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NOTE

Immunology

Functional comparison of the human epidermal growth factor receptor and telomerase reverse transcriptase promoters in canine tumor cells

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ABSTRACT. We previously showed that the promoter region of the human epidermal growth factor receptor (*hEGFR*) gene elicits high transduction efficiency, with transgene expression restricted to canine breast tumor cells. However, it was unclear whether this promoter induces tumor cell-specific transgene expression in canine urothelial carcinoma cells. Furthermore, compared with studies in human cancer cells, the utility of the telomerase reverse transcriptase (*TERT*) gene promoter for therapeutic transgene expression in canine cancer cells has not been evaluated thus far. Here, we compared the activity of these promoters in canine mammary tumor and urothelial carcinoma cells. Our results showed that compared with the *TERT* promoter, the *hEGFR* promoter was more useful as a tumor-specific promoter to induce efficient transgene expression in canine tumor cells.

KEY WORDS: canine breast tumor, canine urothelial carcinoma, gene therapy, human epidermal growth factor receptor promoter, human telomerase reverse transcriptase promoter

Dogs are among the most popular companion animals worldwide. In recent years, the life expectancy of dogs has been extended by the wide use of high-quality commercial pet foods, improvements in environmental hygiene, and advances in veterinary medical science. Cancers is the leading cause of death in dogs [9], and mammary tumors are the most common tumor type in female dogs [2, 6]. In these tumors, the epidermal growth factor receptor (EGFR) is overexpressed [3, 11]. Moreover, EGFR overexpression has also been reported in canine urothelial carcinoma (CUC) of the bladder, the most common urogenital cancer in dogs [14].

Although solo or combinatorial therapeutic approaches, such as surgery, radiotherapy, and chemotherapy, are used to treat these cancers, such treatments may induce serious side effects. Furthermore, resistance often develops, resulting in shortened disease-free survival. Thus, there is an urgent need for additional treatment approaches to alleviate suffering and death caused by cancers. Cancer gene therapy might be a promising alternative strategy with improved efficacy. In particular, suicide gene therapy, i.e., the introduction of pro-apoptotic genes into cancer cells and the induced overexpression of functional proteins from these genes to promote the apoptosis of tumors, as well as cancer-immuno-gene therapy, i.e., the introduction of immunostimulatory genes into cancer cells and the production of proteins from these genes to prevent immune escape and activate immune cells, are promising approaches for cancer gene therapy [4, 5, 13, 18].

Choosing the optimal promoter, i.e., a tumor-specific promoter, is a key factor for achieving successful tumor-specific transgene expression. In a previous study, we evaluated tumor cell-specific transgene expression by a human *EGFR* (*hEGFR*) promoter-driven adenovirus vector in a canine mammary tumor cell line and showed the potential viability of using the *hEGFR* promoter for gene therapy in canine mammary tumors [15]. However, little is known about whether the *EGFR* promoter can induce efficient, tumor cell-specific transgene expression in CUC cells, even when EGFR is overexpressed in these tumors. Studies have shown that the human telomerase reverse transcriptase (*hTERT*) promoter is an appropriate candidate for gene therapy in human cancers. TERT, the catalytic submit of telomerase, is expressed in most tumor cells, but not in normal cells; therefore, the *hTERT* promoter is expected to be highly active in tumor cells. In fact, the hTERT promoter has been successfully applied to express various therapeutic transgenes in tumor cells only [7, 8, 10, 12, 17, 19]. Although *hTERT* promoter-driven tumor-specific gene expression has been extensively studied in human cancer cells, the utility of the *hTERT* promoter for the expression of therapeutic transgenes in canine cancer cells has not been evaluated yet.

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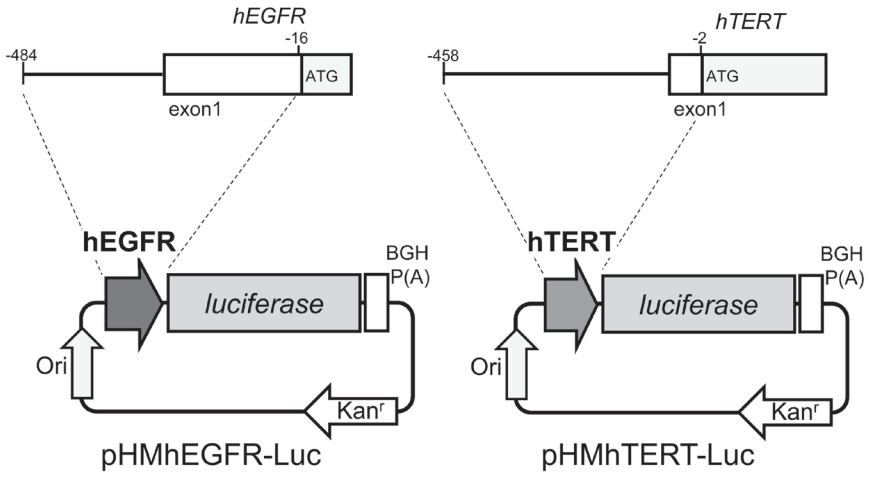

Fig. 1. Schematic representation of reporter expression plasmids used in this study. Numbers in this schema show the number of bases upstream of the translation initiation site of each gene. ATG, translation initiation codon; BGH P (A), bovine growth hormone polyadenylation signal; Kan^r, kanamycin resistance; Ori, origin of replication.

Therefore, in this study, we investigated tumor cell-specific gene expression driven by the *hTERT* promoter in canine mammary tumor cells and CUC cells. We also examined transgene expression by the *hEGFR* promoter in CUC cells and compared the promoter activation of the *hEGFR* promoter with that of the *hTERT* promoter in both cell lines.

MDCK canine kidney cells were obtained from the Health Science Research Resources Bank (Ibaraki, Japan), and canine breast tumor (CBT) cells and CUC cells were purchased from Cosmo Bio (Tokyo, Japan). All cell lines were cultured in Dulbecco's modified Eagle's medium (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 100 U/m*l* penicillin/streptomycin. Cells were cultured in an atmosphere containing 5% CO_2 at 37°C. CBT cells were cultured in Celltight C-1 dishes (Sumitomo Bakelite, Tokyo, Japan).

The *hEGFR* promoter-containing plasmid pHMhEGFR was constructed as described previously [15]. The *hTERT* promoter, located immediately upstream from the ATG translation initiation site (positions −458 to −2, corresponding to positions −378 to +78 from the transcription start site) [16], was cloned from human genomic DNA by polymerase chain reaction (PCR) using PrimeSTAR Max DNA Polymerase (Takara Bio, Kusatsu, Japan) and the following primers: forward primer, 5′-CGGAATTCATCATGGCCCCTCCCTCG-3′ and reverse primer, 5′-GCTCTAGACGCGGGGGTGGCCGG-3′. The PCR-amplified product was digested with *EcoR*I/*Xba*I and inserted into pHMCMV10 lacking the CMV promoter/enhancer and intron A [15] to generate pHMhTERT. The coding region of the firefly luciferase reporter gene was inserted into pHMhEGFR or pHMhTERT cut with *BstX*I and *Acc*65I, respectively, to obtain pHMhEGFR-Luc and pHMhTERT-Luc (Fig. 1). Nucleotide sequences of all PCR-amplified fragments were sequenced by Fasmac Co., Ltd. (Atsugi, Japan). Before transfection into mammalian cells, plasmid DNA was purified using NucleoBond Xtra Midi Plus EF to remove endotoxins (Takara Bio).

Prior to transfection, CBT cells were seeded at 1.0×10^4 cells/well in Celltight C-1 96-well plates, and CUC or MDCK cells were seeded at 1.0×10^4 cells/well in 96-well plates. DNA transfection into cells was performed using the X-tremeGENE HP DNA Transfection Reagent (Sigma-Aldrich, St. Louis, MO, U.S.A.) with 100 *n*g of reporter plasmid (pHMhEGFR-Luc or pHMhTERT-Luc). We used 100 *n*g of Cy3-labeled control DNA (Label IT Plasmid Delivery Control, Cy3; Takara Bio) as transfection controls. After the cells were incubated for 20 hr, luciferase activity was measured with a Dual Luciferase Reporter Assay System (Promega, Madison, WI, U.S.A.) according to the manufacturer's instructions. The fluorescence intensity of Cy3 was measured using a PowerScan plate reader (DS Pharma Biomedical, Suita, Japan). Luciferase activity was normalized for transfection efficiency by the fluorescence intensity of Cy3-labeled control DNA.

Statistical analyses were performed using two-way analysis of variance followed by Bonferroni's post-hoc tests. Data are represented as the mean ± standard deviation. Differences with P values of less than 0.05 were considered statistically significant.

We previously demonstrated that in CBT cells, the *hEGFR* promoter induces efficient expression of the downstream-located gene [15]. In this study, we first evaluated whether the *hTERT* promoter could induce high transcriptional activity in CBT cells, similar to the *hEGFR* promoter. CBT and MDCK cells (used as "normal cells") were transfected with either pHMhEGFR-Luc or pHMhTERT-Luc (Fig. 1), and the enzyme activity of luciferase was measured to confirm promoter-driven gene expression. As shown in Fig. 2, high luciferase activity was detected in CBT cells transfected with pHMhEGFR-Luc. In contrast, MDCK cells transfected with pHMhEGFR-Luc exhibited weak luciferase activity (Fig. 2). This low level of luciferase expression in MDCK cells was not due to low efficiency of plasmid transfection, because the luciferase activity was normalized for transfection efficiency according to the fluorescence intensity of Cy3-labeled control short-length DNA transfected into cells. Thus, small DNAs, such as plasmids or oligonucleotides, could be transfected into both CBT and MDCK cells, and the low level of luciferase expression in MDCK cells in this experiment could be due to the inactivation of the *hEGFR* promoter rather than failure or low

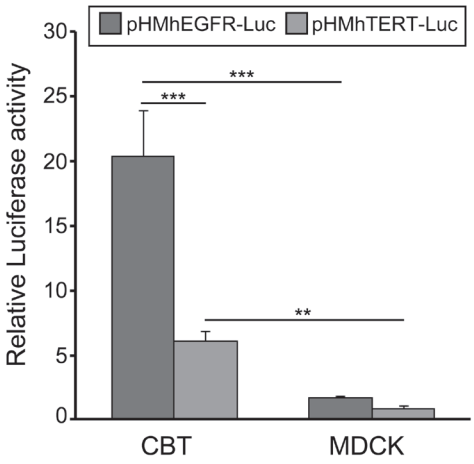

Fig. 2. Comparison of luciferase expression driven by the *hEGFR* and *hTERT* promoters in CBT and MDCK cells. Data are expressed as means ± standard deviations (n=4). ***P*<0.01, ****P*<0.001. The data are representative of three independent experiments.

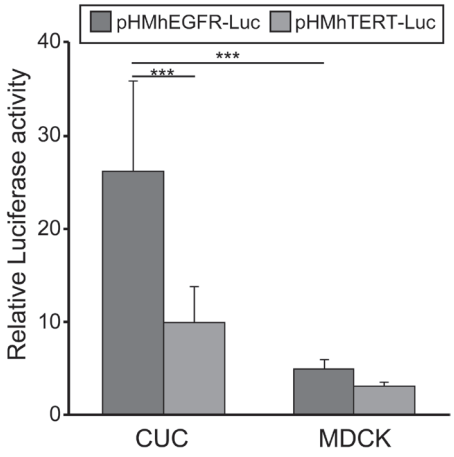

Fig. 3. Comparison of luciferase expression driven by the *hEGFR* and *hTERT* promoter in CUC and MDCK cells. Data are expressed as means ± standard deviations (n=4). **P*<0.05, ****P*<0.001. The data are representative of three independent experiments.

efficiency of transfection. Furthermore, we detected higher luciferase activity in CBT cells transfected with pHMhTERT-Luc than in MDCK cells (Fig. 2); thus, *hTERT* promoter-driven luciferase gene expression in CBT cells was significantly higher than that in MDCK cells. These results suggested that the *hTERT* promoter was active in CBT cells but relatively quiescent in normal canine cells. However, the transcriptional activity of the *hTERT* promoter was lower than that of the *hEGFR* promoter in CBT cells. Compared with the *hTERT* promoter, the relative luciferase activity in the *hEGFR* promoter was about fourfold higher in CBT cells (Fig. 2). In MDCK cells, the *hEGFR* promoter seemed to be more highly activated than the *hTERT* promoter, but no significant differences were found (Fig. 2).

We next examined whether these promoters exhibited high transcriptional activity in CUC cells. CUC and MDCK cells were transfected with either pHMhEGFR-Luc or pHMhTERT-Luc, and the enzyme activity of luciferase was measured 20 hr after transfection. As shown in Fig. 3, the *hEGFR* promoter showed significantly higher transcriptional activity in CUC cells than in MDCK cells. These results showed that the *hEGFR* promoter was active in CUC cells and CBT cells. *hTERT* promoter-driven luciferase gene expression was also observed in CUC cells transfected with pHMhTERT-Luc (Fig. 3). Compared with the *hEGFR* promoter, the relative luciferase activity of the *hTERT* promoter was about 2.6-fold lower in CUC cells (Fig. 3). The transcriptional activity of the *hTERT* promoter was higher in CUC cells than in MDCK cells, although no significant differences were detected (Fig. 3). Although we did not determined whether the *hTERT* promoter could induce the expression of downstream genes as efficiently as the canine *TERT* promoter could, Arendt *et al.* showed that the *hTERT* and canine *TERT* promoters transcribed genes at similar levels in canine cells [1]. Taken together, our results indicated that these promoters were active and elicited efficient gene expression in CBT and CUC cells; moreover, as a tumor-specific promoter, the *hEGFR* promoter is more useful than the *hTERT* promoter. In this study, we only examined the utility of tumor-specific promoters in canine cancer cells. In an earlier study, we showed that a fusion protein containing a core part of an oxygen-dependent degradation (ODD) domain of hypoxia-inducible factor-1 alpha fused to caspase-3 could be stably expressed in CBT cells inducing cell death under hypoxic conditions [15]. Because most solid tumors propagate under hypoxic conditions, tumor-specific stable expression may also occur by the combined use of the *hEGFR* promoter and ODD-fused proapoptotic/therapeutic proteins in the treatment of CUC. Further studies are needed to explore this aspect. Furthermore, we demonstrated the utility of promoters in canine tumor cells *in vitro* only, and further *in vivo* studies, such as studies using canine tumor-bearing animal models, are necessary for determining which genes should be inserted downstream of the *hEGFR* promoter for suitable treatment of canine cancers.

CONFLICT OF INTEREST. None of the authors have any conflict of interests.

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REFERENCES

1. Arendt, M. L., Nasir, L. and Morgan, I. M. 2010. The human and canine TERT promoters function equivalently in human and canine cells. *Vet. Comp. Oncol.* **8**: 310–316. [Medline] [CrossRef]
2. Benjamin, S. A., Lee, A. C. and Saunders, W. J. 1999. Classification and behavior of canine mammary epithelial neoplasms based on life-span observations in beagles. *Vet. Pathol.* **36**: 423–436. [Medline] [CrossRef]
3. Bertagnolli, A. C., Ferreira, E., Dias, E. J. and Cassali, G. D. 2011. Canine mammary mixed tumours: immunohistochemical expressions of EGFR and HER-2. *Aust. Vet. J.* **89**: 312–317. [Medline] [CrossRef]
4. Cross, D. and Burmester, J. K. 2006. Gene therapy for cancer treatment: past, present and future. *Clin. Med. Res.* **4**: 218–227. [Medline] [CrossRef]
5. Das, S. K., Menezes, M. E., Bhatia, S., Wang, X. Y., Emdad, L., Sarkar, D. and Fisher, P. B. 2015. Gene therapies for cancer: Strategies, challenges and successes. *J. Cell. Physiol.* **230**: 259–271. [Medline] [CrossRef]
6. Gilbertson, S. R., Kurzman, I. D., Zachrau, R. E., Hurvitz, A. I. and Black, M. M. 1983. Canine mammary epithelial neoplasms: biologic implications of morphologic characteristics assessed in 232 dogs. *Vet. Pathol.* **20**: 127–142. [Medline] [CrossRef]
7. Gu, J., Andreeff, M., Roth, J. A. and Fang, B. 2002. hTERT promoter induces tumor-specific Bax gene expression and cell killing in syngenic mouse tumor model and prevents systemic toxicity. *Gene Ther.* **9**: 30–37. [Medline] [CrossRef]
8. Huang, T. G., Savontaus, M. J., Shinozaki, K., Sauter, B. V. and Woo, S. L. 2003. Telomerase-dependent oncolytic adenovirus for cancer treatment. *Gene Ther.* **10**: 1241–1247. [Medline] [CrossRef]
9. Inoue, M., Hasegawa, A., Hosoi, Y. and Sugiura, K. 2015. A current life table and causes of death for insured dogs in Japan. *Prev. Vet. Med.* **120**: 210–218. [Medline] [CrossRef]
10. Irving, J., Wang, Z., Powell, S., O'Sullivan, C., Mok, M., Murphy, B., Cardoza, L., Lebkowski, J. S. and Majumdar, A. S. 2004. Conditionally replicative adenovirus driven by the human telomerase promoter provides broad-spectrum antitumor activity without liver toxicity. *Cancer Gene Ther.* **11**: 174–185. [Medline] [CrossRef]
11. Kim, N. H., Lim, H. Y., Im, K. S., Kim, J. H. and Sur, J. H. 2013. Identification of triple-negative and basal-like canine mammary carcinomas using four basal markers. *J. Comp. Pathol.* **148**: 298–306. [Medline] [CrossRef]
12. Lanson, N. A. Jr., Friedlander, P. L., Schwarzenberger, P., Kolls, J. K. and Wang, G. 2003. Replication of an adenoviral vector controlled by the human telomerase reverse transcriptase promoter causes tumor-selective tumor lysis. *Cancer Res.* **63**: 7936–7941. [Medline]
13. Litzinger, M. T., Foon, K. A., Tsang, K. Y., Schlom, J. and Palena, C. 2010. Comparative analysis of MVA-CD40L and MVA-TRICOM vectors for enhancing the immunogenicity of chronic lymphocytic leukemia (CLL) cells. *Leuk. Res.* **34**: 1351–1357. [Medline] [CrossRef]
14. Mutsaers, A. J., Widmer, W. R. and Knapp, D. W. 2003. Canine transitional cell carcinoma. *J. Vet. Intern. Med.* **17**: 136–144. [Medline] [CrossRef]
15. Okamoto, M., Asamura, A., Tanaka, K., Soeda, T., Watanabe, K., Mizuguchi, H. and Ikeda, T. 2016. Expression of HIF-1α ODD domain fused canine caspase 3 by EGFR promoter-driven adenovirus vector induces cytotoxicity in canine breast tumor cells under hypoxia. *Vet. Res. Commun.* **40**: 131–139. [Medline] [CrossRef]
16. Takakura, M., Kyo, S., Kanaya, T., Hirano, H., Takeda, J., Yutsudo, M. and Inoue, M. 1999. Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells. *Cancer Res.* **59**: 551–557. [Medline]
17. Takeda, T., Inaba, H., Yamazaki, M., Kyo, S., Miyamoto, T., Suzuki, S., Ehara, T., Kakizawa, T., Hara, M., DeGroot, L. J. and Hashizume, K. 2003. Tumor-specific gene therapy for undifferentiated thyroid carcinoma utilizing the telomerase reverse transcriptase promoter. *J. Clin. Endocrinol. Metab.* **88**: 3531–3538. [Medline] [CrossRef]
18. Wang, J., Yang, W. K., Yang, Y., Wei, S. J., Yang, D. M., Whang-Peng, J., Chen, Y. M., Ting, K. L. and Ting, C. C. 1998. Paradoxical effect of GM-CSF gene transfer on the tumorigenicity and immunogenicity of murine tumors. *Int. J. Cancer* **75**: 459–466. [Medline] [CrossRef]
19. Yao, X., Yoshioka, Y., Eto, Y., Morishige, T., Okada, Y., Mizuguchi, H., Mukai, Y., Okada, N. and Nakagawa, S. 2007. TERT promoter-driven adenovirus vector for cancer gene therapy via systemic injection. *Biochem. Biophys. Res. Commun.* **362**: 419–424. [Medline] [CrossRef]