

***Bifidobacterium longum* as a gene delivery system for cancer gene therapy**

Research Article

Minoru Fujimori¹, Toshiyuki Nakamura¹, Takayuki Sasaki¹, Kazuyuki Yazawa¹, Jun Amano¹, Yasunobu Kano³, Shun'ichiro Taniguchi²

¹Department of Surgery and ²Molecular Oncology and Angiology, Angio-Aging Research Division, Center on Aging and Adaptation, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto, Japan

³Department of Molecular Genetics, Institute of Molecular and Cellular Biology for Pharmaceutical Science, Kyoto Pharmaceutical University, 1 Shichono-cho, Misasagi, Yamashina-ku, Kyoto, Japan

***Correspondence:** Minoru Fujimori, M.D., Ph.D.; Department of Surgery; Shinshu University School of Medicine; 3-1-1 Asahi, Matsumoto 390-8621, Japan; Telephone: 81-263-37-2657; Fax: 81-263-37-2721; e-mail address: minoru1@hsp.md.shinshu-u.ac.jp

Key words: cancer, prodrug-enzyme therapy

Abbreviations: 5-fluorocytosine, (5FC); 5-fluorouracil, (5FU)

Received: 15 November 2001; accepted: 28 November 2001; electronically published: February 2004

Summary

A fundamental obstacle in cancer gene therapy is the specific targeting of therapy directly to a solid tumor, and no systemic delivery system yet exists. A strain of domestic bacteria, *Bifidobacterium longum*, which is nonpathogenic and anaerobic, selectively localized to and proliferated in solid tumors after systemic application. We propose a novel approach to cancer gene therapy in which anaerobic bacteria of the genus *Bifidobacterium longum* (*B. longum*) are used to achieve tumor specific gene delivery and prodrug-enzyme therapy. This is the first demonstration that *Bifidobacterium longum* can be utilized as a specific gene delivery vector for gene therapy on solid tumors.

I. Introduction

A fundamental obstacle in cancer gene therapy is the specificity to a solid tumor, and yet no systemic delivery system exists. Hypoxic or necrotic regions are characteristic of solid tumors in many murine and human tumors, including the majority of primary tumors of the breast, uterine cervix (Moulder et al, 1984). Hypoxic regions are characteristic of many solid tumors and gene therapy that targets to hypoxic tumor cells is currently being investigated (Dachs et al, 1997). Kimura and colleagues demonstrated that anaerobic bacteria of the genus *Bifidobacterium* could selectively germinate and grow in the hypoxic regions of solid tumors after intravenous injection (Kimura et al, 1980). The genus *Bifidobacterium* is a Gram-positive anaerobe and is one of the domestic, nonpathogenic bacteria found in the lower small intestine and large intestine of humans and some other mammalian animals (Gorbach et al, 1967). We

propose a novel approach to cancer gene therapy in which anaerobic bacteria of the genus *Bifidobacterium longum* (*B. longum*) are used to achieve tumor specific gene delivery and prodrug-enzyme therapy.

II. Results

A. Selective growth of *B. longum* in tumor tissues

The number of two kinds of *B. longum* organisms per gram of various tissue at various time intervals after intravenous administration of bacilli into mice bearing Lewis lung cancer. At 168 hour, tumors had approximately sixty thousands bacilli per gram of tumor tissue regardless of the bacterial strain. In contrast, the number of bacilli in non-malignant tissues, such as the liver, spleen, kidney and lung, began to decrease immediately after injection and were below detectable

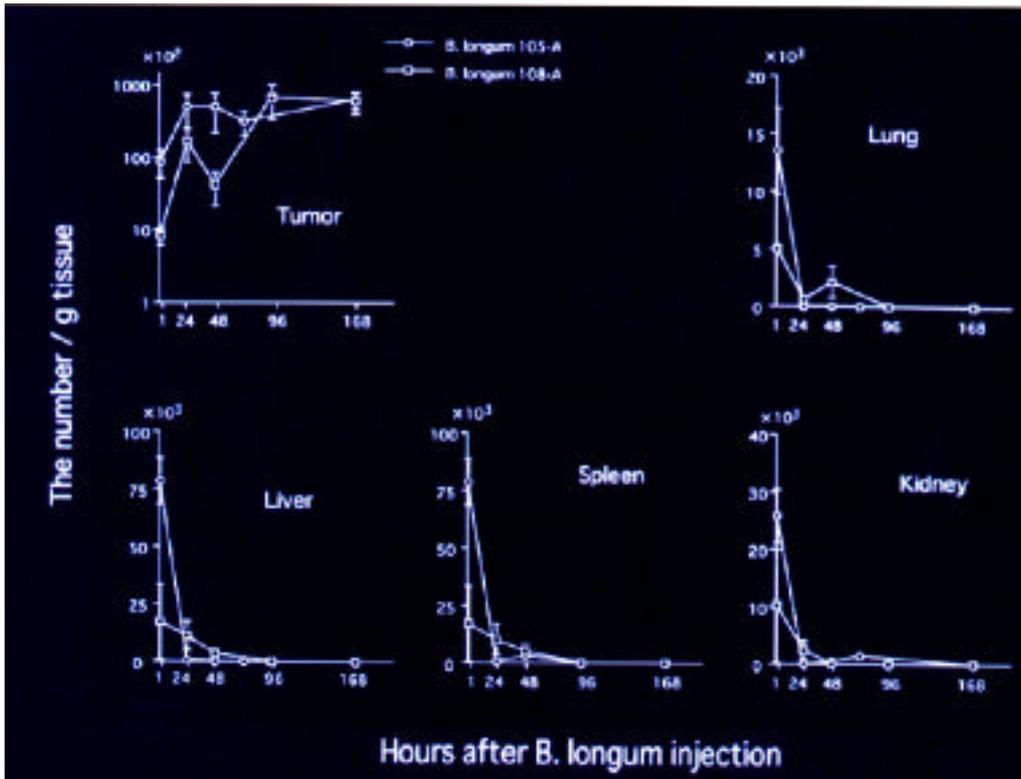


Figure 1. Organ distribution of *B. longum* 105-A and 108-A after a single i.v. administration of $5-6 \times 10^6$ viable bacilli into Lewis lung cancer-bearing mice.

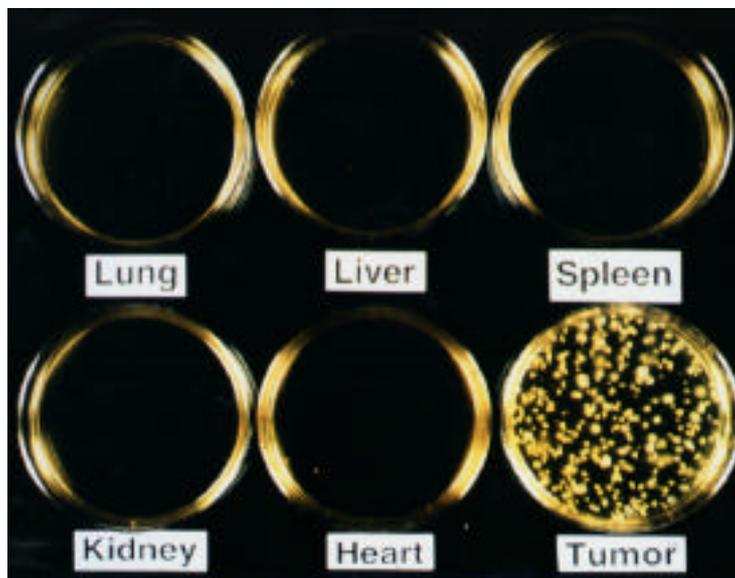


Figure 2. Comparison of the number of genetically engineered *B. longum* 105-A in both tumor and normal tissues from rats after 168 hr injected of about 2×10^8 viable bacilli. After homogenization of removed tumor and tissues, 100 μ l samples were plated on each of the dishes and cultivated for 3 days

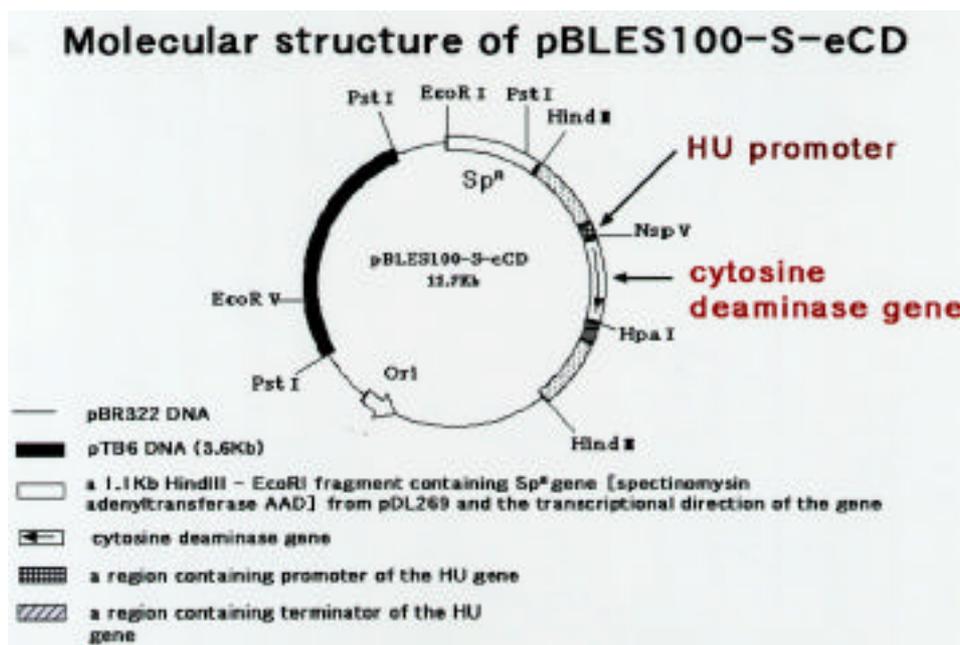


Figure 3. Molecular structure of pBLES100-S-eCD

levels after 168 hours with *B. longum* 105-A and after 96 hours with 108-A (Figure 1).

Colonies were recognized on the agar plate inoculating the tumor tissue only. Same result obtained DMBA induced mammary tumor of rats (Figure 2).

B. Prodrug-enzyme therapy using genetically engineered *B. longum*

B. longum is effective as hypoxic tumor specific vector, and it seems to be able to utilize for prodrug-enzyme therapy. We chose to use the combination of cytosine deaminase and 5-fluorocytosine (5FC) in initial studies of the feasibility of this strategy. The cellular toxicities of 5FC are a results of its deamination by the enzyme cytosine deaminase to give 5-fluorouracil (5FU). We constructed pBLES100-S-eCD that includes HU gene promoter and cytosine deaminase gene in shuttle vector pBLES100. It has been proven that HU gene that encodes histon like DNA binding protein highly express in *B. longum*. Cytosine deaminase gene was ligated with HU gene promoter, and inserted into pBLES100. Figure 3 is molecular structure of pBLES100-S-eCD. *B. longum* were cultured anaerobically, and expression of the cytosine deaminase was analyzed by western blot method. Figure 4 is western blot analysis of cytosine deaminase. The lane in the left is recombinant cytosine deaminase as positive control. The expression of cytosine deaminase is recognized only in transfected *B. longum*. 5-fluorocytosine (5FC) was added in the culture solution of wild and transfected *B. longum*, the 5-fluorouracil (5FU) concentration was measured time-dependent. Figure 5 is

time-dependent 5FU concentration in the culture solution. In transfected *B. longum*, 5FU concentration rises with the passage of the time. It was confirmed that produced cytosine deaminase had the enzyme activity. Wild and transfected *B. longum* were injected into the DMBA induced mammary tumor of rats directly. Figure 6 is intratumoral concentration of 5FU. In transfected *B. longum* injected group, 5FU concentration was significantly more high-dense compare with control group. 55 days after injection of wild type *B. longum*, size of

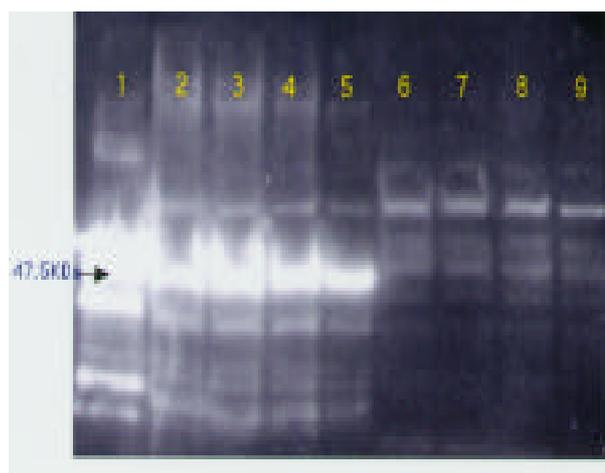


Figure 4. Western blot analysis of cytosine deaminase. Lane 1, recombinant cytosine deaminase as positive control; Lane 2,3,4,5, transfected *B. longum*; Lane 6,7,8,9, wild type *B. longum*

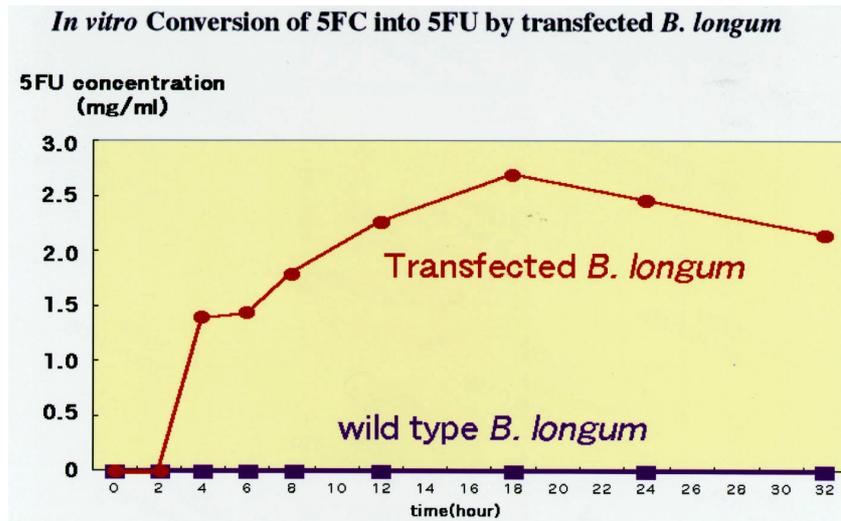


Figure 5. Time-dependent 5FU concentration in the culture solution of wild and trasfected *B. longum*

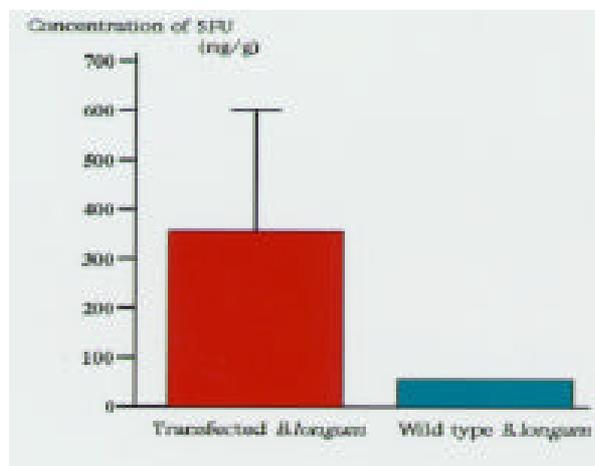


Figure 6. Intratumoral concentration of 5FU.

tumor increased remarkably. In transfected *B. longum* injected group, the tumor size decreased (Figure 7). Hematoxylin-eosin staining of the tumor in wild type *B. longum* injected group indicated that tumor cells were viable and proliferated. In transfected *B. longum* injected group, there are fibrosis and cytoplasm contains vacuoles and eosinophilic granules. Cancer cells tend to shrink away from stroma (Figure 8).

III. Discussion

A crucial difficulty for cancer gene therapy is the lack of specificity of current delivery systems. In this report, we observed a distribution of viable bacilli throughout the body, but after 96 to 168 hr they were detectable only in the tumor tissue after i.v. inoculation of *B. longum* to tumor-bearing mice and DMBA-induced mammary carcinoma in rats. It has been suggested that the only requirement for success of this gene therapy strategy

should be the presence of hypoxia in the treated tumors. This gene delivery system is not only tumor specific, but also nontoxic. Some investigators have described the availability of anaerobic bacteria such as clostridia (Fox et al, 1996; Lemmon et al, 1997) or Salmonella (Low et al, 1999) as a gene delivery vectors, but the pathogenicity of these organisms in humans likely precludes their use (Hone et al, 1992). Conversely, Bifidobacterium strains constitute almost the entire flora of the stools of breast-fed infants and are widely used for the preparation of fermented milk products. The nonpathogenicity and importance of these microorganisms are now generally acknowledged. To be able to exploit the potential of these organisms for cancer gene therapy, detailed knowledge is required about such basic biological phenomena as cellular metabolism, gene expression, protein secretion and genetics.

However, studies on Bifidobacterium at the molecular level are severely limited in the absence of an efficient

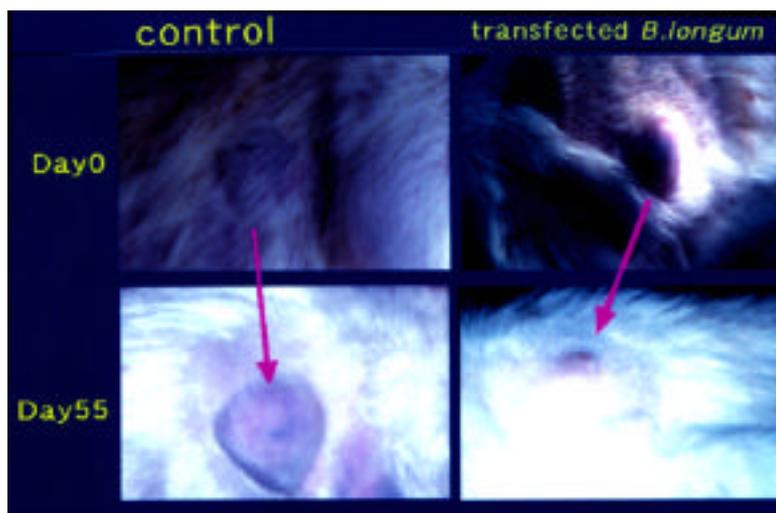


Figure 7. Tumor of DMBA induced rat mammary tumors. Left, Day0 and 55 days after injection of wild type *B. longum*; Right, Day0 and 55 days after injection of transfected *B. longum*.

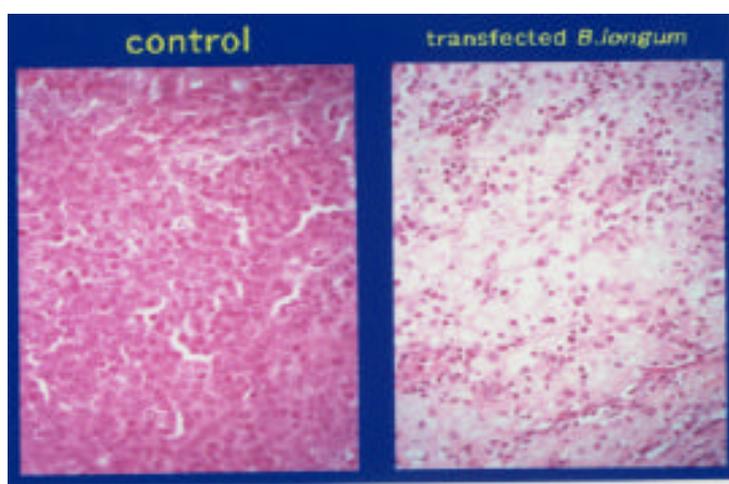


Figure 8. Hematoxylin-eosin staining of DMBA induced mammary tumor. Left, Wild type *B. longum* injected; Right, Transfected *B. longum* injected group.

transformation system. Recently, Matsumura and colleagues developed a system for convenient and reproducible genetic transformation of *B. longum* (Matsumura et al, 1997).

We have demonstrated the tumor-specific germination of Bifidobacteria with transfected *B. longum*. These results strongly suggest that *B. longum* can be utilized as a highly specific gene delivery vector for cancer therapy.

One of the major limitations of conventional chemotherapy is the toxicity associated with the lack of specificity of drugs for tumor cells over normal tissues. We proposed a new approach involving the genetically engineered *B. longum* for prodrug-enzyme therapy to use the combination of cytosine deaminase and 5-fluorocytosine (5FC).

In summary, *B. longum* is accumulated in the hypoxic tumor, and it is effective as novel gene delivery system. Transfected *B. longum* by pBLES100-S-eCD produced cytosine deaminase in the hypoxic tumor, and it was confirmed to be effective for prodrug-enzyme therapy.

IV. Materials and Methods

A. Animals

Male C57BL6 mice, 6 to 8 weeks old, and female Sprague-Dawley rats, 6 weeks old were used in this study. As transplanted tumors, Lewis lung cancer and B16-F10 melanoma were used in mouse model. About 5 hundreds thousands tumor cells were inoculated into the right thigh muscle of mice. The solid tumors were obtained two weeks after. As autochthonous tumor, the rats were administered 10 mg of 7,12-dimethylbenz[a]anthracene (DMBA) by intragastric gavage weekly for two weeks. At 23

weeks after the first dose of DMBA, 89% rats developed mammary tumors.

B. Bacteria

B. longum 105-A and 108-A were anaerobically cultured and five to six millions bacilli were injected intravenously.

C. Selective growth of *B. longum* in tumor tissues

Animals were injected *B. longum* into tail vein. This number was generally five to six million bacilli per mouse, and two hundreds million per rat. At 24, 48, 96, 168 hours after injection of *B. longum*, mice were killed. Tumor and normal tissues were excised and homogenized thoroughly. The diluted tissue homogenates were cultured under anaerobic conditions. On day 3 of culture, the number of colonies per dish was determined.

D. Prodrug-enzyme therapy using genetically engineered *B. longum*

pBLES100-S-eCD that includes HU gene promoter and cytosine deaminase gene in shuttle vector pBLES100 was constructed. It has been proven that HU gene that encodes histon like DNA binding protein highly express in *B. longum*. Cytosine deaminase gene was ligated with HU gene promoter, and inserted into pBLES100. pBLES100-S-eCD were transfected directly into *B. longum* 105-A by electroporation.

Transfected *B. longum* were cultured anaerobically, and expression of the cytosine deaminase was analyzed by western blot method.

5FC was added in the culture solution, and the 5FU concentration was measured time-dependent. Add 5FC quantity was 25 mg per five to six millions bacilli.

Wild and transfected *B. longum* were injected into the DMBA induced mammary tumor of rats directly. Both wild type and transfected *B. longum* injected group, rats were administered 500 mg per day of 5FC by intragastric gavage. Intratumoral concentration of 5FU was measured, and size of the tumor was compared wild type *B. longum* injected group with transfected group.

References

Dachs GU, Patterson AV, Firth JD, Ratcliffe PJ, Townsend KMS, Stratford IJ, Harris AL (1997) Targeting gene expression to hypoxic tumor cells. **Nat Med** 3, 515-520.
Fox ME, Lemmon MJ, Mauchline ML, Davis TO, Giaccia AJ, Minton NP, Brown JM (1996) Anaerobic bacteria as a delivery system for cancer gene therapy: in vitro activation

of 5-fluorocytosine by genetically engineered clostridia. **Gene Ther** 3, 173-178.

Gorbach SL, Plaut AG, Nahas L, Weinstein L, Spanknebel G, Levitan R (1967) Studies in intestinal microflora. II. Microorganisms of the small intestine and their relations to oral and fecal flora. **Gastroenterology** 53, 856-867.

Hone DM, Tacket CO, Harris AM, Kay B, Losonsky G, Levine MM (1992) Evaluation in volunteers of a candidate live oral attenuated *Salmonella typhi* vector vaccine. **J Clin Invest** 90, 412-420.

Kimura NT, Taniguchi S, Aoki K (1980) Selective localization and growth of *Bifidobacterium bifidum* in mouse tumors following intravenous administration. **Cancer Res** 40; 2061-2068.

Lemmon MJ, van Ziji P, Fox ME, Mauchline ML, Giaccia AJ, Minton NP, Brown JM (1997) Anaerobic bacteria as a gene delivery system that is controlled by the tumor microenvironment. **Gene Ther** 4, 791-796.

Low KB, Ittensohn M, Le T, Platt J, Sodi S, Amoss M, Ash O, Carmichael E, Chakraborty A, Fischer J, Lin SL, Luo X, Miller SI, Zheng LM, King I, Pawelek JM, Bermudes D (1999) Lipid A mutant *Salmonella* with suppressed virulence and TNF induction retain tumor-targeting in vivo. **Nat Biotech** 17, 37-41.

Moulder JE, Rockwell S (1984) Hypoxic fractions of solid tumors: experimental techniques, methods of analysis, and survey of existing data. **Int J Radiat Oncol Biol Phys** 10, 695-712.

Matsumura H, Takeuchi A, Kano Y, (1997) Construction of *Escherichia coli*-*Bifidobacterium longum* shuttle vector transforming *B. longum* 105-A and 108-A. **Biosci Biotechnol Biochem** 61, 1211-1212.



Minoru Fujimori, M. D., Ph. D.