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Serum Soluble Fas (sFas) levels might be a discriminator between precancerous and Cancerous Cervical lesion in North Indian Population- A case control study

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***Abstract*- Background:** One of the best characterized apoptosis triggering systems is the CD95/Fas/APO-1 pathway. Aim of the study was to evaluate the serum sFas levels in cervical neoplasia and localized cervical cancer patients and examines its role as a discriminator between precancerous and cancerous cervical lesion.

Methods: A case control study was conducted in Department of Obstetrics & Gynaecology, in collaboration with Pathology, CSM Medical University, Lucknow. 5ml venous blood samples were drawn into sterile vials, prior to treatment being administered. sFas levels were measured with an enzyme linked immunosorbent assay in histopathological proven 45 cases of Cervical Intraepithelial Neoplasia (CIN I, II and III), 30 cervical cancer patients (FIGO Stage I and II) and 30 cervical cytology negative healthy volunteers (Pap Smear).

Results: In the present study we observed a significant positive correlation between sFas levels of healthy controls, CIN I, CIN II, CIN III and early invasive localized cancer patients (stage I and II) ($p<0.001$). It was observed that the three stages of precancer, and stage I and II of cervical cancer although had small difference in their mean sFas levels yet, can be precisely discriminated on the basis of mean sFas level alone. Risk classification matrix obtained using the discriminate function analysis, showed that sFas levels in plasma of patients with CIN I, II and III could helped in better predicting the risk of developing invasive cervical cancer in individual patient. sFas level was able to validate about 98% of cases correctly.

Conclusions: This study demonstrates that sFas levels could be used as a discriminator between precancerous and cancerous cervical lesion. The clear discrimination between precancerous and cancerous cervical lesions by sFas can be used in quantification of the risk to have a better understanding of natural history of disease.

***Index Terms*-** sFas, CIN, Cervical Cancer

I. INTRODUCTION

This study was necessary because it is of clinical use, it helps to understand the natural history of disease (from CIN →malignancy). Serum soluble Fas levels could be used as discriminator between precancerous and cancerous cervical lesions.

The aim of our study was to evaluate the serum sFas levels in Cervical Intraepithelial Neoplasia patients and localized cervical cancer patients using Enzyme Linked Immunosorbent Assay technique and examine its role as a discriminator between precancerous and cancerous cervical lesion.

It is the second most common cancer among women worldwide. Approximately 500,000 women worldwide develop cervical cancer each year.[1]

Human papillomavirus is the single most important etiologic event in the pathogenesis of cervical carcinoma is a well known fact. However, not all women who are infected with the high risk HPV (HPV subtypes 16 and 18) develop cervical carcinoma. It often takes years or decades before persistent dysplasia eventually develops into frank malignancy. This, suggest that other cofactors must be present in the pathogenic pathway between Cervical Intraepithelial Neoplasia and carcinoma.

The fact, that spontaneous regression frequently occurs in CIN type I or II lesion but a part of CIN III progress to invasive carcinoma suggests that selection of cell population with high malignant potential may be closely linked to progression of cervical neoplasia. Apoptosis is an important regulatory mechanism that counteracts uncontrolled growth of malignant cells. In fact, premalignant cervical lesions exhibit reduced apoptotic indices of undifferentiated basaloid cells compared with normal cervical cells[2], which correlates strongly with the probability of progression to squamous cell carcinomas. Fas/FasL pathway, a member of the tumor necrosis factor receptor superfamily, is the best characterized death receptor pathway inducing apoptosis in normal and malignant cells [3].

Fas is a type I transmembrane protein which upon triggering by Fas ligand, induces apoptosis through a series of interacting proteins. Fas is characterized by a highly conserved death domain that is responsible for activating the death signal upon activation by Fas ligand[4]. A multimolecular complex of proteins called the death- inducing signaling complex is triggered by receptor ligand cross linking[5]. The apoptotic signaling is propagated by procaspase-8 and further by active caspase-8 and caspase-3[6,7]. Caspase-mediated proteolysis of specific protein targets is central to the execution of apoptosis.

Fas can be present both as a cell surface protein and as a soluble protein called soluble Fas (sFas). sFas is a splice variant generated by alternative mRNA splicing and lacks a transmembrane domain[8]. Papoff *et al* [9] have reported that sFas suppresses Fas-mediated apoptosis by competitive binding with FasL. Increased sFas levels in colorectal cancer, bladder, breast, renal cell carcinoma and melanoma have been documented[10-14]. sFas has been investigated as a prognostic marker in gynecological malignancies by some workers who have correlated it with more invasive, advanced stage tumors [15,16]. Considering the need of present era and long natural history of cervical neoplasia, where women with cervical intraepithelial neoplasia grade 1 (CIN-I) are normally maintained under observation, since almost 60% of these cases revert spontaneously[17]. It is essential to have a biomarker which could predict the propensity of conversion of the precancerous cervical lesion to cancerous cervical lesion. Screening through Pap smear followed by the colposcopy of the women with abnormal pap smear and women with unhealthy cervix, and finally the confirmation of the nature of cervical lesion with cervical biopsy (gold standard), could diagnose the preinvasive, microscopic and macroscopic stages of cervical cancer. But none of these methods could predict the natural history of the disease process. So there is a need for a simple and low cost complementary prognostic marker which could assist in determining or predicting the risk of developing cervical cancer.

Involvement of sFas as a discriminator between precancerous and cancerous cervical lesion has not been documented as yet. We hypothesized that the levels of serum sFas in cervical neoplasia may be used as a prognostic marker. This marker could assist in predicting the risk of developing cervical cancer and may add useful information in the natural history of cervical cancer along with the screening techniques, colposcopy and biopsy and thus taking the treatment decision and follow up.

II. METHODS

A case control study conducted in Department of Obstetrics & Gynaecology and in collaboration with Pathology, CSMMU, Lucknow. Venous blood samples of all patients were drawn into sterile vials after detection of Cervical Intraepithelial Neoplasia and Cervical cancer, prior to treatments being administered. Blood samples were kept at 4 degree centigrade, centrifuged at 6000 rpm for 15 min, and then immediately frozen at -20 degree centigrade until assayed.

The study group consisted of 45 women with colposcopic and histopathologic proven diagnosis of CIN (Cervical Intraepithelial Neoplasia), 30 women with clinical and histopathological proven diagnosis of the early invasive cancer of uterine cervix (FIGO stage I and II) All women (N=75) in the present study were diagnosed and treated at Department of Obstetrics and Gynecology, CSMMU, Lucknow. The control group comprised of 30 healthy female volunteers who were cervical cytology negative. All the subjects in this study were enrolled after taking an informed consent. The age of cancer patients, CIN patients and control group ranged from 35 to 68, 26 to 70 and 26 to 65 years, respectively. CIN patients were classified into grade I-mild dysplasia, grade II-moderate dysplasia and grade III-severe dysplasia. CIN I was diagnosed in 15 women, CIN II in 15 women and CIN III in 15 women. Patients with deranged liver function test were excluded from the study. It is important to mention that patients included in this study did not receive any prior treatment (chemotherapy, radiotherapy, and surgery). The ethical clearance for this study was given by Institutional Ethics Committee, Office of the Research Cell, CSMMU (erstwhile King George's Medical University), Lucknow, U.P. (*Ref. Code: XXX IX ECM/B-P9* dated 24/10/09). The socio-demographic profile of patients in the study has been shown in Table 1.

Table 1: Distribution of cases in different clinical stages of precancerous and cancerous cervical lesions

Variable	Control	CIN	Stage 1 & 2
Age			
20 – 34 yrs.	10%	16.66%	3.45%
34 – 45 yrs.	50%	46.67%	44.83%
≥ 46 yrs.	40%	36.67%	51.72%
Religion			
Hindu	80%	96.67%	100.0%
Muslim	20%	3.33%	–
Locality			
Urban	60%	63.33%	31.03%
Rural	40%	36.67%	68.97%
Education			
Literate	56.67%	56.67%	31.03%
Illiterate	43.33%	43.33%	68.97%
SE Class			

High	–	13.33%	6.90%
Middle	56.67%	63.33%	41.38%
Low	43.33%	23.34%	51.72%
Diet			
Veg	50%	60%	72.41%
Non – Veg.	50%	40%	27.59%
Tobacco Addiction			
Non-addicted	80%	63.33%	37.93%
Tabacco addicted	20%	36.67%	62.03%
Parity			
P0 – 2	33.33%	20%	10.34%
P3 – 6	56.67%	70%	75.86%
P≥ 7	10%	10%	13.80%
Abortion			
None	50%	56.67%	96.55%
≥ 1	50%	43.33%	3.45%
Contraception Stage			
Non User	50%	40%	82.76%
T.L.	40%	50%	10.34%
Temporary Methods	10%	10%	6.90%
BMI			
≤ 18	16.67%	20%	37.93%
18.1 – 24.9	70%	70%	48.28%
≥ 25	13.33%	10%	13.79%

III. Intervention (Fas specific ELISA)

The level of sFas in the serum was determined with a double antibody sandwich Enzyme Linked Immunosorbent Assay kit for quantitative detection of human sFas (Bender Med System, Vienna, Austria) according the producer's protocol. Briefly, the samples were measured and standards were incubated in wells coated with anti-Fas polyclonal antibody. After washing, a peroxidase-conjugated anti-Fas monoclonal antibody was added to each microwell and incubated. After another washing, the peroxidase substrate was mixed with the chromogen and allowed to incubate for an additional period of time. An acid solution is then added to each well to terminate the enzyme reaction and to stabilize the developed colour. The absorbance of each well is then to be measured at 450nm using a microplate reader.

Summary of sFas levels in different clinical stages of precancerous and cancerous cervical lesions is given as Mean±SD. The mean of different clinical stages were compared by one way analysis of variance followed by Neuman-Keuls test for individual comparison. Level of significance used is 0.05 for testing the null hypothesis. The discriminatory study of the precancer and cancer cervical stages on the basis of sFas levels was done by linear discriminate functional analysis. The correct classification of cases has been shown in risk matrix (Table 3).

IV. Results

The mean value of sFas was in increasing order of risk in all the clinical stages of precancer and cancer cervical lesions (Figure 1, Table 2). The mean sFas levels of different clinical stages were tested by one way analysis of variance followed by Neuman Keuls test for individual comparisons. The F value in the analysis was 177.0, which was significantly higher ($p<0.001$) than the characteristic $F_{4,100,5\%}=2.46$. This signifies that the mean concentration of sFas was significantly different between two clinical stages.

The significance of individual comparison between each pair of clinical stages has been shown in Table 3. It can be seen that each pair of clinical stages differed significantly with the probability of $p<0.001$. The significance of clinical stages at such a high level indicate that the precancer and cancer clinical stages were distinct and the mean sFas level were quiet apart at each clinical stage. It signifies that the mean sFas level increases monotonically in each stage of precancerous and cancerous

cervical lesions. In other words Enzyme Linked Immunosorbent Assay test for sFas precisely defined the stages of precancerous and localized cervical cancerous lesions.

On using the discriminate function analysis for the clinical stages we obtained the risk classification matrix (Table 4), which shows the correct classified cases in their own stages as well as misclassified cases into other clinical stages. The risk classification matrix shows the 100% correctly classified cases on the basis of sFas levels was observed in control and CIN I cases. A deviation in the percent correct classification was found in the rest of the clinical stages (i.e., CIN II, CIN III and Stage I & II). Three cases of CIN II have been classified as CIN I on the basis of sFas level, while one case of CIN II has been classified as CIN III on the basis of sFas levels. Six cases of CIN III have been classified as Stage I & II on the basis of sFas levels whereas eight cases of Stage I & II have been classified as CIN III. These misclassified cases have propensity either to regress or to progress. It is interesting to observe that the Enzyme Linked Immunosorbent Assay method for measuring sFas level is fairly sensitive in discriminating the 3 stages of precancer and stage I & II of cancer. These stages though have small difference in their mean sFas levels, yet they can be precisely discriminated on the basis of mean sFas level alone.

To validate the study 20 patients were diagnosed and evaluated clinically/pathologically. The distribution of diagnosis through DFA has been given in Table 5. The present study revealed that sFas is a potential marker for diagnosing the clinical stages of precancerous and localized cervical cancerous lesions. sFas concentration was able to validate about 98% of cases correctly.

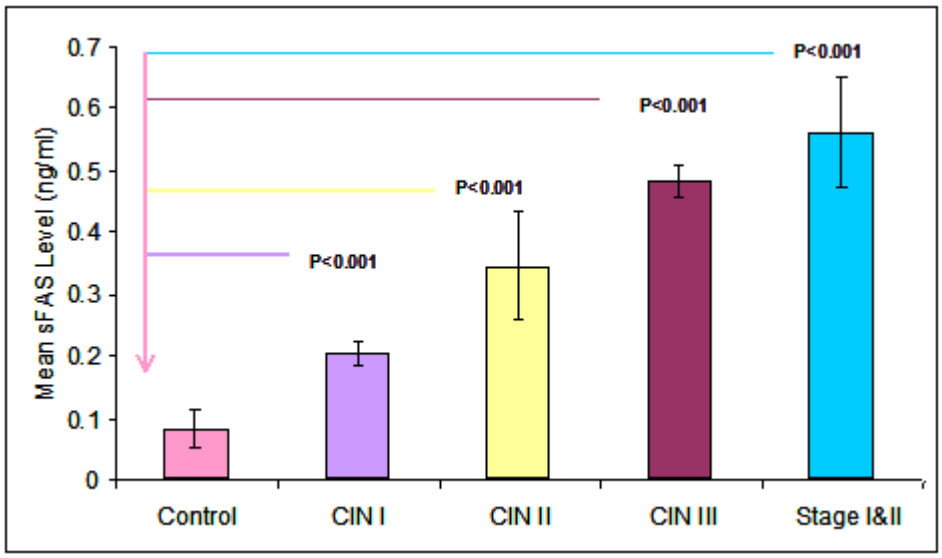

Figure 1: Mean sFAS level in different clinical stages of precancerous and cancerous cervical lesions: the mean sFas level here shows a positively increasing trend with the clinical severity of precancerous stages (CIN I, CIN II, CIN III) and cancerous stages I & II. The difference in mean sFas levels at each stage is statistically significant ($p<0.001$)

Table 2: sFAS Levels in Different Clinical Stages

	Control	CIN I	CIN II	CIN III	Stage I & II
n	30	15	15	15	30
sFas (Mean ±SD)	0.082 ± 0.031	0.204 ± 0.02	0.345 ± 0.088	0.482 ± 0.027	0.56 ± 0.088
95% CI range (ng/ml)	0.0712 – 0.094	0.193 – 0.215	0.297 – 0.394	0.467 – 0.497	0.527 – 0.592

Table 3: The significance of Individual comparison between each pair of clinical stages

Control vs CIN I	P<0.001
Control vs CIN II	P<0.001
Control vs CIN III	P<0.001
Control vs Stage I & II	P<0.001
CIN I vs CIN II	P<0.001
CIN I vs CIN III	P<0.001
CIN I vs Stage I&II	P<0.001
CIN II vs CIN III	P<0.001
CIN II vs Stage I & II	P<0.001
CIN III vs Stage I& II	P<0.001

Table 4: Risk matrix of classification of patients of different clinical stages as obtained from Discriminant Function Analysis (DFA)

	Control	CIN I	CIN II	CIN III	Stage I & II	%age
Control	**30**	**0**	**0**	**0**	**0**	**100.0**
CIN I	**0**	**15**	**0**	**0**	**0**	**100.0**
CIN II	**0**	**3**	**10**	**01**	**1**	**66.7**
CIN III	**0**	**0**	**0**	**9**	**6**	**60.0**
Stage I & II	**0**	**0**	**0**	**8**	**22**	**73.3**

Table 5: Distribution of 20 patients diagnosed and evaluated clinically and pathologically on the basis of sFas level

		Clinically			
		CIN I	CIN II	CIN III	Stage I & II
sFAS	**CIN I**	**1**			
	CIN II		**1**		
	CIN III			**5**	**1**
	Stage I&II			**1**	**11**

V. DISCUSSION

The natural history of cervical cancer is characterized by stepwise progression from a histologically normal cervix to cervical intraepithelial neoplasia to frank invasive cancer[18]. In the past considerable attention has been devoted to determine the potential of CIN lesions to progress, and thus varying recommendation have been made for intervention or observation based on differing interpretation of these data. It was estimated that 60% of CIN I lesion regress, 10% progress to CIN III and just 1% progress to frank malignancy [19]. Some studies estimated the risk of progression of untreated CIN II to CIS at more than 90% after 14 years of follow up [20]. Conversely, a meta-analysis of 15 studies spanning almost 35 years yielded an estimate of progression for CIN II of 20%, with 5% progressing to invasive cancer, 40% regressing and 40% persisting unchanged [19]. Even

after so many years of exhaustive research although it has been proved that CIN I can progress to invasive cancer and even higher grade precancerous lesions sometimes may regress. But, even after establishing the diagnosis of CIN I, CIN II or CIN III, it is still a million dollar question that which of these lesions are going to progress and which will regress.

Owing to the existence of premalignant condition and long latent period in which premalignant lesion or occult cancer can be detected, makes the cervical cancer as the only gynaecological cancer that satisfies the well recognized WHO criteria for screening. The screening for cervical cancer and its precursor lesions currently employs the Pap smear, but this test is subjective and has relatively low sensitivity. The combination of the Pap test with HPV molecular detection achieves significant improvements in sensitivity for detection of cervical cancer, but the last technique is not routinely employed due to methodological and economical reasons. Alternatively, the use of p16INK4a has been proposed as a marker for progression [21,22]; however, disadvantages of this method are that it is mainly confined to biopsies and it is also subjective depending on the pathologist's experience. Similarly colposcopy and cervical biopsy (gold standard) although can establish the diagnosis at given point of time but cannot predict the course of that lesion in future.

Considering the role of apoptosis resistance in tumor progression and considering the need of present era, the objective of present study was to design a simple, low cost, easy and feasible to perform especially in low resource states and a minimally invasive test which may serve to predict more reliably, the risk of developing cervical cancer. Based on sFas levels found in serum we were able to reach the goal of identifying a prognostic marker for precancerous cervical lesions. In the present study, we observed a significant positive correlation between sFas levels of healthy controls, CIN I, CIN II, CIN III and early invasive localized cancer (stage I & II) patients. Data obtained from 45 CIN patients showed that sFas concentration in plasma of women with CIN I, CIN II and CIN III could help in better predicting the progressive and regressive nature of cervical lesions and the risk of developing invasive cervical cancer in individual patient. The clear discrimination between precancerous and cancerous cervical lesions by sFas can be easily used in quantification of the risk, to have a better understanding of the natural history of disease.

Our study is in accordance to the study of Lemarroy et al[23] where they also found the same positive correlation of sFas in control, CIN and cervical carcinoma patients. In addition they demonstrated an inverse relation between the induction of apoptosis in jurkat cells and the sFas levels and the preinvasive and invasive stage of cervical neoplasia. Hence, they concluded that sFas levels could be an important prognostic tool for cervical cancer. However, sFas concentration in the present study is lower than that of study by Lemarroy *et al.,* amongst various study groups. This difference could possibly be explained based on the differences in ethnicity and dietary habits. sFas concentration is lower among the subjects of Indian origin.

We have found that sFas levels were elevated in the serum of women with cervical cancer as compared to healthy controls. Our data is in agreement with other studies, where elevated levels of sFas in serum of women have been observed in bladder cancer[11], breast cancer[12], renal cell carcinoma[13], head & neck carcinoma[24], locally advanced unresectable rectal cancer[25] and also in autoimmune rheumatic disease[26]. All these studies have reported much higher level of sFas (greater than 1 upto 60ng/ml), while our samples showed that the level of sFas in pre-cancer and cancer stages of cervix was in the range of 0.15-0.7ng/ml. They all formed distinct group with pre-cancer and cancer stages with small deviation in values. Concerning gynecological tumors, Konno et al., examined the relevance of sFas levels as a prognostic factor in patients with gynecological malignancies in terms of the survival rate; they observed that patients diagnosed with cervical carcinoma have a better survival rate when they have serum sFas levels lower than 1.5ng/ml before therapy than those with a level higher than 1.5ng/ml[15].

Looking at the sum of our data, we conclude that sFas concentration present in the serum of women could be an important discriminator for cervical neoplasia. However, a follow up study must be made to confirm whether, the natural history or course of cervical lesions found in women diagnosed with CIN I, CIN II and CIN III, was according to our prediction based on sFas concentration or not. Additional studies are also required to identify the mechanism of sFas induction in invasive cervical cancer.

VI. CONCLUSION

It can be concluded that sFas can be used as a discriminator to differentiate between healthy controls, precancerous and cancerous (Stage I & II) cervical lesions. Combining of sFas measurement with Pap smear, colposcopy followed by biopsy will help in better predicting the natural history of cervical neoplasia especially in preinvasive and early invasive stages of cervical cancer.

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