A pilot study of human interferon ß gene therapy for patients with advanced melanoma by in vivo transduction using cationic liposomes

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Running head: IFN-ß gene therapy for melanoma

Abstract
Background Cationic liposomes containing HuIFNß gene (IAB-1) had been used for the clinical trial for glioma patients. HuIFNß gene therapy showed much higher anti-tumor activity compared to administration of HuIFNß protein for melanoma. These results suggest HuIFNß gene therapy is an attractive strategy for treatment of melanoma.
Methods Stage IV or stage III melanoma patients with cutaneous or subcutaneous metastatic lesions, were enrolled in this pilot study.IAB-1 was dissolved by sterile PBS at the concentration of 30 μg DNA/ml, and was injected into cutaneous or subcutaneous metastatic nodules three times a week for two weeks and the effect on the injected and non-injected metastatic lesions was evaluated.
Results Clinical responses were as follows (5 patients): mixed response (MR) and no change (NC) in each one patient, and progressive disease (PD) in 3 patients. In the MR patient, IAB-1 injected lesion disappeared clinically and histopathologically and one half of IAB-1 non-injected skin metastases were transiently inflamed and mostly regressed. In the responded non-injected lesions of this patient, histopathologically, infiltration of CD4 positive T cells was observed around the melanoma cells in the dermis, which expressed HLA Class II antigen. Adverse events due to this gene therapy were not recognized in any of the patients.
Conclusions The efficacy of this gene therapy was generally insufficient, however, some immunological responses were recognized in one patient. No adverse events were observed. HuIFNß gene therapy could be an attractive strategy for treatment of a variety of malignancies including melanoma, though some modifications should be required.

Mini-abstract
A pilot clinical trial of HuIFNß gene therapy was performed for 5 advanced melanoma patients having cutaneous metastases without any adverse events. Some immunological responses were recognized in one patient.

Keywords: interferon ß, gene therapy, malignant melanoma, clinical trial

Introduction

The significant increase in the incidence of malignant melanoma in recent years and the lack of effective therapy for metastasized melanoma have stimulated interest in developing alternative therapeutic approaches in this neoplasm. Among them, human interferon ß (HuIFNß) has good inhibitory effect on melanoma cells, and usage of natural HuIFNß for melanoma patients has been approved and is now widely used in the treatment of melanoma patients in Japan. However, in most cases, melanomas show limited clinical responses to HuIFNß[1].

Our recent study revealed that human melanoma nodules subcutaneously transplanted to nude mice disappeared after injections of cationic liposomes containing HuIFNß gene [2]

and HuIFNß gene therapy showed much higher anti-tumor activity compared to administration of HuIFNß protein. These results suggest HuIFNß gene therapy is an attractive strategy for treatment of melanoma.

In this clinical study, we used IAB-1, the cationic liposomes containing HuIFNß gene, which had been used for the clinical trial for glioma patients in Nagoya University Hospital[3]. In the present study, IAB-1 was injected into cutaneous or subcutaneous metastatic melanoma nodules three times a week for two weeks. The effect on the IAB-1 injected and non-injected skin metastases was evaluated along with the effect on the visceral metastatic lesions. Adverse events of this treatment were also assessed.

This is the first clinical study using HuIFNß gene for patients with advanced melanoma.

Patients, Materials and Methods

Ethical considerations

This study was approved by the Ethical Committee of Shinshu University School of Medicine, the Biosafety Committee of the Ministry of Health, Labor, and Welfare of Japan. All the patients gave informed consents in a written form according to the Declaration of Helsinki before enrolling in this clinical study.

Patients

Melanoma patients in Stage IV or stage III (based on the 2002 UICC/AJCC staging system) who had failed to respond to prior therapies including surgery, chemotherapy and immunotherapy and had at least one cutaneous or subcutaneous metastatic lesions, were enrolled in this study (Table 1). Inclusion criteria were normal hepatic and renal function and life expectancy of more than 6 months. Patients with brain metastases, severe myelosuppression, bleeding tendency, liver and renal dysfunction were excluded. Eligibility of each candidate was approved by the Committee of Gene Therapy in Shinshu University Hospital.

Cell lines

U251SP human glioma cell line derived from the Memorial Sloan-Kettering Cancer Institute (New York, NY) and RPM-EP human melanoma cell line from Harvard Medical School (Boston, MA)[3] were maintained in RPMI 1640 medium (Nipro Co., Osaka Japan) containing 10% heat inactivated fetal calf serum (Gibco BRL, Maryland, USA) and 50 IU/ml of penicillin and 50 μg of streptomycin (Gibco BRL) at 37℃ in a 5% CO_2 atmosphere.

Cationic liposomes containing HuIFNβ gene plasmid (IAB-1)

Clinical-grade freeze-dried cationic liposomes containing HuIFNß gene plasmid (pDRSV-IFNß), IAB-1, were prepared as described elsewhere [3] in the Human Gene Therapy Vector-Producing Facility established in Nagoya University Hospital. The quality was guaranteed by BML Co., Ltd (Tokyo, Japan) with measuring the concentration of HuIFNß protein in the supernatant (100μl) of $3x10^3$ of U251SP human glioma cell line or $3x10^3$ of RPM-EP human melanoma cell line at 48 hours after adding IAB-1 (150ng DNA/ml) to the medium. Concentration of HuIFNß protein in 6 samples of U251SP or RPM-EP was higher than 793 IU/ml or 492 IU/ml, respectively.

IAB-1 was dissolved by sterile PBS at the concentration of 30 μg DNA/ml at the time of local injection.

Study design

The study was performed as a pilot clinical trial. IAB-1 was injected into cutaneous or subcutaneous metastatic nodules three times a week every other day for 2 weeks. Each injected dose was as follows: 10μg DNA (0.33 ml of IAB-1) for nodules less than 1 cm in diameter and 30μg DNA (1 ml of IAB-1) for

nodules 1 to 2 cm in diameter. A half dose of IAB-1 was injected in the center of the metastatic nodules and the rest was injected around the nodules. The sizes of IAB-1 injected and non-injected metastatic nodules were measured with caliper three times a week for 6 weeks (by the end of observation period). The size of visceral matastatic lesions were also evaluated with CT scan just before the treatment and at the end of observation period. All the IAB-1 injected metastatic skin lesions were resected and investigated histopathologically after the observation period (Fig.1).

Laboratory examination

Peripheral blood cell count, blood chemistry and urinalysis were checked three times a week during the treatment and once a week for the observation period. In order to evaluate the dynamics of the plasmid DNA and HuIFNß, the level of plasmid DNA was investigated by PCR amplification of a plasmid DNA locus and that of HuIFNß by ELISA in patients' sera and urine periodically at Department of Molecular Neurosurgery, Nagoya University Graduate School of Medicine.

Efficacy of IFNß gene therapy

The clinical response was evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST criteria) [5]. Patients were deemed to have had a 'mixed response' if some of their tumors showed regression by > 25% of the pretreatment mass while others showed progression by 25% of the pretreatment mass or new metastases appeared[6].

Biopsy specimens were evaluated by routine histology with hematoxyline and eosin staining and by immunoperoxidase staining with the Envision+ system (DAKO, CA、USA). The anti-CD3 polyclonal Ab(DAKO), anti-CD4 mAb(NOVOCASTRA, Wetzlar, Germany), anti-CD8 mAb (DAKO), anti-CD56 mAb (Nichirei Bio, Tokyo, Japan), anti-CD68 mAb (DAKO), anti-MART1(COVANCE, CA, USA), anti-HMB45(DAKO), anti-HLA-Class I mAb HC-10 (7), and anti-HLA class-II mAb LG-II-612.14(8) were used for the immunohistochemical assay. Apoptotic melanoma cells were detected in these specimens by the TUNEL method, using in situ detection kit (Chemicon International, Inc, CA, USA).

Safety evaluation of IFNß gene therapy

Clinical symptoms and the data of laboratory examination were carefully observed for safety of this treatment. Any adverse effects were evaluated according to NCI-CTC criteria.

Results

Patient characteristics

Melanoma patients enrolled in this clinical study were summarized in Table 1. Five patients (3 males, 2 females; aged 33-73 years) were enrolled in this study during two years from July 2003 to June 2005. Patient 4 (the only stage III patient) had initially received resection of the primary lesion on his right heel along with right groin lymph node dissection (no metastases were detected in the lymph nodes), but one year later, many in-transit metastases appeared on his right legs. Visceral metastases had not been confirmed before treatment.

Others (patient 1, 2, 3, 5) were all patients with distant metastases (stage IV). Patient 1 received resection of the primary lesion on his left buttock along with lymph node dissection of left groin and of intrapelvis (pT3aN1bM0, Stage IIIB). Adjuvant chemotherapy (DAV: dacarbazine, nimustine, vincristine) had been given, but skin metastases appeared on the left buttock and thigh 4 months later. Tiny skin metastases (4 lesions on left buttock, 37 on left thigh) (Fig. 2A, 3A) and metastases of mediastinal lymph nodes and bilateral lungs had been confirmed before initiation of the gene therapy. Patient 2 had received amputation of his right big toe bearing the primary melanoma along with right popliteal and groin lymph nodes dissection (pT4bN3M0, Stage IIIC). Although adjuvant chemotherapy (DAV) had been given, many skin metastases appeared on his right lower legs a half year later. Right iliac lymph node metastases and a total of 601 skin metastases on his right legs were observed. Patient 3 received resection of primary melanoma on her occipital skin along with left posterior neck lymph node dissection (metastases were detected) (pT4aN1bM0, stage IIIB). Although adjuvant chemotherapy (DAV) had been given, 6 years later, skin metastases on her forehead and metastases to liver and lungs appeared. Before initiation of the gene therapy, metastases to bilateral lungs, liver, kidney, intraorbit, spinal cord, skin and lymph nodes were detected. Patient 5 had received resection of primary melanoma on her left heel along with right groin and iliac lymph node dissection (pT3aN0M0, Stage IIA). Adjuvant chemotherapy (DAV) had been given. However, 6 years later, skin metastases appeared on her right groin and thigh. Skin metastases and metastases of right iliac, para-aortal and postperitoneal lymph nodes were detected before initiation of the gene therapy.

The total amount of injected IAB-1 was 10 μg (patient 1: tumor size 5.4 x 3.2 mm , patient 5: 8.8 x 7.8 mm) or 30 μg (patient 2, 3, 4). 10 μg DNA of IAB-1 was injected into three metastatic lesions in patient 2 (tumor size: 8.6 x 7.2 mm, 7.0 x 6.7 mm, 10.3 x 10.2 mm) and patient 4 (tumor size:4.8 x 4.7 mm, 4.5 x 3.7 mm, 5.2 x3.7 mm) and 30 μg DNA into one lesion in patient 3 (tumor size: 22.6 x 16.7 mm) (Table 1).

Evaluation of efficacy

One patient (patient 1) showed mixed response (MR), one

patient (Patient 5) no change (NC), and three patients (patient 2, 3, 4) progressive disease (PD) (Table 1). MR patient has PD by RECIST criteria due to development of new metastases. He died of brain metastases at 11 months after gene therapy.

Findings in skin lesions of patient 1 (MR)

In this patient, 4 skin metastases existed on the grafted skin at the excised primary site on the left buttock and 37 tiny skin metastases on the left thigh. IAB-1, 10 μg DNA, was injected into one of the nodules (5.4 mm in the maximum diameter) on the left buttock (Fig.2A). Redness had appeared around the injected lesion after second injection and then scale-crust appeared on the surface of the lesion and the nodule gradually flattened and became a scar lesion on day 8 after the final injection. The nodule disappeared on day 31 (Fig. 2B).

Redness was also recognized around a half number of tiny papular skin metastases on the left thigh after 4th injection and scale-crust appeared on the surface and the papules finally flattened (Fig. 3B). However, new metastases appeared on the region and the number of the skin metastases increased gradually and was 57 on 18 days after the final injection. Each visceral metastatic lesion was slightly enlarged compared to those before treatment on day 31 after the final injection.

IAB-1 injected skin lesion was excised on day 31 and IAB-1 non-injected lesions on day 3 and day 31 after the final injection. Histopathology of the IAB-1 injected nodule showed marked dermal fibrosis and infiltration of many clear cells between collagen bundles in the dermis (Fig. 2C). These clear cells were MART-1 negative and CD68 positive, confirming that these were lipid containing macrophages (Fig. 2D). No melanoma cells were detected by immmunohistochemistry using anti-HMB45 and anti-MART-1 antibodies in the dermis , and thus this IAB-1 injected metastatic nodule was judged as complete response.

Histopathological examination of IAB-1 non-injected lesions on 3 days after the final injection showed epidermal hyperplasia and dense lymphocytic infiltration around the melanoma cell nests in the dermis (Fig. 3E). Melanoma cells expressed HLA-class II antigen(Fig. 4A), and the infiltrating lymphocytes were mainly CD4 positive (Fig. 4B). Apoptotic cells were identified by the TUNEL assay(Fig. 4C).(Before this gene therapy, we excised one of the cutaneous metastases and investigated the tissue with immunological staining. Melanoma cells did not express HLA-class II antigen, and lymphocytic infiltration was only minimal, and most of them were CD8 positive T cells.) Histopathology of IAB-1 non-injected flattened skin lesions on 31 days after the last injection showed degenerated MART-1 positive melanoma cells were identified in mid-dermis. Lymphocytic infiltration was detected around the nests and even among the melanoma cells (Fig. 3F). Melanoma cells still expressed HLA-class II antigen, and infiltrating lymphocytes were mainly CD8 positive T cells. CD4 positive T cells, prominently detected on 3 days after the final injection, were hardly found in this stage. HLA-class I antigen were expressed on melanoma cells consistently before

and after the treatment in this patient.

Findings in skin lesions of patient 2, 3 and 4 (PD)

In patients 2 and 4, IAB-1 injected skin lesions were temporarily flattened by a week after the final injection, however, they then gradually enlarged the size, and became larger compared to those before treatment with the exception of one skin lesion in patient 4 on day 31 after the final injection. In patient 3, no response was observed on the IAB-1 injected skin lesion. The IAB-1 non-injected skin lesions in these patients enlarged the size and increased in number. Histopathological findings of IAB-1 injected sites in patient 2 and 4 showed the necrotic tumor cell nests surrounded by lipid containing macrophage, lymphocytes, and neutrophils in the dermis. Melanoma cells invaded into subcutaneous fat (patient 2) and into subepidermal areas (patient 4). In patient 3, melanoma cell nests in subcutaneous fat were surrounded by lipid containing macrophage.

Expression of HLA-class I antigens on the melanoma cells were weak in patient 3, and melanoma cells in patient 2 and 4 showed no expression of HLA-class I.

Findings in skin lesions of patient 5 (SD)

In patient 5, the size of IAB-1 injected and non-injected skin lesions did not change and no new metastatic skin lesions appeared. HLA-class I antigen was not expressed on melanoma cells in this patient.

Findings in visceral metastases

The sizes of the visceral metastases were enlarged in patients 1, 2, 3 and new metastatic visceral lesions were detected in patient 2. No change of the visceral lesions was observed in patient 5. New metastatic visceral lesions were not detected in patient 4 (Table 1).

Detection of plasmid DNA & HuIFNß in sera and urine of the patients

HuIFNß protein was temporarily detected in sera of patient 1 and 2, and plasmid DNA in sera in patient 1 (Table 2). In urine, neither HuIFNß nor plasmid DNA was detected in any of the patients.

Evaluation of safety

Anaphylactic reaction, high fever, and infection and/or bleeding at injection sites, had been thought to be possible adverse clinical events of this gene therapy, however, there were no adverse events including fever or depressive state, sometimes seen in administration of HuIFNß protein. No abnormal laboratory data attributed to this gene therapy were seen in all the treated patients.

Discussion

This is the first clinical study using HuIFNß gene for patients with advanced melanoma. HuIFNß gene is located at chromosome 9p21, which is often defective in melanoma cells[9]. Autocrine interferon secretion, rather than exogenous interferon, has been found to strongly inhibit proliferation of melanoma cells[10]. Therefore, induction of HuIFNß gene into 9p21 defected melanoma cells can increase sensitivity to HuIFNß. In addition, HuIFNß shows anti-proliferative and apoptotic effects on melanoma cells in a dose dependent fashion[11]. In our previous experiment, 5 daily direct injections of $1x\ 10^6$ IU of HuIFNß protein could not decrease the size of cutaneous metastatic lesions of melanoma[1]. One reason of this unresponsiveness was due to the low level of HuIFNß protein, because serum concentration of HuIFNß drops to less than 2 units/ml at one hour after intramuscular or subcutaneous injection with 6×10^6 units [12]. To maintain higher concentration of HuIFNß within the lesion may be crucial to inhibit the tumor growth. Concerning these two mechanisms, i.e. increasing sensitivity to HuIFNß and maintaining higher local concentration of HuIFNß, HuIFNß gene therapy is considered to be attractive treatment for melanoma patients.

Cationic liposomes have been used as a safer alternative to virus vectors in experimental and/or clinical trials of gene therapy for melanoma [13, 14, 15]. HuIFNß gene therapy using cationic liposomes was started for glioma patients in Nagoya University Hospital in Japan since April, 2000 [3]. In order to check the efficacy of the same gene therapy for melanoma, we injected IAB-1 locally six times to nude mice bearing human melanoma nodules. As reported in the previous paper[2], we confirmed the efficacy of the therapy against melanoma. Thereafter, we planned to start the present clinical trial to examine effects of IAB-1 for advanced melanoma patients.

Five melanoma patients were enrolled in this clinical study. Adverse events and abnormal laboratory data attributed to IAB-1 injection was not seen in any of the patients, although IFNß (patient 1, 2) and plasmid DNA (patient 1) were detected in sera temporarily during injection period.

The efficacy of this gene therapy was generally insufficient; one MR, one NC, three PD. However, in the MR patient, IAB-1 injected lesion (left buttock) completely disappeared, and a half number of IAB-1 non-injected lesions (left thigh) were flattened. In addition, histopathologically, CD4 positive T cells infiltrated around the tumor cells expressing HLA-Class II antigen in association with epidermal hyperplasia and many apoptotic melanoma cells even in IAB-1 non-injected lesions, suggesting induction of immunological responses to melanoma cells. The mechanism was not elucidate, but it would be possible that macrophage, CD4 positive and CD8 positive T cells activated by HuIFNß at IAB-1 injected site, infiltrate into non-injected lesions and secrete various cytokines such as IFN-γ and heparin-binding EGF-like growth factor[16], an autocrine growth factor for human

kerationocyte[17], and that these cytokines could increase HLA Class II expression on melanoma cells and induce epidermal hyperplasia. It is not certain many apoptotic melanoma cells were induced by direct effect of HuIFNß. Melanoma cells of patient 1 solely expressed HLA-Class I antigen strongly among the enrolled 5 patients, and systemic immunological response was exclusively observed in this patient. This result may suggest the importance of cytotoxic T lymphocytes (CTL) response for induction of systemic response, although induction of NK cells were verified with this gene therapy in the system of B16 mouse melanoma that expressed low level of major histocompatibility complex (MHC) class I antigen[18]. CTL-based immunological reaction may be crucial for inducing systemic response with this gene therapy. Thus, it may be important to investigate abnormalities in the antigen processing machinery and in the chaperon molecules in melanoma cells [19]. Further studies will be required to define the antitumor mechanism including pharmacokinetics of IAB-1, direct effect of HuIFNß and susceptibility of HuIFNß gene transferred melanoma cells through this gene therapy.

Among 5 glioma patients treated with the same gene therapy in Nagoya University Hospital, 2 patients had PR, 2 patients had NC. Thus, this gene therapy seems to be more effective for glioma patients [3]. In the clinical study for glioma patients, surgical treatment was combined with the gene therapy, which could not be compared to the present study for advanced melanoma.

Repeated cationic multilamellar liposome-mediated gene transfers enhanced the transduction efficiency against murine melanoma cell lines and experimental subcutaneous melanoma due to the increased number of cells expressing transgenes [20]. Therefore, in order to get much more efficacy in the gene therapy, a possible next step may be increase of the number of administration of HuIFNß gene. Combination with other therapies such as dendritic cells or with other cytokines may be effective. Safety of the HuIFNß gene therapy was suggested by this study. Thus, HuIFNß gene therapy seems still an attractive strategy for treatment of a variety of malignancies including malignant melanoma.

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Figure legends

Fig. 1 Schedule of the clinical study of the interferon ß gene therapy

Fig. 2 IAB-1 injected lesion on the left buttock of patient 1. Clinical findings just before IAB-1 injection(A) and 31 days after the final injection(B). Histopathological finding of the specimen resected on 31 days after final injection. HE stain (x200) (C), immunostaining with CD68 (x150) (D)

Fig. 3 IAB-1 non-injected lesions on left thigh of patient 1. Clinical findings just before IAB-1 treatment (A), 3 days after the final injection (B), and 31 days after the final injection (C). Histopathological findings of the specimen resected before the treatment (D), on 3 days (E) and on 31 days (F) after the final injection (x100).

Fig. 4 Immunostaining of the specimen resected on 3 days after the final injection. Expression of HLA-Class II antigen (x100)(A) and CD4 antigen (x100)(B). The TUNEL staining of apoptotic melanoma cells of the specimen resected on 3 days after the final injection (x100)(C) and the specimen before the treatment (x100) (D).

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Appendix

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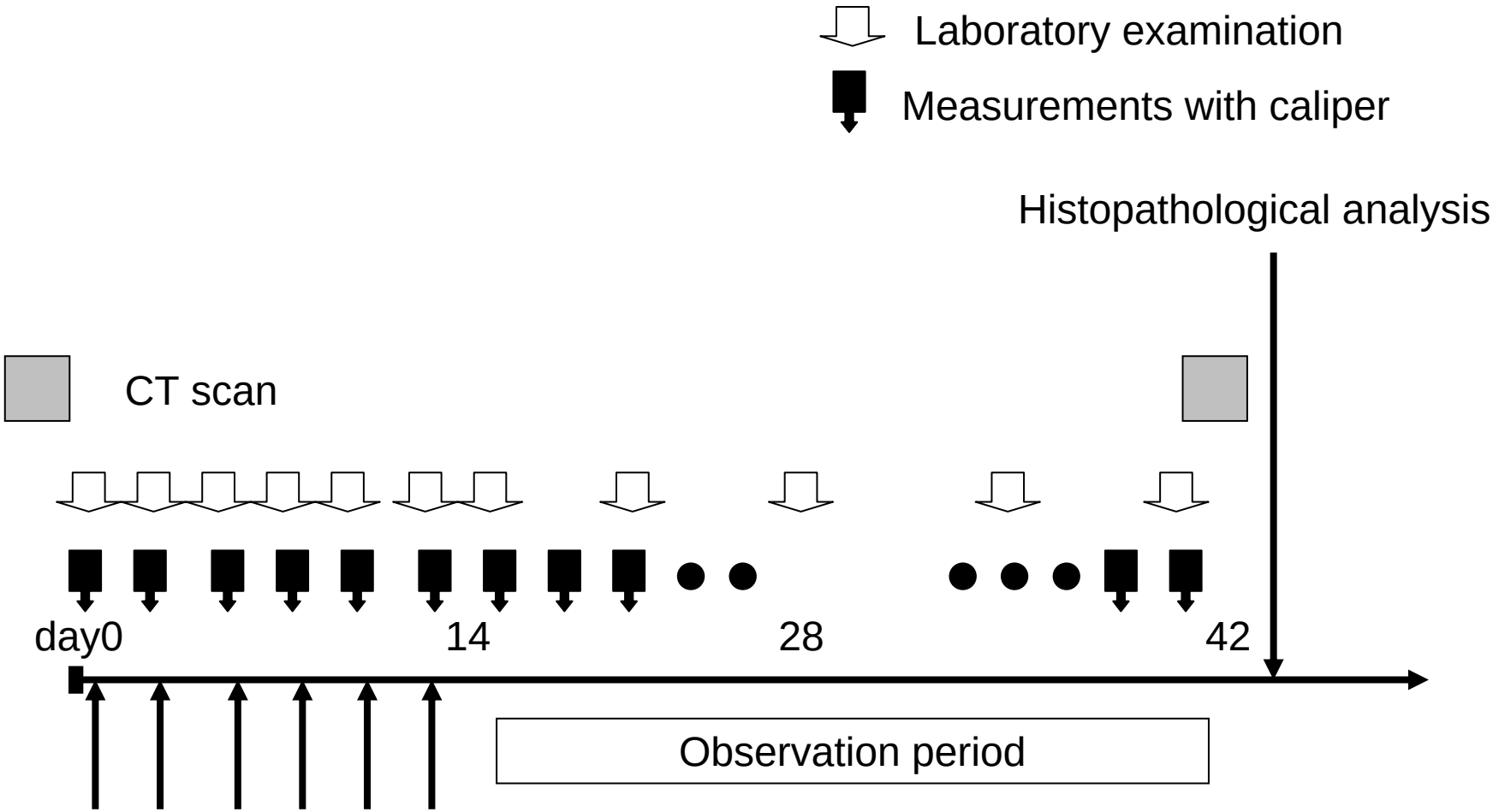
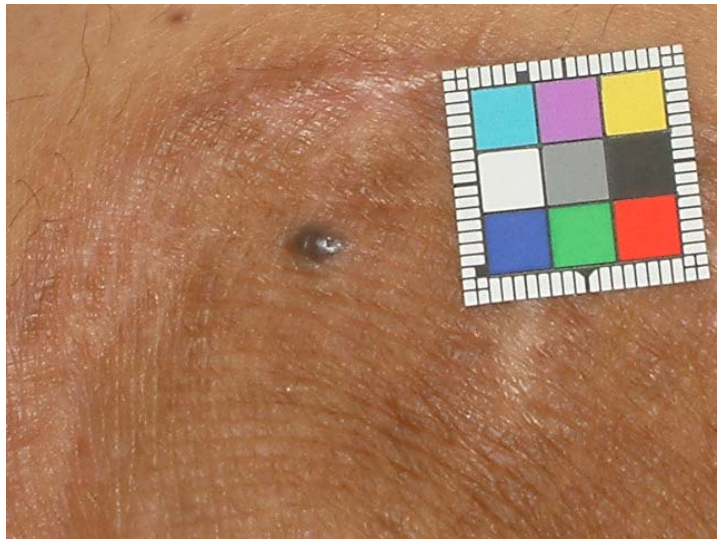
Fig. 1 Schedule of the clinical study



Local injections of IAB-1
into metastatic skin lesions
every other day (six times)

Fig. 2



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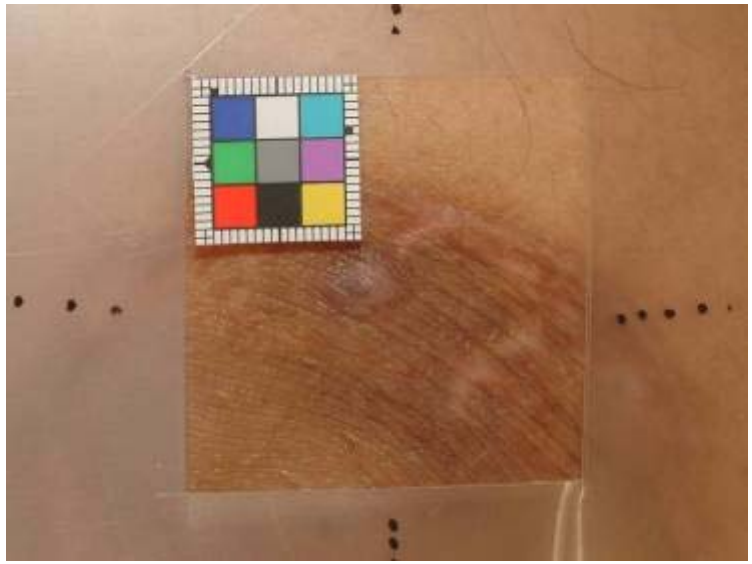

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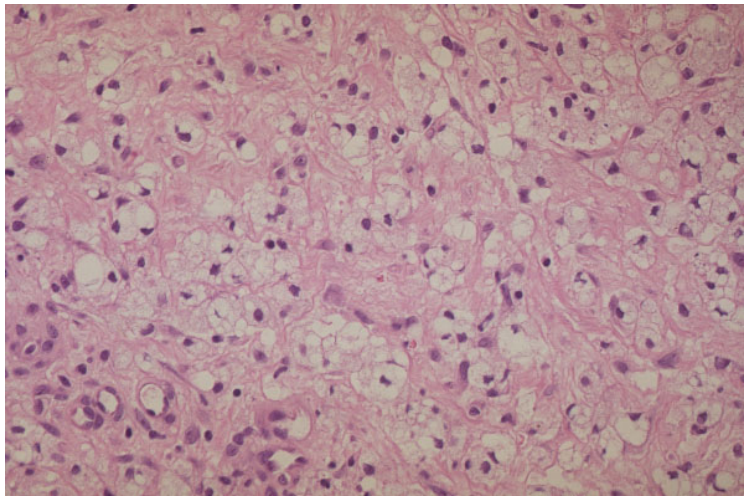

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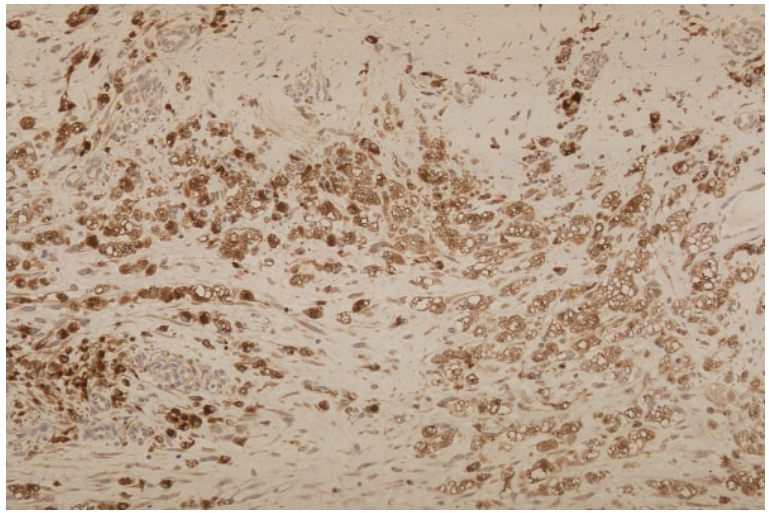
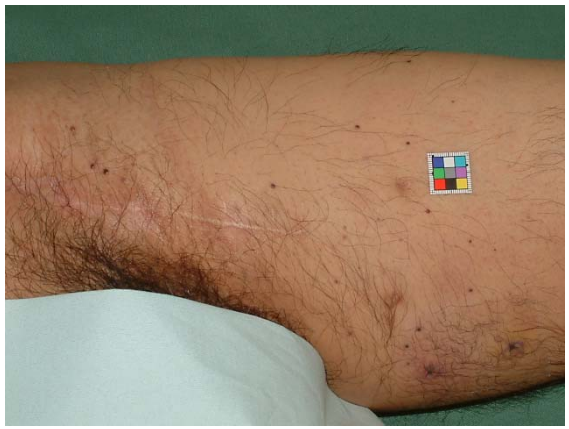


D

Fig. 3



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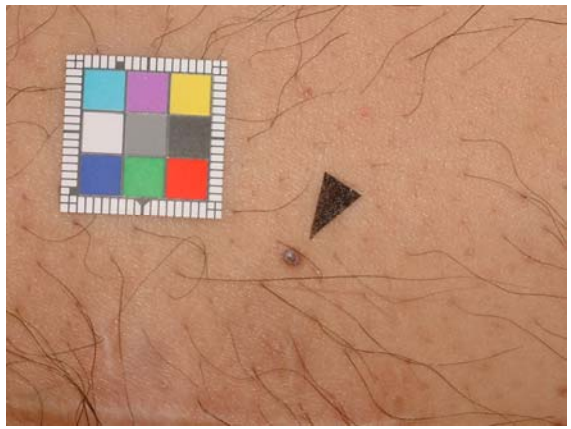

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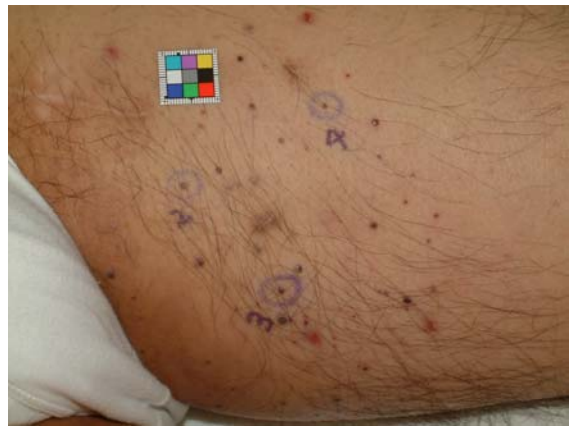
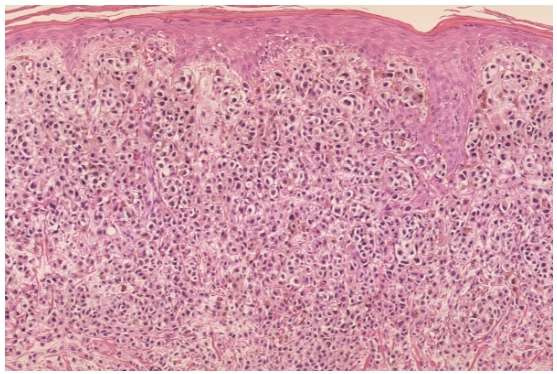


C



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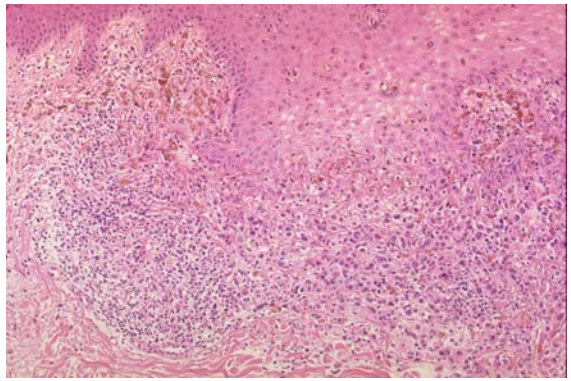

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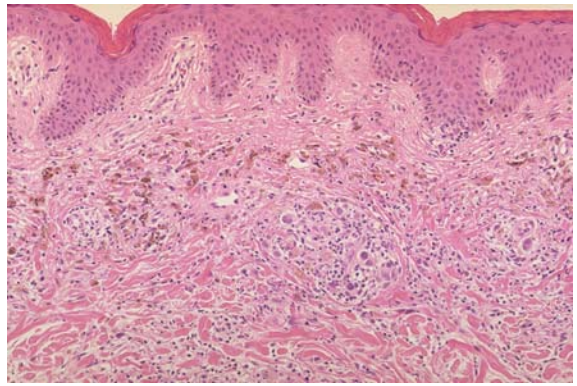
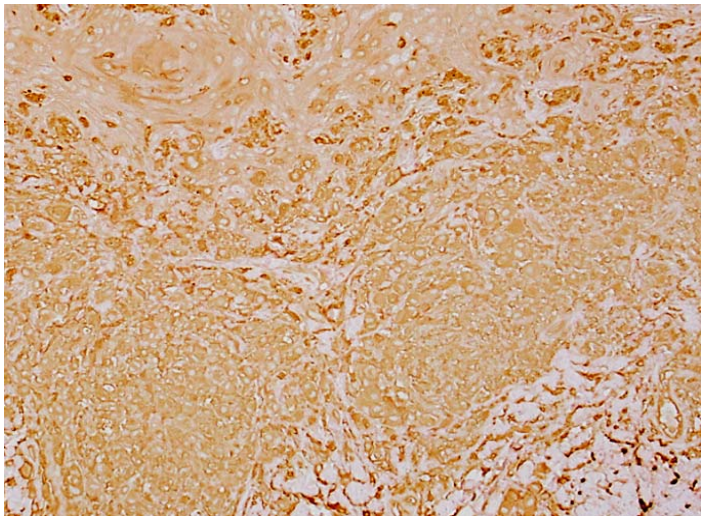


F

Fig. 4



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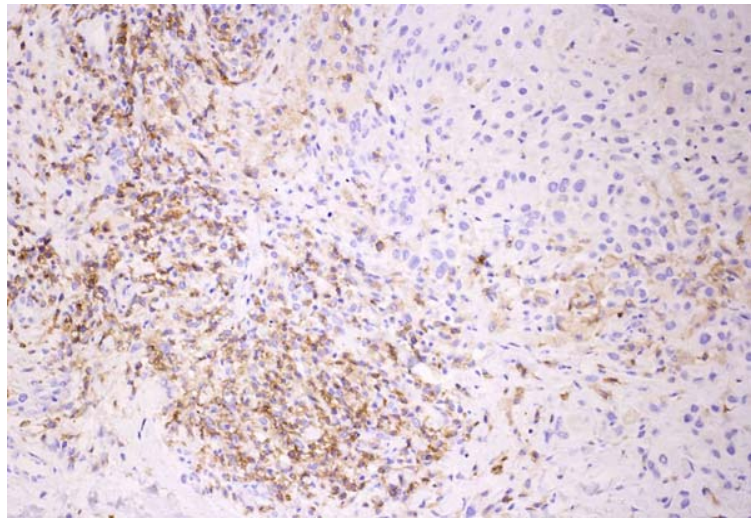

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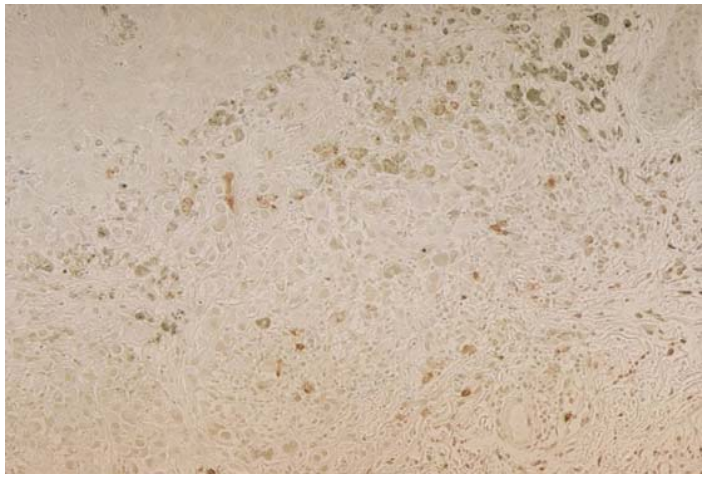

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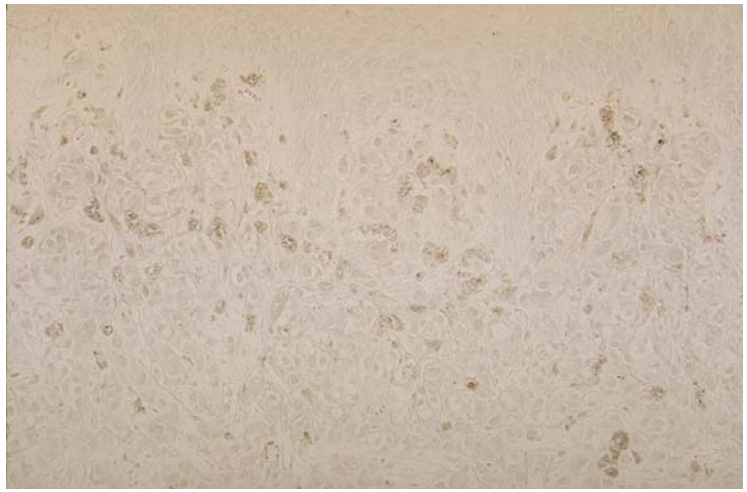

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