様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

平成　３０　年　　５　月　３０　日現在

機関番号：　１４３０１
研究種目：　基盤研究(C)（一般）
研究期間：　2015～2017
課題番号：　１５Ｋ０８１３４

研究課題名（和文）計算解剖学および遺伝学的手法を用いた妊娠初期ヒト胎児における新しい診断基準の確立

研究課題名（英文）New diagnostic criteria in early human embryo using computational anatomy and genetic techniques

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交付決定額（研究期間全体）：（直接経費）　　3,800,000円

研究成果の概要（和文）：本研究は、所有するヒト胎児標本について、三次元画像所見を計算解剖学的手法により形態学的に解析、また、標本から遺伝学的データを抽出し、形態学的診断と組み合わせ、有用な診断基準を確立しようとするものである。中枢神経系について三次元モデルを作成し、脳の各部分のセグメンテーションを行った。このデータはランドマーク設定の後、幾何学的形態測定学（GM）に応用可能である。また、古標本からのDNA抽出については、長期間ホルマリンに液に浸漬されていたため、DNA変性が進行したためかシークエンス解析を実施できるレベルの抽出DNAは得ることが困難であった。

研究成果の概要（英文）：This study includes morphological analyses of three-dimensional images of human fetus by computational anatomy, extraction of genetic data from the specimen, and combination of morphological diagnosis with genetic data. Three-dimensional models was created from MR images for the central nervous system and segmentation of each part of the brain was performed. This data can be applied to Geometric Morphometrics (GM) after landmark setting. In addition, we tried DNA extraction from the old specimens, which are soaked in formalin solution for a long time. It was difficult to obtain extracted DNA capable of carrying out sequence analysis because of denaturation DNA.

研究分野：　解剖学、発生学

キーワード：　ヒト発生学　計算解剖学

１．研究開始当初の背景

(1) The morphology of fetal organs changes significantly throughout the development process; in particular, the central nervous system continues to undergo dramatic changes, even after the fetal period. Since the 1990s, magnetic resonance imaging (MRI) has been used to analyze the fetal developing brain. MR images are obtained from specimens of a wide range of sizes using an appropriate MRI instrument. In 1994, Smith used MRI to study embryonic development, and this technique was also applied to a human embryo using a 1.0 Tesla (T) Magnetic resonance microscope (MRM) in 1996 (Smith et al., 1994, 1996; Smith, 1999). Subsequently, MRI techniques have been improved to observe fetal structure in greater detail. For example, Matsuda et al. (2007) developed a super-parallel MRM equipped with 2.34T and made it possible to image four embryo specimens simultaneously. As a result, they acquired T_1-weighted images of 1204 embryos with sufficiently high resolution of 40–150 μ m^3. Thus, owing to advancements in imaging modalities, 3D models from the obtained image data show greater structural detail (Huang et al., 2009; Shiraishi et al., 2015).

Recently, 3D models of the developing brain have also been generated using USG (Guitierrez-Becker et al., 2013). If the developing brain is segmented into different areas on MR images, and 3D visualization models of the regionalized brain are created, these data will be helpful for comparisons in the clinical setting and for research using USG.

(2) Molecular genetic analysis is a major tool for elucidating the mechanisms of congenital anomalies in recent years. Deoxyribonucleic acid (DNA), which is required for genetic tests, has been extracted from viable cells of patients using fresh materials, but DNA has been difficult to extract from fixed materials. The “Kyoto Collection of Human Embryos” at Kyoto University was started in 1961 and now comprises over 44,000 embryos, through the collaborative effort of several hundred obstetricians. In most cases, the embryos were from healthy women during the first trimester of pregnancy, which were terminated for social reasons under the Maternity Protection Law of Japan. Because the attending obstetricians did not examine the aborted materials before donation, the collection is unbiased by embryo condition (e.g., normal or abnormal, live or dead) and can be considered representative of the total intrauterine population in Japan (the characteristics of the Kyoto Collection are shown in Table 1). Embryos were preserved in tissue sections or as whole specimens, fixed in formalin or Bouin’s solutions. Previous histological analyses of the Kyoto Collection have revealed that congenital anomalies were more frequently seen in the intrauterine population than at birth. For example, holoprosencephaly, malformations characterized by specific brain and face dysmorphia (DeMyer et al. 1964; Edison & Muenke 2003), was encountered at greater rates (1/250 or higher) *in utero* than in newborns (1/10,000–20,000) (Matsunaga & Shiota 1977). To date, however, the collection has not been analyzed genetically.

２．研究の目的

(1) The first purpose of this study is to obtain consecutive MR images of human prenatal brains from the embryo to fetus stages using three MRI instruments (2.34-T, 3.0-T, and 7.0-T), to make 3D models of human brain, and to register landmarks for further study by computational anatomy.

(2) The purpose of the present preliminary study was to modify the DNA extraction protocol for wet preparations of tissues preserved in formalin or Bouin’s solution for long term. Additionally, we aimed to validate the DNA quality and quantity resulting from this modified protocol. If the Kyoto Collection can provide suitable DNA for genetic analyses, the resultant data should greatly advance research on the genetic background of human congenital anomalies in the embryonic stage.

３．研究の方法

(1) We divided the samples into three groups by size and applied a suitable MRI apparatus and method for stabilization conforming to the corresponding MRI apparatus for each group (Table 1).

Table 1 MRI apparatus, specimens, and methods of fixation

Group	I (Embryo) CS13–23 (5.2–23.4 mm)	II (Fetus) CRL (33.6–86 mm)	III (Fetus) CRL (116–225 mm)
Device	2.34 T MR microscope (Super-parallel MR Microscopy)	7 T preclinical MRI (BioSpec 70/20 USR, Bruker Biospin MRI Gmbh, Ettlingen, Germany)	3 T clinical MRI (Siemens Healthcare, Erlangen, Germany)
Voxel size	40–150 μm	35.4–109.4 μm	200 μm
Specimens	•CS13 5.2 mm (#52233)	•37.2 mm (#52002)	•116 mm (#53591)
CRL and CS	•CS14 6.1 mm (#31431)	•56.5 mm (#52201)	•145 mm (#53524)
	•CS15 6.8 mm (#30867)	•86 mm (#F226)	•170 mm (#53590)
	•CS16 8.3 mm (#34584)		•205 mm (#53588)
	•CS17 9.5 mm (#38341)		•225 mm (#53570)
	•CS18 11.9 mm (#33377)		
	•CS19 13.4 mm (#33249)		
	•CS20 17.8 mm (#32816)		
	•CS21 20.5 mm (#33988)		
	•CS22 21.2 mm (#36135)		
	•CS23 23.4 mm (#52817)		
Method of fixation	NMR test tube with 10% formalin solution	Plastic container with absorbent cotton and formalin solution	Plastic container with agarose gel

(雑誌論文④より引用　Cited from Ref 4.)

Group I: MRI data were obtained by using a 2.34T MRM (Super-parallel MRM) at the Institute of Applied Physics, University of Tsukuba.
Group II: MRI data were obtained using a 7T preclinical MRI (BioSpec 70/20 USR; Bruker Biospin MRI Gmbh, Ettlingen, Germany) at the Biomedical Engineering Laboratory, Kyoto University.
Group III: MRI data were obtained using a clinical 3T MRI (Siemens Healthcare, Erlangen, Germany) at Kyoto University Hospital.

Group I MRI data, which were Softimage Image files, were analyzed using ImageJ (Wayne Rasband, National Institutes of Health, USA). In contrast, Group II and III data, which were Amica Paint Image files, were analyzed using OsiriX (version 6.0.1 64-bit; Pixmeo, Bernex-Swizerland) to determine data integrity. Next, all data were processed using Amira software (version 6.0.1; Visualization Sciences, Berlin, Germany). Segmentation of the developing brain was performed manually using MRI data based on previously published reference books. To register landmarks, we chose sagittal sections of MR images because it was easy to identify the morphological minutia as the basis of landmarks.

(2) As a preliminary test, DNA was isolated from formalin-fixed-mouse and human embryo tissue samples, using DNA FFPE tissue kits from various suppliers to determine the most appropriate kit. Thereafter, we chose one of the DNA extraction kits.

Tissue samples were processed for DNA isolation using the QIAamp DNA FFPE Tissue Kit (Cat. No. 37625) (QIAGEN, Tokyo, Japan), with modifications of the manufacturer protocol to improve efficiency. The DNA extraction procedure was divided into six stages: wash, lysis, heat treatment, binding to the membrane, elution, and reparation. The wash, lysis, and reparation stages were modified.

4．研究成果

(1) We could divide the developing brain into a maximum of six areas using the identified nine landmarks. The definitions of the nine landmarks are shown in Table 2.

Table 2 Nine landmarks and the corresponding locations

Landmark	Description
pc	The deepest point of dorsal constriction of the mesencephalon (near the posterior commissure)
mb	The upper end of the ventral swell of the diencephalon (mammillary body)
ig	The dorsal constriction between the mesencephalon and rhombencephalon (isthmic groove)
ir	The ventral swell of the mesencephalic vesicle upon the isthmus rhombencephali (isthmic recess)
cp	The dorsal root of the telencephalon (near the choroid plexus)
pa	Until CS17, the innermost region of the ventral curve between the telencephalon and diencephalon After CS18, the difference in signal intensity between the basal telencephalon and preoptic area
cn	The caudal trough of the rhombic lip extending to the isthmic canal (cerebellar notch)
pt	The innermost point of the pontine flexure (pontomedullary trench)
C1	The upper region of C1

(雑誌論文④より引用　Cited from Ref 4.)

As the brains grew, the segmented areas increased; three periods were identified: until Carnegie stages (CS) 14, until CS18, and after CS19. MR images and landmarks for each period are shown in Figure 1. Until CS14, there were three brain vesicles: the prosencephalon, mesencephalon, and rhombencephalon. Four landmarks were identified on MR images (Fig. 1A, B): the posterior commissure (pc), mammillary body (mb), isthmic groove (ig), and isthmic recess (ir). The pc and mb segmented the developing brain into two areas: the prosencephalon and mesencephalon. The ig and ir made up the boundary between the mesencephalon and rhombencephalon. After CS15, two new landmarks were placed: the choroid plexus (cp) and preoptic area (pa), and these landmarks segmented the prosencephalon into the telencephalon and diencephalon (Fig. 1D, E). After CS19, an additional two landmarks were placed: the cerebellar notch (cn) and pontomedullary trench (pt) and segmented the rhombencephalon into the cerebellum, pons, and mylencephalon. Then, we excluded the tegmentum of the rhombencephalon and the fourth ventricle from the area of the rhombencephalon (Fig. 1G, H).

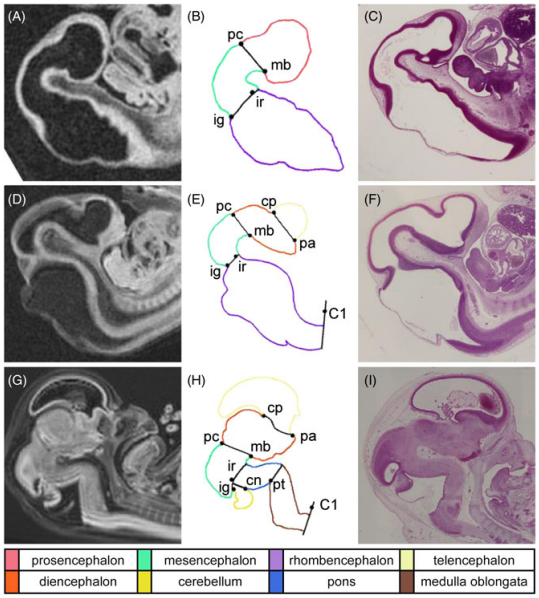

Figure 1. Landmarks and borderlines. A, B, C: CS14. C: Sample #13835. D, E, F: CS17. F: Sample #4429. G, H, I: CS23. I: Sample #9026. A, D, G: MR images. B, E, H: landmarks on MR images. The names of landmarks are shown in Table 2. C, F, I: serial sections of each stage in correspondence with the MR section. (i): tegmentum of the rhombencephalon. (ii): fourth ventricle.

(2) Next we reconstructed 3D segmented models of the developing brain. 3D models of embryonic brains are shown in Figure 2A, and eight models of fetal brains are shown in Figure 2B. The models were composed of the cerebral parenchyma and ventricle (after CS19,

excluding the fourth ventricle) without the cranial nerve system in order to simplify the images. From 3D visualization models, the growth of each area could be visually observed. In particular, the telencephalon and the cerebellum became very large after the brain reached a CRL of 116 mm and could be assessed visually (Fig. 3).

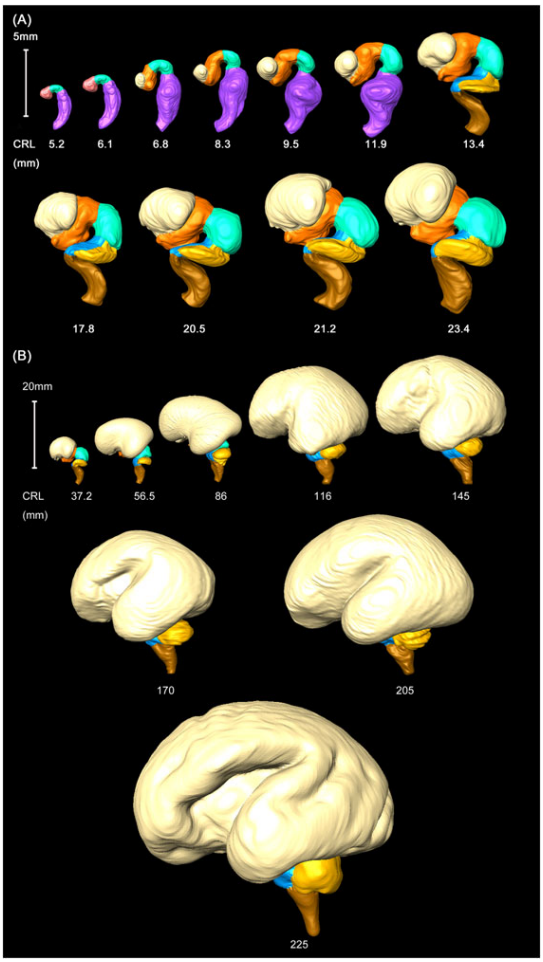

Figure 2. Lateral view of nineteen 3D models of the developing brain ranging from 5.2 to 225 mm. **A**: Embryonic brains ranging from 5.2 to 23.4 mm. Samples were at CS13 to CS23 and could be divided into three periods. In CS13 and CS14, the models were segmented into three areas. After CS15, there were four areas. Moreover, after CS19, there were six areas. **B:** Fetal brains ranging from 37.2 mm to 225 mm.

(3) We successfully extracted DNA from long-term and short-term fixed tissues, regardless of whether Bouin's or formalin solutions were used. The quantity and quality of the DNA extracted from samples fixed in Bouin's solution were significantly lower than samples fixed in formalin, regardless of fixation duration. When examining formalin-fixed samples alone, the quantity and quality of DNA using the manufacturer's protocol depended on the preservation duration.

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

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〔産業財産権〕

○出願状況（計０件）

○取得状況（計０件）

〔その他〕
ホームページ等（該当なし）

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