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Original Research Paper

Immunohistochemical analysis of epidermal growth factor receptor in human uterine leiomyomas in relation to endometrial hormonal pattern.

Pathology

KEYWORDS: EGFR protein, Leiomyoma, Immunohistochemistry

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ABSTRACT

Aim: To analyze the expression of EGFR and find the significance of upregulation of EGFR in uterine leiomyomas in different phases of endometrium. Materials and methods: A total of 50 leiomyoma specimens were selected of which 20 cases showing proliferative phase in endometrium, 20 cases showing secretory phase in endometrium and 10 cases of normal myometrium used as control. Haematoxylin and Eosin slides of all the tissues were evaluated.The formalin fixed, paraffin embedded blocks were sliced in 3-4 µm thickness for IHC. The Avidin Biotin complex (ABC) detection system was used. Immunoreactivity was regarded as positive when brown staining was localized in the cytoplasm of leiomyoma cells.Results: The intensity of immunostaining in leiomyoma and matched myometrium in both phases of menstrual cycle was observed and graded as 0,1+,2+ and 3+. Conclusion: EGFR protein expression in leiomyoma cells predominated in the secretory phase compared to that in the proliferative phase. So it is likely that progesterone may participate in leiomyoma growth through the induction of EGFR protein in leiomyoma cells. Therefore the abundant expression of EGFR protein in leiomyoma may be one of the molecular basis for the enhanced growth of leiomyoma relative to that of normal myometrium in the uterus.

INTRODUCTION

Uterine leiomyoma is the most common benign smooth muscle cell tumor of the myometrium, occurring in as many as 30% of women over 35 years of age.[1] Prevalence rate for uterine fibroids approximately is 1 in 20 or 5 % or 13.6 million people globally. As per the Country/Region Extrapolated Prevalence Population Estimated to have uterine fibroid in India is 53 million in a total population of 1.2 billion.[2]Leiomyomas are a frequent cause of menorrhagia, dysmenorrhea, pelvic pain, reduced fertility, and recurrent pregnancy loss.A growing body of evidence suggests that the action of sex steroids may be mediated in part by local growth factors produced by the target cells.[3],[4],[5]However, the mechanisms of ovarian steroid hormone actions in the regulation of leiomyoma growth are not well defined yet.

EGF, a 6045-kDa protein with 53 amino acid residues and three intramolecular disulfide bonds, signals via its transmembrane EGF-receptor (EGF-R also known as ErbB1 or HER1) to regulate key processes of cellbiology such as proliferation, survival, and differentiation during development, tissue homeostasis and tumourigenesis[6]. The EGF-R is one of the most versatile signalling units in biology. EGF-induced activation of EGF-R results in phosphorylation of specific tyrosine residues within the cytoplasmic receptor tail. These phosphorylated tyrosine residues in turn serve as docking sites for proteins containing Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domains that coordinate the activation of multiple downstream signalling pathways. In mammals, canonical EGFR activation involves the binding of seven peptide growth factors including EGF, TGF-a and heparin-binding EGF (HB-EGF).[7]

EGF and EGF-RmRNAhave both been identified in myometrial and leiomyoma cells[8] and immunolocalization of both proteins has also been described in the cytoplasm of smooth-muscle cells of leiomyomas and matched myometrium [9]. Interestingly, EGF-positive staining signals in leiomyoma tissue from the proliferative phase is significantly decreased compared with matched myometrium [9]. *Myometrial* and leiomyoma cells express an EGF-R isoform with a molecular mass of 133 kDa[10][8]. Moreover, leiomyoma and myometrium contain specific, high-affinity binding sites for EGF [11]. EGF is mitogenic for both cultured myometrium and leiomyoma cells [12][13]; and it has been demonstrated that EGF plays a crucial role as a local growth factor in regulating leiomyoma growth [8][13]. A role for EGF in leiomyoma growth is also supported by the fact that the selective EGF-R blocker AG1478 is able to block leiomyoma cell proliferation [14]. Shushan et al. 2007also demonstrated that leiomyoma cell growth is effectively blocked by TKS050, a new EGF-R inhibitor. TKS050 induced cell cycle arrest and apoptosis in a dose- and time-dependent manner. This newly developed inhibitor may be useful as a possible alternative therapy for leiomyomas in the years to come [14]. Recently, NADPH oxidase-derived ROS (reactive oxygen species) have been shown to be critical component of the MAP kinase mitogenicpathway of EGF signalling in leiomyoma smooth muscle cell proliferation. This ROS production is inhibited by the NADPH oxidase inhibitor (DPI), suggesting the presence of the NADPH oxidase system and its importance in mitogenic signalling pathways in leiomyoma smooth muscle cells. The development of new therapies for the treatment and/or prevention of uterine leiomyomas should take advantage of the discovery of the NADPH oxidase-derived ROS acting in EGF signalling pathways leading to cell proliferation [15].

MATERIALS AND METHODS

This study was conducted at the Meenakshi Medical College and Research Institute Hospital, a rural tertiary care hospital with an annual volume of above 1,00,000 patients over one year period. The Institutional Medical Ethics Committee approved this study. Fifty cases were selected from August 2013 until July 2015 and it was diagnosed by a single histopathologist to avoid inter-observer variations.

Uterine leiomyomas and adjacent normal myometrial tissues were obtained from women with regular menstrual cycles who underwent abdominal hysterectomy for medically indicated reasons at Meenakshi Medical College and Hospital. The mean age group was 39 years, and none had received hormonal therapy for at least three cycles before surgery. Informed consent was obtained from each patient before surgery for the use of uterine tissues for the present studies. Each uterine specimen was examined by a pathologist for histological examination and dating of the endometrium. Endometrial tissues were obtained from the extirpated uteri, and the day of the menstrual cycle was determined by endometrial histological dating according to the method of Noyes et al.[16] and the patient's last menstrual period. Histological features of all cases studied with hematoxylin and eosin. A total of 50 uterine leiomyomas and myometrial tissues were collected of which 50% were from

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proliferative phase and 50% from secretory phase of menstrual cycle.

INCLUSION CRITERIA
Cases from August 2013 to July 2015 were selected.

EXCLUSION CRITERIA
Samples were excluded if accurate menstrual cycle dates could not be assigned, if unexpected pathology was found eg - endometrial hyperplasia and leiomyoma variants..

IMMUNOHISTOCHEMICAL STUDY
Haematoxylin and Eosin slides of all the tissues were evaluated, and for each case the best paraffin block with highest tumour content were chosen in order to prevent artefacts staining.These formalin fixed, paraffin embedded blocks were sliced in 3-4 µm thickness for IHC. The Avidin Biotin complex (ABC) detection system was used on specimens of formalin fixed, paraffin embedded tissue section.

Scoring system
Immunoreactivity was regarded as positive when brown staining was localized in the cytoplasm of leiomyoma cells. The intensity of immuno staining was evaluated by repeated staining of the same specimens.

Grading
(–) : No immunostaining
(1+) : Weak but definitely detectable immunostaining
(2+) : Moderate immunostaining
(3+) : Intense immunostaining

The extent was semiquantitatively estimated with a range of 0% to 100%. Percentages were estimated by counting at least 50 cells and then establishing the ratio of immunoreactive cells to total number of cells multiplied by 100; percentages were rounded to the nearest 10%. When less than 10% of cells were positive a score of 0 was used, 10 % to 30% cell positivity was scored as 1 , 31% to 60 % positivity was scored as 2 , and more than 60% positive cells was labelled as 3.

STATISTICAL ANALYSIS
Statistical analysis was carried out using SPSS version 19.0 (IBM SPSS, US) software with Regression Modules installed. All data was entered into a Data Collection Proforma Sheet (Annexure I) and were entered into Excel (MS Excel 2010). Results are represented in the form of tables and bar charts.

RESULTS
A total of 50 uterine leiomyomas and myometrial tissues were collected of which 25 were from proliferative phase and 25 from secretory phase of menstrual cycle. Hysterectomy specimen with endometrial hyperplasia, leiomyoma variants, ovarian pathology and cervical pathology were also not included in the study. Immunohistochemical study was done using EGFR antibody on all 50 cases selected.

Leiomyomas are seen in the women of child bearing age, most commonly occurring in the 4thdecade. The mean age being 39years. The youngest patient in our study was 25 years old, and the oldest was 54 years old.

Though leiomyoma is a disease of low parity, in our study we have noted it to be more common in para-II women In our study, most of the patients presented with menstrual disturbances followed by pain abdomen and dysmenorrhea. Rare symptoms included vaginal mass and infertility.

In our study it was noted that the size of the fibroid uterus varied from a few centimeters to 30 weeks of gravid uterus. It is seen that, about 38% were of the size of 16 weeks gravid uterus, 42% were of the size between 16-20 weeks, and huge fibroids of >20 weeks were encountered in 20% of the patients. The heaviest fibroid weighed 3.5 kg.

All the leiomyomata were corporeal, no extrauterine fibroids were encountered. Among the uterine about 98% were in the body & 2% were in cervix. Intramural fibroid was the commonest variety comprising about 74% of the cases, 10% submucous, 14% subserous and 4% cervical fibroid was encountered In our study we selected our cases in accordance to proliferative and secretory phases of normal menstrual cycle. All other pattern of endometrium was excluded. For our case we selected 50% of each proliferative and secretory phase.

Table 1.EGFR Expression in Leiomyoma & Myometrium (Proliferative phase)

S. No	Cases	EGFR Antibody	
		Positive	Negative
1	Leiomyoma	26%	74%
2	Myometrium	24%	76%

Leiomyoma cases showed 26% positivity while normal myometrium showed 24% of positivity. Thus indicating that EGFR expression remained unchanged in leiomyoma and myometrium during proliferative phase as illustrated in above mentioned table.

Table 2.EGFR Expression in Leiomyoma & Myometrium (Secretory phase)

S. No	Cases	EGFR Antibody	
		Positive	Negative
1	Leiomyoma	85%	15%
2	Myometrium	18%	82%

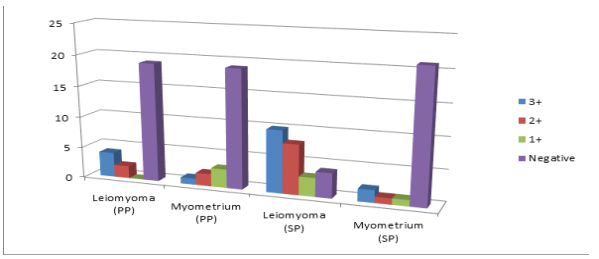
Leiomyoma cases showed 85% positivity while normal myometrium showed 18% of positivity. Thus indicating that EGFR expression was more prominent in leiomyoma than in myometrium during the secretory phase as illustrated in above mentioned table.

Table 3.Comparison of intensityof EGFR Expression in Leiomyoma and Myometrium in Proliferative Phase

S.no	Intensityof EGFR antibody	Proliferative phase	
		Leiomyoma	Myometrium
1	3+	4	1
2	2+	2	2
3	1+	0	3
4	Negative	19	19

Table 4.Comparison of intensity of EGFR expression in Leiomyoma and Myometrium in Secretory phase

S. No	Intensity of EGFR Antibody	Secretory phase	
		Leiomyoma	Myometrium
1	3+	10	2
2	2+	8	1
3	1+	3	1
4	Negative	4	21



Graph.: Comparison of internsity of EGFR staining in leiomyoma and myometrium in both phases of menstrual cycle

Comparison of immunohistochemical staining for EGFR protein showed that leiomyoma cells in the secretory phase of the menstrual cycle showed more predominant immunostaining than the leiomyoma cells in the proliferative phase. However, there was not much difference in the intensity of immunostaining for EGFR protein in myometrial smooth muscle cells between the proliferative phase and the secretory phase of the menstrual cycle.

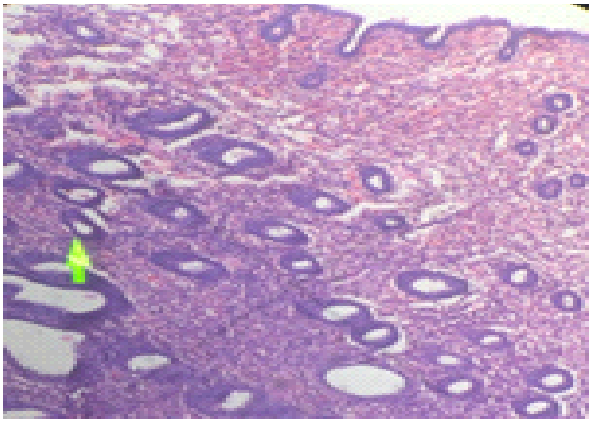


Fig 1a. Photomicrograph of Proliferative phase(H&E, magnification 400X)

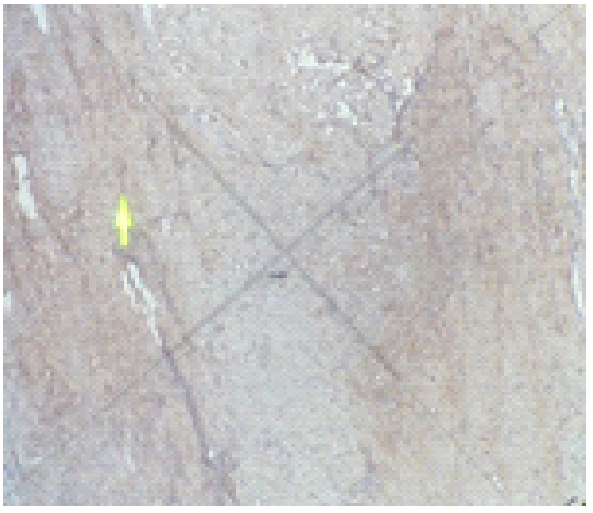


Fig 1b-Photomicrograph of EGFR expression in leiomyoma in proliferative phase(IHC,magnification 400X)

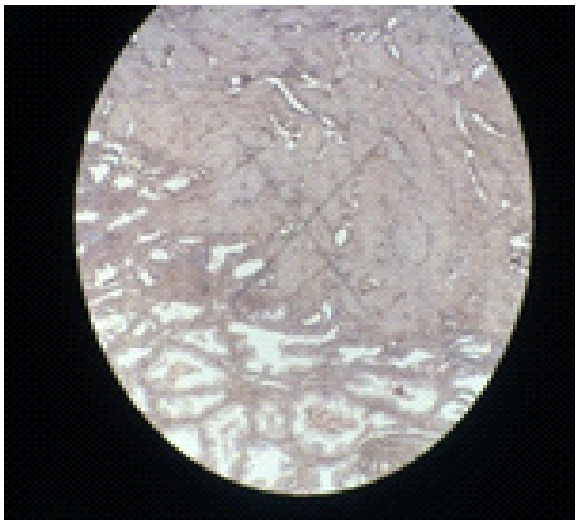


Fig 1c. Photomicrograph of EGFR expression in myometrium in proliferative phase (IHC, magnification 400X)

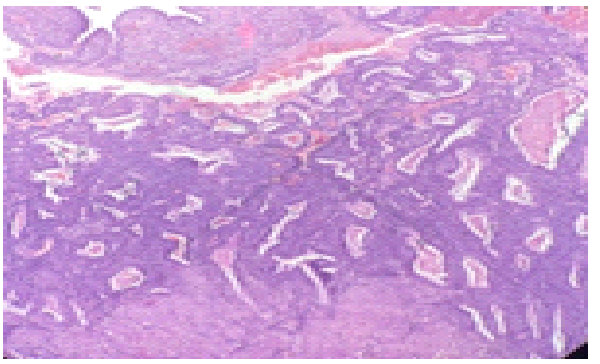


Fig 2 a. Photomicrograph ofsecretory phase(H&E, magnification 400X)

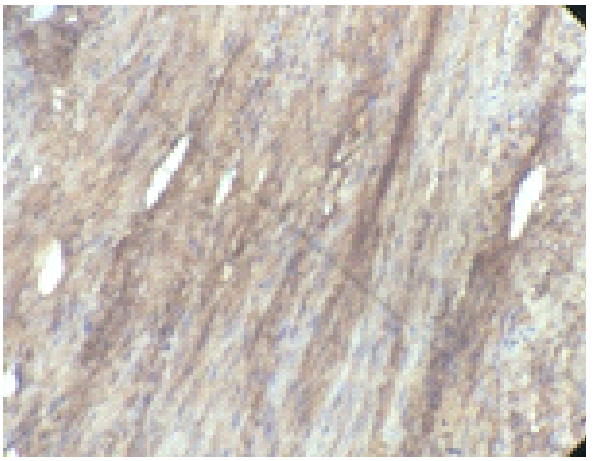


Fig 2.Photomicrograph of EGFR expression in leiomyoma in secretory phase (IHC, magnification 400X)

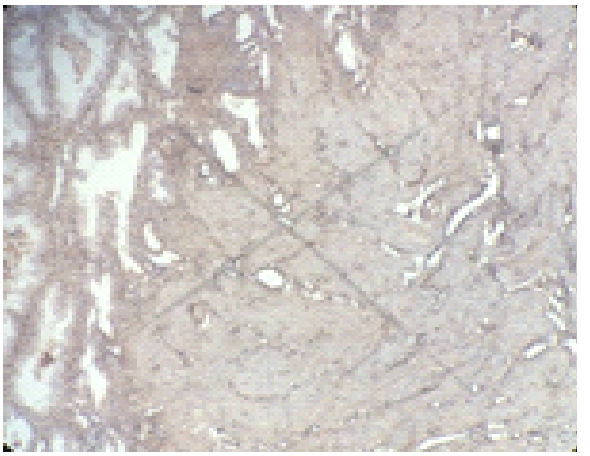


Fig 2c.Photomicrograph EGFR expression in myometrium in secretory phase(IHC,magnification 400X)

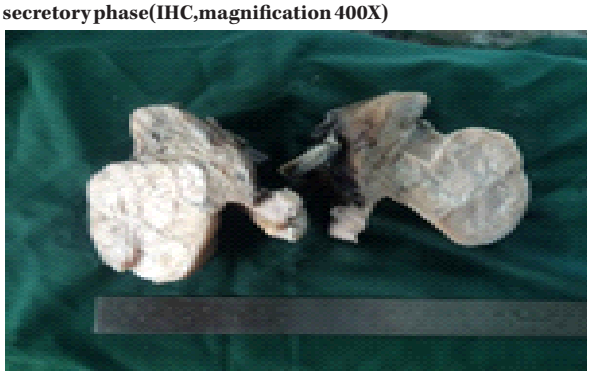


Fig 3. Gross photograph of fibroid uterus.

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CONCLUSION

Taking together our data on EGFR staining in leiomyoma and normal myometrium in relation to secretory and proliferative phase of menstrual cycle and the results from the literature on the expression of EGFR we conclude that there is an abundance of EGFR protein expression in leiomyoma relative to the normal myometrium of the same individual uterus and that EGFR protein expression in leiomyoma cells predominated in the secretory i.e. progesterone dominated phase of the menstrual cycle compared to that in the proliferative phase. So it is likely that progesterone may participate in leiomyoma growth through the induction of EGFR protein in leiomyoma cells. Therefore the abundant expression of EGFR protein in leiomyoma may be one of the molecular basis for the enhanced growth of leiomyoma relative to that of normal myometrium in the uterus.

The limitations of our study using immunohistochemical analysis alone should be acknowledged; for completion, it may require further validation using other methods including cell culture, immunoblotting, molecular and cytogenetic methods for understanding the underlying molecular mechanisms that determine EGFR over-expression and other pathways in development of uterine smooth muscle tumors. It is worth mentioning that, our results may contribute to clarify the sequential evolution in the development and treatment of uterine leiomyoma.

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