Role of Bcl-2 Expression in the Diagnosis of Uterine Leiomyoma

Wageih S. Elnaghy[1], Abdel Khalek H.S.[1], M. O. Ramdan[1], Dareen Abd Elaziz Mohamed[2], Sarah A. Hamam[3], Radwa Mahmoud Elsharaby[3], Shereef Lotfy El Shwaikh[4] and Ahmed Mahmoud El Khyat[4]

[1]Medical Microbiology and Immunology Department, Faculty of Medicine, Tanta University, Tanta, Egypt.
[2]Pathology Department, Faculty of Medicine, Tanta University, Tanta, Egypt.
[3]Clinical Pathology, Department, Faculty of Medicine, Tanta University, Tanta, Egypt.
[4]Obstetrics and Gynaecology Department, Faculty of Medicine, Tanta University, Tanta, Egypt.
wighwigh535@gmail.com

Abstract: Bcl-2 (B-cell lymphoma/leukemia-2) is a protein that inhibits apoptosis. Uterine leiomyoma is a benign cancer in the smooth muscles of the uterus and affecting about 30% of women more than 35 years. The current work aimed to evaluates the techniques of both immunohistochemical and an enzyme-linked immunosorbant assay (ELISA) to measure Bcl-2 in the leiomyomatous tissue and in the healthy myometrium (control) and compares the data with that of Western Blot technique (WB). This study included 36 women attended Obstetric and Gynaecology Department, Tanta Faculty of Medicine, from August 2017 to February 2018. Twenty four were examined clinically and by ultrasound for detection of uterine fibroids which subsequently were confirmed by histopathological examination. Twelve patients were having normal myometrium free from fibroid but carrying other lesions. The myometrium obtained after surgery were examined at the Histopathology Department, Tanta Faculty of Medicine, where ELISA and Western blotting were done in the Medical Microbiology & Immunology Department, Tanta Faculty of Medicine. No significant variations were found among the control and study cases concerning ages, gravidity, abortions and parity. A highly significant variation in Bcl-2 status in the myometrium and the leiomyomtous tissues in the same group and similarly, amongst the control myometrium and leiomyomatous tissues. In addition, non-significant differences were reported amongst Bcl-2 contents either of the control myometrium and confirmed cases or in the proliferative and secretory endometrium. The same tissue lysates from 30 thirty-six women were analysed by both ELISA and WB methods. Merely 3 samples were less than 40U/mg by ELISA from the control group not contained Bcl-2 band. The remaining of the samples indicated to a clear Bcl-2 band, which vary in intensity depending on the level of Bcl-2. It is concluded that the high expression of Bcl-2 in leiomyoma comparative to that of normal myometrium might be considered as one of markers for detection of leiomyoma progression depending on the molecular bases.

[Wageih S. Elnaghy, Abdel Khalek H.S., M. O. Ramdan, Dareen Abd Elaziz Mohamed, Sarah A. Hamam, Radwa Mahmoud Elsharaby, Shereef Lotfy El Shwaikh and Ahmed Mahmoud El Khyat. **Role of Bcl-2 Expression in the Diagnosis of Uterine Leiomyoma.** *Life Sci J* 2018;15(11):7-12]. ISSN: 1097-8135 (Print) / ISSN: 2372-613X (Online). http://www.lifesciencesite.com. 2. doi:10.7537/marslsj151118.02.

Key words: W.B, western blot technique. ELISA Enzyme linked Imunosorbent assay.

1. Introduction

Apoptosis (programmed cell death) is a natural complex physiological mechanism series of events that eliminate damaged or unwanted cells from multicellular organisms. Morphologically, it is characterized by cell shrinkage, membrane blebbing and nuclear condensation. The family of cystien proteases known as caspases, are the mediators of apoptosis [(1)].

Bcl-2 is a protein that inhibits apoptosis. Several researchers have postulated that the growth of uterine leiomyoma depends mainly on oestrogen.

The protein encoded by Bcl-2 gene is perhaps the most well characterized among these genes via its supporting role. Current work have demonstrate a strong connection between estrogen receptor expression and Bcl-2 in normal and epithelium of breast cancer. Bcl-2 has the oncogenic function by blocking apoptosis. Bcl-2 and Bcl-x are related proteins that playing an important role in inhibition of apoptosis; while Bax, Bak, and Bim a class of proteins were found to promote apoptosis. the unequal in the ratio of anti- and proapoptotic Bcl-2 class members that incline scale towards survival can provide tumour cells more resistant to a extensive diversity of cell death stimuli as well as chemotherapeutic agents. According to the hypothesis that an increased expression of Bcl-2 oncoprotein in smooth muscle cells of uterine Leiomyoma comparative to that of healthy myometrium may be considered as one of the biomarkers for detection of neoplasm progression in the uterus [(2,3)].

Generally, uterine leiomyoma is a benign tumour in the smooth muscle of the uterus and predominate in about 30% of women during the reproductive age (their ages more than 35 years) and enlarged in size

along pregnancy stage and declined after menopause. They increase in size during each menstrual cycle under the influence of cytokines, ovarian steroid hormones, and growth factors as well as epidermal growth factor (EGF), transforming growth factor β (TGF- β), interleukin 8, and endothelin which seen to be over expressed in leiomyoma versus the normal myometrium. In addition, extra cellular matrix components, like fibronectin, collagen, matrix metalloproteinases, proteoglycans and tissue inhibitors of metalloproteinases which appear to participate in the pathogenesis of leiomyoma. As regard their location, leiomyomas can be sub-serosal, sub-mucosal, or intra-mural, however some types may be located for example largely intra-mural with a sub-mucosal extension. Although majority of these neoplasms are asymptomatic, but usually manifest as menorrhagia, pelvic pain, compression of adjacent organs, recurrent miscarriages and obstructive labour in female patients. Mostly treatment for uterine leiomyomas is hysterectomy which after caesarean section, is the second choice.

The biology of leiomyomas now being better understood consequently, new medical treatment options are becoming available. Long acting gonadotrophin releasing hormone agonists in patients with uterine leiomyomas significantly reduce the size of this tumor [(4)].

Uterus- sparing thereby relics an up-to-date issue yet in cases of women no longer to be pregnant for different reasons, hysterectomy still the mainly commonly applied surgical technique. There is steady investigate for alternatives to hysterectomy as it's quite invasive for the patients and overwhelming for uterus before intended pregnancy [(5)].

Aim of the Work

In the current work, we used an enzyme-linked immunosorbent assay (ELISA) to determine Bcl-2 protein in the control myometrium and leiomyoma tissues and also, to evaluate the data of ELISA with that of Western Blot method (WB).

2. Materials and Methods

Patients & specimens

The present study was performed on 36 women attended Obstetric and Gynaecology Department, Tanta Faculty of Medicine from August 2017 to February 2018. Twenty four women were diagnosed clinically and by ultrasound as uterine fibroids and these fibroids were examined histopathologically after surgery (hysterectomy or myomectomy) to ensure diagnosis. Twelve women with normal myometrium and having other pathology than fibroid. Non of the patients received any preoperative hormonal therapy for 6 months prior to surgery. The myometrial and/or leiomyomatous specimens obtained after surgery were carried on ice to the laboratory at the Histopathology Department, Faculty Of Medicine, Tanta University; where they were washed with cold saline and divided into 2 portions; one of which was fixed in 10% formalin for histopathological and immunohistochemical staining for Bcl-2 antibody (1:50; Dako, Carpinteria, USA)), which was performed by the standard biotinstreptavidin-peroxidase method. It was graded as (0) for no immunostaining, (+1) for weak but definitely detectable immunostaining, (+2) for moderate immunostaining, and (+3) for strong immunostaining.

The other portion of the sample was stored in citrate sucrose dimethylsulphoxide buffer pH 7.4 at -80ºC for subsequent determination of Bcl-2 in cell lysate by ELISA and western blotting in the Medical Microbiology & Immunology Department, Faculty of Medicine, Tanta University.

Preparation of cell lysates [(6)]

All steps were carried at 4ºC. Tissues were washed with ice-cold saline and homogenized on ice in 10 mmol/L HEPES buffer (pH 7.5) containing 10 mmol/L K2EDTA, 50 mmol/L NaCl, 5 mmol/L bezamidine, 10 ml/L Triton X-100, 10 mmol/L 2-mercaptoethanol, 0.39 mmol/L phenylmethyle-sulphonyl fluoride (PMSF) and 5mg/L aprotinin with a homogenizer (Art Miccra- D3, Italy) for 60 seconds each, separated by a pause for 1 min. The homogenate was incubated in the lysing buffer on ice for 30 min, with vortex-mixing every 10 min. The homogenate was filtered and then centrifuged at 20 000 g for 20 min with a Hettich (universal 16L-Germany) and the resulting supernatents (lysates) were frozen at - 80ºC till time of use. The protein concentration was determined by Bradford's method (1976), using bovine serum albumin as a calibrator.

Quantitative measurement of Bcl-2 protein in cell lysate by ELISA [(7)]:

Bcl-2 protein was measured in cell lysate with a monoclonal antibody-based ELISA kit (Oncogene Science Products USA). The results were obtained as units (U) bcl-2/ml and then were expressed in U/mg protein, where one unite of Bcl-2 equals 5.6X104 cells of an internal control cell line (HL60).

Detection of Bcl-2 by Western Blot tachnique [(8)]:

Sixty micrograms of cell lysate proteins in loading buffer (50 mml/L tris, 20g/L sodium dodecyl sulphate, 100 ml/L glycerol, 100 mmol/L beta-mercaptoethanol and 0.05% bromephenol blue solution, pH 6.8) were boiled for 5 min and separated by 10% sodium dodecyle sulphate-polyacrylamide gel electrophoresis. The gels were transblotted to nitrocellulose filters in tris-glycine buffer (25 mmol/L tris, 192 mmol/l glycine, 200 ml/L methanol, pH 7.4) for 5 hours at 60 volts. The nitrocellulose sheets were washed and unoccupied binding sites were saturated

with 50g/L bovine serum albumin in tri-buffered saline buffer (50 mmol/L tris, pH 7.5, 150 mmol/L NaCl, 2 mmol/L EDTA) for 1 hour at room temperature. Then the filters were incubated with phosphate buffer saline and mouse monoclonal anti Bcl-2 antibody diluted 1:300 (by volume) overnight at 4ºC, then with rabbit anti-mouse IgG alkaline phosphatase conjugate diluted 1:500 for 90 min at room temperature. These two steps was separated by 3-5 min wash in phosphate buffer saline. Finally, the filters were incubated with alkaline phosphatase substrate solution at room temperature until the developed bands were of desired intensity. Then the reaction was stopped by 200 ml of 0.5 mol / L EDTA (pH 8) and 50 ml of phosphate buffered saline. By comparison of the molecular weight marker we could identify the band of Bcl-2 protein (M.W.= 26 KDa). Statistical analysis was done using t test.

3. Results

Immunohistochemical results [Figs. 1, 2 & 3]:

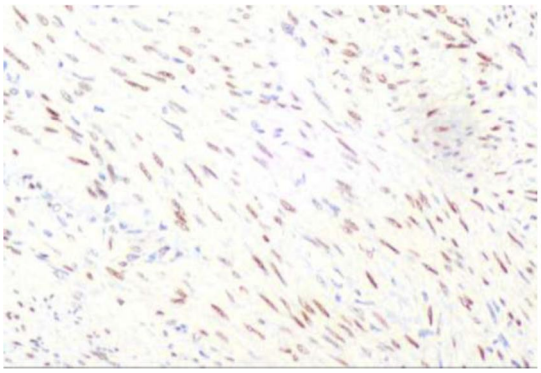

Fig (1): Bcl-2 in uterine leiomyoma showed strong expression (X200).

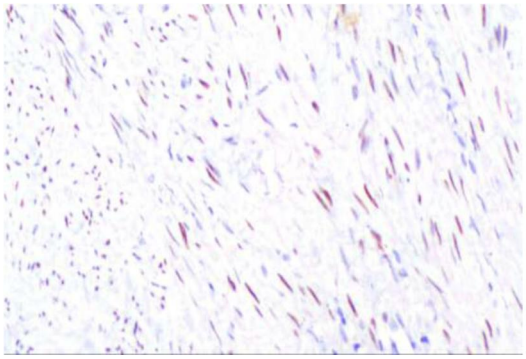

Fig (2): Bcl-2 in studied myometrium showed moderate expression (x200).

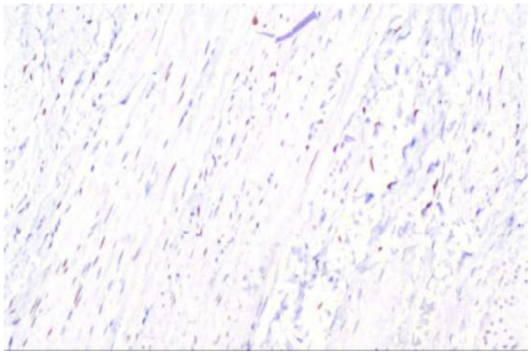

Fig (3): Bcl-2 in normal myometrium showed mild expression (x200).

All studied leiomyoma cases 24/24 (100%) showed intense expression of Bcl-2 (+3) while Bcl-2 showed moderate expression (+2) in 10/24 cases and mild expression (+1) in 14/24 cases of myometrium from which leiomyoma developed (studied myometrium). On examination of control myometrium, Bcl-2 showed negative expression in 10/12 cases however it showed mild expression in 2/12 cases, there were significant differences on regarding Bcl-2 expression in three studied groups.

Table (1): Clinical data of the studied and control groups

	Study cases (n=24)	Controls (n=12)	Significance	
	Mean ± SD	Mean ± SD	T	P
Age	42.23 ± 3.16	44.75 ± 4.55	1.15	> 0.05
Gravidity	5 ± 1.63	5.6 ± 1.72	0.77	> 0.05
Parity	4.12 ± 1.29	4.72 ± 1.24	1.15	> 0.05
Abortions	0.62 ± 0.70	0.54 ± 0.73	0.33	> 0.05

Table (2): Bcl-2 content of leiomyoma studied and control myometrium.

Tissues	Bcl-2 (mean ± SD) U/mg protein	T	P
Leiomyoma (n=24)	292.72± 112.33	6.32	< 0.001
Studied myometrium (n=24)	105.91± 49.56		
Control myometrium (n=12)	75.13± 46.31		

On examination of endometrium, there was no significant alterations in Bcl-2 protein expression among secretory and proliferative stages in the tested myomatous group.

Table (1): Tabulates the clinical results of the the control and study group. There was a non-significant differences were recorded among control and the study groups including age, gravidity, parity and abortions.

The results in table (3) revealed to a highly significant variation in the content of Bcl-2 of the leiomyomtous and the myometrium tissues from affected cases (the same origin of myometrium) and too among the control myometrium and leiomyomatous tissues. Also, in table (3), a non-significant differences were recorded between Bcl-2 content of the control and studied myometrium.

Table (3): Bcl-2 content of the myometrium of the studied and control groups

Tissues	Bcl-2 (mean ± SD) U/mg protein	T	P
Studied myometrium (n=24)	105.93± 49.51	1.68	> 0.05
Control myometrium (n=12)	75.36± 46.26		

Table (4) pointed to a non-significant variation was recorded in Bcl-2 contents of secretory and proliferative endometrium in control and studied groups.

Table (4): Bcl-2 content of proliferative and secretory endometrium in studied and control groups.

Group	Endometrial pattern	Bcl-2 (mean ± SD) U/mg protein	T	P
Studied Endometrium	Proliferative (n=10)	95.85± 43.8	0.71	> 0.05
	Secretory (n=14)	111.68± 53.23		
Control Endometrium	Proliferative (n=3)	43.13± 14.35	1.72	> 0.05
	Secretory (n=9)	103.95± 53.41		

The thirty-six women (studied and control groups) were analysed by both ELISA and WB techniques where we used the same tissue lysates. Only 3 samples of the control group showed no Bcl-2 band (their level by ELISA were less than 40 U/mg). The rest of the samples showed the band, which differs in intensity according to the concentration of Bcl-2.

4. Discussion

A novel suggestion that the failure of apoptosis might be involved in the pathogenesis of many human diseases like cancer, autoimmune disease as well as viral infection [(6)].

Uterine leiomyomata are benign, monoclonal tumours of the smooth muscle cells of myometrium and are considered the commonest neoplasm comprising 11.3% of all gynaecological surgeries. Although these tumours are not malignant, but are the basis for various reproductive and gynaecological ailments and one of the leading cause of hysterectomies done worldwide. Despite the fact that they are the cause of such morbidity to the females, the aetiology of these neoplasms is still poorly understood with scarce related epidemiological data available. The Bcl-2 oncogene may be a widespread "cell death suppressor" gene that directly control the processes of apoptosis in the cells and tissues [(7)].

Some investigators found a strong relationship among over expression of Bcl-2 and tumorgenesis in several studies in human tissues. This is depend on the information that increasing in the cell life span could increases the risk of secondary genetic changes leading to malignant transformation [(8)].

Bcl-2 oncoprotein levels have been assessed in various conditions according to account of its important role in cell development, maturation and the path to terminal differentiation with increased levels reported in cells with prolonged life such as duct cells, basal keratinocytes and cells responsive to hormones like myometrium and endometrium etc. Matsuo et al. (2018) and Gao Z et al, (2001) stated that an increased level of Bcl-2 protein in uterine leiomyomata as compared to the normal myometria were observed with significance [(9), (11), (12)].

The prognostic significance of Bcl-2 expression has reported in variety of malignancies; Most of these malignancies demonstrated a favorable prognoses in Bcl-2 positive tumor. Human Bcl-2 protein by

blocking apoptosis plays an important role in the growth of tumours [10].

The results in the current research revealed to a non-significant difference in Bcl-2 protein expression among secretory and proliferative stages in the tested myometrium group as recorded in table (4), this data coordinate with that of Toa et al., 1977 and Gompel et al., 1994, this may be explained by high estrogen level in myomatous cases where many investigators have suggested that estrogen plays an important role in development of uterine leiomyoma (Andersen et al., 1993). In the control group, the insignificant difference in the level of Bcl-2 (table 4) may be due to the relatively small number of cases [4), (13), (14), (15)].

In the present study it was noticed that Bcl-2 level in the tissues of myomentous was significantly higher (<0.001) than in the myometrial group (control), however done in Pakistani patients stated an up-regulation of Bcl-2 levels in nearly 50% of the leiomyomata. The main level of Bcl2 protein in the leiomyomatous tissues was greater than in the adjacent normal myometrium but showed no statically significant difference P (0.001) [10].

Some authors carried their research for the purpose of estimation of Bcl-2 protein expression in leiomyomas and in the normal myometrium by using immunohistochemical methods (Matsuo et al., 1997). The obtained data from the present study are in agreement with the results of Khurana et al., 1999.

In the current work, we have searched for a suitable method for estimation of Bcl-2 in uterine tissue lysates, easy to performed and not-time consuming or not coasty. The precision and specificity of ELISA for detection of Bcl-2 level in the uterine tissues has been assessed and demonstrated that it will be suitable for the measurement of Bcl-2 in lesser volume of cell lysates. Quantity of Bcl-2 antigen was established by western blot technique. The obtained data revealed that the sensitivity of ELISA in identification of the positive cases is more accurate than the method of western blot technique. Therefore we endorsed the use of ELISA for measurement of Bcl-2 level or immunohistochemical study.

In conclusion: We concluded that the high Bcl-2 oncoprotein expression in leiomyoma in comparison with healthy myometrium in the uterus might be one of the molecular bases for identification of enhanced development of leiomyoma. We hopefulness that an anti-Bcl-2 therapy can be applied in selected cases to avoid surgical interference in cases of m multiple huge fibroids in cases of infertility.

References:

1. Report compiled by Office of Research on Women Health, NIH, DHHS. Status of research on uterine fibroids (leiomyomata uteri) at the National Institute of Health 2006. Available online.
2. Kumar V, Abbas AK, Fausto N. Cellular Adaptation, Cell injury and Cell death. In: Pathological bases of disease. 7 Ed. Philadelphia, Pennsylvania 19106; WB Saunders 2004; 26-32.
3. Eisinger SH, Meldrum S, Fiscella K, Le Roux HD, Guzick DS. Low dose Mifepristone for uterine leiomyomas. ACOG 2003; 101:2.
4. Andersen, J.; Grine, E. and Carla, L. Y. (1993): Expression of connexin 43 in human myometrium and leiomyoma. Am. J. Obstet. Gynecol., 169: 1266-1269.
5. Niger R, Sonek J, Croon C, Ventolini G (2006): Pregnancy-related changes in the size of uterine leiomyomas. J repord Med 51:671-4.
6. Vaux D. L., Haecker, G. and Strassor, A. (1994): An evolutionary perspective on apoptosis. Cell, 76: 777-779.
7. Korsmeyer S. J. (1992): Bcl-2 an antidote to programmed cell death. Cancer Surveys 15: 105-108.
8. Tao, X. L., Tilly, K. L. and Maravei, D. V. (1997): Differential expression of members of Bcl-2 gene family in proliferative and secretory endometrium: Glandular epithelial cell apoptosis with increased expression of bax. J. Clin. Endocrinol. Metab. 82: 2738-2746.
9. Bonder K, Bonder-Adler B, Czerwenka K, Hudelist G, Kimberger O, (2003): Bcl-2 expression in a primary leiomyosarcoma of other ovary: a case report, Wien Klin Wochenschr 115:191-195.
10. Alenzi FQ & Waeens Anb (2003): Cellular and molecular therms in apoptosis. Wien kiln wochenscher 115:563-574.
11. Matsuo Y, Yuzo Maeda, Hiroki Takahashi, Nozomu Nakai, Takeshi Yanagita, Nanako Ando, Nanako Ando, Tomotaka Okubo, Kenta Saito, Kazuyoshi Shiga, Takahisa Hirokawa, Masayasu Hara, Hideyuki Ishiguro & Shuji Takiguchi: (Apigenin induces apoptosis by suppressing Bcl-xl and Mcl-1 simultaneously via signal transducer and activator of transcription 3 signaling in colon cancer) Published online on: March 7, 2018, https://doi.org/10.3892/ijo.2018.4308, Pages: 1661-1673.
12. 1Gao Z, Matsuo H, Wang Y, Nakago S, Maruo T. Up-regulation by IGF-I of proliferating cell nuclear antigen and Bcl-2 protein expression in human uterine leiomyoma cells. *Journal of Clinical Endocrinology and Metabolism*. 2001; 86(11):5593–5599.
13. Gompel, A., Sabourin, J. C. and Martin, A (1994): Bcl-2 expression in normal endometrium

during the menstrual cycle. Am. J. Pathol., 144: 1195-1202.
14. Tao, X. L., Tilly, K. L. and Maravei, D. V. (1997): Differential expression of members of bcl-2 gene family in proliferative and secretory endometrium: Glandular epithelial cell apoptosis with increased expression of bax. J. Clin. Endocrinol. Metab. 82: 2738-2746.
15. Andersen, J.; Grine, E. and Carla, L. Y. (1993): Expression of connexin 43 in human myometrium and leiomyoma. Am. J. Obstet. Gynecol., 169: 1266-1269.
16. Matsuo, H., Maruo, T. and Samoto (1997): Increased expression of bcl-2 protein in human uterine leiomyoma and its up-regulation progesterone. J. Clin. Endoc. Metab., 82:293-299.
17. Khurana, K. K., Singh, S. B. and Tantum, A. H. (1999): Maintenance of increased bcl-2 expression in uterine leiomyomas after GnRH agonist therapy. J. Reprod. Med. 44,6: 487-492.

11/17/2018