

Immunohistochemical Expression of Bcl-2 and Microvessel Density in Uterine Fibroids in Saudi Patients

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ABSTRACT

Objectives: This study assessed the prognosis of leiomyomata in Saudi patients through determining the area per cent of Bcl-2 immunohistochemical expression and the microvessel count as expressed by CD34, aiming to exploit an antibody-based therapy rather than surgical management. Recent studies have focussed on the function of proto-oncogene and tumour suppressor gene products in directing cell fate. In particular, an explosion of research interest has centred on the role of Bcl-2 in controlling the survival and death of cells, and CD34 as an indicator of the new vascularization of the lesion.

Methods: Thirty fibroid tissue specimens were collected from patients aged 30 to 50 years who underwent hysterectomy or myomectomy for the resection of symptomatic single or multiple operable uterine fibroids, and adjacent normal myometrial tissue was obtained (20) at Medina Maternity Child Hospital. Tissue sections (5 μ m) were cut and stained with haematoxylin and eosin for general histological examination. Other sections were immunostained with CD34 and Bcl-2 antibodies. Area per cent of Bcl-2 expression and microvessel count were measured by a stereological procedure with image analyser.

Results: The area per cent of Bcl-2 expression showed significantly higher expression of Bcl-2 in leiomyoma (1.37 ± 0.016) as compared with normal myometrium (0.31 ± 0.007) [$p = 0.045$]. The mean number of microvessels, as expressed by CD34, was significantly higher in leiomyoma (17.9 ± 0.49) as compared with normal myometrium (7.7 ± 0.40) [$p = 0.040$].

Conclusion: The study demonstrated that leiomyomata of Saudi patients expressed upgrading of Bcl-2 and proved the increased vascularity of this tumour.

Keywords: Bcl-2 oncoprotein, CD34, leiomyoma, uterus

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INTRODUCTION

Uterine fibroid or leiomyoma is a benign tumour that originates from the smooth muscle layer (myometrium) of the uterus. Fibroids are often multiple, and if the uterus contains too many leiomyomata to count, it is referred to as diffuse uterine leiomyomatosis. Fibroids are the most common benign tumours in females and are typically found during the middle and later reproductive years. While most fibroids are asymptomatic, they can grow and cause heavy and painful menstruation, painful sexual intercourse and urinary fre-

quency and urgency. Some fibroids may interfere with pregnancy (1).

Growth and location are the main factors that determine if a fibroid could lead to symptoms and problems (2). A small lesion can be symptomatic if located within the uterine cavity, while a large lesion on the outside of the uterus may go unnoticed. There are four types of fibroids: a) intramural fibroids located within the wall of the uterus and may, with time, expand inward, causing distortion and elongation of the uterine cavity; b) subserosal fibroids located underneath the mucosal (peritoneal) surface of the uterus; c) submucosal fibroids located in the muscle beneath the endometrium of the uterus, distorting the uterine cavity and usually – even small lesions – cause bleeding and infertility; d) cervical fibroids located in the wall of the cervix.

Fibroids that cause heavy vaginal bleeding lead to anaemia and iron deficiency. Due to pressure effects, gastrointestinal problems are possible such as constipation,

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compression of the ureter and hydronephrosis. Fibroids may also present alongside endometriosis, which itself may cause infertility. In very rare cases, malignant growths, leiomyosarcoma, of the myometrium can develop. Most fibroids do not require treatment unless they cause symptoms. After menopause, fibroids shrink and only symptomatic fibroids are treated (3). Medication of fibroids aiming at shrinking the tumour is usually carried out through ultrasound fibroid destruction, surgically aided methods to reduce blood supply of fibroids, myomectomy or radio frequency ablation and even hysterectomy.

Uterus-sparing therapy remains an up-to-date topic even in cases of women who no longer desire pregnancy. Myomectomy remains the most frequently used surgical technique. There is a constant search for alternatives to myomectomy as it is quite invasive for the patient and devastating for the uterus before planned pregnancy (4). Angiogenesis plays a central role in local tumour growth (5). The vasculature of uterine leiomyoma was investigated using colour Doppler ultrasound (6).

Recent studies have focussed on the function of proto-oncogene and tumour suppressor gene products in directing cell fate. In particular, an explosion of research interest has centred on the role of Bcl-2 in controlling the survival and death of cells (7). Bcl-2 protein plays an important role in the growth of tumours through preventing the normal course of apoptotic cell death (8), either by extending the life span of certain cells or promoting cell replication (9).

CD34 is a haemopoietic stem cell marker which is expressed on vascular endothelial cells (10). This factor not only stains the endothelial cells of the newly formed blood vessels, but also the lymphoid cells involved in fibroid degeneration. It might be considered as an identifying factor for other endothelial progenitor cells (11).

This study assesses the prognosis of leiomyomata in Saudi patients through determining the area per cent of Bcl-2 expression and the microvessel count as expressed by CD34, aiming to exploit an antibody-based therapy rather than surgical management.

SUBJECTS AND METHODS

Thirty fibroid tissue specimens were collected from patients aged 30 to 50 years who underwent hysterectomy or myomectomy for the resection of symptomatic single or multiple operable uterine fibroids, and adjacent normal myometrial tissue was obtained ($n = 20$) at Medina Maternity Child Hospital.

Tissue samples were collected and immediately fixed in 10% neutral buffered formalin at 4 °C overnight, then dehydrated and processed to wax using routine laboratory techniques. Tissue sections (5 µm) were cut and stained with haematoxylin and eosin [H&E] (12) for general histological examination. Other sections (5 µm) were cut into slides coated with 3-aminopropyltriethoxy-silane (Sigma Chem-

ical, Poole, UK) for immunohistochemical staining for CD34 and Bcl-2.

Immunohistochemical staining was performed by the avidin/biotin immunoperoxidase method with the use of a polyvalent immunoperoxidase Kit (Omnitage, Lipshow, MI) as previously described (13). A mouse monoclonal antibody to human Bcl-2 protein was used as primary antibody in the present study. To assure the specificity of the immunological reaction, corresponding control sections were subjected to the same immunoperoxidase method, except that the primary antibody was replaced by immune murine IgG at the same dilution as the specific antibody.

Consecutive 5–7 µm paraffin embedded tissue sections were subjected to immunostaining according to the streptavidin peroxidase (SP) methods. The tissue sections were deparaffinized and then endogenous peroxide was blocked by incubating the slides with 30 ml/L hydrogen peroxide (H₂O₂) for 10 minutes at 37 °C. After being thoroughly washed with distilled water three times (two minutes each time), the slides were heated in the jar containing antigen retrieval solution (0.01 mol/L citrate buffer, pH 6.0) in an oven at 92–98 °C for 15 minutes for the retrieval of the antigens and cooled to room temperature. After being washed with phosphate-buffered saline (PBS; 0.01 mol/L, pH 7.4) for five minutes, the sections were further blocked by goat serum for 20 minutes at 37 °C to reduce non-specific antibody binding and then incubated separately with primary antibodies (mouse-anti-human CD34) at 4 °C overnight. After being washed three times (three minutes each time) in PBS, the sections were incubated with the biotin-labelled goat anti-mouse IgG at 37 °C for 30 minutes, washed again with PBS, followed by incubation with streptavidin-peroxidase complex for 30 minutes at 37 °C. Staining was visualized with 3,3'-diaminobenzidine (DAB) for 10 minutes at room temperature. Finally, the sections were counterstained by haematoxylin solution (14).

The intensity of immunostaining was evaluated by grading as (-) for no immunostaining, (+) for weak but definitely detectable immunostaining, (++) for moderate immunostaining and (+++) for intense immunostaining (7).

Morphometric measurements were carried out at the Research Unit, Faculty of Medicine, Taibah University, KSA. Tissue sections were examined and the images captured using an image analyser (Leica Q Win standard, digital camera CH-9435 DFC 290, coupled to photomicroscope, Germany).

Computerized imaging analysis systems were introduced in order to minimize subjectivity in quantifying the positive Bcl-2 immunohistochemical stained cells and the microvessel counting. Area per cent of Bcl-2 expression was measured and expressed per area of the view field. Microvessels were counted in 200 × fields. Any endothelial cell or endothelial cluster positive for CD34 (brown yellow staining) was considered to be a single countable microvessel (15). For each specimen, five high power fields were

randomly selected, photographed and stored. The digitalized pictures were examined by two investigators on a high-resolution colour display.

The obtained results and the calculated parameters regarding the Bcl-2 expression and microvessels were statistically analysed using SPSS (version 13) statistical package. Independent *t*-test was used and the resulting data expressed as mean \pm standard error. Statistical significance was considered with *p*-value < 0.05 .

RESULTS

The study assessed the prognosis of leiomyomata in Saudi patients through determining the expression of Bcl-2, CD34 and the vascular pattern in the tumour.

Histological results

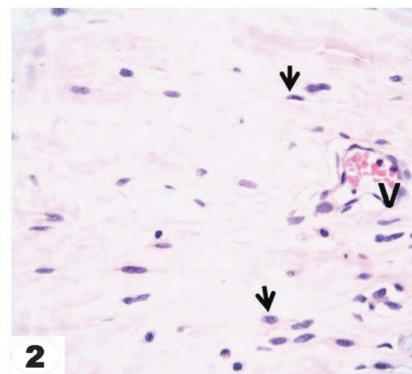
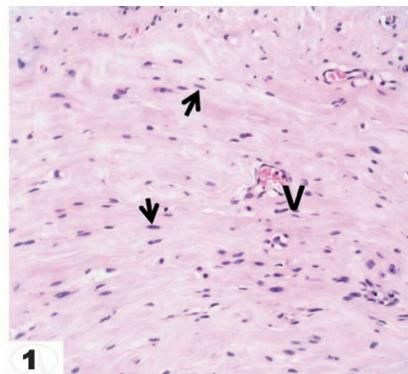
Light microscopic examination of normal myometrium stained with H&E showed that it is formed by interlacing bundles of smooth muscle cells with elongated, cigar shaped nuclei and eosinophilic cytoplasm. Blood vessels could be observed between the muscle bundles (Figs. 1, 2).

Histological examination of leiomyomata showed that it is formed by tightly packed smooth muscle cells with abundant eosinophilic cytoplasm and spindled nuclei having pale chromatin. The cells are arranged in different directions, forming whorls embedded in a fibrous stroma. Enlarged blood vessels and fibroblasts could be seen (Figs. 3, 4).

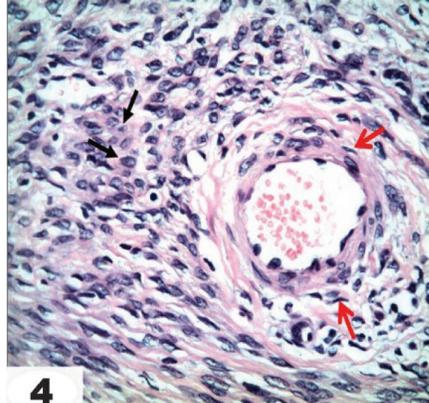
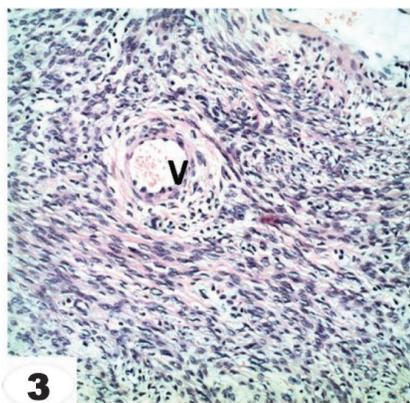
Immunohistochemical expression of Bcl-2 in leiomyoma attained positive grades, while negative grades were achieved in normal myometrium (Figs. 5, 6). CD34 labelling in the endothelial cells of blood vessels was higher and more intense in leiomyoma when compared to normal myometrium (Figs. 7, 8).

The area per cent of Bcl-2 expression showed that the expression of Bcl-2 was more pronounced in leiomyoma (1.37 ± 0.016) when compared with normal myometrium (0.31 ± 0.007). This difference was highly significant ($p = 0.045$), as shown in Fig. 9.

The mean number of microvessels using CD34 revealed significantly higher number of blood vessels in leiomyoma (17.9 ± 0.49) compared with normal myometrium (7.7 ± 0.40) [$p = 0.040$] (Fig. 10).



Figs. 1, 2: Photomicrographs of sections from human myometrium showing that it is formed by interlacing bundles of smooth muscle cells with elongated, cigar shaped nuclei (arrows). Blood vessels can be observed (V). (H&E; 1 \times 200, 2 \times 400)



Figs. 3, 4: Photomicrographs of sections from human leiomyoma showing that it is formed by tightly packed smooth muscle cells (black arrows) with eosinophilic cytoplasm and spindled nuclei. The smooth muscle fibres form whorls and are embedded in a fibrous stroma. Enlarged blood vessels (V) and fibroblasts (red arrows) can be seen. (H&E; 3 \times 200, 4 \times 400)

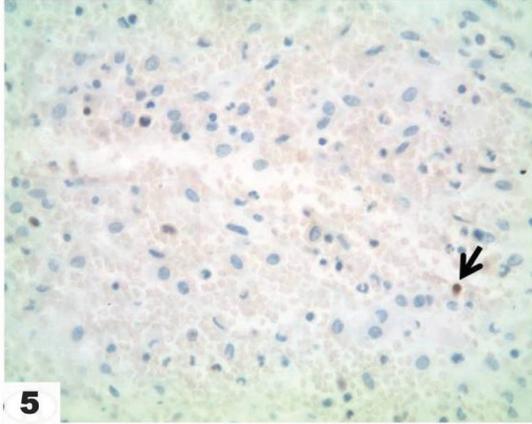


Fig. 5: A photomicrograph of immunohistochemical staining for Bcl-2 protein in human myometrium. The immunoeexpression of Bcl-2 in normal myometrial cells was only slightly observed (arrow). (Immunoperoxidase reaction of Bcl-2; $\times 400$)

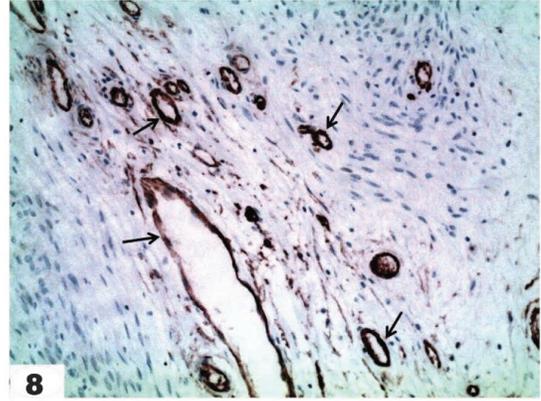


Fig. 8: A photomicrograph of immunohistochemical staining of leiomyoma with CD34. Higher vascularity of leiomyoma was obvious as compared to normal myometrium (arrows). (Immunoperoxidase reaction of CD34; $\times 200$).

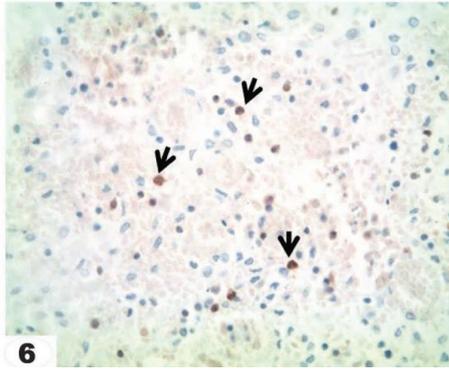


Fig. 6: A photomicrograph of immunohistochemical staining for Bcl-2 protein in leiomyoma. The leiomyoma cells showed pronounced immunostaining for Bcl-2 protein (arrows). (Immunoperoxidase reaction of Bcl2; $\times 400$)

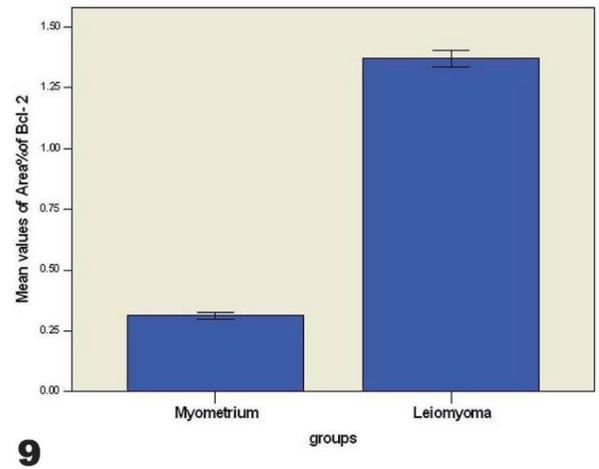


Fig. 9: Mean values of area per cent of Bcl-2 in myometrium and leiomyoma. Values are presented as means \pm SEM.

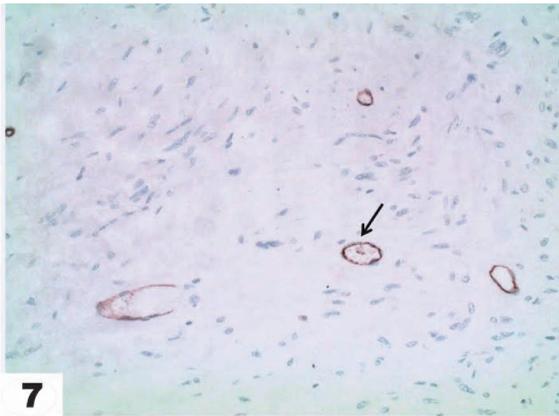


Fig. 7: A photomicrograph of immunohistochemical staining of myometrium with CD34. Blood vessels were highlighted by staining endothelial cells with antibody against CD34 (arrow). (Immunoperoxidase reaction of CD34; $\times 200$).

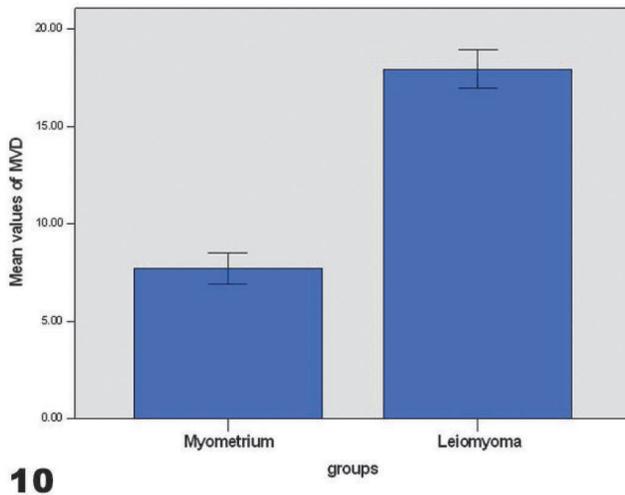


Fig. 10: Mean values of microvessel counting in myometrium and leiomyoma. Values are presented as means \pm SEM.

DISCUSSION

Uterine leiomyomata are considered the commonest neoplasm of all gynaecological conditions. These tumours are not malignant, but are the basis for various reproductive and gynaecological ailments and one of the leading causes of hysterectomies worldwide (16). In the present study, light microscopic examination of leiomyomata showed tightly packed smooth muscle cells with whorled appearance and elongated, cigar-shaped nuclei. Similarly, it was reported that leiomyomata were composed of smooth muscle cells separated by variable amounts of fibrous connective tissue with whorl-like appearance (17). Uterine fibroids are characterized by two primary histologic features: the proliferation of smooth muscle cells and the production of a collagenous matrix. The collagenous component of fibroids is variable in quantity; one subtype of leiomyoma, the cellular leiomyoma, usually displays little extracellular matrix, consisting primarily of closely packed fascicles of smooth muscle cells, while many fibroids contain abundant fibrous matrix, which may even exceed the smooth muscle component itself (18).

Bcl-2 attracted the attention of scientists as unique among cellular genes for its ability to block apoptotic cell death in multiple contexts. Its over-expression in transgenic models and accumulation of cells due to evasion of normal cell death mechanisms raise the thoughts that Bcl-2 is a cell survival gene (19–21).

The grading of Bcl-2 in leiomyomata shows a long debate over the years. It was demonstrated by Matsuo *et al* that there was greater abundance of Bcl-2 protein in leiomyomata of Japanese patients relative to the normal myometrium of the same individual uterus (7). They explained that the action of Bcl-2 on cellular survival is further exemplified by its ability to block apoptotic cell death (7). Similarly, Yin *et al* observed that there was approximately

1.4-fold higher Bcl-2 levels in leiomyomata of American patients than those in the normal myometrial tissues in 44.4%, but they found that 27.7% of their leiomyomatous tissues expressed lower Bcl-2 levels as compared to the normal myometrium (22).

In the present study, and after measuring the area per cent of Bcl-2 expression in specimens of Saudi patients, the expression of Bcl-2 in the leiomyomata showed significant difference ($p = 0.045$) when compared to normal myometrium.

In line with the present data, it was found that Bcl-2 concentration in myomatous tissues was statistically higher than that of the control myometrium group [$p < 0.001$] (23). However, another study done on Pakistani patients depicted an up-regulation of Bcl-2 levels in nearly 50% of the leiomyomata. The mean level of Bcl-2 protein in the leiomyomatous tissue was greater than that of the adjacent normal myometrium but showed no statistically significant difference [$p = 0.399$] (24).

Several investigators reported significantly higher Bcl-2 protein levels in leiomyomata during the secretory phase rather than the proliferative phase of the endometrium. They presumed that progesterone could cause a reciprocal rise in Bcl-2 protein levels favouring an apoptosis inhibited environment within the tumour area (22, 24).

Knowledge of vascular differences between leiomyomata and normal myometrium could aid the understanding of the variability of symptoms reported by women with fibroids and help define the mode of action of some of the current medical managements. It could notably provide knowledge of the future utility of angiogenesis inhibitors for treatment (25).

Expression of CD34 in leiomyomata in the present study was higher and the number of the newly formed vessels showed a highly significant difference ($p = 0.040$) when compared to normal myometrium. These differences in vascular density between myometrium and uterine leiomyomata could represent a difference in angiogenesis and vascular remodelling in these vascular beds. This difference in angiogenesis might be the result of changes in the balance between the factors that promote or inhibit the angiogenesis. It was presumed that during the development of leiomyoma, the pre-existing blood vessels underwent regression and new vessels invaded the tumour from the periphery (26), where intense angiogenesis, probably promoted by growth factors secreted by the tumour, led to the formation of vascular capsule responsible for supplying blood to the growing tumour (27).

On the other hand, using CD31, it was found that the myometrium was more vascular than fibroids of differing sizes (28). This might be due to differences in vascular markers used. It was reported previously that CD31 and CD34 may stain different groups of microvessels, or selectively stain some endothelial cells (29).

CONCLUSION

The study demonstrated that leiomyomata of Saudi patients expressed upgrading of Bcl-2 and proved the increased vascularity of this tumour as expressed by CD34. The study recommends further investigations on the tumour vascularity and the use of anti-Bcl-2 therapy to avoid surgical treatment of fibroids, particularly if multiple and/or huge, especially in infertile patients.

Further studies on tumour vascularity could lead to novel treatments for these common tumours. Also, the abundant expression of Bcl-2 oncoprotein in leiomyoma relative to that of normal myometrium may be one of the molecular bases for the enhanced growth of leiomyoma.

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The authors declare that there is no conflict of interest related to this study.

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