ORIGINAL ARTICLE

Expression of Bcl-2 Oncoprotein as an Apoptosis Inhibitor in Uterine Leiomyomata

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ABSTRACT

Background: Bcl-2 oncoprotein has an important role in cell development, maturation and acts as an anti-apoptotic factor in various neoplastic processes.
Aims: With the hypothesis that an increased expression of Bcl-2 oncoprotein in smooth muscle cells of uterine leiomyoma relative to that of normal myometrium may be one of the molecular bases for the enhanced growth of the neoplasm, its levels in uterine leiomyoma and adjacent normal myometrium were measured through enzyme linked immunosorbant assay (ELISA).
Methods: Tissue homogenates extracted from the surgical specimens of uterine leiomyomata and adjacent normal myometria of 32 female patients presenting with symptomatic single or multiple fibroids. Bcl-2 oncoprotein and total protein concentrations were measured utilizing a double sandwich ELISA technique.
Results: It was observed that the concentration of Bcl-2 protein (p=0.399) and the total proteins (p= 0.049) was higher in leiomyomata than in the adjacent normal myometria.
Conclusion: Bcl-2 protein is credibly expressed in uterine leiomyomatous cells in our female patients. Therefore, it may be exploited for an antibody-based targeted therapy in selected cases to avoid uncontrolled growth and surgical treatment of small sized leiomyomata, particularly if they are multiple and in patients desiring to conserve fertility.
Key words: Uterine leiomyoma, Myometrium, Bcl-2 oncoprotein, Enzyme linked immunosorbant assay

INTRODUCTION

Uterine leiomyomata, the benign myometrial smooth muscle tumours, grow more commonly during reproductive years, increase in size during pregnancy and regress after menopause. They increase in size during each menstrual cycle under the influence of ovarian steroid hormones, cytokines, and growth factors[1]including transforming growth factor beta (TGF-beta), epidermal growth factor (EGF), interleukin 8, and endothelin which have been shown to be over expressed in leiomyoma verses the normal myometrium. In addition, extra cellular matrix components, such as collagen, fibronectin, proteoglycans, matrix metalloproteinases, and tissue inhibitors of metalloproiteinases seem to play pivotal role in the pathogenesis of leiomyoma[2]. As regard their location, leiomyomas can be sub-serosal, sub-mucosal, or intra-mural, however some types may be combined for example largely intra-mural with a sub-mucosal extension[3]. 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. Most common treatment for uterine leiomyomas is hysterectomy which after caesarean section, is the second most frequently performed major surgical procedure with potential hazards in women of reproductive age in United States[4]. Women consider uterus as an important organ representing their feminity[5]and psychological implications such as depression and anxiety are observed in patients after hysterectomy[6]. Alternative surgical treatments for preserving uterus include myomectomies, cryotherapy, myolysis, and uterine artery embolisation that was first reported in 1995[7,8]. As the biology of leiomyomas now being better understood, new medical treatment options are becoming available[9]. Long acting gonadotrophin releasing hormone agonists in patients with uterine leiomyomas significantly reduce the size of this tumour. However serious side effects are bone loss and increased blood lipid levels[10]. Another non surgical treatment with low dose of mifepristone that binds and inhibits progesterone receptors with fewer side effects is in practice[11]. Apoptosis or programmed cell death is a complex series of events that eliminate damaged or unwanted cells from multi-cellular organisms. Morphologically, apoptosis is characterized by cell shrinkage, membrane blebbing

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and nuclear condensation. The family of cystien proteases, known as caspases, are the mediators of apoptosis[12]. Bcl-2 is an oncoprotein named after B cell lymphoma and was discovered in human follicular lymphoma. Bcl-2 proto-oncogene is unique by being localized to inner mitochondrial membrane and it has the oncogenic function by blocking programmed cell death. There are two classes of Bcl-2 related protein, one includes Bcl-2 and Bcl-x that inhibits apoptosis; the other class includes proteins such as Bax, Bak, and Bim, they promote apoptosis[13]. Imbalances in the ratio of anti- and proapoptotic Bcl-2 family members that tilt scale towards survival can render tumour cells more resistant to a wide variety of cell death stimuli, including chemotherapeutic drugs. Various agents that interfere with the action of Bcl-2 or other anti-apoptotic Bcl-2 family proteins, acts as a chemo sensitiser that would make it easier for conventional anti-cancer drugs to commit cell death. Anti sense DNA oligonucleotide targeted against Bcl-2 mRNA has been reported to reduce bcl-2 protein levels[14]. With the hypothesis that an increased expression of Bcl-2 oncoprotein in smooth muscle cells of uterine leiomyoma relative to that of normal myometrium may be one of the molecular bases for the enhanced growth of the neoplasm, the levels of Bcl-2 oncoprotein in uterine leiomyoma and adjacent normal myometrium were measured through enzyme linked immunosorbant assay (ELISA) which may presumed to be exploited for an antibody-based targeted therapy in selected cases to avoid surgical treatment of leiomyoma especially in infertile females.

MATERIALS AND METHODS

This was a cross-sectional descriptive study comprising of thirty two female patients (mean age 40 years, age range 30-50 years) who underwent hysterectomy or myomectomy for the resection of symptomatic single or multiple, operable uterine fibroids at the Obstetrics and Gynaecology Departments of Sir Ganga Ram Hospital Lahore, Sheikh Zayed Hospital Lahore, and Jinnah Hospital Lahore from January 2007 to June 2007. The patients were selected through convenient sampling technique Cases with history of co-morbidity or taking medication /therapy for the neoplasm i-e follow up cases or, if any, necrotic tissue specimens were excluded. All selected patients gave written informed consent.

Relevant clinical data of these patients including age, menstrual history, obstetrical history, history of infertility, presenting complaints, tumor location, and type of surgical procedure were recorded in specially designed proformas. Findings of the baseline laboratory investigations and specific diagnostic modalities including abdomino-pelvic or transvaginal ultrasonography, contrast studies e.g hysterosalpingography and computerized tomography in selected cases, were also documented. Gross observations took account of size, site, shape, consistency, appearance, presence of capsule and degenerative changes, if any, related to the tumor. Microscopic features tabulated included patterns of growth, nuclear features, mitoses, necrosis, and stromal reaction.

The hysterectomy and the myomectomy specimens were obtained and processed at the Morbid Anatomy and Histopathology Department of University of Health Sciences Lahore. The specimens were packed in ice to transfer to the laboratory where they were washed with cold saline. Tissue sections of 32 uterine leiomyomata (from both hysterectomy and myomectomy specimens) and 27 adjacent normal myometria (from hysterectomy specimens only) were taken and divided into two portions. One portion was fixed in 10% formol saline for histopathological examination and the other half of the sample was preserved in a storage buffer at -80^0C, for subsequent determination of Bcl-2 oncoprotein and total protein concentrations in the tissue homogenates extracted from the surgical specimens utilizing a double sandwich ELISA technique. The formol saline fixed tissues were processed through ascending grades of ethanol, cleared in xylene and embedded in paraffin wax for preparing tissue blocks. At least five sections of 4-7µm from each leiomyoma and the adjacent normal myometrium were cut by rotary microtome and collected on frosted glass slides for hematoxylin and eosin (H&E) and Gomori's Trichrome staining. The slides were then examined under the microscope to report the morphological features seen in each submitted sections of leiomyoma and the adjacent normal myometrium.

Preparation of Cell Lysates: About 10 mmol/l of HEPES buffer of pH 7.5 and a Lysing buffer was prepared according to the kits' instructions (BMF 44/3) and kept at room temperature. Frozen samples were washed with cold saline. About 1.5gm of tissue was taken in a falcon tube and 5cc of HEPES buffer was added followed by homogenization under ice using an electric homogenizer for 60 seconds followed by a pause of 60 seconds and the process was repeated till the proper homogenate was obtained[15]. 0.75ml of homogenate was pipetted out in a 2ml epindrof tube and 0.75ml of lysing buffer was added in it. This mixture was kept on ice for 30 minutes with. vortex mixing every 10 minutes. The homogenate was centrifuged at 14,000 RMP at -4°C for 20 minutes and the resultant supernatant (lysate)

was frozen at -80^{0}C in small aliquots until they were to be used for Sandwich ELISA based quantitative determination of Bcl-2 protein according to the manufacturer's protocol.. Total protein analysis was also carried out on the supernatants of the tissue homogenates with spectrophotometric detection according to Biuret method.

Statistical analysis: The data was entered and analyzed using SPSS 17.0. Mean ± S.D was given for quantitative variables. Frequencies and percentage was given for qualitative variables. Pearson Chi Square and Fisher Exact test were applied to observe associations, if any, between the qualitative and the quantitative variables. A p-value of < 0.05 was considered as statistically significant.

RESULTS

A total of 32 sections from uterine leiomyomata and 27 sections from the adjacent normal myometria were examined in this study both histologically and through ELISA based determination of Bcl-2 and total protein concentration. As regards the age of the female patients, it ranged from 30-50 years, (mean age: 40 years) with 6% (n=2) cases between the age group of 50-59 years, 59% (n=19) cases between the age group of 40-49 years and 34% (n=11) between 30 and 39 years of age. Clinical history was more or less similar in all cases (Table:1).

Parity of the 32 patients ranged from no live birth to a maximum of 7 live births. Mean parity was found to be 3.8 with n=1 live birth in 3.1% (n=1) patients, n=2, n=3 and n=4 live births in 15.6% (n=5) patients each, n=5, and n=6 live births in 9.4% (n=3) patients each and n= 7 live births in 12.5% (n=4) patients.

Regarding abortions, about 56.2% (n=18) patients presented with a varying history with n=1 abortion reported in 28.1% (n=9) cases, n=2 abortions in 21.8% (n=7) cases and n=3 and n=4 abortions reported in 3.1% (n=1) cases each.

In context of the number of leiomyomata seen in a single patient, we came across that the majority, 56.3% (n=18), of the patients had multiple (more than 6) leiomyomata, 25% (n=8) patients had two leiomyomata and 18.7% (n=6) patients had one leiomyoma only. When the sites of uterine involvement by leiomyoma was observed in each patient, we found that 3.1% (n=1) patients each presented with a subserosal and a leiomyoma in the broad ligament and 21.8% (n=7) patients had intramural fibroids. Amongst these, the uterine fundus and uterine corpus were involved in 6.2% (n=2) and 15.6% (n=5) cases respectively with uterine cervical myoma seen in none.

The surgical maneuvers included abdominal hysterectomy in 65.6% (n=21) patients, vaginal hysterectomy in 18.7% (n=6), hysteroscopic myomectomy in 9.4% (n=3), and vaginal myomectomy in 6.2% (n=2) patients. Surgical resection of the tumours turned out to be curative without any risk of recurrence in 84.3% (n=27) patients who underwent hysterectomy.

As regards the gross morphological characteristics of the leiomyomata, the smallest and the largest tumour was 2cm^2 and 60cm^2 in size with most of them, 87.5% (n=28), ranging between 22cm^2-45cm^2 in size. All of them were spherical to irregularly lobulated, surrounded by a pseudo-capsule, firm in consistency with degenerative changes seen both on gross as well as microscopic examination.

Keeping with the histological observations, the neoplastic tissue in all the leiomyomata comprised of fascicular, sheet like and whirling arrangements of the benign appearing smooth muscle cells with occasional mitotic figures and frequently seen apoptotic bodies **(Fig:1)**. The intervening stroma appeared as an irregular arrangement of sheets of collagen fibers among the neoplastic smooth muscle cells **(Fig:2)**. Concordant with the gross observations, degenerative changes were seen as vacuolar degeneration in 28.1% **(Fig:3)**, cystic degeneration in 21.8% and hyaline degeneration in 12.5% **(Fig:4)** of the leiomyomata.

The site distribution of the myomas in relation to the age, parity, infertility, clinical symptoms, size and number of fibroids, did not show much variation except that pelvic heaviness, to some extent, was predominantly seen in subserosal leiomyomata than the intramural ones (15.7% vs 10.9%; p=0.219).

Similarly, no significant association was found between the size, site, number or the degenerative changes within the leiomyomata and age, parity, infertility or clinical symptoms in the patients (p>0.05 in all associations tested).

Bcl-2 and Total Protein Concentration Assay: When Bcl-2 concentration was assessed in different leiomyomata and the adjacent normal myometria employing the principle of sandwich ELISA, we came across that the mean level of Bcl-2 protein (49.90 ± 42.12 S.D) in the leiomyomatous tissue was greater than that of the adjacent normal myometrium (42.33 ± 20.77 S.D) in 68.8% (n=22) cases, but showed no statistically significant difference (p=0.399) between the two. Rest 31.2% (n=10) leiomyomatous tissues expressed lower levels of Bcl-2 (39.44 ± 31.59 S.D) when compared with the normal myometria. However, the total protein concentration in the leiomyomata and the adjacent normal myometria measured through spectrophotometric method depicted a significant (p= 0.049) higher protein levels

in the leiomyomata when compared to the adjacent normal myometrial tissue (1.36 ± 0.10 VS 1.24±0.32).

A significant association was observed between the Bcl-2 concentration and the size of the leiomyomata (p=0.032) but no such associations could be set up between the Bcl-2 and the total protein levels in relation to other variables like age, parity, infertility, clinical symptoms of the patients and site, number or degenerative changes within the leiomyomata (p>0.05 in all associations tested).

Table:1: Pattern of Clinical History in Study Subjects

Degenerative Changes	% of Patients (n)
Vacuolar degeneration	28.1% (n=9)
Cystic degeneration	21.8% (n=7)
Hyaline degeneration	12.5% (n=4)
More than one degenerative change	34.4% (n=11)

This table shows the pattern of clinical history in study subjects. Note that menorrhagia was the most common presenting symptom.

Fig. 1: This figure shows the fascicular pattern of neoplastic smooth muscle cells with apoptotic bodies and occasional mitotic figures in a uterine leiomyoma. (H&E: 20X x 10X).

Fig 2: This figure shows the irregular arrangement of sheets of collagen fibers among the neoplastic smooth muscle cells in a uterine leiomyoma. (Trichome: 20X x 10X).

Fig 3: This figure shows the vacuolar degeneration in a uterine leiomyoma. (H&E: 20X x 10X).

Fig. 4: This figure shows the hyaline degeneration in a uterine leiomyoma. (H&E: 20X x 10X).

DISCUSSION

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[16].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[17].

Bcl-2 oncoprotein levels have been assessed in various tissues on 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[18].

Based on the investigations by Matsuo et al. and Gao Z et al, an increased level of Bcl-2 protein in uterine leiomyomata as compared to the adjacent normal myometria were observed with significance[19,20]. This study was also designed to assess the Bcl-2 oncoprotein levels in the tissue homogenates extracted from the uterine leiomyomata (myomas, fibroids, fibromyomas) and the adjacent normal myometria of the same uteri employing sandwich ELISA method. Apart from that, the total protein concentration in the same tissues was also determined using spectrophotmetry. To the author's knowledge, the study on Bcl-2 levels and that too on the tissue homogenates of uterine leiomyomata and the adjacent normal myometria has not been performed previously in Pakistan The method for tissue homogenization was adopted from the protocol devised by Eskenazi B and colleagues[15] and the procedure was standardized optimally according to the requirements.

As regards the clinical aspects of these neoplasms observed, the leiomyomas are clinically apparent in 20-40% women of reproductive age group[21], but pathological examination of the hysterectomy specimens suggested a prevalence of 77% in our study cases. The leiomyomata were more frequently seen in our patients within the mean age of 43 years (range: 30-50 years) while similar findings, with an average age of 43.7 years, were reported by Marina and Hartman[16,21]. Also a much variation in the size ($2cm^2$ to $60cm^2$) and number (>6 to single) of the leiomyomata were observed in all study patients.

Previously it was presumed that the growth of the leiomyomata is enhanced significantly in the presence of oestrogen during proliferative phase of the menstrual cycle as compared to the progesterone released more in the secretory phase. Thereafter, it is recognized in the literature that the size of the leiomyoma also increases with increasing progesterone that in turn is presumed to cause a reciprocal rise in Bcl-2 protein levels favouring an apoptotis inhibited environment within the tumour area. This was supported by various investigators who reported significantly higher Bcl-2 protein levels in leiomyomata during secretory phase rather than the proliferative phase of the endometrium[22,23].

In the present study, increased Bcl-2 protein levels were found in uterine leiomyomata (68.8%) as compared to the adjacent normal myometria, however, difference was not significant (p=0.399). Similar findings were observed by Ping Y and colleagues[22], who reported an approximately 1.4 fold higher Bcl-2 levels in leiomyomata than that of the normal myometrial tissues in 44.4% patients (p=0.12) while 27.7% leiomyomatous tissues expressed lower Bcl-2 levels as compared to the normal myometrium. Another study by Wei et al[24] depicted an up-regulation of Bcl-2 levels in nearly 50% of the leiomyomata.

In contrast to the present study, Kalman et al. found this differential expression of Bcl-2 in leiomyoma and adjacent myometrium to be significant (p=0.01)[26]. Similar results were observed by Matsuo and Maruo et al[19,23].

Apart from the neoplastic cells, the uterine leiomyomata also express large amounts of extracellular matrix proteins containing collagen, fibronectin, and proteoglycans arranged in a disarrayed fashion that was demonstrated in the present study by Trichome staining of the histological tissue sections. It was found that the levels of total protein in uterine leiomyomata were significantly higher (p=0.049) than the adjacent normal myometrial tissues reflecting an increased number of different tissue proteins found in these neoplasms that may also be playing a potential role in the pathogenesis of these otherwise benign tumours.
Scientists at the University of North Carolina, Chapel Hill General Clinical Research Center are developing strategies to prevent fibroid and devise new therapies that may reduce the need for radical surgical procedures like hysterectomy. The profuse release of Bcl-2 protein and its oncogene by the smooth muscle cells of the uterine leiomymata in our females, therefore, may form a potential therapeutic target for controlling the neoplastic proliferation of small sized leiomyomata especially if they are multiple and presenting in infertile females or those who prefer to conserve the fertility.

CONCULSION

Supporting the previous literature, we have also come across that Bcl-2 oncoprotein is credibly expressed in leiomyomatous cell lines in our female patients and exhibit relatively distinct rising patterns in especially the large sized tumours. Therefore, it might constitute a potential candidate gene, for devising new adjuvant monoclonal antibody-based targeted immunotherapy in selected patients to avoid surgical treatment and to conserve fertility. More prospective analyses should be followed in our population, relating the progesterone effects and the serum and/or immunohistochemical expression of this oncogene that might suggest its prognostic implication as an antiapoptotic factor in relation to the tumour burden.

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