Varlitinib induced differential Protein Expression analysis on oral carcinoma cell line: A therapeutic approach

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Research Article

Keywords: Proteomic analysis, varlitinib, mass spectrometry, signaling pathway, EGFR, OSCC

Posted Date: October 28th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1003477/v1

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Abstract

Receptor-ligand complex mediated signaling significantly contributes in cellular activities such as growth, proliferation, differentiation, and survival. However, augmented expression of signal transducing receptors and ligands is the most frequent molecular event and major hallmark of oral carcinogenesis. Among these receptors, Epidermal Growth Factor Receptor (EGFR) with intracellular tyrosine kinase activity is the most frequently overexpressed molecule by Squamous epithelial cells of oral cavity. Aberrant EGFR mediated signaling has laid the foundation of targeted therapy thus providing rationale for the conducted study. We have selected EGFR pathway as targeted intracellular signaling cascade in Oral squamous cell carcinoma (OSCC). Deactivating EGFR by blocking the binding sites is likely to result in prevention of intracellular downstream signaling. In this context, Tyrosine Kinase Inhibitors (TKIs) have come into play. Quinazolines (aromatic heterocyclic compounds) and their derivatives have shown promising clinical outcomes. Present study focused to investigate anti-EGFR potential of quinazoline derivative, varlitinib—a pan-EGFR inhibitor on oral squamous epithelial cell lines. We performed proteomic analyses to identify differential expression pattern of proteins in SCC-25 cells in response to varlitinib treatment. Identified proteins include Binding Immunoglobulin Protein (BiP), Heat Shock Protein 7C (HSP7C), Protein Disulfide Isomerase 1 A (PDIA1), Vimentin (VIME), Keratin type I Cytoskeletal 14 (K1C14), and β-Actin (ACTB). Among these, five proteins were found to be downregulated upon varlitinib treatment whereas only Keratin type I Cytoskeletal 14 was upregulated. Differential expression of proteins and possible role of varlitinib as potential antitumor drug in oral carcinoma is discussed.

1. Introduction:

Oral squamous cell carcinoma (OSCC) constitutes a major proportion with more than 100,000 cancer related fatalities worldwide in 2020[1]. The pathophysiology of OSCC in part involves Epidermal Growth Factor Receptor (EGFR) and associated signaling pathway [2, 3]. EGFR is activated upon ATP mediated phosphorylation and subsequent receptor dimerization resulting in stimulation of pleiotropic downstream signaling cascade. These include Ras/Raf/MAPK and, PI3K/Akt, that control cell proliferation, differentiation, migration, and survival[4]. Dysregulation of EGFR and receptor mediated aberrant signaling cascade has been significantly involved in the development and progression of OSCC, resulting in invasion, metastasis, and angiogenesis [2–4]. High expression of EGFR has been often associated with chemotherapeutic resistance towards the drugs being currently used in clinical settings such as methotrexate, 5 fluorouracil, cyclophosphamide etc. [5, 6]. Other therapeutic strategies including radiotherapy have not gained substantial clinical outcomes for health improvement and overall survival [7]. These clinical findings draw attention towards pressing need to develop new, affective, and improved therapies for oral epithelial malignancy.

Studies based on signaling mechanisms led scientists to follow targeted therapeutic approach. Identification of EGFR as an oncogene has made EGFR pivotal drug target for the treatment of OSCC [8, 9]. Compelling investigations on EGFR therapeutic ventures have shown two main approaches
targeting EGFR i.e., monoclonal antibodies (mAbs) and Tyrosine Kinase Inhibitors (TKIs). Each of this approach has distinct mechanism; mAbs (anti-EGFR antibodies) block the extracellular ligand binding domain whereas, TKIs target intracellular tyrosine kinase domain thus, preventing RTK activity [8]. Amongst TKIs, quinazolines have been significantly important. Quinazoline based drugs such as gefitinib, erlotinib, lapatinib, etc. are currently being clinically and pre-clinically tested for several epithelial malignancies including OSCC [8, 10].

Varlitinib is an oral, pan, quinazoline based TK inhibitor of Human Epidermal Receptor (HER) with maximum efficacy in sub molar potency and minimal side effects[11]. Earlier, we reported anticancer effect of varlitinib on oral cancer cell line SCC-25 and identified that varlitinib mediates its action via MAPK/EGFR pathway [12]. Focus of current study was to investigate protein expression pattern in SCC-25 squamous epithelial cell line in response to varlitinib treatment using proteomic approach.

2. Materials And Methods:

2.1. Cell Culture and Drug Incubation:

Human SCC-25 cell line (ATCC® CRL-1628) Manassas, VA, USA) was cultured in Dulbecco’s Modified Eagle’s Medium and Ham’s F-12 Medium (DMEM/F-12). Medium was supplemented with 10% heat-inactivated fetal bovine serum, 400 ng/ml hydrocortisone, 20 mmol/L glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were grown in humidified environment at 37°C and 5% CO2. Later, they were sub-cultured in 1:4 ratio using 0.05% trypsin-EDTA and were seeded in 96-well microtiter plates (Corning, NY, USA) for the next 24 h. The cells were then incubated with different concentrations of varlitinib (1, 5, 10, 20, 30, 40, 50, 60 and 70µM) for 24, 48, and 72 h. Untreated SCC-25 cells incubated for similar time duration without drug were used as control.

2.2. Cell Proliferation assessment:

After varlitinib treatment, SCC-25 cell viability was determined using MTS assay (Cell Proliferation assay kit, Abcam) as per manufacturer’s instructions. Briefly, MTS reagent (20 µl) was added in each well of 96-well microtiter plate, followed by 2 hours incubation at 37°C. The optical density of each well was then measured with a microplate reader (Beckman Coulter, California, USA) at 520 nm. The percentage cytotoxicity was calculated as a growth percentage of cells relative to untreated controls. All assays were performed in triplicate.

2.3. Two-Dimensional Gel Electrophoresis (2D-GE):

Total protein was extracted from varlitinib treated and untreated SCC-25 cells using lysis buffer (Urea, Glycerol, NP-40, Ampholyte buffer 3-10, 0.5M Tris-HCL pH 6.8, 0.5M DTT, 0.25M EDTA). Total protein concentration in cell lysate was estimated using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) as per manufacturer’s protocol.
~85µg protein from 50µM varlitinib treated cells for 24 hours and untreated cells was dissolved in rehydration buffer containing 6M Urea, 2M Thiourea, 4% CHAPS, carrier ampholyte (Bio-Rad, Hercules CA, USA) and applied on immobilized pH-gradient (IPG) gel strips (7 cm. pH 3-10; Bio-Rad) at room temperature. The strips were rehydrated overnight. Subsequently, first dimension IEF was carried out using Multiphor II system (GE Health-care, England, UK) at 20°C. The total voltage applied was 10,000 V/h with constant current of 2 mA. Following the IEF, focused strips were equilibrated in equilibration buffer A (6M Urea, 50 mM Tris pH 8.8, 30% Glycerol, 2% SDS with 10mg/ml DTT) and buffer B (0.5M Tris-HCl pH 6.8, 12M Urea, 10% SDS, 60% glycerol, 25mg/ml Iodoacetamide) for 30 minutes each. IPG strips were then loaded on to 12% SDS-Polyacrylamide gels. SDS-PAGE was run using Protean Cell system Bio Rad at constant voltage of 60 V. Protein spots were stained using Coomassie Brilliant Blue R-250.

**a) Image Analysis:**

Differential expression pattern of proteins from 50 µM Varlitinib treated, and control gels were analyzed via PDQuest Gel Analysis Software Version 8.0.1 (Bio-Rad). We identified differentially expressed protein spots among control and treated gels. Spot intensities were calculated using PDQuest software followed by excision of differentially expressed spots via EXQuest spot cutter (BIO-RAD) for subsequent identification.

**2.4. MS/MS Analysis for protein identification:**

Differentially expressed protein spots were digested using trypsin. Briefly, protein spots were de-stained with 50mM Ammonium Bicarbonate, and 50% Acetonitrile (ACN) thrice for 15 minutes followed by drying via speed-vac concentrator (Eppendorf, Hamburg, Germany). Desiccated samples were rehydrated using 10 mM DTT and incubated for 30 min at 37°C to reduce disulfide linkages mediated by cysteine. Reduced cysteine residues were exposed with 50 mM iodoacetamide in dark for 30 minutes at room temperature. Protein spots were enzymatically digested with 2ng/µL of sequence grade trypsin (Promega). Resulting peptide fragments were obtained with 25mM Ammonium bicarbonate, 10% (v/v) formic acid and acetonitrile (1:1) used in equal proportions. Subsequently, the peptide fragments were vacuum dried (Speed-Vacuum concentrator Eppendorf) and were re-suspended in 0.1% formic acid. For MS/MS analysis, peptides were subjected to Impact II UHR QqTOF, Bruker Mass Spectrometer. Protein identification was carried out using MGF files through Matrix Science search engine. Peptide fragmentation finger printing was opted for MASCOT search and the selected parameters were Carbamidomethyl (C) for fixed modification, Oxidation (M) for variable modification, maximum 2 missed cleavages were selected, peptide mass tolerance of 20 ppm and fragment mass tolerance 0.5 Da with p < 0.05 was selected. Protein identification of digested peptides was carried by using Swiss-Prot data base with MASCOT search engine.

**2.5. Bioinformatics:**

We analyzed PPI (protein-protein interaction) of proteins identified in the current study using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database with median confidence (scores greater than 0.4).
3. Results:

3.1. Cytotoxic potential of varlitinib on SCC-25 cells:

Approximately 5000 cells were cultured in each well of 96 well plate. After 24 hours cells were incubated with different concentrations of varlitinib (µm Conc.) for 24, 48 and 72 hours. Cytotoxic activity of varlitinib on human oral epithelial SCC-25 cellswas identified by MTS cell-proliferation assay. SCC-25 cells attained 50% cell growth inhibition (median dosage affecting 50% cell population) when exposed with 50 µM varlitinib for 24 hourswith respect to their control (Figure 1), (p < 0.05). We observed linear pattern of cytotoxicity in terms of dose and incubation time (Figure 2). We chose 50µM as cytotoxic dose for further proteomic analysis.

3.2. Analysis of differential expression of proteins in human SCC-25 cell line:

Comparison of protein expression levels in SCC-25 cell line treated with 50 µM varlitinib for 24hours was examined by treating cells along with respective control. Proteins extracted from treated and untreatedcells were subjected to 2D gel electrophoresis (2D-GE)to compare differentially expressed proteins. Maximum proteins were found in the pl range(s) of 4-7 and 7-8.5with molecular weight more than 25 kDa. Respective gel images of untreated control and 50 µM varlitinib treated SCC-25 cells are shown in Figure 3.

In this study, we identifiedsix differentially expressed spots via PDQuest softwarein a proteomic map of OSCC cells. The differential expression of these proteins was statistically significant (Figure 4) among control versus varlitinib treated gels. We found five proteins that happen to be downregulated in varlitinib treated SCC-25 cellswhereas oneprotein was found to be up regulated.

3.3 Identification of differentially expressed proteins:

Differentially expressed spots were submitted to Mass spectrometric analysis and the resulting data was analyzed using MASCOT protein identification search engine. A list of identified proteins with their molecular weight, pl, score, percent coverage and expression levels are given in Table 1. Relative spot intensities from spot 1 to spot 6 are given in Figure 4.

3.4. Correlation of identified proteins with oral carcinogenesis:

Interaction analyses of differentially expressed proteins identified via Mass spectrometric analyses were conducted using STRING network analysis tool. Based on protein interaction and pathway(s) data, obtained through STRING database and PubMed literature searches, we constructed a putative model of protein interaction networksinvolved in cellular regulating activities. Through experiments, databases, text
mining and interaction with 2nd node methods in STRING, we found correlation of all differentially expressed proteins ACTB, K1C14, HSPs and VIME (Figure 5).

4. Discussion:

OSCC remains one of the deadliest malignancies in the past couple of decades possibly due to paucity of appropriate diagnostic and treatment modalities. Proteomic analyses with advances in mass spectrometry has paved the way by exploring novel biomarkers that could be used to timely and accurately diagnose OSCC. The aim of this study is to identify altered expression of proteins (that could be clinically useful) in OSCC upon varlitinib treatment. Cytotoxicity assessment of varlitinib showed dose and time dependent inhibitory action on cell proliferation. Growth inhibitory potential (in terms of reduced cell survival) was attained at 24 hours of treatment.

To determine the effects of varlitinib on protein expression levels in SCC-25 cells, we performed 2D-GE (two-dimensional gel electrophoresis) followed by Mass spectrometric analysis to identify differentially expressed proteins. The study exhibited 6 different proteins spots in total from varlitinib treated as well as control group of SCC-25 cells. We observed significant fold changes in protein expression levels for 6 protein spots after 24 hours of treatment. The catalogue of these identified proteins using mass-spectrometry after treatment is shown in Table 1. Each protein is briefly described in the subsequent paragraphs.

We identified cytoskeletal protein (actin), extracellular matrix protein (keratin), heat shock proteins and molecular chaperones (Bip and HSP7C respectively).

The first group includes proteins that are correlated to evasion of apoptosis such as BiP, HSP7C and PDIA1. Binding immunoglobulin protein (BiP, also known as Glucose Regulated Protein-78; GRP-78) is a member of HSP 70 family hence encoded by HSPA5 gene [13]. BiP is an Endoplasmic Reticulum (ER) residing molecular chaperone protein. It is responsible for mediating folding, translocation of proteins, initiating unfolded protein response (UPR) and ER-associated degradation (ERAD). BiP is of utmost importance for regulating homeostasis [13]. Under stress condition(s), BiP undergoes several changes in terms of function, expression and activation which blocks translocation of proteins to ER. Subsequently causing impaired degradation resulting in pathological conditions such as cancers, cardiovascular as well as neurodegenerative disorders [13]. BiP is an established master regulator of cancer and has been found to be over-expressed in wide variety of cancers including lung cancer, liver cancer, breast cancer etc. [13, 14]. Increased expression of BiP inactivates pro-apoptotic markers such as BIK and BAX and inhibits apoptosis [13, 15]. BiP, through signal transducer, activates intracellular kinases thus activating cellular pathways that promote cell proliferation and survival [16]. Moreover, BiP plays a critical role in OSCC progression by inducing enhanced proliferation, chemoresistance and metastasis [17]. BiP implicitly activates MAPK and PI3K signaling cascades facilitating cancer cell progression [18]. BiP pathogenesis is evidenced through different studies [16–19]. It has been suggested that downregulation of BiP can be potentially linked with impeded tumor formation and growth resulting in apoptotic
induction and improved survival. Our study shows down-regulation of BiP in response to varlitinib treatment suggesting therapeutic potential of the drug.

Protein disulfide isomerase 1 (PDIA1) - a dithiol-disulfide oxidoreductase is also an ER residing molecular chaperone [20, 21]. PDIA1 primarily catalyzes disulfide bond formation via its oxidizing, reducing and isomerizing capabilities thus mediating protein folding, translocation to ER and degradation thus contributing to maintain cellular homeostasis [20, 21]. Deregulated PDIA1 expression and/or enzyme activity is often associated with different human diseases such as CVDs, neurodegenerative disorders, and different cancer types [21]. Higher expressions of PDIA1 have been demonstrated in brain cancer [22], lymphoma [23], colorectal cancer [24], breast cancer [25], often conferring metastasis, invasiveness and chemoresistance. The mechanisms causing increased expression of PDIA1 and associated pathological outcomes are poorly understood [24]. However, PDIA1 is considered as an upstream regulator for balancing Reactive Oxygen Species (ROS) as well as controlling the activity of a metalloprotease ADAM17 which in turn acts as an intermediate in EGFR signaling [25]. ADAM17 activation is based on change in redox balance – an effect which is important for growth factor dependent signaling in cancer cells. It is reported that gene silencing or knocking out PDIA1 results in induction of apoptosis and decreased cell proliferation [24]). Our proteomics data revealed upregulation of PDIA1 in control SCC-25 cells while varlitinib treated group showed reduction in PDIA1 expression levels. The results remained consistent with previously conducted studies.

Another interesting protein found to be decreased in varlitinib treated cells was HSP7C. HSP7C is a 71 KDa heat shock related protein which is constitutively expressed in cells and regulates trafficking and folding of nascent proteins. It has been frequently observed in human breast cancer [26] autoimmune retinopathy [27] etc. Increased expression of HSP7C due to heat, oxidative stress and hypoxia promotes cell survival, apoptotic evasion from proteolytic stress related rapid and abnormal cell growth [28]. Our proteomics data showed increased expression of HSP7C among SCC-25 cells which is in consensus with the already performed studies [28, 29]. The expression was found to be decreased following 24 hours varlitinib treatment of SCC-25 cells confirming its oncogenic role.

The second group of proteins belong to the family of intermediate filament proteins including Vimentin and cytoskeletal Keratin Type 14. Vimentin plays key role in cellular, physiological, structural, mechanical, biological, and developmental processes. Vimentin is known to provide phosphorylation site(s) for kinases thereby regulating signal transduction [30]. Vimentin has been found to be present in wide range of cells including mesenchymal cells and is typically associated with Epithelial to mesenchymal transition - a prominent feature associated with tumor progression [31]. Increased vimentin expression is often associated with breast cancers [31], gastrointestinal [31] and prostate cancer [32]. Knock down clones of these proteins have shown decline in carcinoma cell proliferation and decreased vimentin expression could likely lead to anti-neoplastic behavior of cells by driving them less aggressive and controlled proliferation [33–35]. We also observed reduced expression of vimentin in varlitinib treated SCC-25 cell line in comparison to control cells. Second protein, Keratin is also a member of the family of intermediate filament and is reported as protein marker of epithelial differentiation generating polarity.
which plays a significantly important role in providing mechanical strength and integrity to epithelial cells [36, 37]. Besides mechanical support and stability, keratins contribute in intracellular signal transduction [36, 37]. We identified Type I Keratin cytoskeletal 14 which is typically expressed in basal layer cells of non-keratinized stratified squamous epithelial lining [36]. K1C14 has been associated with carcinogenesis and it is usually overexpressed in different cancers [37, 38]. However, we found up-regulated expression of K1C14 among varlitinib treated group of SCC-25 cells with respect to their counter control. Our results are contradictory to previous reports and needs to be explored further to identify the exact pattern of K1C14 in response to TKI mediated treatment. A possible reason for this contradiction in expression pattern of keratins could be modified expression of genes and proteins (such as cell cycle regulator biomarker p21), associated with carcinogenesis [36]. This, in turn, may result in responding differently to varlitinib mediated targeted therapy. However, alterations at genetic or protein level are key players behind progression / pathogenesis of oral carcinogenesis [36] resulting in apoptotic resistance [38, 39].

Lastly, ACTB Human Actin, cytoplasmic 1 is one of the six isoforms of actin protein, which is considered a highly conserved and ubiquitously expressed [40] protein. Actin is essentially involved in cellular activities such as motility, development etc. [40]. Actin has integral part in survival for most of the cells since it provides mechanical support and force to drive cellular activities as well as motility. Cancer cells also undergo locomotion by moving from one location in the body to another via making contacts with neighboring cells [41], resulting in migration. ACTB has been found to be upregulated during different cancers including prostate, breast, and colorectal cancers [42]. In the present study, ACTB showed increased expression in control SCC-25 cells whereas 50 µM 24 hours prolonged treatment resulted in downregulation of ACTB. This agrees with previous reports [43, 44]. Protein-protein interaction via STRING 9.1 with experimental databases (including GO, KEGG etc.) and text-mining as active methods of predicting protein associations indicated that all identified proteins interacted with each with an exception of KR1T14.

5. Conclusion:

This study focused on varlitinib mediated differential expression of proteins involved in EGFR signaling cascade in SCC-25 cells. Our data indicated that varlitinib might be considered as a potent therapeutic agent with promising anti-proliferative capability concomitantly resulting in suppression of anti-apoptotic proteins resulting in cessation of oral carcinogenesis.

Declarations

Funding

Partial funding by Dean Science Grant, University of Karachi, Karachi

Conflict of Interest

Authors declare that there is no conflict of interest.
Data Availability
On request

Consent for Publication
All authors agree with publication

Author Contribution
FT performed bench work and wrote the draft, AI contributed in cell culture studies, ZH conducted in-gel digestion and two D electrophoresis, BS performed MS analysis, AA and ZH did data interpretation, SZ conceived the project, supervised the study and finalized the manuscript.

Code Availability
Not Applicable

Cell lines
HumanSCC-25 cell line (ATCC® CRL-1628) Manassas, VA, USA

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Figures

![Figure 1](image_url)

**Figure 1**

SCC-25 cells cytotoxicity pattern before and after varlitinib treatment. (A) Control (B) 50µM Varlitinib treatment for 24 hours.
**Figure 2**

The effect of different concentrations of Varlitinib (1 μM, 5 μM, 10 μM, 20 μM, 30 μM, 40 μM, 50 μM, 60 μM and 70 μM) on SCC-25 cells after 24-, 48- and 72-hours treatment. Cytotoxicity was determined by MTS assay. Each bar represents mean percent cytotoxicity± S.D. for each concentration respectively. Data sets were analysed for statistically significant differences by one way ANOVA. * represents statistically significant difference (P < 0.05) between treated and untreated cells.

**Figure 3**

Representative images of two-Dimensional gel electrophoresis from untreated control (A) and 50 μM varlitinib treated (B) SCC-25 cells using Immobilized pH Gradient (IPG) strips 3-10 NL. Identified protein spots are encircled and labelled.
Figure 4

Spot intensities of differentially expressed identified proteins from untreated and varlitinib treated SCC-25 cells. Error bars indicate ± SD whereas * indicates statistical significance (p<0.05).

Figure 5
Protein-Protein interaction analysis of identified proteins as observed through STRING. Identified proteins include PH4B (PDIA1); Protein di-sulfide isomerase 1, KRT14; Keratin type 14, VIM; Vimentin, HSPA8; Heat shock cognate 71 kDa protein, HSPA5; Endoplasmic reticulum chaperone BiP and ACTB; Beta actin. Nodes represent proteins while edges show protein association.