Relation between IFN-γ, STAT3 and pSTAT3 gene expression in different grader of cervical cancer

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Abstract

Objective

Analyzing the serum and expression of IFN-γ, STAT3 and pSTAT3 in different grades of cervical cancer.

Method

Totally 116 cases of Precancer, cancer, , and control samples were collected from 2007 to 2012, all cases undergone PAP (papanicolaou stain) test for general screening purpose, circulating serum and punch was collected in all patients for Elisa, HPV(Human Papilloma Virus) DNA analysis using PCR, expression of IFN-γ(Interferone-γ) by RT-PCR and STAT3(Signal Transducer and Activator of Transcription), pSTAT3 were analyzed in cancer, precancer compare with the control. Baes on histopathology and PCR results, patients were categorized.

Result

We observe that elevated level of circulating IFN-γ levels were seen ($p<0.001$) (0.233 ± 0.28 pg/ml) (0.380 ± 0.367 pg/ml) and (0.034±0.020pg/ml), whereas IFN- γ mRNA ($p=0.01$) CINI4/14, 28.5%. CINII13/18,72.2%.CINIII 11/25, 44.00%, cervical cancer 23/43, 53.48% and control is 2/16, 18.75%, Farther we analyze the STAT3 and pSTAT3 gene expression ($p=0.001$), (4/14, 28.57%.11/18,61.1%.17/25,68.0, 12/43, 27.9% and 2/43, 12.5%) and ($p<0.001$) 42.%,13/18,72.2%.20/25,80.0%,36/43,83.7% and 2/43, 12.5%) interestingly we found that, statistically significant association between precancer, cancer as compare to control.

Conclusion

This result suggest that, IFN-γ and STAT3 gene is strong predictor of different grade of cervical cancer prognosis,

Key word; HPV, IFN-γ, STAT3 and pSTAT3 in cervical cancer patients.

Introduction

Cervical cancer is fourth most common cancer affecting women in worldwide as compare to the breast cancer, colorectal and lung cancer (Bleotu *et al.*, 2013, Concha-Benavente *et al.*, 2016), past one Decca it very much understood that cervical cancer is associated with Human papilloma virus(HPV), however the mechanism of virus is not clear. Accumulating data suggest that, inflammation of HPV is associated with suppression of cellular immunity(Song *et al.*, 2015), through tumor specific CD^+ cells is consistently associated with elevated level of Th1 and Th2 cytokines (Bleotu *et al.*, 2013, Scott *et al.*, 2013), associated with STAT1 signaling pathway(Gough *et al.*, 2010), these gene were regulated by the epidermal growth factor receptor(EGFR) and Receptor tyrosine-protein kinase (erbB-2) receptors were activate the Signal transducer and activator of transcription (STAT1 and STAT3) (Qi *et al.*, 2013). Whereas The IFN-γ is multifunction cytokine secreted by T cell and NK cells under inflammatory condition such as HPV (Kim *et al.*, 2014), IFN-γ acts as two face of a single coin, antitumor and cell survival activity(Zaidi & Merlino, 2011), it may be the cross link between the IL-6 and IFN-γ pathway, but exact mechanism of cancer prognosis is not clear. In addition evidence suggested that STAT3 is transcription factor of the STAT family, activated by tyrosine phosphorylation at carboxyl-terminus (Y705) and serine phosphorylation at transactivation domain(S272)(*STAT3 signaling.pdf*), phosphorylation of a tyrosine residue Tyrosine 705 (Tyr705) in response to IL-6 and EGFR receptor (Wang *et al.*, 2013) and regulation by micro-RNA in pathophysiological condition(Haghikia *et al.*, 2012), therefore for the identification of Phosphorylation of STAT3 in cervical cancer patients indicate favorable prognosis. Whereas the resent data suggest that, polymorphic site changes IFN-γ production in different grade of cervical cancer that influence the HPV malignant progression (do Carmo Vasconcelos de Carvalho *et al.*, 2012). Continuous activation leads to pathological aspect. Therefore the analysis of IFN-γ is most powerful cytokines as compare to IFN-α(Grenz *et al.*, 2013). There is a considerable literature showing that, elevated levels of IFN-γ were associated with Oral cancer(Grenz *et al.*, 2013) ovary cancer(Chen *et al.*, 2013) breast cancer(Ning *et al.*, 2010), in similar manner over expression of STAT3 were seen in cervical cancer(Shukla *et al.*, 2013), The aim of this study is HPV inflammation mediate the activation of IFN-γ through the phosphorylation of STAT3 in different stage of cervical cancer along with control.

Materials and methods:-

Clinical sample preparation

Case control study involved collection of tissue and blood sample form 100 freshly diagnosed different grade of cervical punch tissue biopsy were dived into three portions one portion is collected Trizol reagent from sigma (Cat#T-9424, Sigma Aldrich), for mRNA analysis, immediately store -70^{o}C until analysis carry out, other portion is used for histology analysis and thread portion is used for the DNA HPV analysis. Simultaneously 5 ml peripheral blood sample were collected all patient, and serum is separated and stored in -20^{o}C until analysis carry out, along with 16 healthy control sample ware collected in Deportment of Gyneoncolgy Dharmshila Cancer Hospital New Delhi during 2007 to 2012, in collaboration with institute of cytology and preventive oncology (ICMR) Noida. All patient were undergone PAP smear screening from 2012 to 2014. The clinical and personal history were collected from standard questionnaires by undertaken on the patient who attending hospital.

PCR Analysis for HPV typing

All tissue biopsy were under gone for the DAN was isolation by phenol chloroform method (Shukla *et al.*, 2010). The PCR for detection of DNA of HPV types 16 and 18 were carried out using type-specific primers (table 1). PCR was performed in a total 25µl containing 2.5 µl 10XPCR buffer, 5 µl dNTPs mix .0.75 µl of magnesium chloride (50mmol/L), 0.5 µl of each primer, 2.5 units of Taq DNA polymerase and 200ng of DNA. The PCR condition were 95°C for 3 min and then,35cylcles of 94°C for 45 s, 60°C

for 20 s and 72°C for 45 s with final extension at 72°Cfor 5 min. the reaction were performed in to a thermocyler (Eppendorf, Germany).

Quantitative PCR (RT-PCR) analysis

Total mRNA Extraction

Total mRNA was extracted from 100 cases of different grade of cervical cancer along with 16 control by punch biopsy using TRIZOLE reagent following by the manufacture instruction and checked the integrity of mRNA by spectrophotometry at 260 nm, Integrity was confirmed by visualization of 28S and 18S bands on 1% agarose gel and then subjected to RT-PCR for detection of IFN-γ mRNA expression. Each transcript which is common housekeeping gene (GAPDH) and acts as internal control. RT-PCR were performed using 0.5-1µg of total RNA sample assessing RT-PCR system cycles performed in Eppendorf thermocycler . IFN-γ gene expressions were initially denatured at 94 °C for 4 min, annealed at 55 °C for 30 sec followed by extension at 72 °C for 30 sec, cycles were repeated upto 35 cycles. 10-15 µl of amplified product was run on 2% agarose gel (Fig-2). The primers used for IFN-γ, MY09, HPV16, 18, GAPDH and β-actin (Table 1) were purchased from Sigma-Aldrich, USA. All primers were checked against the available Genbank Database accessions to ensure the no cross reactivity with other human sequences.

Immunohistochemistry.

Respective tissue biopsy section were stained with haematoxyline and eosin for histology conformation of different grade of cervical cancer, same section were carry out in Immunohistochemistry(IHC) analysis was performed to evaluate the expression of STAT3 and pSTAT3. Five micrometer section where undergone antigen retrieval method and dilution. 1:100 of primary monoclonal antibody against STAT3 and pSTAT3 oncoprotein (Santa Cruz Biotechnology), The sections were incubated with primary antibody in a humid chamber at room temperature for 60 min, the slides were washed three times in phosphate buffered solution (PH 7.4) and further incubated with a biotinylated secondary antibody for 30 min at room temperature, antigen antibody complex were detected by substrate 3 3' diaminobenzidine hydrochloride (0.1%) from Sigma in a freshly prepared solution of PBS with (0.05% hydrogen peroxide). The slides were counterstained with haematoxylin and then examined by light microscopy (Fig-3).

Cytokine (IFN-γ) Quantification by ELISA

A total 43 patients with cervical carcinoma, 57 patients with cervical intraepithelial neoplasia (clasification) (CIN I), moderate dysplasia (CIN II) and severe dysplasia (CIN III)) and 16 healthy control for the cytokine quantitation. IFN-γ levels were estimated by enzymatic method, were measured in duplicate at 450-570 nm by standard coupled enzymatic procedure, according to the manufacturer's instruction (Biolegend San Diego USA and e-bioscience San Diego USA) by using Gene-5 ELISA reader. The cytokines conentration were estimated from standerd curves (Fig-1).

Statistical Analysis

The data analysis were performed using SPSS software 14.0 for windows (SPSS INC, Chicago, IL). The analysis of variance (ANOVA) was applied for comparisons between mean age of cancer pre-cancer and normal. Chi square and Fisher Exact test was employed to see the association among different histopathological grades of tissue biopsies. Spearman's correlation was carried out to look into the relationship between molecular markers. $p<0.05$ was considered as statistically significant.

RESULT:-

Expression of IFN-γ in HPV inflammation

Out of 116, 74 cases were infected with HR-HPV 16(85.5%) and 17 cases HPV 18 (19.72%), the mean age of ± SD pre-cancer, cancer and control, 44.94 ± 9.96, 55.86 ± 9.61 and 40.87 ± 11.03 years respectively (table1) , The differences in the mean age amongst the groups were found to be statistically significant (p<0.001). high risk and low risk of HPV16/18 DNA analysed according to histology diagnoses. Our data is reported that HPV increased according to age in epthelial lision in different grads of pre-cancer were 06 out of 14 CINI, 12 out of 18CINII, 19 out of 25 CINIII and 37 out of 43 in CaCx lesion and control 16. the most prevalancetype observed in CIN and SCC groups. 42.9%, 66.7%, 66.7%, and 86.0% ,(p<0.001), followed by HPV18 0%, 0%, 24% and 25.6% (p=0.007) were found respectivly. Forther statistically copairsum with pre-cancer with caner HPV16 64.9% and 86.0% ware p=0.017 similarly HPV18 10.5% and 25.6% ware p=0.047. resent data suggest that, cerculating quantitative analysis of plasm IFN- γ is key role of cervical cancer prognosis (Hu *et al.*, 2015), interestingly we found that, it was observed that, the mean IFN- γ levels of pre cancer patients (0.233 ± 0.28 pg/ml) were observed to be higher as compare to cervical cancer (0.380 ± 0.367 pg/ml) or control subject (0.034±0.020pg/ml). The difference in the mean value were found statistically significant (p<0.001). increasing the concentration of precancerous stage as compare to cancer (CINI 13/14, 92.85%, CIN II11/18,61.11%,CIN III 14/25,56.00% and cancer 17/43, 39.5%), result suggest that statistically significant association between different cancer state (p=0.04) as compare to control (2/16, 12.5%), the slandered value ELISA of IFN- γ 0.07pg/ml. Also, the present study was carried out to evaluate the tissue expression levels epithelium and sub epithelial zone of IFN- γ mRNA in patient in different grade of cervical carcinoma and their comparison with healthy controls. We found that, statistical significant association between the different grades of cervical cancer that, increasing mRNA levels were seen in precancerous stage as compare to cancer and controls (CINI 4/14, 28.5%. CINII13/18,72.2%.CINIII 11/25, 44.00%, cervical cancer 23/43, 53.48% and control is 2/16, 18.75%) were seen. Further in the same tissue section we analyzed the expression levels of STAT3 and pSTAT3. We found that,The increasing the expression levels of STAT3 were seen in the only in precancerous patients compare to cervical cancer and control (4/14,28.57%.11/18,61.1%.17/25,68.0, 12/43,27.9% and 2/43,12.5%). Whereas in the pSTAT3 there is decreasing expression level were seen in the precancerous state as compare to cervical cancer and control (6/14,42.%,13/18,72.2%.20/25,80.0%,36/43,83.7% and 2/43,12.5%) , there is statistically significant association between the precancerous and cancer patients STAT3 (p=0.001) and pSTAT3 (p<0.001)

Discursion

Immune mechanisms plays important role in pathogenesis of development of cervical cancer and its sequel. Very five studies shows that, evaluating role of immune progression of IFN- γ and STAT3 gene expression in high risk HPVs, that may secrete the large amount of IFN-γ (Grabowska *et al.*, 2015). Whereas the stimulation of IFN-γ activate JAK- STAT signaling pathway (Fasler-Kan *et al.*, 2013), resulting mainly from the effect of antigen presenting cell (APC) will increasing the expression of MHC1 molecule (Martini *et al.*, 2010). It may leads to the increasing percentage of nuclear expression of STAT3 and pSTAT3 (David *et al.*, 2011). Accumulating data suggest that, amplification of STAT3 and pSTAT3 is 20.0% and 10.0% of most of adenocarcinoma including cervical cancer(Shukla *et al.*, 2010, Tierney *et al.*, 2014), it may be lead to single nucleotide poly morphism(Yuan *et al.*, 2015), Methylation rate in cervical cancer as compare to precancerous state(Ma *et al.*, 2014). Interestingly our study found high level overexpression of pSTAT3 as compare to STAT3 have seen in malignant and intermediate tumors when compared with benign tissue, precancerous CIN I (28.5%), CINII (61.1%) and (CIN III 68.0 %) as compare cervical cancer (27.9%). There is increased rate of conversion rate of Stat 3 (tumor suppressor) in to pSTAT3(oncogenic) CINI (42.9%), CIN II

(72.2%),CIN III(80.0%) and cervical cancer (83.7%) in tissue may be responsible for high risk of cervical cancer,

In other hand the circulating IFN- γ was positively and significantly associated with cervical cancer prognosis(Telesheva *et al.*, 2012), increasing the higher level IFN- γ also seen in different cancer(Put *et al.*, 2015, Tanji *et al.*, 2015), that may indicate the prognosis of cervical cancer. Some controversy report suggest that however resent data shows did not observe any such association (Sarhan *et al.*, 2015). Interestingly our study also investigated that, increasing the serum and tissue level of IFN- γ seen in the precancer state as compare to the cancer (47.41% 14.65% and 24.13% and 19.82%), some report also shown that, increasing the serum concentration is good prognosis of cervical cancer(Li *et al.*, 2015). In similar way our study found high level overexpression of pSTAT3 as compare to STAT3 have seen in malignant and intermediate tumors when compared with benign tissue (56.1%,27.9%, 68.4%, and 83,07%) (table 2). Long term high risk of HPV incubation invers association change in the immune mechanism finally some more study is required to investigate HPV inflammation.

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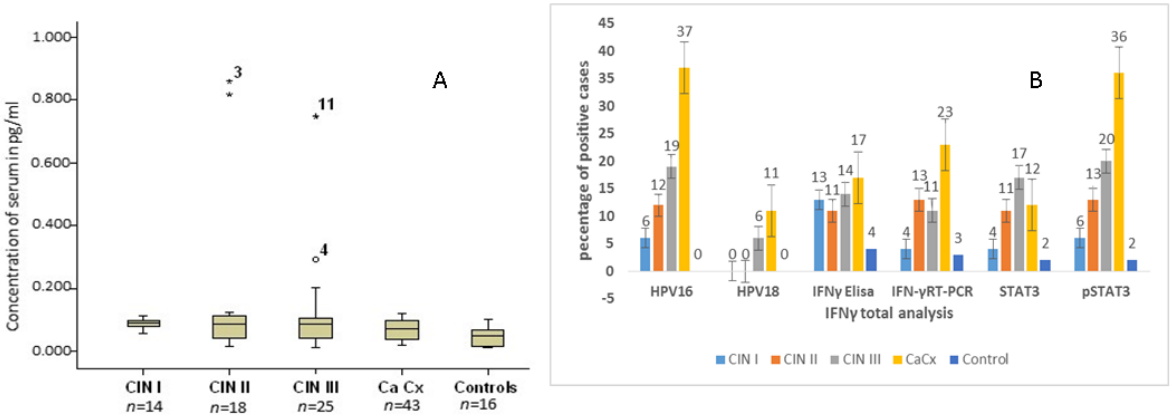

Figure-1. Comparison of plasma IFN-γ levels between cancer, pre-cancer and healthy controls. The central box represents the value from the lower to upper quartile. Significance (p<0.004) assessed SPSS 14.0 (A). The percentage of positive patients in different parameter in pre-cancer and cancer patients as compare to control (B).

Figure-2, RT-PCR expression levels of IFN-γ gene expression showing 110bp amplimer seen. Lane 1 is 100bp marker, lane 2-6 is precancer , lane 7-11 is cancer and lane 12-15 is normal samples

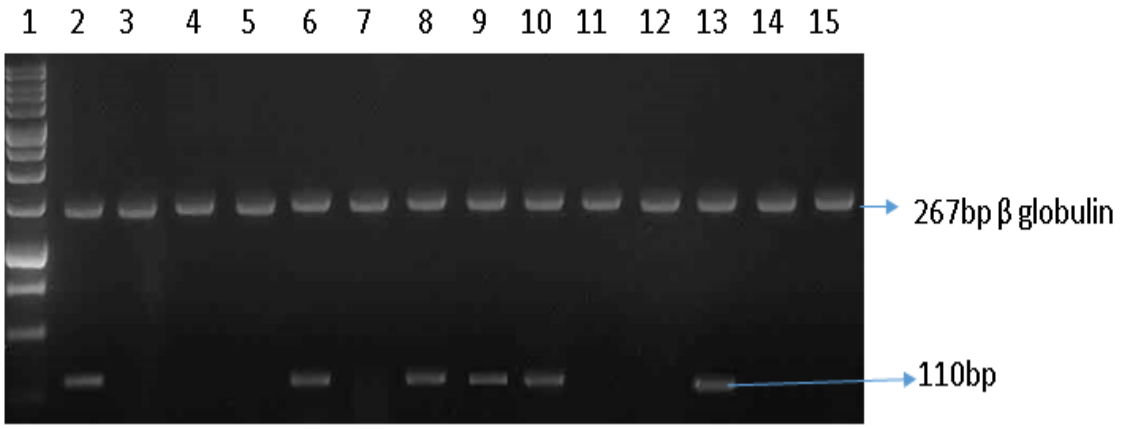

Figure-3: Immunohistochemistry (Semi-quantitative) analysis of STAT3 (A-E) and pSTAT3 (Tyr 705) (F-J). The samples were marked into 3 grade based on the percentage of positive cells Grade 1 ≤ 25% (mild) positive cells (A and F),Grade 2, 26- 50% (moderate) positive cells (B and G),Grade 3,51-75%(sever) positive cells .(D and I), control slides (E and J), image magnification 200X

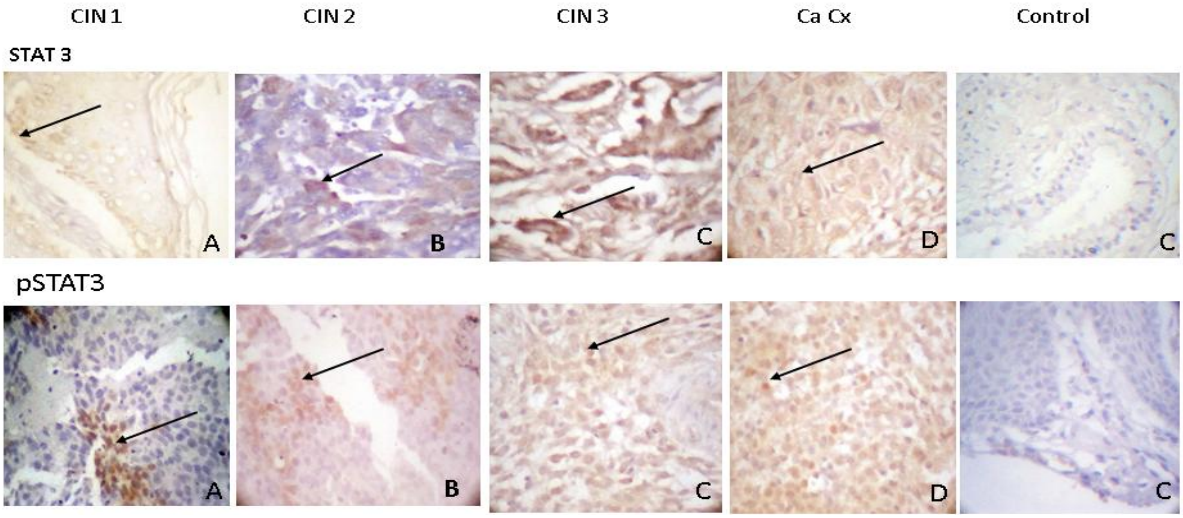

Table-1: Relationship between grades of cancer with different parameters

Grades	n	HPV16(%)	HPV18(%)	IFNγ-ELISA(%)	IFNγ-RT-PCR(%)	STAT3(%)	pSTAT3(%)	Meanage ± SD
CIN 1	14	06 (42.9)	00 (0.0)	13(92.85)	4 (28.57)	4(28.57)	6(42.9)	44.4 ± 10.5
CIN 2	18	12 (66.7)	0.0 (0.0)	11(61.11)	13(72.22)	11(61.1)	13(72.2)	44.9 ± 9.9
CIN 3	25	19(66.7)	06(24.0)	14(56.00)	11 (44.00)	17(68.0)	20(80.0)	45.9 ± 9.5
Ca Cx	43	37 (86.0)	11(25.6)	17(39.53)	23(53.48)	12(27.9)	36(83.7)	55.9 ± 9.7
Normal	16	0	0	2(12.50)	3(53.48)	2(12.5)	2(12.5)	40.9 ± 9.7
		P<0.001	P=0.007	P=0.04	P=0.01	P=0.001	P<0.001	
	116	63.79%	13.79%	46.55%	40.51%	39.65%	66.37%	

Table 2 : Comparison between the pre-cancer and cancer in different parameters.

Tissue type	n	HPV16(%)	HPV18(%)	IFNγ ELISA(%)	IFNγ RT-P PCR(%)	STAT3(%)	pSTAT3(%)	Meanage ± SD
Pre-cancer	57	37 (64.9)	6(10.5)	38(47.41)	28(24.13)	32(56.1)	39(68.4)	44.94 ± 9.96
Cancer	43	37(86.0)	11(25.6)	17(14.65)	23(19.82)	12(27.9)	36(83.7)	55.86± 9.61
control	16	00	00	2(12.50)	3(53.48)	2(12.5)	2(12.5)	40.87± 11.03
		P=0.017	P=0.047	P=0.010	P=0.005	P=0.005	P<0.080	
	116	63.79%	14.65%	44.82%	43.96%	37.93%	64.65%	

Table-1 Primer sequences

Cytokine	Primer sequences	Product length (bp)	Nucleotide position
IFN-γ	5'- ACG AGA TGA CTT CGA AAA GCT G-3'	112 bp	397-509
	5'- TTT AGC TGC TGG CGA CAG TTC-3'		
HPV16	5' -AAG GCC AAC TAA ATG TCA C-3'	217bp	7763-7781
	5' -CTG CTT TTA TAC TAA CCG G-3'		7757-7775
HPV18	5' -CACTTCACTGCAAGACATAGA-3'	321bp	66-86
	5' -GTTGTGAAATCGTCGTTTTTGA-3'		368-387
β globin	5' -GAA GAG CCA AGG ACA GGT AC-3'	267bp	1-20
	5' –CAA CTT CAT CCA CGT TAC ACC -3'		253-268
GAPDH	5'-TGGATATTGTTGCCATCAATGACC-3	460bp	159-183
	5'-GATGGCATGGACTGTGGTCATG-3'		598-619
MY09	5'-GCMCAGGGWCATAAYAATGG-3	450bp	
	(M= A+C,W=A+T,Y=C+T,R=A+G)		
	5'-CGTCCMARRGGAWACTGATC-3'		