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Apoptosis and Angiofibrosis in Diabetic Tractional Membranes after VEGF Inhibition: Results of a Prospective Trial. Report no. 2

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Abstract

Purpose—We sought to characterize the angiofibrotic and apoptotic effects of vascular endothelial growth factor (VEGF)-inhibition on fibrovascular epiretinal membranes in eyes with traction retinal detachment due to proliferative diabetic retinopathy.

Methods—Membranes were excised from 20 eyes of 19 patients (10 randomized to intravitreal bevacizumab, 10 controls) at vitrectomy. Membranes were stained with antibodies targeting connective tissue growth factor (CTGF) or VEGF and co-labeled with antibodies directed against endothelial cells (CD31), myofibroblasts, or retinal pigment epithelium markers. Quantitative and co-localization analyses of antibody labeling were obtained through immunofluorescence confocal microscopy. Masson trichrome staining, cell counting of H&E sections, and TUNEL staining were performed.

Results—High levels of fibrosis were observed in both groups. Cell apoptosis was higher ($p=0.05$) in bevacizumab-treated membranes compared to controls. The bevacizumab group had a non-significant reduction in co-localization in CD31-CTGF and cytokeratin-VEGF studies compared to controls. VEGF in extracted membranes was positively correlated to vitreous levels of VEGF; CTGF in extracted membranes was negatively correlated to vitreous levels of CTGF.

Conclusions—Bevacizumab suppresses vitreous VEGF levels, but does not significantly alter VEGF or CTGF in diabetic membranes that may be explained by high baseline levels of fibrosis. Bevacizumab may cause apoptosis within fibrovascular membranes.

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Keywords

bevacizumab; CTGF; cytokine; diabetic retinopathy; fibrosis; membrane; retinal detachment; myofibroblasts; RPE; VEGF

INTRODUCTION

Diabetic retinopathy is the leading cause of blindness in the working-age US population¹. The most severe form of retinopathy, proliferative diabetic retinopathy (PDR), is defined as the growth of new blood vessels (i.e. neovascularization) on the optic nerve head and/or retina with a predilection for the major vascular arcades in the posterior pole. The natural history of neovascularization, as well as treatment with panretinal photocoagulation or intravitreal anti-VEGF agents, results in progressive fibrosis, ultimately leading to fibrovascular membranes that can contract and cause vision-threatening traction retinal detachment (TRD)²⁻⁵. Vascular endothelial growth factor (VEGF) plays a critical role in the formation of neovascularization^{6,7}, whereas connective tissue growth factor (CTGF) potentiates the generation of fibrosis^{8,9}. Tipping of this balance from angiogenic to fibrotic membranes in eyes with PDR has been linked to VEGF and CTGF, respectively, and is termed the 'angiofibrotic switch'¹⁰. Studies purporting the role of these growth factors in the angiofibrotic switch were based on cross-sectional studies of aqueous and vitreous samples from eyes with PDR and varying severity of fibrovascular membranes^{9,10}. As anti-VEGF agents are given to eyes before surgery for TRD from PDR (and in eyes with PDR not needing immediate surgery¹¹) to purportedly facilitate removal of membranes by decreasing bleeding thereby decreasing complications and improving outcomes¹², it is important to understand the biologic bases mediating the anti-angiogenic and pro-fibrotic mechanisms at the molecular and cellular levels as the former may be helpful during surgery but the latter may worsen the TRD. This might help clinicians better prevent complications from relative indiscriminate use of anti-VEGF drugs and foster development of novel agents that could allow more precise in targeting of the right signals at the right time in eyes with PDR.

In our first report from 2012 of a randomized, double-masked controlled trial examining the effect of VEGF inhibition on vitreous levels of VEGF and CTGF in eyes with severe TRD from PDR, we found that eyes given bevacizumab had suppression of VEGF and had vitreous VEGF levels that correlated positively with CTGF levels². Most of these eyes had advanced TRDs with membranes that were predominantly fibrotic at their pre-operative baseline examination. However, six of the 10 eyes randomized to receive intravitreal bevacizumab 1.25 mg had mixed neovascular-fibrotic membranes pre-operatively; by the time of surgery (average 4.8 days after injection) 83% of these eyes had regression of the neovascular component, leaving completely fibrotic membranes for intra-operative dissection. Nearly all eyes in both study arms had extraction of the diabetic membranes for subsequent histologic analysis to examine the effect of bevacizumab on cells and growth factors previously implicated in angiofibrosis. The goal of the present study was to compare the effect of VEGF inhibition to sham treatment on these membranes vis a vis: 1) VEGF and CTGF content, 2) co-localization of these cytokines with endothelial cells (CD31), myofibroblasts (α -smooth muscle actin), and retinal pigment epithelium (cytokeratin)

markers, 3) morphologic alterations and 4) apoptosis. It also allowed us to determine how well vitreous levels of these growth factors correlated to the amount of signal measured in their respective membranes.

METHODS

Patients and Tissue Preparations

Description of the trial has been previously described and registered under www.clinicaltrials.gov NCT#01270542. Informed consent was obtained from all subjects for this study, which had institutional review board approval and adhered to the tenets of the Declaration of Helsinki. In summary, 20 eyes with severe diabetic TRD requiring vitrectomy at Los Angeles County Hospital, University of Southern California Medical Center (LAC +USC Medical Center; Los Angeles, CA) were randomized to receive intravitreal bevacizumab 1.25 mg or sham intravitreal injection 3–7 days prior to vitrectomy. Epiretinal fibrovascular membranes were surgically excised from 19 eyes. Tissues were snap frozen in liquid nitrogen and 9 μ m sections were cut with a cryostat and stored at -80°C . Laboratory investigators were masked regarding which eyes were treated with bevacizumab throughout all experiments until statistical analysis was performed.

Immunofluorescent double labeling of tissue with VEGF or CTGF with CD31, smooth muscle actin (SMA), or cytokeratin

Thawed tissue sections were air-dried, fixed in acetone (5 minutes), and washed with phosphate-buffered saline (PBS; pH 7.4) for 5 minutes. Sections were blocked with 1% bovine serum albumin (BSA) in 1X PBS for 1 hour, and then incubated with anti-human CTGF (R&D, Minneapolis, MN) or anti-VEGF (Abcam, Cambridge, MA) antibody overnight at 4°C . Sections were washed the next day and incubated with the appropriate secondary Cy5 antibody (Invitrogen, Carlsbad, CA), for 30 minutes. For double labeling, the sections were returned to 1% BSA for 1 hour and then incubated with one of the following: anti-CD31 (Abbiotec, San Diego, CA), anti-smooth muscle actin (SMA; Abcam, Cambridge, MA), or anti-cytokeratin (CK; pan-cytokeratin; Dako, Carpinteria, CA),¹³ and incubated for 1 hour at room temperature. The appropriate secondary FITC (fluorescein isothiocyanate) conjugated antibody (Vector Laboratories, Burlingame, CA) was then applied for another 30 minutes. After each step of the incubation, sections were washed with PBS three times for 5 minutes each. Sections were mounted with medium for fluorescence with DAPI (Vector, Burlingame, CA), and were examined with a confocal laser scanning microscope (LSM510; Carl Zeiss Meditec, Inc., Thornwood, NY).

Automated pixel count of the intensity of VEGF, CTGF, CD31, SMA, and CK signal per standard area as well as co-localization analyses using pixel overlap was performed using Volocity software version 5.4 after thresholding. Two to four sections taken from different areas of each patient's membrane were averaged to generate each of these pixel intensity distributions.

Cell fibrosis, density, and apoptosis measurements

Representative sections (n=2–4) from each patient's epiretinal membrane were stained with Masson's trichrome to evaluate the intensity of fibrosis and vascularity in the membranes via collagen deposition with an Olympus BX41 fluorescence microscope. Each section was assigned a semi-quantitative grade for amount of fibrosis, intensity of fibrosis, and vascularity from + to +++ based on morphology. Vessel density estimation was represented as the number of vessel per area of the membrane¹⁴.

All images from each membrane were taken by an Olympus BX41 fluorescence microscope. Quantitative assessment of nucleated cell density in hematoxylin-eosin stained membranes was performed using 'Image Analyze Menu' from Image J¹⁵ (<https://imagej.nih.gov/ij/>) version 1.46n. H&E stained color images were converted to greyscale. Using a pre-set threshold and binary, all nucleated cells were selected based on their distinct features such as cell size, shapes and staining features; the same threshold and binary were used for all images. The total number of nucleated cells of each image were measured automatically by running 'Analyze-Analyze Particles.' Similar procedures were applied to measure the area of membrane in pixels from each image ('Analyze-Measure Area'); areas were converted to mm² with a scale according to the magnification used while capturing the images. Nucleated cell density was expressed as mean number of cells per mm² in each membrane $\pm$ SD for each group.

Apoptosis in membranes was detected by fluorescent terminal dUTP nick-end labeling (TUNEL assay, Roche Applied Science, Indianapolis, IN). 9 μ m cryo-sections were fixed in 4% paraformaldehyde and stained for nuclear DNA fragmentation according to the manufacturer's protocol and was expressed as the number of TUNEL positive cells per total cell nuclei in the membrane.

Fewer eyes were available for the Masson Trichrome and TUNEL stains due to varying sizes of extracted tissue (see results for n); only eyes with adequate amount of tissue were stained, counted, and analyzed. All other analyses had a minimum of 9 membranes counted in each group.

Six to nine random fields from two to three sections per patient were analyzed in Image J. All estimations were done in a masked fashion by two expert graders.

Statistical Analysis

Data were analyzed using F-test and paired student t-test to compare the bevacizumab group to controls. Results are expressed as mean $\pm$ SEM, and P 0.05 was considered statistically significant. Correlation between vitreous and membrane levels of CTGF and VEGF were performed using the R statistical computing environment (R Core Team [2013]. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria; <http://www.R-project.org/>).

RESULTS

Co-expression of VEGF and CTGF in diabetic membranes with CD31, smooth muscle actin (SMA), and cytokeratin (CK)

Positive staining of VEGF and CTGF was seen in all membranes (Figure 1) but there was no statistically significant difference seen in the bevacizumab group compared to the sham group (n for each group detailed in Table 1; Figure 2).

Positive staining of VEGF, CTGF, CD31, and cytokeratin was detected in all membranes sections (Figure 1). Intravitreal bevacizumab given within 3–7 days before TRD surgery did not significantly decrease VEGF or CTGF expression in membranes, i.e. eyes that received bevacizumab still expressed VEGF in membranes. Immunoreactivity intensity of CD31-CTGF and cytokeratin-VEGF in membrane sections in the preoperative bevacizumab group had a greater than 25% reduction (non-significant) in co-localization in the CD31-CTGF (bevacizumab: 517.04, 95% CI 184.03–850.05; controls: 827.68, 95% CI 266.52–1388.84; $p=0.35$) and cytokeratin-VEGF studies (bevacizumab: 1751.19, 95% CI 1213.99–2288.40; controls: 1661.42, 95% CI 1025.30–2297.55; $p=0.84$) compared to controls. There was also no significant change in VEGF (bevacizumab: 1736.92, 95% CI 1482.90–1990.94; controls: 1949.30, 95% CI 1552.41–2346.18; $p=0.39$) or CTGF expression (bevacizumab: 1279.19, 95% CI 833.25–1725.12; controls: 1378.88, 95% CI 477.73–2280.03; $p=0.78$) between the treated and controls in membranes.

Vitreous levels for VEGF and CTGF found from our first report² were compared to the levels found in membranes of the same patients; a negative correlation was found for CTGF ($p=0.049$) while a modestly positive correlation was found for VEGF, although this relationship did not reach statistical significance ($p=0.061$; Figure 2).

Morphometric assessment and cell penetration

Based on hematoxylin and eosin stained histological sections, there was no difference in mean number of nucleated cells in the membranes receiving bevacizumab (316.94/mm²) and controls (313.18/mm²; $p=0.97$; figure 3).

Assessment of collagen deposition by trichrome staining

Collagen deposition, an indicator of fibrosis, was evaluated in the membranes using Masson's trichrome staining (Figure 4). Grossly, high levels of collagen were observed in both groups. Semi-quantitative assessment showed a trend toward higher intensity of collagen deposition in the bevacizumab group (bevacizumab: 0.82, 95% CI 0.72–0.89; $n=4$ versus controls 0.72, 95% CI 0.45–0.99; $n=2$; $p=0.20$). On the other hand, the grade of vascularity was lower (but not statistically significant) in membranes after bevacizumab (mean=0.62, 95% CI 0.55–0.84) compared to controls (mean=0.89, 95% CI 0.58–1.14; $p=0.15$).

Detection of apoptotic cells by TUNEL staining in membranes

Despite a relatively low number of eyes with membranes adequate for analysis (due to tissue depletion from above studies), the density of TUNEL positive cells (i.e. number of TUNEL

positive cells to total DAPI positive cells) was significantly higher ($p=0.05$) in bevacizumab-treated fibrovascular membranes (20.02%, 95% CI 13.66–26.37, $n=4$) compared to controls (9.75%, 95% CI 9.44–10.07; $n=2$) (Figure 5).

DISCUSSION

In this randomized controlled study we found that fibrovascular membranes from eyes with diabetic traction detachments treated with and without preoperative intravitreal bevacizumab had histologically detectable growth factors associated with angiogenesis (VEGF) and fibrosis (CTGF). VEGF levels in the vitreous published in the first report from this study using ELISA were positively correlated at a modest level to VEGF in membranes of the same patients while CTGF levels in vitreous were negatively correlated to CTGF content in the membranes². Apoptotic cells, detected by TUNEL staining, were more numerous in eyes treated with bevacizumab compared to controls.

The utility of intravitreal bevacizumab for inducing neovascular regression in eyes with severe PDR has been demonstrated in numerous studies^{2,3,16–18}, but the exact mechanism underlying the conversion of neovascularization to fibrosis is unclear. Previous reports have documented that fibrovascular membrane constituents include collagen, myofibroblasts¹⁴, abnormal vascular endothelial cells, perivascular cells, stromal cells¹⁹, and M2 subgroup macrophages²⁰. Vitreous fluid from PDR patients with membranes show elevated levels of several key cytokines involved in angiogenesis and fibrosis such as VEGF, CTGF, TGF-beta^{2,9,21–23}, sCD163. Kuiper and others have described the balance of VEGF and CTGF as having a major role in the angiofibrotic switch whereby the decreasing activity of VEGF is associated with increasing CTGF, resulting in the transformation of a membrane that is predominantly neovascular to a more fibrotic phenotype^{9,14,21,22,24–26}. Thus, while administering anti-VEGF agents may decrease bleeding at surgery and peri-operative complications for eyes with TRD from PDR, the angiofibrotic switch could accelerate fibrosis and worsen the TRD, putting the eye at further risk for vision loss.

We previously showed that within 6 days of intravitreal bevacizumab injection, vitreous VEGF levels were significantly reduced and there was a clinically observable alteration in diabetic membranes, however CTGF levels were unchanged². Here we quantified VEGF and CTGF in membranes extracted from the same patients, and performed co-localization studies with antibodies directed against CD31, and SMA, and cytokeratin. Kubota et al²⁷ also compared membranes from eyes that received bevacizumab compared to controls and while our findings are consistent with theirs regarding similar vascular endothelial cell density between the bevacizumab and control membranes, Kubota et al found that VEGF content was lower in the membranes of bevacizumab treated eyes compared to controls. Though we had over 50% more subjects in our study compared to Kubota et al, the vast majority of eyes in our study (and all eyes in the control group) had completely fibrotic, avascular membranes, thus it may be difficult to detect a difference in VEGF signal due to the already low signal in the control membranes. In addition, that VEGF membrane signal does not change with decreased vitreous VEGF after bevacizumab implies that VEGF is still being produced in these membranes. A significant change in co-localization from VEGF inhibition was not seen in CD31, SMA, or cytokeratin containing cells, thus the inhibition of

vitreal VEGF is likely due to decreased production from the retina, not the membranes. Alternatively, bevacizumab may only bind to VEGF released from cells into the vitreal and not change VEGF gene expression in PDR membranes, so the vitreal concentration is attenuated but the VEGF membrane content is unaffected. Notably, comparison of vitreal ELISA levels for VEGF and measurement of VEGF in membranes from the same patients shows a modest positive correlation between vitreal and membrane VEGF levels.

Based on our initial report of high aqueous CTGF levels at baseline in both study groups², it is not surprising that membrane CTGF levels were not different. Similarly, the co-localization of CTGF was also unaffected by bevacizumab. All cases recruited in this trial had significant fibrosis which was confirmed by the Masson trichrome staining of membranes in our study expressing high levels of collagen content in both groups. This may explain the relative resistance of seeing a definite angiofibrotic switch from bevacizumab suggested histologically by other groups¹⁴. Moreover the negative correlation between CTGF in the vitreal and extracted membranes suggests that aqueous and/or vitreal CTGF levels may not be an ideal biomarker for estimating CTGF levels in the posterior segment.

Notably, we expand on the number of cases that Kohno et al reported regarding TUNEL-positive cells in membranes²⁸ and found a significantly higher amount of apoptotic cells in the bevacizumab treated eyes compared to controls, but this result should be interpreted with caution as the number of sections available for analysis was low. VEGF is known to have pro-survival effects on endothelial cells that are mediated by the phosphatidylinositol (PI)-3-kinase-Akt pathway²⁹ as well as expression of Bcl-2 and A1 in endothelial cells³⁰. Consequently, inhibition of VEGF would downregulate these anti-apoptotic mechanisms, accounting for the higher amount of apoptosis observed in the bevacizumab group. While increased endothelial cell apoptosis in the bevacizumab group would seem to be at odds with similar expression of vascular endothelial cell marker CD31 content between the two groups, it is interesting to note that other groups have similarly found stable levels of endothelial cell markers despite bevacizumab^{31,32}. Alternatively, since the TUNEL stain is a general marker for apoptosis and not specific for endothelial cells, it is possible that there are downstream effects to VEGF inhibition that resulted in apoptosis of non-endothelial cells, however this would need to be explored in further detail for confirmation. Regardless, this report supports the notion that bevacizumab inhibition induces contraction of blood vessels rather than obliteration of them^{31,32}.

In conclusion, our study demonstrates that intravitreal bevacizumab at 1.25 mg administered within one week of surgery significantly suppresses vitreal VEGF levels, but does not significantly alter VEGF or CTGF expression in fibrotic diabetic tractional membranes. Still, since many patients with end-stage PDR often have co-morbidities that make them prone to cancellation on the day of surgery, we recommend judicious use only in patients who already have full medical clearance so their surgery is not delayed past 1–2 weeks after anti-VEGF at which point the risk of worsening TRD increases. Bevacizumab may result in apoptosis within fibrovascular membranes but (at least in the short interval between injection and surgery) did not show a significant change in total nucleated cells or a subset of vascular endothelial cell content, myofibroblasts, or cytokeratin. Despite our sample size being small and the results therefore inherently limited, this study provides a unique histopathologic

point of view along with correlation to ocular fluid levels from the effect of VEGF inhibition (positive for VEGF but negative for CTGF) on severe diabetic membranes due to PDR. To further confirm our preliminary findings, investigations involving a larger sample size with appropriate randomization and control group will be of value. In addition, studies that better elucidate the timing of when CTGF increases during the pro-fibrotic aspect of the angiofibrotic switch would be useful as CTGF inhibition may have a role in reducing intraocular fibrosis.

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Summary statement

While bevacizumab suppresses vitreous VEGF levels in eyes with diabetic traction retinal detachment, VEGF and CTGF content is unchanged in fibrovascular membranes extracted from the same eyes. Bevacizumab may cause apoptosis within fibrovascular membranes.

Figure 1.

Representative hematoxylin and eosin (H&E) and immunofluorescence images from 4 different patient's membranes with co-labeling of antibodies for (A) CD31 (Green)-CTGF (Red) and (B) cytokeratin (Green)-VEGF (Red) antibodies. Note the H&E-stained sections do not correspond precisely to the cytokeratin-labeled sections. While intravitreal bevacizumab did not significantly decrease CTGF (A-top panels) or VEGF (B-top panels) expression in membranes compare to sham group, VEGF was still expressed in membranes of eyes given bevacizumab (B, middle panels). Scale bar=100µm.

Figure 2. VEGF and CTGF levels in membranes were plotted against their respective vitreous levels (log-transformed). There was a modest positive correlation (but not statistically significant) between vitreous and membrane with VEGF (A) and marginal negative correlation observed with CTGF (B). * indicates $P \leq 0.05$ which was considered statistically significant.

Figure 3.

(A) H&E stained histological sections demonstrated the morphology of membranes with and without bevacizumab. (B) Mean nucleated cell density was not different in the bevacizumab treated membranes compared to controls. Scale bar=200um, “—” represents the mean of nucleated cell density in the bar graph.

Figure 4.

Fibrosis was evaluated in diabetic fibrovascular membranes using Masson's trichrome staining for collagen (Blue stain) deposition in controls (n=2) and membranes after bevacizumab (n=4); (A) High baseline levels of fibrosis were observed in both control and bevacizumab membranes. Arrows indicate vessels that were counted for vascularity measurement; (B) Hematoxylin and eosin (HE) stained sections show the morphology of membranes after different treatments. (C) Semi-quantitative assessment of intensity of collagen deposition and (D) Vascularity in the membranes three weeks after bevacizumab

was not significantly different compared to controls. Membranes = Fibrovascular membranes. Student's *t* tests were used to compare two groups. Scale bar=200um.

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Figure 5.

Apoptosis was quantified using TUNEL assay in control and bevacizumab membranes (A), where red indicates TUNEL positive cells and blue indicates DAPI positive nucleated cells. (B) Quantitative results of TUNEL assay demonstrated significantly increased percentage of total DAPI-stained cells that were also TUNEL positive ($p=0.05$) in bevacizumab-treated membranes ($n=4$) compared to controls ($n=2$). Scale bar=200 μ m. Results were expressed as mean $\pm$ SEM, * indicates $P \leq 0.05$, which was considered statistically significant.

Table 1

Co-expression of VEGF and CTGF with CD31, SMA, and cytokeratin in membranes extracted from eyes with TRD/PDR treated with bevacizumab or sham injection 3–7 days prior to surgery

Antigen	Bevacizumab (mean pixels $\pm$ SD)	Controls (mean pixels $\pm$ SD)	p-value
CYTO-CTGF	n=9	n=6	
CTGF	1111.4 $\pm$ 335.2	1136.1 $\pm$ 480.7	0.91
CYTO	1368.6 $\pm$ 505.6	1151.4 $\pm$ 171.0	0.33
% colocalized	40.75 $\pm$ 12.98	38.40 $\pm$ 18.39	0.78
CYTO-VEGF	n=9	n=8	
VEGF	1751.2 $\pm$ 866.7	1661.4 $\pm$ 918.0	0.84
CYTO	1342.9 $\pm$ 529.1	1273.8 $\pm$ 542.6	0.79
% colocalized	27.31 $\pm$ 12.44	37.81 $\pm$ 14.14	0.12
CD31-CTGF	n=9	n=8	
CTGF	517.0 $\pm$ 509.7	827.7 $\pm$ 809.8	0.35
CD31	945.6 $\pm$ 832.8	1127.4 $\pm$ 885.8	0.67
% colocalized	23.78 $\pm$ 10.50	34.75 $\pm$ 18.22	0.14
CD31-VEGF	n=10	n=8	
VEGF	714.4 $\pm$ 145.3	547.1 $\pm$ 239.8	0.17
CD31	591.6 $\pm$ 150.4	594.0 $\pm$ 175.7	0.98
% colocalized	26.71 $\pm$ 13.26	25.31 $\pm$ 9.18	0.8
SMA-CTGF	n=10	n=8	
CTGF	1364.1 $\pm$ 1136.7	1457.1 $\pm$ 1439.3	0.88
SMA	1173.3 $\pm$ 837.2	1325.2 $\pm$ 1122.3	0.75
% colocalized	08 $\pm$ 13.4534.1	32.48 $\pm$ 22.29	0.85
SMA-VEGF	n=9	n=6	
VEGF	760.6 $\pm$ 241.4	896.0 $\pm$ 216.8	0.29
SMA	680.5 $\pm$ 183.9	778.8 $\pm$ 124.9	0.28
% colocalized	23.19 $\pm$ 19.19	22.92 $\pm$ 11.02	0.98

Membranes = Fibrovascular membranes.

SMA=alpha-smooth muscle actin.

Cyto=cytokeratin.

Student paired t test was used to compare two groups.