# Longitudinal Diffusion-Weighted MRI Study of the Tumor Tissue Destruction Process Induced by Novel Attenuated Salmonella Typhimurium Expressing Protein Drugs

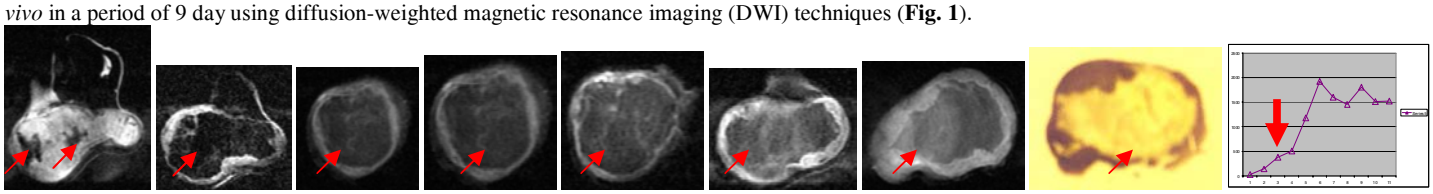
**Q. He[1,2], V. Chibisov[1,3], G. Ramus[1,2], H. Liu[1], K-T. Yung[1], and H. Zhu[1,4]**

[1]Radiology, Univerisity of PIttsburgh, Pittsburgh, PA, United States, [2]Bioengineering, Univerisity of PIttsburgh, Pittsburgh, PA, United States, [3]Bioengineering, Duke University, Duham, NC, United States, [4]Physics, Univerisity of PIttsburgh, Pittsburgh, PA, United States

**INTRODUCTION**. Recently, the attenuated *Salmonella typhimurium* strain, VNP20009, was introduced by auxotrophic mutations and deletion of the Salmonella msbB gene.[1-3] The latter prevents the addition of a terminal myristoyl group to the lipid A domain of the lipopolysaccharide in Salmonella and reduces the host $TNF_\alpha$ induction and the possible septic response of the host.[2] As compared to the wild-type *Salmonella typhimurium*, the virulence of VNP20009 is reduced to >10,000-fold, possessing an excellent safety profile based on the phase-I clinical trial.[4,5] VNP20009 is genetically stable *in vitro* and *in vivo*, and can serve as non-toxic bacteria. In mice, VNP20009 can be rapidly cleared from the blood by antibiotics, from a peak level of 1.0 x $10^6$ cfu/mL to an undetectable level in 24 h. A variety of tumors were found to selectively accumulate attenuated *Salmonella typhimurium* in animal tumor tissues.[6,7] The bacteria can be easily manipulated to introduce therapeutic genes and proteins for cancer treatment.[1,9-13] Although the attenuated *Salmonella typhimurium* did not cause tumor regression, it stopped tumor growth in animal model studies, prevented metastasis, and improved animal survival.[14-16] In general, the bacterial-based therapy can be combined with chemo- and radiation therapy with enhanced therapeutic effects in animal tumor models.[17-20] However, in a human melanoma trial, poor tumor tissue infiltration of the VNP20009 strain was observed, which raised an issue as how useful the bacteria-based therapy would be in human cancer treatment. In this presentation, we show evidence that therapeutic protein expressions in VNP20009 by further genetic engineering can introduce bacterial infiltration in Lewis lung carcinoma (LLC) tumors. LLC is a murine animal tumor model that eliminated the parent VNP20009 in immunocompetent mice C57BL/6J, a process similar to that in the human melanoma. We followed the time course of the bacterial-based cancer therapeutic process *in vivo* using diffusion weighted MRI (DWI).

**METHODS.** Lewis lung carcinoma LLC cells (ATCC) were inoculated subcutaneously at one foot and/or on flank of the C57BL/6J and SCID CB-17 mice. When the tumor diameter reached ~0.7-1cm, the therapeutic bacterial strains were injected into the tumors. Started from third day after bacteria injection, diffusion weighted images were acquired with a diffusion-weighted spin-echo sequence on a GE 3T MRI scanner in multi-slice mode to cover the entire tumor. Solenoidal coils or gap resonators were constructed in-house to fit the tumor size with optimal filling-factors. The animals were anesthetized with mixed ketamine, xylene, and acepromazine and positioned in the magnet center on a Flexiglass cradle. A warm water blanket was positioned underneath the animal to maintain physiological temperature. TR = 2000ms, TE - 50 to 100 ms, slice thickness = 1 mm, and FOV = 3-5 cm. The maximum gradient applied was 4 G/cm. A data matrix of 256 x 192 was acquired for each imaging plane. The LLC tumors were harvested after the last MRI experiment, fixed for 24hrs in 10% buffered formalin, embedded in paraffin, sectioned into 4 μm slices and mounted on slides for tissue staining of bacteria (Gram staining), macrophage (immunohistochemistry), apoptosis (TUNEL) and H&E.

**RESULTS AND DISCUSSION**. In our previous investigation of attenuated *Salmonella typhimutium* as a delivery mechanism in cancer gene therapy, the attenuated *Salmonella typhimutium* was observed to consume lactic acid in Colon 38 murine tumor model during the early phase of bacterial growth, reaching the minimal tumor lactate level at ~7hrs. At ~24 hrs, the lactate signal monitored by the magnetic resonance spectroscopy eventually went back to the initial level of tumor lactate prior to bacterial administration.[21] Thus, the attenuated Salmonella may use elevated lactic acid as a carbon source for nutrient supply in the tumor tissues. Therefore, bacteria may be used to deplete the nutrient source for tumor cells. For this purpose, we expressed in VNP20009 the recombinant methioninase that was demonstrated to have anti-cancer effect due to depletion of methionine, which is absolutely required for the growth of many tumors. This experiment led us to an interesting observation -- the attenuated *Salmonella typhimurium* expressing mathioninase not only infiltrated in the LLC tumors in C57BL/6J mice but also induced massive tumor tissue destruction (Fig. 1). In addition, the original Salmonella strain VNP20009 that was not able to survive in the LLC tumors in the immunocompetent C57BL/6J mice could now infiltrate the LLC tumors implanted in the immunodeficient CB-17 SCID mice. Therefore, the VNP20009 was likely eliminated by the host immune response in LLC on C57BL/6J mice. Similarly, VNP20009 might be eliminated by human immune response in the human melanoma trial. After expression of methioninase, however, the new attenuated salmonella strain successfully infiltrated the LLC tumors in C57BL/6J mice, overcoming the host immune barrier. Similar results were observed with VNP20009 expressing $TNF_\alpha$ or a combination of Methioninase and $TNF_\alpha$, and with a MR reporter gene myoglobin (Mb). Massive tumor cell apoptosis and macrophage activation were revealed by immunochemical staining. The rapid tumor tissue destruction induced by bacteria was monitored longitudinally *in vivo* in a period of 9 day using diffusion-weighted magnetic resonance imaging (DWI) techniques (**Fig. 1**).



*03/13/06* (a) *03/15/06* (b) *03/16/06* (c) *03/18/06* (d) *03/19/06* (e) *03/20/06* (f) *03/22/2006* (g) *03/22/2006* (h) (i)

***Fig. 1*** *(a-g) In vivo diffusion weighted MRI (DWI) presented areas of LLC tumor tissue destruction induced by Salmonella (methioninase, $TNF_\alpha$ Mb). (h) Gram staining of the tumor tissue after the last DWI experiment. (i) The growth curve of the flank LLC tumor implanted on a C57BL/6J mouse. Salmonella (rMETase, $TNF_\alpha$ Mb) was injected at the time indicated by the arrow on day 3.*

**CONCLUSIONS**. The Salmonella strains expressing methioninase and/or $TNF_\alpha$ have overcome the host immune barrier and infiltrated LLC tumor tissues that usually eliminated the parent strain VNP20009 in C57BL/6J mice. These new therapeutic bacteria have induced cancer cell apoptosis, macrophage activation, and rapid tumor tissue destruction, which can be effectively monitored by DWI. In addition, the new bacteria can migrate to distant tumor sites and, therefore, may be used to treat cancer metastasis. **ACKNOWLEDGMENTS**. The work was supported from NIH (grant R21CA80906).

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