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ORIGINAL ARTICLE

THE SUICIDE GENE INTRODUCTION USING TWO RECOMBINANT ADENOVIRUS VECTORS WITH HER2 PROMOTER AND Cre/loxP SYSTEM

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Abstract We examined the therapeutic efficacy for the suicide gene introduction using two recombinant adenovirus vectors with HER2 promoter and Cre/loxP system in human gastric cancer cell lines, MKN-7 and MKN-28. HER2 protein level was more expressed in MKN-7 than in MKN-28. Next, we constructed a Cre recombinae expression vector in HER2-producing cell specifically, AxHER2NCre and AxCALNCD expressing cytosine deaminase (CD) gene under the control of the CAG promoter by the Cre switching system. Much higher CD messenger RNA (CD mRNA) and CD protein expression were induced in cells by the double infection method than by AxCALNCD only. Furthermore, CD mRNA and CD protein expression were induced higher in HER2-overexpressing cell line, MKN-7 than in MKN-28. We examined the efficacy of cell growth inhibition using 5-fluorocytosine (5-FC) as anti-tumor prodrug. Inhibition effect was dose-dependent at each cell line and rate was more in MKN-7 in comparison with MKN-28. This system can be applied for HER2-overexpressing cancer specific gene therapy.

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Key words: HER2 promoter; Cre/loxP system; suicide gene; CD/5-FC.

原　著

Cre/loxP システムを利用したアデノウィルス二重感染法による自殺遺伝子誘導

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抄録　HER2 プロモーターを用いて，Cre/loxP システムを利用したアデノウィルス二重感染法による自殺遺伝子導入を計画し，治療効果を検討した．胃癌細胞株 MKN-7 で，同種株 MKN-28 よりも HER2 蛋白の発現が大であった．次に，HER2 発現腫瘍細胞特異的に Cre を発現する AxHER2NCre 及び Cre 存在下に自殺遺伝子 Cytosine deaminase(CD)を発現する AxCALNCD の二つのアデノウィルスベクターを作製した．各細胞に，ベクターを二重感染させた群(誘導群)では AxCALNCD のみを感染させた群よりも CD mRNA 及び CD 蛋白の発現量が大であった．5-fluorocytosine(5-FC)をプロドラッグとした抗腫瘍効果を検討したところ，各細胞において濃度依存的に細胞増殖抑制効果が認められ，HER2 高発現株である MKN-7 では MKN-28 よりも強い抑制効果を認めた．本方法は，他の HER2 発現腫瘍細胞においても適応できるものと考えられた．

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キーワード：HER2 プロモーター；Cre/loxPシステム；自殺遺伝子；
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Introduction

HER2 is a tyrosine kinase and a member of epidermal growth factor receptor subgroup. HER2 is overexpressed in a number of types of cancers frequently[1-4], including non-small cell lung cancer, breast cancer, pancreatic cancer, gastric cancer, ovarian cancer. Expression of this gene in noncancerous tissue is not comparable to that in tumor cells. Thus, the expression of HER2 is also related to the poor prognosis[5, 6]. Hence, the HER2-overexpressing tumor should be an important therapeutic target.

Adenovirus vector is a highly efficient, and is widely used for gene transduction. However, recombinant adenovirus infects various cell types and induced immune responses and inflammation when administered at a high dose. Therefore, 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[7].

Consequently, tissue or tumor specific promoters are used to regulate the gene expression. However, activity of tissue specific or tumor specific promoters are relatively low[7-9]. To enhance the expression level by the tumor specific promoters, the two recombinant adenovirus with hepatocarcinoma-specific α-fetoprotein (AFP) promoter or colonic cancer-specific carcinoembryonic antigen (CEA) promoter and Cre/loxP system were developed[10-12]. In the previous study[13], we verified that a 'regulator' recombinant adenovirus AxHER2NCre express Cre-recombinase under the control of HER2 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. LacZ gene expression by the HER2 promoter was remarkably enhanced and maintained its specificity using the Cre/loxP regulation system in vitro.

CD is a microbial enzyme that can convert the anti-fungal agent 5-FC into the anti-tumor agent 5-fluorouracil (5-FU)[14]. The expression of CD should only be required for a period several days to two weeks. Therefore, delivery systems that yield short-time episomal gene expression using adenovirus should be suitable for CD/5-FC suicide gene therapy[9]. In this study, we examined whether the combination of CD and 5-FC was efficient for suicide gene therapy in HER2-producing tumor cells without loosing its specificity.

Materials and Methods

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

Generation of recombinant vectors. The 555bp promoter fragment was excised from pGLHER2/neu555, and subcloned into pCANCre[15] after removing CAG promoter to yield pHER2/neuNCre. To construct a cosmid vector, pAxHER2/neuNCre, the HER2/neu-Cre-poly (A) expression cassette from the pHER2/neuNCre was ligated to the SwaI site of pAxcw cosmid. A cosmid vector pAxCALNCD was constructed by ligating the CD fragment into SwaI site of the pAxCALNw cassette [15] (Fig. 1A, 1B). To produce recombinant adenovirus, AxHER2NCre and AxCALNCD, the expression cosmid pAxHER2/neuNCre, pAxCALNCD and adenovirus DNA terminal protein complex were transfected into 293 cells by calcium phosphate precipitation, respectively[16]. Recombinant adenovirus were isolated from single plaque and expanded in the 293 cells. The virus

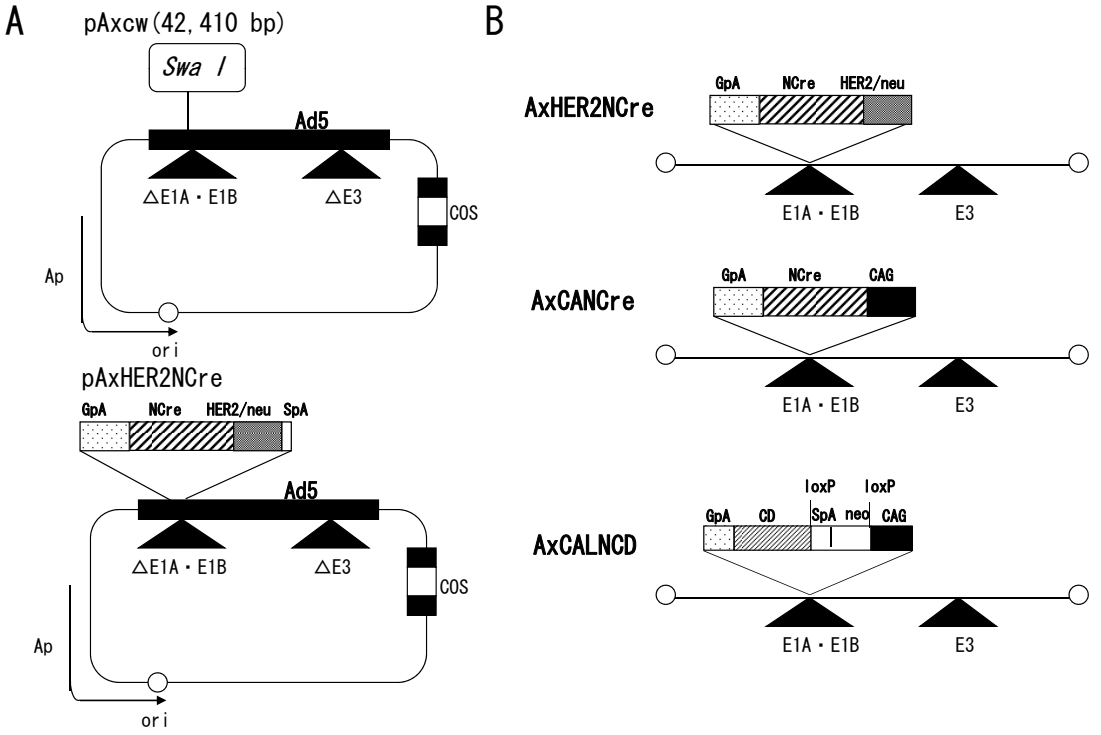

Fig. 1 A: Structure of cosmids. **B**: Cre-mediated activation units of CD gene and recombinant adenovirus. Also shown is the position of the SwaI restriction endonuclease site. SpA, SV40 early poly (A) site; HER2/neu, 555bp HER2/neu promoter; GpA, rabbit β-globin poly (A) site; loxP, loxP site; ▲, deletion of an adenovirus sequence. A cassette cosmid bearing an expression cosmid (pAxHER2/neuNCre and pAxCALNCD) was cotransfected into 293 cells together with adenovirus DNA terminal protein complex to produce recombinant adenovirus, AxHER2NCre and AxCALNCD.

Table 1 Sequence of TaqMan probe and primers for CD gene and glyceraldehydes-3-phosphate dehydrogenase (GAPDH)

	Sequence	Corresponding cDNA sequence
TaqMan Probe	FAM-CACGCCTGTTCCGCTTGCTGAAAAT-TAMRA	340-364
Forward Primer	FAM-ACGGCAATGCACTCCTATAACG-TAMRA	306-327
Reverse Primer	FAM-CGGGTTGGCGACAAAGTTAA-TAMRA	392-373
GAPDH	JOE-CAAGTTCCCGTTCTCAGCC-TAMRA	243-262

solution was stored at −80℃. The virus titer was measured by plaque assay on 293 cells as described[17].

Real-time PCR. Using TaqMan real-time PCR, the samples were checked for expression rate of CD mRNA. Using the Primer Express Software (Applied Biosystems, Foster City, CA), we designed specific primers and fluorescence-labeled probes (TaqMan probes; Applied Biosystems) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene (internal standard; GenBank Accession No. B1977763) (Table 1). Total RNA from each group (the double infection group and the single infection group) was extracted using an RNeasy Mini Kit (QUIAGEN, Inc., Palo Alto, CA). The RNA sample was subjected to reverse transcription using TaqMan Reverse Transcription Reagents

(Applied Biosystems) to prepare cDNA. Reverse transcription was performed under the following condition: 25℃ for 10 min, 48℃ for 30 min and 95℃ for 5 min. Then, cDNA was subjected to 40 cycles of the PCR using a TaqMan PCR Reagents Kit, with each cycle consisting of reactions at 50℃ for 2 min, 95℃ for 10 min 15 s, and 60℃ for 1 min. Threshold cycle (Ct), was obtained from PCR reaction curves monitored by the ABI PRISM 7700 (Applied Biosystems) for expression rate of CD mRNA and GAPDH, followed by calculation of differences between each group. The Mann-Whitney *U* test was used to test the significance of differences. $P<0.05$ was regarded statistically significant. For each combination, experiments were repeated three times.

Immunoblot analysis. During the exponential growth phase, cells were plated in 6-well culture plates (Becton Dickinson, SanJose, CA) at a density of 5×10^5 cells/well. 24h later, the culture medium was aspirated, and the cells were infected with recombinant adenovirus, AxHER2NCre and AxCALNCD at MOI (multiplicity of infection) 5. One was the double infection group (AxHER2NCre and AxCALNCD), other single infection group (AxCALNCD only). After 48h culture, the protein was collected and immunoblot analysis was performed. Equal amounts of protein from each extract (40μg per lane) were separated using 10% polyacrylamide gels and transferred onto PDVF membranes, Immobilon P (Millipore, Eschborn, Germany). After having blocked with 5% dried milk in TBS (10mM Tris-HCl, pH 7.5, 150mM sodium chloride), the membranes were incubated with the primary antibody, anti-Cytosine Deaminase sheep monoclonal antibody (Biogenesis) for 24h at 4℃. After washing, the membranes were incubated with 4μl (per 4ml) of rabbit anti-sheep Ig-AS (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 the Software, CS-Analyzer (ATTO). Date are expressed as relative light units per microgram protein. For each combination, experiments were repeated three times.

MTT-dye reduction assay. Cells were seeded in 96-well plates (100μl/well at a density of 3×10^5 cells/ml), grown for 24h, and exposed to various concentrations (0, 6.25, 12.5, 25, 50, 100μg/ml) of 5-FC after infection with AxHER2NCre and AxCALNCD at MOI 5 or AxCALNCD only at MOI 5 or neither. The survival fraction was estimated with MTT [3- (4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide] dye reduction assay as described by Mosmann[18] with some modifications. Briefly, after 72h incubation with 5-FC, MTT solution (10mg/ml in phosphate-buffered saline) was added (10μl/well). Plates were further incubated for 4h at 37℃. Thereafter, the formazan crystals formed were dissolved by adding 100μl of 0.04 *N* HCl in 2-propanol. Absorption was measured with a plate-reader, Titertek Multiscan (Titertek, Huntsrill, AL) at 540nm. Cell growth inhibition rate (Inhibition rate) was expressed by the ratio of median optical density (O.D) at 540nm in the following equation.

Inhibition rate (%) = 100-[(O.Dw/O.Ds)×100]

O.Dw: median optical density of cells infected with AxHER2NCre and AxCALNCD.

O.Ds: median optical density of cells infected with AxCALNCD only.

For each concentration and combination, experiments were repeated two times. RPMI1640 medium (100μl) with 10μl of MTT solution and 100μl of 2-propanol was used as blank solution.

Results

Induction of CD mRNA and CD protein by the double infection method. In our previous

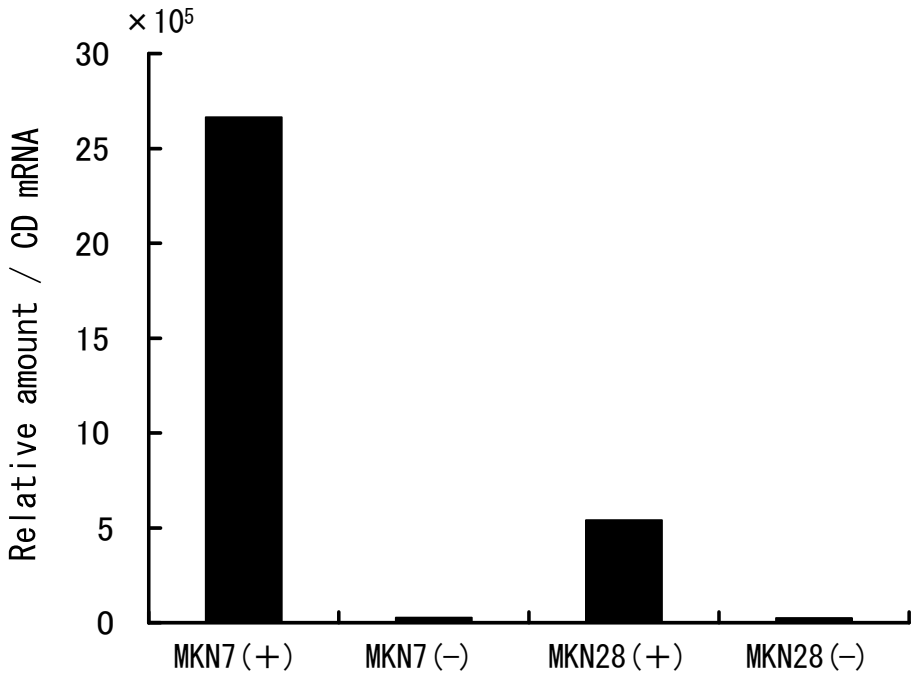

Fig. 2 Relative amount of mRNA for CD are shown. (+), the double infection group (AxCALNCD+AxHER2NCre); (-), the single infection group (AxCALNCD only).

study[13], HER2 protein levels in human gastric cancer cell lines, MKN-7 and MKN-28, were measured. HER2 protein level was higher in MKN-7 than MKN-28. After infection with the double infection group (AxHER2NCre and AxCALNCD) or the single infection group (AxCALNCD only) respectively, total RNA extracted from each cell line was tested by real-time PCR. CD mRNA was induced more in cells infected with the double infection as compared to cells infected with the single infection, and expression rate of CD mRNA was higher in MKN-7 than in MKN-28 (Fig. 2). Similarly, protein from each cell line after infection with each group was tested by western blotting. A single band due to CD protein was induced in each cell line between 116kd and 205kd. Much higher CD protein was induced in cells by the double infection method than cells by the single infection. Furthermore, CD protein was induced higher in HER2-overexpressing cell line, MKN-7, than in low HER2-expressing cell line, MKN-28 (Fig. 3).

Efficacy of cell growth inhibition. In HER2-overexpressing cell line, MKN-7, the inhibition rate by 6.25, 12.5, 25, 50 and 100μg/ml of 5-FC was 19.4, 21.9, 30.2, 32.6 and 33.8% respectively. In low HER2-expressing cell line, MKN-28, the inhibition rate at each concentration of 5-FC was 18.4, 19.7, 20.8, 22.4 and 24.7% respectively. Inhibition effects were dose-dependent. Inhibition rate by 5-FC was higher in MKN-7 compared to MKN-28, especially at 25 and 50μg/ml of 5-FC (Fig. 4). AxHER2NCre by itself did not change optical density of cells at each concentration of 5-FC in both MKN-7 and MKN-28 (data not shown).

Discussion

HER-overexpression has been observed in the various patients, making HER2 a diagnostic marker. Among the gastric cancer cell lines we tested, HER2 protein levels were high in

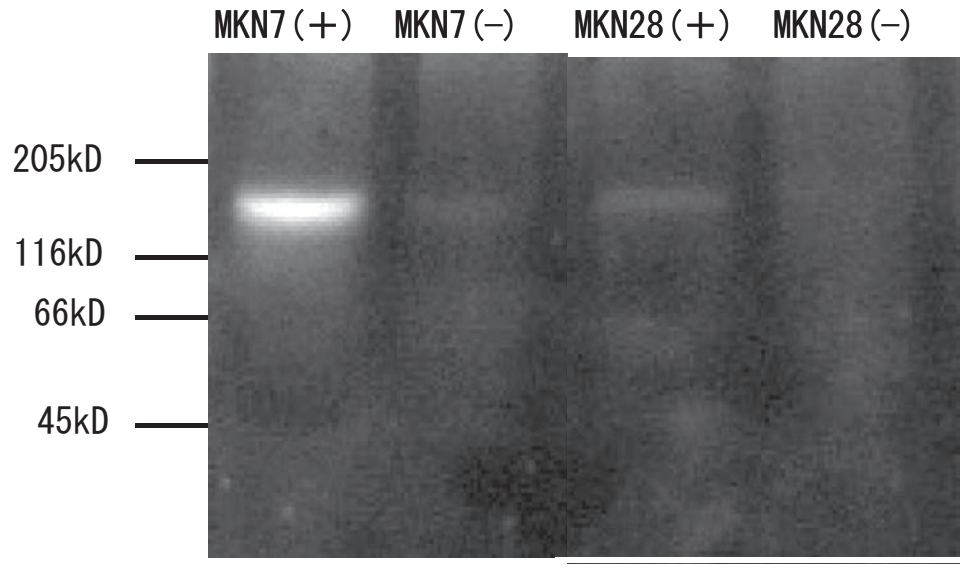

Fig. 3 Expression of CD protein in human gastric cancer cell lines. (+), the double infection group (AxCALNCD+AxHER2NCre); (-), the single infection group (AxCALNCD only).

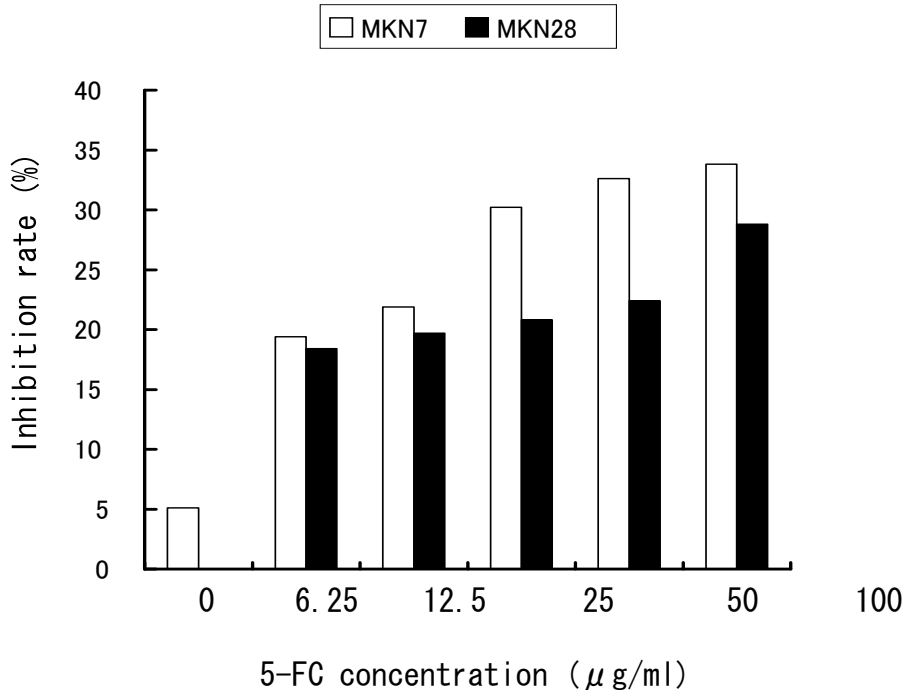

Fig. 4 MTT dye-reduction assay. Cell growth inhibition rate (inhibition rate) at various concentrations of 5-FC. Inhibition rate=100-[(O.Dw/O.Ds)×100]. □, MKN-7; ■, MKN-28.

MKN-7 and low in MKN-28. These results were consistent with a previous report[19].

CAG promoter, consisting of cytomegalovirus IE enhancer, chicken β-actin promoter and rabbit β-globin polyadenylation [poly (A)] signal[20], is known as a quite strong promoter and applied widely for gene transduction systems. In previous study, by the double infection of the

AxHER2NCre and AxCALNLZ, we obtained quite high efficiency of gene transduction, which was comparable to the combination of AxCAN-Cre and AxCALNLZ[13].

Recently, cancer specific gene therapies using two recombinant adenovirus vectors with AFP promoter or CEA promoter and Cre/loxP systems were reported[21]. Those systems had distinct advantages compared to the single viral vector infection method. The HER2 promoter and Cre/loxP system, we established, exhibited high efficiency and specificity. In this study, we used suicide gene, cytosine deaminase (CD), as the 'target' gene. The CD/5-FC system has been developed as suicide gene therapy due to the advantage of the system as strong bystander effect, and its effectiveness in vitro and in vivo had been demonstrated in many cancer cell lines[22-28]. We constructed two recombinant adenovirus vectors AxHER2NCre expressing Cre in HER2-producing cells and AxCALNCD expressing the CD gene under the control of CAG promoter by the Cre switching system. It was found that the double infection of human gastric cancer cells followed by exposure to 5-FC, was cytotoxic to cells in vitro.

Generally, 5-FC as anti-fungal drug is taken into fungus cell and CD converts the nontoxic prodrug 5-FC to the cytotoxic agent 5-FU. 5-FU is anabolized to the active metabolites 5-fluorouridine triphosphate (FUTP) and 5-fluorodeoxyuridine monophosphate (FdUMP) by several steps of an enzyme reaction series and is catabolized to dihydrofluorouracil (FUH_2) by dihydropyrimidine dehydrogenase (DPD). FUTP is incorporated into RNA (F-RNA) and impairs the multiple functions of RNA, whereas FdUMP blocks the catalytic activity of thymidylate synthase (TS) by forming a ternary covalent complex with its cosubstrate, 5,10-CH_2-FH_4, which inhibits DNA synthesis. Conversely, DPD is the first and rate-limiting enzyme of the chain of reactions that regulate 5-FU catabolism. Meanwhile, Mammalian cells cannot convert 5-FC to 5-FU because of a lack of CD. Despite CD expression, a number of tumor cells are 5-FC-resistant, which may be attributable to the lack of an active cytosine transport system in mammalian cells[29]. For this reason, the toxicity of 5-FC is low for mammal. But the cells at rather high dose 5-FC showed morphological changes due to toxicity of 5-FC itself in each cell line (data not shown). This result showed that we need a suitable dose of 5-FC to take efficient and specific therapeutic efficacy.

The double infection with AxHER2NCre and AxCALNCD rendered HER2-producing cells such as MKN-7, HER2-overexpressing cell line, and MKN-28, low HER2-expression cell line more sensitive to 5-FC compared with the single infection with AxCALNCD only. The HER2 promoter and Cre/loxP system, we established, exhibited high efficiency and specificity using suicide gene CD as the 'target' gene. Tumor-specific expression of CD gene by the double infection method will help to decrease potential damage of nontumorous surrounding tissue when the vectors are directly injected into tumors. Research group constituted from members of The New York hospital Cornell medical center and the Rockefeller University hospital have been collaborating on a clinical first phase test using the CD revelation vector, severe hazardous phenomena have not been recognized[30]. In addition, a clinical test of the gene therapy using the CD revelation vector for metastatic hepatocellular carcinoma will soon start in Japan too. Hereafter, if these tests are executed in various medical institutions, and the safety and efficacy are identified, the gene therapy using CD/5-FC gene system will be able to become a one of the choices in 'order-made therapy'. In HER2-overexpression cancer, this system, we established, using two recombinant adenovirus vectors with HER2 promoter and Cre/loxP system will be able to be expected tumor-specific

efficacy. This system might be applicable for HER2-producing cancer specific gene therapy.

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