**Injection of Naked Decoy Oligodeoxynucleotide against Nuclear Factor-kappa B into a Murine Osteosarcoma in a Spontaneous Pulmonary Metastasis Model**

Takao Matsubara1, Koji Akeda1, Akinobu Nishimura1, Katsuyuki Kusuzaki1, Akihiko Matsumine1, Ken Shintani1, Haruhiko Satonaka1, Yuichi Kasai1, Takefumi Gemba2, Atsumasa Uchida1

1Orthopedic surgery, Mie University, Tsu, Japan; 2AnGes MG, Inc., Osaka, Japan

**Introduction:** Osteosarcoma (OS) is the most common malignant bone tumor in children. Despite an aggressive combination of chemotherapy and surgery [1], pulmonary metastasis occurs in approximately 40-50% of patients with OS, who have a poor prognosis with limited therapeutic options. Therefore, developing a new therapy to effectively suppress pulmonary metastasis is critical. It has been increasingly accepted that the activation of nuclear factor-kappa B (NFkB) in many types of malignant tumors plays a critical role in tumor progression as well as metastasis [2]. Recently, a new type of synthetic nucleic acid preparation, double-stranded oligodeoxynucleotides (ODNs) “decoys” corresponding to the cis sequence of NFkB, has been developed [3] and applied experimentally both in vitro and in vivo with subsequent modulation of NFkB target gene expression [4].

We hypothesized that transfection of NFkB decoy ODN (decoy) into OS cells would effectively inhibit pulmonary metastasis. Using an in vitro alginat-encapsulated tumor spheroid model, we had successfully transfected ‘naked’ NFkB decoy ODN into a murine OS cell line with a high metastatic potential to the lung (LM8). Pre-transfected NFkB decoy ODN effectively suppressed pulmonary metastasis in an alginat bead transplantation model in vivo.

In this study, with clinical application as a goal, we have directly injected ‘naked’ NFkB decoy ODN into LM8 tumor and examined the biodistribution and inhibitory effects of decoy in a murine spontaneous pulmonary metastasis model.

**Materials and Methods:** Cell Preparation: A murine OS cell line with high metastatic potential to the lung (LM8) was established from the original Dunn cell line by in vivo selection [5]. LM8 cells were seeded at a density of 2.0x10^6 cells on 175 cm^2 culture flasks and cultured in DMEM supplemented with 10% fetal bovine serum.

**Sequence of NFkB decoy oligodeoxynucleotide:** Phosphorothioate double-stranded ODN, for which sequences have been reported [3], was a gift from AnGes, MG Inc. NFkB decoy ODN: 5′-CCTTGAAGGGATTTCCCTCC-3′ and 3′-GGAATTTCCCTAAAAGGGAGG-5′.

In vivo pulmonary metastasis: Thirty four C3H mice (male, 5-weeks-old) were used in this study. Trypsinized LM8 tumor cells (1.0x10^7) suspended in DMEM were injected into the subcutaneous (dorsal skin) of mice. One week after inoculation, 1 μg (n = 10) or 10 μg (n = 10) of NFkB ODN in 100 μl (PBS) was injected into the newly formed tumors using a 28G needle. For the vehicle control (n = 10), PBS (100 μl) was injected into the tumor. Body weight and tumor volume (minor axis)× (major axis)/2 mm^3 were measured every week. After four weeks from the injection, the animals were sacrificed, and the lungs were removed. Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. The sections (5 μm) were cut, and stained with Haematoxylin and eosin (H-E). On the maximal area of each tissue, the number of tumor nodules (metastasis) was counted microscopically.

Biodistribution of fluorescein-labeled NFkB decoy ODN: To examine the distribution of decoy ODN in vivo, FITC-labeled decoy ODN (10 μg) in PBS (100 μl) was injected into the tumor as described above. On day-3 (n = 2) and -14 (n = 2) after the injection, the animals were sacrificed, and the tumors were removed. Cryosections (8 μm) were cut, and the samples were imaged using fluorescent microscopy. Nuclei were counter-stained with propidium iodide.

**Statistical Analysis:** The association among the variables was determined by one way ANOVA with Fisher’s PLSD post hoc test. P values less than 0.05 were considered significant.

**Results:** Distribution of NFkB decoy ODN: On day-3, the fluorescent intensity was mainly identified at the periphery of the tumor (Fig. A). A high-magnification image revealed that fluorescence was found in the cells marginal to the tumor (tumor capsule) and in the normal cells surrounding the tumor, although no fluorescence was found in the parenchyma of the tumor (Fig. B). On day-14, the fluorescent intensity remained at the periphery of the tumor (Fig. C, D).

**Discussion:** To examine the clinical usefulness of the synthetic nucleic acid preparation “NFkB decoy ODN”, we have directly injected NFkB decoy ODN into a tumor (murine osteosarcoma) without using any reagents in a spontaneous metastasis model. The results of our study demonstrate that decoy ODN was not transfected into the tumor cells (LM8) but was transfected into the cells marginal to the tumor. No inhibitory effects on pulmonary metastasis by the injection of decoy were observed. Therefore, in the development of nucleic acid drugs, it is important to elucidate the underlying differences in the transfection efficiency of decoy ODN between tumor cells and normal cells. Based on our study, alternative transfection methods, such as using transfection reagents to improve transfection efficiency of decoy ODN into tumor cells, should be performed.


**Acknowledgements:** This study was sponsored by grants from the Ministry of Education, Culture, Sports, Science and Technology (Japan).