Abstract. Colorectal cancer (CRC) is one of the most common malignant tumors worldwide. Loss of imprinting (LOI) of the insulin-like growth factor 2 (IGF2) gene is an epigenetic abnormality phenomenon in CRC. Recently observed association of CRC with cluster of differentiation 147 (CD147) could provide a novel approach for gene therapy. In the present study, we investigated the feasibility of using adenovirus-mediated siRNA targeting CD147 based on the IGF2 LOI system for targeted gene therapy of CRC. A novel adenovirus-mediated siRNA targeting CD147, rAd-H19-CD147mirsh, which was driven by the IGF2 imprinting system, was constructed. The results showed that the EGFP expression was detected only in the IGF2 LOI cell lines (HT-29 and HCT-8), but that no EGFP was produced in cell lines with maintenance of imprinting (MOI) (HCT116). Moreover, rAd-H19-CD147mirsh significantly inhibited the expression of CD147, decreased cell viability and invasive ability, and increased sensitivity to chemotherapeutic drugs only in the LOI cell lines in vitro. Furthermore, mice bearing HT-29 xenografted tumors, which received intratumoral administration of the rAd-H19-CD147mirsh, showed significantly reduced tumor growth and enhanced survival. We conclude that recombinant adenovirus-mediated siRNA targeting CD147 based on the IGF2 LOI system inhibited the growth of the LOI cells in vitro and in vivo, which would provide a novel approach for targeted CRC gene therapy.

Introduction

Colorectal cancer (CRC) was the third most common malignant tumor in 2013 (1). Every year, there are more than 1.2 million new cases and nearly 0.7 million people die of this disease, principally due to the tumor relapse and metastasis (2,3). However, carcinogenesis is a complicated biological process, in which most of the underlying molecular mechanisms are unclear (4). Although limited evidence of molecular biology has been used to explain why several genes are involved in carcinogenesis and development of CRC, many efforts are being made to develop some new interventions that target tumor-specific genes by constructing tumor-selective replicating viruses.

Cluster of differentiation 147 (CD147) or extracellular matrix metalloproteinase inducer (EMMPRIN) is a glycosylated cell surface transmembrane protein of the immunoglobulin superfamily (IgSF), also known as basigin (BSG) (5-7). CD147 has a broad tissue distribution, involved in many physiological processes. Previous studies have indicated that aberrant CD147 expression is observed in several cancers, including CRC (8-14), and that increased CD147 expression is seen in CRC and is associated with poor prognosis (15). In addition, CD147 is also involved in multidrug resistance (MDR) of cancer cells (16), and thus changes in its expression levels can be used to predict tumor relapse and patient outcome (11,14). Despite the importance of CD147 in CRC, whether it is feasible to target CD147 for gene therapy in CRC is still unknown.

Genomic imprinting is an epigenetic modification of a gene, which is mono-allelic expression (17). Some genes are expressed in only one allele that is known as maintenance of imprinting (MOI). In contrast, the reactivation of the silenced allele of an imprinted gene (which leads to expression of both paternal and maternal alleles) is loss of imprinting (LOI). Insulin-like growth factor 2 (IGF2) is identified as the first endogenous imprinted gene, which is expressed only in paternal alleles, and regulated by the enhancer, DNA differentially methylated domain (DMD) and promoter (18).

Upregulated expression of IGF2 has been detected in CRC, indicating that IGF2 LOI may serve as a potential biomarker for diagnosis of CRC (19,20). In our previous studies, we found...
that IGF2 LOI is present in both colorectal tissues and cancer cell lines (HCT-8 and HT-29), but that such a phenomenon is not observed in normal cells (21-23). Importantly, we successfully constructed a recombinant adenovirus that carries the IGF2 imprinting system and it is specially expressed in the IGF2 LOI tumor cells, with the greater potential for targeted gene therapy for CRC (21-23). In this study, we constructed the adenovirus-mediated siRNA that targets CD147 and carries the IGF2 imprinting system, and investigated its effects on the efficacy of gene therapy for CRC as a novel therapeutic strategy.

Materials and methods

Cell culture. HT-29, HCT-8 and HCT116 (human colon cancer cell lines) were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). The HEK293 cell line (human embryonic kidney cells containing the E1A region of the adenovirus) was obtained from Microbix Biosystems, Inc. (Ontario, Canada). HEK293, HT-29, HCT-8 and HCT116 cell lines were, respectively, maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (all from HyClone, Logan, UT, USA). Cells were incubated in a humidified incubator at 37˚C with 5% CO₂. Both HT-29 and HCT-8 cell lines were characterized by IGF2 LOI, whereas HCT116 cell line was known to possess IGF2 MOI (21-23).

Plasmid construction and incorporation into adenoviral vectors. The original adenoviral shuttle plasmid used in this study was pYr-mir30-shRNA which included the enhanced green fluorescent protein (EGFP) gene sequence, provided from the Changsha Yingrun Biotechnology Co., Ltd., Hunan, China. The 808–828 fragment of CD147 gene was selected as the RNAi target site, and the scrambled control sequence was also synthesized from the Changsha Yingrun Biotechnology Co., Ltd. These oligonucleotides were annealed and subcloned into the BsaI sites of the pYr-mir30-shRNA vector. These recombinant vectors were designated as pYr-mir30-CMV-control-shRNA and pYr-mir30-CMV-CD147mirsh, respectively. The IGF2 imprinting system, enhancer-DMD-H19 sequence (1798 bp), and pYr-mir30-CMV-CD147mirsh, respectively. The IGF2 imprinting system, enhancer-DMD-H19 sequence (1798 bp), and pYr-mir30-CMV-CD147mirsh, respectively. The IGF2 imprinting system, enhancer-DMD-H19 sequence (1798 bp), and pYr-mir30-CMV-CD147mirsh, respectively. The IGF2 imprinting system, enhancer-DMD-H19 sequence (1798 bp), and pYr-mir30-CMV-CD147mirsh, respectively.

Analysis of EGFP expression in the constructed adenovirus. HT-29, HCT-8 and HCT116 cells were, respectively, infected with rAd-control, rAd-CMV-CD147mirsh, and rAd-H19-CD147mirsh with multiplicity of infection (MOI) of 10 plaque forming units (PFU)/cell. EGFP expression was examined at 48 h after infection using an Olympus microscope with a fluorescent filter set (excitation 450–490 nm).

Analysis of the expression of CD147 mRNA in virus-infected cells by real-time quantitative PCR (RT-qPCR). The CD147 mRNA expression was determined by RT-qPCR. The HT-29, HCT-8 and HCT116 cells were infected with rAd-control, rAd-CMV-CD147mirsh and rAd-H19-CD147mirsh (10 PFU/cell), respectively. After 24 h, total RNA was extracted from the three cell lines with TRIZol (Invitrogen Life Technologies) according to the manufacturer's instructions. Following treatment with DNase I (Takara Bio, Inc., Otsu, Japan) at 37˚C for 30 min, RNA quantification was performed using spectrophotometry. The RNA (1 µg) was subsequently incubated with 1 µl of Oligo(dt) primer (50 µM), 1 µl of Random 6 mers (100 µM), 1 µl of PrimeScript™ RT Enzyme Mix I, 4 µl of 5X PrimeScript™ Buffer and RNA-free dH₂O, and first-strand cDNA synthesis was performed in a total volume of 20 µl. The primer sequences used for CD147 and β-actin are listed in Table I. The PCR reactions were performed in a LightCycler apparatus using real-time PCR Master mix SYBR-Green I (Toyobo Biotech Co., Ltd., Osaka, Japan). Thermocycling was done in a final volume of 25 µl containing 1 µl of cDNA sample, 0.5 µl of the up-primer, 0.5 µl down-primer, 12.5 µl of SYBR-Green Real-Time PCR Master mix, and 10.5 µl of dH₂O. After 15 sec at 95˚C to denature the cDNA and to activate Taq DNA polymerase, the cycling conditions were as follows: 40 cycles consisting of denaturation at 95˚C for 5 sec, annealing at 60˚C for 5 sec, and extension at 72˚C for 30 sec. The Ct used in the real-time quantification PCR was defined as the PCR cycle number that crossed an arbitrarily chosen signal threshold in the log phase of the amplification curve. To verify the fold change of CD147 gene expression, calculated Ct values were normalized to Ct values of β-actin amplified from the same sample (ΔCt= Ct_ΔCD147 - Ct_β-actin), and the 2^ΔΔCt method was used to calculate changes in gene expression. Each sample had
Antisense: 5'-CCGTTCATGAGGGCCTTGTC-3'

Analysis of CD147 protein expression by western blot analysis. The CD147 protein expression was evaluated by western blot analysis. Cells were harvested and lysed by three cycles of freeze/thaw at -80°C. Total protein was separated by 10% SDS-PAGE gels, and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% skimmed milk powder (soluble in TBST buffer solution) was used at room temperature under sealed conditions for 2 h, the membrane was incubated with mouse anti-CD147 primary antibodies (1:500) and rabbit anti-human β-actin primary antibodies (1:500) at room temperature for 2 h, followed by secondary antibodies conjugated to horseradish peroxidase in a 1:2,000 dilution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h at room temperature. The proteins were visualized by ECL detection system (Boster Inc., Wuhan, China).

Analysis of the cytotoxic effect of the adenoviruses by CCK-8. Cytotoxicity was assessed by Cell Counting Kit-8 (CCK-8) (Beyotime, China). Cells were seeded in 96-well plates at a density of 1x10^5 cells/100 µl/well, and then infected with adenoviruses at 10 PFU/cell for 24, 48 and 72 h, respectively. After addition of 10 µl of CCK-8 into the medium, each plate was measured at 450 nm with a microplate reader (Bio-Rad Laboratories, Richmond, CA, USA).

In vitro invasion assay. Transwell plates (Corning Inc., Acton, MA, USA) were coated with basement membrane Matrigel (20 mg/ml; Becton-Dickinson, Franklin Lakes, NJ, USA) for 4 h at 37°C. After the Matrigel was solidified, 1x10^5 cells were seeded onto the Matrigel and infected with adenoviruses at 10 PFU/cell for 24 h. Cells that migrated through the permeable membrane were fixed with 100% methanol for 10 min. The membrane with cells was soaked in 0.1% crystal violet for 10 min and then washed with distilled water. The number of cells attached to the lower surface of the polycarbonate filter was counted at x400 magnification under a light microscope. Each assay was carried out in triplicate and repeated three times.

Drug sensitivity assay. To assess the sensitivity to the cancer drug, HT-29, HCT-8 and HCT116 cells (1x10^5 cells/well) were seeded in triplicates on 96-well plates and then infected with rAd-control, rAd-CMV-CD147mirsh, and rAd-H19-CD147mirsh, respectively. After 24 h, the cells were treated with cisplatin or oxaliplatin (Sigma, St. Louis, MO, USA) with varying concentrations at 0.1, 1 and 10 µM for 48 h, respectively. The cytotoxicity was assessed using CCK-8 assay as described above. The absorbance was measured with a microplate reader at 450 nm (Bio-Rad Laboratories).

Treatment of tumor-bearing nude mice with the recombinant adenovirus. HT-29 cells were trypsinized to a single cell suspension and resuspended in 10^5 cells/100 µl PBS, then subcutaneously injected into the flank area of adult (8-week-old) athymic male nude mice. The protocol was approved by the Experimental Animal Center of University of Yangzhou, Yangzhou, China. Two weeks after injection of HT-29 cells, the developed tumors were measured in two dimensions, then rAd-H19-CD147mirsh were injected into the growing tumor, and rAd-CMV-CD147mirsh, and rAd-control served as viral vector controls. Each adenoviral vector (a total dosage of 10^9 PFU/mouse) was injected into a growing tumor from three directions for 3 consecutive days, and the tumor volume was observed for 28 days. Tumor dimensions were measured, and the tumor volume was calculated according to the formula (width)^2 x length x 0.5. Mice were euthanized 30 days post-inoculation. Harvested tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4 µm, and stained with hematoxylin and eosin (H&E). Mouse survival was recorded in a separate experiment. Animal experiments were performed in accordance with the Institutional Guidelines for Animal Care by the Nanjing Medical University, China.

Statistical analysis. Statistical analysis of data was conducted by SPSS software. Experimental data are presented as the mean ± standard deviation (SD) and assessed by Student's t-tests and one-way ANOVA at a significance level of P<0.05.

Results

Construction and characterization of the recombinant adenovirus. The recombinant adenovirus rAd-H19-CD147mirsh was successfully constructed in this study, and the control virus, rAd-CMV-CD147mirsh and rAd-control, were also constructed. After infection of cells with rAd-control, rAd-CMV-CD147mirsh, and rAd-H19-CD147mirsh (10 PFU/cell) for 24 h, respectively, we tested the applicability of the expression system through detection of the expression of EGFP, and observed that the expression of EGFP protein was positive in the three cell lines (HT-29, HCT-8 and HCT116) as shown in Fig. 1.

The recombinant adenovirus-mediated gene silencing inhibits CD147 expression in colon cancer cells. We also examined CD147 mRNA and protein expression in these cell lines (HT-29, HCT-8 and HCT116) after infection with rAd-control, rAd-CMV-CD147mirsh and rAd-H19-CD147mirsh (10 PFU/cell), respectively. Cells were harvested to determine CD147 mRNA expression by RT-PCR at 24 h after infection, and CD147 protein expression by western blot analysis at 48 h after infection. As shown in Fig. 2, a significantly reduced CD147 mRNA and protein expression was seen in HT-29, and HCT-8 cells (LOI) treated with rAd-CMV-CD147mirsh, and rAd-H19-CD147mirsh compared with rAd-control, respectively (P<0.01). The same results were also seen in HT-29 cells (P<0.05),

<table>
<thead>
<tr>
<th>Target</th>
<th>Primers</th>
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<tr>
<td>CD147</td>
<td>Sense: 5'-CCATGCTGGTCTGCAAGTCAG-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'-CCGTTCATGAGGGCCTTGTC-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>Sense: 5'-CTGGAACGGTGAAAGGTGACA-3'</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5'-AAAGGACCTCCTGAACACGCA-3'</td>
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Table I. Primers of CD147 and β-actin for real-time PCR.
but not in HCT-8 cells (P>0.05), which were both infected with rAd-CMV-CD147mirsh vs. rAd-H19-CD147mirsh. Interestingly, the expression of CD147 was significantly reduced in HCT116 cells (MOI) when treated with rAd-CMV-CD147mirsh vs. rAd-control, but there was no marked difference when treated with rAd-H19-CD147mirsh vs. rAd-control, suggesting that the recombinant adenovirus carrying IGF2 imprinting system could be specially expressed in the LOI cells.

CD147 silencing reduces the invasive ability of tumor cells in vitro. To examine whether the downregulation of CD147 in tumor cells could affect their invasive ability, we performed a Matrigel Transwell analysis in vitro. The results showed that the number of HT-29 and HCT-8 cells that passed through the Matrigel was markedly reduced when infected with rAd-CMV-CD147mirsh or rAd-H19-CD147mirsh vs. rAd-control (P<0.05), the same results were observed with the HT-29 cells passed through the Matrigel when infected...
with rAd-CMV-CD147mirsh vs. rAd-H19-CD147mirsh (P<0.05), but that there was no marked difference in the HCT-8 cells infected with rAd-H19-CD147mirsh vs. rAd-CMV-CD147mirsh as measured with the number of the cells that passed through the Matrigel-coated filter (P>0.05). Similarly, the number of the filtered cells was significantly decreased in HCT116 when infected with rAd-CMV-CD147mirsh vs. rAd-control group (P<0.05), but not infected with rAd-H19-CD147mirsh vs. rAd-control group (P>0.05) as shown in Fig. 3.

Inhibition of the growth of colon cancer cells by CD147 silencing. We were interested in examining whether CD147 silencing would effectively suppress tumor cell proliferation. For this purpose, the growth inhibition and cytotoxicity of tumor cells by CD147 silencing were investigated in the cell lines by CCK-8 assay. As shown in Fig. 4, the colon cancer cells (HT-29, HCT-8 and HCT116) infected with rAd-CMV-CD147mirsh for 48 h displayed decreased viability (P<0.05). The rAd-H19-CD147mirsh induced CPEs as efficiently as did rAd-CMV-CD147mirsh only in the LOI cells (HT-29 and HCT-8), but not in the MOI cells (HCT116) infected with rAd-CMV-CD147mirsh.

Increased sensitivity to the chemotherapeutic drug in colon cancer cells by CD147 silencing. CD147 has been found to be over expressed in tumor cells resistant to multiple drugs, and thus could confer resistance to some (if not all) antitumor drugs. In order to test whether CD147 silencing could affect the sensitivity of the tumor cells to the cancer drugs in colon
In the study, we investigated the sensitivity of colon cancer cells to the antitumor drugs cisplatin and oxaliplatin. The proliferation of colon cancer cells (HT-29, HCT-8, and HCT116) was analyzed by CCK-8 assay. The proliferation of HT-29 and HCT-8 cells infected with rAd-CMV-CD147mirsh and rAd-H19-CD147mirsh were significantly downregulated compared with rAd-control (P<0.05). However, rAd-H19-CD147mirsh group had no significant difference compared with rAd-control group (P>0.05). Oxaliplatin did not show any effects on the sensitivity when treated with rAd-CMV-CD147mirsh and rAd-H19-CD147mirsh, respectively, compared with rAd-control in all infected groups (P>0.05).
CD147 silencing inhibits the tumor formation in nude mice. Because the use of RNAi can effectively reduce the proliferative ability of colon cancer cells in vitro, we investigated the antitumor efficacy of RNAi in vivo. Every 4 days after the date of vaccination, we measured the length and width of the tumors and calculated their volume. As shown in Fig. 6A, tumor growth of the HT-29 cells was slower when infected with rAd-H19-CD147mirsh or rAd-CMV-CD147mirsh than with rAd-control groups after 12 days, respectively (P<0.05), but there was no significant difference between rAd-CMV-CD147mirsh and rAd-H19-CD147mirsh group (P>0.05). Moreover, the average duration of survival for mice treated with rAd-control, rAd-CMV-CD147mirsh and rAd-H19-CD147mirsh were 41, 93 and 85 days, respectively, as shown in Fig. 6B. The results showed that the survival time of the mice was obviously prolonged when injected with rAd-CMV-CD147mirsh or rAd-H19-CD147mirsh vs. rAd-control (P<0.05).

**Discussion**

CD147, a multifunctional glycoprotein, forms homo-oligomers in a cis-dependent manner in the plasma membrane (24), is commonly overexpressed in many tumors, and is associated with tumor progression and invasion (25,26). Previous studies have indicated that CD147 can regulate colon cancer growth by mediating tumor-host interactions (27), and that CD147 is associated with the lymph node metastasis in CRC patients. This suggests that CD147 contributes to the progression of CRC and may serve as a diagnosis marker of CRC (28).

In general, only paternal $IGF2$ alleles expressed and maternal alleles closed, which is known as the MOI, however, the maternal $IGF2$ allele abnormally expressed, triggered by the abnormal binding of insulator CTCF to DMD, which was caused by the impaired function of CTCF or the hypomethylation status of DMD (29,30). $IGF2$ LOI as a hallmark of various human neoplasms has been widely investigated (31,32). In addition, upregulation of $IGF2$ has been detected in CRC, indicating $IGF2$ LOI may serve as a potential biomarker in diagnosis of CRC (19,20).

Based on the above findings, we have constructed the recombinant adenovirus carrying $IGF2$ imprinting system, which is specially expressed in $IGF2$ LOI tumor cells (21-23). Also based on the association of CD147 with the development of colon and its high expression in CRC, we constructed the adenovirus-mediated siRNA targeting CD147 that carries the $IGF2$ imprinting system Ad-H19-CD147mirsh, recombinant adenovirus rAd-CMV-CD147mirsh as a positive control, and rAd-control as a negative control, respectively. Initially the utility of our expression system was tested using EGFP reporter
assays. The results showed that the EGFP was detected in both LOI and MOI cell lines (HT-29, HCT-8 and HCT116) infected with rAd-CMV-CD147mirsh or rAd-control, respectively. The EGFP was detected only in LOI colon cancer cell lines (HT-29 and HCT-8), with no marked changes due to increased time and multiplicity of the infections, suggesting that the recombinant adenovirus carrying IGF2 imprinting system could be specifically expressed in the LOI colon cancer cells. We used RT-PCR and western blotting to detect CD147 expression at mRNA and protein levels, respectively. The results showed that the expression of CD147 was reduced significantly in the LOI colon cancer cell lines (HT-29 and HCT-8) infected with rAd-CMV-CD147mirsh or rAd-H19-CD147mirsh, respectively, compared with rAd-control. Moreover, the rAd-H19-CD147mirsh exerted a potent inhibitory effect on HT-29 cells, which was similar to rAd-CMV-CD147mirsh, but this inhibition was impaired with HCT-8 cells, possibly due to the difference in the ability of adenovirus infection across the different cells. All of the above results indicate that the adenovirus-mediated siRNA targeting CD147 that carries the IGF2 imprinting system is practicable and effective.

To examine whether the downregulation of CD147 in colon cancer cells could affect their proliferation and invasive ability, we performed CCK-8 and Matrigel Transwell analysis in vitro. The results revealed that CD147 silencing reduced the proliferation and invasive ability in both MOI and LOI cell lines, but that there was no significantly change of proliferation and invasive ability in MOI cell line (HCT116) infected with rAd-H19-CD147mirsh. This suggests that CD147 may play a potential role in promoting proliferation and invasion of colon cancer cells.

MDR occurred in tumor cells, which is the main cause of treatment failure and mortality in colon cancer patients, abnormal expression of CD147 was also observed in many MDR cancer cells (33), and the CD147 could decrease the sensitivity of the cancer cells to certain chemotherapeutic drugs (34,35).

In the present study, we found a possible role of CD147 in drug resistance in colon cancer cells. Our results demonstrated that CD147 silence increased chemosensitivity to cisplatin in both MOI and LOI colon cancer cells, but that there was no significant difference in response to oxaliplatin in the cells after CD147 silencing. In addition, there was no effect in MOI cells (HCT116) infected with rAd-H19-CD147mirsh vs. control, because the high expression of CD147 could not be inhibited, indicating that the expression of CD147 is closely related to drug resistance in colon cancer cells. Therefore, CD147 regulated the sensitivity of colon cancer cells to the cancer drug, but the underlying mechanism is still unclear. We also carried out animal experiments to validate the results. After use of the colon cancer cell line HT-29 as a model, we observed that the currently developed therapeutic strategy of CD147 silence can significantly inhibit the tumor formation and potentiate the death of the cancer cell death in vivo.

In conclusion, our results show that the adenovirus-mediated siRNA targeting CD147 carrying the IGF2 imprinting system, rAd-H19-CD147mirsh, confers a significant anti-tumor effect by inhibiting CD147 expression, in particular in the LOI colon cancer cells, but neither had effect in MOI colon cancer cells nor toxic side effects on the normal cells (i.e., IGF2 MOI). In terms of aberrant expression of CD147 and IGF2 LOI in the cancer cells, the use of recombinant viruses in the context of the IGF2 LOI system and upregulated CD147 shows promise as a novel approach for targeted CRC gene therapy.

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