Measles virus selectively blind to signaling lymphocyte activity molecule has oncolytic efficacy against nectin-4-expressing pancreatic cancer cells

Mutsumi Awano,¹ Tomoko Fujiyuki,¹ Koichiro Shoji,¹ Yosuke Amagai,¹ Yoshinori Murakami,² Yoichi Furukawa,² Hiroki Sato,¹ Misako Yoneda¹ and Chieko Kai¹

¹Laboratory Animal Research Center, The Institute of Medical Science, The University of Tokyo, Tokyo; Divisions of ²Molecular Pathology; ³Clinical Genome Research, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

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Pancreatic cancer is one of the most intractable cancers and has a devastating prognosis; over the past three decades the 5-year survival rate has been <10%. Therefore, development of a novel anticancer treatment for pancreatic cancer is a matter of urgency. We previously developed an oncolytic recombinant measles virus (MV), rMV-SLAMblind, that had lost the ability to bind to its principal receptor, signaling lymphocyte activity molecule (SLAM), but which selectively infected and efficiently killed nectin-4-expressing breast and lung cancer cells. In this study, we analyzed the antitumor effect of this virus against pancreatic cancer. Nectin-4 was expressed on the surface of 4/16 tested pancreatic cancer cell lines, which were efficiently infected and killed by rMV-SLAMblind in vitro. The intratumoral inoculation of rMV-SLAMblind suppressed the growth of KLM1 and Capan-2 cells xenografted in SCID mice. The sequence analysis of MV isolated from the tumor revealed that the designed mutation in the H protein of rMV-SLAMblind had been stably maintained for 47 days after the last inoculation. These results suggest that rMV-SLAMblind is a promising candidate for the novel treatment of pancreatic cancer.

Materials and Methods
Cells. We used 16 pancreatic cancer cell lines (PKI, PK9, PK45P, PK45H, PK59, KLM1, SUIT-2, SNU-324, SNU-410, ¹Laboratory Animal Research Center, The Institute of Medical Science, The University of Tokyo, Tokyo; Divisions of ²Molecular Pathology; ³Clinical Genome Research, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan
PANC1, MiaPaCa-2, Capan-2, Hs766T, KP-1N, PSN-1, and AsPC-1. PK1, PK9, PK45P, PK45H, PK59, KLM1, SUIT-2, and MCF7 human breast cancer cells were obtained from the Cell Resource Center for the Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Miayagi, Japan). SNU-324 and SNU-410 were obtained from the Korean Cell Line Bank (Seoul, Korea). PANC1, MiaPaCa-2, and Capan-2 were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). AsPC-1 was obtained from DS Pharma Biomedical (Osaka, Japan). COBL human lymphoid cells were previously established in our laboratory. KLM1 cells (10^6) or Capan-2 cells (10^6) were suspended in 50 L HBSS (Invitrogen) and the suspension were mixed with 50 L Matrigel HC (BD Biosciences) before s.c. injection. For the KLM1 group, mice were inoculated with rMV-EGFP-SLAMblind (6) or firefly luciferase (rMV-Luc-SLAM-blind)(6) was propagated in MCF7 cells. Viral particles were harvested as reported previously (14) and stored at −80°C as aliquots. Virus titers were determined as the 50% tissue culture infectious dose (TCID50) by the Reed–Muench method using MCF7 cells. Measles virus infection of MCF7 cells was detected by fluorescence of EGFP or by immunostaining using anti-MV N protein mAb as previously described (6).

**Flow cytometry.** The expression of nectin-4, SLAM, and CD46 was analyzed by flow cytometry according to previously reported methods (6,14). The following antibodies were used: anti-human SLAM mAb (clone 7D4; Biolegend, San Diego, CA, USA); anti-human CD46 mAb, anti-nectin-4 goat polyclonal antibody, mouse control IgG1, and goat control IgG (all from R&D Systems, Minneapolis, MN, USA); and Alexa 488-conjugated anti-mouse or anti-goat antibody (Invitrogen). Cells were analyzed on a BD FACSVerse (BD Biosciences, San Diego, CA, USA). To compare the expression level of nectin-4, the mean fluorescence intensity of cells stained with anti-nectin-4 antibody was calculated using FlowJo software version 9.7.5 (Tree Star, San Carlos, CA, USA).

**Viruses.** rMV-SLAMblind encoding enhanced GFP (rMV-EGFP-SLAMblind) (6) or firefly luciferase (rMV-Luc-SLAM-blind) (6) was propagated in MCF7 cells. Viral particles were harvested as reported previously (14) and stored at −80°C as aliquots. Virus titers were determined as the 50% tissue culture infectious dose (TCID50) by the Reed–Muench method using MCF7 cells. Measles virus infection of MCF7 cells was detected by fluorescence of EGFP or by immunostaining using anti-MV N protein mAb as previously described (6).

**Cell viability assay.** Cell viability was analyzed by the water-soluble tetrazolium (WST-1) assay as previously reported (14). The viability of infected cells was expressed as a percentage of the mean of quadruplicate absorbance values obtained for the infected cell population divided by that of the uninfected cell population.

**Assessment of in vivo oncolytic activity.** All animal experiments were approved by the Experimental Animal Committee of The University of Tokyo (Tokyo, Japan). Five-week-old female SCID mice were purchased from Clea Japan (Tokyo, Japan). KLM1 cells (1 × 10^6) or Capan-2 cells (1 × 10^6) were suspended in 50 μL HBSS (Invitrogen) and the suspensions were mixed with 50 μL Matrigel HC (BD Biosciences) before s.c. injection. For the KLM1 group, mice were inoculated with rMV-EGFP-SLAMblind (6) or firefly luciferase (rMV-Luc-SLAM-blind) (6).
inoculated intratumorally with $10^6$ TCID$_{50}$ of rMV-EGFP-SLAMblind ($n = 7$) or HBSS ($n = 7$) at 18 days post-implantation. Virus or HBSS treatment was then repeated after a further 8, 16, and 37 days. The mice of the control group and those of the rMV-EGFP-SLAMblind inoculated group were killed at 70 and 84 days, respectively, after the first inoculation. For the Capan-2 group, mice were inoculated intratumorally with $10^6$ TCID$_{50}$ of rMV-EGFP-SLAMblind ($n = 8$) or HBSS ($n = 9$) at 22 days post-implantation. Virus or HBSS treatment was then repeated after a further 8, 17, and 24 days. The mice of both the control group and rMV-EGFP-SLAMblind inoculated group were killed at 50 days after the first inoculation. Tumor diameters were measured with callipers and tumor volumes were calculated based on the formula $V = \frac{4}{3}\pi r^3$.

**Histological analyses.** The excised tumors were fixed in 4% paraformaldehyde overnight. The tumors were then dehydrated sequentially in 10% and 20% sucrose solutions. A small piece of each tumor was imbedded in Tissue-Tek O.C.T. compound (Sakura Finetek Japan, Tokyo, Japan) and then frozen. Slices of 6-μm thickness were sectioned with a cryostat (Leica CM1900; Leica Microsystems, Wetzlar, Germany). After staining of the nuclei with Hoechst 33342 (Cambrex, East Rutherford, NJ, USA), the frozen sections were observed with a confocal microscope.

**Sequence analyses.** Tumor segments were homogenized in 1 mL RPMI-1640 medium (Invitrogen) containing 2% FBS, freeze-thawed three times and centrifuged at 1940 g for 10 min. The supernatants were added to MCF7 cells and cultured with RPMI-1640 medium containing 2% FBS at 37°C for 4 days. Total RNA was extracted from virus propagated in MCF7 cells using TRizol LS (Invitrogen) according to the manufacturer’s instructions. The purified viral RNAs were reverse transcribed for 10 min at 30°C, then 60 min at 42°C followed by 15 min at 72°C, using random primers and PrimeScript Reverse Transcriptase according to the manufacturer’s instructions (Takara Bio, Otsu, Japan). The region containing the mutation R533A was amplified using the forward primer 5'-ACA AGT TGC GAA TGG AGA CA-3' and the reverse primer 5'-GGC TAT CTA GGT TGA ACT TCA-3'. All PCRs were carried out in a total volume of 50 μL containing 1 μL cDNA using Phusion High Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA). Cycling conditions were denaturation at 98°C for 30 s, followed by 30 cycles of 98°C for 10 s, 55°C for 15 s, and 72°C for 45 s. After electrophoresis in agarose gel, the 0.7-kb products were purified from the gel using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) according to the manufacturer’s protocol. The purified products were sequenced with an ABI Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA).

**Visualization of in vivo replication of rMV-Luc-SLAMblind in Capan-2 xenografts.** Five-week-old female SCID mice were purchased from Clea Japan. A total of six mice were s.c. implanted with Capan-2 cells as described above. At 12 days post-implantation, the mice received a single intratumoral injection of $10^6$ TCID$_{50}$ of rMV-Luc-SLAMblind or HBSS as a control. Bioluminescence analysis was undertaken at 1, 2, 3, 4, 5, and 7 days post-infection (dpi). Before each imaging, mice received an s.c. injection of 2 mg d-luciferin (Gold Biotechnology, St. Louis, MO, USA) diluted in 200 μL PBS. Images were acquired using the Xenogen IVIS 100 system (Xenogen, Alameda, CA, USA). Luciferase activity was quantified as total photons/s/cm²/sr using Living Image software version 2.5 (Xenogen).

**Statistical analysis.** To assess the statistical significance of differences, Student’s t-test was carried out. P-values <0.05 were considered significant.

**Results**

**Expression of nectin-4 on surface of pancreatic cancer cells.** To analyze the expression of nectin-4 on the surface of pancreatic cancer cells, 16 cell lines derived from 14 patients were analyzed by flow cytometry. Nectin-4 expression was observed in four of the 16 cell lines, namely KLM1, Capan-2, PK1, and PK9 (Fig. 1a). Based on the mean fluorescence intensity, the highest expression of nectin-4 was observed in KLM1 cells (Fig. 1b). We also analyzed for the presence of the MV receptors SLAM and CD46,8,28,29 SLAM was not expressed in these cell lines, but it was expressed in COBL as a positive
control. CD46 was expressed in all of these cell lines (Fig. 1c).

Because rMV-EGFP-SLAMblind uses only nectin-4 as a receptor, we selected these four cell lines for further analyses.

**Infectivity and cytotoxicity of rMV-SLAMblind against pancreatic cancer cell lines.** To investigate the susceptibility of the pancreatic cancer cells to rMV-SLAMblind, nectin-4-positive cell lines (KLM1, Capan-2, PK1, and PK9) and a nectin-4-negative cell line (PSN-1) were inoculated with rMV-EGFP-SLAMblind. When KLM1, Capan-2, PK1, and PK9 cells were infected with rMV-EGFP-SLAMblind at an m.o.i. of 1, a time-dependent increase of fluorescence from rMV-EGFP-SLAMblind and syncytium formation was observed (Fig. 2a). Because EGFP expression is proportional to virus proliferation, increased fluorescence indicates virus growth. However, rMV-SLAMblind barely infected PSN-1 cells and no syncytium was observed (Fig. 2a).

We next examined the ability of rMV-SLAMblind to kill these cell lines by WST-1 assay. The cell viability decreased after infection with rMV-EGFP-SLAMblind in all nectin-4-positive cell lines, but did not in PSN-1 cells (Fig. 2b). These results indicated that rMV-EGFP-SLAMblind infected and killed pancreatic cancer cells expressing nectin-4.

rMV-SLAMblind replicated most efficiently in KLM1 cells, with a mortality of almost 100%. Although PK1 and PK9 cells expressed nectin-4 at a higher level than Capan-2 cells (Fig. 1a,b), the killing effect of rMV-EGFP-SLAMblind on those cell lines was weaker than Capan-2 cells (approximately 40% of PK1 and PK9 vs. 60% of Capan-2) (Fig. 2b).

**Oncolytic activity of rMV-SLAMblind against pancreatic cancer in vivo.** We analyzed the oncolytic activity of rMV-SLAMblind against pancreatic cancer using a mouse xenograft model. KLM1 cells and Capan-2 were chosen because of the strong cytotoxic effect of rMV-SLAMblind on these cell lines *in vitro*. KLM1 and Capan-2 cells were transplanted s.c. into C.B-17/isc-scid mice. When the presence of tumor was confirmed, 10⁵ TCID₅₀ of rMV-EGFP-SLAMblind was injected intratumorally to KLM1 and Capan-2 mice and repeated after 8, 16, and 37 days or 8, 17, and 24 days, respectively. In the mice given rMV-EGFP-SLAMblind, tumor growth was suppressed (Fig. 3a). In fact, excised tumor volume was significantly smaller than those of control mice (Fig. 3b,c).

Treatment with rMV-EGFP-SLAMblind effectively suppressed tumor growth in xenograft models both of KLM1 and Capan-2 cells, strongly suggesting potent antitumor effects of rMV-SLAMblind.

To examine virus distribution and replication in the xenografts, we inoculated rMV-SLAMblind expressing luciferase into mice transplanted with Capan-2 cells intratumorally. Luciferase activity was detected restrictedly in the tumor mass (Fig. 4a), and its level (indicating viral replication) increased from 1 dpi to 2 dpi and then decreased (Fig. 4b). Simultaneously, the tumor size was reduced (Fig. 4c). These results...
We previously reported that rMV-SLAMblind infects CD46-positive normal human cells at a very low efficiency, and that rMV-SLAMblind as well as its parental MV-HL, efficiently infects and kills breast cancer cells using nectin-4 as a receptor, but not CD46. Thus, the expression of nectin-4 on the cell surface is crucial for the oncolytic activity of rMV-SLAMblind. We showed that nectin-4 was upregulated on pancreatic cancer cell lines, as had been previously reported for breast, lung, and ovarian cancers, although this upregulation was present on only 4/16 pancreatic cell lines tested in this study (Fig. 1a). Conversely, it has been reported that nectin-4 was strongly expressed in 69/123 clinical samples from pancreatic cancer patients. Therefore, rMV-SLAMblind has the potential to be therapeutically applicable to approximately half of pancreatic cancers.

rMV-EGFP-SLAMblind infected and was cytotoxic to all of the nectin-4-expressing pancreatic cancer cell lines but did not show any cytotoxicity to a nectin-4-negative pancreatic cancer cell line (Fig. 2a). This result is consistent with our previous report that rMV-SLAMblind does not infect and kill nectin-4-negative colon cancer cell lines. rMV-EGFP-SLAMblind showed the strongest cytotoxicity to KLM1 cells, which showed the highest expression of nectin-4. Therefore, the efficiency of rMV-SLAMblind infection seemed to be related to the level of nectin-4 expression on the cell surface. A similar tendency was reported in our previous study using lung cancer cell lines. Although PK1 and PK9 cells also express nectin-4, these two cell lines showed slower cytotoxicity to rMV-SLAMblind. The virus growth in PK1 and PK9 cells after virus infection was lower than in KLM1 cells, but as high as in Capan-2 cells (data not shown). Efficiency of cell killing after infection may depend on cell state or condition. The unknown mechanism remains to be clarified.

Whereas rMV-SLAMblind showed lower cytotoxicity against Capan-2 (cytotoxicity, 62%) than that against KLM1 (cytotoxicity, 96%) cell lines in vitro, the virus showed high antitumor effect for both these two cell lines in vivo. Therefore, it is expected that rMV-SLAMblind has a high tumor inhibitory effect for xenograft pancreatic cancer cells when the cell killing rate in vitro is more than 60%. A similar tendency was reported in our recent study using two different colorectal cancer cell lines. The administration of rMV-SLAMblind in xenograft models showed an antitumor effect on the two cell lines, whereas the cytotoxicity of rMV-SLAMblind in vitro was only 40% for one and 90% for the other.

The KLM1 cell line was established as a highly metastatic variant, by repeated passage of PK1 cells within mice. Interestingly, the cytotoxic effect of rMV-SLAMblind was much stronger towards KLM1 cells than PK1 cells (Fig. 2b). Changes in expression of cellular factors after metastasis and the altered cell features may provide better conditions for virus replication.

It has been reported that nectin-4 expression correlated significantly with levels of both ki67 and vascular endothelial growth factor expression, which suggests that nectin-4 expression contributes to tumor proliferation and angiogenesis. Actually, patients with a high nectin-4 expression level had a poorer postoperative prognosis than those with low expression in human pancreatic cancer. Therefore, rMV-SLAMblind is expected to contribute to treatment of malignant cancer.

Pancreatic cancers are stroma-rich and have few blood vessels. Thus, delivery of anticancer agents to these cells is difficult. Furthermore, continuous treatment with anticancer agents is problematic owing to their side-effects on normal
cells. For these reasons, conventional chemotherapies have failed to show significant antitumor effects against pancreatic cancer. In contrast, once infection is established, rMV-SLAMblind multiplies and shows a continuous antitumor effect against the neighboring cancer cells by cell-to-cell infection. In fact, the persistence of residual virus evident in the excised tumor tissues and serum: potential role as a serum biomarker.

For the duration of this study, the sequence of the rMV-SLAMblind H protein was stable in mouse xenografts. In future, it will need to be established using immunocompetent animal models whether the virus is able to revert.

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Disclosure Statement

The authors have no conflict of interest.

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