

ORIGINAL ARTICLE

Moderate transgenic expression of CBR1 is not sufficient to prolong life expectancy in an SV40 T-antigen-induced gynecological carcinogenesis mouse model

Yuka Kadonosawa¹⁾, Minako Yokoyama¹⁾, Yota Tatara²⁾, Ichiro Miyoshi³⁾,
Yoshihiko Araki⁴⁾, Yoshihito Yokoyama¹⁾ and Hodaka Fujii⁵⁾

Abstract

Background: High expression of carbonyl reductase 1 (CBR1) in ovarian cancer inhibits tumor growth and metastasis, although its long-term antitumor effects have not been investigated in detail.

Methods: Transgenic mice (Tg-mice) overexpressing the mouse *Cbr1* (CAG-*mCbr1*) transgene (Tg) (CAG-*mCbr1* Tg-mice) were crossed with carcinogenesis model mice (mouse oviduct-specific glycoprotein (*mOvgp1*)-T-antigen (TAg) Tg-mice), which overexpress simian virus 40 (SV40) TAg in reproductive organs, and the resulting individuals were evaluated. Survival was evaluated with a cutoff of 24 weeks. Indicators of tumor size (testicular diameter in males, abdominal circumference in females) and body weights were evaluated at 12–13 weeks.

Results: There were no significant differences in survival between CAG-*mCbr1*/*mOvgp1*-TAg double Tg (dTg)-mice and *mOvgp1*-TAg Tg-mice ($P = 0.992$). Male CAG-*mCbr1*/*mOvgp1*-TAg dTg-mice tended to have higher survival rates than in *mOvgp1*-TAg Tg-mice. Although, female *mOvgp1*-TAg Tg-mice tended to have higher survival rates than for CAG-*mCbr1*/*mOvgp1*-TAg dTg-mice, female body weights and male testicular diameter tended to be larger in *mOvgp1*-TAg Tg-mice than in WT mice.

Conclusions: The lack of significant differences may be attributed to the strong carcinogenic effect of SV40 T-antigen. Although whether CBR1 has antitumor effects was not established, it may be involved in tumor growth rather than in metastasis.

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Key words: carbonyl reductase 1; reproductive organs; crossbreeding experiment; transgenic mice.

Introduction

Carbonyl reductase 1 (CBR1) is a NADPH-dependent monomeric cytosolic enzyme expressed in the skin, intestine, liver, kidney, and ovary¹⁾, with broad specificity for carbonyl compounds²⁾. CBR1 reduces carbonyl compounds such as anthracyclines, daunorubicin, doxorubicin, and prostaglandins¹⁾. The expression level of CBR1 regulates the malignant potential of cancer cells. Downregulation of CBR1, which is associated with decreased expression of E-cadherin and activation

of matrix metalloproteinases, promotes cell proliferation and tumorigenesis in ovarian, cervical, and endometrial cancers *in vivo*^{3–5)}. In previous reports, we showed that ovarian cancers with low CBR1 expression have a worse prognosis than those with high CBR1 expression³⁾. Additionally, decreased expression of CBR1 promotes proliferation and metastasis⁴⁾, whereas increased expression inhibits ovarian cancer cell proliferation⁵⁾.

The effects of CBR1 have been examined *in vitro* and mostly in piecemeal studies, and much

¹⁾ Department of Obstetrics and Gynecology, Hirosaki University, Graduate School of Medicine

²⁾ Department of Stress Response Science, Center for Advanced Medical Research, Hirosaki University Graduate School of Medicine

³⁾ Institute for Animal Experimentation, Tohoku University Graduate School of Medicine

⁴⁾ Division of Microbiology, Department of Pathology and Microbiology, Nihon University School of Medicine

⁵⁾ Department of Biochemistry and Genome Biology, Hirosaki University Graduate School of Medicine

Correspondence: M. Yokoyama

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✉ : Minako Yokoyama Department of Obstetrics and Gynecology, Hirosaki University, Graduate School of Medicine

E-mail: mnk0704@hirosaki-u.ac.jp

remains unknown regarding the long-term antitumor effects of CBR1. We previously generated CAG-*mCbr1* transgenic mice (CAG-*mCbr1* Tg-mice)⁶⁾. The Tg-mice were crossed with reproductive tract tumor model mice to evaluate the inhibitory effects of CBR1 on carcinogenesis. Here, the *mOvgpl*-TAG Tg-mouse⁷⁾, which overexpresses SV40 T-antigen (TAG) in reproductive organs, was used as the reproductive tract tumor model mice. In this study, we evaluated the tumor size and survival rate of the mice obtained from the crosses and investigated the long-term antitumor effects of CBR1.

Materials and methods

Mouse strains

CAG-*mCbr1* Tg-mice were described previously⁶⁾ and express *mCbr1* under the control of the CAG promoter. The Tg-mouse strain used in this study has the CAG-*mCbr1* transgene (Tg) integrated into the X chromosome and expresses the *mCbr1* Tg in multiple tissues including reproductive organs. The *mOvgpl*-TAG Tg-mouse, which overexpresses SV40 TAG in reproductive organs, was described previously⁷⁾. In this model, mice develop reproductive tract tumors starting at 5 weeks of age. Male Tg-mice also develop tumors in reproductive organs⁸⁾. Because female *mOvgpl*-TAG Tg-mice develop gynecological cancers⁷⁾, female CAG-*mCbr1* Tg-mice were mated with male *mOvgpl*-TAG Tg-mice.

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University (4111) and Hirosaki University (M19024). This study conformed to the ARRIVE guidelines. All methods were followed in accordance with relevant guidelines and regulations.

Genotyping

To genotype the mice, gDNA was extracted from the tail of each mouse using DNeasy Blood & Tissue Kits (QIAGEN, Venlo, Netherlands) and subjected to PCR using KOD FX (Toyobo Life Science, Osaka, Japan). A region of CAG-*mCbr1* Tg was amplified using the following primer pair: 5'-gccttttatggtaatcgtgcgagaggg-3' and 5'-gctccttgacagacatctcgggt-3'. A region of *mOvgpl*-TAG Tg was amplified using the following primer pair: 5'-gaaaatggaagatggagtaaa-3' and 5'-aatagcaaagcaagcaagagt-3'. The amplification protocol for detection of CAG-*mCbr1* Tg consisted of initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 98°C for 10 s, annealing at 66°C for 30 s, and extension at 72°C for 1 min. The amplification protocol for detection of *mOvgpl*-TAG Tg consisted of initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 30 s, and extension at 68°C for 1 min.

Survival analysis

Mice born from the crosses were genotyped for classification into four groups: mice retaining both CAG-*mCbr1* and *mOvgpl*-TAG Tgs [CAG-*mCbr1*/*mOvgpl*-TAG double Tg (dTg)-mice], mice retaining either CAG-*mCbr1* or *mOvgpl*-TAG Tg (CAG-*mCbr1* Tg-mice or *mOvgpl*-TAG Tg-mice, respectively), and mice retaining neither Tg [wild-type (WT) mice]. Among them, CAG-*mCbr1* Tg-mice showed no difference in survival compared with WT mice. The survival of CAG-*mCbr1*/*mOvgpl*-TAG dTg-mice and *mOvgpl*-TAG Tg-mice was compared. Survival days were counted from birth to 24 weeks. Differences between the two groups were determined by the log rank test, and $P < 0.05$ was considered statistically significant. All statistical analyses were performed using IBM SPSS ver. 29 statistical software (IBM, Chicago, IL, USA).

Analysis of mice

Body weights and indicators of tumor size (testicular diameter in males and abdominal circumference in females) were measured in 12–13-week-old mice. Differences between CAG-*mCbr1*/*mOvgpl*-TAG dTg-mice ($n = 10$), *mOvgpl*-TAG Tg-mice ($n = 10$), and WT mice ($n = 10$) were determined by one-way ANOVA followed by Tukey's test. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using IBM SPSS ver. 29 statistics software (IBM).

Protein extraction and mass spectrometry

After obtaining body weights and tumor diameter measurements, mice were euthanized, and testes and epididymis were removed. Each organ was homogenized on ice in radioimmuno-precipitation (RIPA) buffer (catalog #182-02451, Fujifilm Wako, Osaka, Japan) containing protease inhibitors (cOmplete Tablets, Mini EDTA-free EASY pack, catalog #4693159001, Roche, Basel, Switzerland). Label-free whole-cell proteome analysis was performed as previously described⁶⁾. Briefly, the amounts of protein in the cell lysate were measured by the bicinchoninic acid (BCA) method. Cell lysates containing 100 μ g and 50 μ g total protein from testes and epididymis, respectively, were precipitated with acetone. The precipitate was then denatured with 50% trifluoroethanol. Disulfide bonds were reduced with dithiothreitol, alkylated with iodoacetamide, and digested with trypsin. After desalting and purification of the resulting peptides, LC-MS/MS was performed using a nanoLC system (Eksigent 400, AB Sciex) connected online to a mass spectrometer (TripleTOF 6600, AB Sciex). DIA-NN software version 1.8.1⁹⁾ was used to extract quantitative data for proteins from the SWATH runs with a library-free workflow. Principal component analysis of the proteome data identified that there were no outlier proteomes in any of the

samples. Because the organs used show endogenous CBR1 expression, proteins whose expression covaries with CBR1 were subjected to Spearman rank correlation analysis (R software) to capture proteomic variation resulting from the transgenic expression of CBR1. Differences between CAG-*mCbr1*/*mOvgpl*-TAG dTg-mice ($n = 10$), *mOvgpl*-TAG Tg-mice ($n = 10$), and WT mice ($n = 10$) were determined by one-way ANOVA and post hoc test and Wilcoxon rank sum test. $P < 0.05$ was considered statistically significant.

Results

Survival analysis

Survival was compared between the CAG-*mCbr1*/*mOvgpl*-TAG dTg-mice group, which included 61 mice (30 males vs. 31 females), and the *mOvgpl*-TAG Tg-mice group, which included 58 mice (29 males vs. 29 females). The results and the survival curves are shown in Figure 1. There was no significant difference between the two groups ($P = 0.992$). There was no significant difference in survival between the two groups both in male and female mice ($P = 0.397$ and $P = 0.345$) (Figures 2 and 3). Some of the male CAG-*mCbr1*/*mOvgpl*-TAG dTg-mice survived for longer periods of time, although the difference was not significant. On the other hand, survival rates tended to be higher for female *mOvgpl*-TAG Tg-mice than for CAG-*mCbr1*/*mOvgpl*-TAG dTg-mice.

Analysis of mice and expression of CAG-mCbr1

The body weights and indicators of tumor sizes were compared between CAG-*mCbr1*/*mOvgpl*-TAG dTg-mice, *mOvgpl*-TAG Tg-mice, and WT mice groups. Tumors at 12–13 weeks of age are shown in Figure 4. Both males and females showed tumor formation in reproductive organs. The female mice in particular showed well-developed tumors that compressed the

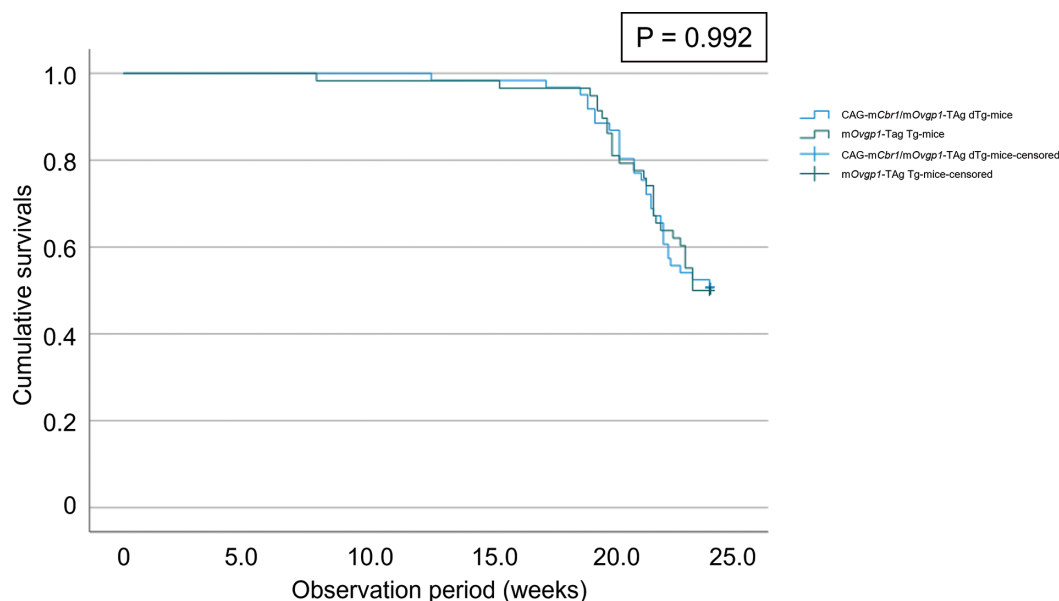


Figure 1 Survival curves of CAG-mCbr1/mOvgp1-TAg dTg-mice. The survivals of CAG-mCbr1/mOvgp1-TAg dTg-mice and mOvgp1-TAg Tg-mice were compared. Survival days were counted from birth to 24 weeks. The blue line indicates CAG-mCbr1/mOvgp1-TAg dTg-mice and the green line indicates mOvgp1-TAg Tg-mice. There was no significant difference between the two groups ($P = 0.992$).

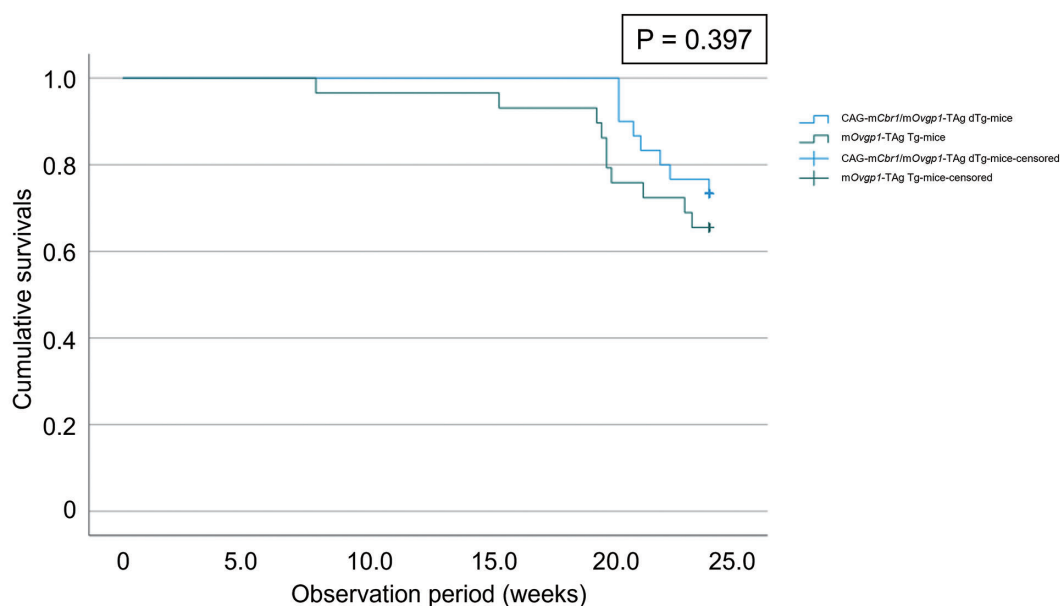


Figure 2 Survival curves of CAG-mCbr1/mOvgp1-TAg dTg-mice (males). The same comparisons as in Figure 1 were made for males only. There was no significant difference between the two groups ($P = 0.397$). Some of the male CAG-mCbr1/mOvgp1-TAg dTg-mice survived for long periods of time.

abdominal cavity. Comparisons of body weights and testicular diameters of males are shown in Figure 5. Testicular diameter was significantly larger in mOvgp1-TAg Tg-mice than in WT mice.

On the other hand, there was no significant difference between CAG-mCbr1/mOvgp1-TAg dTg-mice and mOvgp1-TAg Tg-mice. Body weights were not significantly different among

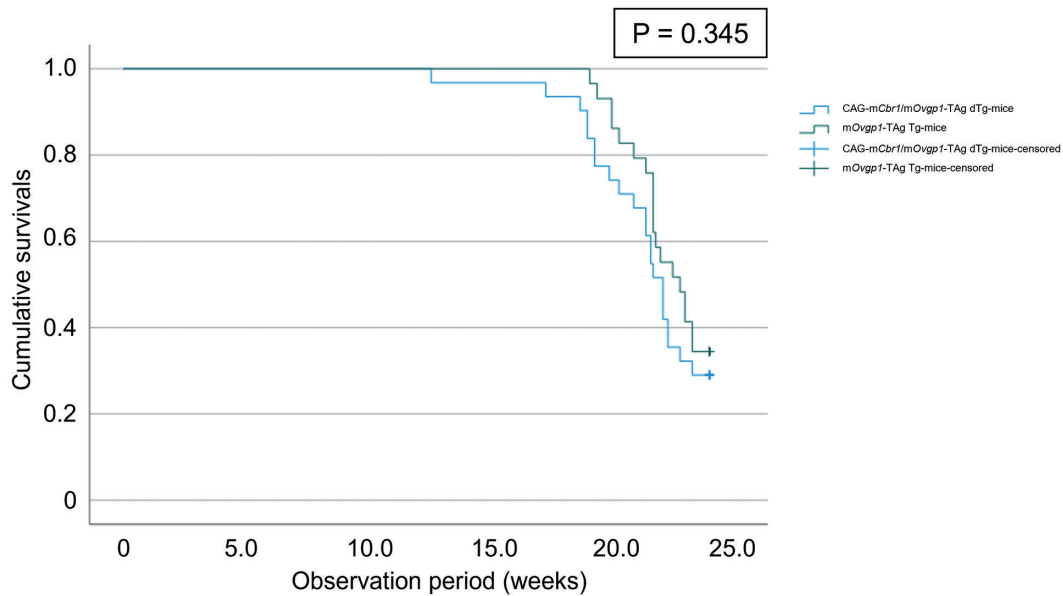


Figure 3 Survival curves of CAG-mCbr1/mOvgp1-TAg dTg-mice (females).

The same comparisons as in Figure 1 were made for females only. There was no significant difference between the two groups ($P = 0.345$).

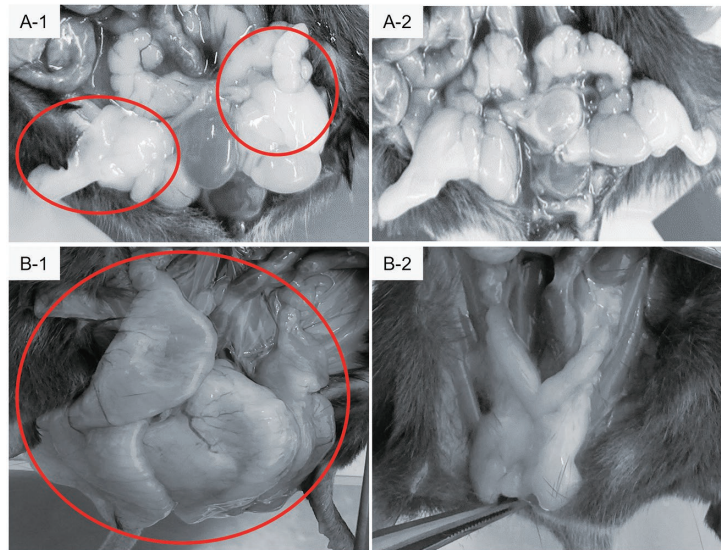


Figure 4 Tumors in the reproductive organs of mice.

mOvgp1-TAg Tg-mice formed tumors at 12-13 weeks of age. Tumor areas are indicated by circles. Males had enlarged testes and epididymis (A-1). Females had enlarged uterus and vagina (B-1). A-2 and B-2 indicate male and female wild type mice, respectively.

the three groups. Comparisons of body weights and abdominal circumference of females are shown in Figure 6. Body weights were significantly larger in mOvgp1-TAg Tg-mice than in WT mice. On the other hand, there was no

significant difference between CAG-mCbr1/mOvgp1-TAg dTg-mice and mOvgp1-TAg Tg-mice. Abdominal circumference was not significantly different among the three groups.

A trend toward relatively higher survival

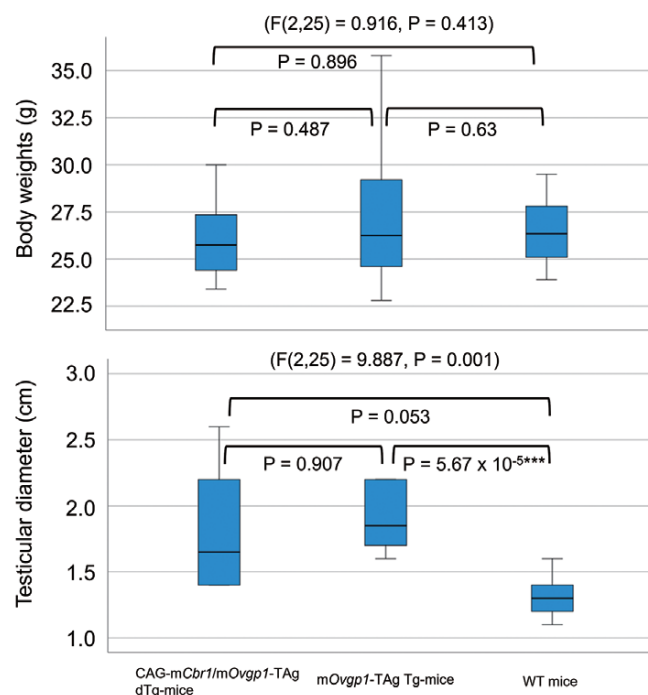


Figure 5 Body weights and indicators of tumor size in the three groups (CAG-mCbr1/mOvgp1-TAg dTg-mice, mOvgp1-TAg Tg-mice, and WT mice) (males).

Body weights and indicators of tumor size (testicular diameter) were measured in 12–13-week-old mice. Differences between the three groups were determined by one-way ANOVA followed by Tukey's test. $P < 0.05$ was considered statistically significant. A trend toward relatively higher weights and larger tumor size in mOvgp1-TAg Tg-mice was observed, although the difference was not significant. *** $P < 0.001$

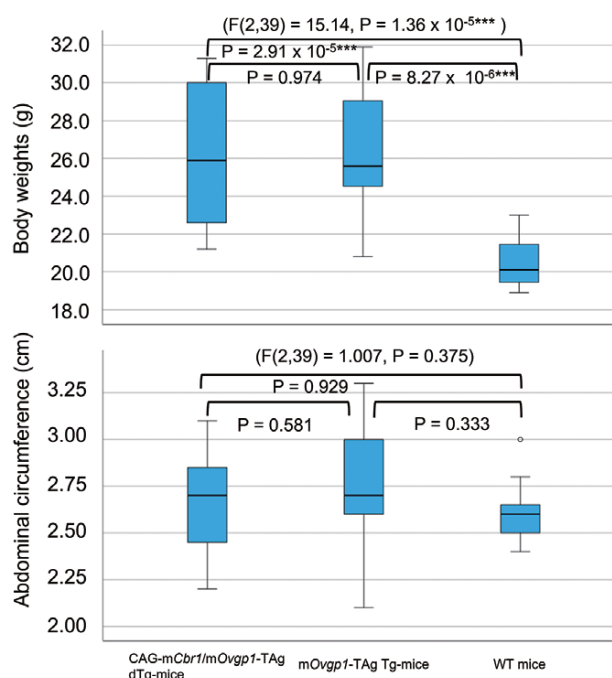


Figure 6 Body weights and indicators of tumor size in the three groups (CAG-mCbr1/mOvgp1-TAg dTg-mice, mOvgp1-TAg Tg-mice, and WT mice) (females).

Body weights and indicators of tumor size (abdominal circumference) were measured in 12–13-week-old mice. Differences between the three groups were determined by one-way ANOVA followed by Tukey's test. $P < 0.05$ was considered statistically significant. A trend toward relatively higher weights and larger tumor size in mOvgp1-TAg Tg-mice was observed, although the difference was not significant. *** $P < 0.001$

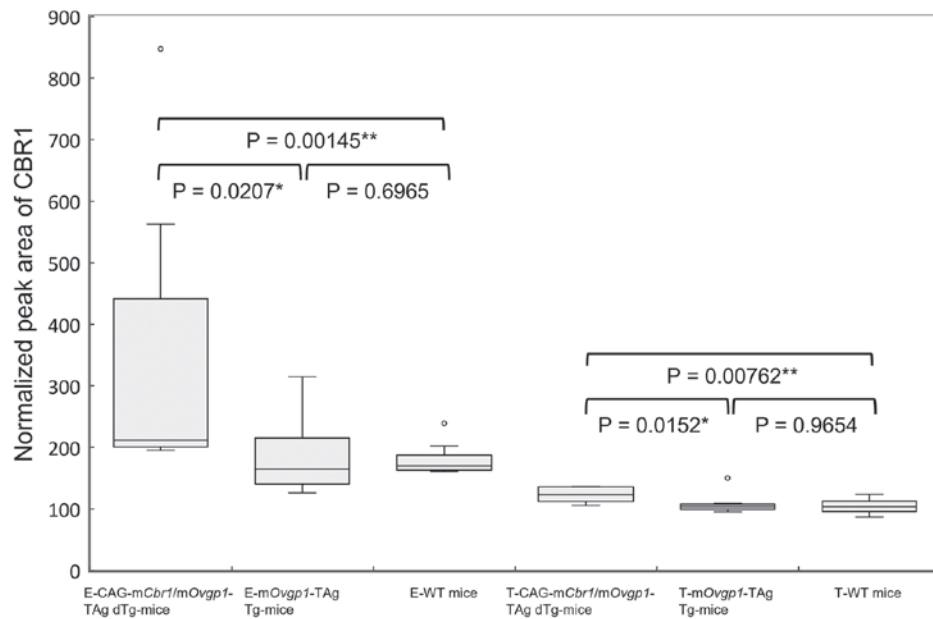


Figure 7 Expression of CAG-mCbr1.

After measurement of body weights and indicators of tumor size, mice were euthanized, and testes and epididymis were removed. CAG-mCbr1 total expression levels of CAG-mCbr1 and endogenous CBR1 were compared. Differences between the three groups were determined by Wilcoxon rank sum test. $P < 0.05$ was considered statistically significant. In CAG-mCbr1/mOvgp1-TAg dTg-mice, CAG-mCbr1 expression was upregulated in the epididymis. E: epididymis, T: testes. * $P < 0.05$, ** $P < 0.01$

rates in males in the CAG-mCbr1/mOvgp1-TAg dTg-mice was also observed. Because mOvgp1-TAg Tg-mice develop tumors in the reproductive tract, cancerous testes and epididymis were removed from male mice, and CAG-mCbr1 expression levels were compared (Figure 7). ANOVA analysis for epididymis and testis, respectively, showed significant differences between CAG-mCbr1/mOvgp1-TAg dTg-mice and mOvgp1-TAg Tg-mice ($P = 0.047$ for epididymis and $P = 0.019$ for testis). However, post hoc test results showed no significant difference between CAG-mCbr1/mOvgp1-TAg dTg-mice and mOvgp1-TAg Tg-mice. ($P = 0.095$ for epididymis and $P = 0.087$ for testis). Due to the large deviation of the data, a nonparametric test, the Wilcoxon rank sum test, was applied, and a significant difference was found between CAG-mCbr1/mOvgp1-TAg dTg-mice and mOvgp1-TAg Tg-mice in both the epididymis and testis ($P = 0.0207$ for epididymis, $P = 0.0152$ for testis) (Figure 7).

Discussion

In previous animal studies, knockdown of CBR1 in ovarian cancer cells increased their proliferation in nude mice⁴⁾, and subcutaneous injection of CBR1-overexpressing ovarian cancer cells into mice inhibited tumor growth⁵⁾. In studies of intraperitoneally injected ovarian cancer cells transfected with the CBR1 DNA-dendrimer complex, CBR1 overexpression inhibited intraperitoneal seeding and increased survival in mice¹⁰⁾. However, these observations are piecemeal and derived from studies of cancer cell lines. Few studies have examined the effect of CBR1 on the course of carcinogenesis. In an *in vivo* study of the intraperitoneal pathology of cancer, we examined the growth and metastatic morphology of ovarian cancer cells using artificial human peritoneal tissue (AHPT) and assessed the metastatic morphology in the

abdominal cavity of mice. The results showed that transfection with CBR1 did not inhibit invasion between mesothelial cells, whereas it inhibited the growth of cancer cells¹¹⁾. These results led us to hypothesize that CBR1 inhibits the growth of cancer cells themselves, rather than inhibiting their adhesion and invasion of the peritoneal cavity. We therefore designed a cross between Tg-mice carrying a cancer-inducing gene and CAG-*mCbr1* Tg-mice.

In this study, differences in survival and tumor size were evaluated in mice obtained by crossing the CAG-*mCbr1* Tg-mice with carcinoma-model mice. Although no significant differences were observed, survival rates tended to be higher for male CAG-*mCbr1*/*mOvgpl*-TAG dTg-mice than for *mOvgpl*-TAG Tg-mice. The aforementioned study using AHPT suggested that CBR1 inhibits tumor cell proliferation, although it does not inhibit cellular activities such as mesothelial invasion and intrastromal migration¹¹⁾, which may have contributed to the survival rate in this study.

However, female mice did not show notable differences in survival between groups, and furthermore survival rates tended to be higher for female *mOvgpl*-TAG Tg-mice than for CAG-*mCbr1*/*mOvgpl*-TAG dTg-mice. Regarding carcinogenesis, female mice showed worsening of the general condition due to the pressure caused by the tumor growth in the uterine ovaries. By contrast, the male reproductive organs are located outside the abdominal cavity and were not associated with compression symptoms as in the females. In this regard, it is important to note that CAG-*mCbr1* Tg-mice are integrated into the X chromosome in the Tg-mouse strain used in this study. Therefore, X chromosome inactivation of Tg might be one of the factors responsible for the differences between males and females. Males express genes necessary for survival on only one X chromosome, whereas in females, one X chromosome is inactivated to prevent the expression of excessive amounts of genes from

the two X chromosomes¹²⁾. Although which X chromosome is inactivated is determined at random in eutherian subclass animals such as mice and humans, once inactivation occurs, the inactivation state of that X chromosome does not change.

The present results do not clearly show whether CBR1 has a marked antitumor effect, although the possibility remains that it may be involved in tumor growth rather than metastasis. Further investigation of the antitumor effect of CBR1 would require the generation of male mice carrying both an oncogene and a CBR1 Tg and crossing these mice with homozygous female CAG-*mCbr1* Tg-mice to exclude the effects of inactivation of the X chromosome. Alternatively, because the carcinogenic property of SV40 TAG was strong in the *mOvgpl*-TAG Tg-mice used in this study, the effect of CBR1 could be assessed by performing crosses with a milder carcinogenesis model.

Conflicts of interest

Y.Y. and M.Y. have filed a patent application for the CAG-*mCbr1* Tg-mice with details as follows: Name: Transgenic non-human animals; Number: Japanese Patent Application No. 2022-009381; Status: Under review; Specific aspect of manuscript covered in the patent application: All the information reported in Reference No. 7.

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