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Investigating Expression of Skin Basal Cell Carcinoma Molecular Biomarkers

Chnoor Mohammed Faqe Qadir[1] and Karzan Ghafur Khidhir[1*]

[1]*Department of Biology, College of Science, University of Sulaimani, Sulaimani, Kurdistan Region- Iraq*
** Corresponding author's e-mail: karzan.khidhir@univsul.edu.iq*

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Abstract

Basal cell carcinoma (BCC) of skin is the most common type of human cancer. It is strongly linked with exposure to UV radiations and tumors mostly develop on the sun-exposed skin. Although BCC rarely metastasizes or causes mortality, it can result in broad morbidity through local invasion and causing tissue destruction. Hedgehog signaling pathway (Hh) have been linked to the development of BCC. The aim of this study was to investigate the expression level of the SHH, PTCH1, GLI1, P53 and SMO genes in BCC skin tissue in order to identify reliable biomarkers for clinical applications in the diagnosis of skin BCC. BCC and healthy skin samples were collected from patients who had pre-operation pathological verification, RNA extracted, cDNA synthesized and RT-qPCR was carried out to analyze the expression levels of SHH, PTCH1, GLI1, P53 and SMO genes. Results of the RT-qPCR analysis revealed significant statistical difference in relative expression of SHH, PTCH1, GLI1, P53 and SMO genes between the BCC and healthy tissue groups. The RT-qPCR analysis also showed that expressions of the target genes were upregulated in BCC skin compared to the healthy skin tissue: GLI1 by 3.1 folds, P53 by 0.844 folds, PTCH1 by 4.64 folds, SHH by 1.75 folds and SMO by 2.46 folds. Results of this study suggest that PTCH1 gene can be used as a molecular biomarker for diagnosing BCC of skin. Further studies of the BCC development signalling pathway genes may lead to improved methods for diagnosis and treatment of this common cancer.

1. Introduction

Basal cell carcinoma (BCC) is the most common malignancy in fair-skinned people and accounts for about two-thirds of all skin cancers [1, 2]. Though BCC rarely metastasizes or causes death, it can result in extensive morbidity through local invasion and producing tissue destruction [3]. Skin cancers can be subdivided in to basal cell and squamous cell carcinoma as well as melanoma [4]. The appearance of BCC is associated with exposure to UV radiations. As a result, tumors develop primarily on the sun-exposed areas of the skin especially in elderly people [5]. Men are more frequently affected by BCC frequently after the age of 50 due to having more outdoor activities. However, a notable increase in the incidence of BCC has been reported in young people, perhaps due to a higher use of tanning beds for cosmetic reasons [6]. Other than exposure to ultraviolet A radiation, ionizing radiation, oral methoxsalen, immunosuppression drugs and chemicals such as arsenic have also been linked to the growth of BCC [7].

BCC is derived from the keratinocytes of the basal stem cell layer of the epidermis in a multistage carcinogenesis process [8]. The first step is initiation which involves carcinogen-induced genetic changes, followed by promotion stage that includes processes in which initiated cells undergo expansion to form visible premalignant lesions termed papillomas. The third stage is progression stage which includes the conversion of papilloma to a tumor [9].

A broad range of therapies are available for the treatment of BCC, like excisional surgery, cryosurgery, radiation therapy, laser surgery, topical medications and oral medications for advanced BCC such as Hh pathway inhibitor, Vismodegib [10].

Cancer may develop as a result of dysregulation of signaling pathways associated with skin's homeostasis and development [11]. At the molecular level, various signaling pathways including those activated by the transforming of Hedgehog signaling pathways (Hh), has been linked to the development of BCC. Mutations in Hh signaling pathway genes, especially those encoding patched homolog 1 (PTCH1), smoothened homolog (SMO), and Gli are believed to play role in the onset and growth of BCC. Any stages of carcinogenesis are associated with differential expression of genes, which is not fully established yet [12, 13].

PTCH is a secreted molecule drawn in tumor genesis and in the embryonic tissue formation and its gene normally works as a tumor suppressor. The gene product of PTCH is a protein with Transmembrane that holds back the discharge of another protein known as smoothened (SMO). When the PTCH binds with the Shh, it signal cell proliferation and SMO is released. To stimulate Hh signal transduction after cellular localization, SMO must be activated. Another transcription factor is Gli which is affected by the vital role played by SMO in its activation [14-16].

This study aimed to investigate the pathologically altered genes in BCC and evaluate the diagnostic reliability of the SHH, PTCH1, GLI1, P53 and SMO genes in the diagnosis of the common skin BCC.

2. Materials and methods

2.1. Sample collection

BCC skin samples of about 0.5-1 cm^2, with pre-operation pathological confirmation, and normal skin tissue were collected from 53 people, at the Sulaymaneyah teaching hospital, who underwent surgical operation between 3 September 2018 and 30 April 2019 with appropriate ethical approval. The samples were placed in RNA*later* stabilizing solution (Sigma-Aldrich, Gillingham, UK) immediately to prevent RNA degradation and stored until analyzed.

2.2. Tissue homogenization and RNA extraction

Disruption and homogenization of BCC and normal skin tissues (50 mg) from all the collected samples were carried out at the Sulaymaneyah central laboratories using Qiagen Tissue Lyser system, then followed by RNA extraction using RNeasy Plus Universal Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions.

2.3. Checking quality and concentration of RNA

RNA concentration from all the samples were measured by spectrophotometric NanoDrop instrument (Thermo Fisher Scientific Inc., Massachusetts, USA), and only samples of RNA with 260/280 ratio bigger than 1.8 and 260/230 near 2.0 were selected for the gene expression investigation. The quality and integrity of the RNA samples were checked by gel electrophoresis through the appearance of two clear bands indicating 18S and 28S ribosomal RNA (rRNA) on 1.5% agarose gels made with 1% ethidium bromide. The samples of RNA with significant degradation were discarded.

2.4. Synthesis of cDNA

To remove any contaminating genomic DNA, mRNA samples were treated with DNase I (Invitrogen, Paisley, UK), and complementary DNAs (cDNAs) were synthesized immediately using QuantiTect Reverse Transcription Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions.

2.5. Real-Time Quantitative PCR (RT-qPCR)

RT-qPCR was carried out to analyze the expression levels of SHH, PTCH1, GLI1, P53 and SMO genes using the QuantiTect SYBR Green RT-PCR Master Mix (Qiagen, Crawley, UK) and real-time cycler instrument (Applied Biosystems Fast 7500, CA, USA). The primers for the target genes (Table 1) were designed newly from the human gene sequence exon nucleotide data. All reactions were carried out in triplicate in 96-well plates. Each reaction mix was prepared in a 25µl reaction volume containing 2x QuantiTect SYBR Green Master Mix (1x final concentration), 0.5 µM forward and reverse primers, 0.5 µl QuantiTect RT Mix, cDNA (<500 ng/reaction, cDNA solutions were diluted 5-fold before RT-qPCR). For

the negative controls, cDNA was replaced with nuclease-free water to ensure that no primer dimers or genomic nucleic acid contaminations existed. The PCR cycling conditions were as follow: one initial activation cycle at 95C for 15 min, followed by 40 cycles of denaturation at 94 C for 15s and annealing 50-60C for 30 seconds and extension 72C for 30 seconds. After each PCR cycle, fluorescence data were collected to generate an amplification plot for determination of the Ct value. The melting curves for each amplification product was assessed by determining the decline in fluorescence from 95 to 60C. A distinct peak showed that no primer dimers affected the detected fluorescence (data not shown).

Table 1: Sequences of the RT-qPCR Primers

Primer	Sequence	Product Size
GAPDH	F: 5'-GAAGGTGAAGGTCGGAGTCA-3' R: 5'-AATGAAGGGGTCATTGATGG-3'	109 bp
SMO	F: 5'-CTTCAGCTGCCACTTCTACGACTTC-3' R: 5'- TCGGGCGATTCTTGATCTCAC-3'	143 bp
SHH	F: 5'-TGCTGCTAGTCCTCGTCTCCT-3' R: 5'-TTTTGGGGTGCCTCCTCTT-3'	90 bp
P53	F: 5'-ACAAGGTTGATGTGACCTGGA-3' R: 5'-TGTAGACTCGTGAATTTCGCC-3'	105 bp
PTCH1	F: 5'-AGCTCAGTGCCGTGCCC-3' R: 5'-CTCCAGGGCAAGCACAGC-3'	131 bp
GLI1	F: 5'-AGGGAGTGCAGCCAATACAG-3' R: 5'-ATTGGCCGGAGTTGATGTAG-3'	171 bp

2.6. Statistical analysis

SPSS software IBM-version 21.0 (Chicago, IL, USA) was used to analyze data of the experimental results expressed as mean ± standard error of the mean (SEM). Groups were compared by grouped T- test. Relative quantity (RQ) used to represent the relative mRNA expression level of the target genes measured by RT-qPCR. The delta-delta-CT (ΔΔCT) method was used to determine the level of expression of SHH, PTCH1, GLI1, P53 and SMO genes. P value less than 0.05 was regarded as statistically significant: * $p< 0.05$, ** $p< 0.01$, *** $p< 0.001$.

3. Results

To investigate relative expression levels of the SHH, PTCH1, GLI1, P53, SMO genes, RT-qPCR was performed on BCC and healthy facial skin samples using specific sets of forward and reverse primers for all the genes of interest.

Expression levels of the SHH, PTCH1, GLI1, P53 and SMO genes were investigated in mRNA of human BCC skin tissues (n=38) and healthy normal skin tissue (n=15) using RT-qPCR analysis. Specific primer set for each target gene and GAPDH, as an internal reference standard, were used. The delta-delta-CT (ΔΔCT) method was used to determine transcript levels of each gene [17].

Relative quantity (RQ) of target genes

Results of RT-qPCR analysis revealed that there is significant statistical difference in relative expression of SHH, PTCH1, GLI1, P53 and SMO genes between the two groups in favor of the BCC tissue Group: GLI1 gene (p=0.018), P53 gene (p=0.033), PTCH1 (p=0.003): SHH gene (p=0.039): SMO gene (p=0.013) (Figure 1). The expressions of the target genes were upregulated in BCC skin tissue compared to the control healthy skin tissue: GLI1 gene by 3.1 folds, P53 gene by 0.844 folds, PTCH1 gene by 4.64 folds, SHH gene by 1.75 folds, SMO gene by 2.46 folds.

The mean relative quantity (RQ) values of mRNA levels of GLI1, P53, PTCH1, SHH and SMO in the BCC (n=38) and normal skin tissues (n=15). The expression levels of GLI1, P53, PTCH1, SHH and SMO were significantly up-regulated in BCC tissues: (A) GLI1: $P < 0.05$, (B) P53: $P < 0.05$, (C) PTCH1: $P < 0.01$, (D) SHH: $P < 0.05$, (E) SMO: $P < 0.05$.

4. Discussion

BCC is the most widespread type of skin cancer affecting humans which does not metastasize in general [3, 18]. The skin BCC commonly found on the face and neck areas among individuals between the ages of 65 and 80. The disease has few mortality rates, however, it is a significant problem in society because the outgrowth frequently localizes in the victim's face [5, 19]. The Shh signaling is an important developmental pathway and its dysregulation have been identified in almost all BCC cases studied which confirm the important role of this developmental pathway in human BCC tumorigenesis [20]. Components of Shh signaling pathway including PTCH, Shh, SMO, GlI, have been linked to BCC development. Abnormalities in other tumor suppressor genes such as p53 were observed to have interactions with the regulatory genes and the Shh pathway in BCC [21]. Knowledge of genes that are implicated in carcinogenesis is vital and can be used to contribute to studies regarding the development of preventative strategies for BCC, as well as its diagnosis and pharmacological therapeutics.

In this study, we have showed that the GLI1, P53, PTCH1, SHH and SMO genes were significantly upregulated in the BCC skin compare to healthy skin tissue. PTCH1 gene had the highest relative expression level among the target genes in human BCC skin tissues compared with the control normal skin tissue ($p=0.003$), followed by SMO, GLI1, P53 and SHH genes. The RT-qPCR analysis also indicated that expressions of the target genes were upregulated in BCC skin tissue compared to the control healthy skin tissue, and the highest fold change of gene expression was recorded for PTCH1 by 4.64 folds.

In a different study, it was shown that products of PTCH gene plays a role in the Shh signaling pathway involving smoothened (SMO) and GLI-1, and RT-PCR detected expression of the genes [22]. Mutations in PTCH gene was also identified in patients with the sporadic basal cell carcinomas [23]. Another study demonstrated mutations in the patched receptor of the Shh signaling pathway as well as aberrant activation of the Shh in sporadic BCCs [24].

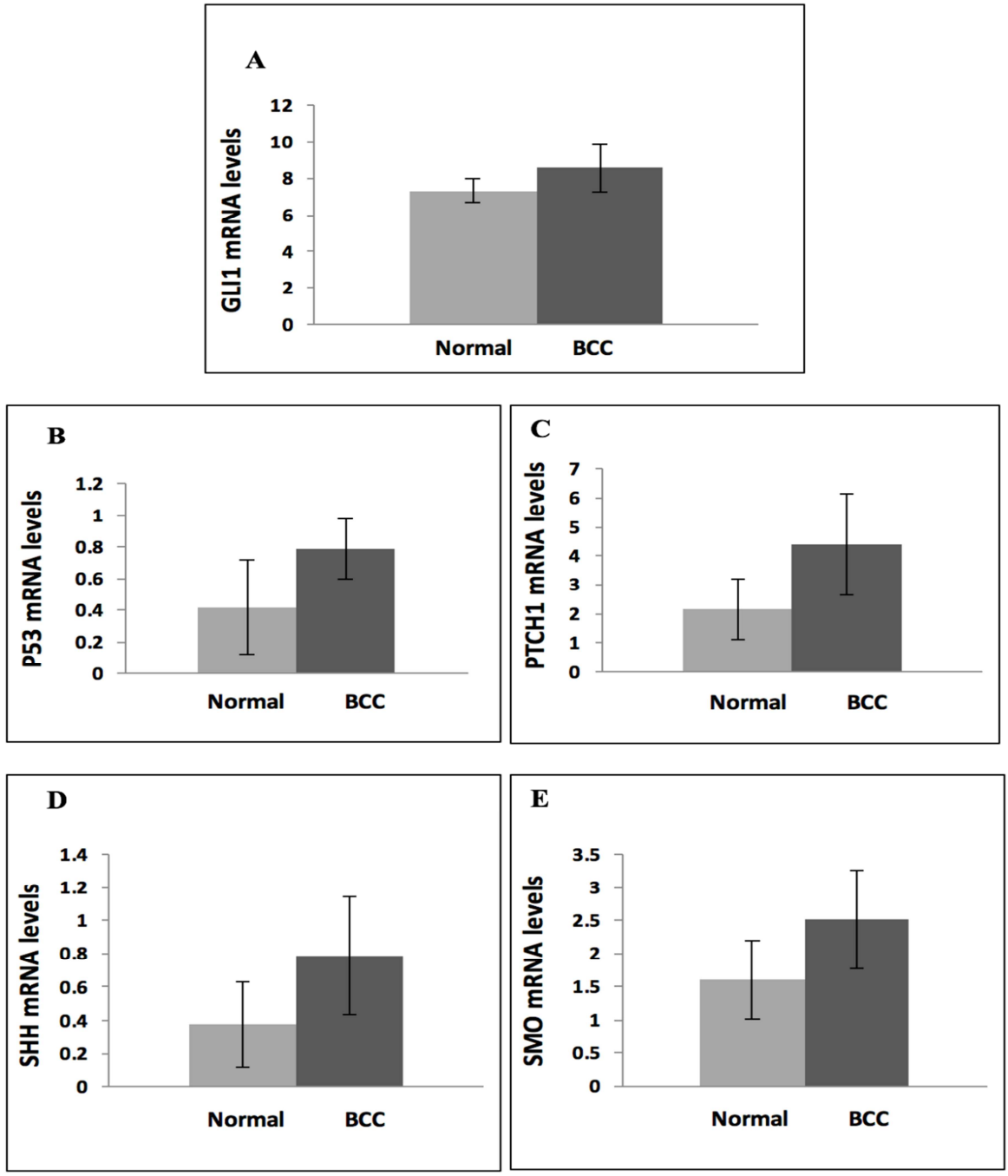

Figure 1. Relative quantity (RQ) of targeted genes.

An investigation found that the expressions of PTCH and SMO mRNA were enhanced in the tumor nests of the nodular BCC while no obvious signals for PTCH and SMO mRNA were detected in the normal human epidermis, appendages, or seborrheic keratosis, indicating that PTCH and SMO mRNA expressions might be associated with BCC tumor progression [25]. Analyses of sporadic basal cell carcinomas in different studies have similarly revealed mutation in Shh pathway genes especially PTCH and SMO [26, 27]. The hedgehog signaling pathway has been exhibited to have potential role in the development of other types of cancer including lung cancer [28]. Different studies showed that large proportion of the participants samples were point mutation among the participants tested for the nucleotide modification in the PTCH gene [29-31].

Separate studies revealed other factors that leads to BCC including the over-expression and mutations of Gli and SMO transcription factors [32-34]. It was shown that p53 is controller of SHH/GLI pathway and it has role in slowing down the GLI action [35, 36]. A p53 mutation at codon 273 was identified in BCC of the ear tissue [37]. Several other studies also showed the role of p53 mutation in skin BCC cases [38-40].

5. Conclusion

The outcomes of this study on local patients showed that expressions of the SHH, PTCH1, GLI1, P53 and SMO genes were upregulated in BCC skin compared to the healthy skin tissue. Our findings indicate that a key regulatory component of hedgehog signalling, PTCH1, plays an important role in the development of BCC and it can be used as a marker gene for diagnosis of skin BCC. The results of this study foster knowledge on BCC and indicate that abnormal proliferation of epithelial cells caused by over expression of Shh pathway component genes might result in BCC tumor development. Further investigation of the molecular mechanisms involved in alterations of the hedgehog signaling pathway that lead to the formation of BCC may lead to effective prevention and therapeutic strategies for this common skin cancer.

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