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ORIGINAL ARTICLE

EFFICIENT GENE TRANSDUCTION IN HER2-EXPRESSING CANCER CELLS BY TWO RECOMBINANT ADENOVIRUS VECTORS WITH HER2 PROMOTER AND CRE/LOXP SYSTEM

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Abstract Tissue-specific promoter has been used for cancer-specific suicide gene therapy, but its transcriptional activity is relatively low. For more efficient gene therapy of HER2-expressing tumor, a double adenovirus infection system was established, in which a 'regulator' vector carried Cre gene under the control of HER2 promoter and 'target' vectors carried target genes activated by Cre. We constructed a Cre recombinase expression vector, AxHER2Cre, for the 'regulator' vector. By the combination of this vector and AxCALNLZ, β-D-galactosidase was induced in 90% and 70% of MKN7 and MDA-MB-453, HER2–overexpressing cell lines, but only about 20% and 10% of MKN28 and MCF7, low HER2-expressing cell lines. By the quantification analysis, the β-galactosidase activities induced by this system were comparable to those by the combination of AxCANCre and AxCALNLZ. These results indicated that Cre/loxP system under the regulation of HER2 promoter could induce efficient gene expression, maintaining the HER2-expression specificity. Breast cancer with HER2 overexpression is treated with trastuzumab. However, refractory or resitance of HER2 positive breast cancer against trastuzumab becomes a severe clinical problem, recently. This system seemed to be another therapeutic option.

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Key words: HER2/c-erbB-2; recombinant adenovirus vector; Cre/loxP system; cancer specific promoter.

原 著

Cre/loxP system を用いた HER2/neu 発現細胞に対する特異的かつ効率的遺伝子導入システムの開発

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抄録 組織特異的プロモーターは癌特異的自殺遺伝子治療において用いられている．しかし，その転写活性は低いものである．HER2 発現腫瘍の遺伝子治療をより効率よく行うために，組織特異的プロモーターの調節のもとに Cre タンパクが働く「制御」ベクターと，Cre タンパクによって活性化される「標的」ベクターをくみあわせた 2 重アデノウイルスシステムが確立されている．これの応用として我々は HER2 プロモーターのもとで働く Cre 発現ベクター AxHER2Cre を制御ベクターとして作製した．標的ベクター AxCALNZ とこのベクターの組み合わせにより，HER2 発現細胞株である MKN7, MDA-MB-453 でそれぞれ90%，70%の細胞にβガラクトシダーゼが誘導された．これに対して HER2 低発現細胞株である MKN28, MCF7 で発現は20%および10%にすぎなかった．
定量分析を行ったところ，このシステムによるβガラクトシダーゼの活性は，強力なプロモーターとして知られる CA プロモーターを用いた AXCANcre と AXCALNLZ の組み合わせに匹敵するものであった．
以上の結果から，HER2 プロモーターの制御下での Cre/LoxP システムは HER2 発現特異性を保ち，かつ，効率的な遺伝子発現を行えることが示唆された．HER2 発現乳癌に対しては Trastuzumab が用いられているが，耐性が問題となっている．このシステムは，そのようなケースの治療におけるあらたな選択肢となりえると考えられる．

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キーワード： HER2/c-erbB-2 遺伝子/タンパク；組換えアデノウイルスベクター；Cre/loxP システム；癌特異的プロモーター．

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INTRODUCTION

HER2 (neu/c-erbB-2) is a tyrosine kinase and a member of epidermal growth factor receptor subgroup. HER2 is frequently overexpressed in a number of types of cancers[1-4], including non-small cell lung cancer, breast cancer, pancreatic cancer, stomach cancer and ovarian cancer. Expression of this gene in noncancerous tissue is not comparable to that in tumor cells. Thus, the expression of HER2 is a tumor specific phenomenon. The overexpression of HER2 protein is also related to the poor prognosis[5,6]. Hence, HER2-overexpressing tumor should be an important therapeutic target. The development of trastuzumab, a humanized monoclonal antibody that binds to the extracellular domain of HER2, has led to a significant improvement in outcomes of patients with HER2-positive breast cancer. However, many patients with HER2-positive metastatic breast cancer do not respond to trastuzumab, or eventually become resistant to it[7].

Adenovirus vector is a highly efficient, and is widely used for gene transduction. But, since recombinant adenovirus infect various cell types, it is desirable to limit gene expression only to tumor cells in order to avoid possible damage to noncancerous tissue when the adenovirus vector is used for suicide gene therapy.

For that purpose, tissue or tumor specific promoters are used to regulate the gene expression. However, the activity of tissue specific or tumor specific promoters is relatively low[8-10]. To enhance the expression level by the tumor specific promoters, the two recombinant adenovirus with AFP promoter or CEA promoter and Cre/loxP system were developed[8),11-13]. A 'regulator' recombinant adenovirus AxA2ANCre expresses Cre-recombinase under the control of AFP promoter. Cre expression by the 'regulator' recombinant adenovirus allows the excisional deletion of a DNA sequence flanked by a pair of loxP site in a 'target' or 'reporter' recombinant adenovirus AxCALNLZ, which in turn, activates a potent nonspecific, but strong promoter to repress the linked lacZ gene at high levels. Using this system, lacZ expression was enhanced 50 times more than single recombinant adenovirus infection method driven by AFP promoter in AFP-producing cells[8].

In this paper, we have tried to develop a novel therapeutic system to overcome trastuzumab resistance.

In this study, we developed the Cre/loxP regulator system to enhance the specific gene expression by HER2 promoter in HER-2 producing tumor cells, and examined whether sufficient transcriptional activity of HER2 promoter could be obtained without loosing its specificity.

MATERIALS and METHODS

Cell lines All cell lines used in this study were obtained from Riken gene bank (Tsukuba). Human stomach cancer cell lines, MKN-7, MKN-28 and MKN-45 were cultured in RPMI1640 medium (GIBCO, Gaithersberg, MD) supplemented with heat-inactivated 10% fetal bovine serum (FBS; GIBCO). Human breast cancer cell lines, MCF-7 and MDA-MB-453, and human embryonic kidney cell line, 293 were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS.

Immunoblot analysis Immunoblot analysis was performed as follows. 10^6 cells were lysed by incubating for 15 min on ice in modified RIPA buffer (50 mM Tris-HCl, pH7.4, 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1mM EGTA, 1 mM PMSF, 1μg aprotinin, leupeptin, pepstatin, 1 mM Na_3VO_4, 1 mM NaF), and the protein content of the supernatant was determined by Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). An equal volume of 2x loading buffer (100 mM Tris-HCl, pH 6.8,

Table I Primers for PCR.

primer	sequence	position
eb1	5'-CCCTTCTTGACCAGTATAGCTGC-3'	-1416 ～ -1438
eb2	5'-AAAGATTCCAGAAGATATGCC-3'	-676 ～ -695
eb3	5'-AAAGATTCCAGAAGATATGCC-3'	-425 ～ -444
eb4	5'-AAAGATTCCAGAAGATATGCC-3'	-246 ～ -265
ebr	5'-AAAGATTCCAGAAGATATGCC-3'	-142 ～ -161

200 mM dithiothreitol, 4% SDS, 10% glycerol, 0.2% bromophenol blue) was added to the supernatant, and this was boiled for 5 min. Equal amounts of protein from each extract (15μg per lane) were separated using 10% polyacrylamide gels and transferred onto PDVF membranes, ImmobilonP (Millipore, Eschborn, Germany). After having blocked with 5% dried milk in TBS (10 mM Tris-Cl pH 7.5, 150 mM sodium chloride), the membranes were incubated with the primary antibody, anti-HER2 mouse monoclonal antibody, Ab-3 (Oncogene Research Products,Cambridge, MA) for 24 h at 4℃. After washing, the membrane was incubated with 12μl (per 6 ml) of horseradish peroxidase-conjugated rabbit anti-mouse IgG, EnVision + (Dako, Carpinteria, CA). Staining was carried out using the ECL kit (Amersham, Buckinghamshire, England), according to the manufacturer's instructions. Signals were measured using Light Capture (ATTO, TOKYO), and analyzed with a Software, CS-Analyzer(ATTO). Data are expressed as relative light units per microgram protein.

Luciferase assay Reporter plasmids were generated as follows. PCR of genomic DNA, which extracted from lymphocytes of a healthy volunteer, was performed using eb1, eb2, eb3 or eb4 as forward primers, and ebr, as a reverse primer (Table I). 1300bp, 555bp, 330bp or 125bp HER2 promoter fragments produced by PCR were subcloned into multicloning site of pGL3-Basic vector (Promega, Madison, WI) which located upstream to the luciferase gene. The nucleotide sequence and direction of the inserts were checked by sequencing. pGL3-Promoter (Promega) and pRL vector (Promega) were used as a positive control or an internal control(Fig. 1).

Plasmids were transfected into cultured cells using Effectin Reagent (Qiagen, Hilden, Germany). The luciferase assay was carried using 'Dual luciferase assay kit' (Promega) as manufacturer's instruction. Signals were measured by Lumat LB9507 (Perkin Elmer , Boston, MA). The luciferase activity observed was expressed by the ratio to the levels obtained in parallel transfections with the parent, promoterless pGL3-Basic plasmid.

Generation of recombinant vectors The 555bp promoter fragment was excised from pGLHER2/neu555, and subcloned into pCANCre[14] after removing CAG promoter to yield pHER2Cre. To construct a cosmid vecter, pAxHER2Cre, the HER2-Cre-poly(A) expression cassette from the pHER2Cre was ligated to the SwaI site of pAxcw cosmid[14] (Fig. 2a, 2b). To produce a recombinant adenovirus, AxHER2Cre, the expression cosmid pAxHER2Cre and adenovirus DNA terminal protein complex were transfected into the 293 cells by calcium phosphate precipitation. Recombinant adenovirus was isolated from single plaque and expanded in the 293 cells. The virus solution was stored at -80℃. The virus titer was measured by plaque assay on 293 cells as described[15]. AxCANCre[14] and AxCALNLZ[14] (Fig. 2c) were kindly provided from Dr I. Saito (Institute of Medical Science, University of Tokyo)

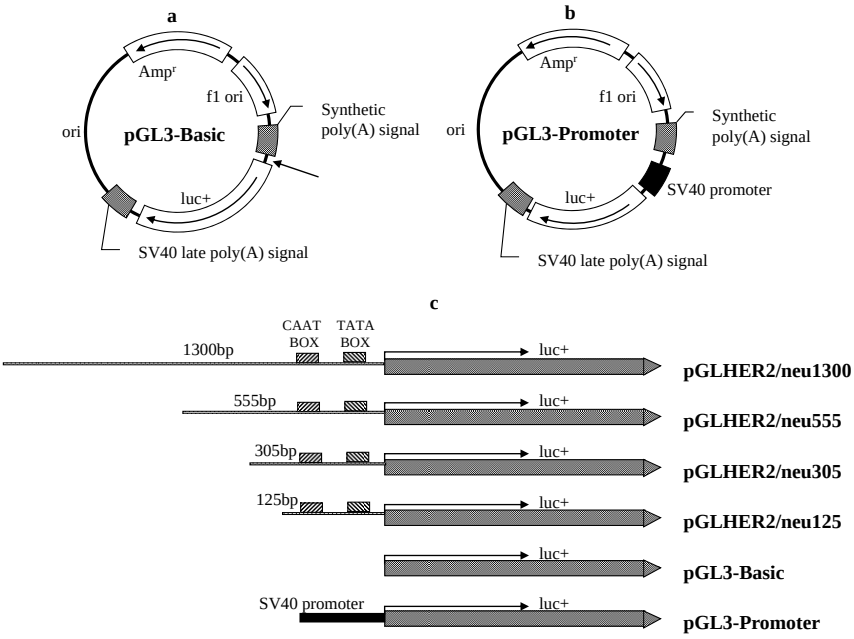

Fig. 1 Circular map of the control luciferase reporter plasmids(a,b) and HER2 promoter deletion constructs(c). Arrow shows the subcloning site of HER2 promoter fragments (a). The regions of HER2 5' flanking region used in each construct are indicated(c). The 3' end point of all construct was the BglII site at -140 which was fused to luciferase gene (Luc+).

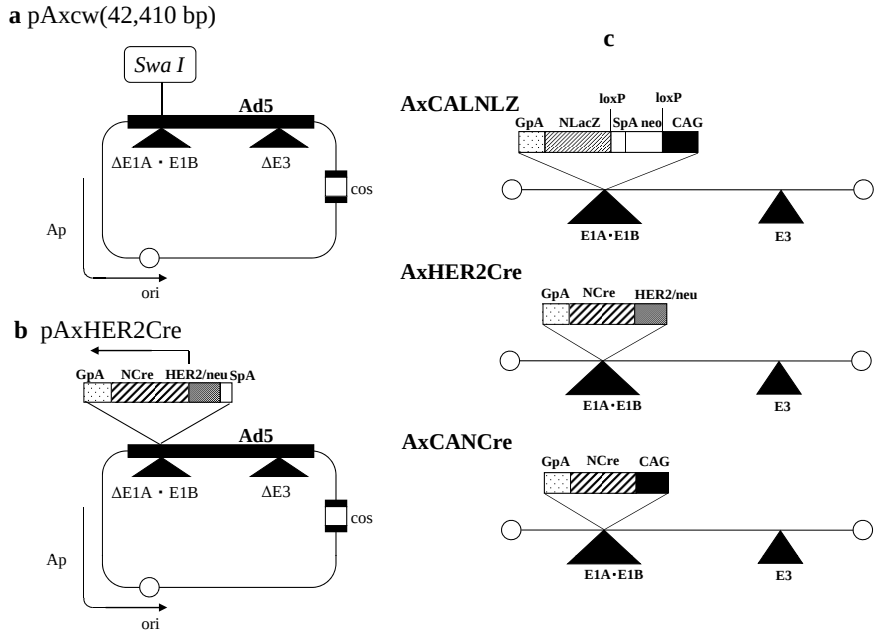

Fig. 2 Structure of cosmids(a,b) and Cre-mediated activation units of LacZ gene and recombinant adenovirus(c). Also shown is the position of the SwaI restriction endonuclease site (a). SpA, SV40 early poly(A) site, HER2/neu, 555bp HER2/neu promoter, GpA, rabbit β-globin poly(A) site, loxP, loxP site, ▲, deletion of an adenovirus sequence.

In vitro transduction by recombinant adenovirus vectors During the exponential growth phase, cells were plated in 6-well culture plates (Becton Dickinson, SanJose, CA) at a density of 5 X 10^5 cells/well. Twenty-four h later, the culture medium was aspirated, and suspension of adenovirus at various MOI (multiplicity of infection) was distributed on to the monolayers.

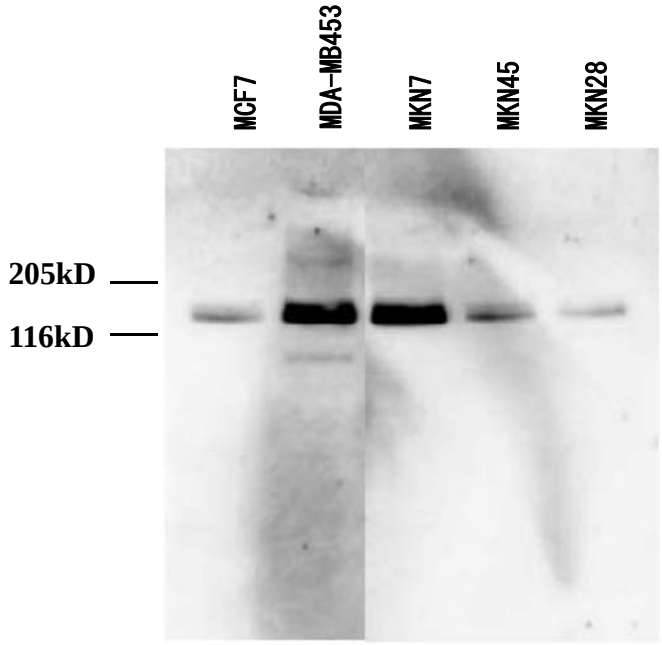

Fig. 3 Expression of HER2/neu protein in human stomach cancer cell lines and breast cancer cell lines.

Table II Production of HER2/neu protein in human cancer cell lines.

cell line	MCF7	MDA-MB-453	MKN7	MKN45	MKN28
Relative light unit	4.94×10^5	2.10×10^6	2.05×10^6	9.44×10^5	2.48×10^5

After 48-h culture, β-D-galactosidase expression was evaluated. For cell staining, the cells were washed twice with phosphate buffered saline, fixed with 0.25% glutaraldehyde and stained with 0.1% 5-bromo-4-chloro-3-indolβ-D-galacto-pyranoside (X-gal, WAKO, Osaka). To quantify β-galactosidase activity, 3 X 10^5 infected cells were disrupted by sonication and 1/10 th volume of lysate was subjected to the color reaction with o-nitrophenyl-β-D-garactopyraniside (ONPG). Absorbance was measured using Titertek Multiscan (EFLAB, Finland). Relative β-galactosidase activity was expressed by the average of OD420 of lysates.

RESULTS

Production of HER2/neu protein in human cancer cell lines HER2/neu protein levels were measured by immunoblotting. In all of cell lines tested, 185 kd bands were detected (Fig. 3). Among stomach cancer cell lines, HER2/neu protein were expressed 8.3-fold and 3.8 fold more in MKN-7 and MKN-45 than in MKN-28 by the quantification (Table Ⅱ). In breast cancer cell lines, MDA-MB-453 expressed 4.3-fold more than MCF-7 (Table Ⅱ).

Transcriptional activity of HER2/neu promoter Except pGLHER2/neu125, all chimeric plasmid, pGLHER2/neu1300, pGLHER2/neu555 and pGLHER2/neu305 showed moderate to high luciferase activity in stomach cancer cell lines. Transcriptional activities by both pGLHER2/neu1300 and pGLHER2/neu555 were 3 to 5-fold higher in HER2-overexpressing cell line, MKN-7, relative to low HER2-expressing MKN-28 cells.

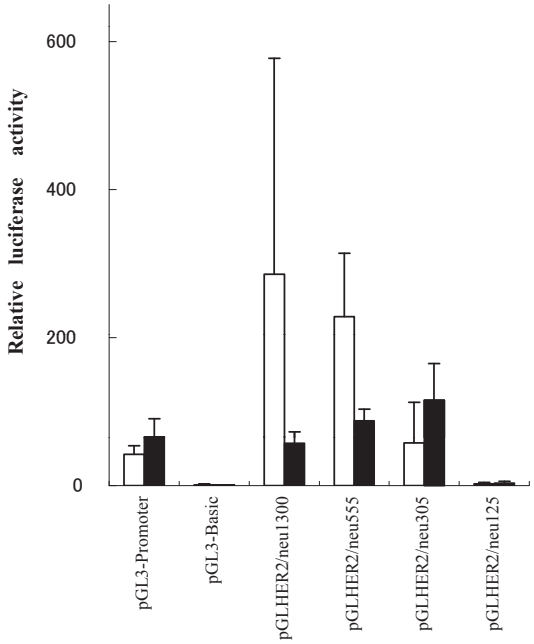

Fig. 4 Luciferase assay. The averages of the triple experiments were shown. □, MKN-7 and ■, MKN-28.

On the other hand, pGLHER2/neu305 showed 2-fold more activity in MKN-28 than in MKN-7 (Fig. 4).

Tranduction efficiency of adenovirus vecters When the LacZ gene expression in MKN7, a HER2-overexpressing stomach cancer cell line, was examined, the double infection of AxHER2Cre and AxCALNLZ (MOI of 50, each) induced intense staining in about 90% of cells. The double infection of AxCANCre and AxCALNLZ (MOI of 50, each) was about 100%. On the other hand, in the case of the MKN28 cells, a HER2-low expressing stomach cancer cell line, the double infection of AxHER2Cre and AxCALNLZ induced only about 20% staining, though double infection of AxCANCre and AxCALNLZ gave about 80%. In the case of breast cancer cell lines, the HER2-overexpressing MDA-MB-453 showed much higher rate of staining cells than low HER2-expressing MCF-7.

β-galactosidase enzymatic activity induced by two recombinant adenovirus vecters with Cre/loxP system At MOIs of 10-25, the mixture of two recombinant adenovirus vectors, AxHER2Cre and AxCALNLZ, showed no significant difference of MKN-7 cells and MKN-28 cells. At a MOI of 50, co-infection of those vectors yielded about 3-fold more β-galactosidase activity in MKN-7 than MKN-28. At a MOI of 100, these two recombinant adenovirus vectors with Cre/loxP system induced markedly high enzymatic activity, comparable to the activity by co-infection of AxCANCre and AxCALNLZ, in MKN-7 cells, though the activity was low in MKN-28 cells(Fig. 5A). In the case of MDA-MB-453 and MCF-7, almost the same results were obtained(Fig. 5B).

DISCUSSION

Among the stomach cancer cell lines we tested, HER2 protein levels were high in MKN-7, moderate in MKN-48, and low in MKN-28. These results were consistent with a previous report[16]. In breast cancer cell lines, HER2 levels were high in MDA-MB-453, and low in MCF-7. These results also agreed with other report[17].

There was a report of marked reduction

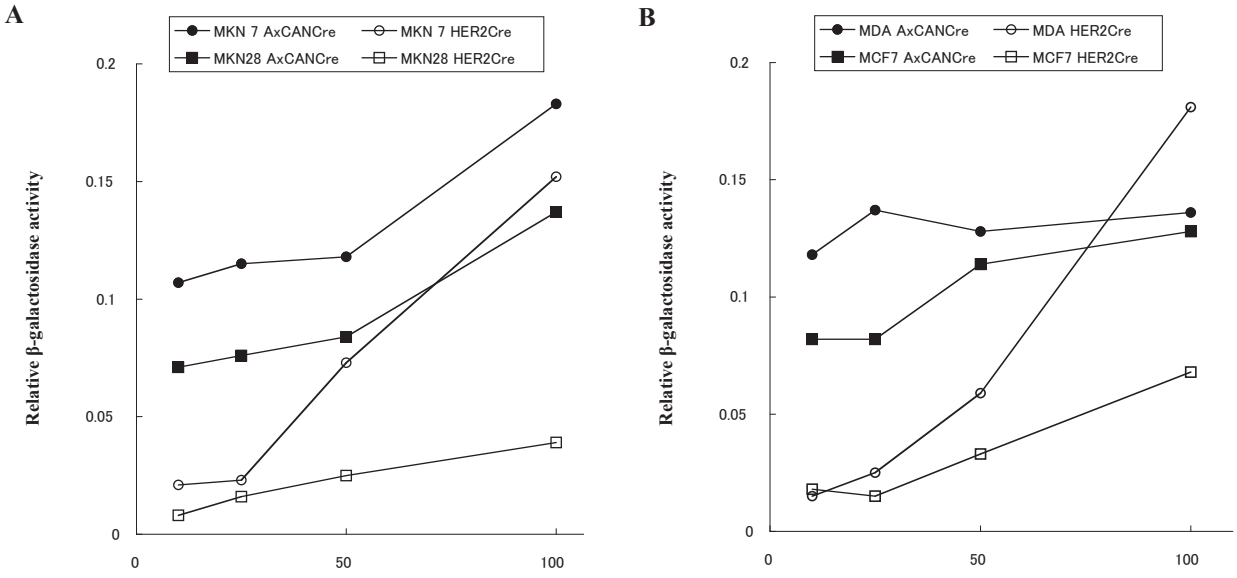

Fig. 5 Quantification of the β-galactosidase activity. Relative β-galactosidase activity was expressed by the average of OD420 of lysates. Stomach cancer cells(A), Breast cancer cells(B).

in CAT activity on deletion from -213 to -100 indicating that an important transcriptional activating elements lies between these two deletion end points in breast cancer cell line, ZR75-1[18]. However, in our results, 125bp promoter, which contained whole sequence of those elements, showed little transcriptional activity, though 305 bp or longer promoter exhibited moderate to high activities in MKN-7. The reason of this result was unclear, but it may be due to the defference of the tissue types from which those cells derived.

OB2-1, which is responsible for up-regulating transcription of HER2 gene, the binding site was shown to lie within 0.5 kb upstream from the transcription start site of HER2 gene and 0.5 kb promoter had the greatest promoter activity[18]. Our results showed that 555 bp and 1300 bp promoter exhibit relatively high transcriptional activity and HER2-expression specificity, coincident with those reports.

CAG promoter is consisting of cytomegalovirus IE enhancer, chickenβ-actin promoter and rabbitβ-globin polyadenylation[poly(A)] signal[19], known as a quite strong promoter, and applied widely for gene transduction systems. By the double infection of the AxHER2Cre and AxCALNLZ, we obtained quite high efficiency of gene transduction, which was comparable to the combination of AxCANCre and AxCALNLZ. The induction rates were also HER2-expression specific in both stomach cancer cell lines and breast cancer cell lines.

The β-galactosidase activity by the co-infection of the AxCANCre and AxCALNLZ was very high even at MOI 10, and the activity did not show marked increase by increase of MOI. The β-galactosidase activity by the co-infection of the AxHER2Cre and AxCALNLZ was increased almost linear up to MOI 100. By the quantification analysis, the activity induced by those recombinant adenovirus vectors was confirmed to be comparable to the combination of AxCANCre and AxCALNLZ. The induced activity was also HER2-expression specific.

Recently, cancer specific gene therapies using two recombinant adenovirus vectors with AFP promoter or CEA promoter and Cre/loxP

systems were reported[20]. Those systems had distinct advantages compared to the single viral vector infection method. The HER2 promoter and Cre/loxP system, we established, exhibit high efficiency and specificity. Using suicide genes, e.g. cytosine deaminase, herpes simplex virus thymidine kinase, as the 'target' gene, this system can be applied for another cancer specific gene therapy.

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REFERENCE

1) Sikola K, Harris J, Helen H, Lemoine N. Therapeutic strategies using c-erbB-2 promoter-controlled drug activation. Ann NY Acad Sci 1994;31:115-24.

2) Slamon DJ, Ckark GM, Wong SG, Levin WJ, Ulrich A, Mcguire WL. Human breast cancer :correlation of relapse and survival with amplification of the HER2/neu oncogene. Science 1987;235:177-82.

3) Hall PA, Hughes CM, Staddon SL, Richman PA, Gullick WJ, Lemoine NR. The c-erbB-2 proto-oncogene in hunman pancreatic cancer. J Pathol 1990;161:195-200.

4) Lemoine NR, Jain S, Silvestre F, Lopes C, Hughes CM, McLelland E, Gullick WJ, et al. Amplification and overexpression of the EGF receptor and c-erbB-2 oncogenes in human stomach cancer. Br J Cancer 1991;64:79-83.

5) Lovekin C, Ellis IO, Locker A, Robertson JF, Bell J, Nicholson R, Gullick WJ, et al. c-erbB-2 oncoprotein expression in primary and advanced breast cancer. Br J Cancer 1991;63:439-43.

6) Tetu B, Brisson J. Prognostic significance of HER-2/neu oncoprotein expression in node-positive breast cancer. The influence of the pattern of immunostaining and adjuvant therapy. Cancer 1994;73:2359-65.

7) Tolaney SM, Krop IE. Mechanisms of trastuzumab resistance in brerast cancer. Anticancer Agents Med Chen 2009;9:348-55.

8) Sato Y, Tanaka K, Lee G, Kanegae Y, Sakai Y, Kaneko S, Nakabayashi H, et al. Enhanced and specific gene expression via tissue-specific production of Cre recombinase using adenovirus-vector. Biochem Biophys Res Commun 1998; 244:455-62.

9) Kaneko S, Hallenbeck P, Kotani T, Nakayashi H, McGarrity G, Tamaoki T, Anderson WF, et al. Adenovirus-mediated gene therapy of hepatocellular carcinoma using gene expression. Cancer Res 1995;55:5283-87.

10) Richards CA, Austin EA, Huber BE. Transcriptional regulatory sequences of carcinoembrionic antigen: identification and use with cytosine deaminase for tumor-specific gene therapy. Hum Gene Ther 1995;6:881-93.

11) Kanegae Y, Takamori K, Sato Y, Lee G, Nakai M, Saito I. Efficient gene activation system on mammalian cell chromosomes using recombinant adenovirus producing Cre recombinase. Gene 1996; 181:207-12.

12) Kijima T, Osaki T, Nishino K, Kumagai T, Funakoshi T, Goto H, Tachibana I, et al. Application of the Cre recombinase/loxP system further enhances antitumor effects in cell type-specific gene therapy against carcinoembryonic antigen-producing cancer. Cancer Res 1999;56:4906-11.

13) Ueda K, Iwasashi M, Nakamori M, Nakamura M, Yamaue H, Tanimura H. Enhanced selective gene expression by adenovirus vector using Cre/loxP regulation system for human carcinoembryonic antigen-producing carcinoma. Oncology 2000;59: 255-65.

14) Kanegae Y, Lee G, Sato Y, Tanaka M, Nakai M, Sasaki T, Sugano S, et al. Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific Cre recombinase. Nucleic Acids Res 1995;23:3816-21.

15) Hersh J, Crystal RG, Bewig BB. Modulation of gene expression after replication deficient recombinant adenovirus-mediated gene transfer by product of a second adenovirus vector. Gene Ther 1995;2:124-31.

16) Kameda T, Yasui W, Yoshida K, Tsujino T, Nakayama H, Ito M, Ito H, Tahara E. Expression of erbB-2 in human gastric carcinomas:relationshio between $p185^{ERBB2}$ expression and the gene amplification. Cancer Res 1990;20:8002-9.

17) Scott GK, Daniel JC, Xiong X, Maki RA, Kabat D, Benz CC. Binding of an ETS-related protein within the DNase I hypersensitive site of the HER2/neu promoter in human breast cancer cells. J Biol Chem 1994;269:19848-58.

18) Hollywood DP, Hurst CH. A novel transcription factor, OB2-1, is required for overexpression of the proto-oncogene c-erbB-2 in mammary tumour lines. EMBO J 1983;12:2369-75.

19) Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 1991;108:193-200.

20) Sakai Y, Kaneko S, Sato Y, Kanegae Y, Tamaoki T, Saito I, Kobayashi K. Gene Therapy for hepatocellular carcinoma using two recombinant adenovirus with α-fetoprotein promoter and Cre/loxP system. J Virol Meth 2001;92:5-17.