Retinoic acid-metabolizing enzyme cytochrome P450 26A1 promotes skin carcinogenesis induced by 7,12-dimethylbenz[a]anthracene

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Abstract. Elevated expression of the retinoic acid-metabolizing enzyme cytochrome P450 26A1 (CYP26A1) has been demonstrated to have an oncogenic function in carcinogenesis. In order to address the oncogenic capacity of CYP26A1 *in vivo*, transgenic mice that ubiquitously overexpressed CYP26A1 driven by the cytomegalovirus promoter were generated in the present study. Since the growth of these animals was normal for ≤15 months and they presented no evident abnormalities, a two-stage skin carcinogenesis analysis was performed. In the CYP26A1 transgenic mice, papilloma formation was observed within 7 weeks after administration of the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA). Development of papillomas in these animals was significantly accelerated when compared with that observed in the control mice following treatment with DMBA in combination with the chemical tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate. In addition, constitutive expression of CYP26A1 increased the susceptibility of these mice to the generation of squamous cell carcinomas caused by treatment with the carcinogen alone. It is thus concluded that CYP26A1 expression promotes skin carcinogenesis initiated by DMBA.

Introduction

Retinoic acid (RA), an active metabolite of vitamin A, is a critical signaling molecule that is involved in the differentiation, proliferation and apoptosis of a wide variety of cell types (1). While essentially all cell types express nuclear RA receptors (RA nuclear receptors and retinoid-X-receptors) and can potentially respond to RA to positively or negatively regulate expression of RA target genes. Disruption of retinoid signaling through mutations in these nuclear receptors has been found in certain types of tumor cells (1). In addition, insufficient RA signaling such as in vitamin A deficiency (VAD) has been linked to increased susceptibility to carcinogenesis (2-8). It is generally believed that cellular responsiveness is primarily determined by intracellular RA levels, rather than by the serum concentration of RA (2). The availability of RA for the cell is regulated by a coordinated balance between vitamin A nutritional status, and RA biosynthesis and catabolism. Thus, it is hypothesized that the concept of 'cellular RA bioavailability' is critical to understand the pathogenesis of diseases that are caused by RA insufficiency (1,2).

To examine the impact of RA depletion, a state of RA deficiency was induced by RA metabolism in cells. Previous studies demonstrated that the expression levels of the RA-metabolizing enzyme cytochrome P450 26A1 (CYP26A1) were elevated in various tumor types, while reduced cellular RA bioavailability caused by CYP26A1 expression served a stimulatory oncogenic effect on carcinogenesis (2). Additionally, it was demonstrated that the cells expressing CYP26A1 gain significant resistance to differently acting apoptogenic factors due to the state of RA insufficiency caused by metabolic inactivation of RA (1). Several studies have also demonstrated that various metabolites of vitamin A are present in cancer patients, as well as that RA metabolism and CYP26A1 expression levels are enhanced in various types of cancer (1-7). These observations are consistent with accumulating evidence suggesting the association of VAD with increased susceptibility to carcinogenesis and elevated risk for a number of human cancer types (8).

The relevance of elevated CYP26A1 expression in human cancer has yet to be elucidated *in vivo*. In order to address the oncogenic role of CYP26A1, transgenic mice that ubiquitously overexpressed CYP26A1 under the control of the cytomegalovirus (CMV) promoter were generated in the present study. A two-stage skin carcinogenesis mouse model was employed, using 7,12-dimethylbenz[a]anthracene (DMBA) as a carcinogen and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as a chemical tumor promoter, which is a widely used model in the study of epithelial carcinogenesis (9,10). In the present study, it was observed that enhanced expression of CYP26A1 increased the susceptibility of skin carcinogenesis initiated by DMBA.

Materials and methods

Mouse room conditions. Animals were kept in the environmental conditions to reduce mouse stress. Mice were maintained in a 12/12 h light/dark cycle to minimize noises, vibrations and odors. Technicians in the animal facility did not enter the mouse room during the dark cycle. The animals were kept in temperatures of 22-24°C with 40-50% humidity. Food and clean water were freely accessible at all times. Transgenic CYP26A1 mice were maintained for up to 15 months to observe phenotypes in the natural course, for example, in the absence of DMBA/TPA treatment. The maintenance and handling of animals were conducted using protocols approved by the Animal Care Committee of Sapporo Medical University School of Medicine (approval no. #12-040).

Generation of CYP26A1 transgenic mice. Generation of CYP26A1 transgenic mice was outsourced to a commercial company (Oriental Yeast Co., Ltd., Kyoto, Japan). Briefly, the mice were generated by microinjection of a linearized plasmid into the pronucleus of a single-cell embryo isolated for super-ovulated JAX C57BL/6J mice (Charles River Laboratories Japan, Inc., Yokohama, Japan), according to the manufacturer's protocol. The plasmid used was a green fluorescent protein (GFP)-tagged open reading frame clone of *Mus musculus CYP26A1* (NM_007811) in a GFP expression vector under control of the CMV promoter (OriGene Technologies, Inc., Rockville, MD, USA). Embryos were implanted into pseudopregnant female: CD-1 ICR mice (Charles River Laboratories Japan, Inc.) and allowed to develop to term. A total of 5 transgenic founder mice were finally identified by evaluating tail DNA samples. Founder mice were back-crossed with JAX C57BL/6J mice for successive generations and their offspring were used in further experiments.

Genotyping of transgenic mice. Genotyping of transgenic animals was performed by polymerase chain reaction (PCR) analysis of tail DNA. The DNA was incubated with 2X Go*Taq* Green Master Mix (Promega Corp., Madison, WI, USA) to amplify the mouse *CYP26A1* and *GFP* sequences in the genomic DNA, using two independent primer sets to confirm the genomic integration of introduced cassettes. The PCR primers used were as follows: CYP26A1 primer set 1, 5'-TTC GGGTTGCTCTGAAGACT-3' (forward) and 5'-TCCTCC AAATGGAATGAAGC-3' (reverse); CYP26A1 primer set 2, 5'-CGGTTCAGCTTCATTCCATT-3' (forward) and 5'-CAG TGGGGCTTGTCTTCATT-3' (reverse); GFP primer set 1, 5'-TGATGGGCTACGGCTTCTAC-3' (forward) and 5'-GTG ATGGGCTACGGCTTCTA-3' (reverse); and GFP primer set 2, 5'-GCTGCCATCCAGATCGTTAT-3' (forward) and 5'-CTT GAAGTGCATGTGGCTGT-3' (reverse). The cycling conditions were as follows: Preheating at 96°C for 3 min, 40 cycles of 30 sec at 96°C, 30 sec at 58°C and 3 min at 72°C, followed by a final elongation of 7 min at 72°C.

Constitutive CYP26A1 mRNA expression examination. To examine the sufficient amount of constitutive *CYP26A1* mRNA expression in the target tissues, reverse transcription-quantitative PCR (RT-qPCR) analysis was performed as described previously (2). Briefly, total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) from the mouse tail samples 3 weeks after birth, and reverse transcription was subsequently performed using the Superscript II Reverse Transcriptase kit (Invitrogen; Thermo Fisher Scientific, Inc.). Samples were incubated at 42°C for 50 min, and the cDNA was then incubated with 2X Go*Taq* Green Master Mix (Promega Corp.) and the appropriate primers (CYP26A1 primer sets 1 and 2, as well as GFP primer sets 1 and 2) to amplify the genes of interest. The cycling conditions were as follows: 20-40 cycles of 30 sec at 96°C, 30 sec at 58°C and 1 min at 72°C, followed by a final elongation of 7 min at 72°C. The expression of each gene of interest was analyzed using cycling parameters that were optimized previously for the detection of linearity, allowing for semiquantitative analysis of signal intensities, as described previously (2). PCR experiments were repeated in triplicate independent reactions to ensure elevated expression of *CYP26A1* in mice.

Skin carcinogenesis model. The chemical carcinogen DMBA (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was dissolved in acetone at a concentration of 5 mg/ml. The chemical tumor promoter TPA (Sigma-Aldrich; Merck KGaA) was dissolved in ethanol at a concentration of 1 mg/ml.

In order to establish the two-stage skin cancer model, transgenic male (n=17) and female mice (n=11) from five different founder lines, as well as control mice (JAX C57BL/6J mice purchased from Charles River Laboratories Japan, Inc.; male, n=5; female, n=4), received a single application of 100 μg DMBA or vehicle (20 μl acetone) only by subcutaneous injection in the dorsal skin at 8-9 weeks of age. Subsequently, the mice were administered 1 μg TPA at 1 week after the single initiation by DMBA. Mice received TPA topically by subcutaneous injection in the dorsal skin twice a week within 20 weeks after administration of DMBA. In the second experiment to verify the observations of the first experiment, the same experiment was repeated according to the same protocol, using transgenic male (n=11) and female mice (n=14), as well as control mice (male, n=4; female, n=4).

In addition, a one-stage skin cancer model was further established in the present study. Briefly, transgenic male (n=9) and female mice (n=11) from the five founder lines, as well as control mice (JAX C57BL/6J mice purchased form Charles River Laboratories Japan, Inc.; male, n=2; female, n=2) were administered 100 μg DMBA in the dorsal skin without subsequent treatment with TPA. In the second line of experiment to verify our observations in the first experiment that was similar design in the two-stage skin cancer model, we separately repeated same experiment in a same protocol, using transgenic male (n=11) and female mice (n=14), as well as control mice (male, n=2; female, n=2).

Examination. The humane endpoint of the present study was to observe the tumor formation and development within 20 weeks after the initiation with DMBA. This is a standard observation period reported to be required for squamous neoplasia such as papilloma formation in a two-stage skin carcinogenesis model

in mice, and to be appropriate by taking action in escaping from the potential pain and distress (9-11).

Potential pain and distress may be intrinsic in the experimental animals since 2 different types of carcinogenesis model were employed. Even in these cases, however, efforts were made to avoid and minimize potential pain and distress of animals. The mice were examined twice a week by palpitation in order to determine skin tumor formation (e.g., tumor size and its number) within 20 weeks after administration of the carcinogen DMBA. Following the final dose of TPA, animals were sacrificed at 20 weeks after DMBA treatment, or indicated time points in the course of the tumor formation experiments, to evaluate tumors histologically. Tumors exceeding a maximum diameter >1 mm were designated as tumor formation in the protocol and measured weekly. Mice were euthanized if skin ulceration of the primary tumor occurred. Formalin-fixed, paraffin-embedded tissue specimens were prepared and stained with hematoxylin and eosin according to a standard protocol, as described previously (2,5,6). The histology of all tumors was examined under a light microscope (Olympus, Tokyo, Japan) independently by a number of trained surgical pathologists, who were officially certified by Japanese Society of Pathology.

Statistical analyses were not conducted during the course of the present study because the goal was to demonstrate the differences in histology of tumor and tumor growth (e.g., onset of tumor formation and development) that were observed in control and CYP26A1 transgenic mice.

Results

Generation of CYP26A1 transgenic mice. The results of our previous study suggested that enhanced CYP26A1 expression may potentially lead to neoplastic transformation of keratinocytes during skin carcinogenesis (5). Thus, the present study assessed the oncogenic role of *CYP26A1* by examining the effect of CYP26A1 overexpression in the skin tissues of transgenic mice.

Genomic PCR analyses revealed genomic integration of the introduced cDNA by detecting sequences of the *CYP26A1* and reporter gene *GFP* in skin tissues of offspring mice (Fig. 1A). There was no positive control sample because, to the best of our knowledge, this is the first report to establish CYP26A1 transgenic animals. In addition, elevated *CYP26A1* expression was observed in skin tissues from different founder lines (Fig. 1B). The expression of *CYP26A1* mRNA varied in the animals; however, mice were not selected for the subsequent experiments according to the levels of *CYP26A1* expression, in order to exclude possible selection bias for a specific phenotype. It has been previously reported that constitutive expression of CYP26A1 was weak but present in basal keratinocytes in human skin (5). However, in the present study, CYP26A1 was not detectable in control animals by RT-qPCR analysis using skin tissue samples (Fig. 1B).

Lack of spontaneous tumor formation in CYP26A1 transgenic mice. CYP26A1 transgenic animals displayed no evident abnormalities for ≤15 months, while male and female mice were viable and fertile. In addition, no macroscopic tumors

were detected by palpitation every month. Furthermore, histological evaluations of various organs, including the skin, mammary glands, head and neck tissues, lung, gastrointestinal tract, liver and central nervous system, of the transgenic mice also presented no evidence of abnormalities, such as hyperplasia, dysplasia and neoplasia, or active inflammation (data not shown).

Two-stage skin cancer model. A two-stage skin cancer model was used in the present study to examine the role of CYP26A1 in enhancing tumor formation and development in combination with exposure to a carcinogenic agent (Fig. 2). The chemical carcinogen DMBA was administered to CYP26A1 transgenic mice, followed by treatment with the chemical tumor promoter TPA. The histology of skin tissues in the control and CYP26A1 transgenic mice (8-9 weeks of age) was normal before the treatment (Fig. 2A). By contrast, the skin treated by DMBA and TPA in control and CYP26A1 transgenic animals demonstrated a varying degree of hyperplastic change, which was characterized by the proliferation of keratinocytes. However, a wide range of aberrant keratinization, dyskeratosis and a significant increase of melanocytes were not observed. Certain tumors arising in the CYP26A1 transgenic mice were composed by thick, interwoven tracts of pigmented basaloid and squamous epidermal cells with formation of pseudo horn cysts, resembling acanthotic type seborrheic keratosis, which is usually common in sunlight-damaged human skin of older individuals.

In CYP26A1 transgenic mice, it was observed that constitutive expression of CYP26A1 induced squamous hyperplasia followed by papilloma formation within 7 weeks after treatment with DMBA (Fig. 2B). Tumor growth was significantly accelerated as compared with the onset of tumor formation following DMBA/TPA treatment in the control mice. In the control mice, papillomas developed at 15-17 weeks after DMBA/TPA treatment, which is consistent with the time reported to be required for papilloma formation in a two-stage skin carcinogenesis model in C57BL/6 mice (11). Microscopic appearance of papillomas is characterized by a papillary and acanthotic growth of well-differentiated squamous cells with hyperkeratinization. Squamous papillomas of the skin that developed in transgenic and control mice were indistinguishable morphologically, although the maximum sizes of tumors detected in these animals were different (Fig. 2A). Mild chronic inflammation was present beneath the epithelium in CYP26A1 transgenic and control mice in the presence of DMBA and TPA (data not shown). An increased incidence of tumor formation was observed in all of the five founder transgenic lines. Despite the clear evidence that CYP26A1 expression leads to early tumor formation, CYP26A1 expression was not sufficient to increase the mean number of tumors (Fig. 2B). These data suggested that CYP26A1 expression promotes skin tumorigenesis induced by DMBA.

One-stage skin cancer model. The present study next examined whether CYP26A1 expression had any effect on the DMBA-induced skin carcinogenesis in the absence of the tumor promoter TPA (Fig. 3). CYP26A1 expression increased the susceptibility of these mice to the induction of papilloma
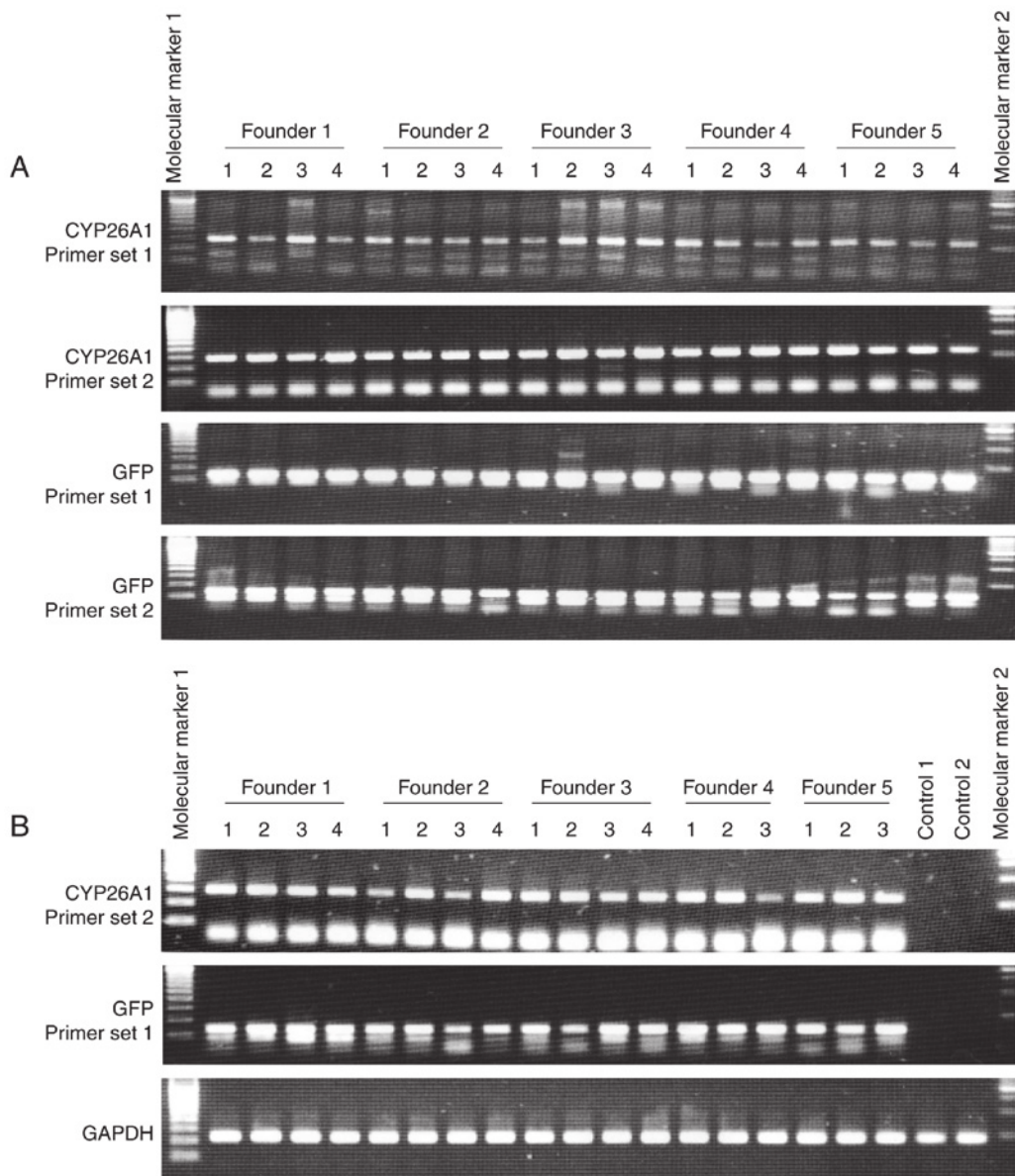
Figure 1. Generation of CYP26A1 transgenic mice. (A) PCR analyses were conducted to examine the genomic integration of introduced cassettes by amplifying *CYP26A1* and *GFP* using different two sets of primers for each gene. (B) Reverse transcription-quantitative PCR analysis demonstrated the constitutive expression of *CYP26A1* and *GFP* in the skin tissues of transgenic mice. Note that the transgenic animals were obtained from all of the five founder mice. CYP26A1, cytochrome P450 26A1; GFP, green fluorescent protein; PCR, polymerase chain reaction.

formation by DMBA treatment alone (Fig. 3A). In CYP26A1 transgenic mice, papillomas appeared at 5-8 weeks, and the increase in the incidence of papillomas was similar to that observed in the two-stage skin carcinogenesis model (Fig. 2B; DMBA/TPA model).

Spontaneous development of invasive squamous cell carcinoma was observed in ~25% of the CYP26A1 transgenic animals at 13 weeks following single treatment with DMBA (Fig. 3B). Squamous cell carcinomas arising in the CYP26A1 mice consisted of nests and strands of atypical epithelial cells with central keratinization, which have abundant eosinophilic cytoplasm and a large round atypical nucleus (Fig. 3C). These cells infiltrated into the subcutaneous fibrous and adipose tissue, while there was a mild chronic inflammatory cell infiltrate at the periphery of the tumors. In addition, scattered squamous intraepidermal neoplasia of the skin was observed in a surrounding area of squamous cell carcinoma, presenting acanthosis consisting of variable atypia of keratinocytes and focal parakeratosis with a loss of cell polarity (data not shown). Although there has been an accumulated body of evidence demonstrating that DMBA promotes the initiation and development of epithelial neoplasia (9,10), the current study also observed the formation of non-epithelial malignancy of the skin, i.e., sarcoma detected in the subcutaneous tissue lining with the intact epidermis (Fig. 3D). These tumors did not demonstrate specific differentiation phenotypes of tumor cells upon immunohistochemical analysis. By contrast, control mice did not develop palpable tumors, or present any definite histological abnormalities in skin tissues within 20 weeks. These observations suggested that CYP26A1 expression predisposes mice to skin carcinogenesis, supporting a stimulatory oncogenic role of CYP26A1 in skin neoplasia.
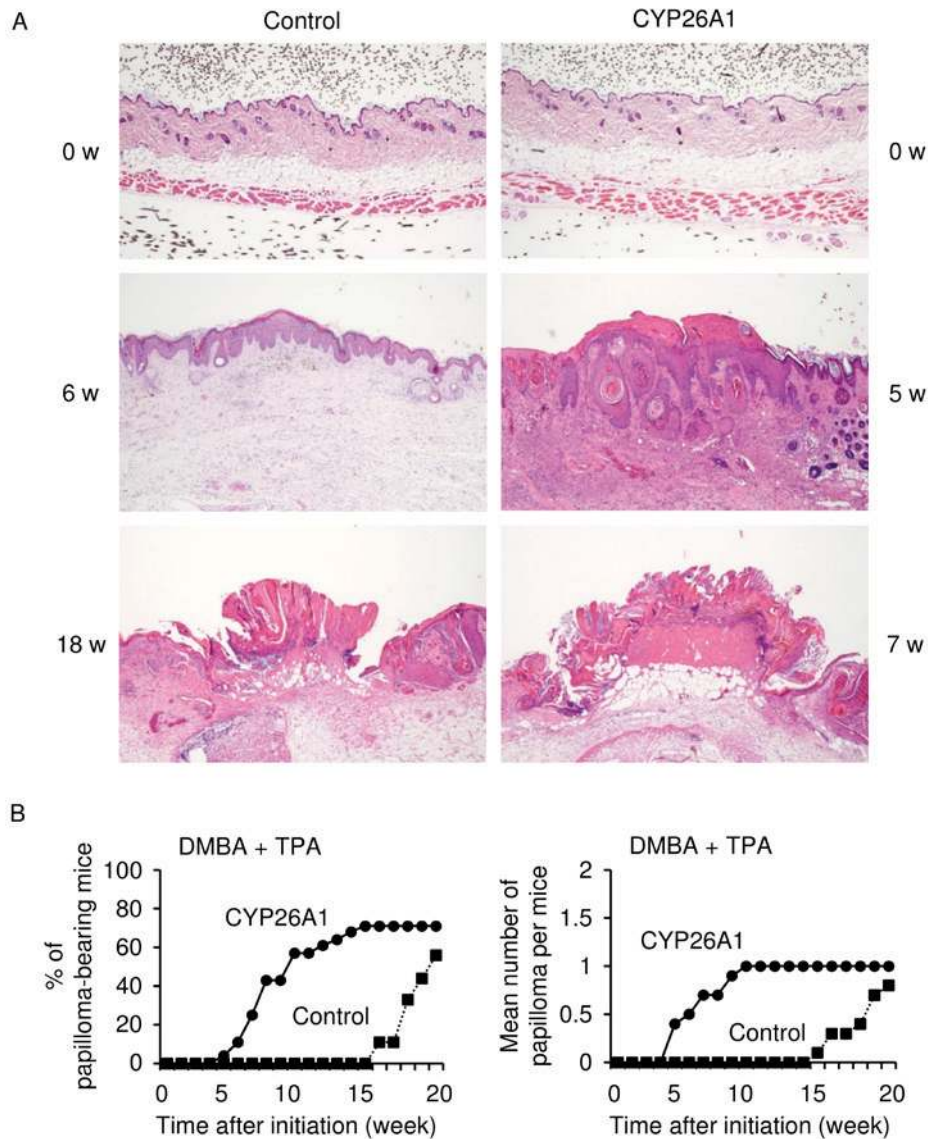
Figure 2. CYP26A1 expression promotes skin carcinogenesis. A two-stage carcinogenesis model demonstrated that CYP26A1 expression promotes tumorigenesis. (A) Representative histological images of hematoxylin and eosin staining (original magnification, x10) of skin tissues at indicated times after initiation with DMBA and TPA. (B) Percentage of mice that developed papillomas and mean number of tumors per mice. CYP26A1, cytochrome P450 26A1; DMBA, 7,12-dimethylbenz[a]anthracene; TPA, 12-O-tetradecanoylphorbol-13-acetate.

Discussion

In the present study, it was demonstrated that enhanced expression of CYP26A1 increases the susceptibility to skin carcinogenesis initiated by DMBA. The skin tissue of CYP26A1 transgenic mice had increased sensitivity to DMBA-mediated carcinogenesis, even when compared with control animals that were subjected to combined treatment with DMBA and TPA. CYP26A1 transgenic animals did not develop spontaneous tumors, or demonstrate any histological abnormalities over the course of the experiments. A possible explanation for these observations is that CYP26A1 overexpression alone was not sufficient for the initiation of epidermal keratinocytes that would lead to the development of a skin tumor. This hypothesis is consistent with our previous results indicating that forced expression of CYP26A1 in non-transformed culture cells did not cause anchorage-independent growth in soft agar (2). However, spontaneous development of cutaneous squamous cell carcinoma in CYP26A1 transgenic animals was observed following single treatment of DMBA in the absence of a tumor promoter. These findings suggested that CYP26A1 expression serves a fundamental role in determining the cellular commitment to carcinogenesis. These data are in agreement with previous observations, suggesting that enhanced expression of CYP26A1 has a stimulatory oncogenic function in neoplasia (2,5-7).

Previous studies unveiled a functional association between CYP26A1-mediated cellular RA insufficiency and enhanced tumorigenicity, implicating *CYP26A1* as a possible candidate oncogene (1,2,5-7). Accumulated evidence suggested that CYP26A1 overexpression may contribute to skin carcinogenesis by causing a state of functional VAD. This hypothesis is based on a mechanistic link between VAD and increased risk for various types of cancer (8). Consistently, a number of
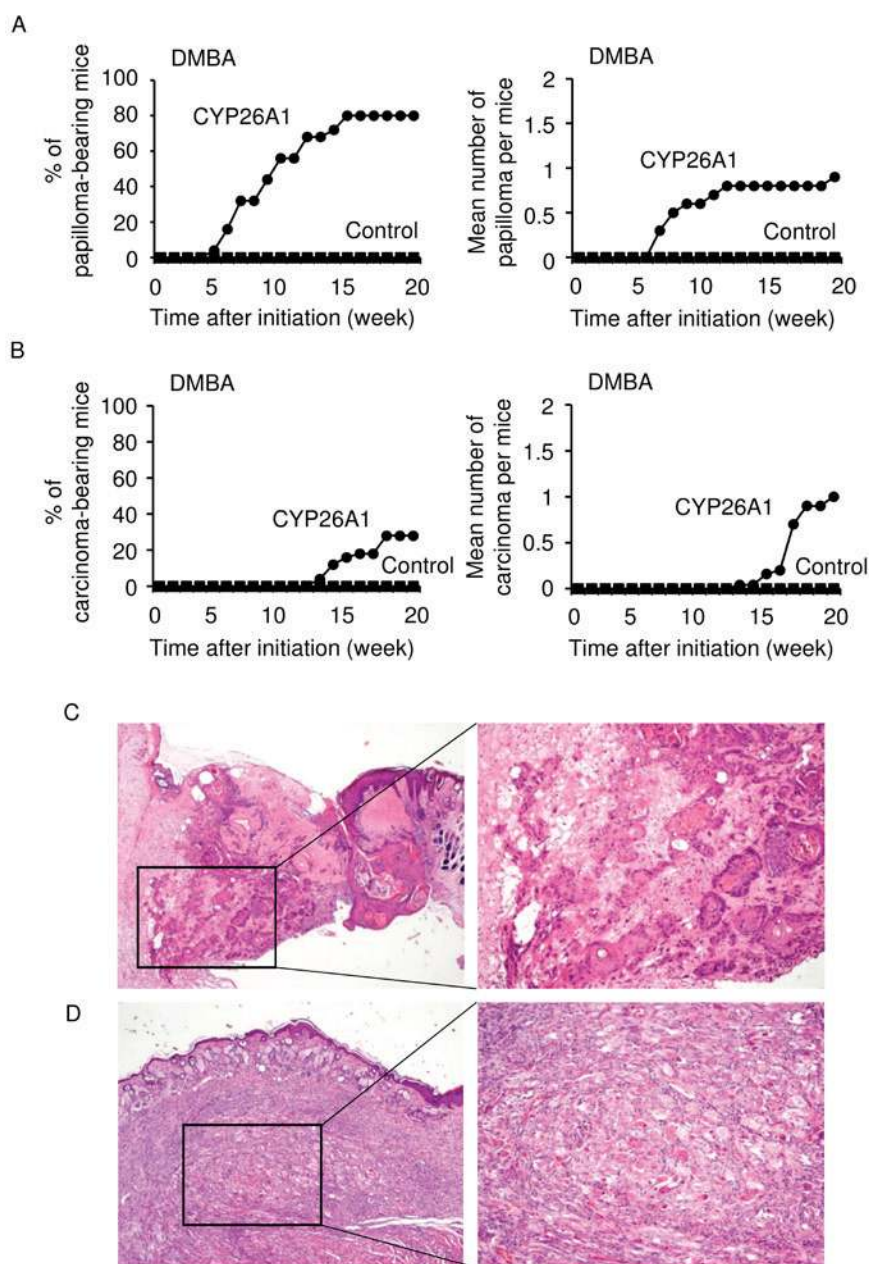
Figure 3. CYP26A1 expression promotes skin carcinogenesis. DMBA-induced one-stage carcinogenesis model demonstrated that CYP26A1 expression predisposes mice to skin carcinogenesis. Percentage (%) of mice that developed (A) papillomas or (B) squamous cell carcinomas and mean number of tumors per mouse. CYP26A1 transgenic mice that were treated with only DMBA developed (C) squamous cell carcinoma and (D) subcutaneous undifferentiated sarcoma. Representative histological images of hematoxylin and eosin staining of skin tissues (original magnification, x10 and magnified views, x40 of the rectangular areas). CYP26A1, cytochrome P450 26A1; DMBA, 7,12-dimethylbenz[*a*]anthracene.

published studies have identified that individuals with VAD accumulate DNA damage at a higher frequency, eventually resulting in increased risk and incidence of cancer (12,13). Conversely, there has been increased interest in the use of RA and synthetic derivatives (known as retinoids) in cancer therapy and dermatology. Several models have been used to demonstrate the effectiveness of retinoids in suppressing carcinogenesis in a variety of tissues, including in the skin, oral cavity, liver, mammary epithelia, bladder and prostate (14,15). These previous studies suggested that the use of several types of RA and synthetic derivatives may be utilized in the treatment of premalignant lesions, as well as in the prevention of secondary tumors and the recurrence of cancer.

It has been demonstrated that CYP26A1 modulates a wide variety of genes to favor cell survival, which results in a selective growth advantage for the cells (1-7). Furthermore, CYP26A1 upregulation alters the gene expression profile already present in tumor cells to potentially generate a wide variety of specific and potent pro-survival signals that render cells resistant to apoptosis. Given the pleiotropic activity of RA, it is plausible that CYP26A1 expression affects a vast array of physiological pathways in regulating various independent signaling molecules associated with tumorigenic events.

In conclusion, the present study clearly provided evidence of the role of elevated CYP26A1 expression in promoting

cutaneous neoplasia induced by DMBA. However, the exact molecular impact of CYP26A1 on carcinogenesis and the underlying regulatory mechanism of CYP26A1 overexpression in human cancer remain to be clarified. Therefore, future studies are warranted to better understand the biological significance of CYP26A1 overexpression in the neoplasia of human malignancies.

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Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MO designed and performed the present study, participated in animal experiments and drafted the manuscript. AT and KT participated in animal experiments and histochemical evaluation of developed tumors in mice. MM and NS participated in data analysis and interpretation, and helped to draft the manuscript. All authors contributed to the manuscript discussion of the article to be published and approved its final version.

Ethics approval and consent to participate

The maintenance and handling of animals were conducted using protocols approved by the Animal Care Committee of Sapporo Medical University School of Medicine (approval no. 12-040).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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