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The Grainyhead-Like Transcription Factor 3 Expression Pattern in Relation to the Caveolin 1/ Mammalian Target of Rapamycin (mTOR) Axis in Non-Melanoma Skin Cancer

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ABSTRACT

Background: Non-melanoma skin cancers (NMSCs), including both basal and squamous cell carcinoma, have increasing incidence worldwide. The grainyhead-like transcription factor-3 (GRHL3) is involved in skin barrier formation and wound healing. Caveolin 1 (cav-1)/mammalian target of rapamycin (mTOR) axis is implicated in different malignancies.

Objective: The study aimed to assess the expression pattern of GRHL3 in NMSCs and its correlation with cav-1/mTOR signaling pathway.

Patients and methods: The study involved twenty NMSC patients. Group1 was subdivided into groups 1a and 1b, which had tumour tissue from basal cell carcinoma (12 patients) and squamous cell carcinoma (8 patients) respectively. Group 2 had tissue of safety margin of same patients, and was also subdivided into 2a, and 2b groups. The levels of cav-1, malondialdehyde, total anti-oxidant capacity, and the gene expression levels of GRHL3, mTOR, autophagy-related gene 7 (ATG7) were assayed in the tumour tissue and the nearby safety margin. The immunohistochemical expression of cav-1 was determined.

Results: The study showed upregulation of the levels of malondialdehyde, and the gene expression levels of mTOR, and ATG7 in tumour tissue compared to tissue of safety margin. On the other hand, it showed downregulation of caveolin 1, total anti-oxidant capacity, and gene expression levels of GRHL3 in tumour tissue compared to safety margin tissue. Both GRHL3 and cav-1 showed significant negative correlation with mTOR and ATG7.

Conclusion: GRHL3 and cav-1 might have tumour suppressor effects. Oxidative stress might trigger mTOR signaling. The rise of the gene expression levels of mTOR and ATG7 indicates autophagy activation in basal and squamous cell carcinoma. Cav-1 might have inhibitory effect on autophagy.

Keywords: Grainyhead-like transcription factor 3, Mammalian target of rapamycin, Caveolin 1, Autophagy-related gene 7, Non melanoma skin cancer.

INTRODUCTION

Skin cancer represents one in every three diagnosed cancer patients. Non-melanoma skin cancer (NMSC) comprises basal cell carcinoma (BCC), and cutaneous squamous cell carcinoma (cSCC) [(1)]. Basal cell carcinoma, which is referred to as rodent ulcer, Jacob's ulcer, basalioma cancer, and basal cell epithelioma, arises from epidermal basal membrane, and it frequently shows local invasion [(2)].

Cutaneous BCC is the most common type of NMSCs followed by cSCC [(3)]. Cutaneous SCC is more aggressive than cBCC, showing a high potential for metastasis [(1)] .

The grainyhead-like transcription factor-3 (GRHL3) is crucial for epidermal wound healing, and neural tube closure [(4)]. GRHL3 has tumour suppressor function for some solid tumours [(5)]. The mammalian or mechanistic target of rapamycin (mTOR) is a serine/threonine kinase, that regulates cell proliferation, metabolism, immune responses, and autophagy [(6)]. Autophagy-related gene 7 (ATG7) is a protein, which controls the cell cycle via affecting p53, and apoptosis in various diseases [(7)]. Based on its role, autophagy can be classified into ATG7-dependent and ATG7-independent [(8)]. Caveolin 1(cav-1) gene encodes a 22 kDa protein [(9)] that occupies plasma membrane invaginations called caveolae [(10)], and modulates glycolytic activities and cholesterol distribution [(9)]. It is also a key regulator of adhesion, migration [(10)], endocytosis, and autophagy [(9)].

The interplay between the cav-1/mTOR signaling pathway and oxidative stress can be evaluated by the assay of malondialdehyde (MDA) and total anti-oxidant capacity (TAC). The interplay between cav-1, mTOR signaling pathway, and oxidative stress has been studied in multiple diseases. However, their role in NMSC hasn't been clearly elucidated. This study aimed to determine the expression pattern of grainyhead-like transcription factor 3 in the NMSC patients and to assess its correlation with cav-1/mTOR signaling pathway in order to elucidate their potential contribution in the disease pathogenesis.

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PATIENTS AND METHODS

The current study involved 12 cBCC, and 8 cSCC patients, presented to Tanta University Hospital, Plastic and Reconstructive Surgery Department. Patients with any other comorbedity were excluded. Tumour tissue of all patients was surgically excised, with safety margin of about 1cm of normal skin followed by reconstruction. The excised tissue was divided into specimens for histopathological examination and biochemical analysis. The present study was carried out on two groups. Group (1) comprised the tumor specimens of the enrolled 20 patients, it was further sub divided into group (1a) including specimens of BCC (12 patients), and group (1b) including specimens of SCC (8 patients). Group (2) comprised the normal tissue of the safety margin obtained from the 20 patients enrolled in this study, which was subdivided into group (2a) including normal safety margins from BCC patients, and group (2b) including normal safety margins from SCC patients.

Inclusion criteria: Patients diagnosed as basal cell carcinoma or squamous cell carcinoma.

Exclusion criteria: Other types of skin carcinoma, and chronic diseases.

Tissue sampling: After resection, frozen samples of the tumour and normal tissues were thoroughly cleaned in cold normal saline 0.9% to remove any remnants of blood. Samples were homogenized in 1.5 ml homogenization buffer per one gram tissue. The homogenization buffer contains 150 mM NaCl ,10 mM Tris pH 7.4, 1% Triton X-100. The lysate was then centrifuged in a cooling centrifuge (at 4 °C) for 5 minutes at 4,000 rpm. Followed by collecting the supernatants, and its storage at -80 °C [11]. Lowry assay was used to assess total protein concentration of homogenate samples [12].

Quantitative real-time polymerase chain reaction (qPCR): TRIzol reagent (Invitrogen, Carlsbad, CA, USA, 15596026) was used for total RNA extraction, whose purity and concentration were determined, using NanoDrop spectrophotometer from NanoDrop Technologies, Inc., Wilmington, NC, USA. Revert Aid H Minus Reverse Transcriptase (Thermo Scientific, #EP0451, Waltham, MA, USA) was used to produce complementary DNA (cDNA), which acts as a template (2 µL) for relative gene expression using SYBR-green qPCR (Thermo scientific, USA, catalog number K0221). Step One Plus real-time PCR system from Applied Biosystem, USA was used. β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) act as housekeeping genes. Relative gene expression was calculated by $2^{-\Delta\Delta Ct}$ method [13]. The used primers were presented in table (1) [14-16].

Table (1): The used primers

Gene	Primer sequence
GRHL3	Forward: 5′-ACGTAGTTTTACCAGTG-3′ Reverse: 5′-AACCCTCCTAGGTGTCAG-3′
ATG7	Forward: 5′-TCG AAA GCC ATGATG TCG TCT T-3′ Reverse: 5-′CCA AAG CAG CATTGA TGA CCA-3′
mTOR	Forward: 5′-GCAGAT TTG CCA ACT TCG G-3′ Reverse: 5′-CAG CGG TAA AAG TGTCCCCTG-3′
β -actin	Forward: 5′-AGG CCA ACCGCG AGA AGATGACC -3′ Reverse: 5′-GAAGTCCAG GGCGACGTAGCAC -3′
GAPDH	Forward: 5′-CATGAGAAGTATGACAACAGCCT-3′ Reverse: 5′-AGTCCTTCCACGATACCAAAGT-3′

Immunoassay of caveolin-1: The amount of cav-1 in tissue samples was assessed, using a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit from MyBioSource, Inc., San Diego, California, USA (CAT# MBS3803931). The findings were quantified at 450 nm, using the ELISA reader from RT-1904C Chemistry Analyzer; Rayto, Atlanta, GA, USA, and were expressed as ng/mg. protein.

Assay of redox status markers (malondialdehyde and total anti-oxidant capacity):

The content of MDA (nmol/mg protein) in the supernatants of tissue homogenate was estimated according to the manufacturer's protocol of Bio-diagnostic kit (Giza, Egypt, CAT# MD 25 29), where absorbance was determined at 534 nm versus blank utilizing semiautomatic BTS-350 Biosystems spectrophotometer.

Total antioxidant capacity (TAC) was measured as described by Erel O [17]. Aliquots from the supernatant of the skin tissue homogenate were added to 150 μL of a cationic 2,2'-azino-bis-(3-ethylbenzot-hiazoline-6-sulphonic acid) (ABTS) solution, and 0.4 M acetate buffer (pH 5.8). The cationic ABTS solution contains 10 mM ABTS ,30 mM acetate buffer, pH 3.6, and 4 mM H_2O_2. After dark incubation for 5 min, a soluble green colored chromogen of a radical cation $ABTS^+$, was formed that can be determined at 734 nm. The compound 6-hydroxy-2, 5, 7, 8-tetramethyl-chloraman-2-carboxylic acid (Trolox) represents a standard. Findings were expressed as μmol Trolox equivalents/g protein.

Histopathological study: Sections from the paraffin blocks were serially cut at 5 µm thickness and placed on glass slides in order to be stained basically with

Hematoxylin & Eosin staining, then examined to confirm the histopathologic diagnosis and to exclude badly fixed tissue and/or cases with extensive necrosis. Representative histopathologic images of SCC and BCC were shown in figure (1).

Immunohistochemical (IHC) expression of cav-1: Five µm thick paraffin sections were deparaffinized in xylene, then rehydrated using ethanol in descending concentrations. After that, the sections were washed in phosphate buffered saline (PBS) and placed in peroxidase-blocking reagent for 10 min. Each section was subjected to the primary antibody (E249)- Caveolae marker ab32577- used in the dilution of 1:100. Afterwards, slides were subjected to horseradish perioxidase polymer reagent for 20 minutes and then counter-stained with hematoxylin. Images were captured using Leica DM500 microscope with built-in Leica ICC50 digital camera. Caveolin 1 immunoreactivity seen in fibroblasts and endothelial cell lining of blood vessel wall acted as an internal control. Referring to the methodology adopted by **Mishra *et al.* [18]** and **Hung *et al.*** [19]. Representative images are displayed in figure (2). The grading of cav-1 expression in tumor cells was as follows:

- Low expression: <10% of tumor cells show cav-1 positive expression.
- Moderate expression: 10-50% of tumor cells show cav-1 positive expression.
- High expression: >50% of tumor cells show cav-1 positive expression.

Ethical approval: Informed consents were provided by all patients for participation in the study. All the procedures followed the recommendations of the Ethical Committee of Medical Research at Tanta Faculty of Medicine with approval code 36264PR241/6/23. The study adhered to the Helsinki Declaration throughout its execution.

Analysis of data

Data were expressed as numbers, percentages, and mean ± standard deviation (SD). Both Student t-test, for comparison between two groups, and Pearson coefficient, for correlation, were used. P was statistically significant if it was ≤ 0.05. The used software was IBM SPSS version 20.0. (Armonk, NY: IBM Corp).

RESULTS

Clinical presentation and demographic data: The group under study included 20 patients: 12 patients had cBCC and the remaining 8 patients had cSCC. The age of the studied group ranged between 37 to 72 years old.The clinical presentation and demographic data were illustrated in table (2).

Table (2): Distribution of the studied groups according to different parameters

	BCC (n = 12)	**SCC (n = 8)**
Gender		
Male	9 (75.0%)	7 (87.5%)
Female	3 (25.0%)	1 (12.52)
Age (years)		
Mean ± SD.	54.08 ± 12.53	57.25 ± 9.39
Clinical presentation		
Nodular	1 (8.3%)	0 (0.0%)
Ulcerative	9 (75.0%)	5 (62.5%)
Pigmented nodular	2 (16.7%)	0 (0.0%)
Marjolin ulcerative	0 (0.0%)	3 (37.5%)
Localiztion		
Facial	12 (100.0%)	4 (50.0%)
Lower limb	0 (0.0%)	3 (37.5%)
Scalp	0 (0.0%)	1 (12.5%)
Grading		
I		2 (25.0%)
II		4 (50.0%)
III		2 (25.0%)

SD: Standard deviation

Histopathological evaluation and Immunohistochemical expression of cav-1:

In the current study, 11/20 (55%) of control group participants showed high cav-1 positive immunoreactivitiy and 9/20 (45%) showed moderate cav-1 reactivity, while SCC cases exhibited low cav-1 expression in 6/8 cases (75%) and the remaining 2 cases (25%) showed moderate cav-1 expression. As regards BCC cases, low cav-1 expression was detected in 6/12 cases (50%) and the other half showed moderate cav-1 immunoreactivity. On analyzing the difference in cav-1 expression in control group cases versus SCC cases and BCC cases, a statistically significant relation was detected in both tests (p-value 0.003 and 0.002) respectively.

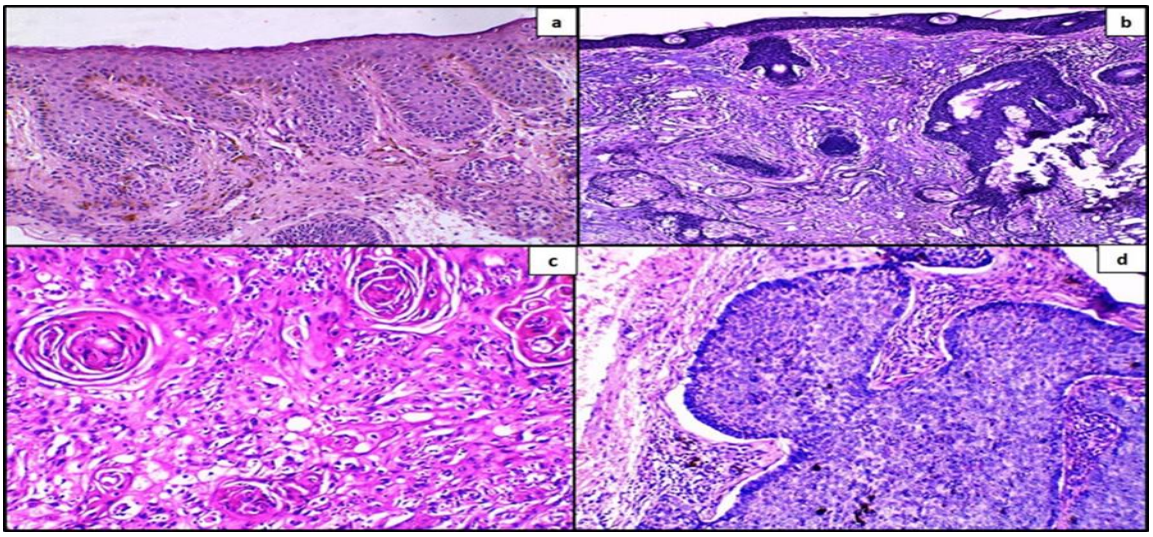

Figure (1): Histopathologic images of SCC and BCC studied cases.

(a) Skin tissue from the safety margin of a SCC case showing intact epidermis with prominent rete ridges and melanocytic hyperplasia in basal cell layer. No malignant epithelial cells could be detected in the dermis. (b) Normal looking skin excised as a safety margin from BCC case showing keratinized stratified squamous epidermal epithelium with adnexal structures of hair follicles and sebaceous glands in the dermis, which was free of any malignant basal epithelial cells infiltration. (c) A case of SCC showed infiltration of the dermis by sheets of malignant cells with moderate degree of differentiation, where keratin pearls are focally detected. The cells were polygonal in shape with eosinophilic cytoplasm and intercellular bridges and exhibit pleomorphic, hyperchromatic nuclei and high nuclear/cytoplasmic (N/C) ratio. (d) A case of BCC showed a basaloid epithelial tumour arising from the epidermis and infiltrating the dermis with clusters of malignant basaloid cells typically exhibiting peripheral palisading with cleft from the adjacent tumor stroma. The cells showed malignant criteria of nuclear pleomorphism, hyperchromasia and high N/C ratio. Besides, increased number of melanocytes were seen within the tumor.

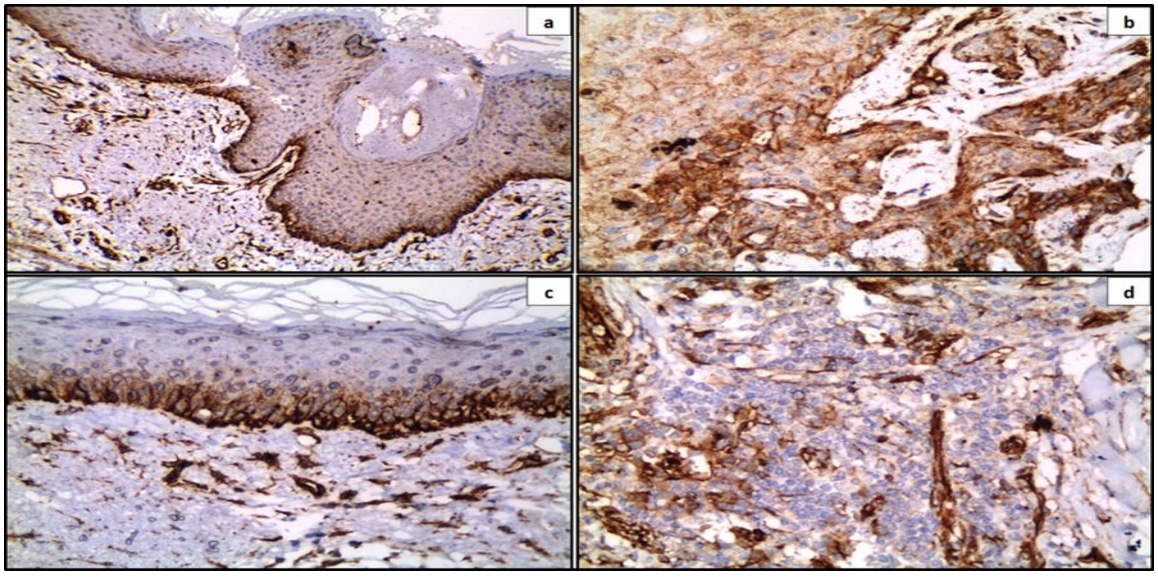

Figure (2): Caveolin1 immunohistochemical expression in studied cases **(**a & c) High cav-1 expression in control group of BCC and SCC respectively (IHCx200), (b) moderate cav-1 expression in BCC (IHCx400) and (d) low cav-1 expression in SCC.

The biochemical analysis within the studied groups: As regards to the comparison between different parameters in cBCC groups, they showed significant upregulation in mRNA expression levels of mTOR, ATG7, and MDA in patients group (1a) relative to group (2a). However, there was significant downregulation in levels of cav-1, mRNA expression levels of (GRHL3), and (TAC), in patients group (1a) compared to group (2a). The cSCC group (1b) showed significant increase in mRNA expression levels of mTOR, ATG7, and MDA compared to the control group (2b). However, there was significant decline in levels of cav-1, mRNA expression levels of (GRHL3), and (TAC) in patients' group (1b) compared to control group (2b), as illustrated in table (3).

Table (3): Comparison between Group (1) and Group (2) according to different parameters in cBCC and cSCC groups

	cBCC (n= 24)		**p**
	Group (1a) (n = 12)	**Group(2a) (n = 12)**	
mTOR expression	3.37 ± 0.70	1.0 ± 0.0	<0.001*
Caveolin 1(ng/mg)	8.93 ± 3.49	30.17 ± 13.79	<0.001*
GRHL3 expression	0.68 ± 0.16	1.0 ± 0.0	<0.001*
ATG7 expression	2.34 ± 0.75	1.0 ± 0.0	<0.001*
MDA (nmol/mg)	5.61 ± 1.54	1.88 ± 0.92	<0.001*
TAC (µmol TE /g)	355.0 ± 107.5	730.0 ± 179.9	<0.001*
	cSCC (n = 16)		**p**
	Group (1b) (n= 8)	**Group (2b) (n= 8)**	
mTOR mRNA expression	2.39 ± 0.54	1.0 ± 0.0	<0.001*
Caveolin 1 (ng/mg)	13.20 ± 5.09	32.23 ± 13.64	<0.005*
GRHL3 expression	0.28 ± 0.15	1.0 ± 0.0	<0.001*
ATG7 expression	3.79 ± 1.07	1.0 ± 0.0	<0.001*
MDA (nmol/mg)	9.11 ± 1.34	1.75 ± 0.90	<0.001*
TAC (µmol TE /g)	307.5 ± 99.53	735.0 ± 192.0	<0.001*

p: p value for comparing Group (1) and Group (2) *: Statistically significant at $p \leq 0.05$

Correlations between numbers of studied parameters: The current study displayed significant negative correlation between the gene expression level of GRHL3 and the gene expression levels of mTOR and ATG7. It also showed significant negative correlation between the caveolin-1 level and the gene expression levels of mTOR and ATG7, as illustrated in table (4).

Table (4): Correlation between different parameters in cBCC and cSCC groups

		Caveolin 1 (ng/mg)	mTOR mRNA expression	mRNA expression levels of (ATG7)	TAC (µmol Trolox equivalent) (TE) /g	(MDA) (nmol/mg)
cBCC (n = 24)						
mRNA expression levels of (GRHL3)	**r**	0.592	- 0.755	- 0.645	0.734	- 0.711
	p	0.002*	< 0.001*	0.001*	<0.001*	< 0.001*
Caveolin 1 (ng/mg)	**r**		- 0.705	- 0.591	0.525	- 0.563
	p		< 0.001*	0.002*	0.008*	0.004*
mTOR mRNA expression	**r**			0.685	- 0.772	0.828
	p			<0.001*	< 0.001*	<0.001*
mRNA expression levels of (ATG7)	**r**				- 0.658	0.730
	p				<0.001*	<0.001*
(TAC) µmol Trolox equivalents (TE) /g	**r**					- 0.618
	p					< 0.001*
cSCC (n = 16)						
mRNA expression levels of (GRHL3)	**r**	0.670	- 0.800	- 0.820	0.775	- 0.954
	p	0.005*	<0.001*	<0.001*	<0.001*	<0.001*
Caveolin-1(ng/mg)	**r**		- 0.606	- 0.553	0.497	- 0.700
	p		0.013*	0.026*	0.049*	0.003*
mTOR mRNA expression	**r**			0.918	- 0.715	0.842
	p			< 0.001*	0.002*	< 0.001*
mRNA expression levels of (ATG7)	**r**				- 0.709	0.796
	p				0.002*	< 0.001*
TAC (µmol Trolox equivalents) (TE) /g	**r**					- 0.780
	p					< 0.001*

r: Pearson coefficient *: Statistically significant at $p \leq 0.05$

DISCUSSION

Skin cancers has high incidence globally [1]. The gene GRHL3 encodes a transcription factor involved in skin barrier formation and wound healing [4]. Both cav-1 and mTOR modulate cellular autophagy [6,9] . The interplay between cav 1 and mTOR has been studied in number of malignancies. The present study aimed to elucidate the expression pattern of GRHL3 in the NMSC patients and its correlation with cav-1/mTOR signaling pathway.

The current study displayed downregulation of the GRHL3 expression level in tumour samples compared to normal skin of same individuals, which aligns with the findings of **Kikulska *et al.*** [20]. **Deng *et al.*** [21] suggested that elevated expression of miR-21, decreases the GRHL3 expression and its direct target, phosphatase and tensin homolog (PTEN), leading to activation of phosphatidylinositol 3-kinase (PI3K)-protein kinase B (AKT) signaling pathway. **Mlacki *et al.*** [22] postulated that GRHL3 has protective role against cSCC.

Significant reduction of cav-1 level in diseased group was detected in the current study. This could be reinforced by the findings of **Gheida *et al.*** **[23]** who declared decline of cav-1 immunohistochemical expression in cSCC and cBCC relative to normal control skin. **Takamura *et al.*** **[3]** documented that overexpression of cav-1 suppressed cellular growth of cSCC in rodents and humans. So, cav-1 could be considered as a tumor suppressor for NMSC [24, 25]. The tumour suppressor effect of cav-1 could be attributed to multiple mechanisms as, cav-1 induces G0/G1 cell cycle arrest via upregulating p53 and p21, and downregulating cyclin D1130 [24]. Cav-1 also sequesters β-catenin resulting in reduction of cyclin D1 level [24, 25]. **Egger *et al.*** **[24]** added that low levels of cav-1 results in rise in cyclin D1 activity, activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and enhancing proliferation. Furthermore, cav-1 downregulation hinders connexin 43 localization, leading to activation of rat sarcoma virus /Activator protein 1 (Ras/AP-1) pathway and accelerating proliferation [3].

The current study showed an increase of mTOR gene expression level in tumour group which can be supported by the findings of **Hoesl *et al.*** [1] who reported activation of mTOR/ myelocytomatosis oncogene (MYC) pathway in NMSC. **Chamcheu *et al.*** [26] attributed the pathogenesis of NMSC to alterations of PI3K/AKT/mTOR signaling. Thus, mTOR contributes to tumour pathogenesis by triggering cellular proliferation and metabolism [27].

In normal tissue, autophagy has tumour suppressor impact as it cleans up misfolded proteins and damaged

organelles. On the contrary, autophagy enhances survival of established tumour cells, as it provides nutrients and energy [28]. ATG7 showed higher expression level in current study, which aligns with significant high immunohistochemical expression in NMSC reported by **Samaka *et al.*** [29]. **Chen *et al.*** [30] added that ATG7 is implicated in radiation-induced fibrosis of skin cancer.

The present study displayed negative correlation between cav-1 and mTOR. Also, there was a negative correlation between cav-1 and ATG7. This is in harmony with the findings of **Xue *et al.*** [31] who documented that caveolin-1 promotes autophagy via inhibiting the Akt/mTOR axis in non-alcoholic fatty liver disease. There was a significant rise in ATG7 expression level in trabecular mesh network with knocked down caveolin 1, which is implicated in primary open angle glaucoma pathogenesis [32].

The current study supported the role of oxidative stress in NMSC pathogenesis, as NMSC tissue showed elevation of MDA and reduction of TAC in comparison with healthy tissue. The reduction of TAC levels aligns with the results of **Karampinis *et al.*** [33]. The elevation of MDA in cBCC was also reported by **Majidi *et al.*** [34]. However, **Williams *et al***. [35] reported rise of the MDA level in cSCC. According to **Fu *et al.*** [36], oxidative stress might lead to autophagic degradation of cav-1, which aligns with the results of the current study. According to **Su *et al.*** [37] oxidative stress triggers mTOR activation.

CONCLUSION

Collectively, in the current study, GRHL3 expression level displayed downregulation in NMSC, accompanied with decline of cav-1 level, and upregulation of mTOR, and ATG7 expression levels, which might contribute to the disease pathogenesis. Based on attained results, GRHL3 and cav-1 might have tumour suppressor effect. The rise in the expression levels of mTOR and ATG7 might give indication of activated autophagy in NMSC. Caveolin 1 might have inhibitory effect on autophagy via inhibiting mTOR and ATG7. Oxidative stress in NMSC might play a role in triggering mTOR signaling pathway. Extensive further studies are needed to confirm such proposals by knocking down of involved genes. It is also recommended to study targeting mTOR and ATG7 for therapeutic interventions.

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