—Original Article—

The phenotype of a pig with monosomy X resembling Turner syndrome symptoms: a case report

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Abstract. The partial or complete loss of one X chromosome in humans causes Turner syndrome (TS), which is accompanied by a range of physical and reproductive pathologies. This article reports similarities between the phenotype of a pig with monosomy X and the symptoms of TS in humans. Born as the offspring of a male pig carrying a mutation in an X-chromosomal gene, ornithine transcarbamylase (*OTC*), the female pig (37,XO) was raised to the age of 36 months. This X-monosomic pig presented with abnormal physical characteristics including short stature, micrognathia, and skeletal abnormalities in the limbs. Furthermore, the female did not exhibit an estrous cycle, even after reaching the age of sexual maturity, and showed no ovarian endocrine activity except for an irregular increase in blood 17β-estradiol levels, which was seemingly attributable to sporadic follicular development. An autopsy at 36 months revealed an undeveloped reproductive tract with ovaries that lacked follicles. These data demonstrated that the growth processes and anatomical and physiological characteristics of an X-monosomic pig closely resembled those of a human with TS.
Key words: Monosomy X, Pig, Turner syndrome

The partial or complete loss of one X chromosome causes Turner syndrome (TS) in humans. This condition occurs once per 2000–4000 female births [1]. The main pathologies of TS include short stature, gonadal dysfunction, and structural deformities such as webbed neck, micrognathia, cubitus valgus, and Madelung deformity [1]. Almost all TS patients experience premature ovarian failure (POF) by age 30 [2].

Monosomy X is not exclusive to humans but has been reported in various animal species [3]. The condition has been recorded most frequently in horses. Compared to normal horses, affected horses are small in stature, and while their external genitalia appear normal, development of the reproductive tract is incomplete; these individuals lack estrus cycles and are infertile [4–8]. In the other species in which monosomy X has been found, such as rhesus monkeys [9], cows [10], buffalo [11], sheep [12], dogs [13, 14], cats [15, 16], llamas [17], and pigs [18], the reported symptoms match those seen in horses. Mice are an exception: While X-monosomic individuals have fewer oocytes in their ovaries and a shorter reproductive lifespan than euploid females, they are anatomically normal and fertile [19, 20].

In an attempt to produce an ornithine transcarbamylase deficiency [OTCD; Online Mendelian Inheritance in Man (OMIM) No. 311250] model, we created a fertile boar with a mutation in the X-chromosomal *OTC* gene (a 5-bp deletion in exon 2, c.186_190delTCTGA) induced by a transcription activator-like effector nuclease (TALEN) [21]. Among the progeny of this male, we identified one female pig with monosomy X (37,XO). Knowledge of the phenotypic characteristics of X-monosomic pigs is still very limited [3]. Thus, we raised this X-monosomic pig with its littermate gilts and monitored its anatomical and physiological changes as it grew to adulthood. We report on the findings of this case here.

Materials and Methods

Animal care

The Institutional Animal Care and Use Committee of Meiji University approved the animal experiments described in this study (IACUC12-0008, 17-0004). All animal care and experimental procedures were performed in accordance with the Japan Act on Welfare and Management of Animals and all applicable regulations. The pigs were housed in a temperature-controlled room, had free access to water, and were provided with commercial feed appropriate for their growth stage (Nosan, Yokohama, Japan) in accordance with the Japanese Feeding Standard for Swine (2013) [22]. The health of all pigs was assessed at feeding (0800 h and 1700 h). For the autopsies, the pigs received an intramuscular injection of 1% mafoprazine mesylate (0.5 mg/kg, DS Pharma Animal Health, Osaka,

Japan) and midazolam (0.1 mg/kg, Takeda Pharmaceutical, Tokyo, Japan), followed by an intravenous injection of sodium thiopental (Nipro ES Pharma, Osaka, Japan), and anesthesia was maintained via the inhalation of isoflurane (DS Pharma Animal Health) while the pigs were exsanguinated.

Chemicals

All of the used chemicals were purchased from Merck KGaA (Darmstadt, Hesse, Germany) unless otherwise indicated.

Chromosomal analysis

For the chromosome counting, metaphase chromosome spreads were prepared from pig peripheral blood mononuclear cells according to standard procedures [23]. The cells were treated with 20 ng/ml colcemid (demecolcine) for 14 h and subsequently harvested. After treatment with 0.075 M KCl for 20 min at 37°C, the cells were fixed by exposure to a 3:1 ratio of MeOH to acetic acid three times, and the fixed cells were spread on slides. A total of 50 chromosome images were captured using a Leica DC350FX cooled charge-coupled device (CCD) camera (Leica, Wetzlar, Germany) mounted on a Leica DMRA2 microscope and analyzed using the Leica CW4000 FISH software.

Computed tomography analysis

Computed tomography (CT) analyses were performed using an Aquilion PRIME CT scanner (Canon Medical Systems, Ohtawara, Japan). Three-dimensional (3D) models of the limbs were created based on the 0.500-mm-thick CT scan slices generated using OsiriX software (Pixmeo SARL, Bernex, Switzerland).

Blood analysis and serum biochemical analysis

Peripheral blood was consecutively collected from the X-monosomic pig (#101) and a littermate (#100) at 15 and 33 months after birth. Blood cell counts were analyzed using an automated blood cell counter (MEK-6550; Nihon Kohden, Tokyo, Japan). Serum biochemistry tests were performed using a dry-chemistry analyzer (FUJI DRICHEM 7000; FUJIFILM, Tokyo, Japan). Serum concentrations of 17β-estradiol and progesterone were measured with enzyme-linked immunosorbent assays (ELISAs) by an external laboratory (Protein Purify, Isesaki, Japan).

Histological analysis

Ovary tissues were fixed in 10% neutral buffered formalin solution (Wako Pure Chemical Industries, Osaka, Japan), embedded in paraffin, sectioned, and treated with hematoxylin-eosin stain using standard methods.

Results

Identification of monosomy X

The DNA analysis performed at birth indicated that the sex chromosome configuration of an apparently female pig (#101) was XO, as this animal did not possess the *OTC* mutation it should have inherited (Supplementary Fig. 1: online only). All of the 50 chromosome samples examined showed 36 autosomes and 1 X chromosome (Fig. 1A). Thus, karyotype analysis confirmed that this pig possessed the 37,XO chromosome composition. We concluded
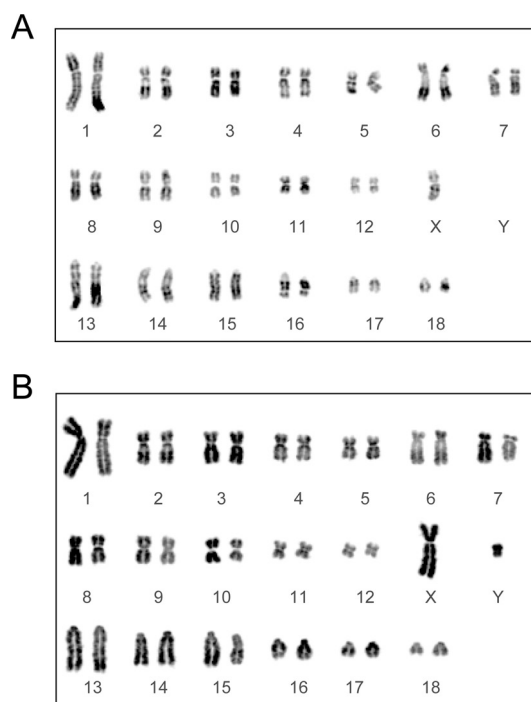

Fig. 1.   Chromosomal analysis of the X-monosomic pig. We analyzed a female pig (#101) born in a litter fathered by a male pig with a knockout mutation in the *OTC* gene on its X chromosome [21]. From the results of the DNA analysis (Supplementary Fig. 1: online only), we predicted this pig to have the sex chromosome configuration of XO. (A) The 37,XO chromosomal image of #101. (B) The 38,XY chromosomal image of a normal male pig.

that, based on the genomic and karyotypic evidence, the female individual #101 had monosomy X.

Growth and anatomical features of the X-monosomic pig

The growth of the X-monosomic (XO) pig was delayed; its weight at 5 months was 69.4 kg, compared to 81.0 ± 4.4 kg for its female littermates (n = 5) (Fig. 2A).

We observed that the lower jaw of the XO pig had a characteristic shape that we diagnosed as micrognathia. Compared to its littermate XX pigs, whose upper and lower jaws lined up when their mouths were closed (Fig. 2B), the XO pig had a lower jaw that was markedly shorter than its upper jaw (Fig. 2C).

Furthermore, we observed reduced limb length in the XO pig (Fig. 2D–2G). Measurements were made based on the CT scans taken at 36 months. The lengths of the humerus, radius, and ulna of the thoracic limbs of the XO pig were 80%, 66%, and 67% (Figs. 3A, 3B, 3F, 3G, Table 1) of the lengths of the corresponding bones of its littermate (Figs. 3B[a]–[c]). In contrast, there was no difference in the length of the third or fourth metacarpal bone or the third or fourth proximal phalanx (Figs. 3B[d]–[g]). Abnormalities were also observed in the lengths of the long bones of the pelvic limbs. The lengths of the femur, tibia, and fibula of the pelvic limbs of the XO pig were 63%, 68%, and 62% (Figs. 3C, 3D, 3H, 3I, Table 1) of the lengths of the corresponding bones of its littermate (Figs.
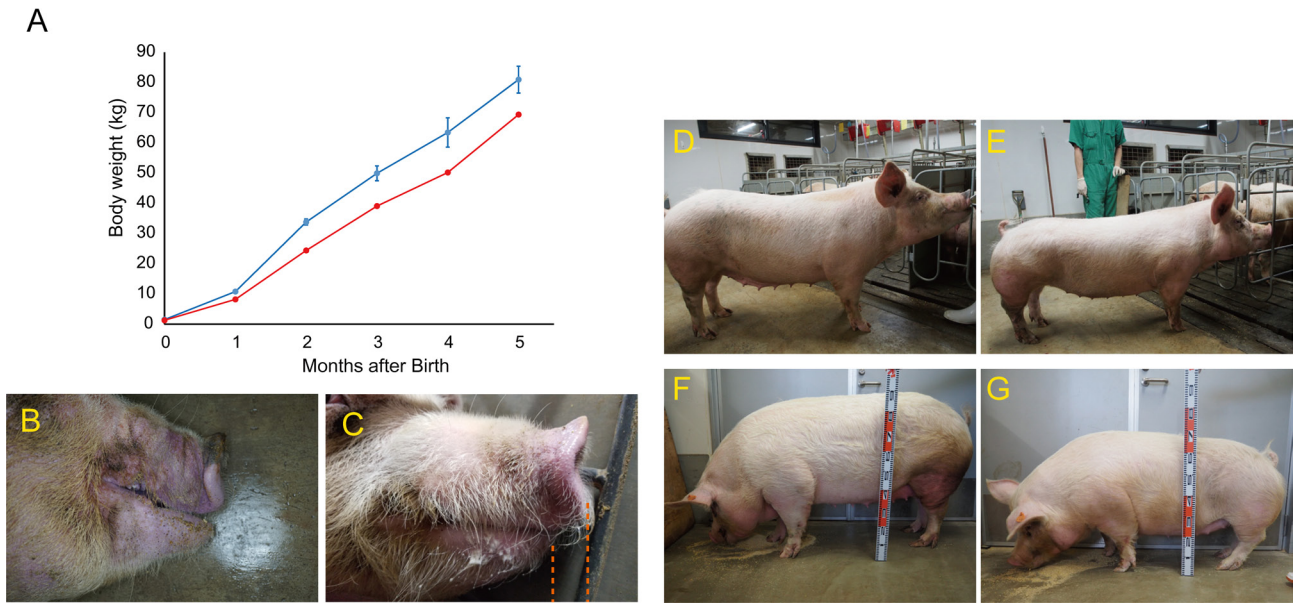
Fig. 2. Growth and external characteristics of the X-monosomic pig. (A) Body-weight changes of the XO pig (red line) and littermate pigs (n = 5, mean ± standard error, blue line). (B, C) Micrognathia observed in the XO pig. In its littermate XX pigs (B), the lengths of the upper and lower jaws matched. However, in the XO pig, the lower jaw was markedly shorter than the upper jaw (C, dotted red line). (D, E) XX (D) and XO (E) pigs at 15 months of age. (F, G) XX (F) and XO (G) pigs at 33 months of age.
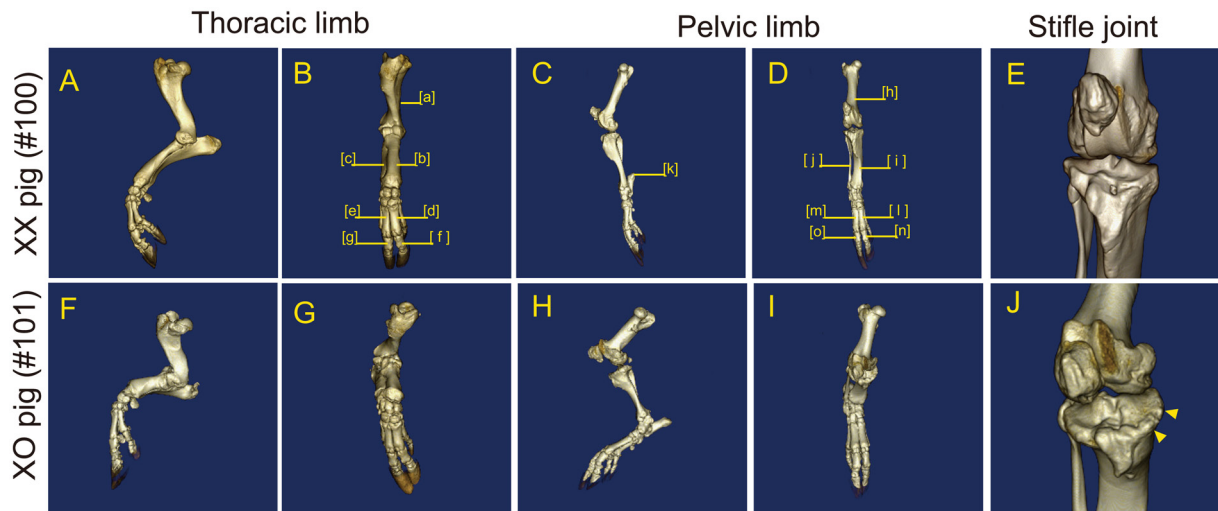

Fig. 3. Deformities in the long bones and knee joints of the X-monosomic pig. (A–J) CT images of the limbs of the XO pig and a littermate XX pig at 36 months of age. The long bones of the XO pig (#101, F–I) were shorter than those of a littermate XX pig (#100, A–D). (A, F) Medial view of the right thoracic limb. (B, G) Anterior view of the right thoracic limb. (C, H) Medial view of the right pelvic limb. (D, I) Anterior view of the right pelvic limb. (E, J) CT images of the knee joint of the right pelvic limb of the XX (E) and XO (J) pigs. Deformities and osteophytes (arrowheads) were observed in the knee joint of the XO pig.

3D[h]–[j]). However, no differences were observed in the lengths of the calcaneus (Fig. 3C[k]), the third and fourth metatarsals, or the third and fourth phalanges (Figs. 3D[l]–[o]).

Additionally, growth-associated changes were observed in the conformation of the pelvic limbs of the XO pig (Figs. 2D–2G). As

shown in Fig. 2, the pelvic limb conformation of the XO pig at 15 months (Fig. 2E) was upright, similar to that of the XX pigs (Fig. 2D). However, by 34 months, this configuration had become slanted (Fig. 2G). An autopsy of the XO individual (at 36 months) revealed the presence of osteophytes on the knee joint (on the end of the tibia)

Table 1. Bone length of the thoracic and pelvic limbs of the pigs

Thoracic limb		#100	#101	Pelvic limb		#100	#101
Humerus	[a]	21.1	17.0	Femur	[h]	24.8	15.5
Radius	[b]	15.6	10.3	Tibia	[i]	21.2	14.5
Ulna	[c]	22.7	15.1	Fibula	[j]	20.3	12.6
3rd metacarpal bone	[d]	6.4	6.1	Calcaneus	[k]	9.6	9.9
4th metacarpal bone	[e]	6.9	7.0	3rd metatarsal bone	[l]	8.0	7.9
3rd proximal phalanx	[f]	3.0	3.3	4th metatarsal bone	[m]	8.4	8.2
4th proximal phalanx	[g]	3.1	3.3	3rd phalange	[n]	3.3	3.9
				4th phalange	[o]	3.9	4.3

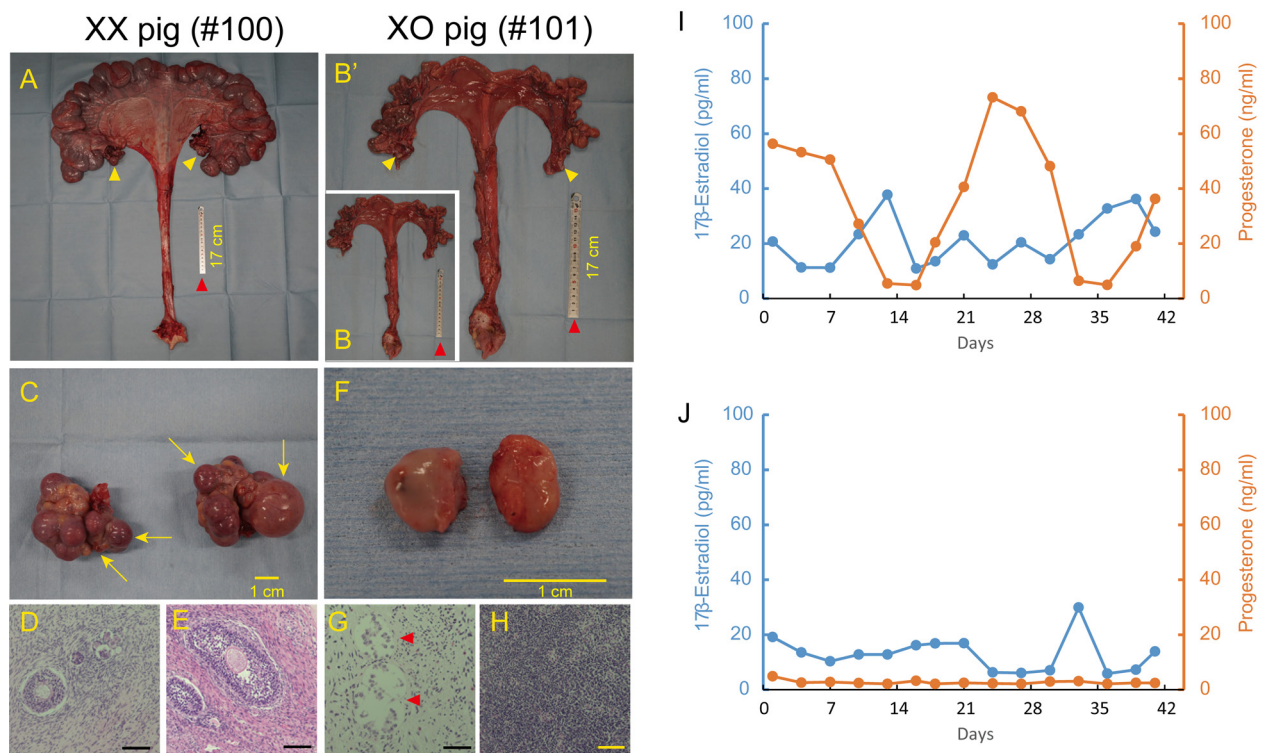[a]–[o]: Each character is linked in Fig. 3.

Fig. 4. Morphological and histological differences in the genital organs of 38,XX and 37,XO pigs. (A, B) Gross size differences in the genital organs of XX (#100, A) and XO (#101, B) pigs. Red arrowheads indicate a 17 cm scale. Inset (B) shows the size of the genital organs of the XO pig on the same scale as those of the XX pig (A). (A, B′) Yellow arrowheads indicate ovaries. (C–H) Presence of corpora lutea (C, arrows) and follicles (D, E, scale bars: 100 μm) indicated cyclic ovarian activity in the XX pig, while functional features were not observed in the ovaries of the XO pig (F–H). (F) The ovaries of the XO pig showed markedly retarded development. (G, H) The ovarian tissue of the XO pig was entirely occupied by interstitial cells, except for atretic follicle-like vacuoles (arrowheads) without oocytes. Scale bars indicate 50 μm (G) and 100 μm (H). (I, J) Hormonal profiles of the XX (I) and XO (J) pigs between 33 and 34 months old.

(Figs. 3E, 3J). It is very likely that pain caused by these osteophytes influenced the limb conformation of the XO pig.

Anatomical, histological, and endocrinological features of the reproductive organs of the X-monosomic pig

The ovaries and reproductive tract of the XO pig were exceedingly underdeveloped in comparison to those of the XX pigs. Upon autopsy at 36 months, the total weight of the genitalia (external and internal) of an XX individual was 2,096 g; in comparison, that of the XO individual was 211 g. The left and right ovaries of an XX individual weighed 14.7 and 18.8 g, while those of the XO pig weighed 0.3 and 0.2 g, respectively. The vagina and uterine horns of the XO individual were also markedly shorter than those of the XX individuals (Figs. 4A, 4B). The vulva of the XO individual was underdeveloped but did not exhibit any structural abnormalities.

While we visually and histologically observed evidence of cyclic

Table 2. Hematological parameters of the pigs

Parameter	#100	#101
Red blood cells (10^4/μl)	833.0	795.0
Hemoglobin (g/dl)	16.7	17.2
Hematocrit (%)	49.4	51.4
Mean corpuscular volume (fl)	59.3	64.7
Mean corpuscular hemoglobin (pg)	20.0	21.6
Mean corpuscular hemoglobin concentration (g/dl)	33.8	33.5
Red cell distribution width (%)	15.0	15.2
White red cells (10^2/μl)	107.0	126.0
Platelet (10^4/μl)	17.0	19.0
Mean platelet volume (fl)	9.4	9.0
Platelet size distribution (%)	12.9	13.7
Plateletcrit (%)	0.2	0.2

ovarian activity in the XX littermates (Figs. 4C–4E), the ovaries of the XO pig evinced clear functional and histological underdevelopment (Figs. 4F–4H). These findings were supported by measurements of blood sex steroids in the XO pig. The dynamics of the serum 17β-estradiol and progesterone levels at 33–34 months in the XX pigs indicated cyclic ovarian activity, but in the XO pig, the only change observed was a slight increase in 17β-estradiol concentration, while progesterone secretion was not detected (Figs. 4I, 4J). Cyclic ovarian activity was also not observed at 15 months in the XO pig (Supplementary Fig. 2: online only).

As for the hematological parameters and biochemical measurements of the XX and XO pigs at 33 months, no clear differences were observed (Tables 2, 3).

Discussion

The discovery of an X-monosomic pig was reported in 1968 by Nes [18]. The characteristics of the three X-monosomic individuals examined were dwarfed stature, short legs, poor development of the ovaries and vulvae, and the lack of estrous cycles. To our best knowledge, this article is the second to report the phenotypic characteristics of an X-monosomic pig.

More than half of the patients with Turner syndrome show chromosomal mosaicism including XO, XX, and XXX [1], reflecting a high prenatal lethality of the 45,XO fetuses [24, 25]. Although our X-monosomic pig is unlikely to possess a mosaic chromosomal composition, the possible lethality of porcine 37,XO embryos/fetuses is yet to be investigated.

The dropout of sex chromosomes with abnormal structures during embryonic development is hypothesized to be a possible mechanism for the occurrence of X-monosomic individuals [26, 27]. The X-monosomic pig we discovered was born via the fertilization of an ovum by sperm containing a mutation (a 5-bp deletion) in the *OTC* gene on the short arm of the X chromosome. A structural change in the X chromosome induced by genome editing may have been the cause of the dropout midway through the development of the embryo. However, among the 34 *OTC*-mutant females that we

Table 3. Serum biochemical parameters of the pigs

Parameter		#100	#101
Electrolytes (mEq/l)	Sodium	145.0	145.0
	Potassium	4.2	4.8
	Chloride	112.0	109.0
Minerals (mg/dl)	Calcium	9.5	9.5
	Inorganic phosphorus	4.6	5.3
	Magnesium	1.9	1.9
Liver function (mg/dl)	Total bilirubin	0.1	0.1
Renal function (mg/dl)	Blood urea nitrogen	12.8	13.2
	Creatinine	2.0	1.5
Metabolites (mg/dl)	Total cholesterol	107.0	103.0
	High-density lipoprotein cholesterol	22.0	36.0
	Triglyceride	48.0	55.0
	Glucose	87.0	93.0
	Uric acid	0.4	0.4
Enzymes (U/l)	Alkaline phosphatase	136.0	116.0
	Alanine aminotransferase	34.0	33.0
	Amylase	1217.0	2137.0
	Aspartate aminotransferase	24.0	35.0
	Lactate dehydrogenase	372.0	438.0
	Lipase	31.0	37.0
	γ-Glutamyltransferase	45.0	19.0
Protein (g/dl)	Total protein	8.4	7.5
	Albumin	4.3	5.2

have created so far, we have only observed one XO individual [21].

A change in the expression levels of the genes on the X chromosome has been proposed to be the cause of TS development in monosomy X [28, 29]. For example, the genes located in the pseudoautosomal region (PAR) of the X chromosome do not normally undergo the inhibition of expression accompanied by X inactivation [30]. However, in monosomy X, the expression levels of these genes are also halved, and it is supposed that this reduction thereby affects the phenotype of the monosomic individual [28, 29].

Expressional reduction of the short stature homeobox (*SHOX*) in the PAR is known to cause wrist deformity and mesomelic short stature concurrent with Léri-Weill dyschondrosteosis (LWD; OMIM No. 249700) and idiopathic short stature (ISS; OMIM No. 300582) [31–33]. It is believed that the commonalities between TS and LWD patients, including micrognathia, a high-arched palate, deformity of the external ear, cubitus valgus, genu valgus, and Madelung deformity, are related to the inhibition of *SHOX* gene expression [34]. In humans, *SHOX* genes are expressed in the mesenchymal cells of the first pharyngeal arch, which later becomes the upper and lower jaws, and in the developing limbs [34]. It is therefore very likely that changes in *SHOX* gene expression levels influence the phenotype of X-monosomic pigs, which is characterized by micrognathia and short limbs. This hypothesis is further supported by the fact that murine sex chromosomes lack *SHOX* and that X-monosomic mice do not present with the aforementioned external deformities.

A large number (690 in total) of protein-coding genes are located on the pig X chromosome [35]. Additionally, it is estimated that, in humans, 15% of the genes on the X chromosome do not normally undergo the X-inactivation-related inhibition of expression [30]. If the same fraction is applicable to pigs, then it is possible that the expression levels of approximately 100 X-linked genes are affected by monosomy X.

However, approximately 123 genes are known to exist on the human X chromosome that are not present on that of the pig [35]. It is possible that these genes have some form of influence on the differences observed between the phenotypes caused by monosomy X in humans and pigs. Further research is needed to clarify the relationship between interspecies differences of X-linked genes and X-monosomic phenotypes observed in humans and pigs.

In the females of many mammalian species, the formation of oocytes ends in the fetal stage. From the prenatal stage until puberty, the primary oocytes in the ovaries are arrested in the prophase of meiosis I. In X-monosomic individuals, the monosomic X chromosome in the primary oocytes cannot pair with its homologous chromosome, causing oocyte apoptosis [36, 37]. For this reason, primordial follicles do not form in the ovaries of X-monosomic individuals [38]. Such individuals consequently experience disrupted follicular development and sex steroid production, even after growth to the age of sexual maturity, and are therefore deficient in the development of reproductive organs.

POF is one of the primary characteristics of TS and represents a considerable risk factor for osteoarthritis [39]. Women with TS experience rapid follicular atresia and normally lose their oocytes in early childhood [39, 40]. A reduction in estrogen secreted from the ovaries then becomes a causal factor for osteoporosis. We suspected a correlation between osteoporosis and the osteophytes at the knee joint observed in the X-monosomic individual we investigated, but micro-CT analysis of the femoral head did not reveal any changes in bone density (data not shown). The passage of more time may be needed for the development of osteoporosis in an X-monosomic pig.

Our analysis revealed many similarities between the phenotype of an X-monosomic pig and the symptoms of TS patients. Although we discovered this X-monosomic pig accidentally, we would be able to provide an animal model for TS research if it were possible to produce cloned animals from its somatic cells (which have been cryopreserved). However, because nearly 99% of 45,XO human fetuses die prenatally [24, 25], cloned XO pig fetuses may also frequently experience lethality. In other words, through the production of cloned XO pigs, we may be able to gain insight into the embryonic/fetal lethality of monosomy X in pigs. If the production of cloned XO pigs proves possible, we believe that research on therapeutic treatments using these animals as disease models may help to improve the quality of life of TS patients.

Acknowledgments

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