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Detection of Smo, Gli2 and Gli3 Among Basal Cell Carcinoma Patients in Sulaimani Province

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Abstract

Basal-cell carcinoma (BCC) is the most common type of cancer in human. Sonic hedgehog (Shh) signaling pathway impairment plays a key role in the pathogenesis of BCC. The specific downstream effector in the Shh pathway leading to cancer development is unclear. However in vertebrates, specific downstream effectors in the Shh signaling pathway including smoothened, frizzled class receptor (SMO), zinc-finger transcription factors Gli2 and Gli3 play an important role in regulating the Shh pathway. SMO, Gli2, and Gli3 family proteins are necessary for adequate and controlled cell proliferation. The expression of SMO, Gli2 and Gli3 signal transducers had not been reported yet in BCC skin of local patients.

The aim of this study was to investigate the expression of SMO, Gli2 and Gli3 in BCC biopsies taken from sun-exposed skin areas of patients in Sulaimani Province. Five BCC skin biopsy specimens were taken from the sun-exposed skin areas which served as material for the study. RNA extracted from the samples, cDNA synthesised carried out and specific primers for each of the SMO, Gli2 and Gli3 genes were designed.

Reverse transcription polymerase chain reaction (RT-PCR) analyses of a series of samples expressed genes for SMO, Gli2 and Gli3 in BCC skin biopsies taken from patients in Sulaimani Province.

These findings support the role of SMO, Gli2 and Gli3 as a signaling component of the Shh–receptor complex. These results establish SMO, Gli2 and Gli3 as potent oncogenes in skin and suggest a pivotal role for these transcription factors in the development of BCC. This method can be used in the diagnosis of BCC and further study of downstream effectors in the Shh pathway may lead to an effective anti-cancer therapy.

Introduction

Basal cell carcinoma is the most common malignancy in humans. Although rarely metastacise, but it is capable of significant local destruction and disfigurement. Skin cancer is accounting for about half of all cancers occur. BCC constitute approximately 80% of all nonmelanoma skin cancers [1].

The highest rate of BCC in the world has been reported in Australia [2]. Exposure to ultraviolet (UV) radiation is generally accepted as the major cause of BCC and the risk of this disease is significantly increased by recreational exposure to the sun during childhood and adolescence [3]. Physical factors, including fair complexion, red or blond hair, and light eye color, influence responsiveness to UV radiation but are also independent risk factors [4]; exposures to ionizing radiation, arsenic, and oral methoxsalen (psoralen) and UV A radiation have also been linked to the development of BCC[5,6].

BCC characteristically arises in body areas exposed to the sun and is most common on the head and neck (80% of cases) [1]. The ideal classification of BCC should be able to identify subtypes which correlate with clinical behavior and treatment requirements. Unfortunately, however, such a classification has yet to be defined. In the interim, the currently most favored classification is one based predominantly on histological growth pattern. This classification contributes to the useful concept of low- and high-risk histological subtypes of BCC. The latter are characterized by an increased probability of subclinical extension and/or incomplete excision and/or aggressive local invasive behavior and/or local recurrence [7].

Shh pathway impairment plays a key role in the pathogenesis of BCC. Shh, SMO, and Gli family proteins are necessary for adequate and controlled cell proliferation. A study showed that the immune-expression of the Shh and SMO proteins significantly increased in the BCC group, as compared with the normal controls [8].

Inappropriate activation of the hh signaling pathway is found in sporadic and familial cases of BCC. Secreted Shh protein binds the tumor-suppressor protein patched homologue 1 (PTCH1), thereby abrogating primarily genes encoding patched homologue 1 (PTCH1)-mediated suppression of intracellular signaling by another transmembrane protein, the G-protein–coupled receptor SMO and he downstream targets of SMO include the GLI family of transcription factors [7].

Shh binds to its receptor patched (PTCH), leading to the activation and repression of target genes via the GLI family of zinc-finger transcription factors. Deregulation of the Shh pathway is associated with BCC due to up-regulation of Gli1 and Gli2[9]. Mutations in Shh pathway genes, PTCH1 and SMO occur in BCC [10].

The zinc-finger transcription factors such as Gli2 and Gli3 are also specific downstream effectors in the Shh signaling pathway. Gli2 and Gli3 contain both activation and repression domains. It has been shown that mutations of the patched (Ptc) gene, a developmental regulator implicated in the signaling pathway via Shh and SMO, play an essential pathogenic role in the development of BCC. Up-regulation of Shh signal transducers, including Ptc, SMO and Shh-interacting protein reported in BCC and Gli2β over expression may lead to the up-regulation of the Shh signaling pathway, thereby inducing BCC [11].

Gli1 and Gli2 are members of the GLI family of zinc finger transcription factors. During embryonic development of vertebrates both genes are transcriptionally activated in response to Shh-signaling and are able to mediate most of the effects caused by activation of the pathway[12, 13].

Green *et al.* 1998 *Year* has established that activation of Shh/Ptc signaling plays a key role in the development of BCC. In Drosophila the effects of Shh signaling are mediated by the transcription factor Cubitus interruptus, which is homologous to the mammalian Gli family of transcription factors.

Sensitive RT-PCR assay was unable to detect Gli-1 transcripts in normal skin but in BCC samples Gli-1 was expressed suggesting that the Gli-1 transcription factor plays a key role in BCC development[14]. Knowledge of BCC molecular pathology has been fuelled by the recent discovery that deregulation of the hh signaling pathway, a key player in embryonic patterning, appears to be fundamental to tumor growth [7].

Results obtained from mouse models studies of BCC have revealed that oncogenic hh signaling can drive BCC-like tumor formation in several different epithelial progenitor populations in skin [15, 16].

Mutations in the p53 tumor-suppressor gene are found in approximately 50% of cases of sporadic BCC and many of these mutations are C→T and CC→TT transitions at dipyrimidine sequences, signature mutations indicative of exposure to UV- B radiation [17].

Inactivation of Ptc alleles results in the formation of tumors and cysts such as BCC, odontogenic keratocysts, and medulloblastomas [18]. Although most BCC are treated surgically, no effective therapy exists for locally advanced or metastatic BCC. A study of vismodegib (GDC-0449), a first-in-class, small-molecule inhibitor of the hh pathway, showed a 58% response rate among patients with advanced BCC [19].

This study was aiming to investigate the expression of SMO, Gli2 and Gli3 in BCC biopsies taken from sun-exposed skin areas of patients in Sulaimani Province.

Materials and Methods

The present study was carried out at the University of Sulaimani, Department of Biology & Faculty of medical sciences.

BCC skin biopsies: Five BCC skin biopsies were obtained from sun-exposed areas of patients from Sulaimani Province who were undergoing elective cosmetic surgery; and the samples had been used for molecular biological analysis. The BCC skin biopsies were stored immediately in RNA stabilization solution; RNAlater (Sigma-Aldrich Ltd., USA), kept at 4°C overnight to allow tissue penetration by the RNAlater, and then stored in freezer (-18° Celsius) until used for analysis.

RNA extraction and cDNA synthesis: RNA was extracted from BCC skin biopsies using RNeasy Mini Kit (Qiagen, Hilden, Germany).

cDNA was synthesized immediately using the avian myeloblastosis virus (AMV) reverse transcription system (Promega, Southampton, UK) and cDNA concentration was checked by spectrophotometer.

Determination of cDNA Concentration by Spectrophotometer: The cDNA samples were diluted 100 times with TE buffer. The optical density (OD) of each sample was measured at wave length of (260 and 280 nm) with a spectrophotometer. The DNA concentration was calculated using the following equation: DNA concentration (µg/µl) = (50 µg/ml x OD at A260 x dłution factor)/1000. The dilution factor was 100 (10 µl of cDNA in 990 µl of high pure water or ddH2O). The final concentrations of extracted cDNAs were 0.6-0.8 µg/µl.

Reverse transcription polymerase chain reaction (RT-PCR): Specific forward and reverse primers for SMO (NM_005631.4), Gli2 (NM_005270.4) and Gli3 (NM_000168.5) were designed from human genome sequence (www.ensembl.org) using the NCBI primer blast tool. RT-PCR was carried out using 50 ng cDNA in a 50 µl reaction volume containing 1.5 mM $MgCl_2$, 0.32 mM dNTPs, 1X Taq DNA polymerase buffer, 2 units of Taq DNA polymerase and 0.3 µM of forward and reverse primers (Smo F:5'-TGTCATCTGACT-ACGCCC-3' and R: 5'-GACATCCAGGCGC-AGCAT -3', Gli2 F: 5'-AAAACGTTCAAGGCAC-CGC-3' and R: 5'-ATGTGCACCACCAGCA-TGTA-3' and Gli3 F: 5'-GTCTATGGGAAGTTC-GGGGA-3' and R: 5'- GCTGAAGGGAGACTC-GGAAG-3') (Sigma-Genosys Ltd., USA). A negative control was used in parallel with each PCR reaction in which the cDNA was replaced with nuclease-free water. Amplification was performed in a thermal cycler (Corbett Research, Australia) using the following conditions: denaturation at 95ºC for 5 min; 36 cycles of 1 min denaturation at 95ºC, 1 min annealing at 49ºC and 1 min extension at 72ºC; and a final extension at 72ºC for 5 min. The PCR products were analyzed directly on 1.5% agarose gel in 1X TBE buffer. The DNA was stained with 0.5 mg/ml Ethidium bromide (EtBr), visualized and photographed under a UV transilluminator.

Statistical analysis

The polymerase chain reaction (RT-PCR) bands were scored in a binary form as presence or absence (1/0) of bands for each sample.

Results

RT-PCR analysis demonstrated the expression of SMO, Gli2 and Gli3 in BCC biopsies taken from sun-exposed skin areas of patients from Sulaimani Province (Figures 1, 2, 3). The results indicated that SMO, Gli2 and Gli3 may play key role in the pathogenesis of BCCs and the method could be used in the diagnosis of BCC.

No band appeared in the negative control when cDNA excluded, indicating that no template contamination occurred during PCR mixture preparations and the bands in the other lanes were resulted from SMO, Gli2 and Gli3 gene transcript amplification.

Agarose gel electrophoresis of RT-PCR analysis showed expected product size for SMO (409 bp) in all the BCC skin biopsies (n=5) with the primers used for detecting smoothened, frizzled class receptor (SMO) (Figure 1). No band appeared in the negative control.

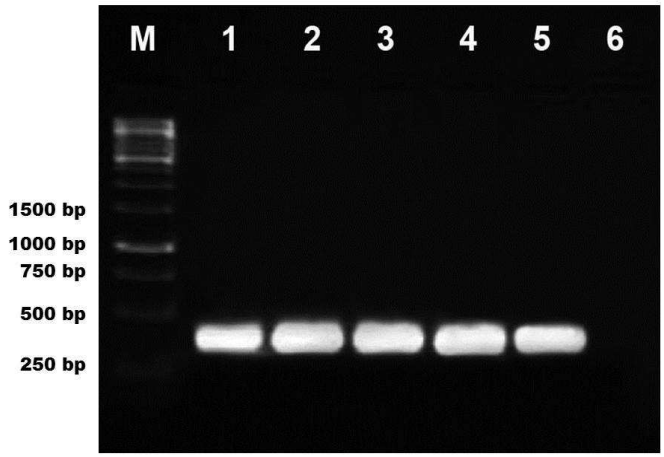

Figure 1. SMO genes expressed in BCC skin biopsies (n=5). M: Marker (1kb DNA ladder), lanes1-5: SMO PCR products of BCCs skin samples (409 bp), lane 6: Negative control. The RT-PCR products were analyzed by agarose gel electrophoresis 1.5%.

RT-PCR reactions using specific primers for zinc-finger transcription factors Gli2 reveals that all the BCC skin biopsies (n=5) expressed expected product size for Gli2 (833 bp) (Figure 2). No band appeared in the negative control. RT-PCR reactions using specific primers for another member of zinc-finger transcription factors Gli3 demonstrated that all the BCC skin biopsies (n=5) expressed the expected product size for Gli3 (658 bp) (Figure 3). No band appeared in the negative control when cDNA was excluded.

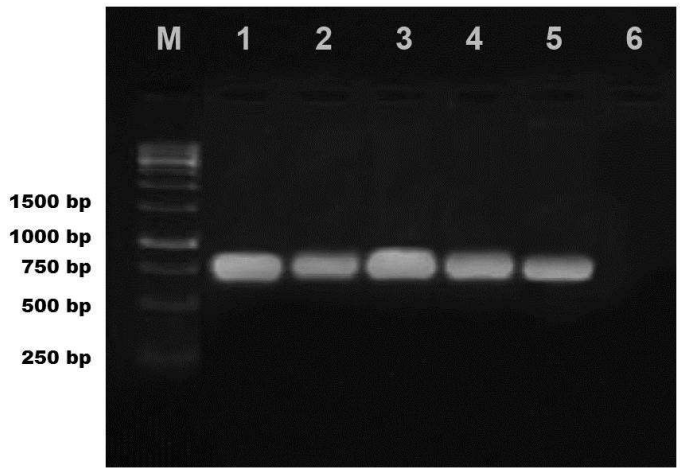

Figure 2. Gli2 gene is expressed in BCC skin biopsies (n=5). M: Marker (1kb DNA ladder), lanes1-5: Gli2 PCR products of BCC skin samples (833 bp), lane 6: Negative control. The RT-PCR products were analyzed by agarose gel electrophoresis 1.5%.

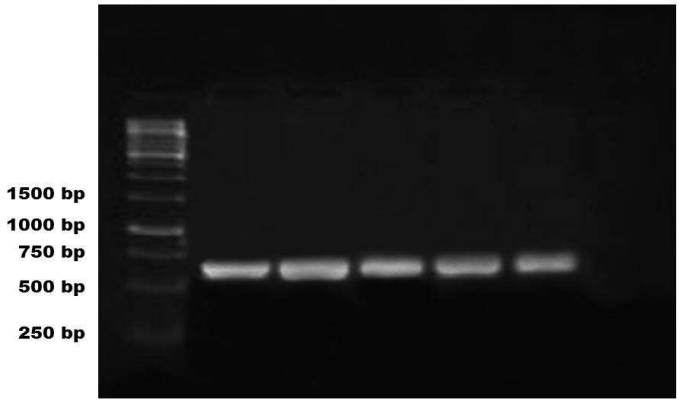

Figure 3. Gli3 gene is expressed in BCCs skin biopsies (n=5). M: Marker (1kb DNA ladder), lanes1-5: Gli3 PCR products of BCC skin samples (658 bp), lane 6: Negative control. The RT-PCR products were analyzed by agarose gel electrophoresis 1.5%.

Discussion

In this study, specific downstream effectors in the Shh signaling pathway leading to BCC development, SMO, Gli2 and Gli3 were identified in BCCs skin biopsies taken from sun-exposed skin areas of patients from Sulaimani Province.

BCC is the most common human malignancy in most of populations in the world and its increasing in incidence is due to an aging population and sun exposure habits. BCC is occasionally aggressive with deep invasion, destruction of adjacent structures, recurrence and, on rare occasions, regional and distant metastases occur [20]. Therefore, investigating the gene transcripts playing role in the Shh signaling pathways of BCC may lead to better understanding of molecular mechanisms involved in the BCC development and can lead to development of a medical treatment for the common cancer, BCC. The realization that aberrant activation of hh signaling is a pathognomonic feature of BCC development has opened the way for exciting progress toward understanding BCC biology [21].

Activation of the Shh pathway has been shown in a variety of human cancers, including, basal cell carcinoma. Downstream effectors of the Shh pathway include SMO and glioma-associated oncogene homolog (GLI) family of zinc finger transcription factors; and both are regarded as important targets for cancer therapeutics [22].

In vertebrates, Shh signaling is mediated by Gli1, Gli2, and Gli3, and the functions of three Gli proteins overlap but also are distinct [12, 13]. Mutations in hedgehog pathway genes, primarily genes encoding PTCH1 and SMO, occur in BCC [10]. All expression of the hh target gene Gli1 is dependent on both Gli2 and Gli3 [13]. Thus investigating the expression of SMO, Gli2 and Gli3 in BCC was chosen for this study.
Agarose gel electrophoresis of RT-PCR analysis showed the expression of SMO in BCC skin biopsies taken from patients from Sulaimani Province (n=5) (Figure 1). No band appeared in the negative control when cDNA excluded, indicating that the bands in the PCR products were resulted from SMO gene transcript amplification and no template contamination occurred. The engagement of SMO in BCCs is supported by another study in which positive results obtained with the orally administered SMO inhibitor GDC-0449 in a trial of patients with locally advanced or metastatic BCC [23].

RT-PCR analysis of BCC skin biopsies taken from patients from Sulaimani Province showed the expression of Gli2 (833 bp) (n=5) (Figure 2). In a study on BCC biopsies, Gli2 expression was determined using an image analysis system (semiautomatic function) [8]. Mouse embryos homozygous for Gli2 mutations display diminished expression of Shh target genes [24, 25].

Shh signaling pathway is sensitive to inhibition by small molecules for the first time noticed from the observation of cyclopia in lambs, induced by the maternal ingestion of corn lilies, followed by the

demonstration that the active compound, cyclopamine, inhibits hh signaling and binds to SMO [26-28]. Studies showed that, in addition to hh inhibition in various in vitro systems, the oral administration of cyclopamine reduced the growth and development of BCC in Ptch1+/– mice exposed to UV irradiation and its topical application to human BCC can induce regression [27, 29].

The region-specific localization of Ptc1, SMO, Gli2, and Gli3 speculate that placental trophoblasts are the hh-responding cells, and Shh acts on these hh-responding cells to activate the signaling pathway and promote the differentiation of them [30].

A study demonstrated that in absence of the Shh signal, the biological activity of Gli2 is suppressed by the N-terminal domain and the existence of such a regulatory mechanism is consistent with the hypothesis that Gli2 is a primary mediator of Shh signaling [31].

In this study, RT-PCR analysis showed the expression of Gli3 (658 bp) (n=5) (Figure 3) in the BCC skin biopsies taken from patients from Sulaimani Province.

Gli3, but not Gli1, is another primary mediator of Shh signaling whereby the N-terminal region modulates the activator function. Therefore, Gli2 and Gli3 proteins appear to be primary mediators of Shh signaling [31]. This further justifies the results obtained by this study on local BCC samples. Gli2 and Gli3 activity leads to Gli1 expression, which positively boosts Gli1 transcription [32]. A study illustrated that Gli2 trafficking links hh-dependent activation of SMO in the primary cilium to transcriptional activation in the nucleus [33]. BCC induced in mice via over expression of Shh in a different study [34]. Therefore, all the studies mentioned above support the results obtained by this study which shows that SMO, Gli2 and Gli3 genes play role in the development of BCC through alteration of Shh signaling pathway.

Conclusion

In conclusion, it can be seen from the present study that SMO, Gli2 and Gli3 genes are expressed in BCC biopsies taken from sun-exposed skin areas of patients in Sulaimani Province. These findings suggest that SMO, Gli2 and Gli3 are potent oncogenes in skin and have pivotal role in the development of BCC. Thus this method can be used in the diagnosis of BCC and further study of molecular mechanisms of BCC development may lead to an effective anti-cancer therapy.

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