The Japanese Society of Toxicology

Fundamental Toxicological Sciences

URL : http://www.fundtoxicolsci.org/index_e.html

Original Article

5-Fluorocytosine induces fetal skeletal malformations in rats by altering expression of Homeobox genes

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(Received February 21, 2020; Accepted March 9, 2020)

ABSTRACT — 5-Fluorocytosine (5-FC) is an antimycotic and teratogenic compound. Oral administration of 5-FC to pregnant rats on gestation days (GD) 9 and 13 was shown to induce thoracolumbar supernumerary ribs (TSR, 14th rib) and abnormal digits, respectively, in fetuses. This study investigated the effects of 5-FC on homeobox genes, which control the anterior-posterior-axis. 5-FC (75 mg/kg) was administered orally on GD9 and GD13, and tissues collected from cranial and caudal regions of TSR sites were analyzed. Following 5-FC administration on GD9, the levels of expression of Hoxa10, which determine the position of the thoracic and lumbar vertebrae, were decreased at GD13. Analysis of hindlimbs 6 hours after administration on GD13 showed decreases in expression of Hoxa11, Hoxd12, and Hoxd13, the Hox genes responsible for limb formation from the proximal to distal, and from the anterior to posterior directions. The present findings showed that altered expression of Hox genes contributes to 5-FC teratogenicity.

Key words: 5-FC (Flucytosine), Homeobox, Thoracolumbar supernumerary rib, Digital malformation

INTRODUCTION

5-FC (5-fluorocytosine, flucytosine) is an orally administered agent used to treat deep-seated mycoses, including fungemia, fungal meningitis, and fungal respiratory infection. 5-FC is selectively taken up by fungal cells and deaminated to fluorouracil (5-FU), which inhibits nucleic acid synthesis in fungi by inhibiting the synthesis of DNA precursors (Polak and Scholer, 1975). 5-FC has several relatively minor side effects, such as nausea, vomiting and diarrhea, as well as more severe side effects, including hepatotoxicity and bone marrow depression (Diasio *et al.*, 1978; Vermes *et al.*, 2000). Although its effects in pregnancy have been assessed by reproductive and developmental toxicity tests (Takeuchi *et al.*, 1976), its toxicological mechanisms are not yet fully understood.

A previous study investigating the origin of thoracolumbar supernumerary ribs (TSR, 14th rib) in rats showed that oral administration of 75 mg/kg 5-FC to pregnant rats on gestation day (GD) 9 induced TSR (Kuwagata *et al.*, 2018a). Furthermore, oral administration of 5-FC to pregnant rats on GD13 induced the formation of abnormal digits. To clarify the mechanisms by which 5-FC induces skeletal abnormalities, this study focused on Homeobox

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(Hox) genes.

Hox genes share a common region, called the Hox domain and consisting of a 180-base pair DNA sequence encoding 60 amino acids. In many vertebrates such as humans and rodents, Hox genes consist of four paralog clusters (a, b, c, d) on different chromosomes. Each cluster consists of 13 genes, although some are defective, resulting in a total of 39 intact Hox genes. Hox genes play important roles in determining the position of limb segments and in the process of limb formation, with the anatomical structure of limbs determined by the timing and position of terminal expression of Hox genes (Hunt and Krumlauf, 1992; Burke *et al.*, 1995). That is, the formation of bones from the cranium to the cervical spine; the thoracic, lumbar, and sacral vertebrae; and the limbs from the proximal to the distal direction is determined by the expression of the relevant number of Hox genes. Changes in expression of Hox genes can result in abnormal skeletal structure, with TSR and limb malformation regarded as associated with abnormal expression of Hox genes at these sites (Wellik and Capecci, 2003; Chen and Capecchi, 1999; Guerreiro *et al.*, 2012).

This study was designed to determine the embryonic stage and area of expression of ribs by assessing the expression of Sox9 (SRY (sex determining region Y)-box 9), which is expressed in mesenchymal cells and is required for cartilage formation (Bi *et al.*, 1999), by whole mount-*in situ* hybridization (ISH). The cranial (anterior) and caudal (posterior) regions of the TSR expression site were separated, and the expression of each Hox gene associated with TSR formation was analyzed. Specifically, 5-FC was administered orally to pregnant rats on GD9, and Hox9 and Hox10 expression was assessed in their fetuses. In addition, 5-FC was administered orally to pregnant rats on GD13; the hind limbs of their fetuses were dissected; and the expression in dissected hind limbs of Hox11-13, genes associated with the development of digits, was analyzed.

MATERIALS AND METHODS

Animal treatment and 5-FC administration

Pregnant SLC:SD rats were obtained from SLC Japan, Inc. (Shizuoka, Japan) and housed individually in an environmentally controlled animal room. Chow (CE-2, CLEA Japan Inc., Tokyo, Japan) and drinking water were provided ad libitum. The day on which sperm were observed was designated as GD0. Fetuses were obtained from two untreated dams each on GD9.5, 10.5, 11.5, 12.5, and 13.5 (19:00-21:00) for whole mount ISH. The number of somites in each fetus was counted under a stereoscopic microscope (Fig. 1).

Other dams were orally administered 75 mg/kg of 5-FC (Tokyo Chemical Industry, Tokyo, Japan), suspended in 0.5% methyl cellulose solution, on GD9 (07:00) or GD13 (08:00), followed by caesarean section on GD13.5 (14:00-15:00). Control groups consisted of dams administered vehicle. Some of the fetuses were used for whole mount ISH and quantitative real-time PCR. All animal experimental protocols conformed to the Guide for the Care and Use of Laboratory Animals of Hatano Research Institute, Food and Drug Safety Center.

Whole mount ISH

Whole mount ISH was performed as described (Motoyama *et al.*, 1998; Shibata *et al.*, 2003), using non-radioactive digoxigenin-labeled RNA probes for Sox9. The templates for transcription of RNA probes were prepared by PCR amplification of Sox9 (NM_011448) mouse cDNA obtained from OriGene Technologies Inc. The Sox9 forward primer, 5'-ACAGACTCACATCTCTC-CTAATGCT-3', contained a T7 sequence at its 5' end, and the Sox9 reverse primer, 5'-CATGTAAGTGAAG-GTGGAGTAGAGC-3', contained a T3 sequence at its 5' end; these primers amplified an 820 bp DNA sequence. PCR reactions were performed using PrimeSTAR GXL DNA Polymerase (Takarabio, Shiga, Japan). The digoxigenin-labelled antisense and sense RNA probes were synthesized using the DIG RNA Labeling Kit (SP6/T7) and T3 polymerase (Roche Diagnostics, Mannheim, Germany). Because the PCR products, excluding the T3/T7 nucleotide sequences, have greater than 94% nucleotide sequence homology to rat cDNA, the antisense probes bound with high affinity to rat Sox9 mRNA. In addition, sense probes were useful as negative controls.

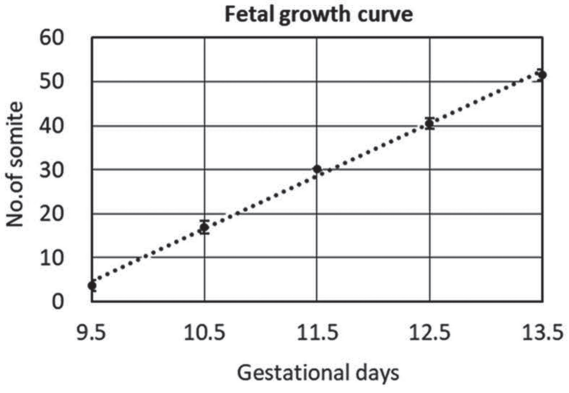

Fig. 1. Fetal growth curve. Relationship between number of somites and gestational days in untreated rats (n = 16-36).

Quantitative real-time PCR

Fetuses were immersed in RNA later (Thermo Fisher, Waltham, MA, USA) and stored at -30°C. Thoracic and lumbar regions of fetuses whose dams (4-5 per group) were administered 5-FC on GD9 were collected under a stereoscopic microscope, as were hind limbs of fetuses whose dams (14-15 per group) were administered 5-FC on GD13. The boundary between the thoracic and lumbar regions was based on the results of Sox9 expression.

Total RNA was extracted from each tissue sample using Isogen (Nippon Gene, Tokyo, Japan), and the quality of each sample was determined from the ratio of optical density at 260 and 280 nm. The concentration of each total RNA sample was adjusted, and reverse transcription was performed using a SuperScript IV First-Strand Synthesis System (Thermo Fisher). The cDNAs were amplified using Power SYBR Green Master Mix (Thermo Fisher) on a real-time PCR system (ABI 7500; Thermo Fisher). The Hoxa9 primers were 5'-aggaagagtaccgaggcaag-3' (forward) and 5'-taaatcactccgcacgctat-3' (reverse), amplifying a 159 bp fragment, and the Hoxa10 primers were 5'-aggactccctgggcaattc-3' (forward) and 5'-gtaagggcagcgtttcttcc-3' (reverse), amplifying a fragment of 83 bp (Huang *et al.*, 2007). Hox11–13 primers were designed using Primer Express software (Thermo Fisher), based on the sequence information in GenBank. The Hoxa11 primers were 5'-gtttttcgagacggcttacg-3' (forward) and 5'-ttctcggcgttcttgtcc-3' (reverse), amplifying a 75 bp sequence; the Hoxd12 primers were 5'-ttctctcagccgtacttgacc-3' (forward) and 5'-gtcttcgggtccgtctttg-3' (reverse), amplifying a 75 bp fragment; the Hoxa13 primers were 5'-ccaaatgtactgccccaaag-3' (forward) and 5'-tctgaaggatgggagacgac-3' (reverse), amplifying an 84 bp sequence; and the Hoxd13 primers were 5'-gcagaacgctctcaagtcgt-3' (forward) and 5'-gttggtcggtacgctgct-3' (reverse), amplifying a 103 bp fragment. The expression of each gene was normalized to that of ActB, which was amplified using the primers 5'-ctggctcctagcaccatga-3' (forward) and 3'-tagagccaccaatccacaca-5' (reverse), yielding a 76 bp fragment. All primers were synthesized by Invitrogen Custom Primers Services (Thermo Fisher). The amplification conditions consisted of an initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 10 sec, annealing at 60°C for 25 sec and extension at 72°C for 30 sec.

Statistical analysis

The mean litter was regarded as a statistical unit. Data are expressed as mean ± S.D. and differences were analyzed by Student's t-test (parametric data) or the Aspin-Welch t-test (non-parametric data). A p-value less than 5% was considered statistically significant.

RESULTS

Effect of 5-FC on fetal survival rates

The numbers of implantations and live embryos on GD13.5 are shown in Table 1. There was no difference in the numbers of implantations or live embryos.

Expression of Sox9 and the locations for dissection

Fetuses of untreated rats were dissected daily on GD9.5, 10.5, 11.5, 12.5, and 13.5, and the numbers of somites of all fetuses were counted under a stereoscopic microscope. The number of somites increased in proportion to gestational days (Fig. 1). Whole mount ISH followed by staining for Sox9 showed clear cartilage on GD13.5 (Fig. 2).

Generation of thoracolumbar supernumerary ribs by 5-FC administration on GD9

To address the mechanisms by which 5-FC administration on GD9 induces TSR, the levels of expression of Hoxa9 and Hoxa10 and their ratios were compared in control and 5-FC treated rats, and in the thoracic (cranial) and lumbar (caudal) regions. The levels of expression of Hoxa9 and Hoxa10 were higher in the lumbar than in the thoracic regions of both control and 5-FC treated rats, with the levels of both, especially Hoxa10, being lower in the fetuses of 5-FC treated than control rats (Fig. 3A, 3C). The ratio of expression of Hoxa10 in the lumbar relative to the thoracic regions was significantly higher in the fetuses of 5-FC treated rats than of control rats (Fig. 3D), whereas the ratio of expression of Hoxa9 in the lumbar relative to the thoracic regions was similar in the two groups of fetuses (Fig. 3B).

Induction of limb dysmorphology by 5-FC administration on GD13

Dissection of the hind limbs showed that the levels of all Hox genes examined (Hoxa11, Hoxd12, Hoxa13, and Hoxd13) were lower in fetuses of rats administered 5-FC than in control fetuses (Fig. 4).

DISCUSSION

Skeletal anomalies, including defects in the skull, vertebrae, ribs, and limbs, are generally irreversible. Individuals with TSR (14th rib) are thought to have fewer clinical problems than those with other skeletal anomalies.

Table 1. Survival rates on GD13.5 of fetuses exposed to 5-FC on GD9 or GD13.

Groups	Administration on GD9		Administration on GD13	
	Control	5-FC	Control	5-FC
Number of dams	8	15	8	11
Number of implantation sites				
Total	111	202	90	155
Mean ± S.D. per dam	13.9 ± 2.0	13.5 ± 1.7	11.3 ± 3.4	12.9 ± 1.5
Live fetuses				
Total	102	171	86	128
Mean ± S.D. per dam	10.2 ± 5.7	10.7 ± 3.4	10.8 ± 3.3	10.7 ± 2.6

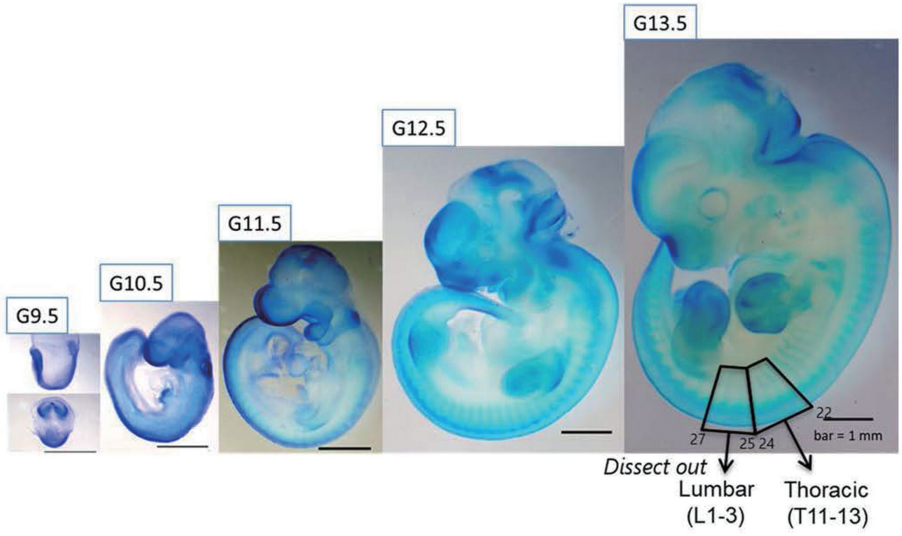

Fig. 2. Expression of Sox9 and locations of dissection. Expression of Sox9 on GD9.5-13.5 in fetuses of untreated rats. The frames show two areas of dissection. The bars indicate 1 mm. The numbers represent the number of somites.

However, the mechanism underlying TSR occurrence remains unclear, interfering with risk assessment following the administration of toxic agents (Chernoff and Rogers, 2004). TSRs that interfere with bodily functions are removed surgically in humans. Their incidence in humans is 3-7% (Nakajima *et al.*, 2014), whereas their incidence among animals differs according to species and strains. For example, the rates of full and short TSR were 1.10% and 8.45%, respectively, in Sprague Dawley (SD) rats, but 2.97% and 49.93%, respectively, in Wistar Hannover (Wistar Han) rats (Kuwagata *et al.*, 2018b), similar to previous findings (Aoyama *et al.*, 2002; Noritake *et al.*, 2013; Ema *et al.*, 2014).

We have reported that TSR (both short and full length) was induced in rat fetuses by single oral administration of 75 mg/kg 5-FC on GD9, without affecting maternal clinical signs, reproductive performance such as the number of implants, or fetal development (Kuwagata *et al.*, 2018a). 5-FC-induced skeletal anomalies were dependent on date of administration, with TSR induced by a single dose of 25 or 35 mg/kg 5-FC on day 11 of pregnancy (Fujii *et al.*, 2019).

To determine the mechanism by which 5-FC induces TSR in this animal model, we analyzed the expression of Hox genes. Cleavage position and analysis times were determined by whole mount ISH of Sox9, a transcription factor of the SRY family that regulates sex determination, cartilage development and many other developmental events. During the fetal period, Sox9 is expressed in mesenchymal cells and regulates the expression of the type II collagen gene, which encodes a major developmentally regulated protein of cartilage (Healy *et al.*, 1999; Hattori *et al.*, 2010). The generation of rib bone cartilage is followed by calcification of the costal cartilage, with the region positive for Sox9 expression indicating premature ribs. The position of somites on whole mount ISH

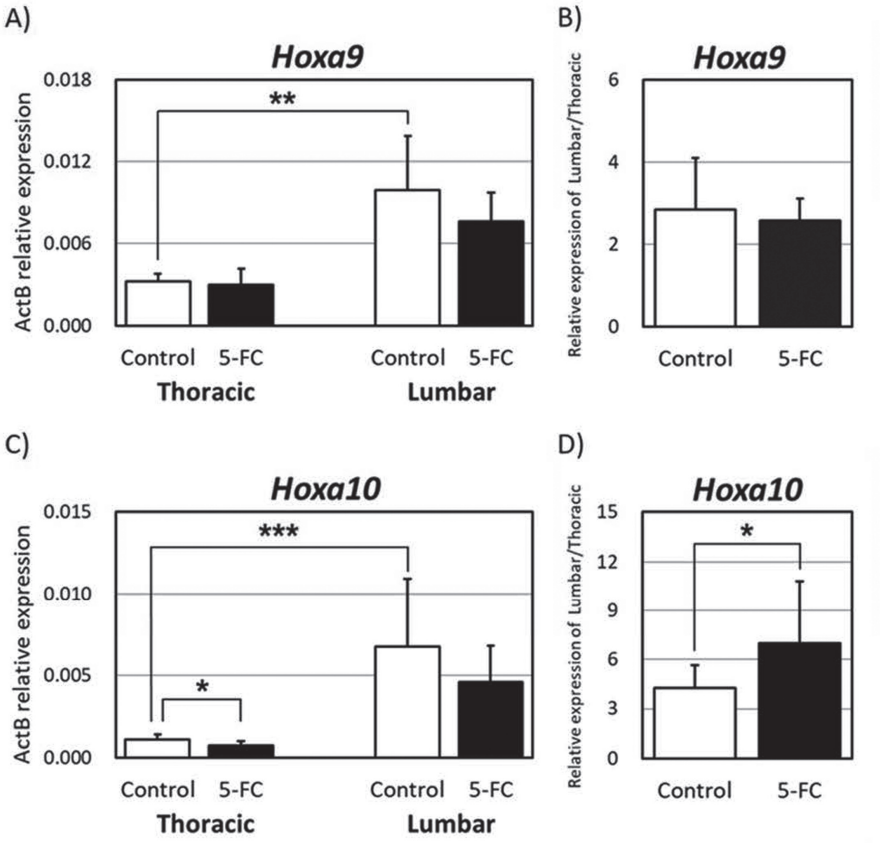

Fig. 3. Expression of Hoxa9 and Hoxa10 by thoracic and lumbar vertebrae. Expression of Hoxa9 (A) and Hoxa10 (C) in areas of thoracic and lumbar vertebrae, as shown in Fig. 2, of control and 5-FC treated rats. Ratio of expression of Hoxa9 (B) and Hoxa10 (D) in lumbar areas relative to thoracic areas in control and 5-FC treated rats. n = 6-10 (1-2 fetuses/litter). *$p < 0.05$, ***$p < 0.001$ by Aspin-Welch *t*-tests

was consistent with that determined by Alcian blue-staining (Dunwoodie *et al.*, 2002).

After determining the thoracic-lumbar boundary area based on Sox9 expression, we dissected out the cranial and caudal regions of the expected sites of TSR expression in fetuses of dams administered 5-FC on GD9, and assessed Hoxa9 and Hoxa10 expression in these dissected tissues. Hox genes play important roles in determining somite position and limb formation, with gene structure determined by the timing of expression and positioning. Hox9 genes are expressed from the thoracic vertebra to the posterior (Chen and Capecchi, 1997, 1999), whereas Hox10 genes are expressed posterior to the boundary between the thoracic and lumbar vertebrae and are involved in rib formation (Guerreiro *et al.*, 2012).

We found that expression of both Hoxa9 and Hoxa10 was significantly higher in lumbar than in thoracic vertebrae. Their expression was lower in fetuses of 5-FC treated than of control dams, especially the expression of Hoxa10 in the thoracic and lumbar regions. The mechanism by which 5-FC induces TSR was assessed by focusing on the relative expression in the lumbar and thoracic regions of each individual. These findings suggested that the gradual shift of Hoxa10 expression in boundary areas is associated with TSR. We therefore evaluated the ratio of Hoxa10 expression in the lumbar relative to the thoracic region in individual rats, finding that the expression of Hoxa10 was significantly higher in the fetuses of 5-FC treated rats than of control rats. Thus, TSRs induced by 5-FC may arise because the posteriorization of Hoxa10 expressed as a gradient cannot prevent the vertebrae from becoming thoracic at the prescribed position of thoracolumbar boundary area.

Hox genes are important for determining the body segment and the thoracic vertebrae/lumbar region, as well as being vital for the skeletal development of limbs. Animal

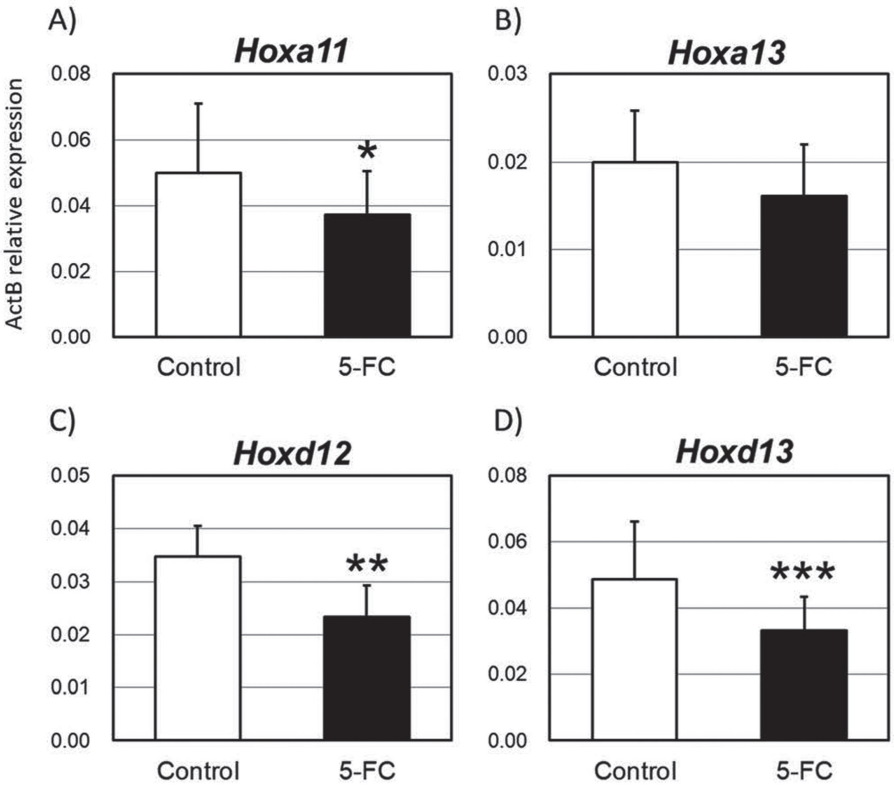

Fig. 4. Expression of Hox genes by fetal hind limbs. Levels of expression of (A) Hoxa11, (B) Hoxd12, (C) Hoxa13, and (D) Hoxd13 by fetal hind limbs of control and 5-FC treated rats. n = 21-25 (1-2 fetuses/litter). *$p < 0.05$, **$p < 0.01$ by Aspin-Welch *t*-tests.

limbs grow by extension of the axial skeleton, with later-acting Hox genes being key to the formation of normal limbs. In particular, Hox11 is expressed distal to and including the radius, Hox12 distal to and including the metacarpals, and Hox13 in the digits. Hoxa subtypes are responsible for limb formation from the proximal to the distal direction, and Hoxd from the anterior to the posterior direction (Johnson and Tabin, 1997).

Thus significant reductions in Hoxa11 and Hoxd13 expression were observed, along with reductions in Hoxa13 and Hoxd12 expression. Hox 11-13 are responsible for limb formation from the proximal to the distal, and from the anterior to the posterior, directions. We also confirmed GD20 embryos exposed to 5-FC at GD13 showed malformed limbs. The results of the present study suggest that altered expression of these Hox genes may cause the abnormal development of limbs.

Administration of 5-FC on GD9 resulted in a posterior-shift of Hoxa10 expression, along with the appearance of TSR, suggesting that altered Hoxa10 expression in the thoraco-lumbar boundary area is a mechanism of TSR formation. Furthermore, administration of 5-FC on GD13 resulted in abnormal limb morphology, along with altered Hox11-13 gene expression. Taken together, these findings indicate that the timing of 5-FC administration is associated with particular types of abnormalities, along with the disruption of particular Hox genes. TSR and digital abnormalities may represent a continuum of axis and appendicular skeletal abnormalities.

However, the mechanisms underlying TSR occurrence following the administration of toxic agents remain unclear, preventing the extrapolation of animal findings to humans and subsequent human health risk assessment. It is also necessary to examine the toxicological meaning in animal experiments.

ACKNOWLEDGMENTS

This work was supported by a grant from the Food Safety Commission, Cabinet Office, Government of Japan (Research Program for Risk Assessment Study on Food Safety; Grant Number 1607).

Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Aoyama, H., Kikuta, M., Shirasaka, N., Hojo, H., Takahashi, K.L., Shimizu, N., Harigae, M., Taguchi, F. and Teramoto, S. (2002): Historical control data on reproductive abilities and incidences of spontaneous fetal malformations in Wistar Hannover GALAS rats. Congenit. Anom. (Kyoto), **42**, 194-201.

Bi, W., Deng, J.M., Zhang, Z., Behringer, R.R. and de Crombrugghe, B. (1999): Sox9 is required for cartilage formation. Nat. Genet., **22**, 85-89.

Burke, A.C., Nelson, C.E., Morgan, B.A. and Tabin, C. (1995): Hox genes and the evolution of vertebrate axial morphology. Development, **121**, 333-346.

Chen, F. and Capecchi, M.R. (1997): Targeted mutations in hoxa-9 and hoxb-9 reveal synergistic interactions. Dev. Biol., **181**, 186-196.

Chen, F. and Capecchi, M.R. (1999): Paralogous mouse Hox genes, Hoxa9, Hoxb9, and Hoxd9, function together to control development of the mammary gland in response to pregnancy. Proc. Natl. Acad. Sci. USA, **96**, 541-546.

Chernoff, N. and Rogers, J.M. (2004): Supernumerary ribs in developmental toxicity bioassays and in human populations: incidence and biological significance. J. Toxicol. Environ. Health B Crit. Rev., **7**, 437-449.

Diasio, R.B., Lakings, D.E. and Bennett, J.E. (1978): Evidence for conversion of 5-fluorocytosine to 5-fluorouracil in humans: possible factor in 5-fluorocytosine clinical toxicity. J. Antimicrob. Agents Chemother, **14**, 903-908.

Dunwoodie, S.L., Clements, M., Sparrow, D.B., Sa, X., Conlon, R.A. and Beddington, R.S. (2002): Axial skeletal defects caused by mutation in the spondylocostal dysplasia/pudgy gene Dll3 are associated with disruption of the segmentation clock within the presomitic mesoderm. Development, **129**, 1795-1806.

Ema, M., Endoh, K., Fukushima, R., Fujii, S., Hara, H., Hirata-Koizumi, M., Hirose, A., Hojo, H., Horimoto, M., Hoshino, N., Hosokawa, Y., Imai, Y., Inada, H., Inawaka, K., Itoh, K., Katsumata, Y., Izumi, H., Kato, H., Maeda, M., Matsumoto, K., Matsuo, S., Matsuoka, T., Matsuura, I., Mineshima, H., Miwa, Y., Nakano, N., Naya, M., Noyori, H., Ohta, T., Oku, H., Ono, A., Shimizu, T., Shimomura, K., Takakura, I., Tanaka, R., Tateishi, T., Tominaga, Y., Uesugi, T., Urakawa, C., Yabe, K., Yamashita, A., Yamauchi, T. and Yokoi, R. (2014): Historical control data on developmental toxicity studies in rodents. Congenit. Anom. (Kyoto), **54**, 150-161.

Fujii, S., Yabe, K., Kariwano-Kimura, Y., Furukawa, M., Itoh, K., Matsuura, M. and Horimoto, M. (2019): Developmental toxicity of flucytosine following administration to pregnant rats at a specific time point of organogenesis. Congenit. Anom. (Kyoto), **59**, 39-42.

Guerreiro, I., Casaca, A., Nunes, A., Monteiro, S., Nóvoa, A., Ferreira, R.B., Bom, J. and Mallo, M. (2012): Regulatory role for a conserved motif adjacent to the homeodomain of Hox10 proteins. Development, **139**, 2703-2710.

Hattori, T., Müller, C., Gebhard, S., Bauer, E., Pausch, F., Schlund, B., Bösl, M.R., Hess, A., Surmann-Schmitt, C., von der Mark, H., de Crombrugghe, B. and von der Mark, K. (2010): SOX9 is a major negative regulator of cartilage vascularization, bone marrow formation and endochondral ossification. Development, **137**, 901-911.

Healy, C., Uwanogho, D. and Sharpe, P.T. (1999): Regulation and role of Sox9 in cartilage formation. Dev. Dyn., **215**, 69-78.

Huang, L., Pu, Y., Hepps, D., Danielpour, D. and Prins, G.S. (2007): Posterior Hox gene expression and differential androgen regulation in the developing and adult rat prostate lobes. Endocrinology, **148**, 1235-1245.

Hunt, P. and Krumlauf, R. (1992): Hox codes and positional specification in vertebrate embryonic axes. Annu. Rev. Cell Biol., **8**, 227-256.

Johnson, R.L. and Tabin, C.J. (1997): Molecular models for vertebrate limb development. Cell, **90**, 979-990.

Kuwagata, M., Senuma, M., Todoroki, M., Kumagai, F., Kumamoto, T. and Ogawa, T. (2018a): Induction of a thoracolumbar supernumerary rib in rat developmental toxicity studies: a short discussion on the critical window. Congenit. Anom. (Kyoto), **59**, 190-192.

Kuwagata, M., Sakai, Y., Tanaka, S., Takashima, H., Katagiri, R., Matsuoka, T., Noritake, K., Senuma, M., Shimizu, T., Hojo, H., Ibi, K., Kudo, S., Oota, T., Ube, M., Miwa, Y., Kajita, S., Uesugi, T., Yabe, K., Tateishi, T., Nakano, N., Taniguchi, T., Yamashita, A., Hirano, T., Kirihata, Y., Sakai, Y., Nishizawa, S., Fujiwara, M., Mineshima, H., Horimoto, M. and Ema, M. (2018b): Historical control data on developmental toxicity studies in rats. Congenit. Anom. (Kyoto), **59**, 125-131.

Motoyama, J., Liu, J., Mo, R., Ding, Q., Post, M. and Hui, C.C. (1998): Essential function of Gli2 and Gli3 in the formation of lung, trachea and oesophagus. Nat. Genet., **20**, 54-57.

Nakajima, A., Usui, A., Hosokai, Y., Kawasumi, Y., Abiko, K., Funayama, M. and Saito, H. (2014): The prevalence of morphological changes in the thoracolumbar spine on whole-spine computed tomographic images. Insights Imaging, **5**, 77-83.

Noritake, K., Ikeda, T., Ito, K., Miwa, Y., Senuma, M., Takashima, H., Tateishi, T., Hisada, S. and Maki, E. (2013): Study for collecting background data on Wistar Hannover [Crl:WI(Han)] rats in embryo-fetal development studies--comparative data to Sprague Dawley rats. J. Toxicol. Sci., **38**, 847-854.

Polak, A. and Scholer, H.J. (1975): Mode of action of 5-fluorocytosine and mechanisms of resistance. Chemotherapy, **21**, 113-130.

Shibata, S., Fukada, K., Imai, H., Abe, T. and Yamashita, Y. (2003): *In situ* hybridization and immunohistochemistry of versican, aggrecan and link protein, and histochemistry of hyaluronan in the developing mouse limb bud cartilage. J. Anat., **203**, 425-432.

Takeuchi, H., Takagi, T., Yoshino, T., Noda, A., Shimizu, M. and Utaka, K. (1976): Studies on toxicity of fluorocytosine (5-FC). Jpn. Pharmacol. Ther., **4**, 101-140.

Vermes, A., Guchelaar, H.J. and Dankert, J. (2000): Flucytosine: a review of its pharmacology, clinical indications, pharmacokinetics, toxicity and drug interactions. J. Antimicrob. Chemother., **46**, 171-179.

Wellik, D.M. and Capecchi, M.R. (2003): Hox10 and Hox11 genes are required to globally pattern the mammalian skeleton. Science, **301**, 363-367.