의학석사 학위논문

Nuclear expression of p53 in mature tumor endothelium of retinoblastoma

망막모세포종의 성숙혈관내피세포 내 p53 단백질의 발현 양상에 관한 연구

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서울대학교 대학원

분자의학 및 바이오제약학과

이 병 주

A thesis of the Master’s degree

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이 논문을 의학석사 학위논문으로 제출함

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Abstract

Purpose: To investigate the p53 expression pattern in tumor cell and mature vascular endothelium of retinoblastoma.

Methods: Each 3 BALB-c nude mice got monocular injection of Y79 or SNUOT-Rb1 cell lines to induce orthotopic retinoblastoma model. Contralateral eye was injected with phosphate buffered saline. Three primary enucleated eyes of retinoblastoma and orthotopic retinoblastoma model were double immunofluorescence stained with anti-p53 and anti-von Willebrand factor (vWF) antibodies. The expression and localization of p53 protein in retinoblastoma cell and tumor vascular endothelium was checked and the ratio of the number of p53 (+) / vWF (+) cells among that of total vWF (+) cells was evaluated at 10 randomly selected high power fields (x400).

Results: In both SNUOT-Rb1 and Y79 model, p53 nuclear

accumulation was observed in most of tumor cells. Some of vWF(+) tumor endothelium demonstrated nuclear p53 immunoreactivity and the ratio was higher in Y79 model (46.1 ± 1.6%) than in SNUOT-Rb1 model (31.4 ± 5.4%). In human retinoblastoma specimen, the percentage of p53 nuclear staining among vWF (+) tumor endothelium was 32.9%. Endothelial p53 nuclear accumulation was identifined only in tumor vascular endothelium, but not in pre-existing retinal vascular endothelium.

Conclusion: The same cytogenetic abnormality as retinoblastoma cell was found in mature tumor vascular endothelium of both human and orthotopic model of retinoblastoma. The incorporation ratio of p53 positive endothelium among tumor vasculature in orthotopic model was different according to cell type.

Keywords: p53, retinoblastoma, tumor vascular endothelium

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Introduction

Retinoblastoma is a hypervascular tumor, the proliferation of which is dependent on the vascular supply of the tumor (1). It is also known that vascular density of human retinoblastoma is closely related to the local invasion and distant metastasis of disease (2). Although local therapy and systemic chemotherapy is widely adopted for ocular salvage, enucleation is still regarded as the treatment of choice in advanced cases of retinoblastoma. Because the inhibitiory effect of several antiangiogenic agents such as anecortave acetate (3), bevacizumab (4) and PEDF (5) on tumor growth have been proven in transgenic or orthotopic murine model of retinoblastoma, vascular targeted therapy is considered as one of the future adjuvant treatment option for retinoblastoma.

Since the differences in gene expression of tumor vascular endothelium from normal endothelial cell had been noted, the

concept of tumor vascularization by sprouting has changed. In human glioblastoma and neuroblastoma, chromosomal analysis using fluorescence in situ hybridization (FISH) revealed that some of tumor vascular endothelium shows cytogenetic abnormality similar to that of tumor cells (6,7). Moreover, direct evidences of tumor vascularization by the endothelial differentiation of cancer stem-like cells were found in glioblastoma (7,8).

The p53 protein is well known as a tumor suppressor, which inhibit tumorigenesis through the activation of apoptosis and induction of cell cycle arrest in various human cancers including retinoblastoma (9). When stained with DO-1 monoclonal antibody, 91.4% of specimens showed positive staining and the immunoreactivity to p53 antibody was prominent in poorly or moderately differentiated areas (9). According to another study which explored the distribution of wild-type p53 in the cultivated retinoblastoma cell lines and human retinoblastoma

tissue using p1801 monoclonal antibody (10), nuclear staining of p53 protein was noted in 89% of human retinoblastoma. In contrast, retinoblastoma cell lines showed cytoplasmic or mixed nuclear and cytoplasmic distribution of p53 protein (10).

Because tumor vascularization is also an important issue in the prognosis and treatment of retinoblastoma and there are increasing evidences of tumor stem-like cell in human and mouse retinoblastoma (11,12), it is necessary to identify the presence of genetically unstable tumor vascular endothelium in retinoblastoma. In this study, to investigate cytogenetic abnormality of tumor endothelium of retinoblastoma, we examined the expression and distribution of p53 protein in mature tumor vascular endothelium of orthotopic retinoblastoma model and human retinoblastoma.

Materials and Methods

1. Retinoblastoma cells

Two kinds of human retinoblastoma cell lines were used in this study. SNUOT-Rb1 (13), which was established by our group, and Y79 cell line (14) (American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI-1640 media (WelGene Inc., South, Korea) supplemented with 10% fetal bovine serum (Gibco-Invitrogen Corporation, Carlsbad, CA) and 1% penicillin-streptomycin solution (Invitrogen Inc. Carlsbad, CA, USA). SNUOT-Rb1 maintained by our group was used at passage 3-7.

2. Orthotopic transplantation model

For the induction of orthotopic retinoblastoma mouse model, cultivated SNUOT-Rb1 and Y79 cell line were injected into the vitreal cavity of BALB/c-nude mice (Samtako, Osan,Korea) as

previously described by our group (13). Three mice were assigned to each treatment group and right eyes of the mice were injected with cultivated retinoblastoma cells (1×10^7)suspended in phosphate buffered saline (4 ° C), using 30-gauge needle. For control, phosphate buffered saline (4 ° C) was injected in the left eyes of the mice in the same manner. Intraocular tumorigenesis was evaluated every week by indirect ophthalmoscope and mices with adequate tumorigenesis were sacrificed and enucleated at 4th week after injection. Mice were maintained and treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

3. Human retinoblastoma tissue

Formalin-fixed, paraffin-embedded human eyeball sections (4 μm) of three patients primarily enucleated for retinoblastoma were obtained from the Department of Pathology, Seoul National University Hospital. The institutional review

board of Seoul National University Hospital approved the study.

4. Double immunofluorescence staining

Formaline-fixed, paraffin embedded blocks of tumor sections cutted into 4 μm thickness (both orthotopic model and human retinoblastoma specimen) were deparaffinized by serial xylene and ethyl alcohol immersion. For antigen retrieval, sections were treated with proteinase K (20ug/ml) under 37℃ for 20 minutes. After permeabilized with Triton-X100 (0.2%), sections were blocked with blocking solution (BioGenex Laboratories, San Ramon, CA, USA). Prepared sections were incubated overnight with primary antibodies at 4 °C and then with fluorescein tagged secondary antibody during 2 hours at room temperature after rinsing. Anti-p53 polyclonal antibody (FL-393; sc-6243-G, Santa Cruz Biotechnology, CA, USA) and anti-von Willebrand factor (vWF) antibody (sc-14014, Santa Cruz Biotechnology, CA, USA), for detecting mature

vascular endothelium, were used as primary antibodies in the concentration of 1:100. For secondary antibody, Alexa Fluor 594-conjugated donkey anti-goat IgG (Molecular Probes, Carlsbad, CA, USA) and Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Molecular Probes, Carlsbad, CA, USA) were used in the concentration of 1:200. Nuclear counter-staining with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) was performed for the discrimination of nuclear or cytoplasmic distribution of p53 protein. After aqueous mounting, the slides were examined under fluorescence microscope (Axio Observer, Carl Zeiss). After confirming the number of total vWF positive endothelium, vWF positive endothelium with p53 nuclear accumulation was independently evaluated by 2 investigators at 10 randomly selected high power fields (x400). Then, the overall ratio of endothelium with p53 nuclear accumulation among all vWF positive tumor vascular endothelium was calculated.

5. Statistical analysis

Statistical analysis was performed using SPSS V.12.0 for Windows. The ratio of vWF positive endothelium with p53 nuclear accumulation among all tumor vascular endothelial cells in SNUOT-Rb1 and Y79 groups was compared using the Mann-Whitney U test. The cut-off value of statistical significance was $p<0.05$.

Results

1. The expression and distribution of p53 protein in orthotopic retinoblastoma model

Four weeks after intravitreal inoculation of retinoblastoma cells, we checked intraocular tumor formation with indirect ophthalmoscopic examination and H&E staining. Adequate tumorigenesis was achieved in both Y79 and SNUOT-Rb1 induced mice model. Although not have been quantitatively analyzed, orthotopic retinoblastoma model induced by SNUOT-Rb1 cells shows more aggressive growth pattern such as relatively rapid growth and occasional extraocular extension compared to the Y79 induced model. We could not find any other significant differences in phenotype between the tumors induced by Y79 and SNUOT-Rb1 cells. In both of the orthotopic retinoblastoma models induced by Y79 and SNUOT-Rb1 cells, strong and uniform immunopositivity against anti-

p53 antibody is observed in the area filled with retinoblastoma cells (Figure 1). In contrast, retina of the contralateral eye did not showed significant immunoreactivity to anti-p53 antibody.

Most of tumor cells demonstrate p53 immunoreactivity and when merged with DAPI nuclear counter-staining, we could find the nuclear location of p53 protein in both Y79 and SNUOT-Rb1 models.

2. Mature tumor vascular endothelium with p53 protein immunoreactivity in orthotopic retinoblastoma model

Some of the vWF immunopositive tumor vascular endothelium was also stained with anti-p53 antisera. In both Y79 and SNUOT-Rb1 model, the nuclear localization of p53 protein in vWF immunopositive tumor vascular endothelium was confirmed by DAPI nuclear counterstaining. The distribution of p53 protein in endothelial cells resembles that in tumor cells

(Figure 2). In contrast to the tumor vascular endothelium in retinoblastoma model, retinal vascular endothelium of contralateral normal eye does not react with p53 antisera.

The ratio of p53 immunopositivity was different from each orthotopic model induced by Y79 and SNUOT-Rb1 cells. The mean percentage of p53 positive endothelium was 31.4 ± 5.4% in 3 eyes of orthotopic transplantation model with SNUOT-Rb1, and was 46.1 ± 1.6% in the other 3 eyes with Y79 cells (Figure 3). The ratio of tumor vascular endothelium with p53 nuclear accumulation was higher in Y79 induced orthotopic retinoblastoma model with a marginal statistical significance ($p = 0.05$).

3. The immunoreactivity of human retinoblastoma specimen against anti-p53 antibody: tumor cells and vascular endothelium

All three patients involved in this study were primarily

enucleated for extensive retinoblastoma which was classified as group D according to the International Intraocular Retinoblastoma Classification. Extraocular extension was not detected in any patients at the time point of enucleation. The clinical and pathological features of the patients were similar to each other (Table 1).

In our study, human retinoblastoma cells demonstrated a variable immunoreactivity against the anti-p53 antibody (FL-393), showing a similar trend with the results of previous study using DO-1 monoclonal antibody [9]. When comparing the distribution of p53 immunopositivity with DAPI nuclear counter-staining, p53 protein in retinoblastoma cell shows mainly nuclear, but some cytoplasmic localization. In cases of cytoplasmic p53 distribution, the immunoreactivity is typically weak. In every specimen, a fraction of vWF positive tumor vascular endothelium presented nuclear accumulation of p53 protein (Figure 4).

In a specimen which had a portion of relatively preserved retinal layers, we found retinal vessels, presumed to be pre-existing and not of neoplastic origin. To verify whether the endothelial p53 immunopositivity exclusively occurs in tumor vessels, we carefully reviewed retinal vessels in the preserved retina. The vWF positive endothelial cells of those vessels do not showed any p53 immunopositivity without exception (Figure 5). The percentage of p53 positive endothelium among total vWF positive tumor vascular endothelium counted by two independent observers is presented in Table 1. The average of percentage reported by each observer ranges from 31.3 to 33.8%.

ID	Age / Sex	Late-rality	Intern-ational classifi-cation	Gross pathology				Percentage of p53 positive endothelium	
				MTD (mm)	AC involve	Choroidal involve	Optic nerve involve	Observer 1	Observer 2
1	8 months / M	B	Group D	15	no	no	no	29.3%	33.3%
2	12 months / M	U	Group D	16	no	yes	no	31.0%	36.2%
3	9 months / M	U	Group D	13	no	no	no	32.4%	35.1%

Table 1. The clinical and pathological characteristics of human retinoblastoma

AC, anterior chamber; B, bilateral; MTD, maximal tumor diameter; U, unilateral

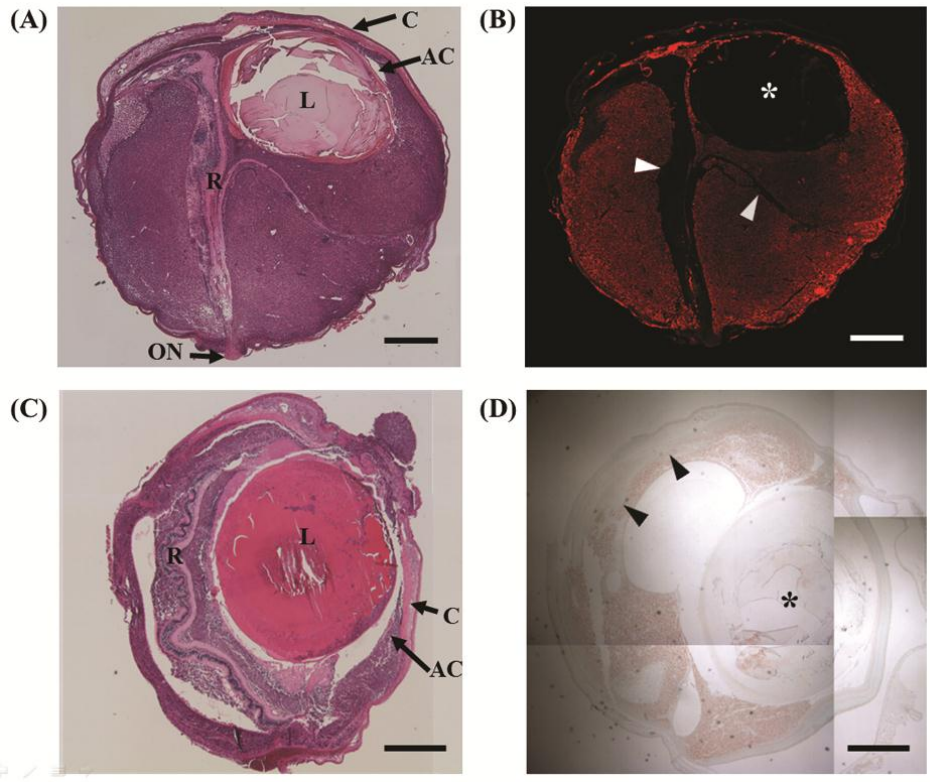

Figure 1. Diffuse p53 expression in an orthotopic transplantation mouse model of retinoblastoma. (A) Tumor mass filling subretinal area, intravitreal cavity and anterior chamber is observed 4 weeks after the intravitreal inoculation of SNUOT-Rb1 cells (H&E staining). (B) In a serial section stained with fluorescein tagged anti-p53 antibody (red), diffuse p53 immunoreactivity of tumor mass is detected except for detached

retina (arrow heads) and crystalline lens (*). (C) After inoculation of Y79, intraocular tumor formation similar to SNUOT-Rb1 model was observed, but tumor volume was relatively smaller in Y79 model. (D) Immunohistochemical staining of Y79 induced model also shows diffuse p53 immunopositivity of tumor mass except for the relatively preserved retina (arrow heads) and crystalline lens (*). AC, anterior chamber; C, cornea; L, lens; ON, optic nerve; R, detached retina. Scale bars: A, B, C and D, 500 μm.

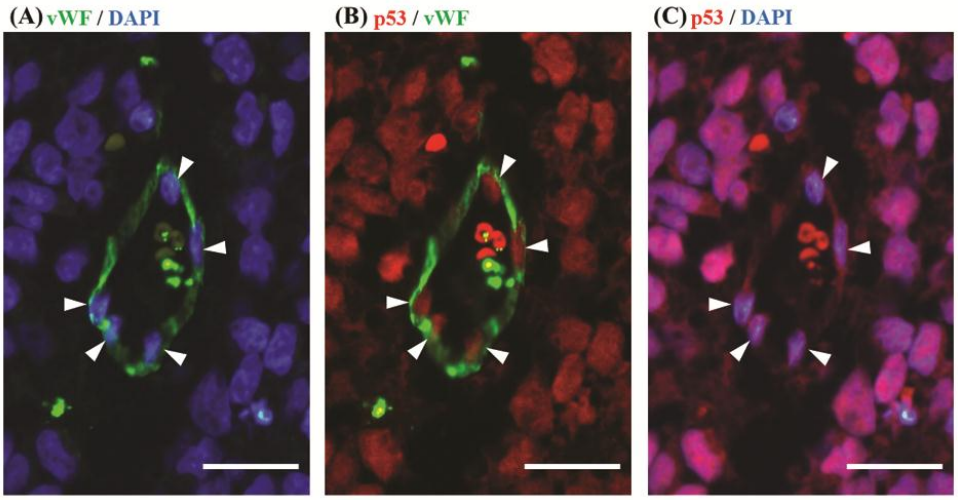

Figure 2. Double immunofluorescence staining with anti-p53 (red) and anti-vWF (green) antibodies demonstrates p53 nuclear accumulation in the mature tumor vascular endothelium of orthotopic retinoblastoma model. After identifying the nucleous of mature tumor vascular endothelium (arrow heads) by DAPI (blue) counterstaining (A), we can detect the nuclear accumulation of p53 in these cells (B and C). Scale bars: 10 μm.

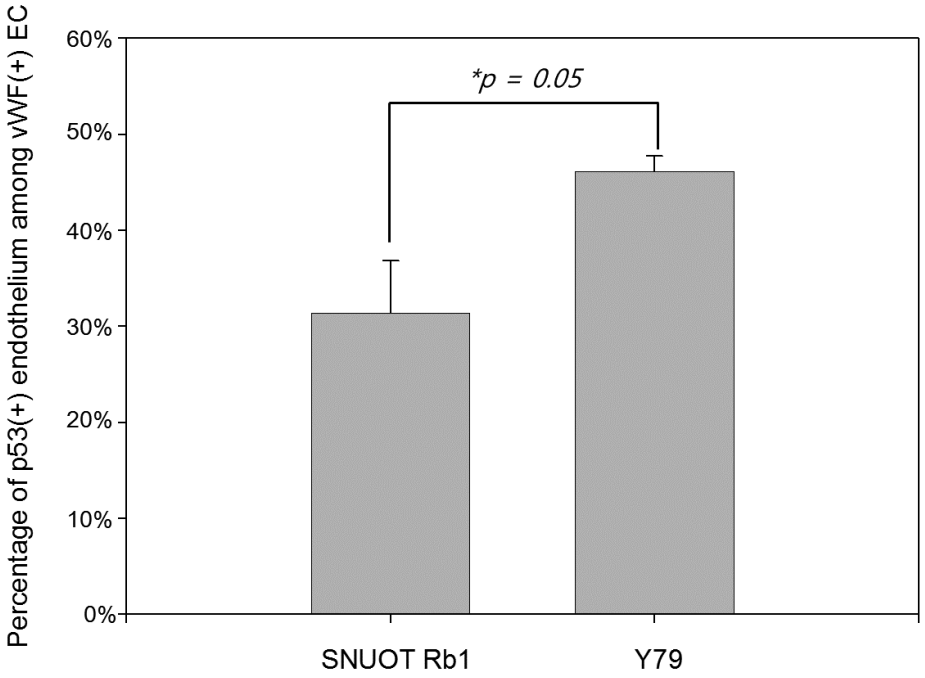

Figure3. Comparison of the percentage of mature tumor vascular endothelium which shows p53 nuclear accumulation in orthotopic retinoblastoma models induced by both SNUOT-Rb1 and Y79 cells. The percentage of p53 immunopositive endothelium is higher in Y79 model with a borderline statistical significance (* Mann-Whitney U test).

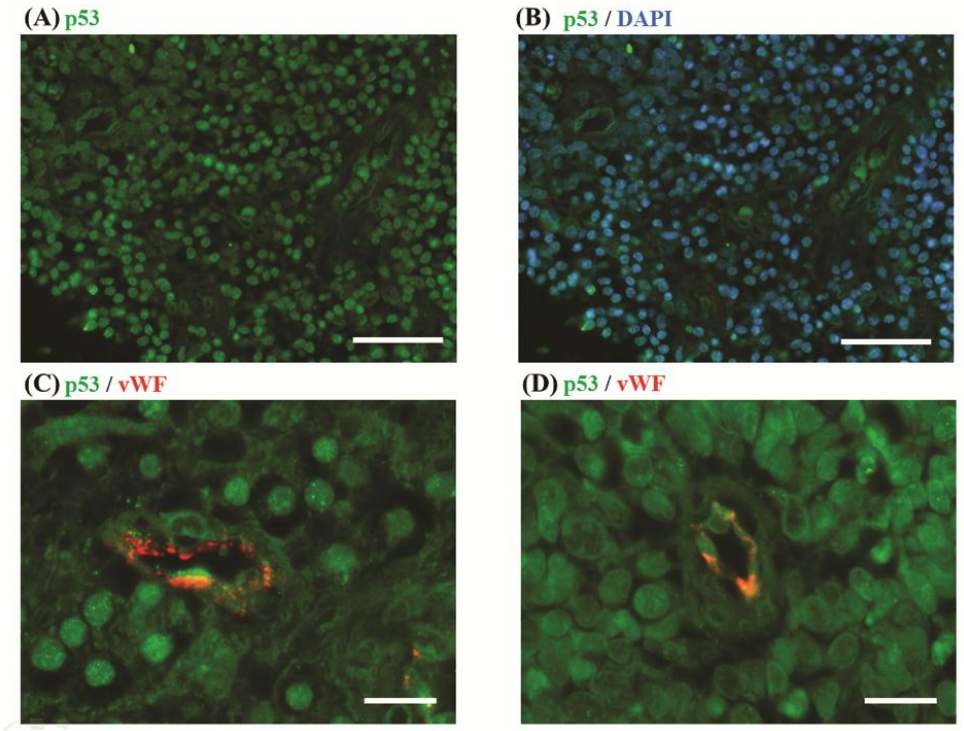

Figure 4. Double immunofluorescence staining with anti-p53 (green) and anti-vWF (red) antibodies demonstrates p53 nuclear accumulation in both tumor cell (A and B) and the mature tumor vascular endothelium (C and D) of human retinoblastoma tissue. Scale bars: A and B, 50 μm; C and D, 10 μm.

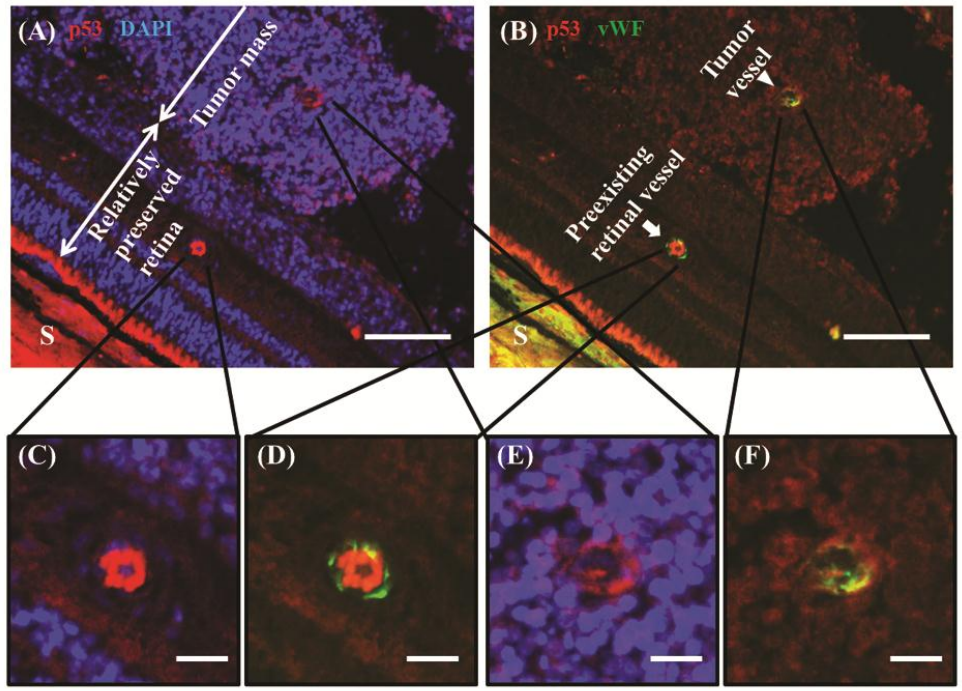

Figure 5. A representative photograph of human retinoblastoma specimen which contains both relatively preserved retina and tumor mass (A and B). When double stained with anti-p53 (red) and anti-vWF (green) antibodies, vWF positive vascular endothelium of presumedly preexisting retinal vessel found in relatively preserved retina does not show any p53 immunopositivity (C and D). In contrast, vWF positive vascular endothelium among tumor mass demonstrates simultaneous p53

immunoreactivity (E and F). S, sclera; vWF, von Willebrand factor. Scale bars: A and B, 100 μm; C, D, E and F, 20 μm.

Discussion

In human retinoblastoma, there found no genetic mutation of p53 in the primary tumor (15). After the report of Laurie et al. (16), it is generally accepted that in some of the human retinoblastoma cases, the p53 protein remains wild-type but is functionally inactivated by abnormally amplified *MDM2* and *MDMX.* According to their results, the amplification of *MDMX* which does not have ubiquitin ligase activity is found in 65% of human retinoblastoma cases (17), whereas that of *MDM2* which results in the ubiquitin-dependent p53 degradation is noted in only 10% of human retinoblastoma (16,18).

Because several previous reports indicated that p53 nuclear staining of tumor cell is observed in most cases of human retinoblastoma (9,10), the authors considered p53 nuclear staining as a putative marker of cytogenetic abnormality in the subjects with retinoblastoma (9,10). Previous study about the p53 expression of normal mouse retina revealed that the whole

layer of retina does not show any significant immunoreactivity against all three kinds of anti-p53 antibodies used for the immunofluorescence assay (including FL-393, which was used in our study) (19). In another study that evaluated the p53 expression of normal mouse eye using anti-p53 driven CAT staining, only the photoreceptor layer showed significant p53 expression throughout the whole retinal layers (20). Because we also evaluated contralateral eye of orthotopic model to rule out the possibility of detecting physiological p53, nuclear staining of p53 could be considered as a putative marker of cytogenetic abnormality.

Cultured retinoblastoma cell lines are known to show cytoplasmic or mixed nuclear and cytoplasmic distribution of p53 protein in vitro. When stained with p1801 monoclonal antibody, p53 protein shows a cytoplasmic localization in Y79 cells (10). However, in this study, both Y79 and SNUOT-Rb1 induced orthotopic retinoblastoma model consistently

demonstrated nuclear accumulation of p53 in tumor cells. It is reported that the gene expression pattern of xenografted human glioma cell is different from that of cell grown in vitro (21). A following study which compared gene expression profile of human pancreatic cancer cells cultured in ectopic and orthotopic condition revealed that organ microenvironment is an important determinant of gene expression profile (22). The authors postulated that tumor microenvironment of orthotopic retinoblastoma model might have induced the molecular change which affect the nuclear localization signal of p53 protein.

Tumor associated endothelial cell which shares the same genetic aberration of original tumor has been noted in several tumors such as lymphoma and renal carcinoma (23,24). More recently, direct evidences for the presence of tumor cell derived tumor associated microvasculature have been piled in human malignancy such as glioblastoma and neuroblastoma (6-8). To our best knowledge, this is the first report about

cytogenetic abnormality of tumor vascular endothelium in human and orthotopic model of retinoblastoma. Moreover, endothelial immunoreactivity against anti-p53 antisera found in the orthotopic retinoblastoma model could be a strong evidence for direct contribution of the retinoblastoma cell to tumor vascularization.

The ratio of tumor derived microvessels has been reported up to 78% in human neuroblastoma (6). According to a report which analyzed tumor-specific chromosomal aberration by FISH assay in tumor vascular endothelium of glioblastoma, 60.7% (range from 20 to 90%) of tumor endothelial cells showed chromosomal aberration (7). In this study, the ratio of p53 nuclear accumulation among mature tumor vascular endothelium was 32.9% (range from 31.3 to 33.8%). Although this is a pilot study involving only a small number of cases, our results show a relatively low mean value and small inter-subject variability compared to that of previous study about glioblastoma. In our

study, lower mean value could be influenced by a low sensitivity of p53 immunostaining for the detection of cytogenetic abnormality. The small inter-subject variability might be related to homogeneous clinical and pathological features of our participants.

In summary, p53 nuclear accumulation in some of mature tumor endothelium as well as tumor cell is consistently observed in both human and orthotopic model of retinoblastoma. Our result implies a role of retinoblastoma cell in tumor angiogenesis and this feature should be considered in upcoming vascular targeted therapy of retinoblastoma. Discrepancy in the ratio of p53 immunopositivity between SNUOT-Rb1 and Y79 cell induced orthotopic model observed in this study might reflect the different biologic properties of each cell line. Previously our group has demonstrated that the doubling time of SNUOT-Rb1 is shorter than that of Y79 (13), and the tumor growth pattern was relatively more aggressive in SNUOT-Rb1

model. During tumor vascularization, tumor vascular endothelium is known to originate from neighboring capillary (25) and bone marrow-derived precursor cells (26). More recent studies revealed the role of endothelial differentiation of tumor stem-like cell in tumor angiogenesis. A more rapidly growing tumor needs more abundant vascular supply, and if we assume that the endothelial differentiation capacity of tumor stem-like cell is limited to a certain level, the proportion of tumor endothelium which derived from tumor stem-like cell could be lower in rapidly growing tumor. Further study comparing the microvascular density in both orthotopic retinoblastoma models could provide a better understanding about the role of p53 positive endothelium in tumor vascularization.

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국문 초록

목적: 망막모세포종의 종양 세포 및 종양혈관을 구성하는 성숙내피세포에서 p53 단백질의 발현양상을 분석하고자 한다.

방법: 망막모세포종 동소위 생쥐 모델을 유발하기 위해 누드마우스(BALB-c nude mice)의 한 쪽 눈에는 Y79 (n=3) 혹은 SNUOT-Rb1 (n=3) 세포주를 유리체강 내로 주입하고, 반대쪽 눈에는 생리식염수를 주입하였다. 망막모세포종 동소위 생쥐 모델과 망막모세포종으로 일차 안구적출을 시행 받은 3안의 인간 망막모세포종 조직에서 항 p53 항체와 항 von Willebrand factor (vWF) 항체를 이용한 이중 면역형광 염색을 시행하였다. 망막모세포종 동소위 모델 및 인간 망막모세포종 조직의 종양세포 및 혈관내피세포에서 p53 단백질의 발현 양상 및 세포내 발현 위치를 확인하였다. 또한, 무작위 선정된 10군데의 고배율 시야(x400)에서 전체 vWF (+) 세포 중 p53 (+) / vWF (+) 세포의 비율을 측정하였다.

결과: Y79 및 SNUOT-Rb1 유발 동소위 모델 모두에서 대부분의 종양세포가 p53 단백질의 핵 내 축적 소견을 보였다. 일부 vWF(+)

혈관내피세포에서 핵 내 p53 단백 발현이 확인되었고, 그 비율은 Y79 세포주 유발 모델 (46.1 ± 1.6%)에서 SNUOT-Rb1 세포주 유발 모델 (31.4 ± 5.4%)에 비하여 높음을 알 수 있었다. 인간 망막모세포종 조직에서는 32.9%의 vWF(+) 혈관내피세포에서 핵 내 p53 단백 발현이 관찰되었다. 이러한 핵 내 p53 단백의 축적 현상은 기존 망막 혈관에서는 관찰되지 않으며, 종양혈관의 내피세포에서만 관찰됨을 확인하였다.

결론: 망막모세포종 동소위 모델과 인간 망막모세포종 조직에서 종양혈관의 성숙혈관내피세포가 망막모세포종 세포와 같은 세포유전학적 이상을 공유함을 알 수 있었다. 또한, 망막모세포종 동소위 모델에서, 종양혈관의 성숙내피세포 중 p53 양성을 보이는 세포의 비율은 각각 사용된 세포주에 따라 다름을 알 수 있었다.

주요어: p53, 망막모세포종, 종양혈관내피세포

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