Assessment of a Capsid-modified E1B 55-kDa Protein-deficient Adenovirus Vector for Tumor Treatment*

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Abstract ONYX-015 and H101 are E1B 55-kDa protein-deficient replicating C group adenoviruses that are currently in clinical trials as antitumor agents. However, their application in cancer gene therapy is limited by the native tropism of C group adenoviruses. This is in part due to low expression of the C group adenovirus receptor (coxsackievirus-adenovirus receptor, CAR) on malignant tumors. An H101-based chimeric virus vector containing sequences encoding the Ad35 fiber domain instead of the Ad5 fiber (H101-F35) was constructed. This modification allowed infection of tumor cells through CD46, a membrane protein over-expressed on tumors. The CAR and CD46 RNA expression was evaluated by RT-PCR method. H101-F35 conferred a stronger cytocidal effect than H101 and ONYX-015 in tumor cell lines that lacked CAR expression (MDA-MB-435 and MCF-7), while the cytocidal effect of H101-35, H101 and ONYX-015 was similar in high-level CAR expressing cancer cell lines (A549, NCI-H446, Hep3B, LNCaP, ZR-75-30 and Bcap-37). In an MDA-MB-435 xenograft mouse tumor model, tumor growth in mice receiving H101-F35 was significantly inhibited compared with mice injected with H101. These results suggest that the chimeric oncolytic adenovirus H101-F35 vector might be a useful candidate for gene therapy of cancer.

Key words adenovirus, transductional control, oncolytic

E1B 55-kDa-gene deleted oncolytic viruses (H101 and ONYX-015) have been tested in human clinical trials [1~6]. Currently, the mechanism for tumor-specific replication of E1B 55-kDa-gene deleted Ads is not entirely clear. The E1B 55-kDa protein is known to bind with cellular p53 and to repress its transcriptional activity. A role for p53 and p53 pathway components is thought to be involved in conferring viral selectivity [7]. On the other hand, a number of groups have argued that ONYX-015 replication is independent of the host cell's p53 status [8,9]. Recent studies suggest a role of Parc (p53-associated parkin-like cytoplasmic protein), which binds to p53 in the cytoplasm and prevents its transport into the nucleus [10]. Differences in Ad infectivity of tumor cells or in expression of anti-apoptotic genes might also account for the discrepancy in different studies with regards to tumor-specificity of ONYX-015[11, 12].

Human coxsackievirus and adenovirus receptor (CAR) are important for efficient C group adenovirus (including Ad2 and Ad5) infection[12]. Recent studies demonstrated a lack or low level of CAR expression on tumors in situ or on freshly isolated tumor cells[13~15]. Low level or lack of CAR expression limits the tumor cell infection and the anti-tumor efficacy of Ad2- and Ad5- based oncolytic adenoviruses[16,17]. To address this problem, numerous strategies for targeting of Ad5 vectors to tumors are currently being explored. These include the use of bispecific conjugates[18~20] and genetic modification of Ad capsid. Approaches to genetic modifications of Ad capsids include alterations of fiber[21,22], hexon[23], penton base[24], or protein Ⅸ[25]. A capsid-modified Ad vector that contains the short-shafted Ad type 35 fiber instead of the Ad5 fiber

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(Ad5F35) appeared to efficiently infect cells expressing low levels of CAR [26,27]. Recently, it is shown that Ad5F35 vectors use CD46 for cell infection[28]. CD46 (membrane cofactor protein, MCP) is a membrane-bound regulatory protein that protects tissues from complement-mediated damage, and is greatly upregulated in all tumors tested so far [29~33], implying that vectors displaying the Ad35 fiber will efficiently transduce malignant tumor cells. Another important feature of Ad5F35 vector is its low affinity to and low infectivity of hepatocytes and Kupffer cells in mice[34] and in baboons[35]. In contrast, systemically applied Ad5 is taken up by normal tissues including normal hepatocytes, and elicits systemic toxicity or systemic inflammatory response[36, 37].

In this study, we have constructed and tested a chimeric H101 virus vector containing the short-shafted Ad type 35 fiber instead of the Ad type 5 fiber (H101-F35). The differences in tumor killing power between H101-F35 and H101 were compared both *in vitro* and *in vivo*.

1 Materials and methods

1.1 Oncolytic adenoviruses

ONYX-015 is an Ad2 based E1B mutant adenovirus in which a deletion of nucleotides 2 496~3 323 and a C to T transition at position 2 022 render the E1B 55-kDa-gene product functionally inactive[38]. The recombinant adenovirus H101 was constructed by Shanghai Sunway Biotech. It was an Ad5 based E1B 55-kDa-gene deficient adenovirus, in which the region of 2 502 bp to 3 327 bp was deleted and there was a C to T transition at position 2 025[1~4].

To prepare H101-F35 employing the short-shafted Ad35 fiber instead of the Ad5 fiber, a pShuttle-H101 plasmid was constructed. A fragment deleted in E1B 55-kDa region from H101 was first amplified by PCR method using primers (CGTGTGTGGTTAACGCCTTT [sense] and ACGACATTAAGTTCCCGGGT [antisense]). The fragment was inserted into pXCI (Microbix, Toronto, Canada) by *Hpa* Ⅰ and *Mun* Ⅰ digestion to generate pXCI-E1a-(E1B 55-kDa-del). pShuttle-H101 was made from pShuttle (Stratagene, CA) modified by *Bsr*G Ⅰ and *Mun* Ⅰ digestion and insertion of the E1a- (E1B 55-kDa-del) cassette excised from pXCI-E1a- (E1B 55-kDa-del).

Pme Ⅰ linearized pShuttle-H101 was recombined with *Swa* Ⅰ / *Pac* Ⅰ digested pAd5F35 in BJ5183 cells. Recombinants were selected on LB agar plates containing 50 mg/L kanamycin. The single colony was grown in 5 ml liquid LB medium containing 50 mg/L kanamycin for less than 8.5 h, in order to minimize the danger of unpredicted recombination events that might go undetected and generate noninfectious adenovirus plasmids. Since pAd-H101-F35 is a low copy number plasmid, overnight plasmid ethanol precipitation at −80℃ was needed after alkaline lysis of the transformed BJ5183 cell pellets and Phenol Chloroform Isopropylalcohol extraction. The pAd-H101-F35 plasmid was identified by size in conjunction with restriction endonuclease analysis. The candidate plasmid was re-transformed into DH5α to preserve the structure of recombinant DNA and to produce large quantities of DNA. Correct plasmid was linearized by *Pac* Ⅰ restriction and transfected into HEK-293 cells. Recombinant viruses were propagated in HEK-293 cells, purified, and plaque titered by standard methods described elsewhere[39~41].

To assess the contamination with $E1B^+$ (wt) Ad, PCR analysis is performed for H101 and H101-F35 using primers for E1a (GACCGTTTACGTGGAG-ACTC [sense] and CAGCCAGTACCTCTTCGATC [antisense]) and primers for a sequence in the E1B region (GCTACATTTCTGGGAACGGG [sense] and GGAACAGCGGGTCAGTATGT [antisense]) according to methods described elsewhere [42,43]. Only virus preparations that contained less than one $E1B^+$ (wt) viral genome in 10^5 genomes were used in these studies.

1.2 Cell lines

We used the following eight cancer cell lines, A549 (lung carcinoma) (ATCC CCL-185), NCI-H446 (lung carcinoma) (ATCC HTB-171), Hep3B (hepatoma) (ATCC HB-8064), LNCaP (prostate carcinoma) (ATCC CRL-1740), MDA-MB-435 (breast carcinoma) (ATCC HTB-129), MCF-7 (breast carcinoma) (ATCC HTB-22), ZR-75-30 (breast carcinoma) (ATCC CRL-1504) and Bcap-37 (breast carcinoma) [44]. All media and tissue culture supplements were obtained from Gibco-BRL (Carlsbad, CA) unless otherwise indicated. All cells were grown in minimum essential medium (MEM) with 10% fetal bovine serum, 2 mmol/L L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mmol/L non-essential amino acids, and 1.0 mmol/L sodium pyruvate, 100 U/ml penicillin, and 100 mg/L streptomycin. Cells were maintained at 37℃ and 5% CO_2.

1.3 Analysis of CAR and CD46 RNA status

The CAR and CD46 RNA statuses of cancer cell lines were analyzed by reverse transcription and polymerase chain reaction (RT-PCR). Total RNA was extracted from semi-confluent cell cultures on 15-cm flasks using the UNIQ-10 total RNA extraction kit (Sangon, Shanghai, China) and analyzed for CAR, CD46 and β-actin RNA with the RNA PCR kit (Takara, Dalian, China) as described by the manufacturer. β-actin RNA was served as an internal control. Briefly, 500 ng of total RNA were reverse-transcribed with random primers (9-mer) and murine leukemia virus reverse transcriptase (30℃ for 10 min followed by 50℃ for 30 min) and amplified by PCR with 50 nmol/L primers using a cycling program (initial step of 95℃ for 120 s, 30 cycles of 95℃ for 30 s and 56℃ for 30 s and 72℃ for 60 s, final step 72℃ for 10 min). The primers used for the analyses were CAR sense (5′ ttgcttgctctagcgctcattggtc 3′), CAR antisense (5′ tcatcacaggaatcgcacccattcg 3′), CD46 sense (5′ gctacctgtctcagatgacg 3′), CD46 antisense (5′ accactttacactctggagc 3′), β-actin sense (5′ caccaactgggacgacat 3′), and beta-actin antisense (5′ tgtcacgcacgatttcc 3′).

1.4 Analysis of the cytocidal effect

The day before Ad infection, 5×10^3 cells were seeded into 96-well plates. Viruses were diluted in culture medium and added to the cells. At the indicated time, cells were washed and incubated with 100 μl phenol-red free MEM (without FBS) containing 20 μl of the CellTiter 96 Aqueous One Solution reagent (MTS assay; Promega, Madison, WI). After 2 h incubation in a 5% CO_2 atmosphere at 37℃, the reaction was stopped by the addition of 10% SDS, and the absorbency in each well was recorded at 490 nm using a plate reader (BIO-RAD, Hercules, CA). The absorbency reflects the number of surviving cells. Blanks were subtracted from all data.

To stain the cells with crystal violet, 4×10^4 cells were seeded on 12-well plates the day before Ad infection. Virus was diluted in culture medium and added to the cells. Four days after virus infection, the medium was removed. Then the cells were washed with phosphate-buffered saline (PBS) and incubated for 15 min in 3% crystal violet in 90% ethanol. After staining, the cells were rinsed three times with water and were air dried for photography.

1.5 Animal studies

Mouse studies were performed in accordance with the institutional guidelines of the Shanghai Second Medical University. Eight- to ten-week-old immunodeficient nude mice (Shanghai Cancer Institute, The Chinese Academy of Science) were housed in specific-pathogen-free facilities. For subcutaneous tumors, a total of 2×10^6 cells in 100 μl of minimum essential medium were injected into the left inguinal regions of nude mice. Subcutaneous tumors were measured with a caliper and calculated as follows: [largest diameter × smallest diameter^2]/2. When tumor volume reached between 80 to 200 mm^3, mice were randomly allocated to different treatment groups (4~6 mice per group). After virus injection, tumor volume was measured twice per week.

1.6 Statistical analysis

The Student *t* test was used throughout the study. Differences between data sets with *P* values of less than 0.05 were considered statistically significant.

2 Results

Transductional targeting is the means to direct adenoviral particles to specific surface receptors expressed on the target cell. In our studies, we utilized pAd5F35 vector that displayed a recombinant Ad5F35 fiber (Figure 1a) [26]. The recombinant fiber gene contained the sequence of human Ad type 35 fiber shaft and knob instead of the human Ad type 5 shaft and knob gene. Ad5F35 fiber has been previously demonstrated to interact with a cellular receptor CD46, which was widely expressed in malignant tumors [29-33]. To generate the pAd-H101-F35 vector, homologous recombination between two fragments in BJ5183 cells was used (Figure 1b). One fragment contained the E1B 55-kDa-gene deficient region from *Pme* Ⅰ digested pShuttle-H101 DNA. Another fragment contained recombinant Ad5F35 fiber from *Swa* Ⅰ and *Pac* Ⅰ digested Ad5F35 vector. An Ad2 based E1B 55-kDa-gene deleted virus displaying Ad2 fiber (ONYX-015) and an Ad5 based E1B 55-kDa-gene deleted virus displaying Ad5 fiber (H101) served as controls (Figure 1a). The control vectors recognized CAR as their cellular receptor.

The cytocidal effect of these oncolytic adenoviral vectors was analyzed in cell lines that expressed low-level CAR but high-level CD46 (MDA-MB-435 and MCF-7) in comparison with cancer cell lines that expressed CAR and CD46 at high levels (A549, NCI-H446, Hep3B, LNCaP, ZR-75-30, and Bcap-37). The expressional levels of CAR and CD46 of these

cell lines were verified by RT-PCR analysis (Figure 2). These cell lines were also selected because of differences in their p53 status, which was thought to affect the oncolytic effect of E1B-55-kDa deleted Ads (Table 1).

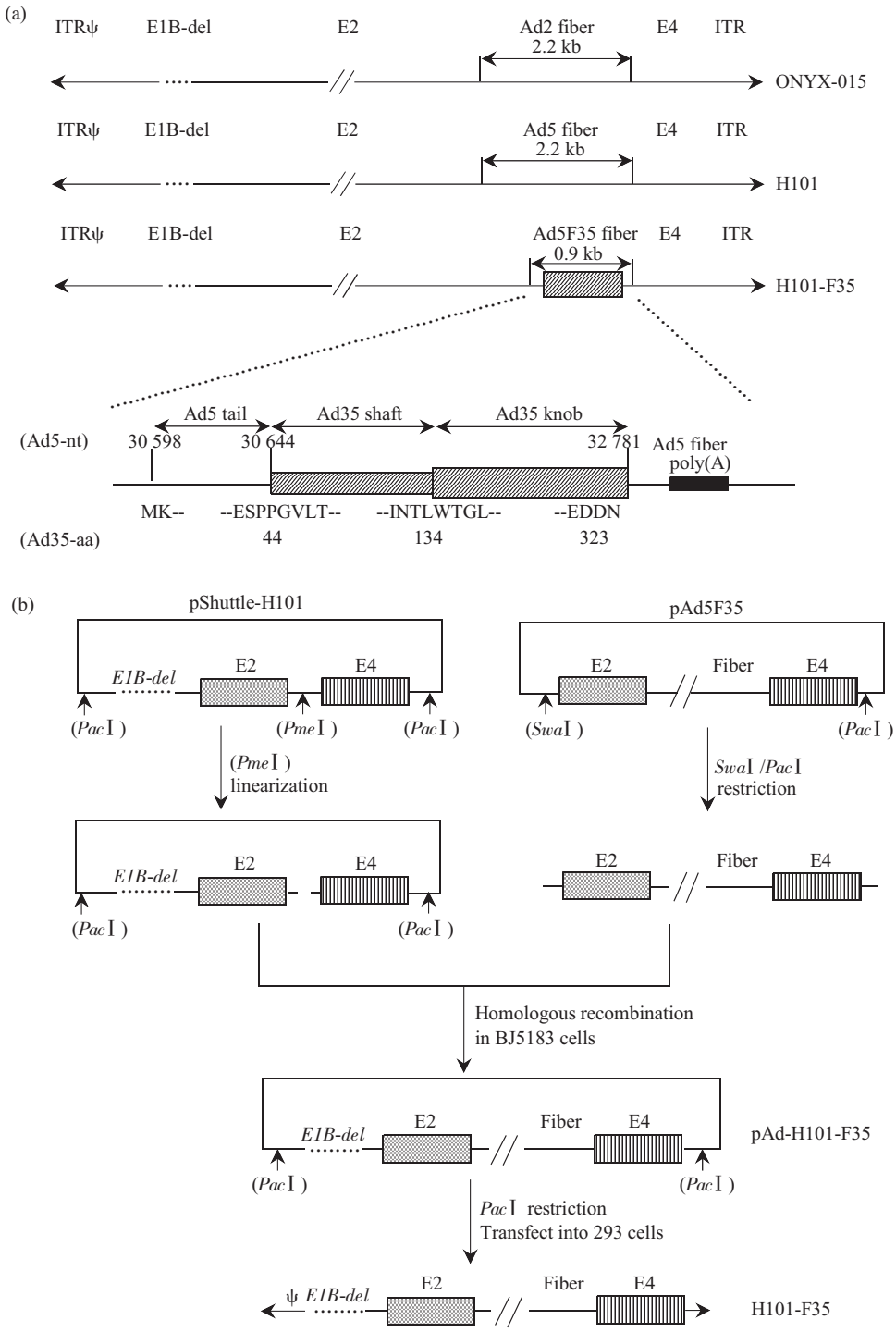

Fig. 1 Viral constructs

(a) Structure of oncolytic virus ONYX-015, H101 and chimeric H101-F35. ONYX-015 is an Ad2 based E1B 55-kDa protein-deficient adenovirus. H101 is an Ad5 based E1B 55-kDa protein-deficient adenovirus. H101-F35 is an H101 based chimeric virus displaying Ad5F35 fiber [26]. (b) Schematic of homologous recombination in BJ5183 cells between *Pme* Ⅰ linearized pShuttle-H101 and *Swa* Ⅰ / *Pac* Ⅰ restricted pAd5F35[26] to produce pAd-H101-F35. H101-F35 virus is obtained after transfect *Pac* Ⅰ restricted pAd-H101-F35 into HEK-293 cells. ITR, inverted terminal repeat.

Table 1 Origin and p53, pRb genotype and CAR, CD46 expression levels of the selected cell lines[1)]

Cell line	Origin	p53 genotype	pRb genotype	CAR expression	CD46 expression
A549	Human lung carcinoma	+ (wt)	+	high	high
NCI-H446	Human lung carcinoma	+ (wt)	–	high	high
Hep3B	Human hepatoma	+ (mut)	–	high	high
LNCaP	Human prostate carcinoma	+ (wt)	+	high	high
Bcap-37	Human breast carcinoma	ND	ND	high	high
ZR-75-30	Human breast carcinoma	+ (mut)	ND	high	high
MDA-MB-435	Human breast carcinoma	+ (mut)	+	low	high
MCF-7	Human breast carcinoma	+ (wt)	+	low	high

[1)]The information about p53 status, pRb status and CAR, CD46 expression level was taken from references [19,44-55]. ND: No corresponding data.

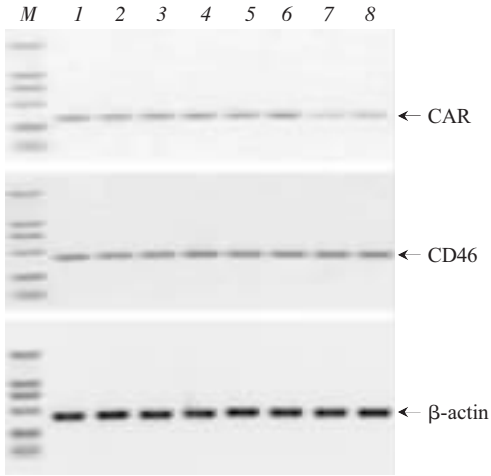

Fig. 2 Evaluation of the CAR and CD46 expression of the tumor cells

M: TAKARA DL-2000 molecular marker; *1*: A549; *2*: NCI-H446; *3*: Hep3B; *4*: ZR-75-30; *5*: Bcap-37; *6*: LNCaP; *7*: MDA-MB-435; *8*: MCF-7. The RNA of the cancer cell lines was analyzed by reverse transcription followed by polymerase chain reaction. The signal for CAR RNA was detected at the position of 325 bp. CD46 RNA was detected at the position of 420 bp. β-actin RNA was detected at the position of 407 bp.

For MTS studies, cell lines were infected with H101-F35 and control viruses H101 and ONYX-015 at an MOI = 200 pfu/cell (Figure 3a). Three days after virus infection, H101-F35 infection resulted in lysis of all cell lines tested, implying that the selected cancer are efficiently transduced and that oncolysis occurred regardless of their p53 status. In contrast, the Ad2 based virus ONYX-015 and Ad5 based virus H101 demonstrated oncolysis only in high-level CAR expressing human cancer cell lines. The oncolytic efficacy of H101-F35, H101 and ONYX-015 was comparable in all high-level CAR and high-level CD46 expressing cancer cell lines. The difference in oncolysis between H101-F35 and H101 was statistically significant in MDA-MB-435 cells and MCF-7 cells ($P < 0.05$). Notably, there was no difference in the oncolytic efficacy of ONYX-015 and H101 in all cancer cell lines tested.

Next, we tested the ability of H101-F35 to induce CPE in tumor cells (Figure 3b). A549, NCI-H446, Hep3B, LNCaP, ZR-75-30, Bcap-37, MCF-7 and MDA-MB-435 are infected with H101-F35, H101 and ONYX-015 at MOIs ranging from 0 to 200 pfu/cell. H101-F35 was more efficient than H101 and ONYX-015 in inducing CPE in MDA-MB-435 and MCF-7 cancer cells. In contrast, there was no difference in the efficiency between H101-F35, H101 and ONYX-015 in A549, NCI-H446, Hep3B, LNCaP, ZR-75-30 and Bcap-37.

The oncolysis over six days after H101-F35 or H101 infection was monitored in MDA-MB-435 cells using MTS method (Figure 4a). Cells started to die at the second day after H101-F35 infection. In contrast, H101 induced cell killing only after the fourth day post virus infection. The differences in oncolysis between H101-F35 and H101 at the second, the third and the fourth day after virus infection were statistically significant ($P < 0.05$).

A xenograft mouse tumor model was used to test the differences between H101-F35 and H101 *in vivo*. An earlier study indicated that the extracellular matrix (ECM) represented a physical barrier to Ad transduction [27]. Since the majority of solid tumors found in patients are encapsulated by ECM [56],

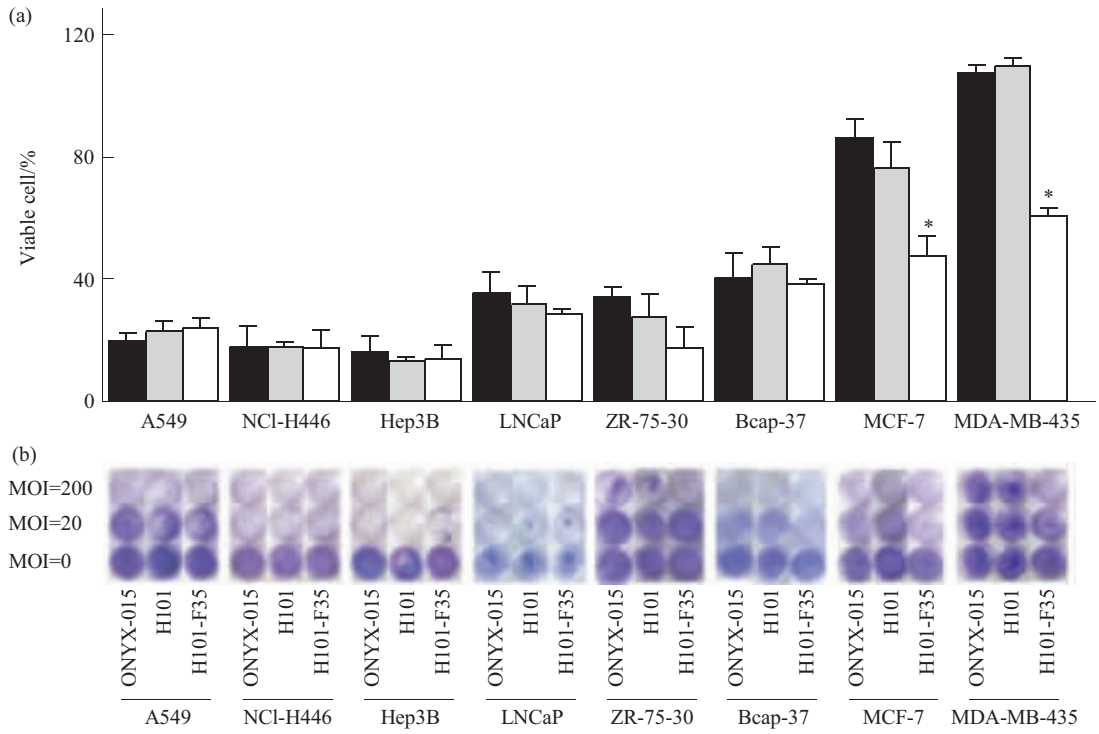

Fig. 3 Analysis of the cytocidal effect

(a) Tumor cells were infected with oncolytic viruses (ONYX-015, H101, H101-F35 or vehicle). At day 3 after virus infection, the percentages of viable cell in virus treated cells (MOI= 200 pfu/cell) versus non-virus treated cells were analyzed using the MTS method. (b) Tumor cells were infected with oncolytic viruses (ONYX-015, H101 and H101-F35) at MOIs ranging from 0, 20 to 200 pfu/cell. At day 4 after virus infection, cells were stained with crystal violet. Notably, ONYX-015 and H101 showed similar cytocidal effect on selected tumor cells, while H101-F35 was more oncolytic in MDA-MB-435 and MCF-7 breast cancer cells. * statistically significant ($P < 0.05$) between H101 treated group and H101-F35 treated group.■: ONYX-015; □: H101; □: H101-F35.

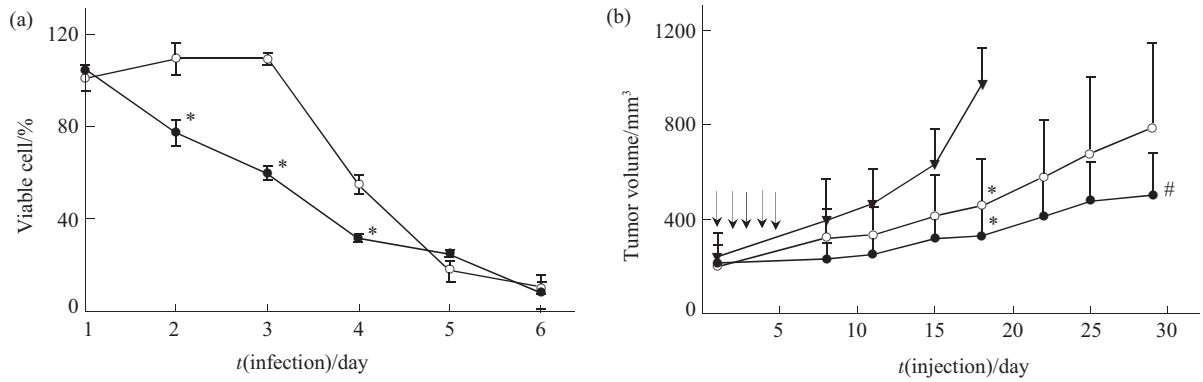

Fig. 4 Analysis of oncolytic efficacy in MDA-MB-435 tumor cells

(a) Tumor cells were infected with oncolytic virus (H101-F35, H101 or vehicle) and the number of surviving cells was analyzed using the MTS method. Shown is the percentages of viable cells in virus infected cells (MOI= 200 pfu/cell) versus mock-infected cells at different time points after infection. Statistical analysis was performed as the difference between H101 treated group and H101-F35 treated group. ●—●: H101-F35; ○—○: H101. (b) Inhibition of growth of subcutaneous tumor xenografts. 2×10^9 pfu of H101-F35 or H101 viruses were daily delivered into pre-established tumors at five consecutive days (indicated as arrows). Tumor size was measured twice per week. Mice were sacrificed when the tumor volume exceeded 1 000 mm^3. Individual data points represent the average volumes of four to six tumors. * Statistically significant ($P < 0.05$) mean tumor size at day 18 compared with that of the vehicle treated control; # Statistically significant ($P < 0.05$) mean tumor size at day 29 between H101 treated group and H101-F35 treated group. ●—●: H101-F35; ○—○: H101; ▼—▼: Vehicle.

intratumoral injection of the oncolytic adenovirus may provide an important accessibility advantage over intravenous injection[57, 58]. Immunodeficient mice were subcutaneously injected with MDA-MB-435 cells. When tumors volume reached between 80 to 200 mm^3, 2×10^9 pfu of Ads are intratumorally administered and tumor growth was monitored over 29 days (Figure 4b). The tumor volumes at day 29 were (501 ±180) mm^3 (for H101-F35), (790 ± 360) mm^3 (for H101) and (1 742 ± 149) mm^3, (for PBS-injected animals). Compared to tumors treated with vehicle (PBS), tumor growth was delayed upon administration of oncolytic virus. It was noted that more significant reduction in tumor growth was achieved in tumors injected with H101-F35 than in tumors injected with H101 at day 29 ($P < 0.05$). This suggested that the increased infectivity conferred by the Ad35 fiber might result in an additional increment in the oncolytic potency.

3 Discussion

The oncolytic efficacy of E1B 55-kDa protein deficient adenovirus depends on the rate of Ad DNA replication. We hypothesize that the status of tumor suppressor gene expression may influence Ad DNA replication and virus oncolytic efficacy. The E1B 55-kDa protein is known to bind cellular p53, to repress its transcriptional activity, and to promote its degradation, thereby leading to an inactivation of p53-mediated checkpoints. Cells containing an intact p53 pathway are thus predicted to inhibit replication of an E1B 55-kDa deficient virus. In contrast, p53-deficient cells would be expected to allow efficient viral replication and subsequent cell killing. However, our studies could not establish a correlation between the oncolytic efficacy and p53 status of test cells, which was in agreement with other studies performed with ONYX-015[8, 9].

The density of adenovirus receptors on tumor cells affects the efficiency of virus internalization, the number of viral genomes that reach the nuclei of infected cells, and hence the level of viral replication and oncolytic effect [48,59]. Ad35 fiber containing Ad vectors infected cells in CAR independent manner and utilized CD46 as a primary attachment receptor [28]. However, Ad35-fiber containing Ad vectors could not efficiently transduce cells with low level expression of CD46 molecules (Lieber A., unpublished observations). In fact, most normal human nucleated cells expressed CD46 in the range of hundred molecules per cell, whereas malignant tumor cells expressed CD46 in the range of hundred thousands or millions molecules per cell[29~33]. After *i.v.* injection of Ad5F35, the inefficient transduction of normal cells was found in baboons, which is a kind of animal model that has CD46 expression patterns and levels that are closely mimic those in humans [60]. Furthermore, the Ad5F35 based vector carrying proapoptotic TRAIL and Ad E1a genes was well tolerated in baboons in a 30-day toxicity study[60]. Since tumors often lack CAR expression but display high levels of CD46 [14~16, 29~33], Ad35-fiber containing vectors might be potentially more efficient in tumor gene therapy trials. The finding that the Ad5F35 based chimeric virus vector H101-F35 efficiently killed tumor cells that are refractory to Ad2 and Ad5 infection, may provide potential implications in tumor gene therapy.

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嵌合型 E1B 55-kDa 蛋白缺陷型腺病毒载体治疗肿瘤的评价 *

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摘要 ONYX-015 和 H101 为可复制 E1B 55-kDa 蛋白缺陷的 C 族腺病毒，它们正作为抗癌药物进行临床研究. 然而它们在癌症基因治疗中的应用却受到了 C 族腺病毒天然特性的制约，部分原因是由于在恶性肿瘤中 C 族腺病毒受体 (coxsackievirus-adenovirus receptor，CAR) 的表达量较低. 构建了一个以 H101 为骨架的含有编码 35 型腺病毒鞭毛区域的基因，替代 5 型腺病毒鞭毛基因的嵌合型腺病毒载体. 这一改动使得腺病毒载体可以通过一种在肿瘤中高表达的膜蛋白 CD46 感染肿瘤细胞. 应用 RT-PCR 方法检测不同肿瘤细胞株中 CAR 和 CD46 表达量的区别. 在 CAR 受体低表达的细胞株中 (MDA-MB-435 和 MCF-7)，H101-F35 表现出比 H101 和 ONYX-015 更强的细胞杀伤效果；在 CAR 受体高表达的细胞株中 (A549，NCI-H446，Hep3B，LNCaP，ZR-75-30 和 Bcap-37)，H101-F35、H101 和 ONYX-015 的细胞杀伤效果则相似. 在荷 MDA-MB-435 肿瘤的裸鼠模型中，注射 H101-F35 的抑瘤效果比注射 H101 的抑瘤效果更明显. 这些结果表明嵌合型溶瘤腺病毒载体 H101-F35 在肿瘤基因治疗中将有很好的应用前景.

关键词 腺病毒，转导调节，溶瘤
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