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Original article

Comparative Analysis of ITGB6 Gene Expression in Oral Cancer

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ABSTRACT

The goal of this study was to determine the quality and quantity of ITGβ6 gene expression in oral squamous cell carcinoma (OSCC). Two Cell lines (H357) and (Vβ6) were used to analyses β6protein and gene expression by using BAC assay, SDS-PAGE gel, and western blotting, agarose gel as well as real- time PCR techniques. The western blotting and real-time PCR studies demonstrated high level of protein expressions in the transected Vβ6 cells line which shown as clear thick band. On the other hand, a protein expression of H357 cells line was less and gives weak band. Therefore, Squamous cell lines from the oral mucosa that express β6 are linked to increased invasion and progression. This highlights the significance of understanding cancer progression mechanisms and the impact of varying expression levels in different tumors.

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INTRODUCTION

Oral Cavity Squamous Cell Carcinoma (OCSCC) is the most prevalent form of Head and Neck Cancer (HNC), ranking 16th globally. It is considered one of the most aggressive malignant tumors due to its tendency to metastasize and its high recurrence rate [1]. In the western world, the incidence of OCSCC ranges from 1% to 4%, while it is more common in less industrialized Asian countries, where it is linked to a high mortality rate [2].

Oral cancer is highly prevalent in Southeast Asian countries, primarily due to the widespread use of tobacco products, particularly in chewable form [3]. However, India has the highest incidence of oral cancer cases, representing one-third of the global burden. This disease is a significant health issue for nations experiencing economic transition. Annually, around 77,000 new cases and 52,000 deaths are reported in India, constituting roughly one-fourth of the global incidences [4]. Although there are numerous treatment options available, the overall 5-year survival rate for oral cancer at all stages is around 50%. Additionally, the rates of metastasis and recurrence continue to be high. Oral cancer patients are primarily treated with surgery in stages 1 and 2, while stages 3 and 4 are managed with surgery combined with adjuvant therapy [5].

Oral squamous cell carcinoma (OSCC) is a predominant malignancy, making up over 90% of all oral cancers. Pain is the most common symptom, with the tongue and floor of the mouth being the most frequently affected areas. In its early stages, OSCC appears as an asymptomatic erytholeukoplastic area. However, in advanced stages, it presents as ulcers and lumps with irregular, rigid margins [6]. Moreover, similar to other types of carcinomas, integrin expression in oral carcinoma varies both between different tumors and within various regions of the same tumor [7].

During cancer progression, tumor cells invade nearby tissues, promote the formation of new blood vessels, alter the extracellular matrix, undergo epithelial-mesenchymal transition, and spread to other parts of the body. This process triggers a persistent inflammatory response that engages various cytokines, developmental pathways, and growth factors typically associated with normal wound healing [8].

The integrin family which consists of transmembrane adhesion receptors is crucial for detecting and adhering to the extracellular environment. Humans possess 24 distinct integrin heterodimers each with unique ligand binding specificities and irreplaceable functions. These integrins undergo intricate structural changes that regulate their ability to bind ligands and initiate various downstream signaling pathways, influencing cell adhesion and dynamics—critical processes for the life and development of multicellular organisms [9].
However, in this study we examined expression of Integrin Subunit Beta 6 (ITGβ6) gene in two cell lines by extracted protein and RNA from these cell lines, in addition the major techniques used in this study to analyses the protein and gene expression are bicinchoninic acid (BCA) assay, sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE gel), western blotting and agarose gel as well as Real Time Polymerase Chain Reaction (RT-PCR) technique.

METHODS

Passage adherent cell line

The medium was removed from the culture container, and the cells were rinsed twice with phosphate-buffered saline (PBS). The cells were then exposed to a trypsin/EDTA solution and incubated at 37˚C. Trypsin activity was stopped by adding growth media. The cell suspension was centrifuged, and the supernatant was discarded. The cells were re-suspended in fresh growth medium and subcultured according to the split ratio before being returned to the incubator

Cell detachment

The cells were washed twice with ice-cold phosphate-buffered saline (PBS) before being harvested using a cell scraper. The harvested cells were then centrifuged at 168g for 15 minutes and stored on ice**.**

Protein extraction

The cell pellets were resuspended in 200 µl of EDTA-free Radio-Immunoprecipitation Assay (RIPA) buffer, which contained 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS, along with protease inhibitors from SIGMAFASTTM Protease Inhibitor Tablets. The mixture was incubated on ice for 30 minutes. DNA was sheared by passing the cell lysate suspension up and down through a sterile needle (Microlance TM 3, Becton Dickinson and Company, Oxford, UK) and a 0.5 ml syringe (Becton Dickinson and Company). The lysate was then transferred to a 1.5 ml Eppendorf tube. The samples were centrifuged at 15,000 x g for 10 minutes at 4˚C, and the supernatant was transferred to a fresh 1.5 ml Eppendorf tube before being stored at -80˚C.

BCA ASSAY

Preparation of standards

A set of BCA standards at a concentration of 2 mg/ml was prepared by performing a series of 1:2 dilutions.

Preparation of the BCA working reagent (WR)

The total volume of WR was determined by:
[(8×3) standards+ (2×4) samples] ×200=6.4ml.

Microtitre procedure

The sample was centrifuged at 13,000 rpm for 10 minutes, and the supernatant was transferred to a clean, labeled Eppendorf tube. 10 µl of each standard and the unknown samples, diluted 1:2 and 1:4, were pipetted into the microplate wells. 200 µl of WR were added to each well, which were then covered and incubated for 30 minutes at 37˚C. Finally, the absorbance at 570 nm (A570) was measured using a plate reader.

Western blot

Appropriate amount of protein samples was mixed with 2x laemmli buffers, and boiled for 10 minutes then centrifuged. 20µl total protein per mini-gel well was loaded from sample A and B. These samples were electrophoresed under reducing conditions in 10% SDS-PAGE.
Protein was electro blotted to nitrocellulose membrane. Blots were probed with antibodies against ITGβ6 c-19: sc-6632 (Santa Cruz Biotechnology, Inc) or monoclonal Anti-β-Actin (Sigma-Aldrich).
HRP-Rabbit Anti-Goat (H+L) conjugate or Anti-Mouse IgG, HRP-Linked Antibody were used as secondary antibodies. Bound antibodies were detected with the enhanced chemiluminescence western blot detection kit system [Thermo scientific]. Then it was developed by using light sensitive x-ray films.

Extraction of total RNA from cell lines

Total RNA was extracted from cell lines using column technology with the isolate RNeasy Mini kit (Bioline Ltd UK). The integrity and concentration of the RNA were determined using a NanoDrop (ND1000). The quality of the RNA was assessed by performing TAE agarose gel electrophoresis at 5%

Protein extraction and quantification

The BCA assay was used to determine the concentration of proteins in both cell lines H357 and vβ6 cells. First step was to draw the curve of BSA protein standard to be used to determine the unknown concentration of protein sample as shown in figure 1.

The graph was drawn in Excel using the data in table 1. Using the equation y= 0.4391x+0.2002. The total amount of protein extracted from the first cell line (H357) was (5.26 mg/ml), whereas in the vβ6 cells the total amount of protein was (5.76 mg/ml). The amount of protein required for western blot was 3.8µg/ml for H357cell line and 3.47µg/ml for the vβ6 transfected cell line. The standard BSA concentrations were prepared at the following mg/ml levels: 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03, and 0.

Table 1. Data used to calculate the average reading of standard BSA.

Three reading of BSA standard			Average
1.0142	1.0808	1.0679	1.0543
0.6712	0.6587	0.6948	0.6749
0.4382	0.4437	0.4508	0.444233
0.3209	0.319	0.3171	0.319
0.2513	0.2449	0.2439	0.2467
0.2219	0.2141	0.2121	0.216033
0.1998	0.1874	0.2039	0.197033
0.1947	0.1888	0.1865	0.19

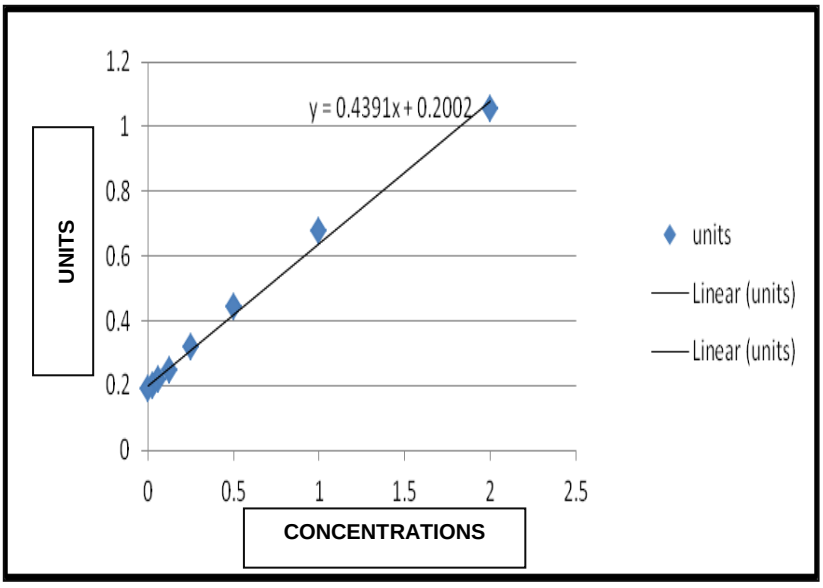

Figure 1. BSA standard, the plot was generated using Excel. It shows the concentration of standard BSA samples vs units. The equation can be used to calculate unknown concentration of protein sample.

Quantitative-PCR (Q-PCR)

After the reverse transcription of the RNA, q-PCR analysis was conducted on cDNA prepared from H357 and VB6 cells. 1 µg of the extracted RNA was used for cDNA synthesis. The primers used were: ITG beta 6 forward primer: $^{5'}$ CTACCTGTGGTGACCCCTGTAAC$^{3'}$ and ITG beta 6 reverse primer: $^{5'}$ GCTTGGCCAGCTGCTGAC $^{3'}$.

The 40 µL PCR reactions included 2 µL of each primer, 16 µL of the reverse transcription product, and 20 µL of SYBR. 10 µLof the H357 mix was added to the first three wells, and 10 µL of the VB6 mix was added to the next three wells of the q-PCR plate. The plate was then placed in the PCR machine for 2.5 hours.

RESULTS

RNA extraction

The amount of RNA extracted from H357 was 898.1µg/ml, while transfected vβ6 cells gives 931.1µg/ml of RNA.

TAE agarose gel electrophoresis

In order to assess the quality of RNA samples that was extracted from the H357 and vβ6 squamous cell carcinoma cell lines, they have been analysed under native conditions on a 1.5% Agarose gel to check for the presence of 28S and 18S RNA signature bands of eukaryotic cells. The result of TAE Agarose gel electrophoresis reveal that the two RNA samples are of high quality as they show the two ribosomal RNA bands as shown in figure.2.

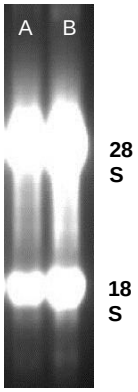

Figure 2. 1.5% Agarose gel show the 28S and 18S RNA bands which indicate that samples A and B are of good quality

RT-PCR

The reverse transcription of the extracted RNA from both cell lines of oral squamous cell carcinoma was carried out. A quantitative PCR analysis of the obtained cDNA (prepared from extracted RNA from H357 cells, which express low levels of IGT beta 6, and vβ6 cells which express high levels).

To analyses the amount of IGT beta 6 transcripts, (i.e. the mRNA encoding the protein) in both samples. The values obtained were normalized to the levels of a housekeeping gene (in this study is U6, although many others can be used) which gives approximately the same result in all samples. The result of q PCR showed that Vβ6 cells line expressed fourfold more ITGβ6 (4.0251) than H357 cell line (0.1) as shown in figure 3.

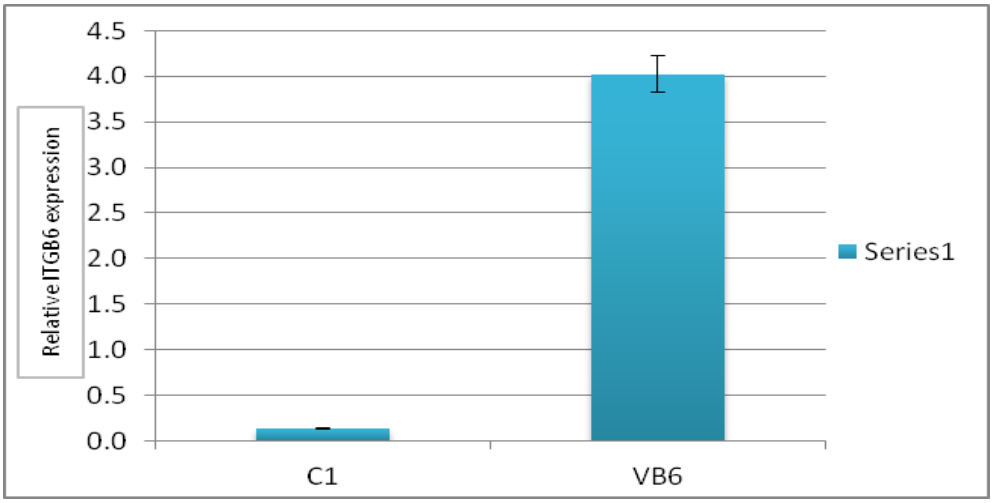

Figure 3. The result of q PCR, Vβ6 cells expressed fourfold more ITGB6 than H357 cell Line.

Western blot

The SDS-PAGE was loaded with pre-stained protein marker, positive control, protein lysate from cell line A, and cell line B and then the SDS-PAGE gel electrophoresis was run for 50 minutes before transferring it to nitrocellulose membrane. The membrane was blocked with skimmed milk powder (5%) then incubated with the anti-beta 6 serum antibodies (1:1000) at 4˚C overnight with agitation.

After washing the membrane 3X in TBST, it was incubated with the secondary antibodies (Horseradish peroxide HRP conjugated anti-rabbit IgG from Sigma). Again, the membrane was rinsed with TBST before developing it on x-ray film and visualised with enhanced chemiluminescence ECL.
The result of the western blot showed that no band was detected for the H357 cell line sample which means that no ITGβ6 was expressed. Whereas the Vβ6 cell line cells were positive for ITGβ6 as determined by immunoblotting with anti-β6 integrin polyclonal antibodies and the western blot demonstrated a band (97 KDa) which mean that ITGβ6 was expressed in this cell line as shown in figure 4.
In addition to the two cell line samples, positive control was included which is β actin to ensure that the protocol is efficient and correct and that the antibodies recognize the target protein. The result of the β actin western blot was positive with a band of 42 KDa, figure 5. A monoclonal anti-β Actin antibody was used as primary antibodies to detect the positive control and anti-muse IgG as secondary antibodies.

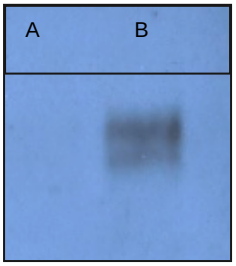

Figure 4. Western blot of H357 sample (A) and vβ6 sample (B). The sample B shows a band which indicates that vβ6 express β6 integrin.

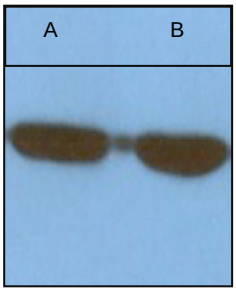

Figure 5. β-actin, positive control for the western blot.

DISCUSSION

Oral cavity squamous cell carcinoma (OCSCC) constitutes more than 90% of cancers found in the oral cavity.Worldwide, it is estimated that there are over 275,000 to 300,000 new cases of OCSCC each year, resulting in more than 150,000 deaths from the disease annually. In the United States, the estimated annual incidence of these cancers ranges from 4 to 4.3 cases per 100,000 individuals [10].
Oral squamous cell carcinoma (OSCC) is one of the most prevalent cancers in the head and neck area, capable of developing in various parts of the oral cavity [11]. One of the key adhesion molecules expressed in OSCC of the head and neck is ITGβ6. Studies have reported varying levels of over expression of certain adhesion molecules in oral SCC, while others show reduced expression [12]. ITGβ6 plays a crucial role in tumor development, as shown in numerous studies. It is found in various types of cancer, including lung, breast, stomach, and oral carcinomas [13]. Our findings reveal that ITGβ6 expression levels were elevated in protein extracted from both cell lines (H357 and vβ6) and analyzed via western blot. Vβ6 cells exhibited a distinct band indicating high ITGβ6 expression, whereas H357 cells showed no expression. The RT-PCR results supported this observation when RNA from both cell lines was examined.
The expression pattern of ITGβ6 in oral cancer differs among various tumors and even within distinct regions of the same tumor [14]. The role of ITGβ6, in cancer progression has been an area of significant research. These molecules are crucial for cell adhesion and signaling, impacting tumor behavior and metastasis [15].
However, the increased expression of ITGβ6 in oral cancer is believed to facilitate malignant transformation, migration on the extracellular matrix, and invasion by promoting the upregulation of metalloproteinases [16]. ITGB6 was regarded as a prognostic marker, as elevated levels of its expression in tumor tissues were significantly correlated with patient outcomes [17]. Because of its important function in advancing oral cancer, ITGβ6 holds potential as a viable target for

therapy. By directing inhibitors or monoclonal antibodies toward ITGβ6, there's a possibility of interrupting its activity, consequently hindering tumor development and spread [18].
For metastasis to succeed, circulating tumor cells (CTCs) need to adhere to the blood vessels in distant organs and then exit into the surrounding tissue. The formation of blood clots is believed to aid in this process by attracting fibronectin, which activates integrins. After extravasation, the interaction of integrins with the extracellular matrix (ECM) in the surrounding tissue determines whether the cancer cells will continue to grow or enter a dormant state [19].
Integrin β1-blocking antibodies achieved an impressive 80% decrease in cancer cell adhesion to the extracellular matrix (ECM). Additionally, the combined use of integrin inhibitors and E-cadherin up regulators successfully reverted metastatic cells from a mesenchymal to an epithelial phenotype, leading to a 70% reduction in invasion [20].
ITGβ6 facilitates invasion in squamous cell carcinoma, and its interaction with HAX1 enables clathrin-dependent endocytosis. This process, in turn, enhances the motility of carcinoma cells in an organotypic culture [21].

CONCLUSION

The presence of ITGβ6 expression in squamous cell lines originating from oral mucosa correlates with increased invasion and progression. This underscores the significance of comprehending the mechanisms driving cancer advancement and the impact of varying expression levels across different tumor types. The comparative analysis of ITGβ6 gene expression in oral cancer highlights its significant role in cancer progression, metastasis, and potential as a prognostic marker and therapeutic target. Future research focusing on the precise molecular mechanisms of ITGβ6 and its interactions within the tumor microenvironment could lead to the development of targeted therapies aimed at improving patient outcomes in oral cancer.

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Conflicts of Interest

The authors declare no conflicts of interest.

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تحليل مقارن للتعبيرالجيني ITGβ6 في سرطان الفم

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المستخلص

كان الهدف من هذه الدراســـة هو تحديد جودة وكمية التعبير الجيني ITGβ6 في ســـرطان الخلايا الحرشـــفية الفموية (OSCC). تم استخدام خطين خلويين (H357) و (Vβ6) لتحليل البروتين β6 والتعبير الجيني باستخدام اختبارBAC ، هلامSDS-PAGE ، والنشاف الغربي، هلام الاغاروز وكذلك تقنيات PCR في الوقت الحقيقي. أظهرت دراسات النشاف الغربي ودراسات PCR في الوقت الحقيقي مستوى عالٍ من تعبيرات البروتين في خط خلايا Vβ6 المقطوعة والتي تظهر على شـكل شـريط سـميك واضـح. من ناحية أخرى، كان التعبير البروتيني لخط خلايا H357 أقل وأعطى نطاقاً ضـعيفاً. ترتبط خطوط الخلايا الحرشفية من الغشاء المخاطي للفم التي تعبر عن β6 بزيادة الغزو والتقدم. وهذا يسلط الضوء على أهمية فهم آليات تطور السرطان وتأثير مستويات التعبير المختلفة في الأورام المختلفة

الكلمات المفتاحية: سرطان الخلايا الحرشفية الفموية(OSCC) ، التعبير الجيني.ITGβ6

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