Published in final edited form as:

Am J Med Genet A. 2011 June ; 0(6): 1367–1373. doi:10.1002/ajmg.a.33627.

Craniorachischisis and Omphalocele in a Stillborn Cynomolgus Monkey (*Macaca fascicularis*)

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Abstract

Nonhuman primates have been a common animal model to evaluate experimentally-induced malformations. Reports on spontaneous malformations are important in determining the background incidence of congenital anomalies in specific species and in evaluating experimental results. Here we report on a stillborn cynomolgus monkey (*Macaca fascicularis*) with multiple congenital anomalies from the colony maintained at the Southwest National Primate Research Center at the Southwest Foundation for Biomedical Research, San Antonio, Texas. Physical findings included low birth weight, craniorachischisis, facial abnormalities, omphalocele, malrotation of the gut with areas of atresia and intussusception, a Meckel diverticulum, arthrogryposis, patent ductus arteriosus, and patent foramen ovale. The macaque had normal male external genitalia, but undescended testes. Gestational age was unknown but was estimated from measurements of the limbs and other developmental criteria. Although cytogenetic analysis was not possible due to the tissues being in an advanced state of decomposition, array Comparative Genomic Hybridization analysis using human bacterial artificial chromosome clones was successful in effectively eliminating aneuploidy or any copy number changes greater than approximately 3–5 Mb as a cause of the malformations. Further evaluation of the animal included extensive imaging of the skeletal and neural tissue defects. The animal's congenital anomalies are discussed in relation to the current hypotheses attempting to explain the frequent association of neural tube defects with other abnormalities.

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Keywords

neural tube defects; schisis association; macaque; cynomolgus monkey; non-human primate; congenital defects; malformations

INTRODUCTION

The common association of neural tube defects (NTD) with other anomalies in humans is well-documented [Toriello and Higgins, 1985; Hall et al., 1988; Hunter et al., 1996; Källén et al., 1998; Stevenson et al., 2004]. In most series, about 15–25% of the NTD cases have additional abnormalities. The more severe and the higher the defect, the more likely there are additional anomalies. Thus, craniorachischisis and encephaloceles have the highest rates of associated anomalies, while lumbar and sacral spinal bifida defects have the lowest [see Stevenson et al., 2004].

Nonhuman primates have been a common animal model for the evaluation of experimentally-induced malformations such as neural tube defects. Reports on spontaneous malformations in these animals are important in determining the background incidence of congenital anomalies in specific species, in maintaining healthy colonies, and in evaluating experimental results. Here we report on a stillborn cynomolgus monkey (*Macaca fascicularis*) with multiple congenital anomalies, including craniorachischisis and omphalocele.

Family History

The Southwest National Primate Research Center at the Southwest Foundation for Biomedical Research (SNPRC/SFBR) in San Antonio, Texas maintains a breeding colony of cynomolgus monkeys, *Macaca fascicularis* (crab-eating or long-tailed macaque), of approximately 1,000 animals. There is no record of previous neural tube defects in the colony. The male infant reported here was the third product of a mating between a 14-year-old sire and an 8-year-old dam. They previously had produced two normal female offspring. The sire and dam were young parents; the average lifespan of cynomolgus monkeys is 37.1 years with males reaching sexual maturity at approximately 4.2 years of age and puberty in females occurring at approximately age 4.3 years [Rowe, 1996]. The gestation period for a cynomolgus monkey is approximately 165 days [Tarantal et al., 1993]. Breeding in this cynomolgus colony is not closely monitored, so the exact gestational period and time of delivery were unknown. The infant was found dead during morning rounds.

Gross Anatomical Examination

At the time of evaluation, the infant weighed 188 g. The normal birth weight for a newborn *Macaca fascicularis* at the SNPRC/SFBR is approximately 300 g (275 – 350 g). The monkey had no skull above the eyes, absent cerebral hemispheres, and an incomplete occipital bone and foramen magnum. The spinal cord was covered only by meninges through the cervical and thoracic regions of the spinal column due to failure of fusion of the cervical and thoracic posterior vertebral arches. The male infant had wide-set protruding eyes, and a flattened nose. Lips, palate, and tongue were all normal. Specifically, the palate was intact and well-formed. Arthrogryposis of all limbs was observed with fingers tightly folded over palms and tight flexures at all the joints. The infant's toes had been partially cannibalized. This is a common finding in stillbirths found in communal cages. Images of the infant at the time of evaluation and with a female stillborn cynomolgus monkey, who had no obvious defects, are shown in Fig. 1. Measurements were taken of the limbs and tail and were compared to those of the female stillborn monkey.

On internal examination the tissues were found to be in an advanced state of decomposition. The four chambers of the heart appeared normal as did the ventricular septum. However, a patent ductus arteriosus and patent foramen ovale were present. The lungs were uninflated and thus consistent with stillbirth. The diaphragm was observed to be intact with no hernia. Approximately 5 mm of intestine protruded from the abdominal wall. The defect was confirmed to be an omphalocele as it was within the umbilical cord and covered with amnion and peritoneum. Malrotation of the gut was also present with areas of atresia; two long string-like appendages were attached at different locations to the gut. One was diagnosed as an intussusception and the other was part of a Meckel diverticulum. Both kidneys and ureters were present and appeared normal with no cysts. Normal male external genitalia were observed and two testes were present in the pelvis.

MATERIALS AND METHODS

Histopathology Examination

The placenta was not available for examination, but an extensive set of tissues was taken from the infant for histopathological examination during necropsy. These included samples from the major organs, regional lymph nodes, skeletal muscle, and genitourinary tract. Three sections of the spine were taken at different levels for evaluation of the neural tube defect. The tissues were fixed in 10% neutral-buffered formalin, processed conventionally, cut at 5 μ , and stained with hematoxylin and eosin.

Radiological Studies

Following the necropsy, a set of whole body X-rays were taken. To obtain more details for the skeletal structures and soft tissue, MRI and volumetric microCT imaging were performed.

MRI Imaging Studies

The body was scanned using a Siemens 3T Trio MRI unit. Images were acquired using a standard pulse sequence with TR = 20 ms, TE 5.79 ms at a slice thickness of 0.5mm (6 averages per slice).

MicroCT Imaging Studies

The infant monkey was packed in a loose sponge in a plastic container and scanned on volumetric microCT at 93 μm^3 voxel resolution using an eXplore Locus RS Small Animal MicroCT Scanner (GE Healthcare, London, Ontario). This volumetric scanner uses a 3500 $\times$ 1750 CCD detector for Feldkamp cone-beam reconstruction. The platform independent parameters of current, voltage and exposure time were kept constant at 450 μA , 80 kVP and 100 ms, respectively. Additional scan parameters include 900 evenly-spaced view angles (views) and 10 frames per view, for a total of 350 min of image acquisition time. Images were reconstructed with the manufacturer's proprietary EVSBeam software. Maximum Intensity Projection (MIP) images were produced on MicroView (<http://microview.sourceforge.net/>, GE Healthcare, London, Ontario).

Cytogenetic Studies

Samples of skin and muscle appeared to be in an advanced state of decomposition. Despite this finding, growth was attempted. The samples were placed in RPMI 1640 with 10% fetal bovine serum and 1% antibiotics and cultured at 37°C for two weeks.

Array CGH

DNA samples from the infant and a sex-mismatched normal cynomolgus control were prepared for array CGH (Comparative Genomic Hybridization) with the conventional manufacturer's protocol using the Spectralchip 2600™ (Spectral Genomics Inc/PerkinElmer, Turku, Finland) bacterial artificial chromosome array. This chip has 2621 human clones spaced approximately every 1 Mb throughout the genome and was used to detect copy number changes including those found in many microdeletion/microduplication syndromes.

RESULTS

Gross Anatomical Examination

Measurements taken of the abnormal male infant were compared with those of the stillborn female monkey from the colony and also compared with measurements from controls in the literature (Table I). The stillborn female had a normal birth weight for full-term newborn cynomolgus monkeys at SNPRC/SFBR, which was at the lower end of the normal weight range in the full-term controls in the ultrasound study by Tarantal and Hendrickx [1989]. The female's limb and tail measurements were within the normal range of measurements compared with controls from the ultrasound study.

Although the exact gestational age was unknown, the affected infant's humeri, femora, and tail were similar in length to that found in the stillborn female in the SFPRC/SFBR colony and also to the full-term controls published by Tarantal and Hendrickx [1989]. The gestational age was, therefore, estimated to be near-term (greater than 155 gestational days). However, due to obvious contractures, the length of the infant's hands and feet was shorter than those of the stillborn female and the Tarantal and Hendrickx [1989] measurements. The weight of the affected infant was much less than that of the stillborn female or the Tarantal and Hendrickx [1989] controls, suggesting maturational delay and death *in utero*.

Histopathology Examination

There were moderate-to-severe post-mortem changes in the tissues that were also compatible with death *in utero*. Three sections of the spine were examined. In the most caudal section, at the posterior end of the craniorachischisis (the level of the upper lumbar spine), the vertebral body was ossified. The lateral aspects of the vertebrae were composed of fibrocartilage with two foci of mineralization on each side. Neither the dorsal spinal process nor the dorsal aspect of the vertebrae showed ossification. Instead, the dorsal aspect of the vertebrae consisted of a continuous concave band of fibrocartilage. A spinal cord and associated nerve roots were present in the spinal canal. In the more cranial sections (mid-thoracic and cervical), the bodies of the vertebrae were wider and less completely ossified. The lateral portions of the vertebrae angled outward as they approached the dorsal surface and were less well-developed and multifocally ossified. The dorsal portions of the vertebrae were nonexistent. Meninges and associated nerve roots were present in the region corresponding to the spinal canal. Although there was significant intrauterine autolysis, remnants of spinal cord tissue were seen within the meninges. There was no observable connective tissue, muscle or skin overlying the meninges.

Examination of skeletal muscle tissue also showed autolysis, but the muscle fibers did not appear inflamed or fibrotic. They had the appearance of normal late fetal muscle tissue with loosely arranged, small, thin fibers.

Radiological Studies

The radiographs confirmed craniorachischisis through the thoracic vertebrae, with short, wide vertebral bodies (Fig. 2). The cervical vertebrae were highly dysplastic, but cervical

ribs were not present. There were 8 thoracic vertebrae with a hemivertebra at the T5–6 level. Only 9 pairs of ribs were present instead of the 12 found in a normal infant. There were 7 lumbar and 29 sacral or caudal vertebrae. Tight flexures were observed at the elbows, wrists, hip, knees, ankles, and digits. Abnormal widening of the vertebral bodies with angulation of the lateral walls and absence of the neural archs were observed through the cervical and thoracic areas. The thumbs and forearms of the monkey were examined separately. Each of the thumbs had two phalanges and an incompletely ossified metacarpal. Both radii appeared bowed.

MRI and MicroCT Imaging Studies

Though the tissue was partly desiccated due to formalin fixation, there was sufficient water to reveal structure in the MRIs. The MRI and volumetric microCT scans enhanced the visibility obtained from the routine radiological studies and clearly confirmed and defined the abnormalities described above (Fig. 3). A three-dimensional 360° view of the microCT scan represented in Fig. 3 is presented as supplementary online material.

Cytogenetic Studies

After incubation for 14 days, no growth was observed in any of the cultures. Therefore, the cultures were harvested and the cells were fixed with 3:1 methanol:acetic acid. Slides were made for Fluorescence *In Situ* Hybridization enumeration probe studies. However, the hybridization was unsuccessful.

Array CGH

The infant's DNA was degraded. This made it difficult to interpret subtle copy number differences. However, separation was good for the X chromosome, indicating "loss" of an X in the male infant compared to the female control, although the background signals were higher than routine analyses. For the autosomes, there were no large areas of copy number gains or losses, and based on the overall resolution, we estimate that no copy number alterations were detected that were greater than 3–5 Mb; the array platform used and the sample quality prohibited interpretation with confidence below this size. Furthermore, we carefully examined the region at human 17q25.3 that has recently been associated with distal arthrogryposis [Lukusa and Fryns, 2010] and found no deviation at this locus or within 1 Mb of this site. In addition, we similarly examined the region of 13q31-34 that has been associated with anencephaly [Tyshchenko et al., 2008] and again found no deviations. The findings from the infant's DNA were therefore interpreted as grossly normal.

DISCUSSION

Isolated NTD are the result of interactions between genetic predisposition and environmental influences [Copp and Green, 2010]. Although a large number of genes have been identified in the mouse that are important in neural tube closure [Harris and Juriloff, 2007; Zohn and Sarkar, 2008], only a few of these have been implicated in human NTD. Two well established environmental factors affecting the occurrence of isolated NTD in human pregnancy are low levels of folic acid and maternal diabetes.

The cynomolgus colony at SNPRC is fed Land O'Lakes Purina Feed, LLC Monkey Diet 15% [5LE0]. The folic acid content in this feed 2.2 PPM or 2.2 mg per kg of diet. An adult female consumes approximately 0.56 – 0.83 g of feed per day and thus consumes approximately 0.1232 – 0.1826 mg or an average of 0.1529 mg folic acid per day. No additional supplement is given to pregnant females. The recommended amount of folic acid supplementation for women at low-risk for neural tube defects is 0.4 mg per day prior to and

during pregnancy, while the amount recommended for women with a history of spina bifida or anencephaly is ten times higher at 4 mg per day [Cheschier, 2003].

A strong environmental influence on the frequency of NTD is diabetes in pregnancy and the causes for this are currently being explored. One possible connection is the expression of *Pax3* and its action on closure of the neural tube. *Pax3* expression is greatly reduced in the presence of excess glucose metabolism and the reduced expression is associated with an increased frequency of neural tube defects [Loeken, 2005]. Other defects may be the result of genetic mutations in the pathway for the conversion of homocysteine to methionine, which is necessary for gene regulation [Cheschier, 2003]. Gestational diabetes and genetic mutation in the methionine pathway have not been explored in the cynomolgus colony at SNPRC/SFBR.

Maturation delay was suggested in the infant due to its low birth weight, patent ductus arteriosus and foramen ovale, and undescended testes. Arthrogryposis in all four limbs with bowing of the radii is indicative of limited or no movement *in utero* and was most probably related to the high NTD. The advanced decomposition of tissues and uninflated lungs were compatible with death prenatally.

The differential diagnosis for a finding of NTD with associated defects such as omphalocele and heart defects includes chromosomal abnormalities, such as trisomy 13 or 18, and single gene mutations leading to conditions such as Meckel syndrome. Because the macaque and human genomes are quite similar, human probes can be utilized to diagnose primate chromosomal aneuploidies [Moore et al., 2007, 1999; Best et al., 1998]. Although metaphase chromosomes were not obtained from culture of the infant's skin and muscle because of lack of growth from the decomposed tissue, the results of the microarray analysis using human probes effectively eliminated aneuploidy, or any copy number changes greater than approximately 3–5 Mb, as a cause of the malformations.

Reports of spontaneous congenital abnormalities in laboratory-raised nonhuman primates, primarily macaques and baboons, have been relatively low compared to the human population. Reviews of Old World species have reported rates that range from 0.3 – 1.6% [Peterson et al., 1997; Hendrickx and Binkerd, 1993; Binkerd et al., 1988; Wilson, 1978]. The rate specifically found in the cynomolgus colony at the California Regional Primate Research Center during a 14 year period (1983–1996) was 0.3% (3/965) and in their rhesus colony during the same period was 0.9% (40/4,390) [Peterson et al., 1997]. The rate in humans is estimated to be approximately ten times greater at 3–6% [Shepard and Lemire, 2007].

The most common spontaneous malformations reported in macaques were in the musculoskeletal, cardiovascular, urogenital and central nervous systems [Peterson et al., 1997; Hendrickx and Binkerd, 1993; Binkerd et al., 1988; Wilson, 1978]. A smaller number of malformations was observed in the gastrointestinal and endocrine systems. Of the central nervous system malformations in simian primates, the most common was anencephaly [Wilson, 1978]. Other reported spontaneous CNS malformations included hydrocephaly, hydranencephaly, acephaly, spina bifida, Arnold-Chiari malformation, and rarely, exencephaly and meningocele [Hendrickx and Binkerd, 1993]. In only a few instances were these malformations reported with other abnormalities, e.g., Binkerd et al. [1988] reported additional anomalies in two infant rhesus monkeys with CNS abnormalities, one with anencephaly and two tails, and the other with exencephaly and multiple defects including hypoplastic limbs.

In contrast, approx 15 – 25% of human infants with NTD have associated defects. The most common ones, found with a frequency of 1 – 6%, are facial clefts, small or absent ears and

eyes, limb deficiencies, cardiac defects, abdominal wall defects, and renal anomalies [Stevenson et al., 2004]. The more cranial and more severe NTD have the highest frequency of associated defects. Thus, NTD such as craniorachischisis and encephalocele have the highest rates of associated anomalies, anencephaly without rachischisis and high spina bifida have intermediate rates, and low spina bifida has the lowest rates.

Several hypotheses have been put forward to explain the frequent association of NTD with other anomalies. Czeizel [1981] has described “schisis association,” in which he proposes common effects on closure during embryogenesis that lead to NTD and associated facial clefting, diaphragmatic hernia, and omphalocele. Opitz and Gilbert [1982] have proposed the midline developmental field concept that gives midline structures a common susceptibility to developmental insults. Alternatively, Seller and Kalousek [1986] suggest that simple mechanical forces and space limitations exerted on the thorax and abdomen as well as neural crest migration disruptions imposed by the NTD may produce secondary anomalies such as omphalocele, diaphragmatic hernia, Meckel diverticulum, and possibly cardiac defects. Stevenson et al. [2004] studied NTD frequencies in South Carolina over a ten year period from 1992 to 2002 and found no compelling evidence to support one hypothesis over the others. They suggest that it is just as plausible to explain the associated anomalies that occur with NTD with Seller and Kalousek’s mechanical force and space limitation theory as it is to explain them with developmental field or closure defects.

Recent advances in the study of developmental patterning in early embryogenesis will help elucidate the causes of such associations [see reviews such as Aulehla and Pourquié, 2010; Diez del Corral and Storey, 2004]. The interactions of various signaling centers and formation of morphogen gradients in the early embryo occur in a temporal, spatial, and tissue specific manner. Left-right, rostro-caudal, and dorso-ventral patterning occur simultaneously in a highly interactive fashion. In light of this, the hypothesis proposed by Martínez-Frías [1995], Martínez-Frías et al. [2000; 1998], and Opitz et al. [2002] based on disturbances to an inductive system during blastogenesis that result in multiple anomalies is quite informative. If the disturbance can be causally identified, the result is identified as a syndrome, while results from unknown disturbances are called associations. These associations are commonly found in human fetuses and infants, have often been defined as “specific associations” with acronyms such as VATER, VACTERL, or CHARGE, and are often lethal [Martínez-Frías et al., 2000, 1998; Martínez-Frías, 1995]. Mutations affecting the expression of proteins such as calreticulin, which has recently been found to be important in the development of the heart and brain and in the closure of the ventral body wall [Rauch et al., 2000], may be found to be the cause of some associations.

The animal we report here fits Czeizel’s schisis association of craniorachischisis with omphalocele, but the infant did not have facial clefting or a diaphragmatic hernia. The animal’s cardiac defects and undescended testes may be related to maturational delay or the former could be considered malformations related to the proposed midline developmental field of Opitz and Gilbert [1982]. In the absence of chromosomal or other genetic causes, the abnormalities in this infant indicate an association of anomalies arising from patterning defects during blastogenesis. Malrotation of the gut, related to left-right patterning defects, and defects in the vertebral bodies with an abnormal number of ribs, related to rostro-caudal patterning defects resulting from abnormal changes in signaling expression in the paraxial mesoderm, point to an early defect of blastogenesis in this animal and not simply to mechanical forces and space limitations that may have occurred secondarily to the initial insult. Studies in non-human primates may shed light on these hypotheses by comparing cases of NTD and associated defects in animals with folic acid supplementation with those animals that have been folic acid deprived. Examination of the effects of folic acid on expression of *Alx-3* and other transcription factors may help identify human candidate genes

for NTD [Lakhwani et al., 2010]. For colony management, an increase in the folic acid supplementation for dams that have given birth to an infant with NTD may be warranted, if subsequent pregnancies are planned.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Dr. John Opitz, University of Utah School of Medicine, Salt Lake City, Utah, and Dr. Roger Stevenson, Greenwood Genetics Center, Greenwood, South Carolina, for their valuable suggestions for the manuscript.

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Fig. 1.
Physical characteristics of infant with craniorachischisis and omphalocele.
a. Face of the infant with no skull above wide-set, protruding eyes and flattened nose. Lips and tongue were normal, and palate was intact and well-formed.
b. The back of the infant shows the considerably shortened trunk as compared with a term stillbirth. Normal measurements were found for the humeri, femora, and tail. Tight flexures are seen at the joints.
c. Left hand of the infant with the fingers tightly folded over the palm, illustrating the tight flexures that were observed in all limbs.
d. The back of the infant showing extent of craniorachischisis through the thoracic vertebrae.
[Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 2. Whole-body radiograph. Note the dysplastic cervical vertebrae and hemivertebra at the T5–6 level.

Fig. 3.

Whole body Maximum Intensity Projection (MIP)-images from a volumetric microCT scan. [A three-dimensional 360° movie of the microCT scan is available at

www.interwscience.wiley.com. Three-D glasses are needed for viewing the movie.]

a. Ventral MIP-view shows craniorachischisis with abnormal widening of the vertebral bodies, angulation of the lateral walls, and absence of the neural arches through the cervical and thoracic areas. Only 9 ribs were present instead of the 12 found in normal *M. fascicularis*.

b. Lateral MIP-view demonstrates the lack of bony elements in the skull above the orbits and clearly shows the tight flexures in the elbows, wrists, hip, knees, ankles, and digits as well as bowing of the radii. The infant's mandible was separated during the necropsy.

Table I

Measurements of the stillborn male cynomolgus monkey with craniorachischisis and omphalocele and a female cynomolgus stillbirth from the colony with no apparent malformations.

Measurements	Stillborn Male with Craniorachischisis/Omphalocele (<i>M. fascicularis</i>)	Stillborn Female with No Apparent Malformations (<i>M. fascicularis</i>)
Birth weight	188 g (Low for SNPRC/SFBR births)	316 g (WNR for SNPRC/SFBR births)
Crown - rump	N/A	180 mm (WNR, Fig. 3) *
Humerus L/R	50/53 mm (WNR, Fig. 4) *	52/52 mm (WNR, Fig. 4) *
Hand L/R	37/38 mm (Low, arthrogryposis)	44/44 mm (WNR, Fig. 4) *
Femur L/R	54/54 mm (WNR, Fig. 4) *	54/54 mm (WNR, Fig. 4) *
Foot L/R	NA (toes cannibalized)/55 mm (Low, arthrogryposis)	66/66 mm (WNR, Fig. 4) *
Arm circumference L/R	45/45 mm (Low, Fig. 4) *	NA (swollen)/47 mm (WNR, Fig. 4) *
Ribs L/R	9/9	12/12
Tail	235 mm (WNR, Fig. 4) *	230 mm (WNR, Fig. 4) *

* WNR (Within Normal Range) or Low (Lower than normal range) when compared with measurements from control animals (Tarantal and Hendrickx, 1989), pp. 140–142 (Figs. 3, 4, 5).