# The growth arrest mechanism of the internal radiotherapy using Holmium-166 in B16 melanoma

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# The growth arrest mechanism of the internal radiotherapy using Holmium-166 in B16 melanoma

Directed by Professor Jong Doo Lee

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by
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A Dissertation for the Degree of Dr. of Philosophy in Medical Sciences by Byoung-Hee Han has been approved by

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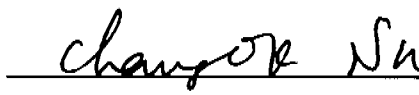

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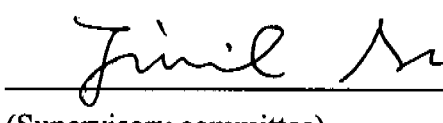

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# 감사의 글

"너는 복의 근원이 될찌라" (창세기 12:2)

살아계신 하나님께서 예수 그리스도의 말할 수 없는 십자가의 크신 은혜와 부활의 권능으로 저를 구원하시고, 박사과정을 마치기까지 믿음의 길을 걸어오게 하신 것을 감사하고 찬양 드립니다. 또한 이기적이고 소시민적인 저를 연대, 이대와 세계 캠퍼스 지성인들의 목자요 복의 근원의 삶을 살게 하신 것을 감사 드립니다. 이 논문의 시작부터 완성에 이르기까지 어느 때보다 각별히 노심초사하시고 수고하시며 지도해주신 이종두 교수님, 관심과 지도를 아끼지 않으신 서창욱 교수님, 이민걸 교수님, 양우익 교수님, 성진실 교수님, 섬세한 손길로 애써주신 연구원 여러분들께 감사를 드립니다. 저를 낳고 헌신적으로 사랑하며 길러주시고 지금은 주님 품에 안겨계신 나의 아버지, 지금까지도 사랑하시며 기도하시는 어머니, 영적 해산과 양육을 위한 사랑의 수고를 감당해오신 김다윗 목자님과 김사라 사모님, UBF 연희 센타의 형제자매들과 평신도 목자님들, 특히 김성보 목자님을 비롯한 주니어 평신도 목자님들, 지난 9년간 사랑 안에서 동역하며 기도해 준 5부 형제자매 동역자들, 항상 기도와 격려를 아끼지 않으신 장모님과 장인어른, 그리고 눈물의 기도와 사랑으로 함께 해 준 사랑하는 아내에게 감사를 드립니다. 박사과정동안 여러모로 지원해주신 삼성 제일병원 방사선과 조병제 선생님, 이영호 선생님, 이경상 선생님을 비롯한 여러 의국원들께 감사 드립니다. 살아계신 하나님께서 제가 일평생 예수 그리스도와 그의 십자가의 은혜와 부활의 권능을 알기 위하여 전심전력하는 삶을 살며, 의사 목자로서의 직분을 충성스럽게 감당하고, 길 잃고 방황하는 캠퍼스의 지성인들을 그리스도의 제자들로 일으켜 세우는 영원한 하나님 역사에 복의 근원의 삶을 살도록 축복해 주시기를 간절히 기도합니다.

저 자 씀

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## Abstract

### The growth arrest mechanism of the internal radiotherapy using Holmium-166 in B16 melanoma

The incidence of malignant melanoma has been substantially increasing over the past two decades. Complete surgical excision of the tumor is the only proved therapeutic strategy that leads to long-term disease free survival and radiotherapy and/or chemotherapy have not been proved to be successful. Actually malignant melanoma has been known to be resistant to radiation. In this study, we evaluated the effectiveness and mechanisms of internal radiotherapy using Holmium(Ho)-166. B16 melanoma cells were cultured at 37 °C in RPMI 1640 media, and 5 x $10^5$ cells suspended in 100 μl of normal saline were inoculated into subcutaneous layer in the back of C57BL/6 mice. To evaluate survival rate a total of 40 mice were divided into 2 groups and 0.3 ml of saline alone (group 1, n=20) and carrier free Ho-166 5 mCi (group 2, n=20) in 0.3 ml of saline were injected intra-tumorally. The survival of the mice after injection of the radionuclide was observed until the mice died. Additionally, 30 mice were prepared with the same method as previously described to evaluate the mechanism of the radiation effect. They were divided randomly into 6 groups and five mice in each group were sacrificed by cervical dislocation just before, $1^{st}$, $2^{nd}$, $3^{rd}$, $6^{th}$, and $14^{th}$ days after the injection of 5 mCi of Ho-166 in 0.3 ml of saline. Change of p53 tumor suppressor and its downstream effector genes were evaluated by using immunohistochemistry and RT-PCR and apoptosis was evaluated by TUNEL staining and flow cytometry. Control group showed rapid tumor growth and the mean tumor volume reached approximately 30 times of its original volume. None of control group was alive until $16^{th}$ day after the injection of normal saline with median survival of 9

days. The median survival was 33 days in the treated group. Histological examination demonstrated wide central necrosis that matched with the area of Ho-166 deposition and viable tumor cells were distributed along the peripheral margin without cytologic features of apoptosis. Expression of bax mRNA increased substantially until 48 hours after treatment and then decreased, however, overall higher than control. But expression of p53 and p21 increased until 48 hours after treatment and then decreased. RT-PCR finding was closely correlated with the results of immunohistochemistry. TUNEL positive nuclei were observed from 2 days until 2 weeks after treatment despite cytologic features of apoptosis was not noted. Flow cytometry also did not demonstrate any results supporting apoptosis. These results suggest that Ho-166 brachytherapy is useful for the local treatment of melanoma and the therapeutic mechanisms of high dose continuous irradiation using beta-emitting agent are necrosis or secondary necrosis with resulting growth arrest.

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Key words: malignant melanoma, internal radiotherapy, Holmium-166, radionuclide, apoptosis, growth arrest

**The growth arrest mechanism of the internal radiotherapy using Holmium-166 in B16 melanoma**

(Directed by Professor **Jong Doo Lee**)

Brain Korea 21 Project for Medical Sciences

*The Graduate School, Yonsei University*

**Byoung Hee Han**

# I. Introduction

The incidence of malignant melanoma has been increasing substantially over the past two decades.[1] Excision of the primary and metastatic lesions is the only therapeutic strategy that could lead to long-term disease free survival. The data from the National Cancer Data Base indicate that additional adjuvant therapy has been applied in only about 3% of all cases because radiotherapy and/or chemotherapy were not proven to be highly successful and there was little evidence to suggest a benefit for any adjuvant therapy.[2-4]

Melanoma has been considered as a radioresistant tumor because of large shoulder on their radiation survival curve,[5-6] therefore external radiotherapy had a limited role as a primary therapeutic modality. However, recent studies demonstrated that varying radiation sensitivity of melanoma cells within the same individual as well as between tumors.[7] In addition, treatment methods such as dose per fraction and total dose would affect the radiosensitivity.[8] The purpose of this study was to evaluate therapeutic effects of high dose internal radiotherapy by intra-tumoral injection of beta-emitting

radionuclide, Ho-166, as an alternative to external radiation in an animal model with B16 melanoma. A patient with widespread subcutaneous metastases of malignant melanoma was also evaluated.

Beta rays have some advantages over gamma or X-rays since maximum soft tissue penetration range of beta rays is much shorter than gamma rays, therefore, higher radiation dose can be delivered to tumor tissue without adverse radiation effect on the surrounding normal tissue. Ho-166 is an ideal radionuclide for internal radiotherapy because it emits not only 94% of 1.76 and 1.86 MeV beta rays but also emits small proportion of 81 KeV gamma rays suitable for gamma camera imaging. The physical half-life is 26.9 hours and maximum soft tissue penetration range is 8.7 mm (average 2.1 mm).[9]

In terms of radiation biology, previous studies have demonstrated that growth arrest and apoptosis induced by activation of p53 and downstream effector genes play an important role in radiation induced cell death mechanisms.[10-12] However, high dose continuous irradiation on tumor using beta-emitting radionuclides has not been extensively evaluated. In this study, underlying biologic mechanisms of this treatment modality were evaluated by the analysis of the expression of p53 tumor suppressor and p53 downstream effector genes using RT-PCR and immunohistochemistry, as well as routine histology, TUNEL staining, and flow cytometry for assessment of apoptosis whether these genes and protein products play an important role in cell death mechanisms of internal radiotherapy using Ho-166.

## II. Materials and Methods

### 1. Animal experiments

B16 melanoma cells were cultured at 37 °C in RPMI 1640 media (Gibco laboratories, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (Gibco laboratories), 2 mL L-glutamine (Gibco laboratories), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco laboratories). Tumor cells (5 x $10^5$) suspended in 100 μl of normal saline were inoculated into subcutaneous layer of the back of C57BL/6 mice and the mice were grown in a specific pathogen free condition with standard diet and water ad libitum. When tumors were grown to be approximately 1 cm of their long axes, tumor volume was approximately 500 $mm^3$. Total 40 mice were randomly divided into 2 groups and 0.3 ml saline alone (group 1, n=20), and carrier free Ho-166 5 mCi (group 2, n=20) in 0.3 ml of saline were injected intra-tumorally to evaluate survival rate. The radionuclide, Ho-166, was prepared by bombarding Ho-165 in a nuclear reactor (HANARO center, Taejon, Korea). The amount of the radioactivity to be injected was determined by computer simulation using the formula provided by Prestwich et al.[13] On the assumption of uniform distribution of injected Ho-166 within 1 cm diameter tumor, radiation dose to the surrounding normal tissue 1, 2, 3 and 4 mm apart from the tumor surface is approximately 120, 36, 10 and 3 Gy/mCi respectively. The survival rate of the mice after injection of the radionuclide was evaluated until death

Additionally, 30 mice were prepared with the same method as previously described for morphological and biological analysis of the radiation effect. They were randomly divided into 6 groups and five mice from each group were sacrificed by cervical

dislocation just before and 1st, 2nd, 3rd, 6th, and 14th days after the injection of 5 mCi of Ho-166 in 0.3 ml of saline. Gamma camera images were obtained with dual-headed gamma camera (ADAC Laboratory, Milpitas, USA) before sacrifice, which confirmed sustained retention of injected radionuclide without leakage.

After gamma camera imaging, the tumor tissues were removed and processed for the flow cytometry, routine histologic examination by hematoxylin-eosin (H&E) staining, immunohistochemical analysis, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining and reverse transcription-polymerase chain reaction (RT-PCR).

Macro-autoradiography was performed to evaluate the distribution pattern of the injected Ho-166, which showed relatively even distribution of the injected radionuclide inside the tumor, however, peripheral margin was devoid of the radioactivity ( Fig. 1).

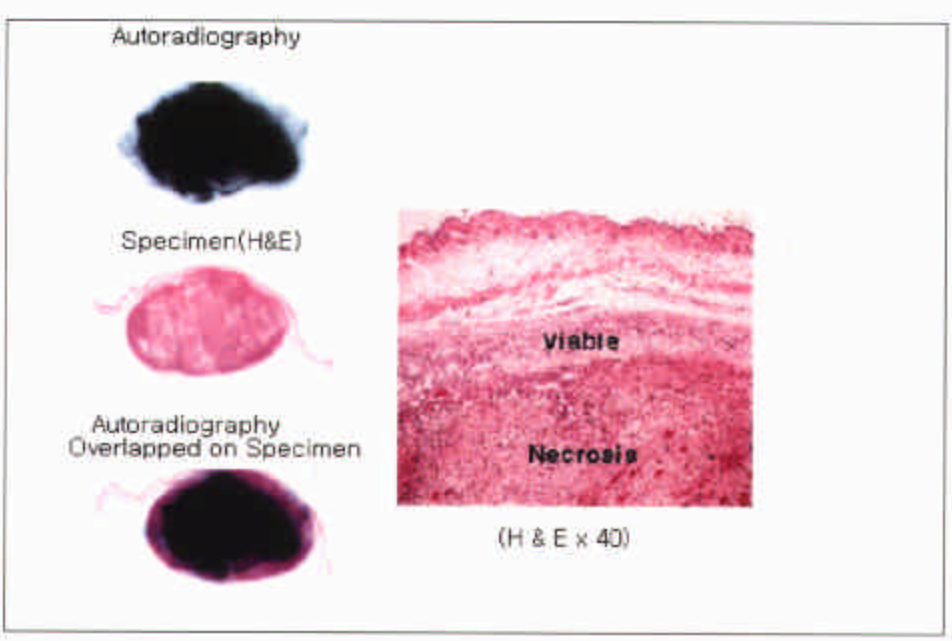

**Figure 1**. Distribution pattern of the injected Ho-166 in tumor. Autoradiographic image (left upper), whole mount histologic image (left mid), and autoradiographic image overlapped on whole mount histologic image (left lower) demonstrate even distribution of injected Ho-166 within the tumor tissue, with sparing of the peripheral margin. Low power microscopy (right) shows tumor necrosis within central part which is well matched with the autoradiographic image but residual viable cells along the peripheral margin is noted.

For visual side-by-side comparison, melanoma cells were inoculated into the bilateral backs of 10 additional mice, and 5 mCi of Ho-166 in 0.3 ml of saline was injected in one side and normal saline in the other side of the tumors. Gross and histological evaluation with H&E staining were performed 2 weeks after injection.

**2. Immunohistochemistry**

Immunohistochemical staining was performed using 4 μm thick formalin-fixed and paraffin-embedded tissue sections. Antibodies used for immunohistochemistry were polyclonal anti-p53 (Santa Cruz, Santa Cruz, CA, USA), monoclonal anti-p21 (BD PharMingen, San Diego, CA, USA), polyclonal anti-bax (BD PharMingen, San Diego, CA), monoclonal anti-cyclin D1 (BD PharMingen, San Diego, CA), and monoclonal anti-proliferating cell nuclear antigen (PCNA) (Dako, Glostrup, Denmark) antibodies. Dilution factors of the antibodies were 1:20 for p53, 1:50 for p21, 1:500for bax , 1:50 for cyclin D1 and 1:100 for PCNA and incubation with primary antibodies was done at 4 °C overnight. Endogenous peroxidase activity was blocked by incubation in 3% $H_2O_2$ for 10 min. Antigen retrieval was performed by microwave pretreatment using

0.01M citric acid buffer (pH 6.0) in a pressure cooker for 15 minutes. After cooling, immunostaining was performed by a commercially available kit (Vector® M.O.M.™ Immunodetection Kit, Vector Laboratories, Burlingame, CA, USA) for monoclonal antibodies and by an EnVision™ kit (Dako) for polyclonal antibodies according to the manufacturer's instructions. Diaminobezidine was used as a chromogen except for the Bax immunostaining for which 3-amino-9-ethylcarbazole was used as a chromogen. The nuclei were lightly counterstained with hematoxylin.

**3. TUNEL stain**

Apoptosis was assessed by the TUNEL method using the ApopTag® Peroxidase *In Situ* Apoptosis Detection Kit (Intergen, Purchase, NY, USA) according to the manufacturer's instructions with some modifications. Sections were deparaffinized in xylene and rehydrated in a graded concentration of ethanol and then in distilled water. They were then treated with proteinase K (20 μg/ml) for 15 minutes at 37 °C and washed in distilled water. After applying equilibrium buffer, the sections were incubated with digoxigenin-dNTP and TdT at 37 °C for 60 minutes. After being washed and endogenous alkaline phosphatase was inhibited, sections were incubated with alkaline phosphatase conjugated rabbit F(ab') anti-digoxigenin (Dako) at 1:20 for 30 minutes at room temperature. They were washed, and the signal was detected by incubation with 5-bromo-4-chloro-3-indolyl-phosphatase/nitroblue tetrazolium (Dako) for 20 minutes before being counterstained with nuclear fast red.

**4. Flow cytometry**

Using the fresh tumor tissue immediately removed from the mice, flow cytometry was performed after separation of viable cells and necrotic debris. The viable cells were labeled with PI and AnnexinV and flow cytometry was performed to evaluate whether expression of Annexin V is present within the viable cell population.

**5. RT-PCR**

Tumor tissues within the peripheral margin containing viable cells were removed from the paraffin blocks and RNA was isolated by paraffin block RNA Isolation kit (Ambion Inc., Austin, Texas, USA) according to the manufacturer's instructions. To synthesize cDNA, 3 μg of total RNA was mixed with 100 ng random hexamer (Phamacia, Uppsala, Sweden), boiled at 70 °C for 10 min, and then quickly chilled on ice. Three μl of 5 X first strand buffer, 1.5 μl of 0.1 M DTT, 2 μl of 10 mM dNTPs and 200 units murine molony leukemia virus-reverse transcriptase (MMLV-RT) (Gibco BRL, Grand Island, NY, USA) were added into the reaction mixture and incubated at 42 °C for 2 hrs. The reaction mixture was boiled at 95 °C for 5 min, quickly chilled on ice, and 20 μl of distilled water was added. To determine that the equal amount of RNA was used, the expression level of beta-actin mRNA was also examined. PCR reaction was performed in 50 μl vol containing 4 μl cDNA, 4 μl of 2.5 mM dNTPs, 1 μl of 10 pmol each up-stream primers, 1 μl of 10 pmol each down-stream primers, 5 μl of 10 X PCR buffer, 30.5 μl distilled water and 2.5 units of Taq polymerase (Solgent, Korea). After initial 3 min incubation at 94 °C, PCR reaction was performed by the following conditions: denaturation at 94 °C for 1 min, annealing at 60 °C (p21; 62°C) for 1 min, and polymerization at 72 °C for 1 min for 40 cycles. The primers used were:

p53 forward: 5'-CAAGTGAAGCCCTCCGAGTG-3',

reverse : 5'-GGCAGCGTCTCACGACCTCC-3',

bax forward: 5'-CAGCTCTGAGCAGATCATGAAGACA-3',

reverse : 5'-GCCCATCTTCTTCCAGATGGTGAGC-3',

p21 forward:5'-AAGACCATGTGGACCTGTCA-3',

reverse :5'-GGCTTCCTCTTGGAGAAGAT-3',

beta-actin forward : 5'-CGTGGGCCGCCCTAGGCACCA-3',

reverse: 5'-TTGGCCTTAGGGTTAGGGGGG –3'.

# III. Results

## 1. Tumor volume changes and survival rates following intra-tumoral injection of Ho-166.

Nine to ten days after inoculation of melanoma cells, mean tumor volume reached 492.5-631.9 $mm^3$. Control group showed rapid growing of the tumor and the mean tumor volume reached approximately 30 times of original volume and none of control group was alive until $16^{th}$ day post injection of normal saline. The tumors of control group were initially round shape with pseudocapsule but progressively enlarged with lobulated contour. The median survival of the control group was 9 days after injection.

Histological examination demonstrated wide central necrosis that was matched with the area of Ho-166 deposition. Viable tumor cells were distributed along the peripheral margin, and these cells became progressively swollen with vacuolization and giant cell formation. Increased melanin pigmentation was noted but morphologic findings of apoptosis such as cellular shrinkage with nuclear condensation and fragmentation were not observed (Fig. 2).

The median survival was 33 days in the treated group (Fig. 3) and 5 mice are still alive until 160 days after treatment.

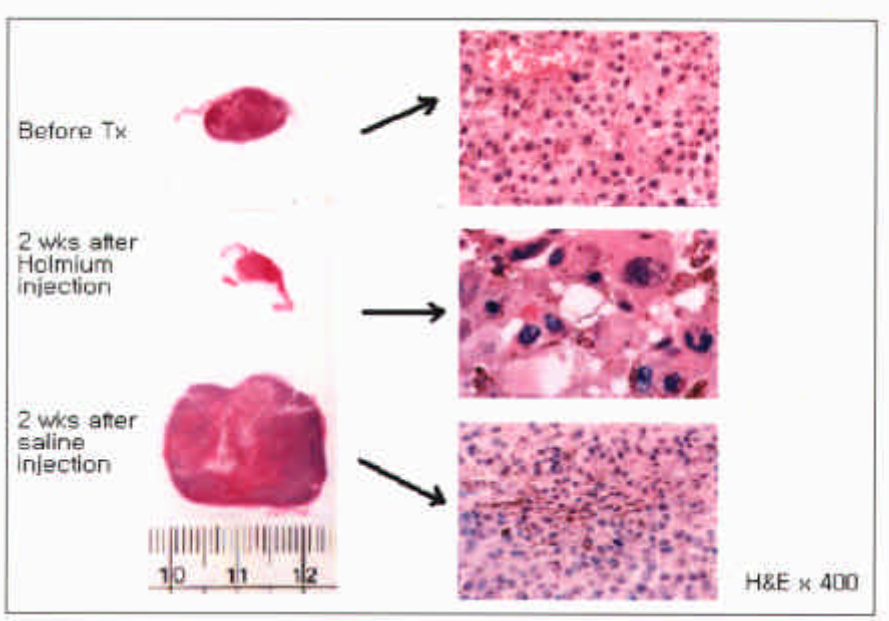


**Figure 2.** Microscopic findings of representative samples of the tumors before and 2 weeks after Ho injection, and 2 wks after saline injection . One cm sized tumor before the injection shows uniformity of the tumor cells (upper), however, Ho treated group shows decrease of tumor size and swelling of tumor cells and increased melanin pigments (mid) in contrast to the untreated group (lower) or without cytologic change of the tumor cells compared with those before treatment.

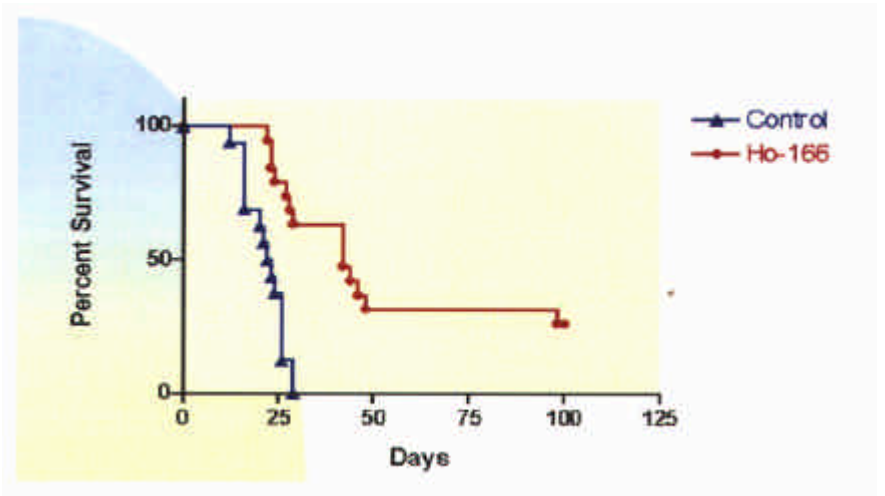

**Figure 3.** Survival curves of the control and treated group.

The median survival of treated group was longer than that of control group and survival rate was also higher in treated group ($p<0.001$). Gross and microscopic evaluation with H&E staining 2 weeks after treatment in mice with melanomas in both sides of the back showed large tumor mass with minimal necrosis or shrinkage in non-treated side but markedly shrunken tumor with extensive necrosis in treated side (Fig. 4).

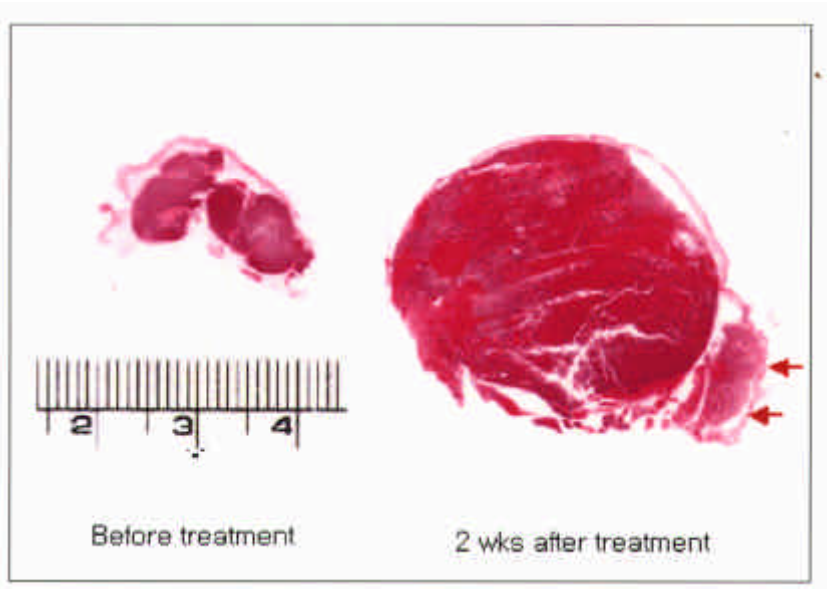


**Figure 4.** Whole mount sections of melanomas in both side of the back of a mouse. A large tumor mass with minimal necrosis in the non-treated side but extensive necrosis with marked shrinkage in the treated side (arrows).

**2. Expression pattern of p53, p21, bax, PCNA and cyclin-D1 proteins following intra-tumoral injection of Ho-166.**

Control group without treatment showed strong positive nuclear reaction for

PCNA and cyclin D1 of viable cells, while expression of p53, p21 and bax protein was not observed (Fig. 5).

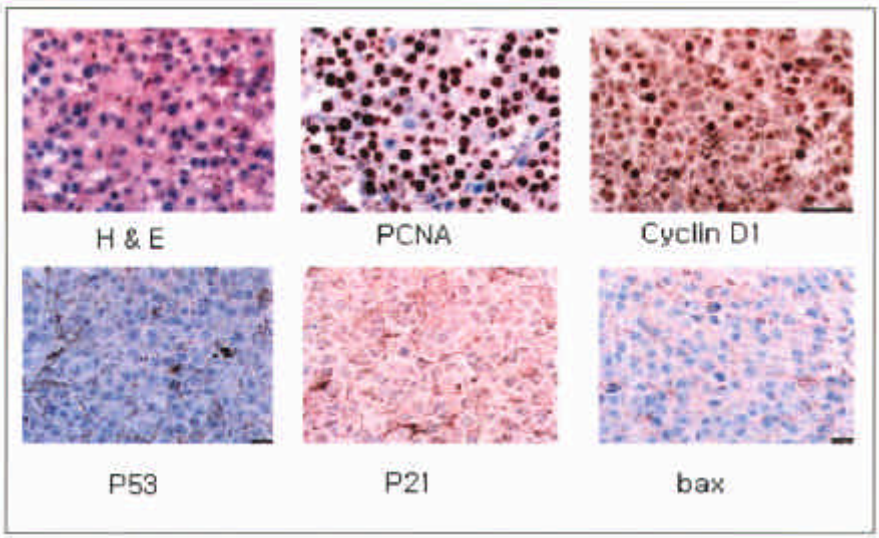


**Figure 5.** H&E and immunohistochemical findings of control group. Representative photos of control group show negative reaction for p53, p21, bax but strong positivity for PCNA and cyclin D1.

Twenty-four hours after treatment, cellular swelling and nuclear enlargement occurred. There was no change of PCNA positivity compared with control but cyclin D1 expression was markedly reduced. Nuclear p53 was strongly expressed in most viable cells, a less strong positivity for p21 was noted, and faint cytoplasmic positive reaction for bax began to appear (Fig. 6). Two days after treatment, swelling of cytoplasm and enlargement of nuclei were more prominent. Necrosis became prominent and increased melanin pigments was noted. Expression of PCNA and p53 persisted without remarkable change but cyclin D1 expression disappeared and expression of p21 and bax increased.

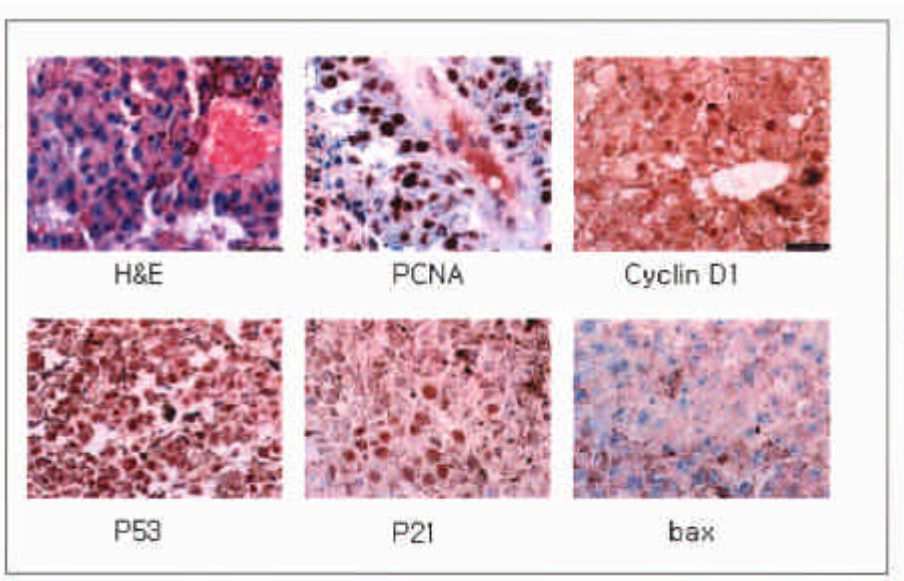


**Figure 6.** Representative photos of twenty-four hours after Ho-166 treatment. PCNA is strongly positive but markedly decreased cyclin D1 reaction was noted. P53 is diffusely positive and a scattered p21 positivity is observed. Weak cytoplasmic bax expression is noted.

Three days after, PCNA was persistently strong positive but p53 expression near totally disappeared and p21 expression was slightly diminished. Cyclin D1 immunreactivity reappeared and bax expression was strong positive. Until 2 weeks after treatment, expression of PCNA, cyclin D1, p21 and bax was seen although the intensity of positive reaction was decreased. Cytoplasmic swelling, vacuolization, enlargement of nuclei and disruption of cell membrane progressed and increased intracytoplasmic melanin pigments persisted (Fig. 7-9).

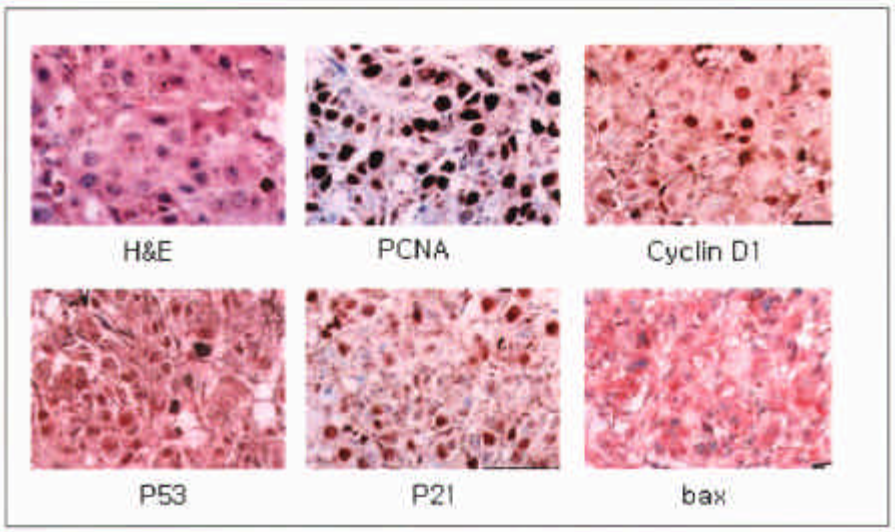


**Figure 7.** H&E and immunohistochemical findings 72 hrs after treatment.PCNA expression is strong positive, cyclin D1 is weakly positive and p53 is markeldly diminished. p21 is also decreased, however, bax expression is increased.

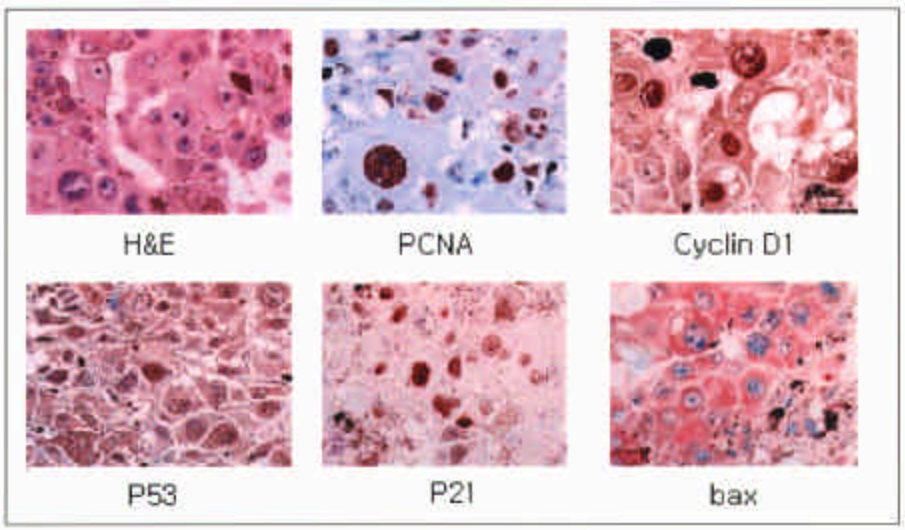


**Figure 8.** H&E and immunohistochemical findings 6 days after treatment. Cytoplasmic and nuclear swelling is prominent. PCNA and cyclin D1 reactivity is persistently positive, p53 is almost negative but p21 is still observed. Bax expression is strong positive.

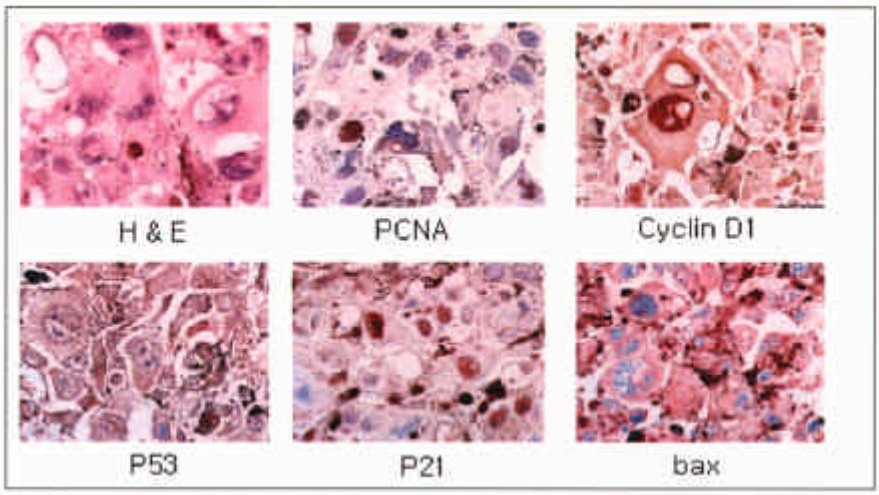


**Figure 9.** H&E and immunohistochemical findings 2 weeks after treatment. Bax shows strong positivity, otherwise similar to those seen on 6 days after treatment.

### 3. RT-PCR

Expression pattern of mRNAs of p53, p21 and bax was closely correlated with those of immunohistochemistry (Fig. 10). Expression of bax mRNA increased substantially until 48 hours after treatment and then decreased, however, overall higher than control. But expression of p53 and p21 increased until 48 hours after treatment and then decreased.

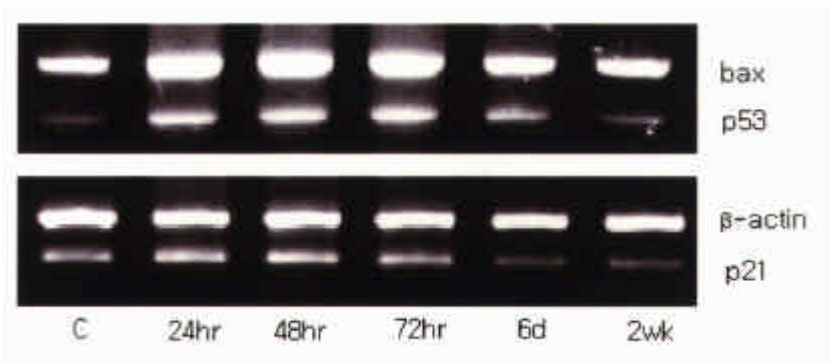

**Figure 10**. RT-PCR finding. Expression of bax mRNA increased substantially until 48 hours and then decreased, however, overall higher than control. Expression of p53 and p21 increased until 48 hours after treatment and then decreased.

**4. TUNEL stain and flow cytometry for the evaluation of apoptosis**

Positive nuclear signals by TUNEL staining were noted from 2 days until 2 weeks after treatment despite morphological features of the positive cells were compatible with radiation induced necrosis rather than those of apoptotic cell (Fig. 11). Flow cytometry also did not demonstrate any apoptotic expression of annexin V in the tumor cells.

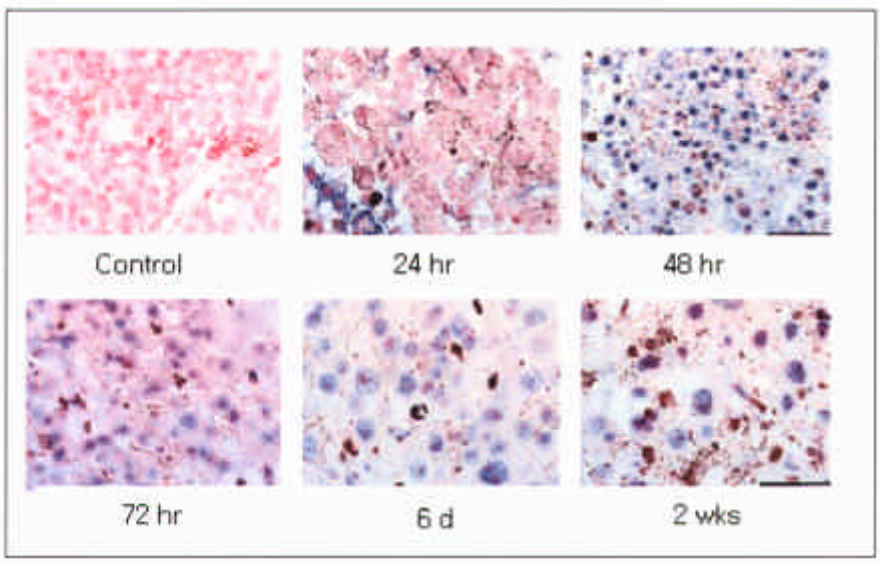


**Figure 11.** TUNEL finding. Positive reaction is demonstrated in the tumor cell nuclei from 48 hours after treatment, however, positively labeled tumor cells show cytoplasmic feature of necrosis such as cytoplasmic and nuclear swelling, membrane disruption and vacuolization.

**5. Clinical illustration.**

An intra-tumoral injection method was attempted in a patient with metastatic melanoma. A 64-year female patient had a melanoma at left sole 11 years ago and a complete excision was performed. Ten years later, inguinal lymph node metastasis developed and extensive node dissection with adjuvant radiotherapy was underwent. A small metastatic nodule was found at left thigh during follow up. Initial ultrasonography of the lesion showed a 1.3 x 1 cm sized homogenous well-defined low echoic nodule. Five mCi of Ho-166 in 0.3 ml saline was injected into the tumor after obtaining an informed consent. The size of the nodule has decreased progressively but a residual nodular lesion was found on follow up ultrasonography (Fig. 12) and a painful metastatic tumor developed at the external genitalia.

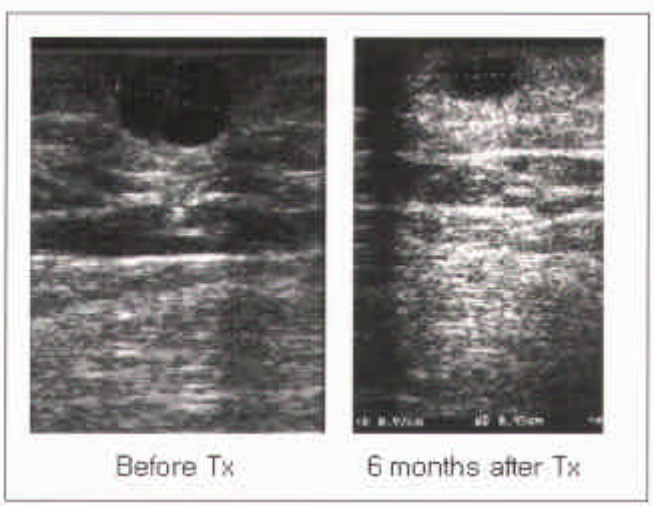


**Figure 12.** Ultrasonography in a 64 year-old female before and after Ho treatment of melanoma . Decreased tumor size is noted until 6 months after treatment.

Six months after the injection of Ho-166, the residual nodule at left thigh and recurrent tumor at the external genitalia were removed. H&E stain and immunohistochemical staining of the treated nodule demonstrated extensive fibrosis and hyalinization but a focal residual cellular tumor admixed with inflammatory cells was found. The residual tumor cells showed ballooning and nuclear enlargement with scattered positive reactions for p53, p21, bax and positive nuclear signals by TUNEL (Fig. 13-15).


**Figure 13.** H&E stained whole mount section of the surgical excision specimen in a 64 year-old female. Viable portion is seen on the peripheral corner of the tumor, however, most areas are necrotic and fibrotic. (V; viable, N; necrosis)

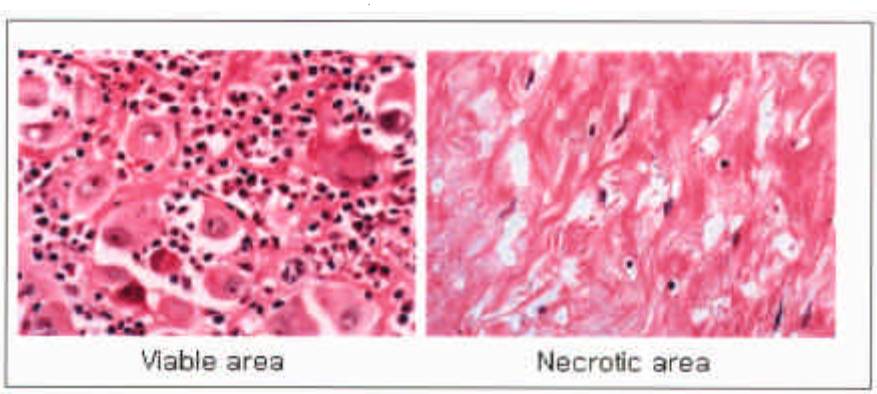

**Figure 14**. H&E stained section of melanoma in a 64 year-old female. The H&E (x400) stained section shows scattered tumor cells with cytoplasmic swelling admixed with many inflammatory cells. Most areas of the tumor are necrotic with fibrosis.

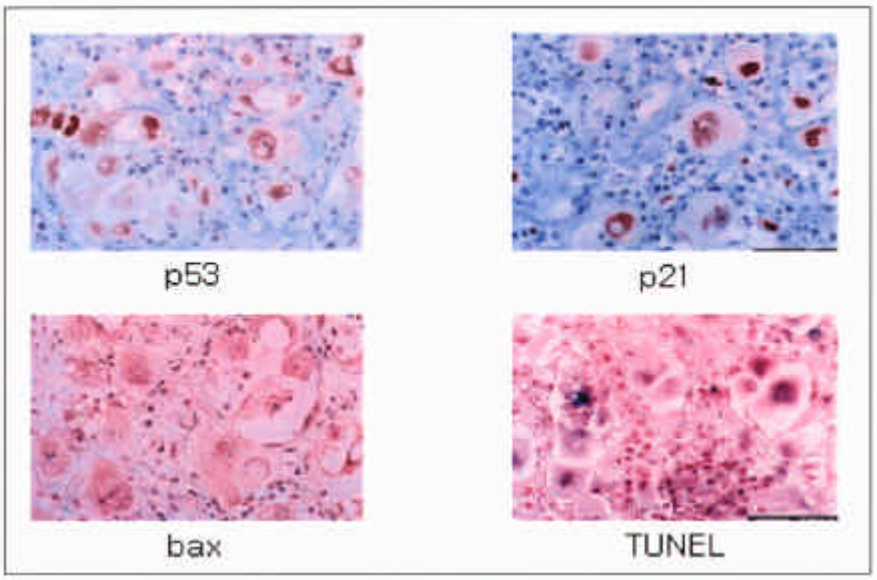


**Figure 15.** Immunohistochemical and TUNEL stains of the residual tumor tissue 6 months after treatment . There are scattered positive nuclear signals for p53 and p21 and by TUNEL and cytoplasmic reaction for bax.

## IV. Discussion

Despite the high incidence of distant metastasis, locoregional control still remains an important goal in the management of melanoma. Conventional external radiation therapy had a limited role in the local control. Therefore high-dose-per-fraction (HDPF) strategy has been proposed to overcome the reparative capacity of the tumor cells with sublethal damage[14-15] and a recent study demonstrated the adjuvant postoperative HDPF regimen was effective in reducing local recurrence in patients

with high risk of locoregional failure.[16] Nevertheless, HDPF scheme is considered to be no better than conventional fractionated regimens at the present time and inherent variability in the radiation sensitivity of malignant melanoma is the main limiting factor for radiation treatment until recently.[17]

In the radiation biological aspect, delivering very intense large dose confined to the tumor tissue can be more effective than conventional or HDPF unless surrounding normal structures are spared. Indeed, stereotactic radiosurgery of cerebral metastatic melanoma with 166 cGy in a single session added to the whole-brain irradiation showed a marked improvement of neurological deficit,[18] which suggests total dose (very-intense-dose) is also an important determinant in the radiation sensitivity of melanoma. However, conventional external radiotherapy has an inherent limitation to deliver an extremely large dose due to its long penetration ranges of high-voltage X rays or high-energy gamma rays, which lead to inevitable radiation hazard to the normal tissues. On the other hand, penetration ranges of the high-energy β- rays or α-particles are much shorter than X or gamma rays, therefore, a much greater fraction of energy can be deposited into the tumor cells without damage to the surrounding normal tissues if large amount of radiotracers can be specifically delivered to the tumors. For an effective targeted therapy, various types of radioimmunoconjugates and methods were developed using anti-melanoma monoclonal antibodies and boron compound labeled with high-energy β or α emitting radionuclides.[19-22] These targeted therapy methods were proposed to deliver a extremely intense tumor radiation dose based on the physical characteristics of β or α rays. Although the results of the radioimmunotherapy or boron captured neutron therapy appeared to be promising, survival benefit is still under investigation. Recently, radionuclide has also been attempted for targeted gene therapy using sodium/iodide symporter gene and

radioiodine in melnoma and hepatoma.[23-24]

In this study, direct intra-tumoral injection of high-energy β-emitting radionuclide, Ho-166, was attempted in animal model and human melanoma. With this method, radiation dose to the central portion of tumor tissue calculated by computer simulation was approximately 800 Gy/mCi and doses to surrounding normal tissue 1, 2, 3 and 4 mm apart from the tumor surface were approximately 120, 36, 10 and 3 Gy/mCi respectively. However, actual tumor dose was higher than 800 Gy/mCi since administered radionuclide was not homogenously distributed with sparing of peripheral rim as seen on autoradiography. Despite the intense radiation dose, injury of surrounding normal tissue was minimal and central tumor necrosis with prolonged survival and growth arrest was observed.

In terms of biological aspect, necrosis has been considered the main cell death mechanism by radiation. Nowadays researches on the change of several molecules involved in cell cycle regulations during radiation treatment have accumulated on their roles. p53 and its down stream effectors play important roles in growth control. Karjalainen et al.[25] demonstrated that high levels of p53 and PCNA were significantly interrelated, and p21 expression was significantly associated with p53 and PCNA levels in cutaneous malignant melanoma. The p21 inhibits DNA synthesis by binding the N-terminus of p21 with CDK-cyclin complex and by binding the C-terminus with PCNA,[26-27] which produce growth arrest of the tumor cells by blocking of DNA replication and G1-S arrest. But reduced expression of p21 in metastatic melanoma and reveals tumorigenicity was reported[28] and the precise role of p21 in tumorigenecity seems to need further investigations. Overexpression of cyclin D1 protein is also a marker of carcinogenesis and cyclin D1 is inversely correlated with nuclear accumulation of p53 and p21.[29] In our study, especially in the clinical case, growth

arrest was evident clinically and morphologic examination of the surgical specimen obtained 6 months after Ho-166 injection showed mainly fibrosis and necrosis with small foci of residual swollen tumor cells. These residual tumor cells with cytologic features of radiation-induced changes exhibited scattered positive reaction for p53, p21, and cyclin D1.

Another important underlying mechanism of radiation induced cell damage is apoptosis. Induction of p53 and caspase-mediated cleavage of p21 was reported to convert tumor cells from growth arrest to undergo apoptosis.[30] Elevated bax and p53 protein levels and positive reaction in TUNEL stain in our study may be interpreted that apoptosis is an important process of radiation responses. However, morphological features are those of necrosis rather than apoptosis and flow cytometry also did not demonstrate a distinct pattern of apoptosis in our study. Although TUNEL stain is one of standard method demonstrating apoptosis, DNA damage in necrotic area may produce false positive reaction on TUNEL stain.[31] Previous reports have described a distinct mode of cell death, secondary necrosis, indicated by parallel occurrence of morphological characteristics of apoptosis and necrosis.[32-33] Although secondary necrosis might take part in Ho-166 induced tumor shrinkage, we think this is a rare possibility due to meticulous microscopic examination and flow cytometry revealed no evidence of apoptosis in the sequentially sampled specimen. However, it is not clear whether secondary necrosis is an important mechanism induced by continuous irradiation using beta-emitting radionuclide. Nevertheless, intratumoral injection of beta-emitting radionuclide, Ho-166, appears to be an alternative radiotherapeutic modality in local control of malignant melanoma.

# V. Conclusion

- Therapeutic, morphological and biological effects of high dose continuous irradiation using beta-emitting radionuclides were analyzed by evaluation of tumor volume and survival rate, analysis of p53 tumor suppressor and p53 downstream effector genes using RT-PCR immunohistochemistry, assessment of apoptosis by TUNEL staining and flow cytometry after intratumoral injection of Ho-166.
- Intratumoral injection of Ho-166 produced extensive central tumor necrosis and growth arrest, which resulted in increased survival rate of mice with malignant melanoma.
- Histological examination demonstrated wide central necrosis that matched with the area of Ho-166 deposition. Viable tumor cells were distributed along the peripheral margin and the cells demonstrated nuclear and cytoplasmic swelling without apoptotic features..
- Expression of bax mRNA increased until 48 hours after treatment and then decreased, however, overall higher than control. But expression of p53 and p21 increased until 48 hours after treatment and then decreased, which were closely correlated with the results of immunohistochemistry.
- TUNEL positive nuclei began to appear from 2 days and persisted until 2 weeks after treatment, but microscopic features of the positive cells were not compatible with those of apoptosis. The results of flow cytometry also did not support the occurrence of apoptosis.
- These results suggest that Ho-166 brachytherapy is useful for the treatment of melanoma and the underlying mechanisms of high dose continuous irradiation using beta-emitting agent are necrosis and growth arrest.

## References


1. Liu T, Soong SJ. Epidemiology of malignant melanoma. Surg Clin North Am 1996;76:1205-1222.
2. Fraker DL. Surgical issues in the management of melanoma. Curr Opin Oncol 1997;9:183-188.
3. Urist MU, Karnell LH. The national cancer data base-Report on melanoma. Cancer 1994;74:782-788.
4. Sondak VK, Wolfe JA. Adjuvant therapy for melanoma. Current Opinion in Oncology 1997;9:189-204.
5. Barranco SC, Romsdahl M, Humphrey RM. The radiation response of human malignant melanoma cells grown in vitro. Cancer Res 1972;31:830-833.
6. Doss LL, Hemula L. The radioresponsiveness of melanoma. Int J Radiat Oncol Biol Phys 1982;8:1131-1134.
7. Geara FB, Ang KK. Radiation therapy for malignant melanoma. Surg Clin North Am 1996;76:1383-1388.
8. Bentzen SM, Overgaard J, Thames HD, Overgaard M, Hansen PV, von der Maase H, et al. Clinical radiobiology of malignant melanoma. Radiother Oncol 1989;16:169-182.
9. Lee JD, Park KK, Lee MG, Kim EH, Rhim KJ, Lee JT, et al. Radionuclide therapy of skin cancers and Bowen's disease using a specially designed skin patch. J Nucl Med 1997;38:697-702.
10. Bae I, Smith ML, Sheikh S, Zhan Q, Sccudiero DA, Friend SH, et al. An abnormality in the p53 pathway following γ-irradiation in many wild-type p53 human melanoma lines. Cancer Res 1996;56:840-847.

11. Rieber M, Straussber-Rieber M. Induction of p53 and melanoma cell death is reciprocal with down-regulation of E2F, cyclin D1 and pRB. Int J Cancer 1998;76:757-760.

12. Coupland SE, Bechrakis N, Schuler A, Anagnostopoulos I, Hummel M, Bornfeld N, et al. Expression pattern of cyclin D1 and related proteins regulating G1-S phase transition in uveal melanoma and retinoblastoma. Br J Ophthalmol 1998;82:961-970.

13. Prestwitch WV, Nunes J, Kwok CS. Beta dose kernels for radionuclides of potential use in radioimmunotherapy. J Nucl Med 1989;30:1036-1046

14. Habermalz HJ, Fischer JJ. Radiation therapy of malignant melanoma. Cancer 1976;38:2258-2262

15. Hornsey S. The relationship between total dose, number of fractions and fraction size in the response of malignant melanoma in patients. Br J Radiol 1978;51:905-909

16. Stevens G, Thompson JF, Firth I, O'Brien CJ, McCarthy WH, Quinn MJ. Locally advanced melanoma. Results of postoperative hypofractionated radiation therapy. Cancer 2000;88:88-94

17. Cooper JS. The evolution of the role of radiation therapy in the management of mucocutaneous malignant melanoma. Hematol Oncol Clin North Am 1998;12:849-862

18. Somaza S, Kondziolka D, Lunsford LD, Kirkwoodd JM, Flickinger JC. Stereotactic radiosurgery for cerebral metastatic melanoma. J Neurosurg 1993;79:661-666

19. Kwok CS, Crivici A, MacGregor WD, Unger MW. Optimization of radioimmunotherapy using human malignant melanoma multicell spheroids as a

model. Cancer Res 1989;49:3276-3281

20. Hamby CV, Chinol M, Palestro CJ, Manzo C, Ferrone S. Improved tumor targeting of rhenium-186-labeled anti-human high MW melanoma-associated antigen monoclonal antibody 763.74 following purification with anti-idiotype monoclonal antibody MK2-23. Int J Cancer 1998;78:486-90

21. Kraeber-Bodere F, Faibre-Chauvet A, Sai-Maurel C, Gautherot E, Fiche M. Campion L, et al. Bispecific antibody and bivalent hapten radioimmunotherapy in CEA-producing medullary thyroid cancer xenograft. J Nucl Med 1999;40:198-204

22. Matalka KZ, Baily MQ, Barth RF, Staubus AE, Soloway AH, Moeschberger ML, et al. Boron neutron capture therapy of intracerebral melanoma using borophenylalanine as a capture agent. Cancer Res 1993;53:3308-3313

23, Mandell RB, Mandell LZ, Link CJ. Radioisotope concentrator gene therapy using sodium/iodide symporter gene. Cancer Res 1999;59:661-668

24. Haberkorn U, Henzee M, Altman A, Jiang S, Morr I, Mahmut M, et al. Transfer of the human NaI symporter gene enhances iodide uptake in hepatoma cells. J Nucl Med 2001;42:317-325

25. Karjalainen JM, Eskelinen MJ, Kellokoske JK, Alhava EM. Kosma VM. $p21^{waf1/cip1}$ expression in stage I cutaneous malignant melanoma: its relationship with p53, cell proliferation and survival. Br J Cancer 1999;79:895-902

26. Mutoh M, Lung FDT, Long YQ, Roller PP, Sikorski R, O'Connor PM. A $p21^{waf1/cip1}$ carboxy-terminal peptide exhibited cyclin-dependent kinase-inhibitory activity and cytotoxicity when introduced into human cells. Cancer Res 1999;59:3480-3488

27. Rousseau D, Cannella D, Boulaire J, Fitzzgerald P, Fotedarr A, Fotedar R. Growth inhibition by CDK-cyclin and PCNA binding domain of p21 ocurs by distinct

mechanisms and is regulated by ubiquitin-proteasome pathway. Oncogene 1999;18:4313-4325

28. Maelandsmo GM, Holm R, Fostad O, Kerbel RS, Florenes VA. Cyclin kinase inhibitior $p21^{waf1/cip1}$ in malignant melanoma. Reduced expression in metastatic lesions. Am J Pathol 1996;149:1813-1822

29. Lee CCR, Yamamoto S, Wanibuchi H, Wada S, Sugimura K, Kishimoto T, et al. Cyclin D1 overexpression in rat two-stage bladder carcinogenesis and its relationship with oncogenes, tumor suppressor genes, and cell proliferation. Cancer Res 1997;57:4765-4776

30. Zhang Y, Fujita N, Tsuruo T. Caspase-mediated cleavage of $p21^{waf1/cip1}$ converts cancer cells from growth arrest to undergoing apoptosis. Oncogene 1999;18:1131-1138

31. Saraste A, Pulkki K. Morphologic and biochemical hallmarks of apoptosis. Cardiovasc Res 2000;45:528-537

32. Paddengerg R, Wulf S, Weber A, Heimann P, Becck LA, Mannherz HG. Internucleosomal DNA fragmentation in cultured cells under conditions reported to induce apoptosis may be caused by mycoplasma endonucleases. Eur J Cell Biol 1996;71:105-19

33. Payne CM, Bjore CG, Schultz DA. Change in the frequency of apoptosis after low- and high-dose X-irradiation of human lymphocytes. J Leukocyte Biol 1992;52:433-440

## 국문요약

# B16 흑색종에서 Holmium-166을 이용한 체내 방사선치료의 성장억제 기전

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악성 흑색종의 발병률은 지난 20여년간 실질적으로 증가해 왔다. 병소의 완전한 수술적 절제가 오랜 기간의 무병생존 (disease free survival) 에 이르게 하는 유일한 치료방법이며 방사선 치료나 화학요법등이 성공적이라는 증거는 없다. 실제로 악성 흑색종은 방사선에 저항성이 높다고 알려져 있다. 본 실험에서는 흑색종에서 Ho-166을 이용한 체내 방사선 치료의 효과와 기전을 알아보고자 하였다. 이를 위해 B16 흑색종 세포를 섭씨 37도에서 RPMI 1640 배지에 배양하였고, 5 x $10^5$개의 세포가 들어있는 100 μl의 생리식염수 현탁액을 C57BL/6 mouse의 배측 피하에 접종하였다. 생존율을 알아보기 위해서 40마리의 mouse를 두 군으로 나누어 제 1군 20마리에서는 생리식염수 0.3 ml를, 제 2군 20마리에서는 Holmium-166 5 mCi가 함유된 생리식염수 0.3 ml를 종양의 내부에 주사하였다. 방사성 동위원소 주사후 mouse의 생존율 mouse가 사망할 때까지 측정하였다. 또다른 30마리의 mouse는 방사선 효과의 기전을 알아보기 위해서 같은 방법으로 준비하였다. 30마리를 5 mCi Ho-166을 함유한 0.3 ml 생리식염수를 주사한 후 무작위로 5 마리씩 여섯 군으로 나누어 1일, 2일, 3일, 6일, 14일째 되는 날 한 군씩 희생시킨 후, p53 종양 억제인자와 p53 downstream

effector gene을 면역조직화학검사와 RT-PCR을 이용하여 알아보았고, 세포고사는 TUNEL 염색과 유세포분석을 이용하여 알아보았다. 대조군은 종양이 빠르게 성장하여 평균종양부피가 초기부피의 약 30배에 달했다. 대조군 중 생리식염수를 주사한 후 16일 이후까지 생존한 mouse는 한 마리도 없었다. 대조군의 median survival은 생리식염수 주사후 9일이었고 Ho-166 치료한 군에서는 33일이었다. 치료한 군의 조직학적 검사에서 광범위한 중심부 괴사를 보였는데, 이는 Ho-166이 침착된 영역과 일치하였고 생존 종양 세포들은 주변부에 분포하였으며 세포고사의 소견은 보이지 않았다. bax mRNA의 발현은 치료 후 48시간까지 증가되었다가 그 이후 점차 감소 하였으나 2주후까지 여전히 대조군보다 증가되었고, p53과 p21의 발현은 치료 후 48시간까지 증가되다가 그 이후 감소하였는데, 이는 면역조직학적 소견과 밀접하게 연관되어 있었다. TUNEL 양성인 핵은 치료 후 2일부터 2주까지 관찰할 수 있었으나 세포고사의 소견은 보이지 않았다. 유세포분석 역시 세포고사의 소견을 발견할 수 없었다. 이러한 결과들을 통해 볼 때, Ho-166 근접치료는 흑색종의 치료에 유용하며, 베타선 방출 동위원소를 사용한 고용량의 지속적인 방사능 조사의 치료 기전은 괴사나 이차적 괴사와 이로 인한 성장 지연에 의한 것으로 생각된다.

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핵심되는 말: 악성 흑색종, 체내 방사선치료, Ho-166 방사선 동위원소,
세포고사, 성장지연