Evaluation of a novel oncolytic Raccoonpox virus expressing the bifunctional *FCU1* suicide gene

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SUMMARY

Oncolytic virotherapy for cancer treatment utilizes naturally occurring or engineered viruses for selective infection and death of cancer cells without any adverse effect on normal cells. Raccoonpox virus (RCNV) is a member of the *Orthopoxvirus* genus of *Poxviridae,* with no known pathogenicity in any mammalian species so far (1,2). Raccoonpox virus has already been used as oncolytic virus in human cancer models (3,4). This study explores the potential of modified RCNV armed with a suicide gene as an oncolytic vector for cancer treatment.

We have generated a TK deleted recombinant Herman strain virus expressing the suicide gene *FCU1* fused with Green fluorescent protein (RCNtk$^-$/*gfp::fcu1*). The *FCU1* gene encodes a bifunctional chimeric protein that efficiently catalyses the direct conversion of the nontoxic 5-fluorocytosine (5-FC) into the toxic metabolites 5-fluorouracil (5-FU), an anti cancer chemotherapy drug, and 5-fluorouridine monophosphate (5-FUMP) (5).

The combined FCU1/5-FC treatment has proven to be successful in various resistant human cancer cells.

The RCNtk$^-$/*gfp::fcu1* vector has been evaluated in numerous therapeutic human cancer cells , where it demonstrated significant tumor selectivity and retained full replication efficiency and its ability to kill human cancer cells.

In vitro studies also demonstrated that the TK deleted Raccoonpox virus expressing FCU1 (RCNtk$^-$/*gfp::fcu1)* displayed reduced replication properties in primary non-transformed human liver cells but still lysed hepatocarcinoma.

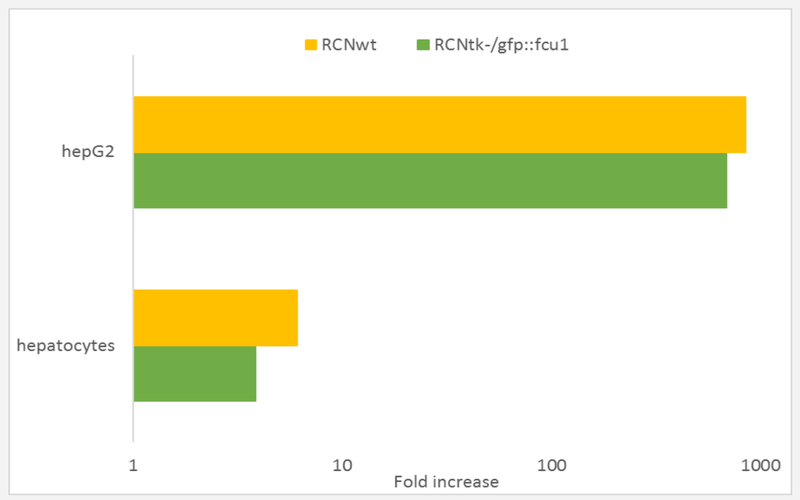
The results demonstrate the increased antitumoral activity of this new modified poxvirus armed with FCU1 and its promising future for cancer treatment.

IN *VITRO* RESULTS

Recombinant RCNTK$^-$/*gfp::fcu1* selectively replicates in tumoral cells

Sequence of recombinant RCN virus and transgene expression by Western blot

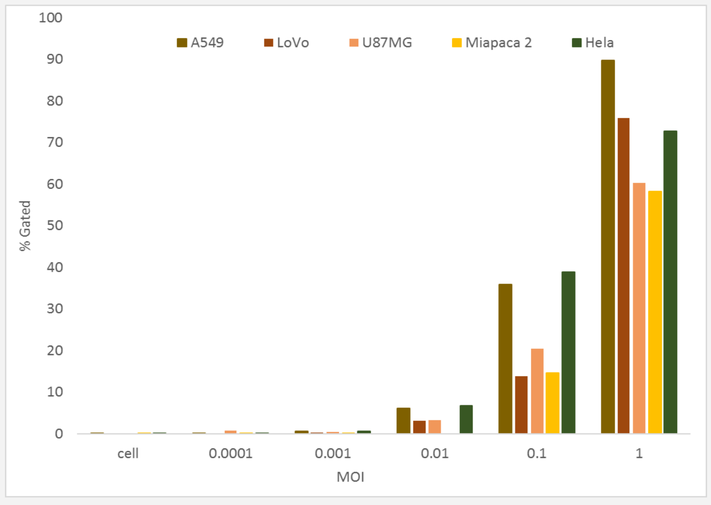
Schematic map of the modified Raccoonpox virus (RCN) expressing the FCU1 gene and detection of the FCU1 protein expression by Westernblot. (a) Schematic representation of viruses sequence. RCNtk$^-$/*gfp::fcu1* contains in the TK locus the indicated transgenes (Green fluorescent protein and FCU1) under the control of the vaccinia synthetic p11K7.5 promoter. (b) Specific detection of the FCU1 protein on Western blot by monoclonal antibody (mAb) 3H1. Lane 1: (left to right), LoVo cells infected with RCN; Lane 2: LoVo cells infected with RCNtk$^-$/*gfp::fcu1*. Molecular weight standards are shown in kDa on the left. The presence of gfp::fcu1 (Mr 72 000) is indicated (with an arrow).



Replication of RCNtk$^-$/*gfp::fcu1* was identified on hepatocarcinoma whereas no viral replication was detecteble on normal primary hepatocytes.

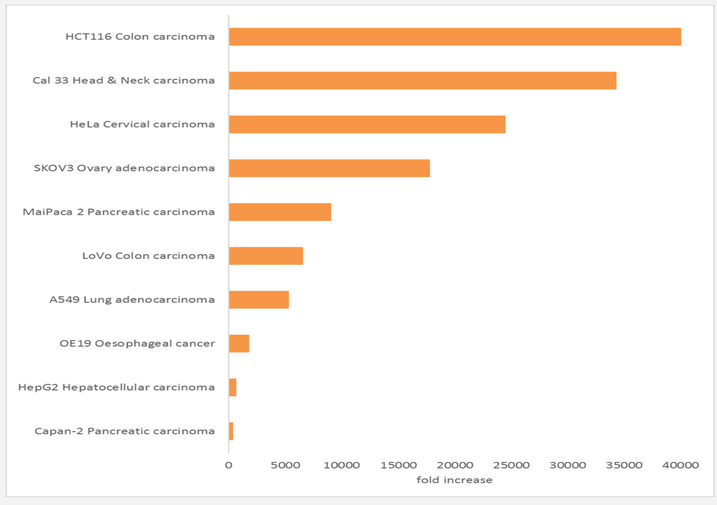
Human tumor cells (HepG2) and human normal hepatocytes were infected with small amount of virus (MOI 0.001= 1E+03pfu/well). 72h post infection plates were freezed and virus titration was performed on vero cells after sonication of the collected samples.

Viral performance: RCNTK$^-$/*gfp::fcu1* infects, replicates and kills a large panel of human tumoral cells



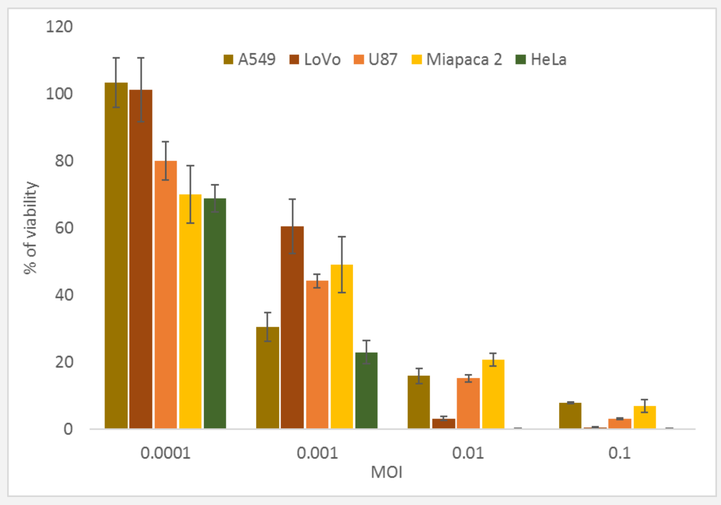
RCNtk$^-$/*gfp::fcu1* vector infects a large panel of human tumor cells

Human tumor cells were infected with RCNtk$^-$/*gfp::fcu1* at MOI 0.0001 to 1. At 16h post infection flow cytometry analysis was performed.



Replication of RCNtk$^-$/*gfp::fcu1 vector* is effective in a large panel of human tumor cells

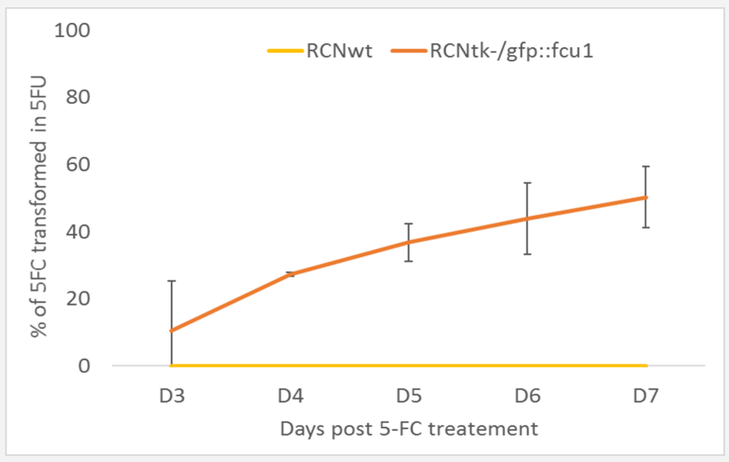
We compared several tumorigenic human cell lines for replication of RCNtk$^-$/*gfp::fcu1*. Human tumor cells were infected with RCNtk$^-$/*gfp::fcu1* at MOI 0.001. At 72h post infection virus titration was performed by plaque assay on vero cells.



RCNtk$^-$/gfp::fcu1 shows oncolytic activity in a panel of human tumor cells

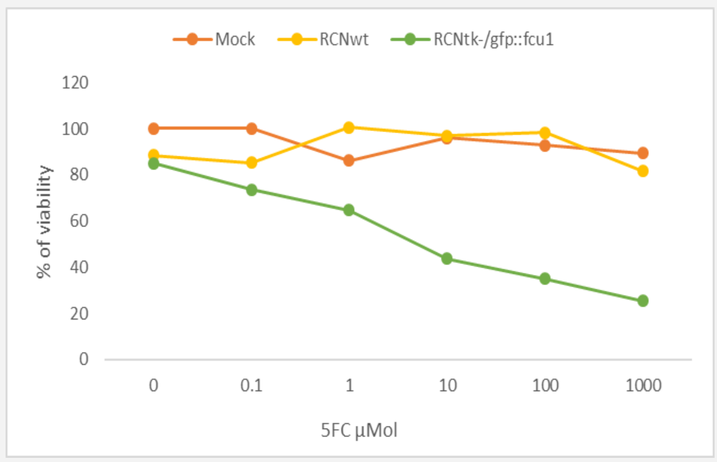
Human tumor cells were infected with small amount of virus (MOI 0.0001 to 0.1) and cell survival was determined 5 days later by Trypan blue staining.

Arming efficiency: RCNtk$^-$/*gfp::fcu1* combined with 5-FC treatment increases the antitumoral activity



Conversion of 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU) and release of 5-FU in the cell culture supernatant.

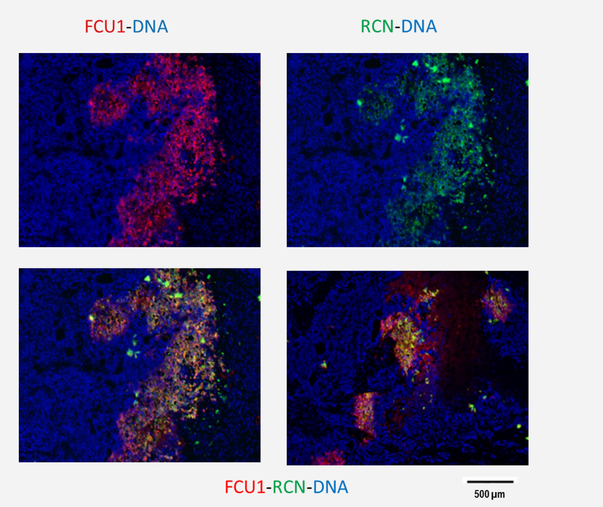
LoVo cells were infected with the indicated vectors (RCNwt and RCNtk$^-$/*gfp::fcu1)* at a multiplicity of infection (MOI) of 0.0001. Two days post infection, cells were incubated with 3 mM of 5-FC. From 3 to 7 days post-infection, the relative concentration of 5-FC and 5-FU in the media was measured by high performance liquid chromatography (HPLC). The results are expressed as the percentage of 5-FU in the media relative to the total amount of 5-FC+5-FU as the mean of triplicate determination.



***In vitro* sensitivity to 5FC: RCNtk$^-$/*gfp::fcu1* shows an increased antitumoral activity by combination of cell lysis and 5-FU cytotoxicity**

Human colorectal lovo tumor cells were infected with both wild type and recombinant virus at MOI 0.0001. At 48h post-infection cells were exposed to various concentration of 5-FC for 4 days before determination of cell viability

In vivo RCN infection and FCU1 gene expression



Tumor tissue staining: the *FCU1* gene is expressed *in vivo*

Lovo cells were injected in s.c. into swiss nude mice. 15 days later, RCNtk-/*gfp::fcu1* was injected into the tumor. At day 5 after infection, viral immunostaining was performed with Rabbit α- Vaccine virus (green) and Goat anti Rabbit IgG Polymer Dextran HRP. *FCU1* gene staining (red) was performed with Mouse monoclonal α–FCU1 and Goat α–Mouse-IgG-Polymer Dextran-HRP.

CONCLUSION

We have shown that RCNtk$^-$/*gfp::fcu1* can replicate *in vitro* in a large panel of human tumoral cells without any impact on its therapeutic index. We also have demonstrated that the expression of the *FCU1* gene with addition of 5-FC prodrug can increase the antitumoral activity of RCNtk$^-$/*gfp::fcu1* vector in the infected tumor cells. Our data showed a clear benefit in combining the oncolytic virotherapy using RCNtk$^-$/*gfp::fcu1* and the prodrug 5-FC for treatment of resistant tumor model.

Future development will focus on the *in vivo* therapeutic activity of RCNtk$^-$/*gfp::fcu1* on a panel of human tumor in murine model in order to confirm these *in vitro* results.

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(3) Evgin L. et al. Potent oncolytic activity of raccoonpox virus in the absence of natural pathogenicity. Molecular Therpay, 2010

(4) Nichols A. et al. Vaccinia Virus Outperfomrs a Panel of other Poxviruses as potent oncolytic agent for the control of head and neck squamous cell carcinoma cell lines. Intervirology, 2013

(5) Erbs, & al, e. (2008). Modified vaccinia virus Ankara as a vector for suicide gene therapy. Cancer gene Ther, 18-28.

XXI International Poxvirus, Asfavirus and Iridovirus Conference, Le Bischenberg, France 1-5 July 2016