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Role of KLF2: New insight in Inflammatory Acne Pathogenesis. KLF2 Predictor of Severity and Outcome in Acne

Summary

Background: Acne is an inflammatory skin condition of pilosebaceous unit. Its pathogenesis is multifactorial with a central role of inflammatory and pro inflammatory cytokines mediators. Downregulated Kruppel like factor 2 (KLF2) leads to rapid secretion of many cytokines that are involved in acne pathogenesis.

Aims: This study aimed at evaluating the level of KLF2 mRNA, clarifying its role in acne pathogenesis and its relation to acne lesion type, degree of severity and outcome.

Patients and Methods: The level of KLF2 mRNA was measured in 100 patients with acne and 50 age and sex matched healthy controls by using quantitative real-time polymerase chain reaction (qRT-PCR).

Results: The value of KLF2 mRNA was lower in acne patients than control group (***P*** <0.001), being lowest in inflammatory acne group (grades III, IV and V) than non-inflammatory acne group (grades I and II) and highest in the control group (***P*** <0.001). KLF2 mRNA was decreased significantly with increased acne severity grade (***P*** < 0.001). KLF2 mRNA was lower in cases healed by scars than those healed by post inflammatory hyperpigmentation.

Conclusions: Decreased serum level of KLF2 is not only a claimed for AV pathogenesis but also a predictor for degree of acne severity and outcome.

KEYWORDS: Acne Vulgaris, Inflammatory Cytokines and KLF2.

INTRODUCTION

Acne Vulgaris (AV) is a chronic inflammatory disease of the pilosebaceous unit. It is classified into two groups: non inflammatory (open and closed comedones) and inflammatory acne (papules, pustules, nodules and cysts), with papules considered an intermediary step between the two ***[1].***

The pathogenesis is multifactorial, with an important role of inflammatory mediators and pro inflammatory cytokines in its pathogenesis ***[2].*** These inflammatory cytokines produced by keratinocytes, macrophages and neutrophils induce hyperkeratinization, comedones and inflammatory lesions because of CD4 T cell activation and migration ***[3].*** During puberty alteration of the sebaceous lipid profile, aggravated by stress and irritation, induce inflammation and AV development. Another player in the pathogenesis is the pro-inflammatory activity of the cutaneous microbiome. Dysbiosis, leads to a disturbed barrier and disequilibrium of skin microbiome ***[4].***

Kruppel like factors (KLF)s share the homology of proteins to DNA-binding domains of Drosophila fly; Drosophila embryos deficient in kruppel die due to abnormal thoracic and abdominal segmentation and appear crippled (kruppel means cripple) ***[5].*** The family of KLF has 18 members, with a wide range of expression profile among several tissues ***[6].*** Each of which contains 3 zinc fingers by that bind to the DNA binding transcription factors, which play role in proliferation, differentiation, development, growth and inflammation ***[7].*** KLFs are widely expressed in various tissues, genes only can encode transcription activators and repressors proteins ***[8].***

Kruppel like factor 2 gene, located at chromosome 19 p13.1, plays an important role in regulating adipogenesis, inflammatory and malignant conditions. The KLF2 is expressed in positive CD4 and CD8 cells and remains highly expressed in both naïve and memory T cells. It has been shown that during T cell stimulation, both KLF2 m-RNA and protein are down regulated ***[7].***

An important process that triggers AV is that Propionibacterium acnes (P. acnes) activates the innate immunity via expression of protease activated receptors (PARs), TNF-α and toll-like receptors (TLRs), and the production of interferon (INF), interleukins (IL-8, IL12, IL-1) and matrix metalloproteinases (MMPs) resulting in subsequent hyperkeratinization of the pilosebaceous unit ***[4].*** Downregulated KLF2 leads to rapid secretion of cytokines such as IL-1β, IL-8, TNF-α and monocyte chemotactic protein-1

(MCP-1) ***[9],*** IL-6 and MMP ***[10].*** Which suggests that KLF2 might be linked to pathogenesis of AV.

THE AIM OF THE STUDY

The aim was to assess the role of KLF2 in pathogenesis of inflammatory acne.

SUBJECTS AND METHODS

This case control study included 100 clinically diagnosed AV patients with different severities and 50 age and sex matched healthy controls. The inflammatory acne patients were 19 males and 31 females (age; 22.50 ± 3.62 years). Non-inflammatory acne patients were 21 males and 29 females (age; 26.08 ± 4.91). Healthy controls were 26 males and 24 females (age; 25.84 ± 4.89 years). Patients were selected from outpatient clinic of Dermatology Department. Written informed consent was taken before the start of the study, which was approved by local Ethics Committee for Human Research in Faculty of Medicine in accordance with Helsinki declaration of the human rights 1975. Patients were excluded if suffering from other systemic inflammatory diseases, autoimmune diseases, infectious diseases or reported previous treatment with oral retinoids or any medications affects the AV severity. Each patient was subjected to complete history taking, general examination and clinical assessment of AV. Severity of AV was assessed by using the Global Acne Severity Scale ***[11].***

Sampling: Two ml venous blood were obtained from each subject, put into sterile tube with EDTA, mixed well and aliquoted into 2 eppendorf tubes. Tubes were kept at -80 °C till the following steps.

Relative quantitation of KLF2 mRNA level:

A. Total RNA extraction: Total RNA extraction was performed with 100 µl EDTA whole blood via Direct-zol RNA MiniPrep (Zymo Research). Digestion with DNase I was performed. Ultraviolet spectrophotometric quantification of RNA by Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). Pure RNA preparations have optical density (OD) ratio at 260/280 nm of 1.9-2.3 ***[12].***

B. Quantitative real-time polymerase chain reaction (qRT-PCR): It was performed on two-steps: **The first step** is conversion of RNA into complementary DNA (cDNA) in a Veriti™ Thermal Cycler (Applied Biosystems), using SensiFAST™ cDNA Synthesis Kit (Bioline Reagents Ltd, United Kingdom). The PCR mix for reverse transcription reaction contained 5ul RNA extract, 4ul TransAmp Buffer (5x), 1ul reverse transcriptase and 10µl

nuclease-free water. The thermal cycler conditions were 25^{o}C for 10 min., 42^{o}C for 15 min. and 85^{o}C for 5 min. **The second step** was quantitation of KLF2 mRNA in a Stepone real-time PCR system (Applied Biosystem, Singapore). Singleplex reactions were done. This step was performed using TransStart Green qPCR SuperMix (TransGen Biotech Co., Ltd). Human GAPDH was the endogenous housekeeping gene. Melting curve analysis was done in each run to confirm specificity of real-time PCR assay. KLF2 primers were; FP: 5'-ACAGACTGCTATTTATTGGACCTTAG-3'&RP:5'-CAGAACTGGTGGCAGAGTCATTT -3'. GAPDH primers were; FP: 5'- TGAAGGTCGGAGTCAACGGATT -3' & RP: 5'-CCTGGAAGATGGTGATGGGATT -3' ***[13].*** The reaction mix contained 10 µl TransStart Green qPCR SuperMix (2X), 0.5 µl FP, 0.5 µl RP, 2 µl cDNA, 0.4 µl reference dye and up to 20 µl nuclease-free water. The thermal profile was holding stage (95^{o}C/10min.), cycling stage that includes 45 cycle, each has denaturation (95^{o}C/15sec.), annealing (53^{o}C/30min.) & extension (72^{o}C/30sec.).

C. Data analysis: According to stepOne software version 2.2.2., the data were produced as sigmoid shaped amplification plots. The control samples were used as calibrators, so their expression levels were set to 1. The relative quantities of human KLF2 mRNA were normalized against that of human GAPDH, so relative quantitation (RQ) was calculated by the equation $2^{-\Delta\Delta CT}$ ***[14]*** Fig. (1).

Statistical Analysis

Data were analyzed using IBM SPSS software package version 20.0 **(**Armonk, NY: IBM Corp**).** Qualitative data were described using number and percent. The Kolmogorov-Smirnov test was used to verify the normality of distribution Quantitative data were described using range (minimum and maximum), mean ± standard deviation (SD) and median Inter Quartile Range (IQR). The used tests were Chi-square test, Monte Carlo correction, F-test (ANOVA), Mann Whitney test, Pearson coefficient, Student t-test and Receiver operating characteristic curve (ROC).

RESULTS

The mean value of KLF2 mRNA was statistically significantly lower in AV patients (0.61) than controls (1.0) (***P*** <0.001) Table (1)**.** The mean value of KLF2 mRNA was statistically significantly lower in inflammatory acne group (grades III, IV and V) than non-inflammatory acne group (grades I and II) and highest in the control group (***P*** <0.001). On comparing inflammatory and non-inflammatory acne groups (***P1*** < 0.001), on comparing

inflammatory acne and controls (***P2***<0.001) However on comparing non-inflammatory acne and controls (***P3***=0.082) Table (2).

There was statistically significant difference in KLF2 level as regards the Global Acne Severity Scale in inflammatory acne group (***P*** < 0.001). The mean value of KLF2 was lowest in grade V (0.22) than in grade IV (0.29) and grade III (0.48), similarly in non-inflammatory acne group mean value of KLF2 was lower in grade II than in grade I but this difference was not statistically significant (***P***= 0.277). Despite that KLF2 mean value was higher in non-inflammatory acne group than in inflammatory acne group Table (3) and Fig. (2).

There was statistically significant difference in KLF2 level as regards pattern of healing in inflammatory acne group (***P*** = 0.016). The mean value of KLF2 was lower in cases healed by scars (0.23) than cases healed by post inflammatory hyperpigmentation (PIH) (0.37), similarly KLF2 mean value was lower in cases healed by scars than those healed by PIH in non-inflammatory acne group but this difference was not statistically significant (***P*** = 0.314). Despite that KLF2 mean value was higher in non-inflammatory acne group than in inflammatory acne group Table (3) and Fig. (3).

There was statistically significant difference as regards effect of diet on KLF2 mRNA level in inflammatory acne group (***P*** < 0.001), The mean value of KLF2 was lower in cases consuming fatty diet (0.20) than carbohydrate (0.4) and protein diet (0.35), also in non-inflammatory acne group the mean value of KLF2 was lower in fatty consumers (0.93) than carbohydrate (0.95) and protein diet (0.98) but this difference was not statistically significant (***P*** =0.271)Table (3) and Fig. (4).

The ROC curve showed that KLF2 level was statistically significant for diagnosis of inflammatory acne (***P*** <0.001) with 98% sensitivity and specificity. The probability of inflammatory acne among those with low KLF2 level is 98% (PPV 98) and that cut off point of KLF2 for diagnosis of inflammatory acne is ≤0.67 relative quantitation (RQ) Table (4) and Fig. (5).

DISCUSSION

Inflammation plays a central role in AV pathogenesis together with androgens are prime orchestrators that lead to increased sebum production and a more inflammatory composition of sebaceous lipids ***[15].*** The P. acnes promotes mixed Th17/Th1 responses by inducing the concomitant secretion of IL-17A and IFN-c from specific CD4 (+) T cells.

Therefore, Th17-related cytokines potentiate acne pathogenesis ***[16].*** High levels of IL1, macrophages and CD4 cells in AV patients compared to apparently healthy subjects suggest that inflammation precedes hyperproliferation in acne pathogenesis ***[15].*** IL-1, through promoting comedone formation also may participate in inflammation and rupture of follicular canal ***[17].***

Naïve lymphocytes are quiescent until encounter specific antigens. KLF2 is expressed in resting lymphocytes (naïve and memory cells) but is downregulated rapidly after lymphocyte activation, suggesting that it may be a quiescence factor ***[18].*** Although the physiological role of KLF2 in cell cycle control in lymphocytes is currently unclear, there is a growing evidence that KLFs regulate migration of inflammatory cells and lymphocytes during both normal homeostasis of immune system and inflammation ***[19].***

Nuclear factor-kappa B (NF-κB) is an important inducer of inflammation through transcription of several proinflammatory cytokines, chemokines, and adhesion molecules. KLF2 is a negative regulator of inflammation which reduces proinflammatory activity of NF-κB ***[7].*** It has been revealed that cellular levels of KLF2 are reduced in chronic inflammatory states. This shows the importance of KLF2 in the biological response to inflammation ***[20].***

Out of all members of KLF family, KLF2 and KLF4 have been detected in vascular endothelium, lymphoid cells, and in skin, gut, kidney, and lung epithelium ***[21].***

In this study, the mean value of KLF2 was lower in acne patients (0.61) than control group (1.0), lower in inflammatory acne group (grades III, IV and V) than non- inflammatory acne group (grades I and II) and highest in the control group (***P*** <0.001). Similar pattern of KLF2 was found by ***Das et al. [10]*** who observed that downregulation of KLF2 and concomitant upregulation of several inflammatory markers as IL-6, TNFα and MMPs (MMP1, 9 and 13) in patients with active rheumatoid arthritis established the inverse correlation between KLF2 levels and inflammatory cytokines expression, ***Wang et al. [13]*** found that KLF2 participates in the inflammatory response of ulcerative colitis by negatively regulating expression of IL6, IL-8, IL-10 and TNF-α levels. These results were supported by ***Nayak et al. [20]*** who stated that deficiency of KLF2 modulates the in vivo response to acute (sepsis) and subacute (skin) inflammation and enhances pro-inflammatory cytokines expression.

Acne pathogenesis entails the development of acne lesions by induction of the secretion of IL1, IL6, IL8 and TNFα ***[22]***. It is well established that the secretion of

proinflammatory cytokines such as IL1, IL8, TNF-α and MMPs contributes to the inflammatory nature of acne ***[2].***

Decreased KLF2 enhance proinflammatory cytokines expression, vascular instability, interstitial fluid accumulation and macrophage infiltration. So, its reduction enhances the process of acute inflammation through augmentation of proinflammatory gene expression and macrophage recruitment to the site of inflammation ***[20].***

van Vliet et al. [23] reported that mammalian KLFs are known to perform critical functions in lipid metabolism and lipogenesis in adipose and non-adipose tissues. Also, ***Ling et al. [24]*** stated that KLF2 has a new function as it inhibits fat build-up.

Sebocytes, in addition to producing sebum, link lipid metabolism with inflammation at a cellular level and hence greatly resemble adipocytes ***[25]***. Sebocyte and adipocyte share the expression of many adipogenic factors during their differentiation ***[26].*** KLF2 dramatically diminish upon adipocyte differentiation as excess KLF2 prevents pre-adipocyte differentiation ***[5].*** Thus, KLF2 may have a role in sebocyte differentiation which may affect acne pathogenesis.

FoxO1 function is a key regulator in the pathogenesis of acne as FoxO1 suppresses lipid metabolism through suppression of the activity of PPARγ. FoxO1 deficiency results in spontaneous T-cell activation ***[27].*** FOXO1 can drive the induction of KLF2 in human T cells as FOXO1 binds the KLF2 promoter in vivo ***[28].***

Same result was found by ***Fathy et al. [29]*** who found that the mean value of TLR2 was significantly increased in acne patients and the expression was still higher among those with predominantly inflammatory lesions than those with predominantly non-inflammatory lesion. Knockdown of KLF2 expression augment the activation of TLR2 through NFkB ***[30].***

As regards Global Acne Severity scale we found that the mean value of KLF2 was lower in grade V acne cases (0.22), grade IV (0.29) and grade III (0.48) than in non-inflammatory acne group grade II (0.93) and grade I (0.96) (***P***= 0.277). These results were supported by ***Wang et al. [13]*** who stated that severe ulcerative colitis patients showed the lowest expression of KLF2, suggesting that KLF2 was negatively correlated with the inflammatory level, illustrated that KLF2 is linked to the severity because the level of cytokines IL6, IL8, IL10, and TNFα was higher in severe than mild ulcerative colitis patients. Also, ***Amr et al. [31]*** found that there was a significant association between PPARγ as a

suppressor marker of inflammation and acne severity graded by global acne grading system (***P*** <0.001) revealing that the PPARγ is less common in patients with severe acne. Overexpression of KLF2 induce PPARs ***[5].***

As regards the pattern of healing of acne lesions, we found that the mean value of KLF 2 was lower in cases healed by scarring (0.23) than in cases healed by post inflammatory hyper pigmentation (0.37) which denotes continuous process of inflammation in cases of scarring. ***Holland et al. [32]*** found that the inflammatory response has been implicated as an important component in the development of scar. Also, ***Saint-Jean et al. [33]*** found that absence of scars is associated with low expression of pro-inflammatory cytokines as TLR2, IL2, TNFα and MMPs. ***Das et al. [34]*** found that over expression of KLF2 inhibits the induction of pro-inflammatory cytokines such as IL2 and TNFα.

As regard the effect of diet on KLF2 mRNA expression, the level was significantly lower in acne patients on high fat than carbohydrate and protein diet, however no literature is available concerning this finding.

CONCLUSIONS

Acne pathogenesis involves many factors that cause inflammation and formation of different types of lesions with different degrees of severities. These factors include the alteration of the KLF2 level. We observed that KLF2 was downregulated in AV patients suggesting a role in the inflammatory acne pathogenesis negative relation was found between serum KLF2 level and AV severity and scar formation. This suggests that KLF2 could serve as a marker of inflammation and scaring in AV.

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RESULTS

Table (1): Comparison between acne patients and control subjects according to KLF2 level

KLF2	Acne patients (n = 100)	Control (n = 50)	t	p
Min. – Max.	0.15 – 1.08	0.88 – 1.17		
Mean ± SD.	0.61 ± 0.37	1.0 ± 0.09	10.099	<0.001
Median (IQR)	0.71(0.20 – 0.96)	0.98 (0.93 – 1.09)		

t: Student t-test

Table (2): Comparison between the three groups according to KLF2

KLF2	Inflammatory acne (n = 50)	Non-inflammatory acne (n = 50)	Control (n = 50)	F	p
Min. – Max.	0.15 – 0.76	0.67 – 1.08	0.88 – 1.17		
Mean ± SD.	0.27 ± 0.16	0.95 ± 0.09	1.0 ± 0.09	578.639	<0.001
Median (IQR)	0.20 (0.16 – 0.28)	0.96 (0.89 – 1.03)	0.98 (0.93 – 1.09)		
Sig. bet. Groups.	$p_1<0.001^*$, $p_2<0.001^*$, $p_3=0.082$				

F: **F for ANOVA test**, pairwise comparison bet. each 2 groups were done using **Post Hoc Test (Tukey)**

p: p value for comparing between the studied groups, p_1: p value for comparing between **inflammatory acne** and **non-inflammatory acne**, p_2: p value for comparing between **inflammatory acne** and **Control** p_3: p value for comparing between **non-inflammatory acne** and **Control**

Table (3): Relation between KLF2 and different parameters

	KLF2							
	Inflammatory acne				Non-inflammatory acne			
	N	Mean ± SD.	Test of Sig.	p	N	Mean ± SD.	Test of Sig.	p
Grading								
I	0	–	$F=11.215^*$	$<0.001^*$	29	0.96 ± 0.08	t=1.099	0.277

II	0	–			21	0.93 ± 0.11		
III	7	0.48 ± 0.24			0	–		
IV	11	0.29 ± 0.15			0	–		
V	32	0.22 ± 0.10			0	–		
Healing								
PIH	15	0.37 ± 0.20	t=2.663*	0.016*	35	0.96 ± 0.10	t=1.017	0.314
SCAR	35	0.23 ± 0.12			15	0.93 ± 0.09		
Diet								
Carbohydrate	13	0.40 ± 0.19		p_1<0.001*	12	0.95 ± 0.03		p_1=0.807
Fatty diet	30	0.20 ± 0.07	F=11.038* p<0.001*	p_2=0.738	20	0.93 ± 0.11	F=1.341 p= 0.271	p_2= 0.697
Protein diet	7	0.35 ± 0.22		p_3=0.029*	18	0.98 ± 0.10		p_3 = 0.241

t: Student t-test F: **ANOVA test** #: Excluded from the association due to small number (n = 1). Pairwise comparison bet. each 2 groups were done using **Post Hoc Test (Tukey).** p_1: p value for comparing between **CHO** and **Fat,** p_2: p value for comparing between **CHO** and **Protein,** p_3: p value for comparing between **Fat** and **Protein**

Table (4): Receiver Operator Characteristics curve (ROC curve) for KLF2 mRNA to diagnose inflammatory acne

Parameter	**Cut off**	**AUC**	**p**	**95% C.I**	**Sensitivity**	**Specificity**	**PPV**	**NPV**
KLF2 mRNA (RQ)	≤0.67	0.999	<0.001*	0.995 – 1.0	98.0	98.0	98.0	98.0

RQ: relative quantitation, AUC: area under the curve, CI confidence interval, PPV: positive predictive value, NPV: negative predictive value

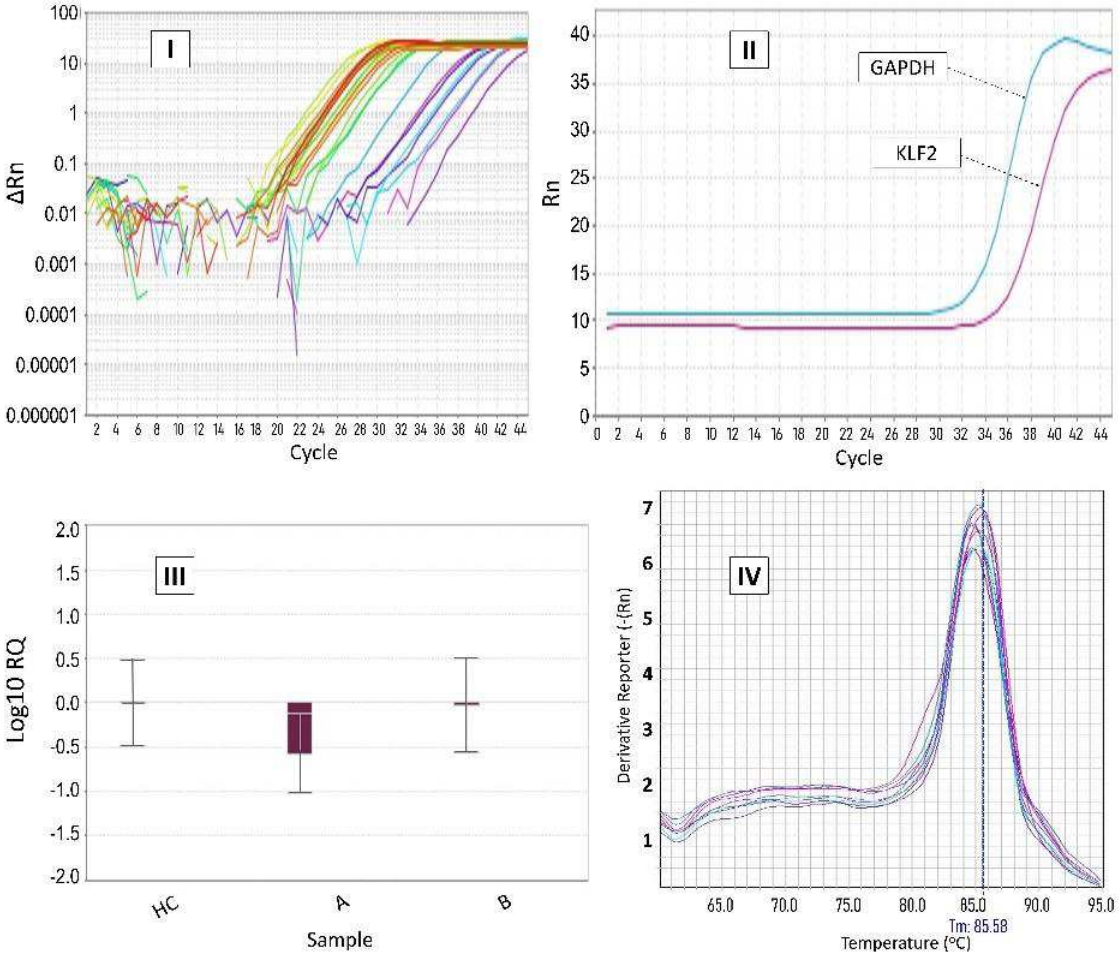

Fig. (1): I: Amplification plot of KLF2 and GAPDH genes in the studied groups, II: Amplification plot of KLF2 and GAPDH genes in a single sample, III: Gene expression plot of KLF2 (violet bars) normalized to that of GAPDH (no bar in the graph), IV: Melting curve of KLF2 (HC: healthy controls, A: inflammatory acne & B: non-inflammatory acne).

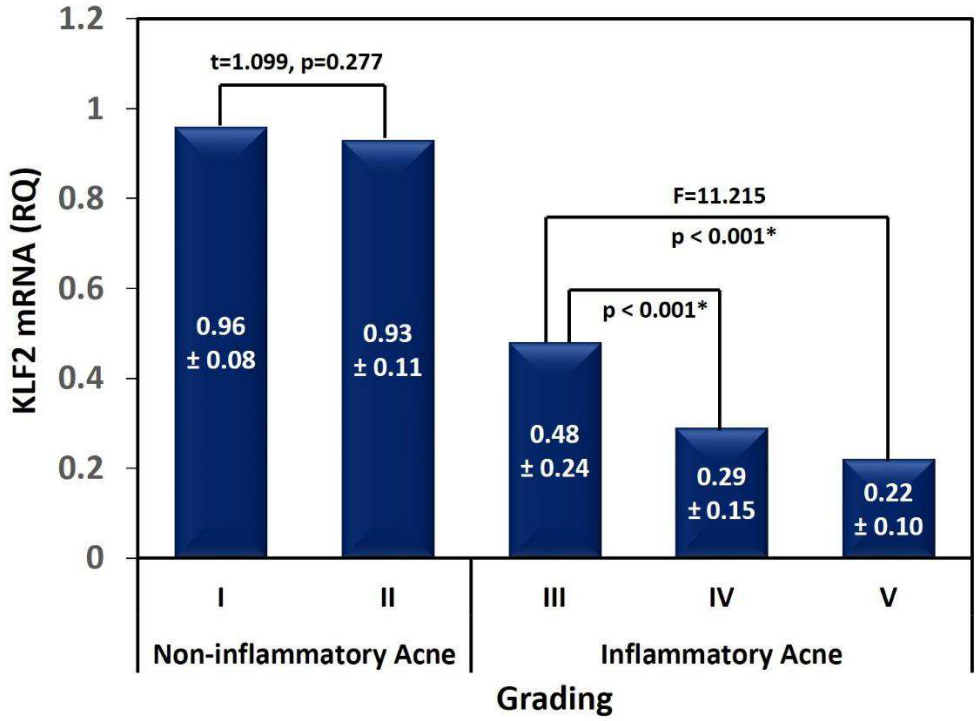

Fig. (2): Relation between KLF2 and Global Acne Severity Grading

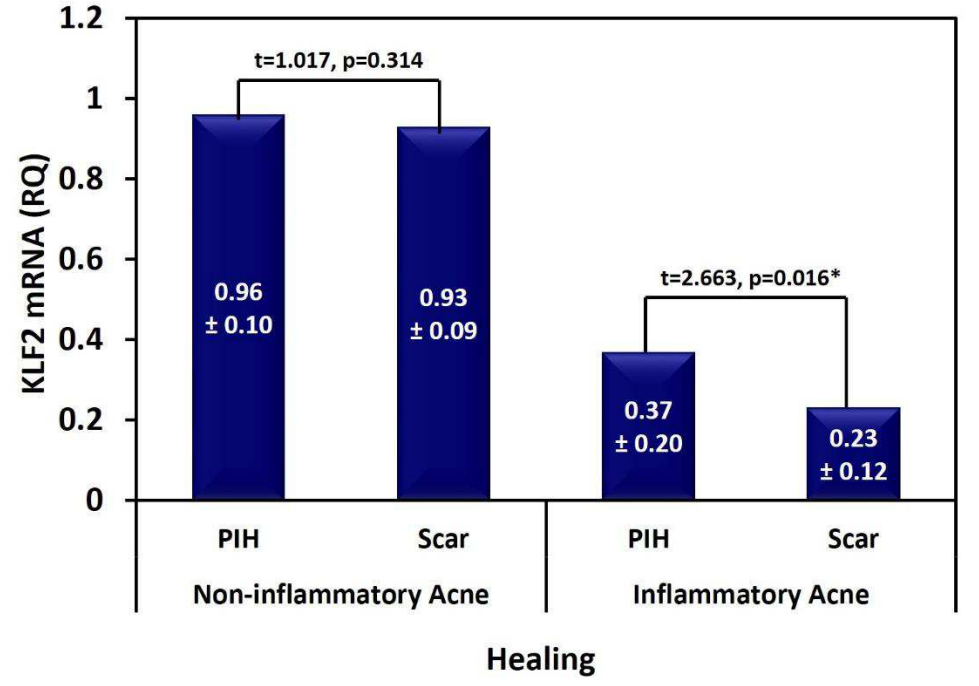

Fig. (3): Relation between KLF2 and Healing in inflammatory and non-inflammatory acne

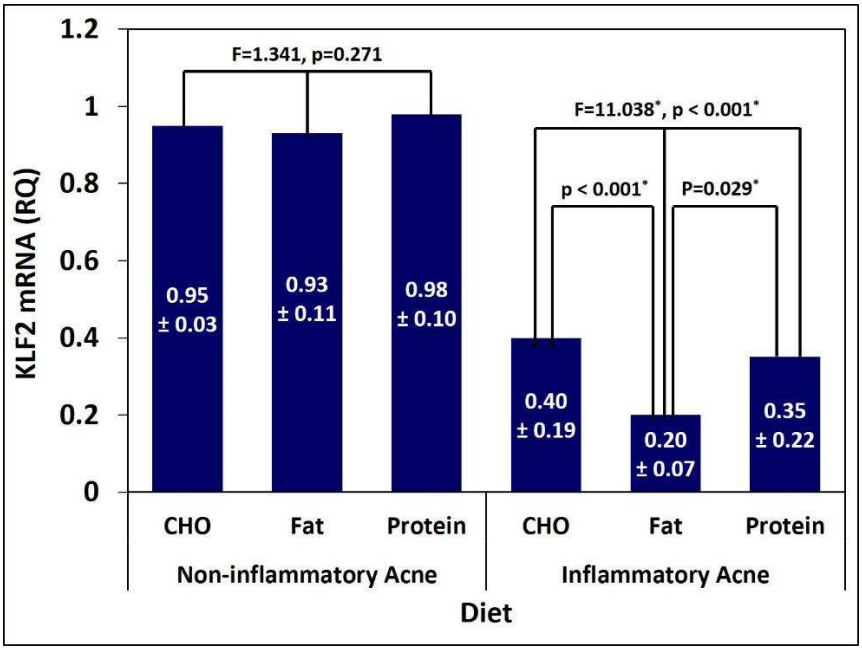

Fig. (4): Relation between KLF2 and Diet in inflammatory and non-inflammatory acne

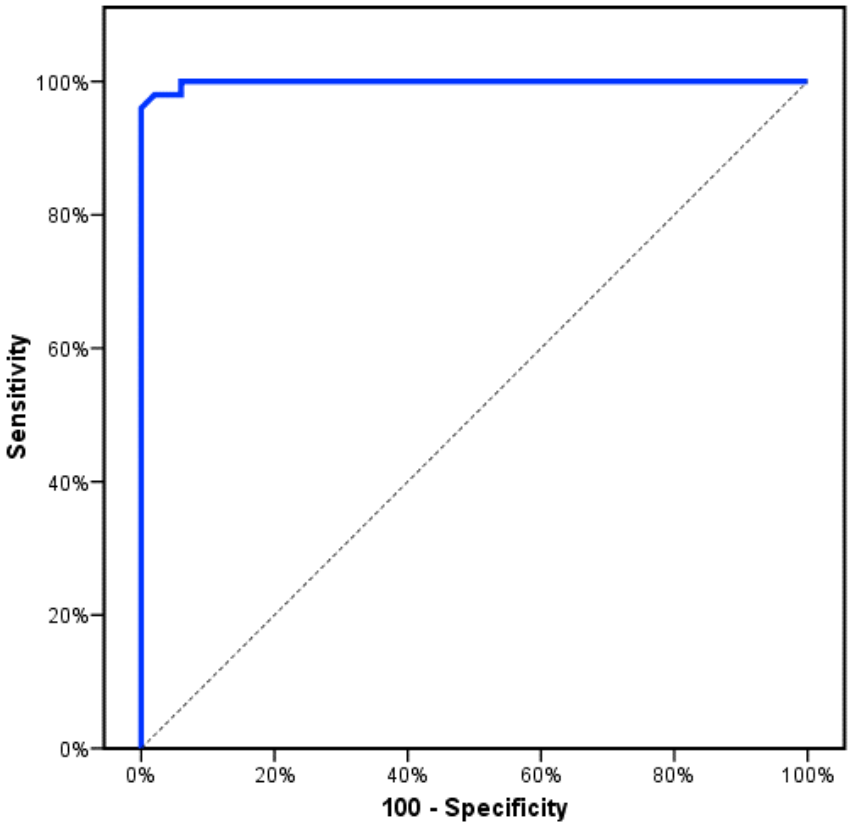

Fig. (5): ROC curve for KLF2 to diagnose inflammatory acne

Cut off was done according to Youden index.