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Expression of Estrogen Receptor-α Protein in the Rat Digestive Tract

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Abstract. *The current study evaluates the expression of estrogen receptor-α (ER-α) protein in the digestive tract and other organs using immunohistochemistry in male and female intact rats. As a result, the expression of ER-α protein was intensively immunoreactive in the nuclei of squamous epithelium of the forestomach connected to the limiting ridge and the anus connected to the anorectal junction. Rat ER-α mRNA signals were also detected in the epithelium of the limiting ridge using in situ hybridization. The incidence of ER-α protein in the limiting ridge decreased with age in both males and females. The incidence of ER-α protein in the anorectal junction strongly decreased with age in males, although the incidence did not decrease with age in females. In conclusion, it was suggested that estrogen may be involved in the proliferation and differentiation of these cells in the limiting ridge of the stomach and anorectal junction of rats.*

It has been noted that estrogen controls the development and differentiation of various cells in the mammary gland and genitalia (uterus, ovary, testis, and prostate) (1). In recent reports of the digestive tract, it was clarified that sex differences are recognized in the development of cancer of the human esophagus (2, 3), stomach (4-8) and colon (4, 5, 9), and in the secretion of human gastric juice (10, 11); the expression of estrogen receptor-α (ER-α) protein was determined in human tissues such as the digestive tract (including stomach), genitalia, endocrine system and nervous system using immunohistochemistry (12).

In rats, it was reported that ER-α immunoreactivity was localized in the uterus, gastric epithelium and enteric nerve (13), and that ER-α mRNA was demonstrated in the gastric muscular layer of the normal stomach by reverse transcription-polymerase chain reaction (RT-PCR) (14). However, there are no reports on studies of the expression of ER-α protein and ER-α mRNA in the normal stomach of intact rats using immunohistochemistry and *in situ* hybridization histochemistry, respectively.

This study is the first report that the strong expression of ER-α protein has been shown in squamous epithelium of the forestomach in the limiting ridge and anus at the anorectal junction following investigations of the expression of ER-α protein in the digestive tract of rats.

Materials and Methods

Animals. The animals were intact and inbred Sprague-Dawley (SD) rats, maintained under filtered air laminar flow in the Division of Laboratory Animal Science, Research Center for Life Science Resources, Kagoshima University. The animals were given a commercial diet (CE-2; CLEA Inc., Tokyo, Japan) and tap water *ad libitum*. Room temperature was maintained at 25±2˚C and 55±10% relative humidity, with a 12 h-light/dark cycle. The use of animals in this research complied with all relevant guidelines of the Japanese government and Kagoshima University (15-18).

Necropsy and tissue preparation. At the ages of 50 (young rats; 8 males and 21 females), 100 (adult rats; 11 males and 20 females) and 300-400 days (old rats; 11 males and 21 females), animals were anesthetized with diethyl ether, exsanguinated from the abdominal aorta, and necropsied. The digestive tract and other systemic organs were removed and fixed in 10% phosphate-buffered formalin. The tissues were then dehydrated, embedded in paraffin, and sectioned. The sections were stained with hematoxylin and eosin, and examined histopathologically.

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Key Words: Rat, estrogen receptor, immunohistochemistry, *in situ* hybridization, limiting ridge, stomach and anorectal junction.

Table I. *Expression of ER-α in the epithelium of stomach in rats.*

	No. [%] of ER-α positive rats		
	Young rats (50 days of age)	Adult rats (100 days of age)	Old rats (300-400 days of age)
Limiting ridge#			
male	8/8 [100%]	7/11 [63.6%]	3/11 [27.3%]^{aa}
female	21/21 [100%]	16/20 [80.0%]^{a}	11/21 [52.4%]^{aa}
Forestomach			
male	1/8 [12.5%]	0/11 [0%]	1/11 [9.1%]
female	6/21 [28.6%]	3/20 [15%]	0/21 [0%]^{aa}
Glandular stomach			
male	2/8 [25.0%]	1/11 [9.1%]	0/11 [0%]
female	0/21 [0%]^{b}	1/20 [5%]	0/21 [0%]

#Squamous epithelium of the forestomach connected to the limiting ridge; $^{a}p<0.05$, $^{aa}p<0.01$: differs from young rats; $^{b}p<0.05$: differs from young male rats.

Table II. *Expression of ER-α in the epithelium of the digestive tract (except stomach) in rats.*

	No. [%] of ER-α positive rats		
	Young rats (50 days of age)	Adult rats (100 days of age)	Old rats (300-400 days of age)
Esophagus			
male	0/9 [0%]	0/11 [0%]	0/11 [0%]
female	1/21 [4.8%]	2/20 [10%]	5/21 [23.8%]
Small intestine			
male	0/9 [0%]	0/11 [0%]	0/11 [0%]
female	0/21 [0%]	0/20 [0%]	0/21 [0%]
Cecum			
male	0/9 [0%]	0/11 [0%]	0/11 [0%]
female	0/21 [0%]	0/20 [0%]	0/21 [0%]
Colon-Rectum			
male	0/9 [0%]	0/11 [0%]	0/11 [0%]
female	0/21 [0%]	0/20 [0%]	0/21 [0%]
Anorectal junction#			
male	9/9 [100%]	1/10 [10.0%]^{a}	1/11 [9.1%]^{a}
female	13/17 [76.5%]	14/18 [77.8%]^{b}	14/18 [77.8%]^{c}

#Epithelium of the anus connected to the anorectal junction; $^{a}p<0.01$: differs from young rats; $^{b}p<0.01$: differs from adult male rats; $^{c}p<0.01$: differs from old male rats.

Immunohistochemistry. Sections from the digestive tract and other systemic organs were examined using standard avidin-biotin complex immunoperoxidase assays. After endogenous peroxidase activity had been blocked with 1% H_2O_2 in methyl alcohol for 20 min, deparaffinized sections were pretreated in 10 mM citrate buffer (pH 6.0) by microwaves for 20 min. After rinsing in PBS, the sections were blocked with Block-Ace (Dainippon Sumitomo Pharma Co., Ltd. Osaka Japan) for 30 min. The sections were incubated overnight at 4˚C with primary antibody-diluted estrogen receptors-α (ER-α; DAKO cytomation, Glostrup Denmark, 1:200). After rinsing in PBS, the sections were incubated with biotinylated anti-mouse immunoglobulin for 30 min, then rinsed again with PBS and incubated with VECTASTAIN Elite ABC KIT (Vector Laboratories, Burlingame CA, USA) for 30 min. To visualize immunoreactivity, 3,3'-diaminobenzidine tetrachloride (DAB) containing 0.02% hydrogen peroxide was used. The sections were then washed, counter-stained, dehydrated, cleared in xylene and mounted (15-17). For ER-α, nuclear stained cells were interpreted as positive cells.

Preparation of digoxigenin (DIG)-labeled probe. The probe for *in situ* hybridization was chosen from the reported sequence of the rat genome (19, 20). The ER antisense oligonucleotide selected was complementary to nucleotides 1405-1435 (estrogen-binding domain) of the rat ER cDNA sequence as follows: 5'-ACA GGA GCT TCC CCG GGT GTT CCA TGG AGC-3'. The ER sense oligo-DNA selected corresponded to the following mRNA sequence: 5'-GCT CCA TGG AAC ACC CGG GGA AGC TCC TgT-3'. This oligo-DNA was completely homologous to the sequence for Sprague-Dawley rats. The oligoprobes were synthesized and purified by gel filtration (GENSET KK, Kyoto, Japan).

In situ hybridization. Deparaffinized stomach sections were rehydrated and pretreated with proteinase K at 37˚C for 10 min, dehydrated, air-dried, and hybridized at 37˚C overnight with DIG-labeled oligoprobes in hybridization solution (DAKO cytomation). The sections were washed twice in 0.5 x SSC (3 M NaCl, 0.3 M sodium citrate dehydrate), then reacted with alkaline phosphatase-labeled rabbit anti-DIG antibody (diluted to 1:100 in 0.1 M Tris-HCl, 0.15 M NaCl (buffer 1, pH7.5)) at 37˚C for 1 h. The sections were then reacted in the dark with 0.04% 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/0.04% 4-nitroblue tetrazolium (NBT) (DAKO cytomation) in substrate buffer. Sections were mounted without counter-staining (19-23).

Statistics. The incidence of ER-α protein expression was tested using a four-fold contingency table (24).

Results

Pathological findings. No abnormal changes were seen grossly or microscopically in the digestive tract or other systemic organs.

Immunohistochemical localization of ER-α. In the limiting ridge of the stomach, forestomach and glandular stomach, the expression of ER-α protein was detected in both males and females with immunohistochemistry (Table I). ER-α protein was detected in the nuclei of basal cells of squamous epithelium of the forestomach connected to the limiting ridge (the junction of the forestomach and glandular

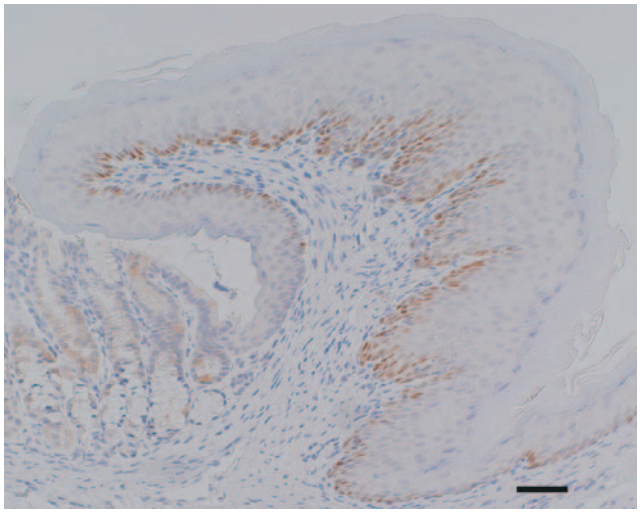

Figure 1. *Immunohistochemistry showing ER-α protein detection in the nuclei of basal cells of squamous epithelium of the forestomach connected to the limiting ridge. (Bar=50 μm)*

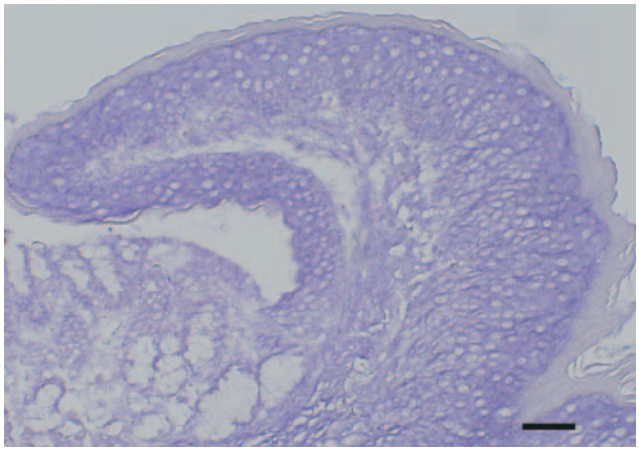

Figure 2. *In situ hybridization showing ER-α mRNA detection in epithelium of the forestomach connected to the limiting ridge. (Bar=50 μm)*

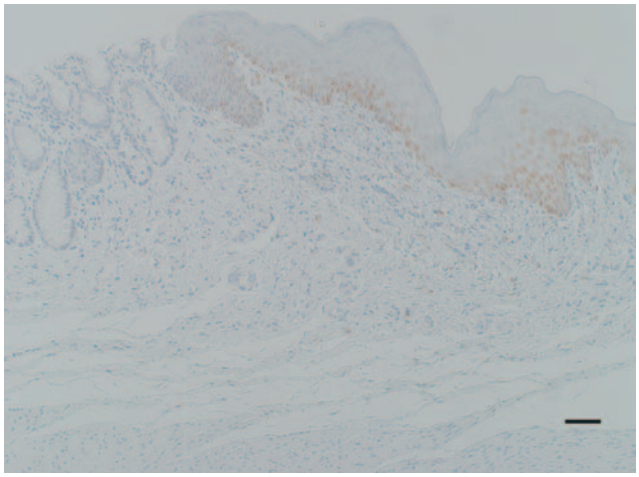

Figure 3. *Immunohistochemistry showing ER-α protein detection in the nuclei of basal cells of squamous epithelium of the anus connected to the anorectal junction. (Bar=50 μm)*

stomach) (Figure 1). Compared with young rats, the incidence of ER-α protein expression in the limiting ridge was significantly low in old male rats ($p<0.01$), and adult and old female rats ($p<0.05$ and 0.01, respectively). In the female forestomach, the incidence of ER-α protein expression was significantly low in old rats compared with young rats ($p<0.01$). In the glandular stomach of young female rats, the incidence of ER-α protein expression was significantly low compared with young male rats ($p<0.05$).

In the anorectal junction, the localization of ER-α protein was also detected in the nuclei of basal cells of squamous epithelium of the anus connected to the anorectal junction in both male and female rats (Table II) (Figure 3). The incidence of ER-α protein expression significantly decreased with age in adult and old male rats ($p<0.01$ and 0.01, respectively); however, the incidence of ER-α protein expression did not decrease with age in female rats.

In the digestive tract, ER-α protein expression was detected in the esophagi of female rats.

ER-α protein expression in other systemic organs such as the mammary gland, genitalia (uterus, ovary, vagina), endocrine system (pituitary, thyroid), nervous system (cerebrum, spinal cord, sciatic nerve), thymus, trachea, liver and bone marrow, was also detected in female rats (Table V).

In situ hybridization for ER-α mRNA in the stomach. The localization of ER-α mRNA was investigated in the stomach and uterus as a positive control of rats using *in situ* hybridization. In the myometrium, ER-α mRNA was demonstrated to be constantly positive. In the stomach, the ER-α mRNA signals were expressed in the epithelium of the forestomach connected to the limiting ridge (Figure 2). The expression of other epithelia of the forestomach was relatively weak; however, no signals were detected in the glandular stomach.

Discussion

This study is the first report that the strong expression of ER-α protein has been shown in squamous epithelium of the forestomach connected to the limiting ridge and the anus connected to the anorectal junction.

In the rat stomach, the forestomach is covered by keratinized stratified squamous epithelium, the glandular stomach is lined with simple columnar epithelium, and the main function of the forestomach is food storage (25, 26).

Table III. *Comparison of immunohistochemical methods of estrogen receptor in rats.*

Source	Strain (animal)	Fixation	Section	Inactivations	Antigen retrieval method	Primary antibody				Secondary antibody
						Supply	Clone	Dilution	Incubation	
Gejima K *et al.* (this study)	Sprague-Dawley rat	10% phosphate-buffered formalin	paraffin section	0.3% H_2O_2 in methanol, 20 min.	microwave for 20 min.	DAKO cytomation	1D5	1:200	4˚C, overnight	mouse
Ueyama T *et al.* (2002)	Wistar rat	4% paraformaldehyde in 0.1 M phosphate buffer	frozen section	3% H_2O_2 in DW, 20 min.	–	Hayashi S (1992)	–	1:10000	4˚C, 48 h	rabbit
Campbell-Thompson M *et al.* (2001)	Sprague-Dawley rat	2% paraformaldehydelysine-periodate	paraffin section	3% H_2O_2 in PBS, 10 min.	microwave for 4 intervals of 2.5 min. and trypsin digestion for 12 min. at room temperature	Greene G (1980)	–	10 μg/ml	10˚C, overnight	rabbit

Table IV. *Comparison of in situ hybridization methods of estrogen receptor mRNA in rats.*

Source	Strain (animal)	Fixation	Section	Pretreatment	Nucleotides	Hybridization	Following treatment
Gejima K *et al.* (this study)	Sprague-Dawley rat	10% phosphate-buffered formalin	paraffin section	proteinase K at 37˚C for 10 min.	1405-1435	at 37˚C overnight	rabbit anti-digoxigenin antibody, 1:100, at 37˚C for 1 h
Ueyama T *et al.* (2002)	Wistar rat	4% paraformaldehyde in 0.1 M phosphate buffer	frozen section	proteinase K at 37˚C for 15 min.	501-540 and 951-990	at 37˚C overnight	–
Campbell-Thompson M *et al.* (2001)	Sprague-Dawley rat	2% paraformaldehyde-lysine-periodate	paraffin section	proteinase K for 20 min.	46-307	at 42˚C overnight	sheep anti-digoxigenin antibody, 1:500

The forestomach is separated from the glandular stomach by the limiting ridge. The limiting ridge of the rat stomach is thought to correspond to the esophagogastric junction in humans (27). In humans, there are no reports that the expression of ER-α protein has been investigated in the esophagogastric junction, although it was reported that ER-α immunoreactivity was noted in the esophagus (12). In the expression of ER-α protein in the squamous epithelium of the anus connected to the anorectal junction, there is also a human report (28) similar to our study.

This study showed that the expression of ER-α protein in the limiting ridge of the stomach decreased with age in both males and females, and that the expression of ER-α protein in the anorectal junction decreased with age in males, although no changes with age were recognized in females. These findings strongly suggest that estrogen plays a role in the proliferation and differentiation of squamous epithelium in the forestomach of the limiting ridge and anorectal junction of rats; however, these findings also suggest that the contribution of estrogen in the proliferation and differentiation of squamous epithelium in the male and female forestomach of the limiting ridge and male anorectal junction decreases with age, and androgen may contribute to proliferation and differentiation instead of estrogen. It would be very interesting to study whether there is a correlation between the high incidence of ER-α protein expression in the limiting ridge of the rat stomach and the high incidence of chemical carcinogen-induced forestomach tumor in the mouse squamocolumnar junction corresponding to the limiting ridge of rats (27).

Table V. *Expression of ER-α in other systemic organs in female rats.*

Organs	No. [%] of ER-α positive rats		
	Young rats	Adult rats	Old rats
Ovary	10/10 [100%]	10/10 [100%]	9/10 [90%]
Oviduct	9/9 [100%]	9/9 [100%]	7/8 [88%]
Uterus			
horn	10/10 [100%]	9/9 [100%]	10/10 [100%]
cervix	10/10 [100%]	9/9 [100%]	10/10 [100%]
Vagina	10/10 [100%]	9/9 [100%]	10/10 [100%]
Mammary gland	10/10 [100%]	10/10 [100%]	10/10 [100%]
Skin (epidermis)	10/10 [100%]	1/9 [11%]**	1/10 [10%]**
Subcutaneous			
hair papilla	10/10 [100%]	7/10 [70%]	10/10 [100%]
adipose tissue	10/10 [100%]	4/10 [40%]**	1/10 [10%]**
Clitral gland	0/5 [0%]	0/4 [0%]	0/6 [0%]
Pituitary gland	10/10 [100%]	10/10 [100%]	10/10 [100%]
Adrenal gland	0/10 [0%]	0/10 [0%]	0/10 [0%]
Thyroid gland	2/5 [40%]	2/8 [25%]	0/9 [0%]*
Submandibular gland	0/5 [0%]	0/5 [0%]	0/6 [0%]
Parotid gland	0/5 [0%]	1/4 [25%]	0/6 [0%]
Lacrimal gland	0/5 [0%]	0/5 [0%]	0/6 [0%]
Harderian gland	0/5 [0%]	0/4 [0%]	0/6 [0%]
Pancreas	0/5 [0%]	0/5 [0%]	0/6 [0%]
Liver (hepatocyte)	7/10 [70%]	4/10 [40%]	5/10 [50%]
Heart	0/5 [0%]	0/5 [0%]	0/6 [0%]
Aorta	0/5 [0%]	0/5 [0%]	0/6 [0%]
Tongue	0/5 [0%]	0/5 [0%]	0/6 [0%]
Lung	1/10 [10%]	0/10 [0%]	0/10 [0%]
Trachea			
epithelium	7/10 [70%]	4/10 [40%]	7/10 [70%]
cartilage	7/10 [70%]	0/10 [0%]**	0/10 [0%]**
Kidney	5/10 [50%]	4/10 [40%]	1/10 [10%]
Urinary bladder	0/10 [0%]	1/9 [11%]	2/10 [20%]
Cerebrum	9/10 [90%]	10/10 [100%]	8/10 [80%]
Cerebellum	0/10 [0%]	0/10 [0%]	0/10 [0%]
Brain stem	1/10 [10%]	0/10 [0%]	0/10 [0%]
Spinal cord	8/10 [80%]	7/10 [70%]	7/10 [70%]
Sciatic nerve	0/5 [0%]	0/5 [0%]	0/6 [0%]
Skeletal muscle	0/5 [0%]	0/5 [0%]	0/6 [0%]
Spleen	0/5 [0%]	1/5 [20%]	0/6 [0%]
Thymus	9/10 [90%]	8/10 [80%]	7/9 [78%]
Lymph nodes	1/5 [20%]	0/5 [0%]	0/6 [0%]
Bone marrow			
sternum	2/5 [40%]	0/5 [0%]	0/6 [0%]
femur	3/5 [60%]	0/5 [0%]*	0/6 [0%]*
Eye	0/5 [0%]	0/5 [0%]	0/6 [0%]

*$p<0.05$, **$p<0.01$: differs from young rats.

In normal tissues of adult humans, Taylor and AI-Azzawi reported ER-α immunoreactivity in the goblet cells of the stomach and small intestine, and in the intestinal glands and submucosal glands of the large intestine and other systemic organs (such as genitalia, endocrine system, nervous system) with the exception of the digestive tract (12). In the normal stomachs of SD rats, Campbell-Thompson *et al.* reported that ER-α immunoreactivity was localized in fundic epithelial cells within the progenitor zone and in parietal cells throughout the glands (13). Moreover, it was reported that ER-α mRNA was detected in the human normal gastric tissue by RT-PCR (29), and in smooth muscle cells in the gastric wall of adult male rats using *in situ* hybridization (14). In this study, the expression of ER-α protein in the digestive tract, except the limiting ridge and anorectal junction, was very slight following intensively positive staining of nuclei of paraffin-embedded various cells, which was accepted as significant, similar to previous reports (12, 30-32). It is possible that the localization of ER-α protein was different from previous reports because of different immunohistochemical methods: the clone of the primary antibody, procedures such as fixation, inactivate endogenous peroxidase, antigen retrieval (Table III), and immunoblotting methods: nucleotides, anti-digoxigenin antibody and procedures such as fixation (Table IV).

In conclusion, it was suggested that estrogen may be involved in the proliferation and differentiation of these cells in the limiting ridge of the stomach and anorectal junction of rats.

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