Detection of p53 and Bcl-2 expression in cutaneous hemangioma through the quantum dot technique

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Abstract. Hemangioma is one of the most common types of infantile vascular benign tumor. The aim of the present study was to investigate the role of B-cell lymphoma 2 (Bcl-2) and tumor protein p53 (p53) in the proliferation and apoptosis of hemangioma cells. A total of 38 paraffin-embedded hemangioma specimens (16 males and 22 females) and another 5 paraffin-embedded healthy surrounding tissue samples, collected between January 2007 and December 2010, were obtained from the Department of Pathology at Renmin Hospital of Wuhan University (Wuhan, China). Immunohistochemistry, hematoxylin and eosin staining, and quantum dot double staining were used to detect the expression of proliferating cell nuclear antigen (PCNA), Bcl-2 and p53 in hemangioma and healthy surrounding skin tissue samples. All hemangioma specimens were classified into proliferative or the involuting stage hemangioma according to Mulliken's criteria and their expression of PCNA. The results of the quantum dot double staining were analyzed using a multi-spectral imaging system. One-way analysis of the variance and the Student-Newman-Keuls q test were performed to statistically analyze the data. There were 24 cases of proliferative stage and 14 cases of involuting stage hemangioma among the specimens. Immunohistochemical analysis results indicated a high expression of Bcl-2 and p53 in proliferative stage hemangioma tissue samples, and low expression in involuting stage hemangioma and healthy tissue samples. Statistical analysis of the results from quantum dot double staining demonstrated that the expression of Bcl-2 and p53 in proliferative hemangioma was significantly increased compared with that in involuting stage specimens ($P<0.05$) and healthy tissue samples ($P<0.05$). No significant difference in Bcl-2 and p53 expression was identified between the involuting hemangioma and healthy surrounding tissue samples. The higher expression of Bcl-2 and p53 in proliferative hemangioma suggests that Bcl-2 may cause an imbalance between endothelial cell proliferation and apoptosis through the inhibition of endothelial cell apoptosis. Furthermore, p53 may promote the proliferation of endothelial cells in proliferative hemangioma.

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Introduction

Cutaneous hemangioma is one of the most common types of benign tumor (1). The majority of types of hemangioma go through three distinct stages, the proliferation stage, the involuting phase and the involuted phase. Hemangioma typically presents itself during the proliferative phase. During the proliferative phase, excessive proliferation of endothelial cells and nuclear hypertrophy occurs, forming lumens or lumps without cavities that are similar in size to a small capillary. The proliferative phase is characterized by rapid proliferation of capillary endothelial cells and the appearance of a vascular anomaly. Subsequently, hemangiomas enter an involuting phase during which the endothelial cells undergo apoptosis, and extracellular matrix fibers and adipose tissue replace the hemangioma. This phase can last between a number of months and several years, following which the hemangioma enters the involuted phase (2,3).

There are two processes that enable the formation of new blood vessels, vasculogenesis and angiogenesis. Vasculogenesis refers to the formation of early blood vessels through the induction, differentiation and aggregation of endothelial progenitor cells (4). Angiogenesis refers to the process of forming new vessels from pre-existing vessels following birth (5). The first vessels in the embryo form through vasculogenesis and following birth angiogenesis becomes the predominant pathway used to develop blood vessels (6). Abnormalities in vasculogenesis and angiogenesis can lead to vascular diseases, of which hemangioma is a common type (7,8).

Folkman (8) reported that an imbalance in the regulation between angiogenic factors and angiogenic inhibitors that causes uncontrolled angiogenesis may result in the occurrence of hemangioma. Therefore, the mechanism of regulation of angiogenesis serves an important role in the pathogenesis of hemangioma. A large number of factors that positively and negatively regulate angiogenesis have different levels of expression and roles in different stages of the development of hemangioma (9-12). Previous studies have demonstrated that multiple angiogenic factors, such as vascular endothelial

growth factor (VEGF), basic fibroblast growth factor (bFGF) and the estrogen receptor, are highly expressed in the proliferative stage of hemangioma development (2,13). The number of mast cells is increased in proliferative hemangioma tissue compared with healthy tissue, which decreases in the involuting stage and can be restored to normal levels in the involuted stage (14).

A previous study demonstrated that the proliferation and apoptosis of endothelial cells serves an important role in the pathogenesis of hemangioma; the onset and involution of hemangioma were identified to be associated with endothelial cell proliferation and apoptosis (15,16). It has been suggested that the proliferation and apoptosis of vascular endothelial cells is associated with the onset and spontaneous regression of hemangioma (17). Specifically, it is thought that when an increased level of apoptosis occurs in endothelial cells during the second year of a child's life, cellular proliferation is offset which results in the initiation of the spontaneous regression of hemangioma. Activation of oncogenes and inactivation of tumor suppressor genes can cause uncontrolled cell proliferation and apoptosis, and induce tumor formation. The results of a previous study revealed that the proliferation and apoptosis of vascular endothelial cells were associated with the regulation of VEGF, transforming growth factor-β, angiostatin and other cytokines (18,19).

B-cell lymphoma 2 (Bcl-2) belongs to the Bcl-2 protein family and serves an important role in regulating cell survival and apoptosis (20,21). Tumor protein p53 (p53) is a common tumor suppressor protein that is frequently activated when the cell is damaged, and which serves a role in various signaling pathways to regulate cell survival and death. p53 can affect DNA repair and cell cycle progression, and serves an important role in the inhibition of tumorigenesis (22-25). If a mutation of the gene encoding p53 occurs, the mutated p53 protein produced cannot trigger apoptosis, resulting in tumorigenesis (26-28). Although Bcl-2 and p53 have been studied in multiple tumor types, few studies investigating their expression in tumor angiogenesis, particularly in the different stages of hemangioma, have been performed. In the present study, immunohistochemical analysis and quantum dot technology were used to measure the expression of Bcl-2 and p53 in the proliferative and involuting stages of hemangioma, and the blood vessels of healthy skin, in order to investigate the role of Bcl-2 and p53 during the proliferative and involuting stages of hemangioma.

Materials and methods

Samples. Formalin-fixed paraffin-embedded samples of archived cutaneous hemangioma tissue samples collected between January 2007 and December 2010 were obtained from the Department of Pathology of Renmin Hospital of Wuhan University (Wuhan, China). There were samples from 38 patients with hemangioma, including 16 males and 22 females, aged between 2 months and 18 years old. There were another 5 samples of healthy skin tissue surrounding the hemangioma. The locations of the vascular tumors included the following: Scalp, eyelids, forehead, back of the ears, neck, arms, back, legs, hands and foot. The patients did not receive any adjuvant therapy prior to surgery.

Reagents. The Ready-to-use anti-human mouse monoclonal antibody directed against proliferating cell nuclear antigen (PCNA), ready-to-use Ultra-Sensitive Immunohistochemical Streptavidin-Peroxidase (S-P) kit, ready-to-use mouse anti-human monoclonal antibody directed against p53, DAB Color Developing Reagent kit and poly-lysine were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (OriGene Technologies, Inc., Beijing, China). Mouse anti-human monoclonal antibody directed against Bcl-2 was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The Quantum Dot Immunofluorescent Double-Staining Reagent kit was purchased from Wuhan Jiayuan Quantum Dot Technological Development Co., Ltd. (Wuhan, China; cat no. K-3001-3) and diethylpyrocarbonate was purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Classification of specimens. All specimens were stained through routine hematoxylin and eosin (H&E) staining. H&E staining was performed following conventional protocol and included dehydration, permeabilization, paraffin-embedding, sectioning and H&E staining (29). Neutral formalin fixed buffer solution (10%) was used for fixing at room temperature for 24 h. PCNA protein was detected using the ready-to-use Ultra-Sensitive Immunohistochemical S-P kit, as described below. Specimens were classified according to Mulliken's criteria (30) combined with their expression of PCNA. The staging of specimens was determined according to the characteristics of their organizational structure. The involuting stage was defined as hemangioma with fatty infiltration, fibrosis or vascular lumen occlusion in >20% of the hemangioma. The proliferative stage was defined as hemangioma without structural changes. Positive reactions of PCNA were presented as brownish-yellow particles in the nucleus, as determined by H&E staining and the immunohistochemistry S-P method. PCNA participates in DNA synthesis, and its expression has repeatedly been confirmed to be closely related to cellular proliferation. As a result of its involvement in cellular proliferation, PCNA can be used in the diagnosis of malignant tumors such as hemangioma. In the present study, PCNA expression was observed to be high in proliferative stage hemangioma tissue, whilst its expression was low in involuting stage hemangioma tissue. A significant difference was found in the positive reaction of PCNA expression between proliferative stage hemangioma tissue and involuting stage hemangioma tissue.

Detection of Bcl-2-, p53- and PCNA-associated antigens through H&E staining and the immunohistochemistry S-P method. Details of the protocol followed have been reported previously (16). Tissue sections were cut into 5-μm thick slices and conventionally dewaxed using xylene for 20 min, anhydrous alcohol for 10 min, and a graded ethanol series of 90, 80 and 70% alcohol (each for 5 min) and finally soaked in distilled water twice for 10 min in order to replace the paraffin in samples with water. Sections were then washed and incubated with distilled 3% hydrogen peroxide for 10 min at room temperature, in order to eliminate endogenous peroxidase activity. Specimens were washed with distilled water and soaked in PBS for 5 min. For antigen retrieval, slices were placed in 0.01 M citrate buffer (pH 6.0), boiled (at 95˚C for 15-20 min), and left to cool for 20 min. Sections were then washed with cold water in order to cool

samples to room temperature, and finally washed with PBS 3x for 5 min each. Following antigen retrieval, tissue sections were blocked with 5-10% normal goat serum (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany; diluted in PBS), incubated at room temperature for 10 min and washed with PBS 3 times (5 min/wash). Following the discard of excessive serum, the following primary antibodies were added: Bcl-2 (dilution, 1:100; cat. no. sc-7382; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), p53 (dilution, 1:100, cat.no. ZM-0408; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.; OriGene Technologies, Inc.) and PCNA (dilution, 1:100; cat. co. ZM-0213; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.; OriGene Technologies, Inc.). Sections were then incubated with these antibodies at 4˚C overnight. The tissue sections were then incubated with primary antibodies for 1 h at 37˚C, and washed with TBS-Tween® 20 (TBST) 3 times for a total of 5 min each. A total of 150 μl biotin-labeled secondary antibody (cat. no. SPN-9001; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.; OriGene Technologies, Inc.) was diluted with 1% bovine serum albumin (BSA; cat. no. ST023)-PBS and added to each of the sections, which were then incubated at 37˚C for 10-30 min. BSA was supplied by Beyotime Institute of Biotechnology (Haimen, China). Sections were then washed 3 times with TBST for 5 min each time. A total of 150 μl streptavidin-labeled horseradish peroxidase-conjugated secondary antibody (diluted with 1% PBS; cat. no. V7023; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.; OriGene Technologies, Inc.) was then added to the first stain solution (DAB Color Developing Reagent kit; cat. no. ZLI-9018; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.; OriGene Technologies, Inc.) and incubated with the sections at 37˚C for 10-30 min. Specimens were washed with tap water, counterstained with hematoxylin and mounted using neutral gum/aqueous mounting medium (cat. no. ZLI-9550; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd.; OriGene Technologies, Inc.) onto the slides.

Detection of Bcl-2, p53 and PCNA through quantum dot double staining. Details of the protocol followed have been reported previously (16). The paraffin-embedded tissue sections were cut into 3-4-μm slices; heated, dewaxed and hydrated. Following microwave antigen retrieval, specimens were washed with TBS. Subsequently, tissue sections were blocked with Immunol Staining blocking buffer (cat. no. P0102; Beyotime Institute of Biotechnology, Shanghai, China) and incubated in a wet box for 30 min at 37˚C. The Bcl-2, P53 and PCNA primary antibodies were incubated at 37˚C for 2 h and subsequently washed with TBST 3 times (5 min/wash). Specimens were blocked with Immunol Staining blocking buffer (cat. no. P0102; Beyotime Institute of Biotechnology) and incubated in a wet chamber for 20 min at 37˚C. Then biotin-labeled secondary antibody (cat. no. YM005; dilution, 1:100; Quantum Dot Immuno-Fluorescent Double Staining reagent kit) was added to the sections, which were incubated at 37˚C for 30 min. Drops of undiluted streptavidin-coated quantum dots (QDs-SA; dilution, 1:50-200; final concentration, 5-20 nmol/l; Quantum Dot Immuno-Fluorescent Double Staining reagent kit) were added to specimens, which were then incubated in a wet box at 37˚C for 30 min and washed with TBST 3 times, (5 min/wash). Following air-drying, tissue samples were mounted on slides using 90% glycerin buffer as the mounting medium. Specimens were visualized using an Olympus IX71 fluorescence microscope (Olympus Corporation, Tokyo, Japan). A wavelength of 545 nm was used to excite the QDs with ultraviolet light, and a wavelength of 605 nm was used to excite QDs with blue light.

Analysis of the results of immunohistochemical and quantum dot staining. Cells were considered Bcl-2-positive if brown-yellow granules were identified in the cytoplasm and/or the cell membrane. Cells were considered PCNA- or p53-positive if brown-yellow granules appeared in the nucleus. No brown-yellow granules were identified in the cytoplasm or nucleus of the negative control group.

Bcl-2 and p53 expression was quantitatively analyzed through a Nuance FX Multispectral Imaging system (Cambridge Research & Instrumentation, Inc., Woburn, MA, USA). For each section, five complete and non-overlapping high magnification (x400) fields were randomly selected for each sample. The average optical density, area of positive staining and total area of all cells in each field was measured, and used to calculate the positive area rate. Positive area rate was calculated as follows: (Total area of positive staining/the total area of cells) x100%.

Statistical analysis. Quantum dot staining results are illustrated as the mean ± standard deviation. One-way analysis of variance and the Student-Newman-Keuls q post hoc test were used to determine the significance of differences in the average optical density and positive area rate between groups. A significance level of α=0.05 was selected. Normality test and variance homogeneity test were conducted prior to testing. Each group met the requirements of normal distribution and homogeneity of variance. All tests were performed using SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

H&E staining. The capillaries in the healthy skin tissue samples had thin walls and were surrounded by 1-2 endothelial cells, which were primarily thin but were thicker around the nuclei (Fig. 1A). In addition, the nuclei of the endothelial cells were flat compared with other cell types (Fig. 1A). A large number of active proliferative endothelial cells shaped like a cord or mass were observed to be gathered in proliferative hemangioma tissue samples (Fig. 1B). There were also irregular gaps in the sinusoid capillaries between endothelial cells, the vessel lumen and large weakly stained nuclei (Fig. 1B). In involuting hemangioma tissue samples, the number of endothelial cells surrounding the capillaries was decreased, vascular lumens were enlarged, and fibrosis of the blood vessels, fatty deposits, lumen occlusion and flat endothelial cell nuclei were present (Fig. 1C).

Detection of PCNA through the immunohistochemistry S-P method. Cells were considered PCNA-positive if brown-yellow granules were distributed diffusely in the nuclei of endothelial cells. In proliferative hemangioma tissue samples, the nuclei of endothelial cells were large and PCNA

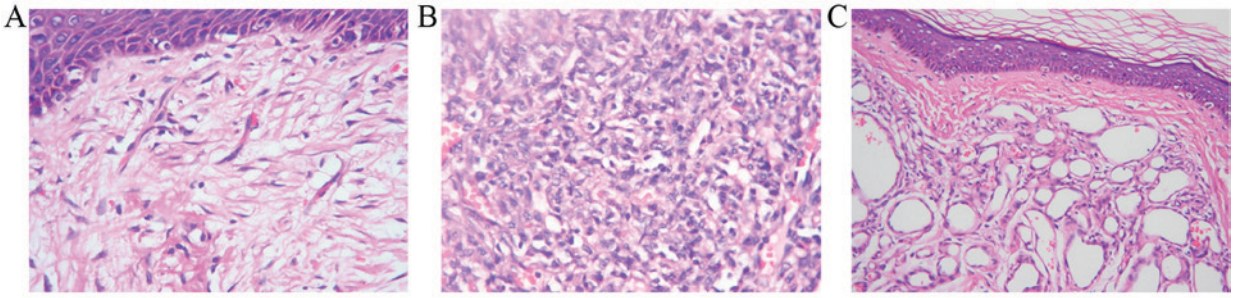

Figure 1. Results of hematoxylin and eosin staining. (A) In healthy skin tissue samples, the cross-section of a capillary consisted of 1-2 endothelial cells with light cytoplasmic staining, and thicker and flat nuclei. (B) In proliferative hemangioma tissue, proliferating endothelial cells were identified, and their nuclei were hypertrophic and lightly stained. (C) In involuting hemangioma tissue, there were few endothelial cells, the vascular lumen was enlarged and the nuclei of endothelial cells were flat. Magnification, x200.

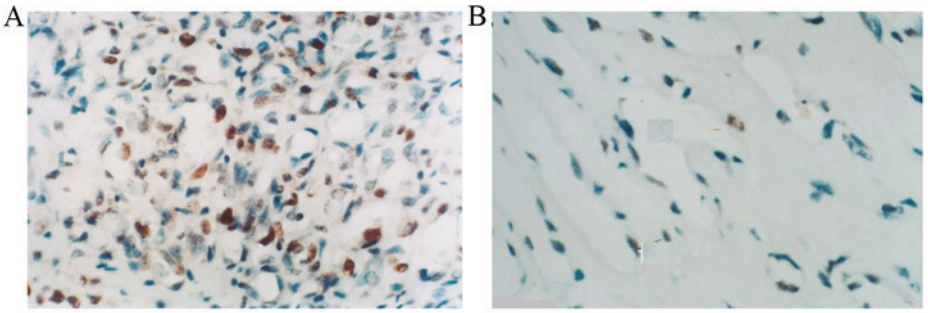

Figure 2. PCNA was detected through the immunohistochemical streptavidin-peroxidase method. (A) Presence of PCNA in proliferative phase hemangioma endothelial cells. The nuclei were hypertrophic and contained brownish-yellow particles, demonstrating strong PCNA expression. (B) Expression of PCNA in involuting hemangioma tissue samples. The nuclei were flat and brownish-yellow particles were sparsely distributed within nuclei, demonstrating weak PCNA expression. Magnification, x200. PCNA, proliferating cell nuclear antigen.

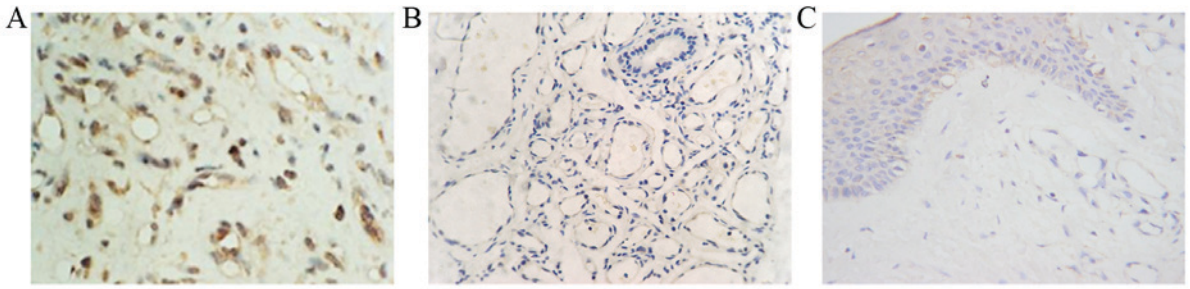

Figure 3. Immunohistochemical streptavidin-peroxidase staining for Bcl-2. (A) Expression of Bcl-2 in proliferative phase hemangioma endothelial cells. Brownish-yellow particles were densely distributed in the nuclei and cytoplasm of endothelial cells, revealing strong Bcl-2 expression. (B) Expression of Bcl-2 in involuting phase hemangioma endothelial cells. Brownish-yellow particles were sparsely distributed in the nuclei and cytoplasm of endothelial cells, demonstrating little or no Bcl-2 expression. (C) Expression of Bcl-2 in healthy surrounding skin tissue samples. Brownish-yellow particles were not identified in the nuclei or cytoplasm of vascular endothelial cells, demonstrating little or no Bcl-2 expression. Magnification, x200. Bcl-2, B-cell lymphoma 2.

expression was high (Fig. 2A). In the involuting hemangioma tissue samples, the nuclei of endothelial cells were flat, there were few nuclei and nuclei contained a small amount of brown-yellow granules, indicating that PCNA expression was low (Fig. 2B).

H&E staining and the immunohistochemistry S-P method were used to detect PCNA. Classification indicated 24 cases of proliferative hemangioma and 14 cases of involuting hemangioma. In addition, 5 cases had healthy skin surrounding the hemangioma and were thus used as the control group.

Detection of Bcl-2 and p53 through the immunohistochemistry S-P method. Numerous brown-yellow granules were identified in the endochylema of endothelial cells in proliferative hemangioma tissue, indicating that Bcl-2 expression was high (Fig. 3A). No brown-yellow granules were identified in the endochylema of endothelial cells in involuting hemangioma samples (Fig. 3B) or cells in healthy skin tissue samples (Fig. 3C), suggesting Bcl-2 was not expressed.

Brown-yellow granules were observed in the endochylema of endothelial cells in proliferative hemangioma tissue, indicating that p53 expression was high (Fig. 4A). No brown-yellow granules were identified in the endochylema of endothelial cells in involuting hemangioma tissue samples (Fig. 4B) or healthy skin tissue samples, revealing weak or no p53 expression (Fig. 4C), suggesting little or no p53 expression.

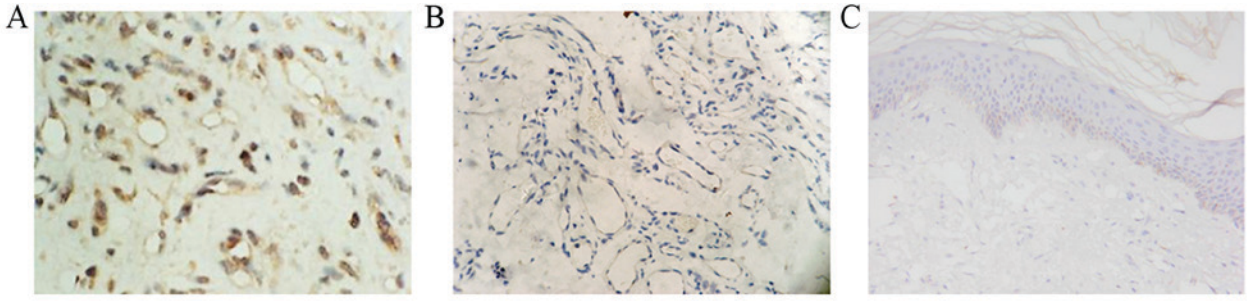

Figure 4. Immunohistochemical streptavidin-peroxidase staining for p53. (A) Expression of p53 in proliferative phase hemangioma. Brownish-yellow particles were densely distributed in the nuclei and cytoplasm of endothelial cells, demonstrating strong p53 expression. (B) Expression of p53 in involuting stage hemangioma. Brownish-yellow particles were sparsely distributed in the nuclei and cytoplasm of endothelial cells, revealing weak p53 expression. (C) Expression of p53 in healthy surrounding skin tissue samples. Brownish-yellow particles were not observed in the nuclei or cytoplasm of vascular endothelial cells, demonstrating weak p53 expression. Magnification, x200. p53, tumor protein p53.

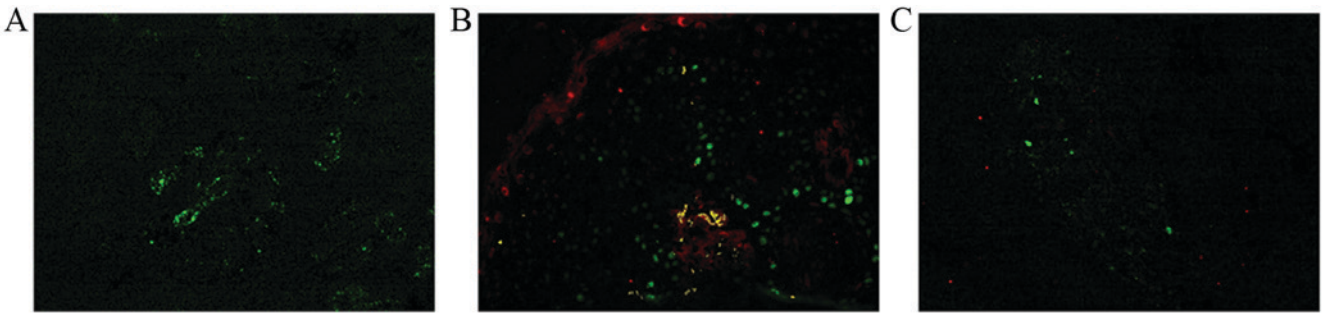

Figure 5. Quantum dot double staining for Bcl-2 and PCNA. (A) There was no Bcl-2 expression (red fluorescence) in the vascular endothelial cells of healthy surrounding skin tissue samples. PCNA was stained a fluorescent green to display the nucleus. (B) In proliferative hemangioma, the cytoplasm and cytomembrane of vascular endothelial cells had strong red fluorescence, demonstrating high Bcl-2 expression. (C) In involuting stage hemangioma, the expression of Bcl-2 was decreased compared with that of the vascular endothelial cells in proliferative phase hemangioma. Magnification, x200. Bcl-2, B-cell lymphoma 2; PCNA, proliferating cell nuclear antigen.

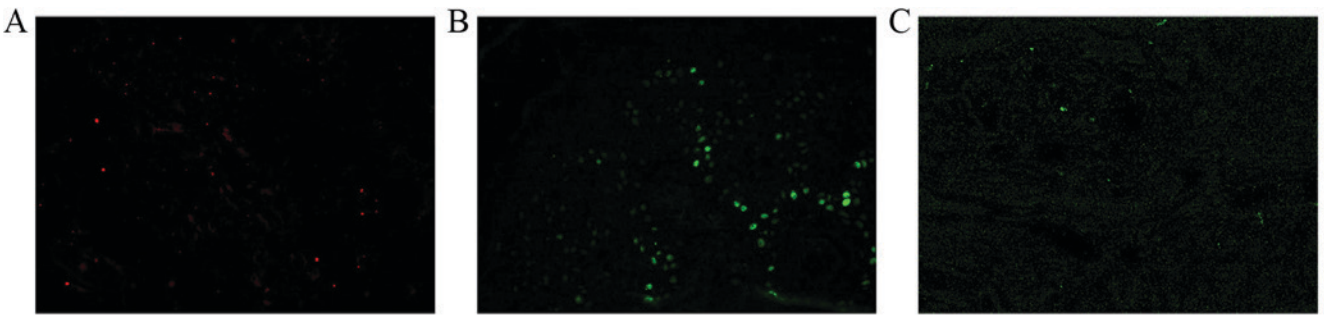

Figure 6. Quantum dot double staining for p53. (A) No p53 expression (green fluorescence) was observed in the vascular endothelial cells of healthy surrounding skin tissue samples. (B) Vascular endothelial cells of the hemangioma tissue in the proliferative stage exhibited strong green fluorescence, demonstrating high p53 expression. (C) Vascular endothelial cells of the hemangioma tissue in the involuting stage exhibited weak green fluorescence, revealing low p53 expression. Magnification, x200. p53, tumor protein p53.

Detection of Bcl-2, p53 and PCNA through the quantum dot double staining method. The presence of PCNA was visible as green fluorescence, and the presence of Bcl-2 was visible as red fluorescence (Fig. 5). No Bcl-2 expression was identified in the vascular tissue cells of healthy skin specimens (Fig. 5A). Bcl-2 was highly expressed in the endochylema and cytomembrane of cells in proliferative hemangioma tissue samples (Fig. 5B). Conversely, Bcl-2 and p53 expression was low in the endochylema and cytomembrane of cells in involuting hemangioma tissue samples (Fig. 5C).

The presence of p53 was visible as green fluorescence in a separate experiment. No p53 expression was observed in the vascular tissue cells of healthy skin tissue (Fig. 6A). However, p53 was highly expressed in the nuclei of proliferative hemangioma cells (Fig. 6B). Conversely, p53 expression was low in the nuclei of involuting hemangioma cells (Fig. 6C).

Statistical analysis of the detection of, Bcl-2, p53 and PCNA through the quantum dot double staining method. As illustrated in Tables I and II, significant differences in the average optical density and positive area rate of Bcl-2 and p53, respectively, were identified in proliferative stage hemangioma tissue samples compared with healthy tissue samples ($P<0.05$). By contrast, no significant differences in the average optical

Table I. Average optical density and positive area rate of B-cell lymphoma 2 in different hemangioma stages determined through quantum dot double staining.

Sample	No. of cases	No. of sections	Average optical density	Positive area rate
Proliferative hemangioma	24	145	0.3941±0.0210[a]	0.4012±0.0227[a]
Involuting hemangioma	14	115	0.1749±0.0195[b]	0.1573±0.0074[b]
Healthy surrounding skin	5	35	0.1704±0.0184[c]	0.1486±0.0057[c]

Results are represented as the mean ± standard deviation. [a]P<0.05 vs. involuting hemangioma; [b]P>0.05 vs. healthy surrounding skin; [c]P<0.05 vs. proliferative hemangioma.

Table II. Average optical density and positive area rate of tumor protein p53 in different hemangioma stages determined through quantum dot double staining.

Sample	No. of cases	No. of sections	Average optical density	Positive area rate
Proliferative hemangioma	24	145	0.4735±0.0307[a]	0.3987±0.0304[a]
Involuting hemangioma	14	115	0.1781±0.0214[b]	0.0954±0.0059[b]
Healthy surrounding skin	5	35	0.1760±0.0197[c]	0.0902±0.0051[c]

Results are represented as the mean ± standard deviation. [a]P<0.05 vs. involuting hemangioma; [b]P>0.05 vs. healthy surrounding skin; [c]P<0.05 vs. proliferative hemangioma.

density and positive area rate of Bcl-2 and p53 were identified between involuting stage hemangioma tissue samples and healthy surrounding skin tissue samples (all P>0.05).

Discussion

Infantile cutaneous hemangioma is a common type of benign tumor, and is characterized by the rapid growth of endothelial cells at birth, followed by a slow involuting phase that lasts for a number of years, and this may lead to the tumor completely disappearing (16). Vascular endothelial cells serve an important role in the development of hemangioma through the modulation of the expression of a series of positive and negative regulators (9-11). It is typically understood that the proliferation and regression of vascular endothelial cells is the primary reason for the growth and involution of hemangioma (31). Apoptosis is the process of spontaneous cell death through gene regulation in order to regulate the development of organisms and maintain a stable environment (32). Under certain conditions, the activation of enzymes, such as endogenous DNA incision enzymes, can induce cell death through a series of signaling cascade reactions via exogenous or endogenous processes. The Bcl-2 protein, encoded by the Bcl-2 gene, serves an essential role in the formation of a variety of malignant tumors. Bcl-2 does not promote cell proliferation, however it inhibits apoptosis, which is the opposite of other members of the Bcl-2 family such as Bad, Bid and Bax that promote cellular apoptosis (33,34). Bcl-2 was first isolated by Tsujimoto *et al* (35) from follicular non-Hodgkin's lymphoma B cells and is one of the most well investigated apoptosis-regulating genes. The results of a study performed by McDonnell *et al* (36) on Bcl-2 transgenic mice demonstrated that high expression of Bcl-2 can inhibit the apoptosis of cells.

From the results of previous studies and the present study, it can be hypothesized that the important role of Bcl-2 serves in inhibiting apoptosis accounts for its association with hemangioma formation. The results of a study by Turner *et al* (37) demonstrated that the expression of Bcl-2 was positively correlated with tumor microvessel density in pituitary tumors. In addition, the results of a study by Gazzaniga *et al* (38) revealed that the expression of Bcl-2 was positively correlated with VEGF and bFGF expression, indicating that Bcl-2 is associated with tumor angiogenesis. Furthermore, a previous study identified higher VEGF and bFGF expression in the proliferative stage of hemangioma compared with the involuting stage (39).

The present study demonstrated that the expression of Bcl-2 in the endothelial cells of proliferative stage hemangioma samples was significantly increased compared with the expression in involuting stage hemangioma samples and healthy skin tissue samples. It has been suggested that Bcl-2 inhibits the apoptosis of endothelial cells, resulting in an imbalance between cell proliferation and apoptosis. An increase in cell proliferation and inhibition of cell apoptosis are involved in the formation and development of hemangioma.

p53 is tumor suppressor protein that is associated with tumor development and serves an essential role in the regulation of cell cycle progression, cell differentiation and apoptosis. Previous studies have revealed that >50% of tumors are associated with p53 gene mutation (40-43). p53 is ~53 kDa and is encoded by the TP53 gene. p53 is important for the maintenance of normal cell growth and inhibition of malignant proliferation. The primary mechanisms of action of p53 are the regulation of the cell cycle and induction of cell death. In the case of DNA damage, increased p53 expression

can induce cell cycle arrest at the G_1 stage to allow for DNA damage repair and if the damage cannot be repaired, then cell apoptosis is induced. p53 has wild-type (wt-TP53) and mutant (mt-TP53) forms. mt-TP53 cannot inhibit cell proliferation and induce apoptosis, enabling malignant cells to proliferate. In a previous study, the expression of wt-p53 in healthy tissue surrounding a hemangioma was low and only mt-p53 was detected through immunohistochemistry and quantum dot analyses (44).

In the present study, the presence of mt-p53 in human hemangioma tissue was detected through immunohistochemical and quantum dot analysis in 38 cases, and relatively accurate staging was conducted through measuring PCNA expression. The results of the current study demonstrated that compared with healthy skin tissue samples there was no significant difference in endothelial cell p53 expression in involuting stage hemangioma. Proliferative stage hemangioma endothelial cell p53 expression was significantly increased compared with involuting stage hemangioma. The expression of p53 was relatively low in the involuting stage, but relatively high in the proliferative stage. These results were consistent with results on the expression of VEGF in different stages of hemangioma (45,46). A number of studies have indicated that p53 can promote the expression of VEGF, increasing tumor angiogenesis; in addition, coexpression and a positive correlation between p53 and VEGF expression have been identified in a number of tumor types (47-49). Therefore, p53 may promote the formation of hemangioma through upregulation of VEGF expression. p53 can influence apoptosis by regulating Bcl-2 and BCL2 associated X apoptosis regulator (BAX) gene expression. p53 can specifically inhibit the expression of Bcl-2 and promote the expression of BAX. p53 is a direct transcription-activating factor of BAX. Therefore, if p53 mutation occurs, it will reduce the apoptosis of cells.

In conclusion, Bcl-2 can cause an imbalance between cell proliferation and apoptosis in endothelial cells through the inhibition of endothelial cell apoptosis. In addition, p53 may promote the proliferation of endothelial cells in proliferating cutaneous hemangioma and contribute to the generation of a large number of vascular endothelial cells. The results of the present study have provided future directions for the study of tumor angiogenesis. Understanding the role of these regulatory factors in tumor angiogenesis may aid in the development of novel treatments to inhibit tumor angiogenesis.

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