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Original Research Article

Association of sFRP4, a novel serum biomarker, in patients with Endometriosis

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Abstract

Introduction: An inflammation mediator, called secreted frizzled-related protein 4 (sFRP4), is closely associated with endometrial tissue. The purpose of the study was to test the feasibility of sFRP4 as a non-invasive diagnostic biomarker in different grades of endometriosis and to study the presence of sFRP4 in the peritoneal fluid of these patients.

Aims and objectives: To correlate serum & peritoneal fluid (PF) levels of sFRP4 in women with endometriosis.

Materials and methods: A hospital based cross-sectional case control study was carried out on women who were admitted for operative laparoscopy for the diagnosis of endometriosis. Based on their grading according to the revised American Society of Reproductive Medicine (rASRM), 22 patients with endometriosis aged 18– 45 years and 21 age matched healthy controls were included in the study. sFRP4 was estimated by ELISA method.

Results: Mean of serum and saline wash of POD levels of sFRP4 in endometriosis patients was found to be 8017.0 ± 2577.8 pg/ml &3718.9 ± 1650.8 pg/ml, respectively & that in serum of controls was 3871.0 ± 2077.8 pg/ml. Serum sFRP4 levels of cases were significantly elevated when compared to controls (p<0.0001). Total serum sFRP4 levels were positively correlated with saline wash of POD sFRP4 (r=0.743, p<0.001).

Conclusion: An increase in serum sFRP4, correlates with peritoneal fluid concentrations, and accompanies with increasing grades of endometriosis.

Keywords: Endometriosis, sFRP4, WNT, Peritoneal Fluid.

Introduction

Endometriosis is a common estrogen dependent gynaecological disorder, characterized by presence of tissue resembling endometrium tissue outside the uterine cavity. [1,2] Revised American Society of Reproductive Medicine (rASRM) classification is the commonly and extensively used classification system used for endometriosis.

It is estimated that endometriosis affects ~10% women in reproductive age in any population globally. [3] Women with the disease are often asymptomatic or have variable clinical presentations, and imaging modalities have low sensitivities for

diagnosis. Due to exclusive reliance upon laparoscopy for a definite diagnosis & staging determination of prevalence and incidence figures has been hampered . [1,4-6] Much research has recently focused on serum biomarkers, including cancer antigen-125 (CA125), leptin, monocyte chemotactic protein-1 (MCP-1), etc, although these have not been useful diagnostic predictors owing to poor sensitivity or specificity & inadequate validation of their accuracy. [7-10] The purpose of the studywas to test the feasibility of a non-invasive diagnostic method for endometriosis, to save patients from the burden of an operation like L/S and cause their treatment to begin earlier.

Secreted frizzled-related protein (sFRP4), expressed in endometrium has been associated with inhibition of endometrial cell proliferation. [11] Several lines of evidence support the hypothesis that WNTs, secreted glycoproteins are known to be stimulated by estrogen, whose signalling pathways control homeostasis of regenerating tissues participating in endometrial stromal-epithelial cell communication and thus mediate their hormonal action. [11-15]. Certain WNT like WNT4 are involved in development of female reproductive system, steroidogenesis and folliculogenesis. [16] Aberrant WNT signalling pathway have been found to be implicated in endometrial carcinomas. [17-19]

The sFRPs are thought to inhibit WNT signaling by binding to the WNT ligand or its frizzled receptor.20 sFRP4 also stimulated by estrogen, is involved in regulation of apoptosis and endometrial proliferation. [11,21] Although the function of sFRP4 in human endometrium has been studied, its role has not been satisfactorily investigated in endometriosis. [21] Absence of WNT signalling has been associated with its degradation by other components. [22] We hypothesize that sFRP4 as a WNT inhibitor might be associated with the pathogenesis of endometriosis. Our study aims at examining the serum levels of sFRP4 in patients of endometriosis and comparing it with the controls. Random blood samples irrespective of the stage of menstrual cycle and timing of the day were collected from patients and controls.

Normally, sFRP4 is secreted by the endometrium stromal cells. Hence it may be presumed that the ectopic endometrium would also secrete it. In endometriosis, there is increased peritoneal fluid volume in the Pouch of Douglas which could mirror the components secreted by the refluxed menstrual spill within the uterine endometrium as well as the ectopic endometrium. Our study aims at analysing the levels of sFRP4 in the blood and peritoneal fluid of symptomatically and laparoscopically diagnosed, and hisptopathologically (HPE) confirmed endometriosis patients.

Methods

A hospital-based cross-sectional, observational, non-interventional case control study was conducted over 18 months duration. Patients who were admitted for diagnostic and operative laparoscopy for suspected endometriosis were included in the study. Endometriosis was graded according to the revised American Society of Reproductive Medicine (rASRM). In this study, 21 patients with endometriosis in reproductive age group(18– 45 years) in different stages and 22 age matched healthy controls in reproductive age group(18– 45 years) were taken. Women with previous history of diabetes mellitus, cardiac disease, immunological disease or any malignancy in any part of the body were excluded from both cases & controls group.

sFRP4 was estimated by ELISA method by Cloud -Clone Corp (Catalog no-SEF878Hu).

Ethical clearance was obtained from the Institutional Ethics Committee (IEC

470/2014). Informed written consent was obtained from all subjects.

From cases and controls both, 5ml blood was collected and centrifuged at 2500 rpm for 5 minutes, serum was separated and stored at -80^{O} C until analysis.

Peritoneal fluid was collected in sterile syringe only from the cases during laparoscopy. It was centrifuged at 2500 rpm for 5 minutes, separated and stored at -80^{O} C until analysis.

This study measures concentration of sFRP4 in saline wash of Pouch of Douglas (POD) instead of pure peritoneal fluid sample. Therefore the exact concentrations is not reported. Peritoneal fluid was not collected from controls.

Statistical analysis

All statistical data were analyzed using IBM SPSS Statistics for Windows (Version 20.0). Independent t test, Spearman and Pearson's correlation, ANOVA was used wherever appropriate. A receiver operating characteristic (ROC) curve was plotted to assess diagnostic performance.

Results

Table 1 shows the sFRP4 levels in women with endometriosis and healthy controls without endometriosis. Women with endometriosis had significantly higher levels (almost two fold) of sFRP4 values in serum as compared to controls (8017.0 ± 2577.8 v/s 3718.9 ± 1650.8 pg/ml ; $p < 0.0001$). There was significant increase in sFRP4 levels in serum with increasing severity grading (rASRM; grade 2-4) of the disease ($p < 0.0001$). (See table 1)

Table 1: Comparison of sFRP4 levels in case and controls

	Cases (serum) total (n=22)	**Cases (PF) total (n=22)**	**Comparison between blood and PF levels (p-value, r value)**	**Controls(serum) Total(n=21)**	**Comparison between serum levels of case and controls (p-value)**
Age		-	-		
All	8017.0± 2577.8	6426.2± 3605.7	r = 0.743*, p < 0.001	3718.9 ± 1650.8	< 0.0001
Grade 2 (n=4)	4654.2± 1525.1	1963.7± 288.7	r = -0.34, p=0.65	-	
Grade 3 (n=14)	8166.0± 1688.4	6678.7± 2919.4	r = 0.369, p=0.19	-	
Grade 4 (n=3)	11805.3± 365.1	11198.0± 249.5	r=0.450, p=0.703	-	

sFRP4 levels in saline wash of POD were 6426.2 ± 3605.7 pg/ml . Levels of sFRP4 in saline wash of POD is lower as compared to serum levels (8017.0 ± 2577.8pg/ml in serum v/s 6426.2 ± 3605.7pg/ml in saline wash of POD; $p < 0.001$). A significant strong positive co-relation($r=0.743^*$, $p < 0.001$) was observed between sFRP4 levels in serum and saline wash of POD indicating that sFRP4 levels in saline wash of POD and serum increase simultaneously and in parallel with the severity of the disease. Hence, their increased levels in saline wash of POD and serum bear significant contribution in pathology of the disease (Pearson's correlation was used). Although saline wash of POD obtained was diluted, it reaches close to serum sFRP4 levels. sFRP4 levels being measured in diluted samples /saline wash of POD will have lower values than the pure peritoneal fluid sample. There was significant increase in sFRP4 levels with severity grading (rASRM) of the disease in saline wash of POD; p=0.006) (see table 1).

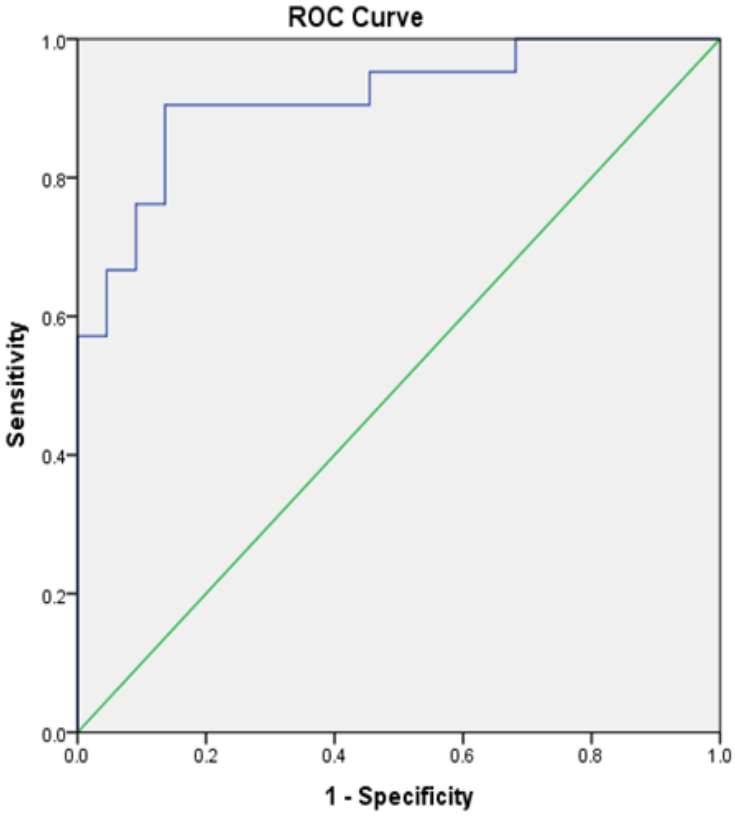

Figure 1: ROC curve for sFRP4

Area	Std. Error	Sensitivity	Specificity	Cut - off point	p-value
0.913	0.04	90	87	5134.5	< 0.0001

Figure 1 shows the ROC curve drawn between cases and controls sFRP4 levels. The ROC curve drawn gives the cut-off value of 5134.5 pg/ml, and sensitivity and specificity of 90 % and 87 % respectively. Area under the curve is 0.913.

Further analysis was done for interpreting the symptoms of endometriosis like dysmenorrhea and pelvic pain with serumsFRP4 levels. We found a significant increase in sFRP4 levels with successive grade severity of dysmenorrhoea (mild - 4654.2 $\pm$ 1525.1, moderate- 8210.8 $\pm$ 1772.3 and severe- 9480.2 $\pm$ 2332.8, p = 0.003). However, women with symptoms of presence of pelvic pain (n = 9) showed a non-significant decrease in sFRP4 levels in serum compared to those with absence of pelvic pain (n = 12) (6010.7 $\pm$2180.4 v/s 9521.6$\pm$1691.5, p = 0.42).

Table 2: Serum levels of sFRP4 with different laparoscopic finding

Laparoscopic finding	No.	Serum sFRP4 (Mean±SD) pg/ml	Serum sFRP4 (min-max) pg/ml
Endometriotic cyst	9	7395.4 ± 2270.1	3265 - 9672
Superficial Adhesions	4	6659.5 ±2054.5	5243 -9608
Kissing ovaries	2	6197.0 ± 3722.2	3565 - 8829
Adhesions to other pelvic organs	3	9649.6 ±1525.8	8706 - 11410
Obliterated POD	2	10843.5±1460.1	9811 - 11876
Frozen pelvis	1	12130.0	12130.0

Table 2. shows the levels of sFRP4 with various morphologic appearance of endometriosis while laparoscopy (Endometriotic cyst, superficial adhesions, kissing ovaries, adhesions to other pelvic organs, obliterated POD, frozen pelvis). Serum sFRP4 mean levels were found to be highest in the frozen pelvis, followed by obliterated POD, deep adhesions to pelvic organs, endometrial cyst, superficial adhesions and kissing ovaries

Discussion

In the present study, we demonstrated that serum sFRP4 levels of cases were significantly elevated when compared to controls (mean 8017 pg/ml in cases v/s 3718.9 pg/ml in controls, $p<0.001$). Additionally, circulating levels of sFRP4 were also significantly raised in cases within grade 3 and grade 4 when compared with that of controls ($p <0.0001$, $p<0.0001$ respectively). sFRP4 in saline wash of POD was raised even in patients with peritoneal wash samples despite being diluted. Total serum sFRP4 levels were positively correlated with saline wash of POD($r=0.743$, $p<0.0001$).

sFRP4 expression has been shown to peak during the proliferative (or estrogen dominated) phase of the menstrual cycle. Quantitative reverse transcription-PCR (RT-PCR) studies done in a study found that in postmenopausal women treated with estrogen for 3months sFRP4 expression in the endometrium increased 9-fold [11]. Downregulation of sFRP4 and associated promoter hypermethylation have been reported in several other cancers. [11]

Lin Jet al in their study determined the linkage and possible variants in those regions of putative associated genes(INHBA, SFRP4 and HOXA10), with endometriosis and reported two novel variants in sFRP4 and one in HOXA10. [21]

sFRP4 is known to promote apoptosis in various human tissues and thus help maintain homeostasis. sFRP4 can also induce cell death in cancer cells. Several studies have shown sFRP4 as a local initiator of apoptosis and that, tumour cells survive by shutting down the expression of sFRP4. The progressive loss of SFRP4 observed from normal tissue to aggressive pattern of tumour has placed this molecule as potential biomarker with prognostic implications.

Saran et al. in their experiment on 4 ovarian cell lines using Western blotting and IHC demonstrated that the chemoresistant cell line expressed lower levels of sFRP4 protein compared to chemosensitive cell lines. [23] This differential expression of sFRP4proteinobservedin chemosensitive and chemoresistant cell lines indicated that sFRP4 has prognostic implication in cancer. Cisplatin was known to selectively target sFRP4 expressing cells and following continuous treatment with cisplatin leading to depletion of sub-population of cells expressing sFRP4 gradually over the time. [23] The remaining population of cells would not express sFRP4 sufficiently and would be more resistant to be killed by chemotherapeutic drugs. The acquired resistance to apoptosis is thus presumably due to loss of sFRP4

expression and is associated with occurrence/development of more malignant and chemoresistant subtype. Here, the treatment itself, is contributing or selecting the tumour to become chemoresistant. The results of our study can also be explained on similar principle. The refluxed endometrium in the peritoneal cavity during endometriosis may require more apoptotic phenomena to occur to clear the peritoneal cavity of useless menstrual debris. So findings of our study that increased expression ofsFRP4 in the serum and peritoneal fluid of cases as compared to controls points to the increased need of apoptotic removal of menstrual debris in cases and this need is facilitated by increased expression of sFRP4 on the tissues. Again, with increase in severity of the disease or increased involvement/reflux of menstrual in the peritoneum, there is even increased need for its apoptotic removal and hence increased expression of this sFRP4 protein. This principle complies with the results of our study that there is proportionate increase in sFRP4 with increasing severity of the disease.

However, based on the above understanding of role of sFRP4 in apoptosis in carcinogenesis, one can speculate that if the decrease in apoptosis facilitates survival and proliferation of cancer tissue, then it is expected to find similar trend in endometriosis (i.e. decreased sFRP4 correlating with presence of disease and higher grade). But the results of our study surprisingly, comes to be completely opposite. i.e. we found increase in sFRP4 with endometriosis subjects as compared to controls. This can be explained/ speculated as follows. The tumour/carcinoma subjects studied in various other studies would initially employ various apoptotic mechanisms including sFRP4 expression for clearance of tumour cells. But tumour cells possessing pervasive uncontrolled proliferative nature being later by suppression of sFRP4 expression. However, endometriosis is not a tumour and uncontrolled growth of endometrial fragments does not take place. So, it may be associated with defensive mechanism by apoptotic removal of excess menstrual debris by increasingsFRP4 expression.

Again, however, increased apoptosis may not always correlate with increased sFRP4 expression, mostly due to the complex nature of interactions between different signalling pathways. Increased apoptosis of Fas-bearing immune cells can result in endometriosis. Increase in sFRP4 may be associated with apoptosis of Fas-bearing immune cells rather than apoptosis of endometrial cells. Or, the inhibitory effect of endometrial cell proliferation bysFRP4 in the endometriosis patients may be damaged. This might be involved in the pathogenesis and development of endometriosis. In this regard, increased expression in sFRP4 could signify a compensatory increase to counter-regulate aberrant WNT signalling, in addition to sPRP4 enhanced expression due to increased estradiol in endometriosis. Although the full mechanisms behind this process are not fully understood, these results suggest a link between endometriosis and increased sFRP4 levels.

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