | | 2 | | 0 | | 0 |

| **Brain classification** | **Brain malformation** | **Total** | | ***De novo* CNV** | | **Inherited CNV** | |
|---|---|---|---|---|---|---|---|
| Midline defects | CCA, microcephaly & cerebellar hypoplasia | 14 | 3 | 4 | 0 | 2 | 0 |
| | CCA & cerebellar hypoplasia | | 1 | | 0 | | 0 |
| | CCA & microcephaly | | 3 | | 2 | | 0 |
| | Holoprosencephaly | | 2 | | 1 | | 1 |
| | CCA & cerebellar vermian hypoplasia | | 1 | | 0 | | 0 |
| | CCA | | 3 | | 1 | | 1 |
| | Cerebellar vermian hypoplasia | | 1 | | 0 | | 0 |
| Posterior fossa malformations | Dandy Walker syndrome | 10 | 4 | 0 | 0 | 2 | 2 |
| | Mega cistern magna | | 3 | | 0 | | 0 |
| | Other | | 3 | | 0 | | 0 |
| Total | | 100 | 100 | 10 | | 13 | |

CCA: corpus callosum agenesis

# 2. Array CGH results

Array CGH was performed in the 100 selected patients. A common pitfall in the analysis of array CGH results is the presence of single clone variations that may not represent true variations of copy number. To overcome this problem and thereby decrease the possibility of false positive results, only those copy number variations that encompassed at least 3 overlapping BAC clones were called.

Also, all copy number variants detected in the regions with high copy number repeats of more than 0.5 times were disregarded and not included in the study. The remaining CNVs were thereafter screened against the Database of Genomic Variants. The CNVs that were included within reported polymorphisms were considered polymorphic. Those CNVs that overlapped with reported polymorphisms on either or both ends were included only if there was more than a total of 100 kbs difference on either or both sides.

All the remaining CNVs were thereafter confirmed by parental studies. When and if the same CNV was present in the parent that was taken as sufficient evidence to the verity of the CNV. In cases where the CNV was not present in either parent, the CNV was confirmed by a second alternate technique such as oligoarray or FISH and in a few cases by both.

The cases presenting with *de novo* CNVs are presented in Table III.3, Figure III.1 and those with hereditary CNVs are presented in Table III.4, Figure III.2.

There are total of 27 CNVs in 22 cases; patients 17926, 19958, 22392 each have two and 21835 has three CNVs. The CNVs range in size from 210 Kb to 11.12 Mb. Parental studies of these cases revealed 14 of the CNVs to be hereditary, 5 inherited from the father and 9 present in the mother. It was assumed reasonable to conclude that all other CNVs disregarding the possibility of non paternity are *de novo*.

Table III.3. Patients with *de novo* CNVs

| patient | sex | Imbalance | Chromosome | Begin (Mb) | End (Mb) | Size | Syndromes | Brain phenotype | Genes |
|---|---|---|---|---|---|---|---|---|---|
| 23376 | M | Deletion | 1p36.32-p36.23 | 4.9 | 15.1 | 10.2Mb | 1p36 microdeletion , OMIM#607872 | Corpus callosum agenesis | *CLSTN1*, *KIF1B* |
| 17652 | M | Deletion | 1q43q44 | 238.6 | 241.5 | 2.95Mb | 1q43q44 microdeletion, OMIM#612337 | Microcephaly, corpus callosum agenesis | ***AKT3, ZNF528*** |
| 21835 | F | Deletion | 2p25.3 | 0.04 | 3.0 | 2.96Mb | Novel CNV | Focal cortical dysplasia | ***MYT1L*** |
| | | Deletion | 2p25.2 | 4.6 | 5.1 | 0.52Mb | Novel CNV | | ***TSSC1*** |
| | | Duplication | 19q13.42q13.43 | 58.8 | 63.7 | 4.92Mb | Novel CNV | | ***TSEN34***, *NLRP12, TNNI3, CDC42EP5* |
| 17926 | M | Deletion | 9q21 | 68.2 | 72.8 | 4.56Mb | Novel CNV | Microcephaly, hydrocephaly | *APBA1*, ***FXN*** |
| | | Duplication | 9q31.1 | 105.2 | 116.3 | 11.12Mb | Novel CNV | | ***FCMD***, *SLC31A2, SLC31A1, AMBP* |
| 20196 | M | Duplication | 12p | Whole arm | | | Killian Pallister , OMIM#601803 | megalencephaly | Many |
| 21573 | F | Duplication | 12p | Whole arm | | | Killian Palllister, OMIM#601803 | megalencephaly | Many |
| 22392 | F | Duplication | 16p13.2 | 10.1 | 12.6 | 2.46Mb | Novel CNV | holoprosencephaly | Many |
| 20375 | M | Deletion | 17p13.3p13.2 | 0 | 3.6 | 3.66Mb | Miller Dieker , OMIM#247200 | lissencephaly | ***PAFAH1B, YWHAE*** |
| 23510 | M | Deletion | 17p13.3p13.2 | 2.4 | 3.5 | 1.10Mb | Lissencephaly, OMIM#247200 | lissencephaly | ***PAFAH1B*** |
| 19673 | M | Deletion | 22q11.21 | 17.4 | 20.0 | 2.64Mb | Di George**,** OMIM#188400 | Polymicrogyria | ***CRKL*** |

Bold: Genes recognized in brain malformation syndromes Underlined: genes identified through network analysis

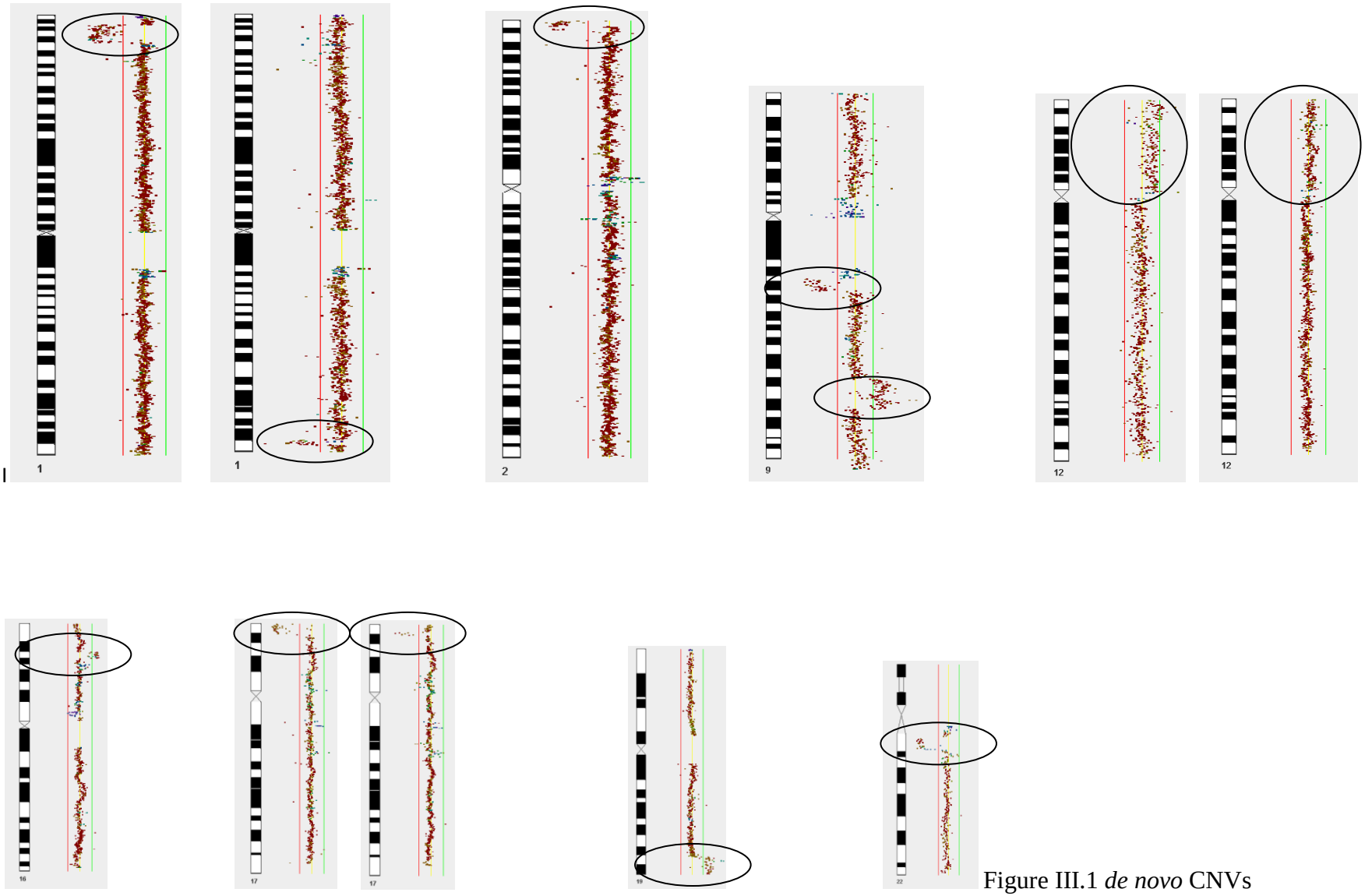


Figure III.1 *de novo* CNVs

Table III.4 Patients with inherited CNVs

| patient | sex | imbalance | origin | Chrom | Begin | End | Size | Brain phenotype | Genes |
|---|---|---|---|---|---|---|---|---|---|
| 17807 | M | Deletion | paternal | 2q36.3 | 228.1 | 228.4 | 0.24Mb | Focal cortical dysplasia | ***SLC19A3*** |
| 20732 | F | duplication | maternal | 5p15.2 | 12.9 | 13.2 | 0.29Mb | Microcephaly | Gene desert |
| 20673 | M | Deletion | maternal | 6p24.3 | 10.5 | 10.8 | 0.26Mb | Cerebellar hypoplasia | *TFAP2A* |
| 22943 | M | duplication | paternal | 7p21.1 | 17.0 | 17.8 | 0.8Mb | Corpus callosum agenesis | *AHR*, *SNX13* |
| 22245 | M | duplication | maternal | 9p24.3 | 2.16 | 3.2 | 1.06Mb | Pachygyria | *SMARCA2*, ***VLDLR*** |
| 23779 | F | duplication | maternal | 9p21.2 | 27.1 | 27.3 | 0.21Mb | Dandy Walker | *TEK*, *MOBKL2B* |
| 17500 | F | duplication | maternal | 10p15.3 | 1.1 | 1.6 | 0.55Mb | Dandy Walker | *IDI1*, *ADARB2* |
| 22748 | F | duplication | paternal | 10q23.31 | 92.1 | 92.6 | 0.56Mb | Focal cortical dysplasia | ***HTR7*** |
| 18986 | M | duplication | paternal | 16p13.11 | 15.4 | 16.3 | 0.87Mb | Microcephaly | ***NDE1*** |
| 21003 | F | duplication | maternal | 18q23 | 73.1 | 73.7 | 0.67Mb | Microcephaly | *GALR* |
| 19958 | F | duplication | maternal | Xp22.33 | 3.79 | 4.08 | 0.29Mb | Pachygyria | Gene deserts |
| | | | | Xp22.32 | 4.7 | 5.1 | 0.39Mb | | Gene deserts |
| 17671 | M | duplication | maternal | Xq21.31 | 85.0 | 85.5 | 0.44Mb | Focal cortical dysplasia | ***DACH2***, *CHM* |
| 22392 | F | duplication | maternal | Xq21.33 | 93.0 | 94.1 | 1.10Mb | Holoprosencephaly | Gene deserts |

Bold: Genes recognized in brain malformation syndromes Underlined: genes identified through network analysis

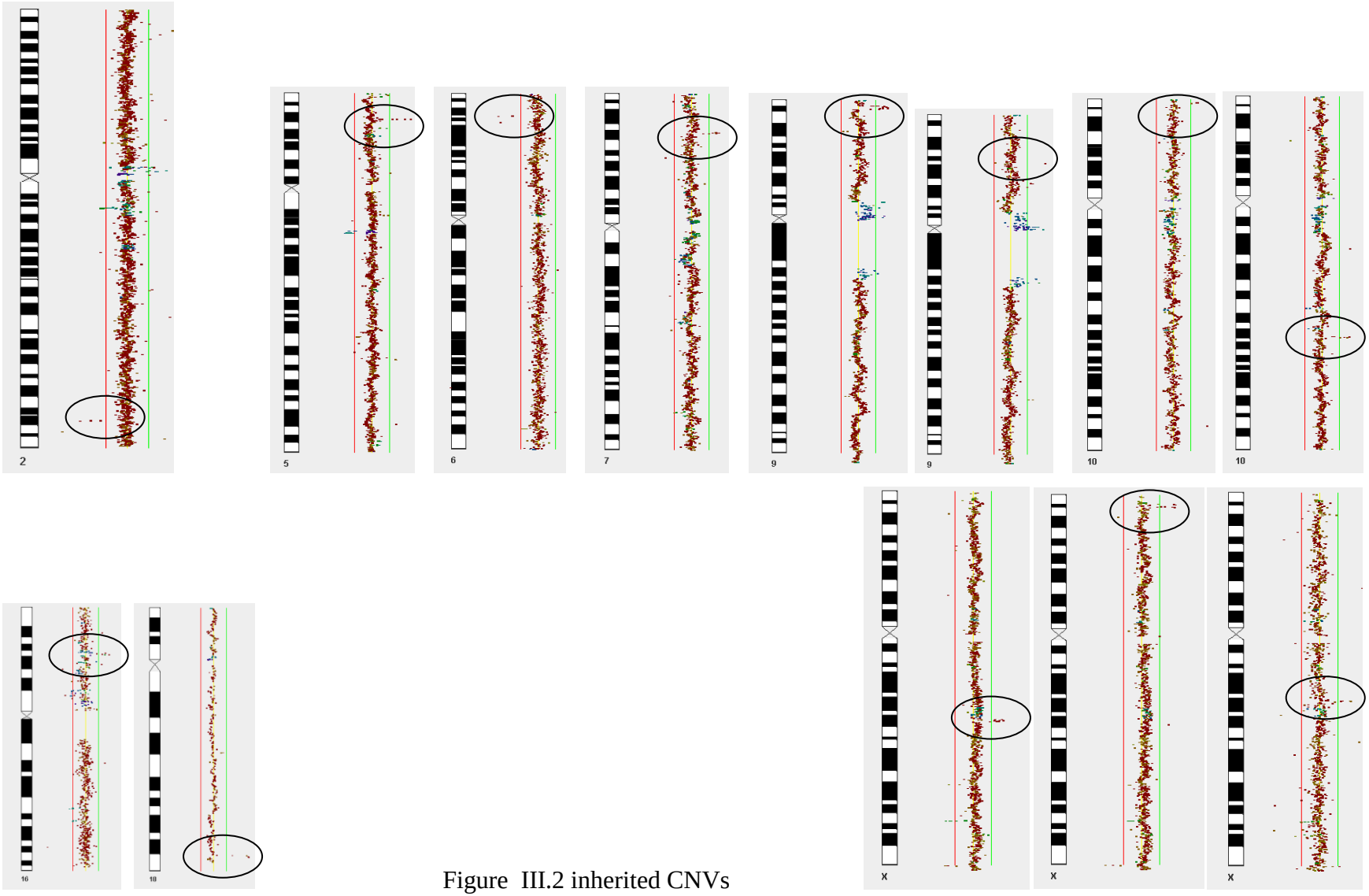



Figure III.2 inherited CNVs

# 3. Alternate techniques for confirmation

## 3.1 Confirmation by oligoarray

Oligoarray was performed to confirm the detected CNV in 4 of the cases with *de novo* CNVs. The results are presented in Table III.6.

Table III.5 Begin and end probes identified in selected patients by oligoarray

| **Case** | **Begin** | **End** |
|---|---|---|
| 17652 | 238482587-238482632 | 241440175-241440234 |
| 20375 | 29169-29223 | 3554483-3554542 |
| 19673 | 16978506-16978553 | 19886024-19886068 |
| 17926 | 16978506-16978553 | 19886024-19886068 |

## 3.2 Confirmation by FISH

As the results of array CGH do not reflect the location and rearrangements within the chromosomal regions FISH was performed in all patients with duplications, or with more than one imbalance which could result from a chromosomal rearrangement. The samples for which FISH was performed and the results obtained are presented in TableIII.6.

Table III.6 Result of FISH in selected patient cases

| **Case** | **Chromosome** | **Clones** | **Dye** | **Results** |
|---|---|---|---|---|
| 21835 | 2 | 2pter, | FITC, | (2pter-,19qter+) X1 |
| | 19 | 19pter, 19qter | FITC, Orange | (19pter+,19qter+) X2 |
| Parents of 21835 | 2 | 2pter | FITC | (2pter+) X2 |
| | 19 | 19pter,19qter | FITC, Orange | (19pter+,19qter+) X2 |
| 17926 | 9q21.11 | N0415N07 | Orange | N0415N07 X1 |
| | | N0419K08 | Orange | N0419K08 X1 |
| | 9q31.1 | C23235 | FITC | 9q31.1(C23235 X2);<br>9q21.11: (C23235 X1) |

| | | | | |
|---|---|---|---|---|
| | | N0703I18 | FITC | 9q31.1(C23235 X2)<br>9q21.11:(C23235 X1) |
| 20196 | 12pter | 12pter | FITC | P08498 X4(7%)P08498 X2(93%) |
| | 12cen | 12cen | Red | D16815 X4(7%)D16815 X2(93%) |
| 22392 | 16p13.13 | RP11228K09 | Red | 16p13.13: RP11228K09 X3 |
| | 16p13.13 | RP11547D14 | FITC | 16p13.13: RP11547D14 X3 |
| 23510 | 17 | LIS1 | Red | LIS1 X1 |
| | | 17pter | FITC | 17pter X2 |
| 21573 | 12 | 12pter, | FITC, | 12pter X4(12%)/12pterX2(88%) |
| | | 12cen | Red | 12cen X3(12%)/12cen X2(88%) |

# 4.Gene enrichment analysis:

Gene enrichment analysis was performed for the CNVs in this cohort and those from a Danish cohort of epilepsy patients with structural brain malformations reported in Kariminejad et al. (2011). Those detected for the CNVs in this cohort are shown in Table III.7

Table III.7 Genes and Molecular functions identified in patients and CNVs

| patient | symbol | Ensemble | Molecular_Function | OMIM phenotype |
|---|---|---|---|---|
| 17807 | HRB | ENSG00000173744 | GO:0001675~acrosome formation | |
| 23510 | PAFAH1B1 | ENSG00000007168 | GO:0001675~acrosome formation | Subcortical laminar heterotopia |
| 20375 | PAFAH1B1 | ENSG00000007168 | GO:0001675~acrosome formation | Subcortical laminar heterotopia |
| 20375 | GGT6 | ENSG00000167741 | GO:0003840~gamma-glutamyltransferase activity | |
| 19673 | GGT3 | ENSG00000133475 | GO:0003840~gamma-glutamyltransferase activity | |
| 23510 | GGT6 | ENSG00000167741 | GO:0003840~gamma-glutamyltransferase activity | |
| 20375 | ENO3 | ENSG00000108515 | GO:0004634~phosphopyruvate hydratase activity | Enolase-beta deficiency |
| 23376 | ENO3 | ENSG00000074800 | GO:0004634~phosphopyruvate hydratase activity | Enolase-beta deficiency |
| 23510 | ENO3 | ENSG00000108515 | GO:0004634~phosphopyruvate hydratase activity | Enolase-beta deficiency |
| 23376 | ICMT | ENSG00000116237 | GO:0004671~protein-S-isoprenylcysteine O-methyltransferase activity | |
| 23376 | TNFRSF25 | ENSG00000215788 | GO:0005031~tumor necrosis factor receptor activity | |
| 23376 | TNFRSF1B | ENSG00000028137 | GO:0005031~tumor necrosis factor receptor activity | |
| 22930 | TNFRSF19 | ENSG00000127863 | GO:0005031~tumor necrosis factor receptor activity | |
| 23376 | SLC2A5 | ENSG00000142583 | GO:0005353~fructose transmembrane transporter activity | |
| 23376 | SLC2A7 | ENSG00000197241 | GO:0005353~fructose transmembrane transporter activity | |
| 21835 | CDC42EP5 | ENSG00000167617 | GO:0007254~JNK cascade | |
| 17926 | AMBP | ENSG00000106927 | GO:0007254~JNK cascade | |
| 23510 | MINK1 | ENSG00000141503 | GO:0007254~JNK cascade | |
| 22930 | TNFRSF19 | ENSG00000127863 | GO:0007254~JNK cascade | |
| 19673 | CRKL | ENSG00000099942 | GO:0007254~JNK cascade | |
| 20375 | MINK1 | ENSG00000141503 | GO:0007254~JNK cascade | |
| 22392 | TNP2 | ENSG00000178279 | GO:0007341~penetration of zona pellucid | |
| 23510 | PAFAH1B1 | ENSG00000007168 | GO:0008088~axon cargo transport | Subcortical laminar heterotopias |
| 23376 | KIF1B | ENSG00000054523 | GO:0008088~axon cargo transport | Charcot-Marie-Tooth disease, type 2A1, 118210 |
| 20375 | PAFAH1B1 | ENSG00000007168 | GO:0008088~axon cargo transport | Subcortical laminar heterotopias |
| 17926 | APBA1 | ENSG00000107282 | GO:0008088~axon cargo transport | |
| 23376 | KIF1B | ENSG00000054523 | GO:0008089~anterograde axon cargo transport | |
| 23510 | TRPV1 | ENSG0000196689 | GO:0007655~chemosensory behavior | |
| 20375 | TRPV1 | ENSG0000196689 | GO:0007655~chemosensory behavior | |

| | | | | |
|---|---|---|---|---|
| 21035 | PRKG | ENSG00000126583 | GO:0007655~chemosensory behavior | Spinocerebellar ataxia 14, 605361 (3) |
| 23510 | ENO3 | ENSG00000108515 | GO:0008243~plasminogen activator activity | |
| 23376 | ENO3 | ENSG00000074800 | GO:0008243~plasminogen activator activity | |
| 20375 | ENO3 | ENSG00000108515 | GO:0008243~plasminogen activator activity | |
| 23376 | RERE | ENSG00000142599 | GO:0008267~poly-glutamine tract binding | |
| 23510 | ATP2A3 | ENSG00000074370 | GO:0015082~di-, tri-valent inorganic cation transmembrane transporter activity | |
| 17926 | SLC31A1 | ENSG00000136868 | GO:0015082~di-, tri-valent inorganic cation transmembrane transporter activity | Friedreich ataxia, 229300 |
| 17926 | FXN | ENSG00000165060 | GO:0015082~di-, tri-valent inorganic cation transmembrane transporter activity | |
| 20375 | ATP2A3 | ENSG00000074370 | GO:0015082~di-, tri-valent inorganic cation transmembrane transporter activity | |
| 17926 | SLC31A2 | ENSG00000136867 | GO:0015082~di-, tri-valent inorganic cation transmembrane transporter activity | Friedreich ataxia, 229300 |
| 20375 | ATP2A3 | ENSG00000074370 | GO:0015085~calcium ion transmembrane transporter activity | |
| 23510 | ATP2A3 | ENSG00000074370 | GO:0015085~calcium ion transmembrane transporter activity | |
| 23376 | SLC2A5 | ENSG00000142583 | GO:0015755~fructose transport | |
| 21835 | TNNI3 | ENSG00000129991 | GO:0016525~negative regulation of angiogenesis | Cardiomyopathy, familial restrictive, 115210 |
| 23510 | SERPINF1 | ENSG00000132386 | GO:0016525~negative regulation of angiogenesis | |
| 23376 | NPPB | ENSG00000120937 | GO:0016525~negative regulation of angiogenesis | |
| 20375 | SERPINF1 | ENSG00000132386 | GO:0016525~negative regulation of angiogenesis | |
| 17926 | PTAR1 | ENSG00000188647 | GO:0018346~protein amino acid prenylation | |
| 17671 | CHM | ENSG00000188419 | GO:0018346~protein amino acid prenylation | Dejerine-Sottas syndrome, 145900 |
| 21835 | CDC42EP5 | ENSG00000167617 | GO:0031098~stress-activated protein kinase signaling pathway | |
| 17926 | AMBP | ENSG00000106927 | GO:0031098~stress-activated protein kinase signaling pathway | |
| 20375 | MINK1 | ENSG00000141503 | GO:0031098~stress-activated protein kinase signaling pathway | |
| 22930 | TNFRSF19 | ENSG00000127863 | GO:0031098~stress-activated protein kinase signaling pathway | |
| 22930 | TNFRSF19 | ENSG00000127863 | GO:0043120~tumor necrosis factor binding | |
| 23376 | TNFRSF25 | ENSG00000215788 | GO:0043120~tumor necrosis factor binding | |
| 23376 | TNFRSF1B | ENSG00000028137 | GO:0043120~tumor necrosis factor binding | |
| 21835 | NLRP12 | ENSG00000142405 | GO:0045409~negative regulation of interleukin-6 biosynthetic process | |
| 17926 | ANXA1 | ENSG00000135046 | GO:0046717~acid secretion | |
| 23376 | ESPN | ENSG00000187017 | GO:0051639~actin filament network formation | Deafness, autosomal recessive 36, 609006 |

# IV. Discussion

The aim of this study was to determine the role of copy number variants in the etiology of structural brain malformations with the optimal goal of discovering genes or regions associated with abnormal brain development. We performed array CGH on 100 consecutive patients with structural brain malformations and analyzed our CNVs and patient cohort by the following aspects:

1. Cohort selection criteria
2. Size and nature of CNVs
3. Specific brain malformations
4. Gene content

## 1. Cohort selection criteria

During the past decade, array CGH has been applied to the detection of genomic imbalances for various cohorts of patients. The detection rate has differed based upon the resolution of the array, the selection criteria of the patients and the analysis criteria. The diagnostic value of array CGH is a matter of great speculation in both clinical and research applications. The advantages and benefits of performing array CGH for cohorts of patients with intellectual disability (ID), developmental delay (DD), multiple congenital anomalies (MCA), and autism (ASD) are being established. There is much consideration as to what selection criteria will yield the highest pathogenic CNV detection rate without adding the burden of extra cost imposed by microarray technologies.

Here we study the role of CNVs in patients with a structural brain malformation. We identified potential causative *de novo* CNVs in 10 patients (10%). We compared our cohort of patients with previous cohorts to determine the significance of a brain structural malformation as selection criteria for performing array CGH in a clinical context.

### 1.1 Array CGH in patients with seizures

Array CGH has been performed for the elucidation and characterization of the possible role of CNVs in seizures. A number of loci have been identified in this capacity including the 15q13.3, 16p13.11 and 15q11.2 (Hannes et al., 2009; de Kovel et al., 2009; Helbig et al., 2009; Dibbens et al. 2009). In a recent series study, Mefford et al. (2010) reported oligonucleotide array CGH findings in 517 patients with mixed types of idiopathic epilepsy. A 139000 oligo-array with approximate resolution of 38 kb throughout the genome was used, with closer and higher resolution in genomic hotspots. The results reveal 46 individuals with 51 imbalances, 60% of which were reported to be deletions. The hereditary state of the CNVs was not determined. Mefford et al. reported at least one rare CNV not present in the control group in 8.9% of the cases.

In our cohort 52 patients have seizures, eleven of whom have CNVs, 5 *de novo* (9.6%) and 6 hereditary (10.5%) totaling 21.1%. [Table III.2] When the data from this cohort was combined with data from another cohort of 69 patients with seizures and brain malformations, 28 (23%) of the 121 patients have CNVs (Kariminejad et al, 2011). This is a significant increase in the rate of CNV detection, suggesting that the presence of a structural brain malformation in patients with seizures is a strong indication of the higher likelihood of a genomic imbalance and the presence of CNVs. Considering that the resolution of the array we used is lower than that used in the Mefford study (2010), it could be expected that the use of a higher resolution array in seizure patients who have structural brain malformations, would correlate with an even higher CNV detection rate.

We find that the presence of a structural brain malformation in patients with epilepsy is a strong indication for CNV screening. We also propose that the CNVs detected in this study may add to the list of potentially causative regions in the etiology of epilepsy.

# 1.2 Array CGH in intellectual disability

In 2003, Vissers et al. reported the first application of array CGH to the detection of CNVs of possible clinical significance in patients with intellectual disability, with or without multiple congenital anomalies, facial dysmorphism, learning disabilities and autism spectrum disorders. They selected 20 patients following normal karyotype results and undetermined clinical diagnosis and reported 2 *de novo* CNVs. Since then many publications have reported

their array CGH findings in patients with nearly similar clinical phenotypes. In the consensus report published by the International Standards for Cytogenomics Array (ISCA) Consortium, in 2010, a total of 31 reports from 2003-2009 were compiled, where array CGH on various platforms had been performed on cases with intellectual/developmental disability with or without other clinical findings. The overall detection rate is 10% with variations from 5.3% to 35% based primarily on the selection criteria and the preliminary work-up of the patients prior to microarray testing. Thereafter, Poot et al. (2010) reported on array CGH in 278 patients selected after karyotyping and screening for subtelomeric rearrangements and common clinically diagnosable microdeletion/duplication syndromes for study on a 3783 clone BAC array. Their patients had mental retardation and multiple congenital anomalies. They report a total of 28 CNVs in 28 patients, 12 *de novo* losses, 8 *de novo* gains, 6 hereditary losses and 2 hereditary gains. The findings of the above studies are summarized in [Table IV.1].

In general, the reports could be categorized according to their genome coverage: whole genome or targeted arrays. Another distinctive feature between the studies is the resolution of the arrays. Earlier studies: Vissers (2003), Tyson (2005), Krepischi-Santos (2006), Engels (2007), Newman (2007), Aston (2008) and Menten (2006), have used whole genome BAC array with resolution of 0.5-1.3 Mb and report detection rates of 10, 13.7, 16.8, 10, 13.8, 12.2, and 13.6 percent respectively, with overall average of 12.8%. In these studies, cases were not screened by other molecular techniques, such as subtelomeric/microdeletion MLPA or FISH, prior to array CGH. In those reports where the patients were initially screened with subtelomeric MLPA or FISH prior to testing, such as the ones by Shaw-Smith (2004), Schoumans (2005) and Rosenberg (2006) detection rates are 14%, 9.8%, and 16%, respectively. Additional screening for microdeletion by MLPA or FISH in the Thuresson (2007) and Poot (2010) report results in 6.3% and 7.2% CNV detection, respectively.

Using higher resolution whole genome oligoarrays of 30-75 kbs, the reports show a higher average overall detection rate of 14.2%: de Vries (2005):10%, Friedmann (2006): 11%, Ming (2006): 20%, Fan (2007): 15%, Hoyer (2007): 9.6%, Wagenstaller (2007): 16.4%, Baldwin (2008): 15.6% and Xiang (2008): 18%.

**Table IV.1 Publications of array-CGH results on patients with intellectual and developmental delay arranged by scope and resolution**

| Author | Year | # | Indication | Pre-Screening | Array | Scope | Resolution | Yield |
|---|---|---|---|---|---|---|---|---|
| Ballif | 2006 | 3600 | ID,DD | | | Targeted | | 5.1% |
| Sharp | 2006 | 290 | ID,DF,MCA | | BAC | Targeted | | 5.5% |
| Shaffer | 2006 | 1500 | Various | | BAC | Targeted | | 5.6% |
| Shaffer | 2007 | 8789 | ID,DD,MCA | | BAC | Targeted | | 6.9% |
| Lu | 2007 | 2513 | ID,DD,DF,MCA,ASD | | BAC | Targeted | | 7.0% |
| Baris | 2007 | 234 | ID,DD,DF,MCA | | BAC | Targeted | | 5.6% |
| Nowakornska | 2008 | 91 | ID,DD | | BAC | Targeted | | 11.8% |
| Shevell | 2008 | 94 | ID,DD | | BAC | Targeted | | 6.4% |
| Engels | 2007 | 60 | ID | | BAC | Whole/targeted | 0.5 Mb | 10% |
| Pickering | 2008 | 822 | ID/DD | | BAC | Targeted | | 7.8% |
| | | 354 | ID/DD | | BAC | Whole | 1 Mb | |
| Miyake | 2006 | 30 | ID,MCA | | BAC | Targeted,backbone | 1.4 Mb | 16.7% |
| LU | 2008 | 372 | MCA | | BAC | Targeted, backbone | | %17.1 |
| | | 266 | MCA | | Oligo | Targeted, backbone | | |
| Wong | 2005 | 102 | ID | 17 ascertained cases | BAC | Telomere | | 18.6% |
| Vissers | 2003 | 20 | ID | Cytogenetics | BAC | Whole | 1 Mb | 10% |
| Shaw Smith | 2004 | 50 | ID | Cytogenetics, Subtelomeric | BAC | Whole | 1 Mb | 14% |
| Tyson | 2005 | 22 | ID | | BAC | Whole | 1.3 Mb | 13.7% |
| Schoumans | 2005 | 41 | ID | SKY FISH, subtelomeric FISH | BAC | Whole | 1.3 Mb | 9.8% |
| Thuresson | 2007 | 48 | ID,DD,MCA | Microdeletion, subtelomeric | BAC | Whole | 1 Mb | 6.3% |
| Aston | 2008 | 1075 | ID, DD, DF, MCA | | BAC | Whole | 1.3 Mb | 5.3% |
| Krepischi-Santos | 2006 | 95 | ID,DF,MCA | Deletions related to phenotype | BAC | Whole | 1 Mb | 16.8% |
| Menten | 2006 | 140 | ID,MCA | | BAC | Whole | 1 Mb | 13.6% |
| Rosenberg | 2006 | 81 | ID,DF | Cytogenetics | BAC | Whole | 1 Mb | 16% |
| Newman | 2007 | 36 | DD,LD,DF | | BAC | Whole | 1 Mb | 13.8% |
| Poot | 2010 | 278 | ID,DD,MCA | Cytogenetics, subtelomeric, microdeletions | BAC | Whole | 0.7 Mb | 7.2% |
| De Vries | 2005 | 100 | ID | Subtelomeric | BAC | Whole | 50 kb | 10% |
| Friedmann | 2006 | 100 | ID | | SNP/Oligo | Whole | 30 kb | 11% |
| Ming | 2006 | 10 | MCA | | SNP/Oligo | Whole | 30 kb | 20% |
| Xiang | 2008 | 50 | ID, DD | | Oligo | Whole | 35 kb | 18% |
| Aradhya | 2007 | 20 | ID,DD,DF,MCA,GR | Clinical, cytogenetics, subtelomeric | Oligo | Whole | 70 kb | 35% |
| Fan | 2007 | 100 | ID,DD | | Oligo | Whole | 35 kb | 15% |
| Hoyer | 2007 | 104 | ID | | SNP/oligo | Whole | 30 kb | 9.6% |
| Shen | 2007 | 211 | ID,DD,MCA | | Oligo | Targeted | 35 kb | 7.6% |
| Wagenstaller | 2007 | 67 | ID | Subtelomeric | SNP/Oligo | Whole | 30 kb | 16.4% |
| Baldwin | 2008 | 211 | ID,DD,DF,MCA,ASD | | oligo | Whole/targeted | 75 kb | 15.6% |

ID: intellectual delay, DD: developmental delay, DF: dysmorphic features, MCA: multiple congenital anomalies, ASD: Autism spectrum disorders,

At the lower end of detection rates are those reports using the targeted BAC array platforms Baliff (2006): 5.1%, Miyake (2006): 16.7%, Sharp (2005): 5.5%, Shaffer (2006): 5.6%, Baris (2007): 5.6%, Lu (2008): 7%, Shaffer (2007): 6.9%, Shen (2007): 7.6%, Shevell (2008): 6.4%, Nowakowska (2008): 11.8% and averaging below 10%.

There is a general overall trend that would suggest that whole genome array platforms of 1 Mb resolution have yielded an average of 10% or higher detection rate in cohorts of intellectual disability with or without additional clinical findings. As expected, there is also a general overall trend that suggests an increased detection rate with the increase in resolution of the arrays, wherein the studies based on the use of oligoarrays yield a higher detection rate. The added value of the increased detection rate is debatable as an updated systematic review and meta analysis of 19 studies and 13926 subjects rates the overall diagnostic yield of causal abnormalities as 10% (95% confidence interval: 8-12%) with an overall false-positive yield of noncausal abnormalities of 7% (95% confidence interval: 5-10%) (Sagoo et al., 2009).

We have used an overlapping 200kb BAC array platform. The theoretical resolution of the array based on the tiling pattern is about 75 kb. This theoretical resolution has been reached in former studies on this platform (Motezacker et al., 2007; Schwarzbraun et al., 2006). Based on the conservative CNV calling procedure applied in this study, the average functional resolution of about 240 kb was inferred by the mean size of the smallest 10% of CNVs identified in this study. Although none of the platforms used are comparable to the one we have employed, the reported resolution of our array lies somewhere in between the resolution of the oligo- and BAC- array platforms used.

The clinical guidelines for pathogenic CNV calling have been revised and are presently limited to those CNVs that occur *de novo* and are not present in either normal parent, those CNVs that are hereditary and are present in a clinically affected parent or vary from the CNV of the parent, and those CNVs that encompass a known contiguous gene syndrome region. Based on these guidelines, we have detected 13 *de novo* CNVs in 10 patients and 1 hereditary CNV in a recognized microdeletion/duplication region (11%). This is compatible with expected rates in unscreened patients with ID/DD/MCA. Our detection rate is inclusive of 3 cases with common microdeletion syndromes, the deletion 22q11 and deletion 17p13.2, 2 patients with Pallister Killian, and 1 patient with subtelomeric CNVs (deletion & duplication resulting from subtelomeric rearrangement). The fact that our array platform has a higher resolution than the more commonly used BAC- arrays, does not seem to have increased

detection rates in comparison to the cohort of patients with intellectual disability and multiple congenital anomalies.

The detection rate of 11% is reached by the clinical concepts presently defined and overlooks inherited CNVs, about which we do not have sufficient information, as possible susceptibility genes or contributory factors. Inclusive of hereditary CNVs we have 22 patients with CNVs (22%) which is on the higher end of reported rates. We cannot disregard the possibility of presence of susceptibility or contributory effect in the etiology of structural brain malformations that would not reflected in *de novo* CNVs.

We believe that although the selection of patients with a structural brain malformation in otherwise intellectually disabled patients does not increase the probability of CNV detection, it is in itself a strong indication for performing the test and yields similar detection rates. Also further studies and data are required to assess the value and possible significance of the hereditary CNVs.

# 2. The size and nature of the CNVs

We find 27 CNVs in 22 patients, where patients 17926, 19958 and 22392 have two and patient 21835 has 3 CNVs. There are a total of 13 *de novo* CNVs in 10 patients and 14 hereditary CNVs in one of the above and 12 other patients.

## 2.1 Comparison of hereditary and *de novo* CNVs

The average size of the *de novo* CNVs, disregarding the 12p arm gains, is 4.28 Mb ranging in size from 1.1 Mb to 11.2 Mb. The smallest hereditary CNV is 210 kb and the largest is 1.06 Mb with an average size of 0.55 Mb overall. There is a great difference in the average size of the *de novo* and hereditary CNVs which reflects the better tolerance for the smaller CNV in unaffected individuals without a notably visible phenotypic implication. Among the *de novo* CNVs we have 5 duplications and 8 deletions, whereas 12 of the 14 hereditary CNVs are duplications. This is evidence of better tolerance for duplications in comparison to deletions, where triplicate copies of the genes may be better tolerated than the decreased copy numbers.

## 2.2 Size of *de novo* CNVs

When looking at the *de novo* CNVs there is a paradox in regard to the size of the CNV and the extent of the brain malformation. In the two 17p13.3 microdeletion cases, the nature and severity of the brain malformation relates directly to the size of the CNV, where the patient with the larger CNV has the more severe form of lissencephaly in comparison to the patient with the smaller CNV. This seems to suggest that the larger CNV is directly responsible for the more severe brain malformation.

Based on the assumption that size will directly influence severity of brain malformation, we would expect to find the most severe brain structural malformation in the largest CNV. One of the largest CNVs in this cohort is the one found in our patient with the 1p36 deletion. Yet, this patient has corpus callosum agenesis without any additional structural brain malformation. The patient with both the deletion and duplication in 9q has a total genomic imbalance of 15.6 Mb with a brain phenotype of microcephaly and corpus callosum agenesis that is categorically similar to that of the patient with the 3.5 Mb 1q43q44 microdeletion. In these cases, the increase in the size of the imbalance, has not contributed to the severity of the brain malformation.

It seems logical to conclude that although the size of the CNV does appear to be correlated with the severity of the general phenotype of a patient, the specific brain malformation/s is affected primarily by the gene content of the genomic segments. The size relationship may hold true in most cases, as there is most probably a higher gene content in larger genomic segments. Brain malformations and their severity will be affected by the number of genes involved in the development and maturation of the brain within the deleted/duplicated segment. In support of this, we know that the variation in the structural brain malformation between the 17p13 microdeletion cases does not derive from the size and is not measured by the size variation but more specifically by the inclusion of the second gene *YWHAE* that seems to affect the brain development and to cause the dysmorphic features.

But this does not appear to be the whole story. Were the structural brain malformation a direct consequence of gene content, we would expect to find the same or similar brain malformation in the CNVs where the copy number of a specific gene or genes is changed. The lack of the consistent presentation of the same structural brain malformation in other patients with the same CNV in some genomic imbalances further complicates the situation.

In patients with the 22q11.2 microdeletion, there is no consistent structural brain malformation, even in patients with nearly identical genomic imbalances. These patients may or may not have a brain malformation, and even in those cases where a brain malformation is noted, it is not always the same malformation. In the 22q11.2 cases, it has been proposed that the brain malformation is the result of a general developmental error rather than the haploinsufficiency of a specific gene/s or region (Robin et al., 2006).

Therefore there appears to be multiple mechanisms by which the CNVs can lead to the brain malformation. The brain malformation can be the direct result of the dosage of a given gene such as in the case of the *PAFAH1B* gene, it can result as the interaction of the dosage of more than one gene within the CNV similar to interaction of *PAFAH1B* and *YWHAE* genes, or ultimately it can result as an indirect result of the gene dosage through interaction with other genes not within the CNV, in the case of the 22q11.2 microdeletion.

# 2.3 Size of hereditary CNVs

The average size of hereditary losses in this study is 250 kbs while the average size of hereditary gains is 520 kbs. This could suggest that the general threshold of tolerance for loss and gain is, in average, different by a factor of 2. This is slightly different from published data generated by studying CNVs in the general population, where benign losses were almost three fold shorter than gains (43 kb vs. 120 kb) (Redon et al., 2006). One reason can be the resolution of the array we have used and its inability to detect the smaller variations. It could also be assumed that the rare CNVs we have detected are not within the range of the common benign variants more commonly present in the general population. More specifically, the present data could suggest that the losses are larger than common benign loss variants and more likely, constitute predisposing changes.

The two detected hereditary losses are 240 and 260 kbs while the smallest *de novo* loss is 1.10 Mb, 4-5 folds larger. The smallest detected hereditary gain containing gene/s is 210 kbs; the smallest *de novo* gain is 2.46 Mb and is more than 10 fold larger. Of course, this is a broad generalization that cannot hold true for losses/ gains that affect a single gene with significant gene dosage or conversely encompass large gene deserts.

It has been argued that losses are under stronger purifying selection, which removes deleterious variants from the population, than gains (Locke et al., 2006; Brewer et al., 1999).

In the study by Redon 2009, there is no significant difference in the frequencies with which benign losses and gains were called ($p>0.05$ using G-test for independence on WGTP data) in the general population. However, in our study, there is not only a significant difference between the average size of the losses and gains, there is also an even more significant difference in their frequency, where 12 of the 14 hereditary CNVs are gains. This could be biased by the fact that we are unable to detect the smaller variations among which losses may be more common. Conversely, the size difference of our rare CNVs in comparison to the benign CNVs most commonly detected in the general population could signify their potential contribution to the phenotype observed in the affected carrier in view of the possibility of incomplete penetrance and variable expression.

# 3. CNVs in specific brain malformations

To determine the possible relation between CNVs and specific brain malformations and/or classification of brain malformations, we looked at the brain malformations and the CNVs detected within each category.

## 3.1 Corpus Callosum Agenesis

We have 11 patients with corpus callosum agenesis as a major finding. Three of these eleven patients, approximately 27%, have *de novo* CNVs which is the highest frequency for a given structural brain malformation in our cohort. There are 7 more patients with corpus callosum and additional brain malformation/s, totaling 18 cases, and if we include the patients with hypogenesis of corpus callosum we have total of 21 cases with abnormalities of the corpus callosum. The frequency of patients with CNVs is 14.2% among these patients. We find that regardless of the categorization, this is the brain malformation with the highest frequency of *de novo* CNVs.

A former study of 63 patients with partial/complete agenesis of the corpus callosum reported chromosomal constitution imbalances in 11% of the patients (Paul et al., 2007). They proposed that the presence of corpus callosum agenesis alone did not correlate with higher probability of chromosomal imbalance and it was only in the presence of another brain

malformation or intellectual disability that there was a higher probability of imbalance indicated. Our findings show that the highest frequency of CNVs in patients is correlated with hypogenesis/ agenesis of corpus callosum with or without other structural malformations. All our patients have intellectual disability, and the added presence of malformations of the corpus callosum shows a direct correlation with the highest CNV frequency in our cohort. The increased detection rate of CNVs in comparison to the former study is most probably a result of the increased resolution of array CGH in comparison to routine cytogenetic analysis.

Within the proposed classification of brain malformations, 28% of all cases with midline defects: malformations of corpus callosum and holoprosencephaly have CNVs, which suggests that there is an increased possibility of CNV detection in these patients. These malformations reflect very early events in brain development and are more closely linked to a variety of genetic factors that are affected by genomic imbalances.

## 3.2 Cerebellar hypoplasia

Of the 8 cases with cerebellar hypoplasia, none have *de novo* CNVs, which is consistent with the fact that to date all genes identified in the etiology of cerebellar hypoplasia are autosomal recessive genes and there are no specific haploinsufficient genes recognized in the etiology of this brain malformation.

## 3.3 Microcephaly

Microcephaly is defined as head circumference with standard deviation of -2 or less. By this definition microcephaly is found in 2% of newborns and constitutes a feature found in more than 400 genetic syndromes (Gilbert et al., 2005). Microcephaly is a clinical feature in multiple microdeletion syndromes such as the 15q11.2, 3q29, 17q12, 17q21.31 to name a few. It is also found in microduplication syndromes such as the 5q24. In all of these instances the CNVs are *de novo*. There usually is not sufficient data to pose evidence for the definitive role of single genes or specific regions in the etiology of the deviation in head size.

There is one well documented instance where hereditary microdeletion/duplications are shown to be associated with abnormal head size. In the 1.35-Mb microdeletion/duplication at chromosome 1q21.1 from position 145-146.35 Mb (NCBI Build 36) 1q21.1 (MIM#612474

&612475), 50-57% of the heterozygous deletion carriers are microcephalic and the majority of duplication carriers are macrocephalic (Brunetti-Pieri et al., 2008). Most often, the imbalance is hereditary with incomplete penetrance and variable expression. Of the eight OMIM genes: *PRKAB2* (MIM#602741), *FMO5* (MIM#603975), *CHD1L* (MIM#613039), *BCL9* (MIM#609004), *ACP6* (MIM#611471), *GJA5* (MIM#121013), *GJA8* (MIM#600897), and *GPR89B* (MIM#612806) included in this region no single gene mutation is known to cause the 1q21.1 microdeletion phenotype, but haploinsufficiency of one or more of the deleted genes likely contributes to the phenotype (Brunetti-Pierri et al., 2008). Brunetti-Pierri et al. suggested that the variation in head size is due to the deletion or duplication of the chromosome 16q22.2 HYDIN paralog that has been inserted into the 1q21.1 region (Davy & Robinson, 2003). Homozygous mutations of the *Hydin* (MIM#610813) gene cause hydrocephalus in mice and microcephaly has been reported in individuals with deletions of 16q22.2 involving the *Hydin* gene (Callen et al., 1998; Fujiwara et al., 1992; Natt et al., 1989)

In our cohort, 42 of the 100 cases have microcephaly, 20 of them with other structural brain malformations. Eight (20%) have CNVs, 5 (13%) inherited and 3 (7%) *de novo*. Cases 17652, 17926 and 23510 with *de novo* CNVs, also have other structural brain malformations, corpus callosum agenesis in the first two and lissencephaly in the latter.

Of the 5 microcephaly patients with hereditary CNVs, patients 22748 and 20673 have additional malformations, focal cortical dysplasia and cerebellar hypoplasia, respectively. Patient 18986 has the 16p13.11 duplication, which is a recognized recurrent microdeletion/ duplication syndrome with variable phenotypes and incomplete penetrance, this is the first case with microcephaly. The hereditary CNVs: dup5p15.2 in patient 20732, del6p24.3 in patient 20673, dup10q23.31 in patient 22748 and dup18q23 in patient 21003 may later constitute microdeletion syndromes similar to the 1q21.1 microdeletion. Further data and patient cases are required.

To date, the genes: *MICROCEPHALIN* (MCPH1), *WDR62* (MCPH2), *CDK5RAP2* (MCPH), *CEP152* (MCPH4), *ASPM* (MCPH5), *CENPJ* (MCPH6) and *STIL* (MCPH7) found to be responsible for true congenital microcephaly of -4 SD or less, are all autosomal recessive. As the majority of our patients in this cohort were not selected according to these criteria, it is unlikely that they would be within this category of microcephaly.

## 3.4 Posterior fossa malformations:

Of the 10 cases with malformations of the posterior fossa, 2 have inherited CNVs. No *de novo* CNVs were detected in this group of patients. The only known CNV linked to Dandy Walker syndrome has been identified by Grinberg et al. (2004) in 7 individuals with *de novo* interstitial losses of 3q. They identified two candidate genes, *ZIC1* and *ZIC4* heterozygous loss of which were sufficient to cause DWM-like cerebellar vermis hypoplasia in mice. We did not find any associated regions in the patients of our cohort.

## 3.5 Lissencephaly/Pachygyria

Of the 19 patients with lissencephaly/pachygyria, 2 (approximately 10%), patient 20375 with deletion and patient 23510, have *de novo* overlapping deletions of 17p13.3p13.2 and 2 (approximately 10%), patient 22245 with dup9p24.3 and patient 19958 dupXp22.33, have inherited CNVs, the latter bearing no genes. The gene content of the two *de novo* and two hereditary CNVs will be discussed further in the following sections.

# 4. Gene Content of CNVs

It becomes apparent that we must consider the CNVs by their content to be able to postulate their role in the etiology of the brain malformations. Taking a look at the *de novo* and hereditary CNVs, we can see that as a result of the size difference there is a considerable difference in gene content. Of the 14 hereditary CNVs, 4 do not carry any genes. And the other 10 overlap or contain 21 UCSC genes (excluding hypothetical proteins), 8 of which are in the CNV in patient 18986 with 16p13.11 duplication. Whereas, there are 17 genes in the smallest *de novo* CNV detected on 16p13.2.

## 4.1 Network analysis for Brain Development genes

In the published analysis of this cohort and a cohort of 69 patients with brain malformations and epilepsy referred to a Danish epilepsy center, bioinformatics research of the data was

performed. A protein-protein interaction network including 1557 proteins (p-value = 0.00000072) was established by using 324 genes related to brain development according to BioMart (Lage et al., 2007; Smedley et al., 2009). The search for candidate gene clustering within the interaction network, or otherwise topological analysis was performed for *de novo* and inherited CNVs separately. It shows that genes mapping to *de novo* CNVs are significantly concentrated within the network (P-value = 0.036), while those genes located within inherited CNVs are not (P-value = 0.11). Separate analysis of this cohort and the Danish cohort revealed that the network concentration reaches significance only in *de novo* CNVs from this cohort (P-value = 0.039 and P-value = 0.31 for genes within *de novo* and inherited CNVs, respectively). The p-values generally become lower when both cohorts are included. This is due to the topological fact that when using both cohorts, the smaller networks supplement each other thereby connecting more genes from the CNVs.
The result of this network analysis confirms the role of the CNVs, most significantly the de novo CNVs, in the etiology of brain malformations.

## 4.2 Brain development genes

Also investigated and reported in Kariminejad et al. 2011, was whether CNVs containing a brain development gene are enriched in the combined datasets when compared to CNVs identified in 2026 healthy controls (Shaikh et al., 2009). Due to the small sample size of the pooled data all the pathogenic CNVs were used for this analysis. Interestingly, the general gene count per base was higher in the potentially disease causing CNVs, which argues for the efficiency of our filtering procedure, but made it necessary to introduce a 1Mb size thresholds for the control CNVs in order to get an acceptable similarity in gene count distribution (P-value = 0.8928). In total, 17 rare CNVs from this study and that of the Danish cohort, and 16 CNVs from the control set were chosen because they all contained at least one gene from the PPI-network (P-value = 1). Again the *de novo* CNVs are significant (p-value: 0.015) while the inherited are not (p-value: 0.51). Although the frequency of CNVs comprising PPI-network genes is nearly the same in both CNV sets, the absolute number of PPI-network genes within the CNVs differs significantly. We found that the rare CNVs identified in our pooled cohorts generally contains significantly more (P-value = 0.011) genes involved in brain development (mean=2.9, median=2) than the CNVs detected in healthy individuals (mean=1.6, median=1). These findings support the increased possible

significance of the *de novo* CNVs in brain structural malformations and the significance of the gene content in determining the pathogenicity of the CNVs.

In many cases the phenotypic effect may not be caused by a single gene alone, but instead may depend on the synergistic action of two or more genes. This idea is fueled by the observation that, while the chance to find one gene involved in brain development is the same for the CNVs in patients and healthy controls, the chance to find two or more of them in one CNV is considerably higher in patients. Yet, the sample size of our comparison was rather small (16 vs. 17 CNVs) in the joint cohorts (Kariminejad et al. 2011), so this finding will have to be verified in other cohorts before strong conclusions can be drawn. Also of interest in this context is the identification of more than one rare CNV in individual patients.

## 4.3 Gene content of *de novo* CNVs

### 4.3.1 *PAFAHB1, YWHAE* in 17p13.3 deletions

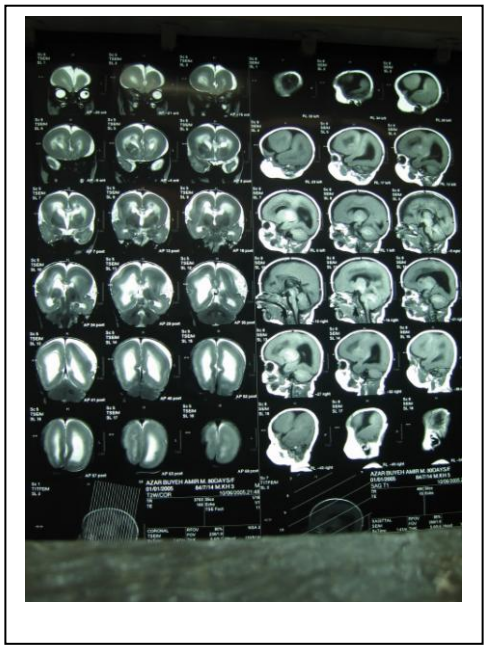
In two patients, 2% of the total number in the cohort and approximately 20% of those patients with pathogenic imbalances, deletions of 17p13 were detected. Overall, we have 19 patients with lissencephaly (partial and complete) and pachygyria. Of the six genes identified that cause or contribute to lissencephaly in humans, *PAFAH1B1* or *LIS1* (17p13.3) (Kato et al., 2003) and *YWHAE* (17p13.3) (Toyo-oka et al., 2003) responsible for classical lissencephaly (OMIM#607432) and Miller Dieker syndrome lissencephaly (OMIM#247200) reside on the short arm of chromosome 17, *RELN* causing Norman Roberts syndrome lissencephaly (OMIM#257320) on 7q22.1 (Zaki et al., 1995), *TUBA1A* caused lissencephaly (OMIM#611603) on chromosome 12q13.12 (Poirier et al., 2007), *NDE1* in lissencephaly with microcephaly (OMIM#614019) on chromosome 16p13 (Backircioglu et al., 2011), *DCX* responsible for lissencephaly and agenesis of corpus callosum (OMIM# 300067) on Xq22.3-23 (Gleeson et al., 1998) *ARX* causing hydranencephaly and abnormal genitalia (OMIM#300215) on Xp21.3 (Kitamura et al., 2002).

*PAFAHB1* is the gene considered to be responsible for normal migration of the neural cells in the 12-14$^{th}$ week of gestation. There is evidence that the heterozygous loss or mutation in this gene is sufficient to cause isolated lissencephaly. In Miller Dieker syndrome, which is

characterized by lissencephaly and additional dysmorphic features, a 400kb commonly deleted region on chromosome 17p13.3 encompassing eight genes *PRP8* (MIM#607300), *RILP* (MIM#607848), *SREC (SCARF1*, MIM#607873), *PITPNA* (MIM#600174), *SKIP* (MIM#607875), *MYO1C* (MIM#606538), *CRK* (MIM#164762), *and 14-3-3-epsilon (YWHAE,* MIM#605066) was determined by Cardoso et al. (2003). Based on comparison of 17p13.3 microdeletion patients presenting the Miller Dieker phenotype with those patients presenting isolated lissencephaly without the Miller Dieker syndrome phenotype, a 258 kb critical region including the 6 genes: *TUSC5* (MIM#612211), *YWHAE, CRK, MYO1C, SKIP* and part of *PITPNA* seems to correlate with the facial features and additional findings of the Miller Dieker syndrome (Mignon-Ravix et al., 2009; Nagamani et al., 2009; Bruno et al., 2010). This region encompasses 5 of the formerly suggested genes and *YWHAE* considered to be involved in the greater severity of the brain malformations in the Miller Dieker syndrome (Toyo-oka et al. 2003 and 2004). *CRK* gene is considered to be the gene responsible for growth regulation (Bruno et al., 2010)

The *PAFAH1B* is the single gene associated with brain malformations found with the highest frequency in our cohort. Two deletions of 17p13.3 were detected in patients 20375 and 23510. The deletion in patient 20375 is a 3.66 Mb deletion of the terminal region of chromosome 17p13 and the deletion in patient 23510 is a smaller 1.2 Mb interstitial and more proximal deletion. They both include the *PAFAH1B* gene. The larger deletion also includes *YWHAE* and *CRK*. Patient 20375 has the classical clinical features and lissencephaly characteristic of the Miller Dieker syndrome, while patient 23510 has lissencephaly without additional clinical features [Figures IV.1 & 2]. The findings in our two patients with 17p13 deletion correlate with the data on this region.

Considering that array CGH detects approximately 50% of cases with PAFAHB1 involvement and that 15% are caused by intragenic duplications and deletions detectable by MLPA, and another 35% by sequencing of the gene (Dobyns and Das, 2009); it could be assumed that another two patients would have a *PAFAHB1* related brain malformation totalling 4 of the 19 patients with lissencephaly/pachygyria, which would suggest the highest rate of single gene involvement.



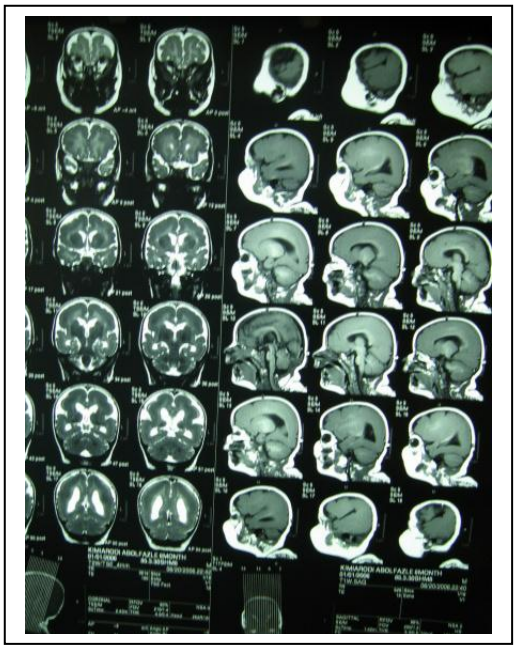


Figure IV.1 MRI of 17p13.3 microdeletion patients: the one on the left belonging to patient 20375 shows a more severe form of lissencephaly than the one on the right belonging to patient 23510.

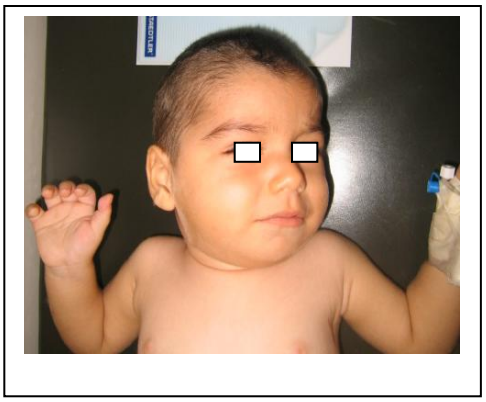


Figure IV.2 Patient 23510 does not exhibit the facial characteristics of Miller Dieker syndrome such as bitemporal narrowing, high prominent forehead, short nose with upturned nares, protuberant upper lip with downturned vermilion border and small jaw

## 4.3.2 AKT3 and ZNF238 in corpus callosum agenesis

The other gene/s that are directly implicated in the brain malformation of the patient are the *AKT3* (MIM#611223) or *ZNF238* (MIM#608433) in the deleted region of 1q43q44 (MIM#612337) that have been postulated as being responsible for corpus callosum agenesis. In a 2 year old boy we identified a 3.5 Mb *de novo* deletion of 1q43q44 (chromosome position). He was born by caesarean section but delivery was complicated by perinatal asphyxia, neonatal hypocalcemia & hypoglycaemia. He presented with myoclonic seizures at the age of 2 months. Brain MRI showed hydrocephalus and agenesis of corpus callosum. At 2 years of age, he was evaluated as having severe psychomotor retardation. His weight was

11 kg (SD -2), height 88 cm (SD-2), and OFD was 42 (SD-2) cm. He had dysmorphic features including microcephaly, malformed ears, bulbous nose, puffy eyelids, round face, short neck which are characteristic for 1q43q44 deletions [Figure IV.3].

Boland et al. (2007) described detailed mapping studies of patients with unbalanced structural rearrangements of distal 1q4. Based on their analysis of 4 patients with microcephaly and agenesis of corpus callosum, the overlapping deletion region was found to lie within a 3.5 Mb region interval with proximal border beyond *RGS7* (MIM#602517) & distal border beyond NP_001013732.

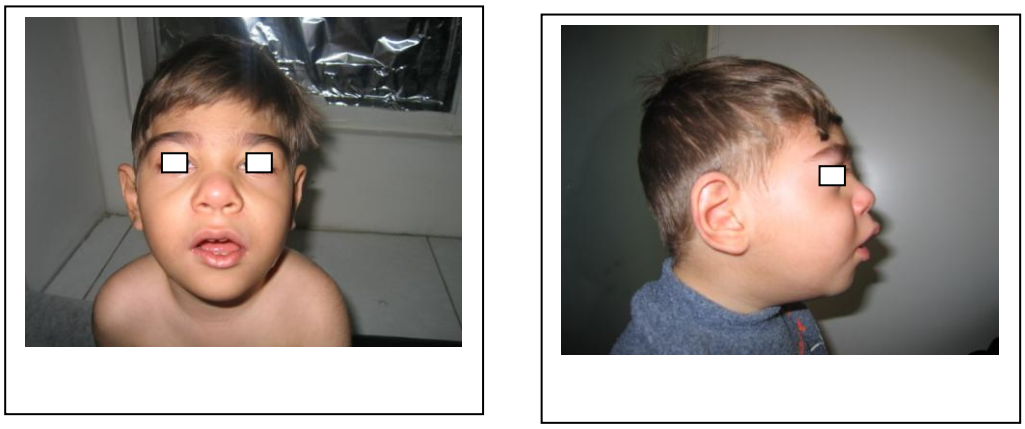


Figure IV.3 Images of patient 17652, the short nasal bridge, puffy eyelids, round face are noted.

Mapping of a balanced reciprocal t(1;13)(q44;q32) translocation in a patient with postnatal microcephaly and agenesis of the corpus callosum demonstrated a breakpoint in this region that was situated 20 kb upstream of *AKT3*, a serine-threonine kinase. The murine ortholog *AKT3* is required for developmental regulation of normal brain size and callosal development. Whereas sequencing of *AKT3* in a panel of 45 patients with agenesis of the corpus callosum did not demonstrate any pathogenic variations, whole-mount in situ hybridization confirmed expression of *AKT3* in the developing central nervous system during mouse embryogenesis. The authors concluded that thus, *AKT3* represents an excellent candidate for developmental human microcephaly and agenesis of the corpus callosum, and suggested that haploinsufficiency causes postnatal microcephaly and agenesis of the corpus callosum.

Hill et al. (2007) defined a 2 Mb overlap region in the interval between *PLD5* and *LOC440742* by putting together the breakpoints of 6/7 of their cases of 1q43q44 deletion who had microcephaly and corpus callosum agenesis. The two regions defined by Hill and Boland

overlap in the 2.0 Mb region defined by Hill which encompasses the *AKT3* and *ZNF238* genes.

Later, van Bon et al. reported another 13 cases with deletion of 1q43q44 region. The first case in their report has microcephaly and a 1.7 Mb deletion, the proximal border of which lies within *AKT3* gene and will contain only the *ZNF238* gene of the 2.0 Mb region specified by Hill et al. Also, Van Bon et al detected deletion of *AKT3* in two (sib pairs) patients and their normal mother arguing against haploinsufficiency of *AKT3* in causing microcephaly. Based on their analysis of breakpoints within the region and the common deleted region in their 11/13 patients with microcephaly and corpus callosum agenesis, they narrowed their region to a 360 kb region including four refseq genes *Corf100, ADSS* (MIM#103060), *C1orf101,* and *Corf121* and not including either of the two genes formerly suggested.

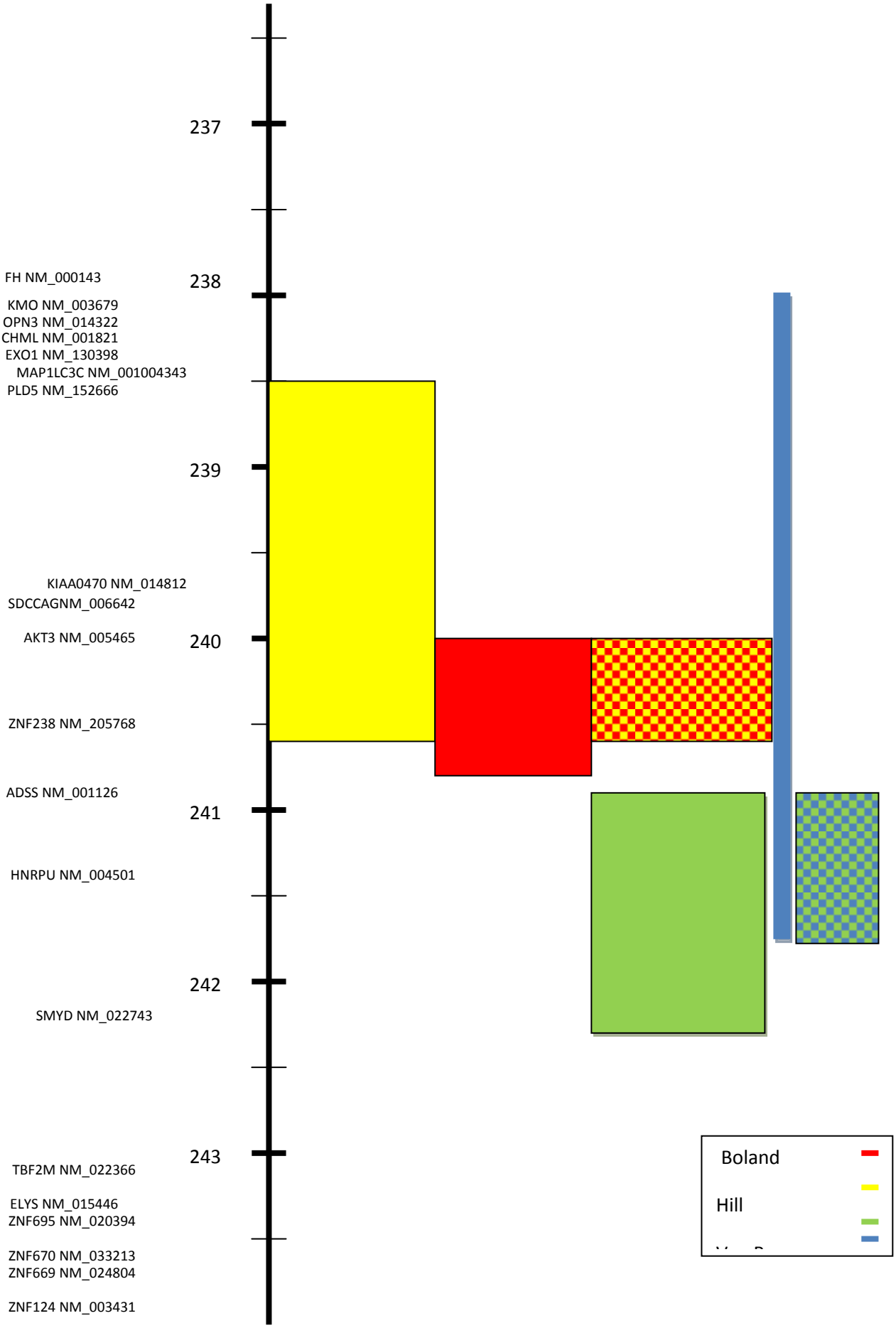
We have compared the critical regions defined by the former studies, Boland et al., Hill et al., and van Bon et al. with the present case [Figure IV.4]. The present deletion includes the previously discussed candidate gene, *ZNF238* for microcephaly and the 360 kb critical region for corpus callosum agenesis further supporting the causative role of this gene and region in the pathogenesis of these features. It overlaps with the critical region defined by van Bon and excludes one of the genes, *Corf121* suggested by their study. Unfortunately, breakpoints do not provide any basis for further characterization of the genes responsible for corpus callosum agenesis and microcephaly but do provide evidence in support of the present knowledge of the region.

## 4.3.3 Known syndromes

In addition to the 17p13.3 microdeletion syndrome cases and the 1q43q44 microdeletion syndromes discussed above, we identified other cases with known syndromes that will be discussed below.

### 4.3.3.1 Tetrasomy of 12p

The single recurrent identical CNV detected in this cohort is 12p whole arm gain, determined to be tetrasomy 12p by FISH analysis. Of the 10 *de novo* CNVs, we have detected 2 cases with mosaic tetrasomy 12p which is compatible with Pallister Killian syndrome (OMIM#601803) [Figure IV.5]. As the cases were selected prospectively and based on the


237
238
239
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242
243
FH NM_000143
KMO NM_003679
OPN3 NM_014322
CHML NM_001821
EXO1 NM_130398
MAP1LC3C NM_001004343
PLD5 NM_152666
KIAA0470 NM_014812
SDCCAGNM_006642
AKT3 NM_005465
ZNF238 NM_205768
ADSS NM_001126
HNRPU NM_004501
SMYD NM_022743
TBF2M NM_022366
ELYS NM_015446
ZNF695 NM_020394
ZNF670 NM_033213
ZNF669 NM_024804
ZNF124 NM_003431
Boland
Hill

presence of a structural brain malformation, it would seem that the 12p tetrasomy should be considered in similar cases and screened for in a clinical setting. It appears that this imbalance is a recurrent cause of brain structural malformations and requires special attention. Despite the fact that not all patients with 12p tetrasomy have the same brain phenotype, which is in part due to the mosaicism consistently present in patients with this chromosomal aberration, the presence of megalencephaly in both of our cases is noteworthy. Regarding the few cases in this cohort with this malformation, a direct correlation of megalencephaly with this chromosomal imbalance is implicated.

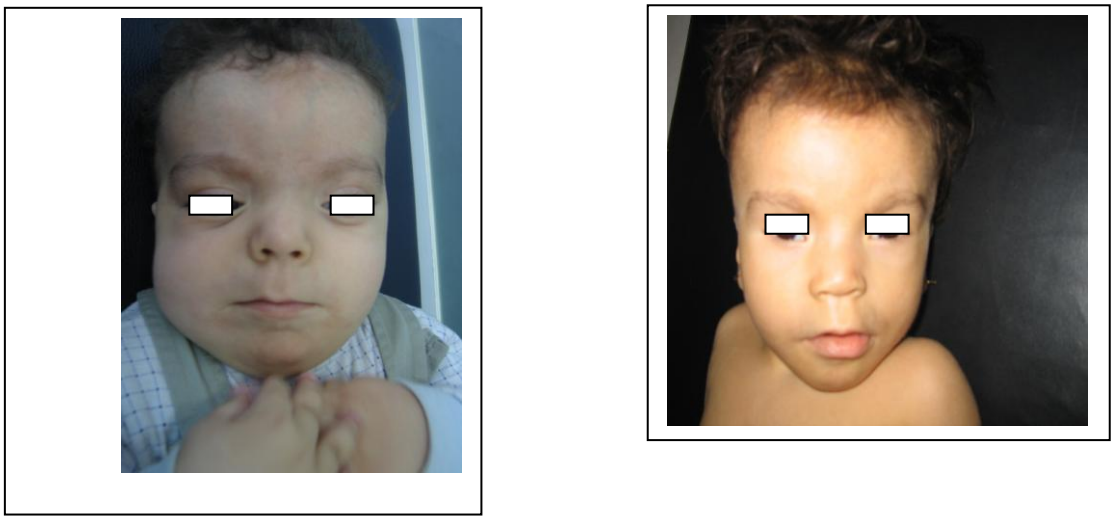

Figure IV.5 Patients 20196 (left) and 21573 (right). Clinical features of Pallister Killian Syndrome including bossing forehead, hypertelorism, flat nasal bridge and long philtrum are noted in both.

An unresolved issue is the ability of array CGH to detect low level mosaicism. There is still insufficient data to evaluate conclusively the limits of mosaicism detection on various array-CGH platforms. Former studies have shown the ability of CGH to detect mosaic patterns of chromosome aneuploidies and even partial imbalances (Cheung et al., 2007) that were present in as few as 6.5% of the lymphocytes studied by FISH. Powis et al. (2007) reported the detection of tetraploidy of 12p by array-CGH.

The detection of the mosaic pattern in these patients is of special interest as this technique would provide an appropriate alternative to the presently used invasive practice of culturing fibroblasts for karyotyping, as most often the 12p tetrasomy is not always present in metaphases derived from peripheral blood. The minimal shift on the array from the normalized median, in the case of patient 21573, is interesting and emphasizes the fact that

visualization of these slight variations from the normalized median requires very good and robust hybridizations [Figure IV.6].

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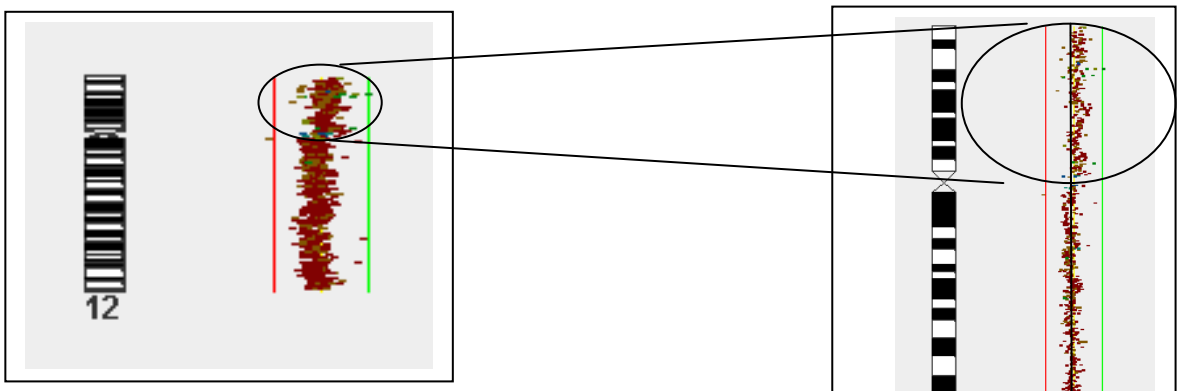


Fig IV.6 Result of array in patient 21573. There is a slight shift in the short arm of chromosome 12 from the normalized median but still within the normal range of standard deviation.

Final interpretation would not have been possible without FISH confirmation. In case 20196, tetrasomy 12p was present in both metaphase and interphase cells with a frequency of 12%. In case 21573 tetrasomy 12p was not seen in any of 50 metaphase spreads scored by FISH but was present in 14 of 150 interphase cells, final frequency of 7% [Figure IV.7]. In terms of DNA, this would mean a mosaic pattern of 24 and 14 percent as the affected cells carry four copies of 12p.

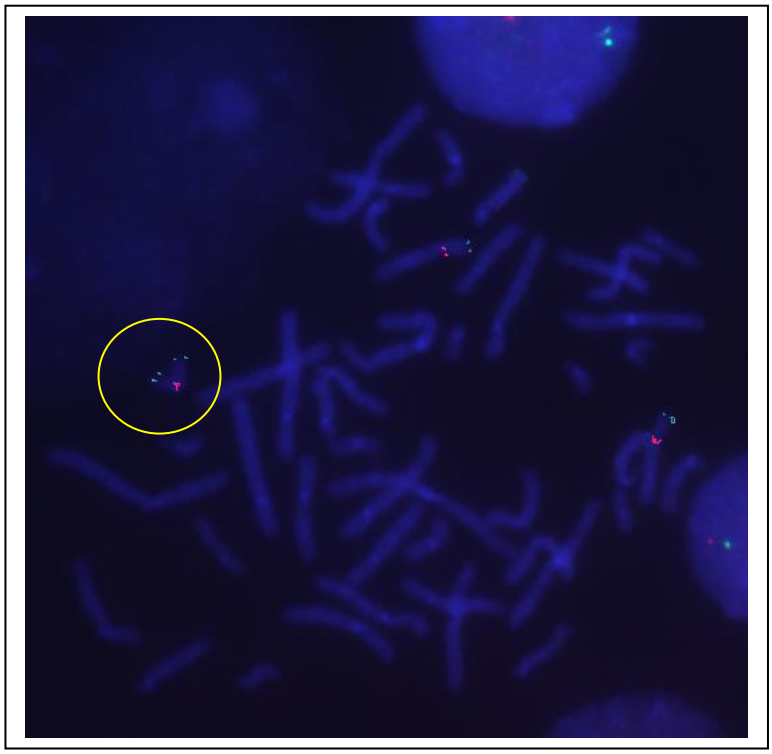

Figure IV.7 The isochromosome 12p is within the circle where the probes for the pter region of 12 are shown in green and the probe for the centromere is shown in red.

### 4.3.3.2 Microdeletion 1p36

1p36 deletion syndrome (OMIM#607872) is characterized by typical craniofacial features consisting of straight eyebrows, deep-set eyes, midface hypoplasia, broad and flat nasal root/bridge, long philtrum, pointed chin, large, late-closing anterior fontanel, microbrachycephaly, epicanthal folds, and posteriorly rotated, low-set, abnormal ears. Other characteristic findings include brachy/camptodactyly and short feet. Developmental delay/intellectual disability, of variable degree, is present in all, hypotonia in 95% and seizures occur in 44% to 58% of affected individuals. Most patients have microbrachycephaly. Other structural brain malformations are not consistent and infrequent in these patients.

Various genes including *DISHEVELLED-1 (DVL1,* MIM#601365) (Bedell et al., 1998), *GAMMA-AMINOBUTYRIC ACID A RECEPTOR DELTA SUBUNIT (GABRD,* MIM#137163) (Windpassinger et al., 2002) and *PROTEIN KINASE C ZETA (PRKCZ,* MIM#176982) genes have been candidated for the neurodevelopmental disorders in the

phenotype. However, all genes are located in the terminal region of 1p36.3 and are not included in the deleted region of many patients with interstitial deletions.

There has been further classification of the 1p36 microdeletion syndrome into terminal and more proximal variant groups (Gajeckam et al. 2007; Kang et al. 2007). Apart from the severity of the phenotype, there may be very little variation in the phenotype between the two

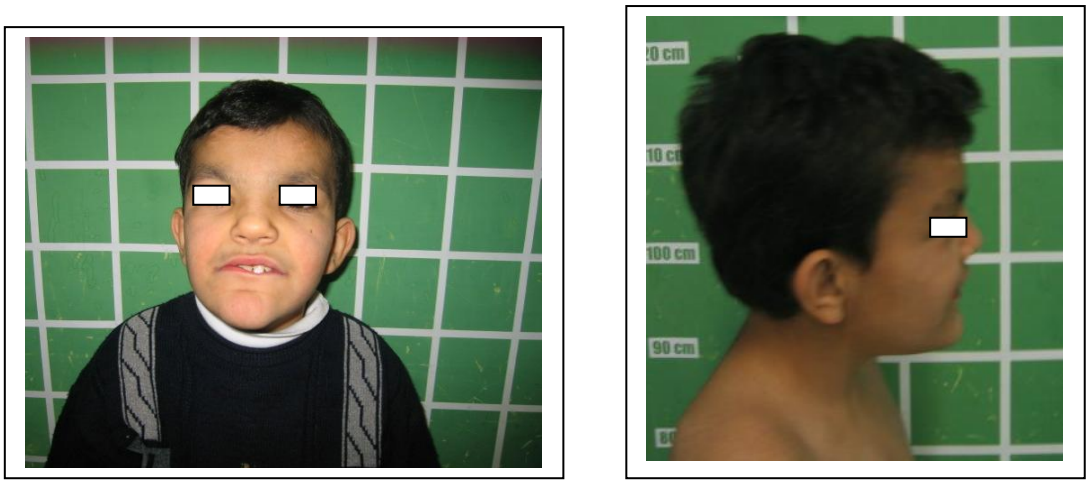

Figure IV.8 The photos show the deep-set eyes, midface hypoplasia, broad and flat nasal root/bridge, and low set ears.

groups and there are major overlapping features even when there is little overlap between the deleted regions. When the microdeletions in two of the 6 patients with highly similar phenotypes were characterized by array-CGH and had no region overlap, Redon et al. (2005) proposed that position effect and not the deleted region is the major pathogenetic factor in the development of the phenotype.

Our patient, case 23379, has an interstitial deletion that does not overlap the common terminal region or any of the genes candidated thus far. The physical position of the CNV would better qualify as the more proximal deletion variant. The clinical phenotype and features have extensive overlap with those of the more distal group [Figure IV.8]. He shares many of the facial features, and clinical findings including a history of seizures, that is medication controlled, a small VSD, and repaired inguinal hernia. He differs in that he has normal for age head circumference and corpus callosum agenesis as revealed by MRI. He does not have hearing loss, or clefting abnormalities, which have been assigned to the more

distal and subtelomeric regions of the 1p. The *KCNAB2* (MIM#601142), a subunit of K+ channel has been considered to be one of the factors responsible for seizures (Heilstedt et al., 2001) in a number of these patients and is within the deleted region of our patient.

The more proximal deletion breakpoints such as those in our patient are not detectable by the commercially available FISH or MLPA probes which target the more distal microdeletions. Most phenotypic evaluations of patients are from the more distal breakpoint and more data on brain malformations may appear as patients with the more proximal breakpoints are molecularly classified by array-CGH technologies.

#### 4.3.3.3 Microdeletion 22q11.2

Microdeletion of 22q11.2 (OMIM#188400) is the molecular event behind the Di George, Velocardiofacial, and CATCH22 syndromes. The microdeletion most often involves a common 3 Mb region that encompasses several genes that have been classified as Di George Critical regions 1-8. Despite the consistency of the deleted region, several brain malformations have been described in rare patients with the deletion 22q11.2 syndrome including agenesis of the corpus callosum, pachygyria or polymicrogyria, cerebellar anomalies and meningomyelocele, with PMG reported most frequently. In a review of 24 patients with 22q11 deletion and brain malformations, the authors concluded that the PMG may be a sequela of abnormal embryonic vascular development rather than a primary brain malformation (Robin et al., 2006). They argue that the phenotype of patients with brain malformations in the 22q11.2 deletion varies with no direct correlation with the deleted segment.

### 4.3.4 Novel genomic imbalances

#### 4.3.4.1 Deletion 2p and duplication 19q

Here we describe a 6 year old female patient with mental retardation and severe autism. She has normal facial features, hypertrichosis of back and limbs [Figure IV.9]. There are 3 CNVs detected in this patient, two on 2p and one on 19q. The breakpoints of the 2p loss have changed from the build 17 to build 18 and where initially they were separated by a 1.6 Mb interval (build 17), they are now considered to be in close proximity (build 18). This would

explain the presence of the 2 intermittent deletions detected in the patient 21835 [Figure III.1]. The deletion/duplication however is the result of a *de novo* subtelomeric rearrangement [Figure IV.10].

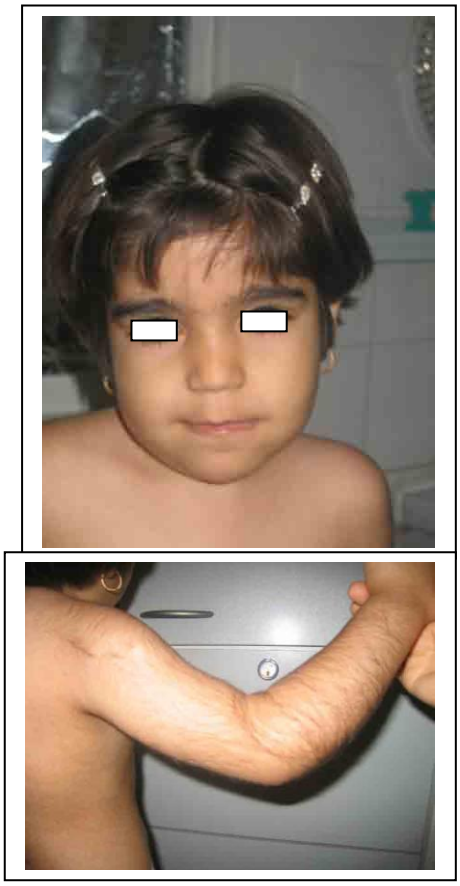

Figure IV.9 The patient has intellectual/developmental delay. The facial features are not dysmorphic and there are no congenital anomalies. The only phenotypic feature is hirsutism.

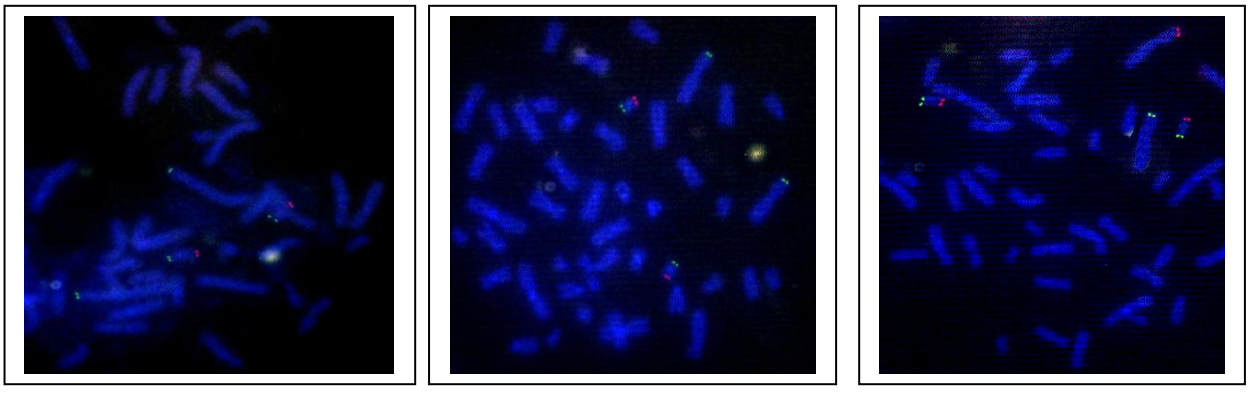

Figure IV.10 FISH analysis of proband (right) and parents (left and middle) show the subtelomeric rearrangement to be of de novo origin. 2pter and 19pter are labeled in green and 19qter is labeled in red.

There are very few reports of terminal 2p deletion and duplication 19q in the literature suggesting that they are rare aneusomies. There are no specific cases of brain malformations linked to the aneusomy.

The deleted region on 2p contains the *MYT1L* (MIM#613084) gene which functions as a panneural transcription factor associated with neuronal differentiation and may play a role in development of neurons in CNS.

The duplicated region on 19q13.3 contains the *TSEN34* (MIM#608754) gene, mutations in which have been recognized to cause autosomal recessive pontocerebellar hypoplasia type 2. However, our patient does not have signs of cerebellar hypoplasia but has a focal cortical dystrophy. The segment of the 19q duplicated region contains a number of leukocyte myocyte killer cell antigens or receptors, a large number of Zn finger genes and many other transcription units. As in recent years there has been increasing evidence for the role of transcription factors, many of these genes can be considered to be plausible candidates for contributing to the patient's mental retardation.

It has been reported that 2.5% of unexplained mental retardation is due to cryptic subtelomere imbalances which are detected using chromosome specific subtelomeric FISH probes (Ravnan et al 2005). This patient and patient 20375 with the terminal deletion of 17p13.3 are the two subtelomeric events in this cohort, constituting 2% of the total.

## 4.3.4.2 Duplication 16p13.2

The patient with the *de novo* tandem duplication of 16p13.2 presents with lobar holoprosencephaly [Figure IV.11] The clefting abnormalities that are believed to parallel the brain malformations are of the mildest form with midline cleft lip, ocular hypotelorism, and flat nose [Figure IV.12]. The child has severe intellectual and developmental delay with history of seizures.

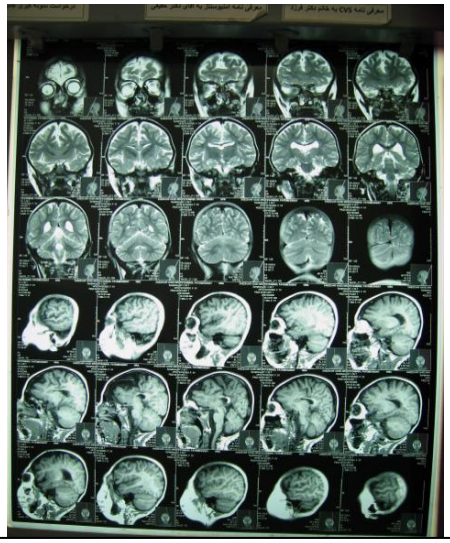

Figure IV.11 MRI revealing lobar holoprosencephaly

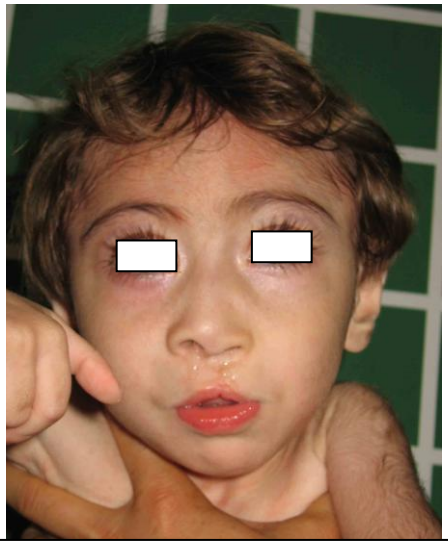

Figure IV.12 Midface clefting and hypotelorism

Holoprosencephaly has been associated with chromosomal abnormalities in all chromosomes, the most frequent in descending order are deletions or duplications of various regions of 13q, del(18p), del(7)(q36), dup(3)(p24-pter), del(2)(p21), and del(21)(q22.3) (Muenke & Beachy, 2001; Dubourg et al., 2007). Many of these regions are known to harbor known genes associated with autosomal dominant nonsyndromic HPE. Significant phenotypic variation exists among individuals with a similar cytogenetic deletion (Schell et al., 1996).

Copy number variants have been identified in 10 to 20% of all individuals with HPE (Bendavid et al., 2009). These CNVs can include loci already known to be associated with HPE, as well other loci whose relationship to HPE is less well understood (Bendavid et al., 2009). Duplications of chromosome 16p have not been associated with holoprosencephaly in the literature. Even patients with mosaic trisomy of whole chromosome 16 have not been reported to have holoprosencephaly; therefore, it would appear that overexpression of the region alone cannot explain the brain malformation. However, the present locus can very well be considered to be one of the loci where the relationship to HPE is less understood and may later contribute to the knowledge of candidate loci for this malformation.

The gene content of the CNV includes 17 RefSeq genes and 4 hypothetical proteins. Of these, the *LITAF* (MIM#603795) and *MHC2TA* (MIM#600005) are among OMIM morbid genes, the first causing autosomal recessive Charcot Marie Tooth syndrome type 1C (MIM#601098) and the latter leading to familial arthritis (MIM#600005). The region

contains many genes with possible involvement in early development of the brain. *GRIN2A* (MIM#138253) is a glutamate receptor believed to be involved in memory and learning. By gene analysis of Array CGH findings in a cohort of MR/MCA patients Poot et al. (2010), report enrichment of genes involved in glutamate receptor in the CNVs. *STANNIN* (MIM#603032), a gene believed to function in response to stress has very high fetal expression. *GSPT1* (MIM#139259), a G1 to S phase transition activating transcription factor is involved in translation termination in response to the termination codons UAA, UAG and UGA; stimulates the activity of *ERF1* (MIM#611888) and is involved in regulation of mammalian cell growth. EMP2, epithelial membrane protein 2, is part of the biological process of multicellular organismal development, cell death and cell proliferation. Another possible gene is the *NUBP1* (MIM#600280) encoding nucleotide binding protein 1 which is involved in the duplication of centrosome. *SOCS* (MIM#611659) is the suppressor of cytokine response and knockout mice models suggest that it has a role for postnatal growth and survival.

### 4.3.4.3 Deletion 9q and duplication 9q

Patient 17926 had microcephaly and agenesis of the corpus callosum. Brain MRI also revealed hydrocephaly and extensive cyst area mostly occupying right hemisphere with communication of lateral ventricles. The child is the first and only child of unrelated couple and was born by Cesarean section. The dysmorphic features include wide forehead, downslanting palpebral fissures, hypertelorism, and bossing wide forehead. The DNSA scan of kidney is mildly decreased and the child has jejunal atresia. Other clinical findings include high myopia, amblyopia and nystagmus. The child has severe intellectual and development delay [Figure IV.13].

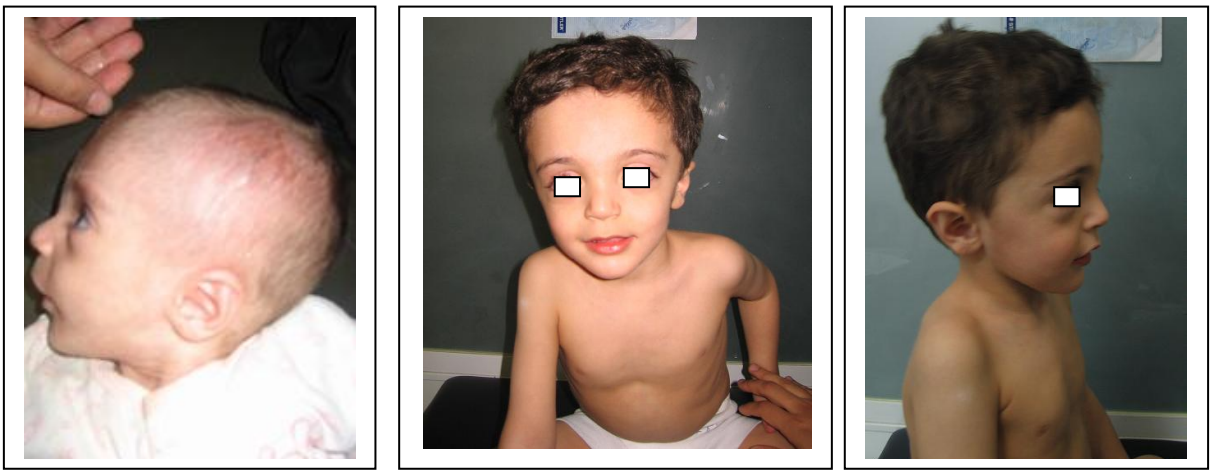

Figure IV.13 Patient at 9 months and again at 2 years.

The CNVs detected by array CGH are relatively large (4.6 Mb deletion and 11.2 duplication) and would not constitute submicroscopic variations. Upon retrospective re-evaluation of the karyotype, the imbalances could still not be called with clarity. To determine the location of the duplicated segment on chromosome 9q, FISH was done and revealed that the duplicated segment had been inserted into the deleted region, thereby masking the detection of the deletion and duplication in routine cytogenetic analysis [Figure IV.14].

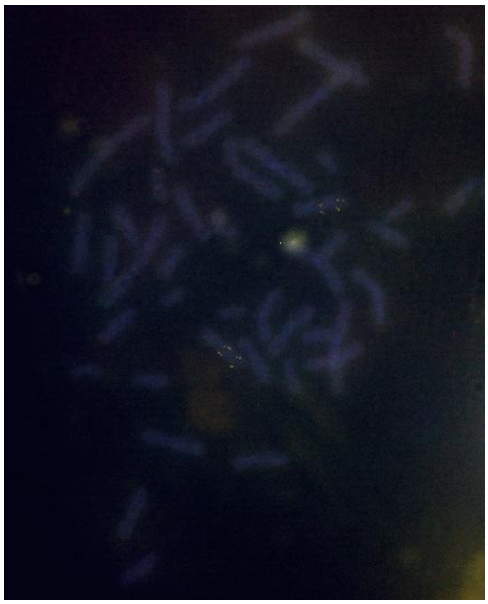

Figure IV.14 FISH results. Two probes were selected from 9q31 region and labeled in green, two probes from 9q21 region were labeled in red. As can be seen, there are red and green signals on one chromosome, and only green signals on the other chromosome.

A review of the literature shows a similar rearrangement reported in 1995, where a duplication of the 9q region is inserted into a region designated as 9q13. At the time

molecular evaluation was not possible and the case does not report a deletion of the 9q21.11-q21.13 region (Hou and Wang, 1995).

Chromosome 9 displays a high susceptibility to breaks. There is extreme polymorphic variability in the chromosome 9 pericentric region where pericentric inversion of chromosome 9, inv(9)(p11q13) is one of the most common chromosome heteromorphisms with a frequency of 1% in the general population. Chromosome 9 is also frequently involved in reciprocal translocations in humans (Cohen et al., 1996). Furthermore, this chromosome is represented more in unrejoined breakage events than expected by chance in human sperm complements (Brandiff et al.,1988; Estop et al., 1991).

Duplication/deletion of the 9q13q21 region is also a common segmental duplication/deletion variant. The rearrangement in this case resembled a duplication variant in cytogenetic study and was therefore overlooked in the karyotype. The present case indicates that a more cautious approach is required in the analysis of what may appear to be a benign duplication of the euchromatic 9q13q21 region.

The deleted segment includes the genes *FRATAXIN* (MIM#606829) and *APBA1* (MIM#602414). *FRATAXIN* is a regulator of mictochondrial iron transport and respiration; trinucleotide repeats within the gene leads to Friedrich Ataxia (OMIM#229300). *APBA1* encodes a neuronal adapter protein that interacts with the Alzheimer's disease amyloid precursor protein (APP) and is considered to be a putative vesicular trafficking protein in the brain. *GDA* (MIM#139260) encodes a guanine deaminase that probably plays a role in microtubule activity and is expressed in the forebrain.

The duplicated region includes the *FUKUTIN* (MIM#607440) gene. The encoded protein is thought to be a glycosyltransferase and plays a role in brain development. Defects in this gene are a cause of Fukuyama-type congenital muscular dystrophy (FCMD), Walker-Warburg syndrome (WWS), limb-girdle muscular dystrophy type 2M (LGMD2M), and dilated cardiomyopathy type 1X (CMD1X).

Also included in the duplicated region are *ACTIN-LIKE 7A* (*ACTL7A*, MIM#604303) and *7B* (*ACTL7B*, MIM#604304). They are members of a family of actin-related proteins (ARPs) which are involved in diverse cellular processes, including vesicular transport, spindle orientation, nuclear migration and chromatin remodeling. They are located within the familial dysautonomia candidate region on 9q31. The protein tyrosine phosphatase, non-

receptor type, is a member of a family of signaling molecules that regulate a variety of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation. The protein interacts with and may be regulated by adaptor protein 14-3-3 beta, a family of proteins of which the YWHAE is also a member.

*CDC26 SUBUNIT OF ANAPHASE PROMOTING COMPLEX* encodes a protein that is highly similar to Saccharomyces cerevisiae Cdc26, a component of cell cycle anaphase-promoting complex (APC). APC is composed of a group of highly conserved proteins and functions as a cell cycle-regulated ubiquitin-protein ligase. APC thus is responsible for the cell cycle regulated proteolysis of various proteins.

The solute carrier family 31 is among the family of copper transporters which are required for the maintenance of a critical copper balance by regulation of the intake, export, and intracellular compartmentalization or buffering of copper. The 2 related genes *ATP7A* (MIM#300011) and *ATP7B* (MIM#606882), responsible for the human diseases Menkes syndrome (OMIM#309400) and Wilson disease (OMIM#277900), respectively, are involved in copper export.

Also present within this region is the *PRP4* (MIM#602338), pre-mRNA processing factor 4 homolog, which is 1 of several proteins that associate with U4 and U6 snRNPs, the *KIF1B* (MIM#605995) gene involved in axon cargo transport and anterograde axon cargo transport, the *ENO3* (MIM#131370) gene responsible for plasminogen activator activity, and the *ESPN* (MIM#606351) gene encoding a protein in the actin filament network formation.

## 4.4. Gene content of hereditary CNVs

In the Redon study (2005) over half (58%) of the 1,447 CNVs overlap known RefSeq genes and they observed that a significantly lower proportion of deletions than duplications overlap with the OMIM database of disease-related genes ($p=0.017$, chi-squared) and RefSeq genes ($p=1.7\times10^{-9}$), and they have concluded that deletions are biased away from genes with respect to duplications.

We find that more than 70% of our CNVs overlap with known RefSeq genes. This is in part due to the larger average size of the CNVs detected in this study and may also further indicate their possible contribution to the phenotype of the affected carriers.

It is estimated that approximately 0.4% of the genomes of unrelated people typically differ with respect to copy number (Kidd et al, 2008). However, it has been shown that some CNVs present in the normal unaffected population are also present in affected members with epilepsy, autism, schizophrenia and intellectual disability in these families, with higher frequency than the general population. Examples of these CNVs are the 15q13.3, 1q21.1 microdeletions. This is presently attributed to the variable expression and incomplete penetrance of these CNVs and the genes they harbor.

The plausibility of the effect of a gene on given genetic backgrounds renders the development of a brain malformation by the inheritance of a well supported hereditary imbalance in a normal parent to an affected offspring possible. Therefore, we have considered the likelihood of the hereditary CNVs in affecting the development of the brain malformation in the offspring by looking at the gene content of these CNVs and the possible genes that could have a role.

## 4.4.1 The 16p13.11 duplication

One of the hereditary duplications, dup16p13.11, detected in this study is a formerly identified microdeletion/duplication syndrome proposed to have variable expression and incomplete penetrance in normal and affected carriers. Deletion of this region has not been identified in normal population samples, but duplications have been reported (Hannes et al., 2009), but do not account for the difference in frequency between the general population and those ascertained by patient referrals.

In an 8 year old male with severe mental retardation, short stature (111 cm), OFC of 48 cm (-2 SD), cortical atrophy and slightly dysmorphic features including a beaked nose and downslanting palpebral fissures, {Figure IV.15] we identified a paternally inherited 870kb 16p13.11 duplication. The duplication was not present in his unaffected sister. Ullmann et al. (2007) reported a 1.5 Mb duplication, spanning the present duplication, in 4 families with autism. The reported duplication was flanked by LCRs and detected in various family

members and exhibited phenotypes ranging from normal to variably affected neurobehavioral phenotypes. Furthermore, to date, the duplication/deletion has been reported in a number of patients with a range of phenotypes including intellectual/developmental delay, autism, schizophrenia and various congenital anomalies. These CNVs may be de novo or inherited from similarly affected, mildly affected or even normal parent. Breakpoints are most often consistent and comprise a 1.5-1.65 Mb region. The overlapping region between the common 1.5 region and the present case includes *C16orf45, LKAP, NDE1, MYH11* (MIM#160745), *FLJ31153, ABCC1* (MIM#158343), *ABCC5* (MIM#605251), *NOMO1* (MIM#609157), *NOMO3* (MIM#609159) and excludes 6 genes from the 1.5 Mb region including the *NTAN1* (which is

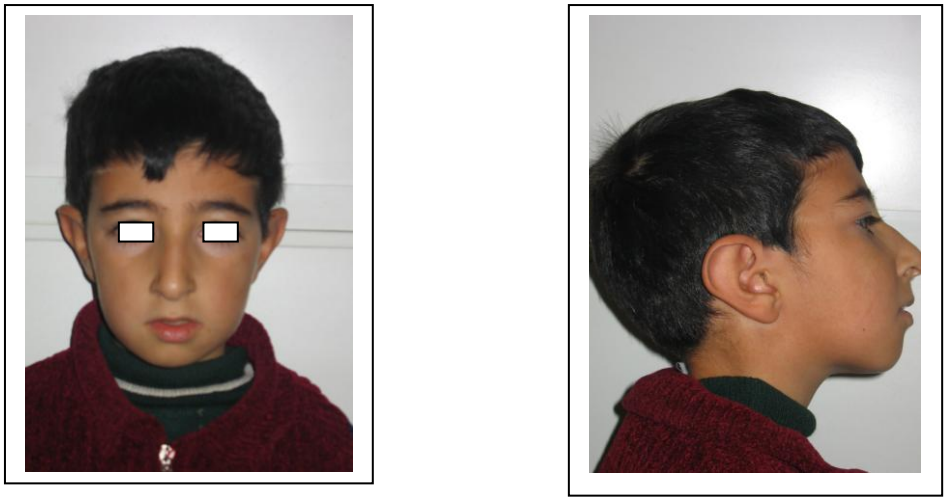

Figure IV.15 Clinical features of patient 19968 show the downslanting palpebral fissures, and beaked nose.

considered to be one of the two candidate genes for the mental/neurobehaviorial disorders of these patients. The distal border of the duplicated region in our patient is in the clone spanning 15.4-15.6 Mb, containing the gene *C16orf45* coding for the hypothetical protein LOC89927 which is highly expressed in the brain. Disruption of the *C16orf45* gene by the duplication can lead to monosomy of the gene and account for the mental retardation in our patient, which is more similar to that of the deletion patients formerly reported.

Copy number variations are frequently mediated by flanking segmental duplications which are more often hot spots for duplications (17.7%) rather than deletions (2.3%) due to non-allelic homologous recombination (NAHR) (Liu et al., 2011). Interestingly, in this case, the

duplication does not involve the flanking LCRs that were detected in the 1.5 Mb duplication/deletions formerly identified.

One of the genes found within this region that is presently known to cause lissencephaly and microcephaly is the *NDE1* gene. The protein product of the gene is highly expressed in the developing human cortex particularly at the centrosome and has a significant role in mitotic spindle assembly during early neurogenesis.

## 4.4.2 Other genes

Regardless of the smaller size and low gene content of the other hereditary CNVs, there are a number of genes within these regions that are already implicated in brain malformations or neurological disorders.

### 4.4.2.1 *VLDLR*

Very low density lipid receptor VLDLR is part of the reelin signaling pathway, which guides neuroblast migration in the cerebral cortex and cerebellum. Mutations in *RELN* will lead to lissencephaly (OMIM#257320). Reln binds directly and specifically to the extracellular domains of Vldlr and ApoER2 (Hiesberger et al., 1999). As blocking of VLDLR and ApoER2 ligand binding correlated with loss of Reelin-induced DAB1 tyrosine phosphorylation, Hiesberger et al. (1999) concluded that RELN acts via VLDLR and ApoER2 to regulate microtubule function in neurons by Dab1 tyrosine phosphorylation.

*VLDLR*-associated cerebellar hypoplasia (*VLDLR*-CH) is characterized by non-progressive congenital ataxia, moderate-to-profound intellectual disability, dysarthria, strabismus, and seizures and it is transmitted in an autosomal recessive pattern.

Patient 23779 with the maternally inherited duplication involving the *VLDLR* gene has pachygyria, a milder form of neuronal migration disorder than that seen in complete disruption of the *RELN* pathway. There is at present no data regarding the duplication or overexpression of the gene and it remains to be seen whether the neuronal migration disorder in our patient could result from the disruption of the reelin signaling pathway by increased VLDL receptor proteins.

### 4.4.2.2. *SLC19A3*

*SLC19A3* (MIM#606152) is a member of the reduced folate family of micronutrient transporter genes, with the highest abundance in mice kidney and brain detected by Northern blot analysis.

Biotin-responsive basal ganglia disease (BBGD)(OMIM#607483), a disorder with childhood onset, presenting as a subacute encephalopathy that progresses to severe cogwheel rigidity, dystonia, quadriparesis, and eventually death if left untreated, is caused by homozygous mutations in the gene. Our patient with the paternally inherited deletion involving the *SLC19A3* gene has focal cortical dysplasia, but does not have any of the other symptoms of the BBGD syndrome.

### 4.4.2.3 Genes with brain expression

In hereditary CNVs of our cohort we also find genes with high or specific brain expression that could be considered as candidate genes, for which there is insufficient data at present. HTR7 is a G protein-coupled receptor for serotonin (Ruat et al., 1993; Gelernter et al. 1995) expressed predominantly in human brain, functioning in adenylate cyclase activation. An association with cognitive function was mapped to 10q22-q24 and *HTR7* (MIM#182137) was within the cluster that was candidate as potential disease-causing genes in the region.

A major 9.5-kb transcript of *ADARB2* (MIM#602065) was detected only in brain with highest expression in thalamus and amygdale, in Northern blot analysis of several tissues. As ADAR3 binds dsRNA and single-stranded RNA (ssRNA) and also inhibits in vitro RNA editing by other ADAR family members, Chen et al. (2000) hypothesized that ADAR3 may have a regulatory role in RNA editing. Patient 17500 has a maternally inherited duplication of 10p15 region spanning this gene with brain phenotype of Dandy Walker malformation spectrum.

## 4.5. GO Term analysis

### 4.5.1 Biological processes

Searching for candidate genes in many of the CNVs identified in this study proved to be time consuming as many of the regions especially in the de novo CNVs encompass a large number of genes, many of which appear to be involved in cellular functions that relate to brain malformations. To systemize this search, we used a bioinformatics approach.

All genes located within the 39 unique chromosomal regions of the combined cohorts published in 2011 by Kariminejad et al., were subjected to enrichment analysis employing GO Terms and Kegg-pathways. Using a p-value cut off of 0.05 we identified significant enrichment of 12 molecular functions and 13 biological processes among the genes localized in the rare CNVs, the ones from this cohort are presented in Table III.7. No significant Kegg-pathways were identified. Many of the pathways and genes identified are unique, and occur in one patient only. However, there are GO terms that appear in more patients and affect genes that are more closely related to developmental process with possible roles in brain development. Based on the fact that four genes in five patients are involved in 'axonal transport', and in the 'c-Jun N-terminal kinase cascade' we propose that these molecular processes play a significant role in the etiology of brain malformations. The other molecular process that was in the joint cohort and is also present in our cohort is the di-, tri-valent inorganic cation transmembrane transporter activity which is represented by three genes in one of our patients.

The 'c-Jun N-terminal kinase cascade' is involved in mitogen stimulated response to stress such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock. This process mediates degeneration and apoptosis in the brain following stress mediated signals and/or DNA damage. The c-Jun N-terminal kinase isoforms JNK1 and JNK2 are found in all cells and tissues, while JNK3 is found mainly in the brain, but is also found in the heart and the testes. JNK activity regulates several important cellular functions including cell growth, differentiation, survival and apoptosis by activation of c-Jun, ATF2, ELK1, SMAD4, p53 and HSF1 and inhibition of NFAT4, NFATC1 and STAT3. *MINK1* (MIM#609426), expressed in the whole brain and amygdale, is upregulated in postnatal cerebral development, and is involved in actin organization and cell adhesion. *CRKL* (MIM#602007) is within the deleted region in di George/VCFS syndrome and is implicated in the etiology of the syndrome. *TNFRSF19* (MIM#606122) is a tumor necrosis factor, highly expressed during embryonic development; *CDC42ep5* (MIM#609171), is involved in actin filament assembly. Interestingly, mutations in *WDR62* (MIM# 613583), encoding a JNK-binding protein, has

recently been reported to cause microcephaly and diverse defects in cerebral cortical architecture (Nicholas et al., 2010; Yu et al., 2010).

In this cohort there are also 4 genes from 5 patients involved in di-, tri-valent inorganic cation transmembrane transporter activity. *FXN* is known to be related to ataxia, *SLC31A1* (MIM#603085) and *SLC31A2* (MIM#603088) are involved in copper transport, ubiquitously expressed, and early embryonic lethality has been shown in *SLC31A1* homozygous mutant embryos and a deficiency in copper uptake in the brains of heterozygous animals (ref 30) while *ATP2B2* (MIM#108733), *TNNI3* (MIM#191044) and *AMBP* (MIM#176870) are expressed in the brain.

Within the rare CNVs, we identified 3 genes from 4 patients involved in axon cargo transport four genes related to "axonal transport" including *PAFAH1B1* and *APBA1* (MIM# 602414). The role of *PAFAH1B1* in the etiology of brain malformations is well documented and has been described above. *APBA1* encodes a neuron-specific protein of the X11 protein family. The protein has a function in synaptic vesicle exocytosis by binding to *STXBP1* (MIM# 602926*;* Munc18-1) (Okamoto and Sudhof, 1997). Interestingly, haploinsufficiency of *STXBP1* has recently been described to cause early-onset epileptic encephalopaties and severe ID (Saitsu et al., 2008). In addition, *APBA1* also forms a protein complex with a multidomain scaffolding protein encoded by the *CASK* gene (MIM# 300172) (Butz et al., 1998). Mutations in *CASK* cause an X-linked brain malformation syndrome with microcephaly, disproportionate brainstem and cerebellar hypoplasia, and severe ID (Najm et al., 2008). In conclusion, *APBA1* might be a novel candidate gene for brain malformations, and haploinsufficiency of *APBA1* is likely to contribute to the ID, microcephaly, and corpus callosum agenesis observed in patient 17926 in this study.

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Table IV. GO terms, Ensemble genes identified in various CNVs

| Patient | Chr | Start | End | Gene name | Ensembl gene | Significant terms |
|---|---|---|---|---|---|---|
| 17926 | chr9 | 71235022 | 71477042 | *APBA1* | ENSG00000107282 | GO:0008088~axon cargo transport |
| 20375 | chr17 | 2444011 | 2535638 | *PAFAH1B1* | ENSG00000007168 | GO:0008088~axon cargo transport |
| 23510 | chr17 | 2444011 | 2535638 | *PAFAH1B1* | ENSG00000007168 | GO:0008088~axon cargo transport |
| 23376 | chr1 | 10193418 | 10364248 | *KIF1B* | ENSG00000054523 | GO:0008088~axon cargo transport, GO:0008089~anterograde axon cargo transport |
| 23376 | chr1 | 9723346 | 9818816 | *CLSTN1* | ENSG00000171603 | GO:0008089~anterograde axon cargo transport |
| 17926 | chr9 | 114953059 | 114966242 | *SLC31A2* | ENSG00000136867 | GO:0015082~di-, tri-valent inorganic cation transmembrane transporter activity |
| 17926 | chr9 | 115023638 | 115068495 | *SLC31A1* | ENSG00000136868 | GO:0015082~di-, tri-valent inorganic cation transmembrane transporter activity |
| 17926 | chr9 | 70840164 | 70878757 | *FXN* | ENSG00000165060 | GO:0015082~di-, tri-valent inorganic cation transmembrane transporter activity |
| 17926 | chr9 | 115862231 | 115880536 | *AMBP* | ENSG00000106927 | GO:0019855~calcium channel inhibitor activity |
| 21835 | chr19 | 60354950 | 60360912 | *TNNI3* | ENSG00000129991 | GO:0019855~calcium channel inhibitor activity |
| 21835 | chr19 | 59,668,022 | 59,676,234 | *CDC42EP5* | ENSG00000167617 | GO:0007254~JNK cascade |
| 17926 | chr9 | 113,901,964 | 113,920,269 | *AMBP* | ENSG00000106927 | GO:0007254~JNK cascade |
| 23510 | chr17 | 683,303 | 4,742,135 | *MINK1* | ENSG00000141503 | GO:0007254~JNK cascade |
| 19673 | chr22 | 19596268 | 19632443 | *CRKL* | ENSG00000099942 | GO:0007254~JNK cascade |
| 20375 | chr17 | 683,303 | 4,742,135 | *MINK1* | ENSG00000141503 | GO:0007254~JNK cascade |

## 4.5.2 Protein-protein interactions

We wanted to find direct protein-protein interactions and shared PPI partners between the different rare CNVs. We found a direct interaction between the protein products of *PAFAH1B1* and *NDE1* located at 17p13.3 and 16p13.11, respectively, and between *YWHAE* and *RGS3* located at 17p13.3 and 9q32, respectively.

Products of both *PAFAH1B1* and *NDE1* genes interact with *DISC1* (MIM# 605210*;* gene disrupted in schizophrenia). Deficiency of the LIS1–NDE1 complex impairs cortical neurogenesis and neuronal migration, (Feng et al., 2000; Pawlisz et al., 2008) whereas DISC1-NDE1 deficiency appears to play a role in neuropsychiatric disorders, including schizophrenia and bipolar affective disorder (Burdick et al., 2008; Hodgkinson et al., 2004; Kamiya et al., 2005; Nicodemus et al., 2010).

*YWHAE* at 17p13.3, which is suspected to contribute to the severe brain malformations in Miller-Dieker syndrome, shares the interaction partner *SRC* (MIM#190090) with *AHR* (MIM# 600253) located in duplication of 7p21.1. *SRC* encodes a proto-oncogene tyrosine-protein kinase and targeted *SRC* knockout studies in mice indicate that this gene plays an important role in embryonic development and suggests that small regulatory changes might produce more subtle effects (Soriano et al., 1991). Furthermore, *SRC* activation also plays a central role in executing glutamate-induced neurodegeneration (Khanna et al., 2007).

# V. Conclusion

To the best of our knowledge, this is the first systematic study of CNVs in a cohort of patients selected based upon the presence of a detectable structural brain malformation. The genomic regions detected in this study include potential novel candidate genes and possible susceptibility loci for predisposition to structural brain malformations, epilepsy and intellectual disability.

In summary, we found that 22 of 100 patients (22%) with various forms of brain malformations, ID, and symptomatic epilepsy carried one or more rare CNVs that may predispose to abnormal brain development. This frequency is higher than frequencies detected in studies of patients with ID or idiopathic generalized epilepsy, which emphasize the importance of testing for CNVs in patients with structural brain malformations. The number of disease associated CNVs in this cohort might be even higher as we have applied very stringent filtering parameters that, without a doubt, have led to the exclusion of several predisposing CNVs.

We find that *PAFAH1B1* is the single gene with the highest actual (10%) involvement in specific structural brain malformations. When considering that CNVs account for about half of all cases of *PAFAH1B1* involvement, it can be assumed that the gene is responsible for 20% of the cases, emphasizing the significance of relevant testing for patients with neuronal migration disorders. The 12p tetrasomy is the single CNV with the highest frequency in our cohort (10%) and we recommend it to be considered in the clinical context and to be specifically screened for, by array CGH, which proves to be an efficient method, in selected patients. We find that among the structural brain malformations in our patients, midline defects (28%) and more specifically corpus callosum agenesis has the highest association with CNVs.

The deletion in our case with the 1q43q44 includes the previously candidated gene, *ZNF238* for microcephaly and the 360 kb critical region for corpus callosum agenesis including the *AKT3* gene, further supporting the causative role of this gene and region in the pathogenesis of these features.

The duplicated region on chromosome 16p13.1 in our patient with holoprosencephaly may encompass a new locus predisposing to or causing this condition.

The fact that hereditary duplication of 16p13.11 in our patient excludes the *NTAN1* gene is further evidence for the possible role of the *NDE1* gene in the phenotype of these patients.

The PPI network analysis also identifies a direct interaction between the protein products of *PAFAH1B1* and *NDE1* located at 17p13.3 and 16p13.11. Products of both *PAFAH1B1* and *NDE1* genes interact with *DISC1*. Deficiency of the LIS1–NDE1 complex impairs cortical neurogenesis and neuronal migration, whereas DISC1-NDE1 deficiency appears to play a role in neuropsychiatric disorders, including schizophrenia and bipolar affective disorder.

We have detected five novel imbalances (as of yet unreported) in three patients, that may provide insight into genes involved in structural brain malformations within these regions.

We propose that *APBA1* might be a novel candidate gene for brain malformations, and haploinsufficiency of *APBA1* is likely to contribute to the ID, microcephaly, and corpus callosum agenesis observed in patient 17926 in this study. *APBA1,* GO term: axon cargo transport, encodes a neuron-specific protein of the X11 protein family, functioning in synaptic vesicle exocytosis by binding to *STXBP1* and forms a protein complex with a multidomain scaffolding protein encoded by the *CASK* gene. Haploinsufficiency of *STXBP1* has recently been described to cause early-onset epileptic encephalopaties and severe ID and mutations in *CASK* cause an X-linked brain malformation syndrome with microcephaly, disproportionate brainstem and cerebellar hypoplasia, and severe ID.

Our analysis of GO Terms and PPI networks suggest that genes involved in “axonal transport,” “cation transmembrane transporter activity,” and in the “JNK cascade” play a significant role in the etiology of brain malformations.

# Genome and gene studies: an outlook

The technological evolution from the karyotype with 5-10 Mb resolution to the BAC array with a 1 Mb or better resolution was a great advancement that proposed and made possible significant accomplishments in the understanding of many disease causing genomic imbalances and led to identification of many of the underlying genes by phenotype-genotype correlation. At the time of this study, microarray technology proposed a practical means for identifying variations in the human genome and thereby investigating as of yet unidentified genes. The major attraction of the array technologies was their ability to do a whole genome scan within one procedure.

Meanwhile, the success of the human genome project, presented sequencing as an attractive technique for identification of disease causing genes in research and diagnostics. A major limitation is the expense and the fact that a whole genome sequence could be time

consuming. The efficacy of sequencing, in identifying genes and mutations, versus all formerly used techniques created an increasing demand to overcome these limitations and has pushed forward high-throughput sequencing. The development of alternative sequencing techniques and what is termed as Next Generation Sequencing is the result of this demand for speedier and less expensive methods.

At present, application of whole genome sequencing for research and diagnostics, although cheaper than in the first generation sequencers, is still expensive and is in most cases, still limited. Whole exome capture (Bamshad et al., 2011) which sequences the approximate 180,000 exons that constitute about 1% of the human genome and translates to about 30 megabases (Mb) in length will provide informative results for the approximately 85% of the disease-causing mutations that occur in exons (Ng et al., 2010). In addition to being able to identify disease causing genes, sequencing the protein coding regions has the potential of being used for predisposition and susceptibility studies in conditions such as Alzheimer disease (Bamshad et al., 2011).

The future promises quicker and more efficient screening of the human genome for disease causing mutations and variations in a clinical context. The concept of targeted exome genome sequencing is an attractive prospect for simultaneous testing of multiple genes that lead to a similar phenotype such as testing for all the genes involved in heterogenous diseases such as Fanconi Anemia, Retinitis Pigmentosa. Targeted exon entrapment and sequencing enables analysis of large genes such as the Dystrophin gene in Duchenne Muscular Dystrophy in one simple assay.

Thus far, whole genome sequencing has been limited to identifying genes linked to so-called Mendelian disorders. It is aspired that the future will bring the possibility to diagnose and predict disease and disease susceptibility through the detection of variations in genome and genes that act as susceptibility factors that will predispose to hereditary or environmental disease. Many of the hereditary CNVs that we have detected may constitute such variations.

Understanding disease causing genes will equip us with new ways to treat, cure, or even prevent the thousands of diseases (HGP website). Elucidation of the function of each human gene will shed light on their role in health and disease conditions. Even today, many therapeutic regimes, are already based on gene expression patterns as represented by the

Mammoprint in breast cancer. This may indicate that we will move towards an era of customized therapy.

The decreasing costs of whole genome assays will definitely bring about the possibility of universal tests that will provide not only data about the specific genes but also a profile that may enable us to shed light on specific disease predisposition in individuals eventhough for complex disorders the search for such markers has been futile.

Despite the fact that sequencing will replace many of the formerly used techniques for gene identification, a more universal approach such as array CGH will probably retain its value as a diagnostic tool for years to come.

# Summary

The proper spatio-temporal expression of structural and regulatory genes is essential to brain development. In an attempt to identify underlying mechanisms, we investigated the role of CNVs in the etiology of brain malformations. We studied genomic variations in 100 prospective patients with various brain malformations by array CGH on a 200 kb overlapping BAC array platform.

We considered only those copy number variations that encompassed at least 3 overlapping BAC clones and differed by more than a total of 100 kbs difference on either or both sides from formerly reported CNVs in the Database of Genomic Variants. These CNVs were confirmed by parental studies or by a second alternate technique such as oligoarray or FISH and in a few cases by both.

We find 27 CNVs in 22 patients, where 3 patients have two and one patient has 3 CNVs. There are a total of 13 *de novo* CNVs in 10 patients and 14 hereditary CNVs in one of the above and 12 other patients. The CNVs range from 150 kb to 10.2 Mb in size. Seven of the de novo CNVs, (microdeletion 1p36, microdeletion 1q43q44, two 12p tetrasomy, 2 deletions of 17p, and one microdeletion 22q11.21), one of the inherited CNVs (microdeletion 16p13.2), directly established the diagnosis of known deletion/duplication syndromes. In three patients one or more *de novo* aberrations not corresponding to know syndromes were identified.

We found that 22 of 100 patients (22%) with various forms of brain malformations, ID, and symptomatic epilepsy carried one or more rare CNVs. This frequency, albeit the stringent exclusion criteria, is higher than frequencies detected in studies of patients with ID or idiopathic generalized epilepsy, emphasizing the importance of testing for CNVs in patients with structural brain malformations.

Tetrasomy12p tetrasomy is the single CNV with the highest occurrence (10%) and *PAFAH1B1* is the single gene with the highest actual (10%) and assumed (20%) involvement in specific structural brain malformations. We find that among the structural brain malformations in our patients, midline defects (28%) and more specifically corpus callosum agenesis has the highest association with CNVs.

We discuss the significance of our findings in the further elaboration of the role of previously candidated genes *ZNF238, AKT3*, *NTAN1*, *NDE1* in the phenotype of these patients.

The five novel imbalances (as of yet unreported) in three patients, may provide insight into genes involved in structural brain malformations within these regions and we propose that *APBA1* might be a novel candidate gene for brain malformations.

Our analysis of GO Terms and PPI networks suggest that genes involved in "axonal transport," "cation transmembrane transporter activity," and in the "JNK cascade" play a significant role in the etiology of brain malformations.

To the best of our knowledge, this is the first systematic study of CNVs in a cohort of patients selected based upon the presence of a detectable structural brain malformation. The genomic regions detected in this study include potential novel candidate genes and possible susceptibility loci for predisposition to structural brain malformations, epilepsy and intellectual disability.

Zusammenfassung

Die richtige räumliche und zeitliche Expression struktureller und regulatorischer Gene ist für die Hirnentwicklung von essentieller Bedeutung. Als Beitrag zur Aufklärung der daran beteiligten Mechanismen haben wir untersucht, welche Rolle submikroskopische Deletionen und Duplikationen (sog. Copy Number Variants, CNVs) in der Ätiologie von Hirnfehlbildungen spielen. Dazu haben wir bei 100 Patienten mit Hilfe der Array CGH-Technologie nach genomischen Varianten gesucht. Für diese Untersuchungen wurde ein Raster aus überlappenden BAC Klonen verwendet, welches das ganze Genom lückenlos überspannt.

Dabei wurden nur solche Genomveränderungen berücksichtigt, die mindestens drei BAC Klone überspannten und sich an einer oder beiden Seiten um mehr als 100 kb von früher geschriebenen und in der Datenbank genomischer Varianten aufgeführten CNVs unterschieden. Diese CNVs wurden durch Untersuchung der Eltern mithilfe von Oligonukleotid-basierter Array CGH oder durch FISH validiert.

In 22 Patienten wurden insgesamt 27 CNVs gefunden. Drei Patienten wiesen zwei und einer drei verschiedene CNVs auf. 13 dieser CNVs wurden nur bei Patienten gefunden, während 14 andere auch bei einem Elternteil vorkamen. Die Größe der CNVs variierte von 150 kb bis 10,2 Mb. Sieben der neuen CNVs (Mikrodeletion 1p36, Mikrodeletion 1q43q44, Tetrasomie 12p (2 mal), zwei 17p-Deletionen und eine Mikrodeletion 22q11.21) und eine der vererbten CNVs (Mikrodeletion 16p13.2) korrespondierten mit bereits bekannten Deletions- bzw. Duplikationssyndromen. Bei drei Patienten wurden *de novo* Veränderungen gefunden, für die vorher kein Krankheitsbezug bekannt war.

Wir fanden, daß 22 von 100 Patienten mit verschiedenen Hirnfehlbildungen, geistiger Behinderung und Epilepsie einen oder mehrere CNVs aufwiesen. Diese unter stringenten Ausschlußkriterien ermittelte Häufigkeit ist deutlich höher als die Häufigkeit von CNVs bei Patienten mit geistiger Behinderung oder idiopathischer generalisierter Epilepsie, was die Bedeutung von CNVs in der Ätiologie struktureller Hirnfehlbildungen unterstreicht.

Zehn Prozent aller Patienten wiesen eine Tetrasomie 12p auf. Damit ist diese Störung die häufigste bei Patienten mit strukturellen Hirnfehlbildungen gefundene Veränderung, und das *PAFAH1B1*-Gen ist am häufigsten an klinisch relevanten CNVs beteiligt. Unter den

strukturellen Hirnfehlbildungen sind Mittelliniendefekte und insbesondere die Corpus Callosum-Agenesie am engsten mit CNVs assoziiert.

Wir diskutieren außerdem die Rolle der früher beschriebenen Kandidatengene *ZNF238*, *AKT3*, *NTAN1* und *NDE1* beim Zustandekommen des Phänotyps dieser Patienten. Bei drei Patienten wurden fünf neue, bisher noch nicht beschriebene genomische Imbalanzen gefunden, die möglicherweise Rückschlüsse auf pathogenetische relevante Gene erlauben. Auf diese Weise haben wir das *APBA1*-Gen als neues plausibles Kandidatengen für angeborene Hirnfehlbildungen identifiziert. Überdies sprechen unsere Untersuchungen dafür, daß Defekte des axonalen Transports, des Kationen-Transports und der JunK-Kaskade bei der Entstehung von Hirnfehlbildungen eine bedeutende Rolle spielen.

Nach unserer Kenntnis ist dies die erste systematische Suche nach CNVs in einer Kohorte Patienten mit Fehlbildungen der Hirnstruktur. Die im Rahmen dieser Studie definierten deletierten oder duplizierten Genomabschnitte sind ein Schlüssel zur Identifizierung weiterer Kandidatengene und Risikofaktoren für strukturelle Hirnfehlbildungen, Epilepsie und geistige Behinderung.

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# List of figures:

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