Original Article

Progression process and safety assessment adaptation of endometrial lesions in ENU-induced 2-stage uterine carcinogenicity in a Tg-rasH2 mouse model

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Abstract: Although acotiamide hydrochloride hydrate (acotiamide-HH) has not been reported to have genotoxic findings in any of the genotoxicity studies or treatment-related toxicological findings in reproductive and developmental studies, suspicious uterine tumorigenesis was observed in the results of a long-term rat carcinogenicity study. To clarify the uterine tumorigenesis of acotiamide-HH, we performed a 2-stage uterine carcinogenicity model in the transgenic rasH2 mouse initiated by N-Ethyl-N-nitrosourea (ENU). This model facilitated the short-term detection of uterine carcinogenic potential, and it appears to be a very useful testing method for assessing the safety of chemicals that may affect uterine tumorigenesis. However, there have not been many reports on this model, and accumulation of case studies using this model is recommended to support its usability. In this study, we performed this carcinogenesis model to not only confirm uterine tumorigenesis of acotiamide-HH but also to confirm the reliability of the model. The results of this study revealed that the endometrial adenocarcinoma found in the long-term rat carcinogenicity study possibly arose spontaneously. Also, we confirmed early induction of a uterine tumor as in previous reports and confirmed that 26 weeks is the appropriate treatment period for this rasH2 mouse model according to time-course observations of uterine tumor development. (DOI: 10.1293/tox.2017-0035; J Toxicol Pathol 2018; 31: 35–41)

Key words: acotiamide-HH, endometrial adenocarcinoma, carcinogenicity, rasH2 mouse

Introduction

Acofide Tablet (generic name: acotiamide hydrochloride hydrate [acotiamide-HH]) is an acetylcholine esterase (AChE) inhibitor discovered by Zeria Pharmaceutical Co., Ltd., and it was the first drug in the world for functional dyspepsia. Fifty animals were assigned to each group in a 104-week rat carcinogenicity study, and the incidence of endometrial adenocarcinoma was 1, 5, 8, and 5 in the control, 200 mg/kg, 600 mg/kg and 2,000 mg/kg groups, respectively. A significant increase of endometrial adenocarcinoma was shown only in 600 mg/kg treatment group[1]. Acotiamide-HH resulted in no observed increases in the incidence of related pre-neoplasm, such as atypical endometrial hyperplasia and endometrial adenoma in rat carcinogenicity studies[2]. No genotoxic findings in any of the genotoxicity studies, and no treatment-related toxicological findings in reproductive and developmental studies suggested that the endometrial adenocarcinoma found in the rat carcinogenicity study possibly arose spontaneously at all doses[2]. We performed a uterotrophic bioassay using immature rats, as mentioned in OECD Guideline 440, to confirm the effect of the sex hormone environment, and no effect of the sex hormone environment was observed in this study[3]. However, the incidence of endometrial adenocarcinoma in the rat carcinogenicity study deviated from the background data of the conducting facility. Therefore, we considered that an additional study was necessary to clarify the causal relationship between the endometrial adenocarcinoma and acotiamide-HH. Only two models have been established for corpus uteri cancer. The first is an ENNG-induced two-stage carcinogenicity model in Donryu rats[4], and the second is an ENU-induced

two-stage uterine carcinogenicity model in Tg-rasH2 mice[5]. CB6F1-Tg-rasH2 mice were originally established by Saitoh *et al.* at CIEA. This Tg-rasH2 mouse carries the human c-Ha-ras gene with its own promoter region, which encodes a prototype c-Ha-ras gene product, p21, that has no capacity for transforming NIH3T3 cells. Approximately 5 or 6 copies of this human c-Ha-ras gene are integrated into the genome of each Tg mouse in a tandem array[6]. Assessment of the carcinogenetic risk for humans is one of the most important studies during the development of new pharmaceuticals, and much discussion occurs regarding the methodology of carcinogenicity studies. Recently, the US, EU, and Japan have agreed, via the ICH S1B Guidelines, that the rat should be recommended as an ideal species for carcinogenicity studies and that the Tg-rasH2 and p53+/− transgenic mouse models are appropriate substitutes[7]. Therefore, we conducted an ENU-induced 2-stage uterine carcinogenicity study in a Tg-rasH2 mouse model to confirm whether acotiamide-HH has carcinogenic potential with respect to endometrial adenocarcinoma or not. For this ENU-induced 2-stage uterine carcinogenicity study in the Tg-rasH2 mouse model, 26-week is an appropriate administration period in general. According to the study design reported by Watanabe *et al.* we injected ENU intraperitoneally on time at 1 week before start 26 weeks of repeated administration of acotiamide-HH and evaluated the variety and frequency of proliferative legions in the uterus. Understanding the progression process and time course of each proliferative legion in the uterus is very important if acotiamide-HH has a potential risk of modifying the enhancement and/or promotion of tumorigenesis in the uterus. Therefore, we set not only a main group but also a satellite group to observe the time course and progression process of proliferative legions in the uterus.

Materials and Methods

Chemicals

N-Nitroso-N-ethylurea (ENU) was purchased from Sigma-Aldrich Corporation Co. (St. Louis, MO, USA), and acotiamide hydrochloride hydrate (acotiamide-HH) was purchased from Zeria pharmaceutical Co., Ltd. (Saitama, Japan). Methyl cellulose was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

Animals

One hundred and forty 6-week-old female transgenic (Tg) mice carrying a human prototype c-Ha-ras gene (Tg-rasH2) were obtained from CLEA Japan Inc. (Tokyo, Japan) for the ENU-induced two-stage carcinogenicity study. The animal room was maintained at 21.0 to 24.5°C with a relative humidity of 46–64%, air ventilation was set at more than 10 air changes per hour, and there was 12 h of lighting (7:00–19:00) per day. Healthy animals with no abnormalities were used for studies after a 4-week acclimation period, with each animal housed in a plastic cage with soft bedding (Hara Shoten, Co., Ltd., Aichi. Japan). CRF-1 feed pellets (Oriental Yeast Co. Ltd, Tokyo, Japan) and tap water were available to mice ad libitum.

Experimental design

This study was conducted in accordance with the rasH2 mouse model previously reported by Watanabe *et al*[4]. Briefly, 120 mg/kg ENU was injected intraperitoneally on the first day of the study. Test compound administration by gavage was then started 1 week after ENU injection. Mice were separated into three groups: 0.5 w/v% methyl cellulose (MC, Wako Pure Chemical Industries, Ltd.) treatment (control), acotiamide-HH 2,000 mg/kg treatment (acotiamide-HH), and satellite. Thirty-six Tg-rasH2 mice were assigned to the satellite group in order to evaluate the time course of uterine endometrium proliferative lesion development, and four or five animals were euthanized under deep anesthesia in weeks 13, 15, 17, 19, 22, 23, and 24. Each mouse underwent necropsy and histopathological examination in order to survey the time course of the uterine tumor development. The time points at which the mice were euthanized were determined based on a previous report by Watanabe *et al.*[5].

In the main study, 50 Tg-rasH2 mice were each assigned to control and acotiamide-HH groups. The duration of treatment with MC or acotiamide-HH was determined to be a period of 26 weeks based on the histological observations of the satellite group. Related to 4 remained Tg-rasH2 mice, 2 Tg-rasH2 mice were used for microbiological monitoring for the animal room, and the other 2 animals were excluded from this study. The dosage of acotiamide-HH, 2,000 mg/kg, was set using the Maximum Tolerated Dose (MTD) during a 2-week repeated dose-finding study in non-Tg mice (data not shown).

Regarding observation parameters, clinical observations were performed daily, and body weights were measured once a week until week 12 and then twice a week from week 13 to the day of necropsy. Food consumption was measured weekly, and the mean consumption volume was calculated daily. All surviving animals were euthanized by exsanguination from the posterior vena cava under diethyl ether deep anesthesia and subjected to gross observation of all organs. Organs and tissue were excised during necropsy and fixed in neutral-buffered 10% formalin solution. Organ weights were measured for the uterus and ovaries (including oviducts) in all surviving animals. In histopathological examinations, both sides of the uterine horns were cut into cross section at 5-mm intervals, and each section was prepared at a 2-micrometer thickness or was cut into as many sections as possible if tumor formation inhibited cutting. These sections were embedded in paraffin, and 8 to 23 pieces of sections were obtained for each animal. The resulting sections were stained with hematoxylin-eosin (HE) and then examined by a light microscopy.

The endometrial proliferation criteria were as follows: presence of multiple cystic lumens with a cuboidal epithelium indicated cystic endometrial hyperplasia, atypical epithelial proliferation to the lumen located only in the lamina propria (and not the muscle layer) indicated atypical hyperplasia, and atypical epithelial proliferation into the

lamina propria and muscle layer indicated adenocarcinoma. The pathological findings were determined by a pathologist certified by the Japanese Society of Toxicologic Pathology. Moreover, all findings and diagnoses were reviewed by a second pathologist who was a certified toxicological pathologist of the Japanese Society of Toxicologic Pathology.

These studies were conducted according to the standards of the DIMS Institute of Medical Science, Inc., Standard for Carcinogenicity Study using Genetically Modified Laboratory Animals (in Japanese) (September 2, 2005) and Rules on Management and Handling of Genetically Modified Laboratory Animals (in Japanese) (April 1, 2007) of DIMS Institute of Medical Science, Inc., Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notice No. 88 of the Ministry of Environment dated April 28, 2006), and Standards for Care and Use of Laboratory Animals of DIMS Institute of Medical Science, Inc. (February 9, 2012).

Statistical analysis

Bartlett test was used to analyze body weight in the dose-finding study with 5% set as the significance level. Since the result was equal variance, a two-sided Dunnett's test was also performed. For the ENU-induced 2-stage uterine carcinogenicity study, two-sided F-tests were conducted to analyze the mean value differences in body weight, food consumption, and organ weight of the control and acotiamide-HH groups. Two-sided Student's t-tests were used in cases of equal variance, and two-sided Welch's tests were used in cases of unequal variance. In the histopathological examinations, the two-sided Student's t-test was conducted owing to equal variances. Two-sided Fisher's exact probability tests were used for incidence difference for gross findings and histopathological examinations, and two-sided Wilcoxon rank-sum tests were used for graded pathological data.

Results

The time course of the histopathological findings in the satellite group are presented in Table 1. One animal was euthanized after observation of decreased locomotor activity at 12 weeks after ENU injection. Additionally, one animal each at weeks 13, 17, and 21 after ENU injection was euthanized for the same reason. Atypical endometrial hyperplasia and cystic endometrial hyperplasia were observed in these animals, and hemangiosarcoma was observed in the animal euthanized at 21 weeks after ENU injection. In some of the remaining animals, both atypical endometrial hyperplasia and cystic endometrial hyperplasia were observed from 13 weeks after ENU injection, with atypical endometrial hyperplasia observed from 23 weeks and cystic endometrial hyperplasia observed from 17 weeks after ENU injection in all animals. Slight to severe endometrial hyperplasia was observed in this group (Fig. 1), but no endometrial adenocarcinoma was observed. Occasionally, hemangioma and hemangiosarcoma were observed after ENU injection in the

Tg-rasH2 mice. On the basis of the results obtained, the timing of necropsy for the control and acotiamide-HH groups was set at 26 weeks after ENU treatment.

In the control and acotiamide-HH groups, animal death and/or morbidity were observed from week 12 in the control group and from week 8 in the acotiamide-HH group after ENU treatment, with the number of pre-end date deaths being 19 and 24, respectively. The numbers of surviving animals in the control and acotiamide-HH groups were 31 and 26, respectively, giving survival rates of 62% and 52% (Fig. 2). The body weights of both groups increased satisfactorily until week 26, although slightly fewer surviving animals were observed in the acotiamide-HH group by the end of the treatment period, and the food consumption levels of both groups were not significantly different during the experimental period (Fig. 3 and 4). In macroscopic observations, characteristic findings such as enlargement of the uterine horn were observed in both groups, but there were no differences in the incidence or the degree of the lesions between the groups. Furthermore, there was no significant difference in the absolute and the relative organ weights in the uterus and ovaries between groups (Table 2). Uterine histopathological results are shown in Table 3. Atypical hyperplasia and cystic endometrial hyperplasia were observed in many animals from both groups, with only a few animals from each group having normal morphology. Endometrial adenocarcinoma was found in five animals in each group (Fig. 5). Endometrial stromal polyps were observed in eight and nine animals in the control and acotiamide-HH groups, respectively. Hemangioma and hemangiosarcoma were also observed in this study, but no statistically significant differences between the control and acotiamide-HH groups were identified.

Discussion

Herein, we evaluated the time course of endometrial adenocarcinoma carcinogenesis in an ENU-induced 2-stage uterine carcinogenicity Tg-rasH2 mouse model. A 22-weeks observation period was set in the report of Watanabe *et al.*, but we set a 26-week observation period in our study. During our observations the satellite group, atypical hyperplasia was observed from week 13 to week 24, and it increased in size with time. However, endometrial adenocarcinoma was not observed. Therefore, we determined that at least 26 weeks were required after ENU treatment to observe stable endometrial adenocarcinoma in the 2-stage Tg-rasH2 mouse model. Regarding other abnormalities in the uterus, cystic endometrial hyperplasia was known to be a spontaneous finding in Tg-rasH2 mice, while endometrial stromal polyps, hemangioma, and hemangiosarcoma were considered dependent on ENU treatment, since it has been reported that they have been induced by MNU treatment[8].

Currently, the animal models capable of evaluating the potential risk of chemicals with respect to endometrial adenocarcinoma carcinogenesis consist of N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG)-induced endometrial adeno-

Table 1. Time Course of Histopathological Findings in the Satellite Group

Treatment			Week at sacrifice [a]	No. of animals	Uterus			
ENU	Group	Dose (mg/kg)			Cystic endometrial hyperplasia	Atypical hyperplasia	Hemangioma	Hemangiosarcoma
+	Acotiamide-HH	2,000	13	4	2 (50)	3 (75)	0	0
			15	5	4 (80)	4 (80)	1 (20)	2 (40)
			17	5	5 (100)	4 (80)	0	2 (40)
			19	5	5 (100)	4 (80)	0	0
			22	5	5 (100)	4 (80)	1 (20)	1 (20)
			23	4	4 (100)	4 (100)	4 (100)	1 (25)
			24	4	4 (100)	4 (100)	0	1 (25)
			MS[b]	4	4 (100)	4 (100)	0	1 (25)

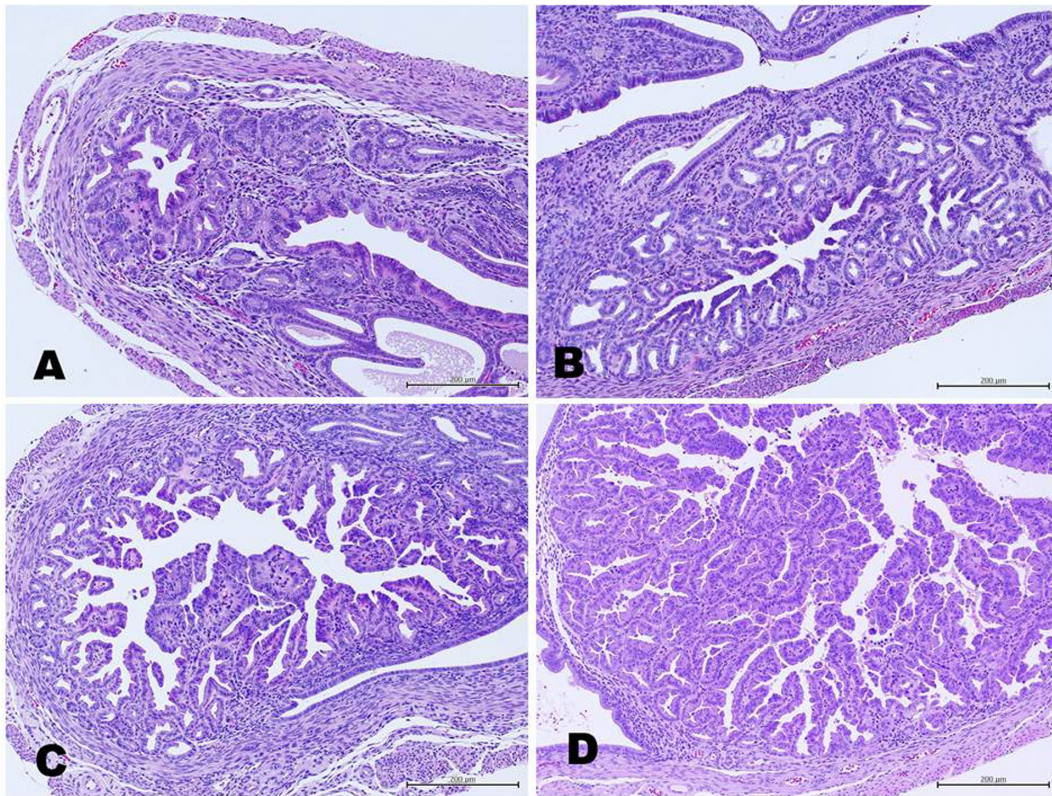[a]After ENU treatment. [b]Sacrificed moribund during the study.

Fig. 1. Histopathological findings of the uterus in the satellite group. Endometrial atypical hyperplasia is shown, HE stain, ×100. A: Slight hyperplasia. B: Mild hyperplasia. C: Moderate hyperplasia. D: Severe hyperplasia.

Table 2. Organ Weights of the Ovaries and Uterus

Treatment		Dose (mg/kg)	No. examined	Bodyweight (g)	Ovaries(g)	Uterus(g)
ENU	Group					
+	Control	0	31	22.59 ± 1.63	0.02636 ± 0.01379 (0.11572 ± 0.05561)	0.3234 ± 0.1159 (1.4245 ± 0.4376)
+	Acotiamide-HH	2,000	26	22.48 ± 2.19	0.02717 ± 0.02326 (0.11876 ± 0.09678)	0.3387 ± 0.1444 (1.4877 ± 0.6069)

Mean ± SD. Relative organ weights are shown in parentheses. *Significantly different from Control group at P<0.05. ** Significantly different from Control group at P<0.01.
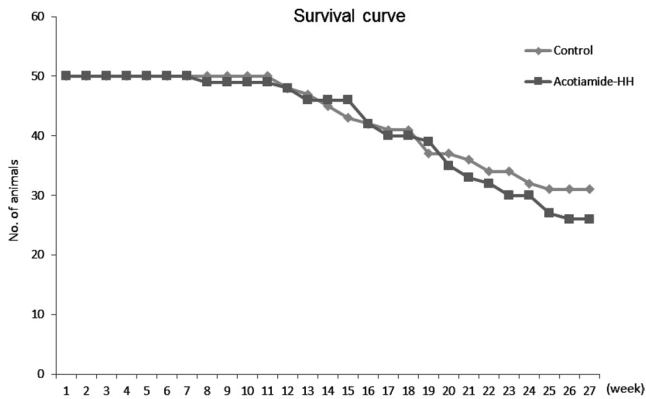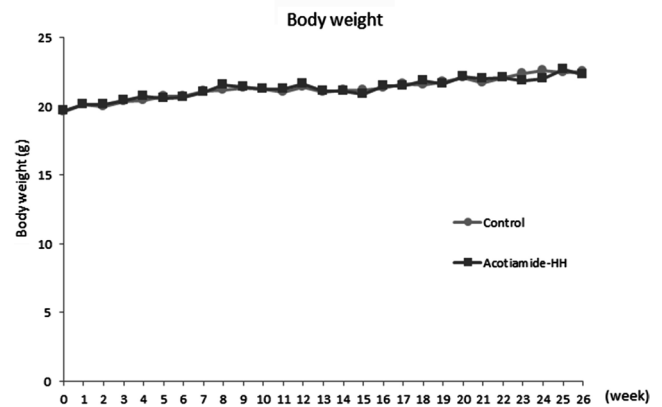
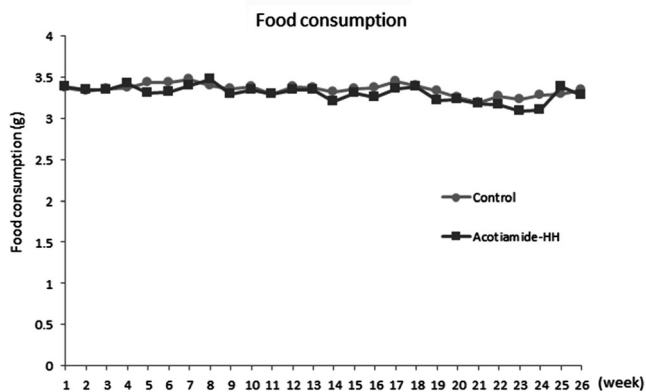**Fig. 2.** Numbers of survival animals

Fig. 3. Body weight change

Fig. 4. Food consumption

Table 3. Histopathological Findings of the uterus

Group	Control	Acotiamide-HH
ENU	+	+
Dose (mg/kg)	0	2,000
No. of animals	50	50
Histopathological findings		
No remarkable changes	3	2
Endometrial adenocarcinoma	5	5
Endometrial atypical hyperplasia	40	43
Cystic endometrial hyperplasia	39	40
Endometrial stromal polyp	8	9
Hemangioma	17	20
Hemangiosarcoma	4	5
Congestion	2	0
Hemorrhage	0	1
Necrosis, focal	1	0
Thrombosis	0	1
Histiocytic sarcoma	0	1

Data were analyzed statistically by two-sided Fisher's probability tests. There were no significant differences in incidence/severity of any findings between the control and acotiamide-HH groups.

carcinoma in the Donryu rat and the 2-stage endometrial adenocarcinoma transgenic mouse model. The Donryu rat is well established as a strain that has a high incidence of spontaneous endometrial adenocarcinoma[9]. It has been pointed out that estrogen plays an important role in the development of endometrial adenocarcinoma, and 20% of Donryu rats showed persistent estrus at 5 months of age; 90% of Donryu rats showed persistent estrus at 10 months of age. This is because Donryu rats develop endometrial adenocarcinoma spontaneously with high incidence[10]. In addition, it has been reported that intrauterus administration of ENNG can induce endometrial adenocarcinoma with high incidence in Donryu rats[4]. Therefore, we considered conducting the model using Donryu rats. However, it was very difficult to obtain sufficient numbers of Donryu rats to conduct this carcinogenicity study, and this model needs ENNG to be treated by intrauterine administration, which requires a special technique. From this standpoint, we thought that it is difficult to conduct the Donryu rat model.

The rasH2 and p53+/− mouse models are well-established short-term alternatives to the 2-year rodent bioassay, and the Tg-rasH2 mouse was accepted as an alternative model to the 2-year bioassay for both genotoxic and non-

genotoxic substances for the evaluation of potential carcinogenic risks by the PMDA, EMA, and FDA at the ILSI/HESI workshop in 2003[11]. In general, p53+/− CBA mice are used for genotoxic molecules, and Tg-rasH2 mice are used for non-genotoxic molecules[12]. In the p53+/− mouse model, only endometrial stromal polyps, endometrial stromal sarcoma, endometrial atypical hyperplasia, and endometrial hyperplasia have been reported, with the key absence of endometrial adenocarcinoma[13]. It has been found that dietary administration of 2.5 ppm of ethinylestradiol (EE) after ENU treatment could enhance the development of endometrial stromal sarcoma, endometrial atypical hyperplasia, and endometrial adenocarcinoma, but these phenomena were not observed in the absence of EE[14]. Also, it was reported that EE inhibited induction of uterine carcinogenesis in the Tg-rasH2 mouse[15]. Regarding acotiamide-HH, no genotoxicity findings and no sex hormone imbalances were observed in
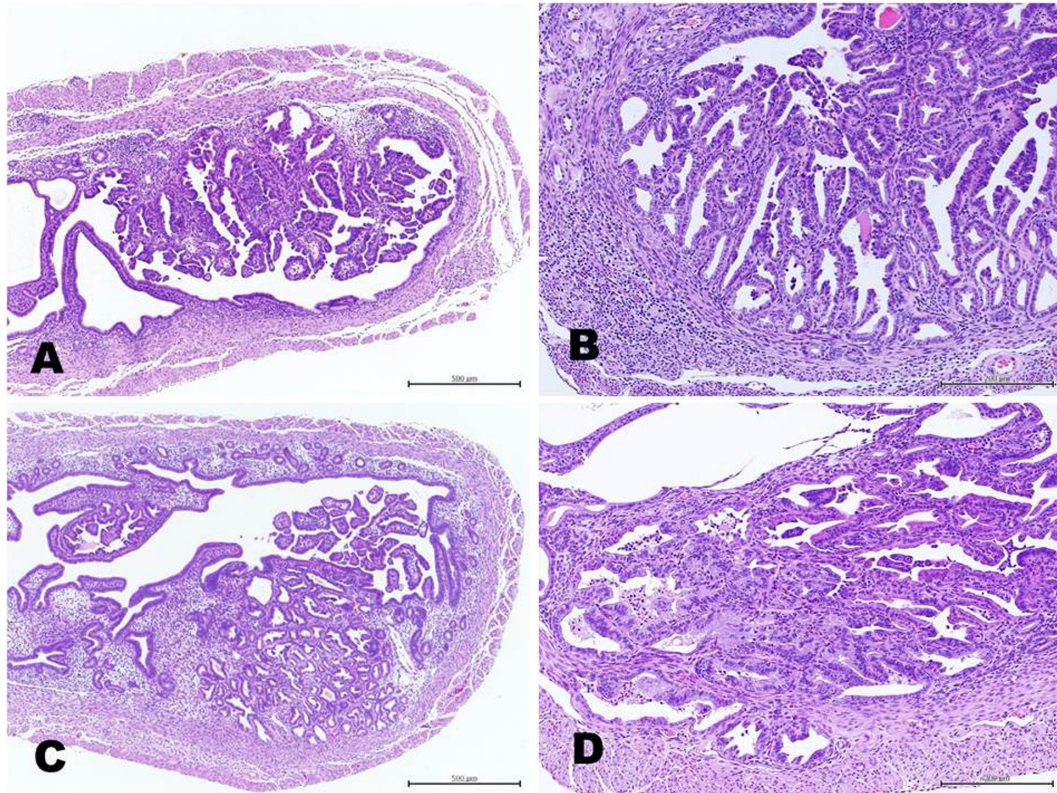
Fig. 5. Histopathological findings in the uterus in the main study. HE stain ×100. A: Focal endometrial atypical hyperplasia (control group). B: Endometrial adenocarcinoma invaded into muscle layer (control group). C: Endometrial atypical hyperplasia (acotiamide-HH group). D: Endometrial adenocarcinoma with papillary proliferation and invasion into muscle layer (acotiamide-HH group).

previous genotoxicity studies and uterotrophic bioassays in rats[3]. Therefore, it was considered that it is appropriate to use this model to evaluate the potential risk of acotiamide-HH with respect to endometrial adenocarcinoma carcinogenesis. Watanabe *et al.* reported that intraperitoneal injection of 120 mg/kg ENU in the Tg-rasH2 mouse could stably induce a high incidence of endometrial adenocarcinoma within a short period of time[5]. Given this, the ENU-induced 2-stage carcinogenicity Tg-rasH2 mouse model was considered the appropriate animal model to evaluate the potential risk of acotiamide-HH with respect to endometrial adenocarcinoma carcinogenesis. To ensure high study accuracy, we assigned 50 Tg-rasH2 mice to each group. No statistically significant differences were observed in survival rate, body weight, food consumption, or the absolute and relative organ weights of the uterus and ovaries. All findings regarding tissues and organs included unscheduled necropsy. Regarding the histopathological findings, such as atypical hyperplasia and endometrial adenocarcinoma in dead animals and unscheduled necropsies, no statistically significant differences between the two groups were observed. These results indicate that acotiamide-HH has no potential for increasing endometrial adenocarcinoma incidence in an ENU-induced 2-stage uterine carcinogenicity study.

Recently, endometrial adenocarcinoma has become one of the most common malignant tumors in women, and it has been reported that sex hormone imbalances are related to tumorigenesis[16]. Indeed, it has been reported that the incidence of endometrial adenocarcinoma in women who have received estrogen treatments was 4.5 times higher than women who had not received them, and it has been established that estrogen plays an important role in the progression of endometrial adenocarcinoma[17]. Acotiamide-HH had no effects on the sex hormone environment in reproductive and development studies. Furthermore, it was shown not to induce any effects on the sex hormone environment in uterine bioassays using immature rats[3]. These results support the conclusion that acotiamide-HH has no carcinogenic potential with respect to endometrial adenocarcinoma in the Tg-rasH2 mouse model and that 26 weeks is an appropriate administration period for this ENU-induced 2-stage uterine carcinogenicity model in the Tg-rasH2 mouse according to our time-course observations of uterine tumor development.

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