Reports of Biochemistry & Molecular Biology
Vol.11, No.3, Oct 2022

Original article

www.RBMB.net

Possible role of LncRNA MEG3-microRNA-21 and endoplasmic reticulum (ER) stress proteins in the Pathogenesis of Psoriasis Vulgaris

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Abstract

Background: Psoriasis is a chronic inflammatory immune mediated disease arising from interaction between genetic risk variants and the environment. Maternally expressed gene3 (MEG3) is a long noncoding RNA (lncRNA) known for gene transcription regulation and inhibiting proliferation. MEG3 competes with microRNA (miRNA-21) influencing cell proliferation and apoptosis balance. Endoplasmic reticulum (ER) stress proteins promote cell survival via unfolded protein response (UPR) influenced by MEG3. We aimed to detect the possible role of MEG3, miRNA-21 and ER stress proteins in pathogenesis of psoriasis vulgaris.

Methods: Human GRP78, ATF6, caspase3 tissue levels were assayed by Enzyme Linked Immunosorbent Assay (ELISA). Assessment of long non-coding MEG3 and miRNA 21 expressions was done by quantitative real time polymerase chain reaction (qRT-PCR).

Results: Expression of MEG3 was significantly downregulated, while miRNA-21 was remarkably upregulated, ER stress proteins GRP78, ATF6, and caspase 3 all showed low levels in homogenized psoriatic lesions when compared to normal skin. miRNA 21 and MEG3 were identified as possible diagnostic markers for psoriasis vulgaris.

Conclusions: MEG3 is barely expressed in psoriatic lesions while miRNA-21 expression is remarkably elevated but when correlated to each other there was unexpected positive correlation. MEG3 and miRNA-21 were identified as possible diagnostic markers for psoriasis. Undifferentiated psoriatic lesions have very weak UPR.

Keywords: ER stress proteins, lncRNA MEG3, Microrna-21, Psoriasis.

Introduction

Psoriasis is a lifelong immune-mediated inflammatory skin disease, the World Health Organization acknowledged psoriasis as a serious non-communicable disease and pointed up the distress related to its misdiagnosis, incompetent treatment, as well as stigmatisation of the disease (1). Psoriasis is characterized by thickening and scaling of the epidermis as resulting from increased proliferation of keratinocytes (KC), it has variable phenotypes, the most common is psoriasis vulgaris or plaque psoriasis, known for erythematous plaques with loosely adherent silvery white scales commonly on the elbows, knees, scalp, and trunk (2). Histological and immunological analyses have revealed that a diverse of Psoriasis clinical symptoms are related to abnormal epidermal hyperproliferation and differentiation. Genetic predisposition and environmental factors are the main contributors to the development of psoriasis. Epigenetic changes are processes

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Received: 29 Mar, 2022; Accepted: 29 Mar, 2022

that change genome activity around DNA without altering DNA sequences while mitotically stable, these changes play crucial role in psoriasis development. Gene expression variation results in the differential regulation of cell signalling pathways. The chief epigenetic processes include DNA methylation, histone post-translational modifications and non-coding RNAs (ncRNAs) (3). The ncRNAs are subdivided into short ncRNAs and long ncRNAs (lncRNAs). Short ncRNAs are less than 200 nucleotides, including small nucleolar RNAs, microRNAs (miRNAs), and other categories of RNA (4), While lncRNAs class is formed of RNA transcripts with no protein-coding capacity and are greater than 200 nucleotides in length (5). LncRNAs play key roles in regulating immune-mediated inflammatory diseases and autoimmunity (6) Dysregulation of lncRNAs has been studied in numerous immune diseases, including systemic lupus erythematosus, rheumatoid arthritis (7) emphasizing the crucial roles of lncRNAs in immune-mediated diseases and their contribution to the pathogenesis of psoriasis by regulating the expression of protein-coding genes (8), still little is known about the association of specific lncRNAs in the prevalence of this disease (7). LncRNA maternally expressed gene 3 (MEG3) is categorized as an imprinted gene found on chromosome 14q32 in human. It is known to be an important regulator in several types of cancers (9), loss of MEG3 expression has been found in diverse types of human tumors (10). Moreover, MEG3 can inhibit cancer cell proliferation or induce apoptosis in vitro and in vivo (11). However, its roles in psoriasis are unclear (12). miRNAs are small single stranded ncRNAs originated from larger primary RNAs and processed into 21-25 nucleotides long mature transcripts, which can regulate expression of their target genes at post-transcriptional level. Dysregulation in the expression of these transcripts contribute to the pathogenesis of psoriasis (13) miRNAs regulate gene expression through binding to the 3′ untranslated region of target mRNAs (14). MicroRNA 21(miRNA-21) is identified as an oncogene, reported to be remarkably upregulated in a wide range of cancers such as breast, gastric and colon cancers (15). Significant elevated level of miRNA-21 was observed in psoriasis lesions compared with non-lesioned skin from psoriasis patients (14). Yet, influence of miRNA-21 on the over proliferation of keratinocytes have not been reported (16). Some reports indicated that MEG3 affected proliferation, apoptosis, and metastasis of gastric and cervical cancer cells by regulating miRNA-21. Nevertheless, the influence of MEG3 on miRNA-21 during the proliferation and apoptosis of psoriasis epidermal cells is still unclear (16). The human epidermis is subjected to constant turnover, a process that requires certain degree of keratinocyte proliferation which is almost particular to the basal layer of the epidermis, KCs are the predominant cell type in the epidermis, they migrate from basal layers to upper layers, where they undergo morphological and biochemical changes toward terminal differentiation. Mechanism of normal epidermal KC differentiation is crucial for understanding a variety of skin diseases characterized by abnormal epidermal KC differentiation, such as psoriasis vulgaris (19). Endoplasmic reticulum (ER) is the organelle responsible for protein synthesis, proper folding, and calcium homeostasis in the cell. Though physiologic levels of ER stress are needed for normal function of skin cells (20), numerous stressors can disrupt ER homeostasis and cause accumulation of unfolded or misfolded proteins inducing pathological consequences, known as ‘ER stress’ (21). Unfolded Protein Response (UPR) initiates to restore normal ER functions via (a) shutting down translation; (b) ubiquitination of misfolded/unfolded proteins via ER-associated degradation (ERAD); and (c) increasing expression of ER chaperones that enhance the efficiency of protein folding (20). The UPR activates transcription factors, including ATF6 which translocates to the nucleus inducing several

UPR genes including glucose-regulated protein 78 (GRP78) (22). GRP78 is a member of the heat-shock protein-70 family that plays a role in proteins folding and assembling in the ER (23). UPR promotes cell adapt stress. However, when the response inadequate and ER stress persists, UPR changes its function from promoting cell survival to promoting cell commit apoptosis through expressing pro-apoptotic factors, such as CHOP and caspase 4/12 (24) which results in activation of cytoplasmic caspase 3 (25). In this study we aimed to spotlight the possible relation between MEG-3/miRNA-21 axis and ER stress proteins in psoriatic lesions.

Materials and Methods

Ethical considerations

The study protocol was approved by the Ethical Committee of Medical Biochemistry and Molecular Biology' and 'Dermatology' departments, Faculty of Medicine "Kasr Alainy", Cairo University, Egypt. Prior to the study a written informed consent was voluntary obtained from participants upon explaining study procedures. The study was conducted according to ethical guidelines of the declaration of Helsinki, October 2013.

Subjects' groups

This is a cross sectional study that included two groups; (a) Patients' group: forty patients (18-70 years) with pure cutaneous psoriasis vulgaris and not on topical or systemic treatment for at least 4 weeks prior to study were recruited from Dermatology outpatient clinic, Faculty of medicine, Cairo University; (b) Control group: included forty healthy subjects of matched age group and gender with no history of chronic dermatological diseases. Patients with any variant other than psoriasis vulgaris e.g., Erythrodermic or pustular psoriasis or Patients with other cutaneous or systemic autoimmune diseases e.g., SLE were excluded. All patients were recruited during the stressful global pandemic of COVID-19 in the period between September 2020 till October 2021.

Tissue sampling and processing

Two punch biopsies 2 mm each were taken under local anesthesia using (Lidocaine®) from the psoriatic plaque in patients and from excess skin after abdominoplasty, breast reduction, brachioplasty operations or minor surgeries in controls.

Enzyme Linked Immunosorbent Assay (ELISA)

Tissue samples in 3 ml phosphate buffer saline (PBS) were homogenized then centrifuged, the supernatant was collected and added to the ELISA kit wells. Tissue levels of human GRP78, ATF6 and, caspase 3 were assayed by commercially available ELISA kits supplied by (SUNLONG BIOTECH Co., Ltd., China). The microtiter plate was pre-coated with an antibody specific to the assayed protein. Standards and samples were then added to the microtiter plate wells combined to the specific antibody then a Horseradish Peroxidase (HRP)-assayed protein conjugated antibody, specific for the assayed protein was added to each microplate well then incubated. Free components were washed away. The chromogenic substrate Tetramethylbenzidine (TMB) solution was added to each well, only those wells that held the assayed protein and HRP conjugated assayed protein antibody had turned blue in color then yellow after adding the stop solution to stop the coloring reaction. The color change was measured spectrophotometrically at a wavelength of 450nm ± 2nm. The optical density (O.D) or absorbance of each calibrator was plotted versus the calibrator concentration to create a calibration curve using GraphPad Prism 9. The concentration of patient samples was then estimated from the calibration curve by interpolation (Fig. 1).

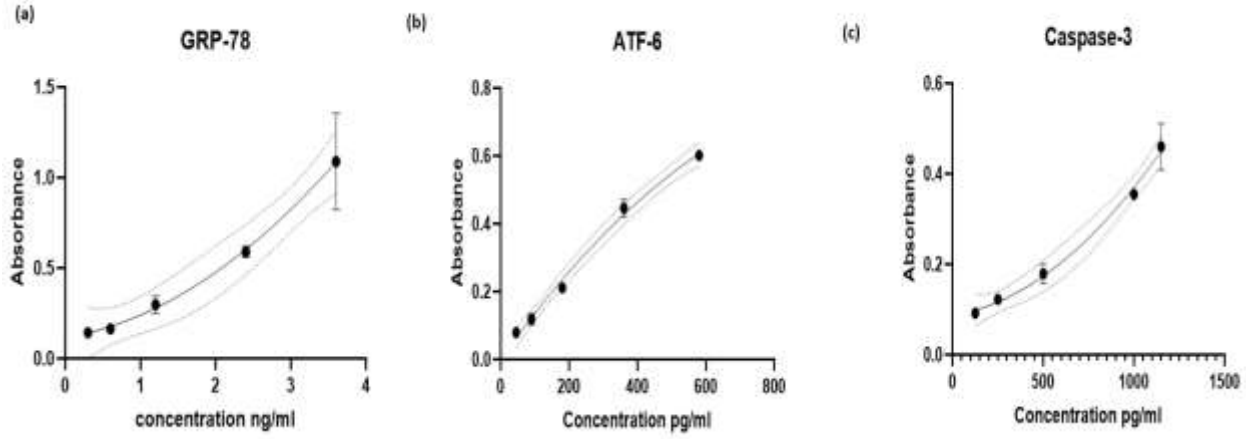

Fig. 1. Calibration curves as by Graphpad Prism 9 used for interpolation of tissue concentration levels of human (a) GRP78 (ng/ml), (b) ATF6 (pg/ml) and, (c) Caspase3 (pg/ml).

RNA Extraction

Total RNA with preserved micro-RNAs were extracted by miRNeasy Mini kit (50) (Qiagen, GmBH, Germany) from homogenized skin samples in 700 µl QIAzol lysis reagent, after incubation of the homogenate at room temperature for 5 minutes, 140 µL chloroform was added to each sample followed by another incubation for 3 minutes and centrifugation for 15 min at 12,000 xg. The upper aqueous phase was transferred to a new tube with 1.5 volume of 100% ethanol added. 700 µl of this mixture were added into Mini column in a 2 ml collection tube, centrifuged at ≥8000x g for 15s at room temperature. After discarding the flow-through, step was repeated twice; once after adding 700 µL of buffer RW, second after adding 500 µl Buffer RPE. Finally, 50 µl RNase-free water was directly pipetted onto the Mini column membrane, centrifuged for 1 min at ≥8000 xg for elution. Extracted RNA was then stored at -80 °C until use.

Reverse transcription (RT) and Quantitative real time PCR (qRT-PCR)

lncRNA MEG3

Extracted RNA was reversely transcribed into complementary DNA (cDNA) using (TransScript® Green One-Step qRT-PCR SuperMix Beijing, China). A final 20 µl preparation of cDNA template, a mix of unique thermostable Moloney murine leukemia virus (M-MuLV) reverse transcriptase enzyme, Taq DNA Polymerase, SYBR Green dye, ROX dye, and MEG3 primers Forward: CTGCCCATCTACACCTCACG, Reverse: CTCTCCGCCGTCTGCGCTAGGGGCT was allowed to react under the following thermocycling conditions: commenced with 45 °C for 5 minutes followed by 94 °C for 30 sec then 40 cycles of denaturation at 94 °C for 5 sec followed by annealing 50 °C for 15 sec and extension at 72 °C for 10 sec as per the manufacturer's protocol, the florescence was detected using thermal cycler (Step One Applied Biosystem, Foster city, USA). Results were normalized to β-actin as reference gene F: GGC GGCACCACCATGTACCCT R: AGG GGCCGGACTCGTCATACT.

microRNA-21

cDNA was reverse transcribed from micro total RNA samples following the manufacturer's instructions of the (TransScript® miRNA First-Strand cDNA Synthesis SuperMix kit Beijing, China). A 20 µl reverse transcription mix has been prepared of micro total RNA, universal miRNA qPCR Primer, RT Buffer M-MuLV, M-MuLV Reverse transcriptase, RNase Inhibitor and nuclease-free water. Mix was the incubated at 37 °C for 60 minutes followed by 5 minutes at

85 °C to stop the RT reaction. Real-time qPCR amplification and analysis were performed using (SYBR Green PCR kit PerfectStart™ Green qPCR SuperMix Beijing, China). A 20 µl mix of Taq qPCR Green Master Mix, forward Primer CAGATCAGCCGCTGCACA, reverse primer TGCCCACCGCACAC, template cDNA and nuclease free water was prepared then florescence was detected using thermal cycler (Step One Applied Biosystem, Foster city, USA) under the following thermocycling conditions: 40 cycles of denaturation at 94 °C for 5 sec followed by annealing 50 °C for 15 sec and extension at 72 °C for 10 sec. Results were normalized to U6 as internal reference gene (5'-GCTTCGGCAGCACATATACTAAAAT-3') (5'-CGCTTCACGAATTTGCGTGTCAT-3').

Statistical analysis

Relative expression (RQ) was calculated using the $2^{-\Delta\Delta Ct}$ method, (a) $\Delta Ct = Ct_{\text{assessed gene}} - Ct_{\text{reference gene}}$ (b) $\Delta\Delta Ct = \Delta Ct_{\text{sample}} - Ct_{\text{internal control gene}}$ (c) $RQ = 2^{-(\Delta\Delta Ct)}$. Data were analysed using the statistical package SPSS version 22, Chi^2 test being used when comparing categorical data. Numerical data was reviewed using mean and standard deviation. Comparisons between the two groups were done independent t test for normally distributed data and Mann Whitney test for non-normally distributed data. ROC analysis was done to detect the diagnostic value miRNA21 and MEG3 for psoriasis patients. Correlations between quantitative variables were done using Pearson and spearman correlation coefficient (26).

Results

Eighty subjects were enrolled in our study, mean age for healthy subjects (37.3 ±13.6), while for Psoriasis patients (41.1 ±17.6), 20 (50%) males and 20 (50%) females in the control group, 15 (35%) males and 26 (65%) females with Psoriasis Vulgaris, there were no significant differences between males and females in psoriasis group regarding studied parameters except that males showed longer disease duration (p value =0.03), mean duration of Psoriasis ranged (1-480) months 129.45±123.3. The mean of Psoriasis Area and Severity Index (PASI) score was 11.96± 3.19 with range (7.3-20.3), it showed significant increase as the age increases (p value =0.012). Our study showed significant decrease in MEG3 expression in psoriatic lesions 0.0012±0.0019 compared to normal skin 1±0 (p value <0.001) which was on contrary to miRNA-21 expression that was markedly elevated in psoriatic lesions 49.57±34 compared to normal skin 1±0 (p value<0.001) (Fig. 2).

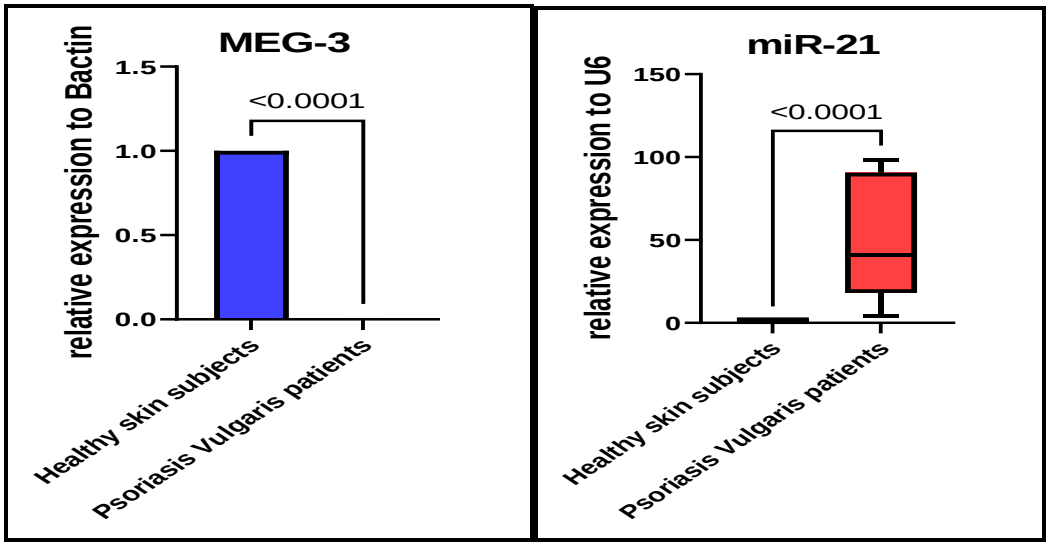

Fig. 2. When compared to healthy skin tissue, MEG3 is barely expressed in psoriatic tissue while miRNA-21 expression is remarkably high is Psoriatic lesions.

MEG3 and miRNA-21 showed positive correlation with (r=0.279, p value= 0.081) ER stress proteins GRP-78, ATF-6, and caspase 3 were all significantly decreased in psoriatic lesions when compared to healthy skin (p value<0.001) (Fig. 3).

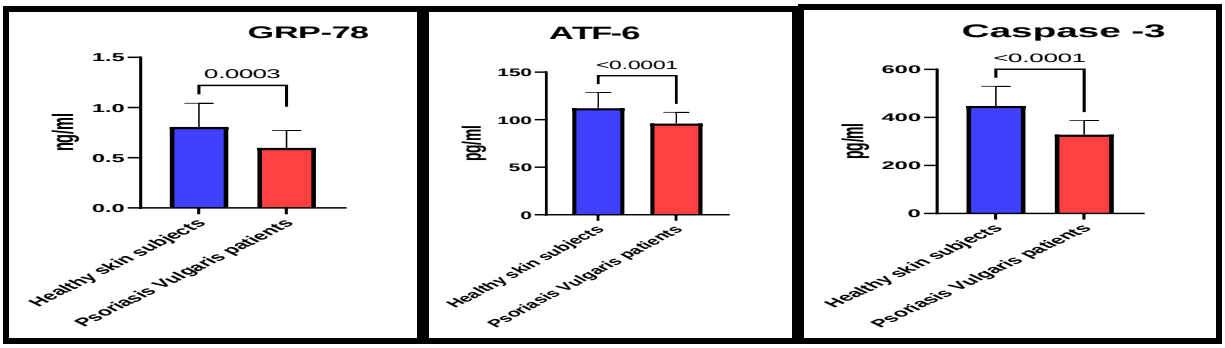

Fig. 3. Human GRP-78, ATF-6 and caspase 3 levels in psoriatic lesions significantly decreased than health skin tissue.

In a trial to find whether MEG-3/miRNA-21 axis and ER stress proteins, age, PASI and disease duration, are correlated or not, we studied their correlation in the Psoriasis group, only miRNA-21 and ATF-6 showed significant inverse correlation (r=-363, p value = 0.02) (Fig. 4), while in the control group any correlation was detected between studied parameters.

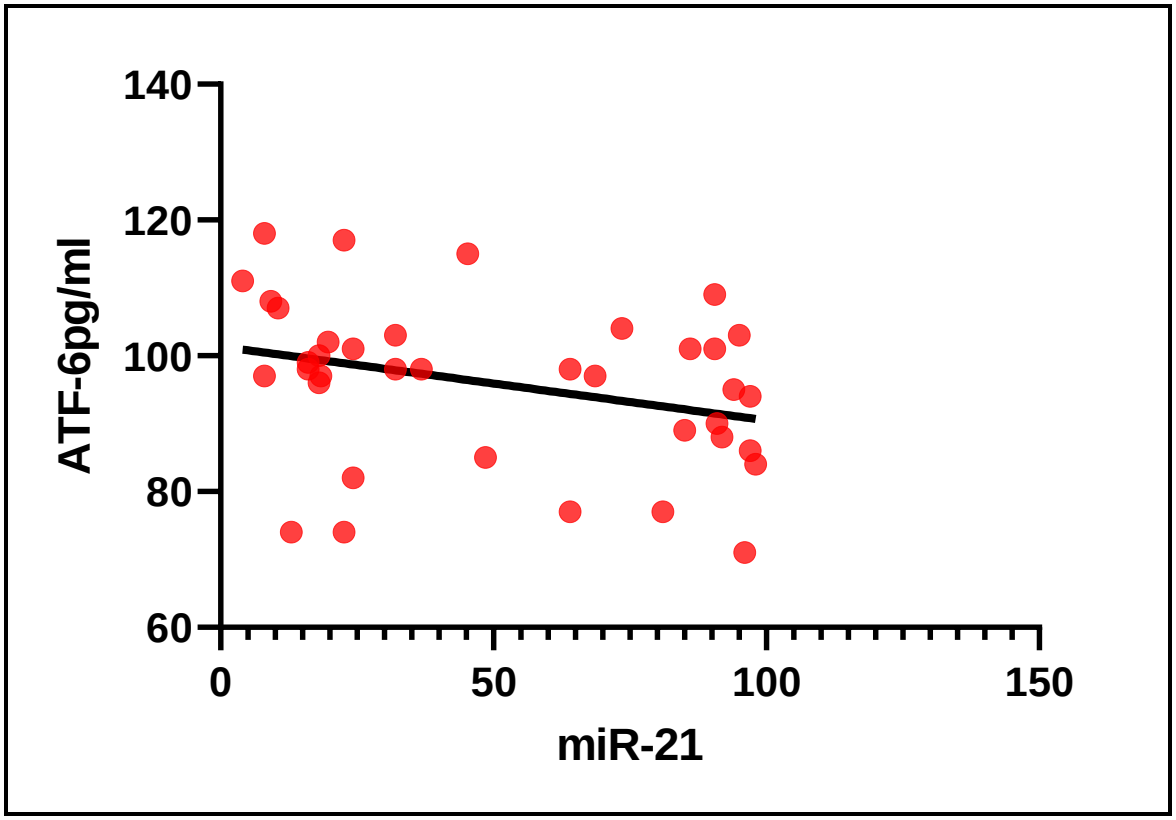

Fig. 4. Inverse significant correlation between miRNA-21 and ATF6.

MEG3/miRNA21 axis could be used as a diagnostic marker for Psoriasis (p value<0.001, AUC=1) at cut off value 6 sensitivity 97%, specificity 99% and accuracy 98% for microRNA 21 and (p value<0.001, AUC=1) at cut off value 0.002 sensitivity 80%, specificity 20% and accuracy 50% for MEG3 (Fig. 5).

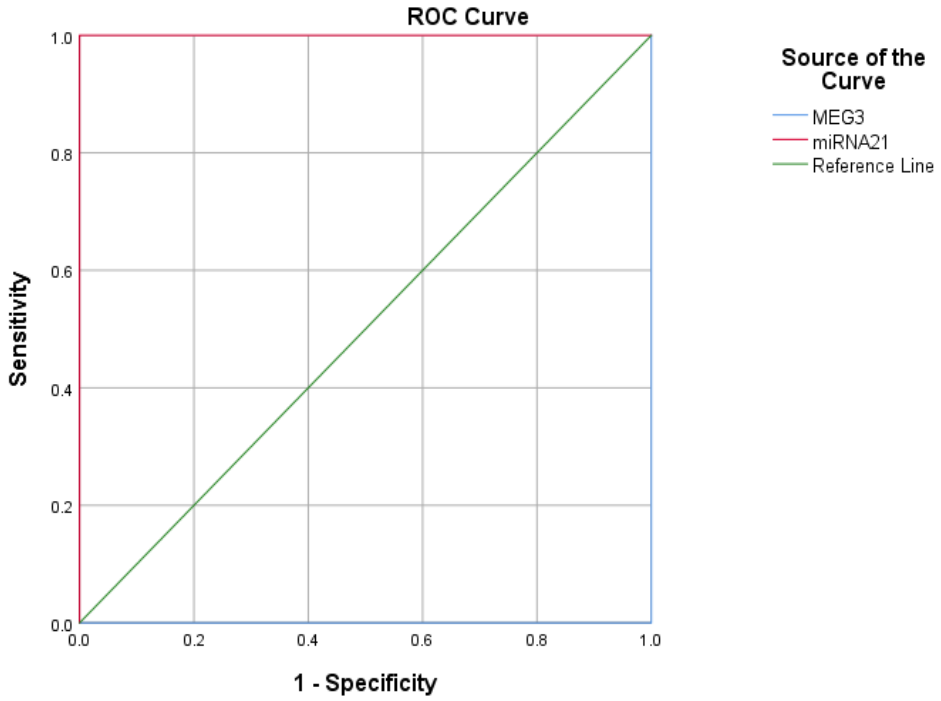

Fig. 5. Specificity and sensitivity of MEG 3 and miRNA 21.

Discussion

Psoriasis is a common, immune-mediated chronic inflammatory skin disorder affecting 1–3% of the population (12). ncRNAs and miRNAs have functional interactions involved in the regulation of immune response and the pathophysiology of inflammatory disorders such as psoriasis. Massive dysregulation of ncRNAs in the psoriasis may take part in the psoriasis pathogenesis (7). LncRNAs play critical functional roles in epigenetic, transcriptional, or post transcriptional regulation by acting upon regulated target genes (27). MicroRNAs, as endogenous 20 –24 nucleotide non -coding small RNAs, are considered as major players in the post -transcriptional regulation of gene expression, which are involved in metabolism, cell proliferation and death (28). ER stress pathways take part in immunity and inflammation and could be both a trigger and/or a consequence of chronic inflammation (29). The present study aimed to detect the possible role of MEG3/miRNA-21 axis and ER stress proteins in the pathogenesis of psoriasis vulgaris. We found that males had significantly longer disease duration compared to females which was on contrary to Hägg et al study where the disease duration was longer for women as compared to men (30). While other studies about gender differences in psoriasis had any statistically significant difference between both genders (31,32). In the current study, expression of MEG3 was significantly lower among psoriasis patients compared to healthy skin group which is in concordance with Jia et al who made an in vitro model for assessment of the role of MEG3 in the pathogenesis of psoriasis using cultured human keratinocytes. Expression of MEG3 was decreased in these cells and in psoriatic skin specimens (16). Regarding other diseases characterized with high proliferation rate such as carcinomas, Huang et al studied the expression of MEG3 in esophageal squamous cell carcinoma tissues and normal esophageal tissues, their results showed significantly low levels of MEG3 expression in esophageal squamous cell carcinoma tissues compared to normal tissues with expression level of MEG3 negatively correlated with prognosis (24). A very recent study found that MEG3 was downregulated in breast cancer (33). These

studies suggests MEG3 plays a critical role in controlling cell proliferation and apoptosis (34). miRNA 21 expression in our study was considerably higher among psoriasis patients compared to control this is supported by a recent study by Navarro et al that demonstrated an overexpression of miRNA-21 in skin abrasions of patients with psoriasis (35), another study emphasized our results showed amended pathological features both in xenotransplants and in a psoriasis-like mouse model after suppressing miRNA-21 which indicated the role of miRNA-21 as a therapeutic target in psoriasis (36). Unexpectedly a non-significant positive correlation between expression of MEG3 and miRNA-21 though Jia et al in their vitro study showed a negative regulatory relationship between lncRNA MEG3 and miRNA-21, suggesting MEG3 should be a competitive endogenous RNA (ceRNA) of miRNA-21 in psoriasis (16)**.** However, the impact of MEG3 on miRNA-21 during the proliferation and apoptosis of epidermal cells in psoriasis remains unclear and needs further studies. Regarding assayed levels of ER stress proteins GRP-78, ATF6 and caspase 3 in the present study, their low levels in cases compared to control go with Sugiura et al study which concluded that the UPR is activated in differentiating KCs compared with proliferating KCs, proved by immunocytochemistry performed on psoriasis vulgaris tissues with anti-GRP78 antibodies, GRP78 staining was weak in the KCs in the epidermis but was intense around the granular layers (19). These results emphasized that undifferentiated KCs had slight activation of the UPR. Expression of miRNA-21 among psoriasis vulgaris group was inversely correlated with expression of ATF6, Zhang et al study on human breast cancer cell line, showed that MEG3 overexpression markedly upregulated the three major UPR members (IRE1, ATF6, and PERK) as well as the proapoptotic caspase 3, suggesting MEG3 activated both UPR and apoptotic pathways, it indicated MEG3 plays a pivotal role in activation of the endoplasmic reticulum (ER) stress pathway, which is linked to apoptosis (37). MEG3 overexpression increased the expression of ER stress-related proteins GRP78, ATF6, and caspase 3 in another study that also found that ectopic expression of MEG3 increased GRP78 expression in hepatoma cells, showing that MEG3-induced ER stress might not be a rare phenomenon in tumor cells (24). Similarly, in our study, a positive correlation was found between expression of MEG3 and GRP78 in psoriatic lesions, yet the correlation was non-significant. In this study a positive but non-significant correlation between MEG3 expression and caspase 3 which is in concordance with Zhang et al where they explored the potential molecular pathways responsible for the observed tumor growth inhibition and apoptosis by MEG3 in human breast cell line. They examined the apoptosis-associated caspase-3 and found marked upregulation of cleaved caspase-3 by MEG3 overexpression, suggesting that the key apoptotic pathway following ER stress (caspase-3) was activated by MEG3 (37). We found that miRNA21 could be a diagnostic marker for psoriasis, from another prospective Sabry et al. study also found that miRNA-21 was diagnostic for adenocarcinoma and adenomas (38). MEG3 was found to be a diagnostic marker for psoriasis in our study. Studies showed lncRNA MEG3 may be a diagnostic marker and prognostic indicator for cervical cancer and has a certain diagnostic value for lymph node metastasis. Low MEG3 levels were revealed to be independent prognostic factors for cervical cancer patients (39).

MEG3 under expression and miRNA- 21 overexpression suggested to promote cell proliferation, undifferentiation and, inhibit apoptosis, suggesting miRNA-21 inhibitors can be a therapeutic target for treatment of psoriasis. Undifferentiated epidermal cells of psoriatic lesions exhibit very weak UPR suggesting UPR inducing agents might be useful to treat disorders with altered epidermal differentiation.

Acknowledgements

All authors declare no conflict of interest, research was funded by Cairo university. Appreciation and gratitude to Medical Biochemistry and Dermatology departments, Faculty of Medicine, Cairo University.

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