EJMO

DOI: 10.14744/ejmo.2020.70327
EJMO 2020;4(4):309–318

Research Article

Mutated USP9X-Associated TRIM33 Inhibition in the Metastasis of Gingivobuccal Oral Squamous Cell Carcinoma

Suchitra Singh, Ajay Kumar Singh

Department of Bioinformatics, Central University of South Bihar, Gaya

Abstract

Objectives: Locoregional abortions of gingivobuccal oral squamous cell carcinoma (OSCC-GB) fabricate a global cancer burden. Ubiquitin Specific Peptidase 9 X-Linked (USP9X), a significantly mutated gene in OSCC-GB, usually aids SMAD family member4 (SMAD4) deubiquitination. The loss of USP9X prevents deubiquitination, which leads to SMAD4 inactivation. The TGF-β/SMAD signaling pathway is continuously regulated by a central transducer (SMAD4 protein). Inactivated SMAD4-deprived TGF-β/SMAD tumor suppressor response promotes the metastasis. USP9X inhibition promotes SMAD4 ubiquitination by E3 ligases TIF1-γ transcriptional intermediary factor1gamma (TRIM33). Overexpressed TRIM33 causes inactivation of SMAD4. The knockdown of TRIM33 inhibits tumor cell invasion. TRIM33 serves as a potential therapeutic target for OSCC-GB.

Methods: We conducted experiments to determine the possible interactions and inhibitions of target TRIM33 PHD-domain (PDB ID 3u5n) with natural as well as synthetic anticancerous drugs through molecular docking and virtual screening approach.

Results: Based on the lowest binding energy and rmsd values, out results suggest the probable synthetic inhibitor lapatanib having a binding energy of −9.22 and rmsd value of 0.00 and the natural inhibitor resveratrol having a binding energy of −8.22 and rmsd value of 0.00.

Conclusion: Our cumulative results formed a basis for investigating resaveratrol and lapatanib as potent drugs for arresting active metastasis. Our findings can accentuate the importance of high-quality investigations in OSCC-GB.

Keywords: Gingivo Buccal oral squamous cell carcinoma (OSCC-GB), TGF-β/SMAD signaling pathway, E3 ubiquitin-protein ligase (TRIM33/TIF1-γ), USP9X, SMAD4

Cite This Article: *Singh S, Kumar Singh A. Mutated USP9X-Associated TRIM33 Inhibition in the Metastasis of Gingivobuccal Oral Squamous Cell Carcinoma. EJMO 2020;4(4):309–318.*

Oral cancer is one of the most chronic head and neck malignancy reported on the national and international levels. Globally, it is the sixth-most common type of cancer.[1, 2] as well as the leading cancer affecting men in India.[3] Several past studies have established the fact that the consumption of tobacco and betel are the main source of this disease. Almost 369.200 cases of oral cancer have been reported worldwide, and approximately 145.328 deaths occurred worldwide in 2012 from this cancer.[4] Oral cancer incidence varies across different regions due to the local geographical, biological, dietary, and environmental factors. Oral cancers can be categorized into squamous cell carcinoma (OSCC), verrucous carcinoma, Kaposi sarcoma, minor salivary gland carcinoma, and lymphomas. About 90% of all cases of oral cancers OSCC predominantly occur in the buccal mucosa, lips, tongue, gingiva, hard palate, and soft palate of the mouth. The degree of oral cancer development determined by the stages, i.e., stages I, II, III, and IV. In western countries, oral cancer usually affects the tongue region, while, in India, it mainly affects the gingi-

Address for correspondence: Ajay Kumar Singh. Department of Bioinformatics, Central University of South Bihar, SH-7, Gaya–Panchanpur Road, Gaya (Bihar) Pin- 824236, India
Phone: +91-9935686230 **E-mail:** ajaysingh@cusb.ac.in
Submitted Date: September 02, 2020 **Accepted Date:** November 16, 2020 **Available Online Date:** December 25, 2020

vobuccal region that constitute the buccal mucosa, retro molar trigone, and lower gum, together known as OSCC-GB. The involvement of buccal mucosa is an aggressive malignancy, with a significant propensity of invasion into the surrounding tissues and metastasis into the cervical lymph nodes.[5] Cervical lymph node metastasis is the most critical prognostic factor of the head and neck cancer, as evidenced by multiple studies.[6] We noted that OSCC-GB substantially occurred at advanced stages (stages III and IV) with high loco regional failure despite the best multimodal treatment and therapy.[7]

Past studies on OSCC-GB identified some significantly and commonly mutated gene specific to OSCC-GB (USP9X, MLL4, UNC13C, ARID2, and TRPM3) as well as some shared genes (with TP53, FAT1, CASP8, HRAS, and NOTCH1).[8] Moreover, various pathways have been reported to be altered in gingivobuccal cancer (including p53 signaling pathways, apoptosis, viral carcinogenesis, neurotrophin signaling pathway, Wnt signaling pathway, PI3K–Akt signaling pathway, dorso-ventral axis formation, axon guidance, MAPK signaling pathway, focal adhesion, cell adhesion molecules, neuroactive ligand-receptor, Notch signaling pathway, and serotonergic synapse).

Recent studies have shown that the alteration rate of USP9X is the most frequent in OSCC-GB patients. USP9X is a deubiquitinating enzyme for SMAD4,[8] which act as an essential or crucial component of the TGF-β signaling pathway.[9] The loss of USP9X prevents deubiquitination of SMAD4, which in turn enhances the tumor progression. The mutation of USP9X results in the overexpression of E3 ubiquitin-protein ligase TRIM33 protein that antagonized the SMAD4 transcriptional and tumor suppressor activity. The overexpression of ubiquitin ligases promotes the metastasis of breast cancer.[10]

The TGF-β/SMAD signaling pathway helps in regulating cell growth, differentiation, apoptosis, and homeostasis. The alteration of the TGF-β/SMAD pathway have been shown to be involved in a variety of human diseases, including cancer, fibrosis diseases, atherosclerosis, cleidocranial dysplasia, and familial primary pulmonary hypertension.[11, 12] Different components of the TGF-β/SMAD pathway are SMAD4, R-SMAD, I-SMAD, ubiquitinating enzyme (TIfig, SMURF), deubiquitinating enzyme (USP9X, USP11, and USP15). Transforming growth factor beta (TGF-β) is a family of cytokines that help regulate a variety of biological processes.[13] For instance, they bind to the serine/threonine kinase domain of type I and type II receptor in the cytoplasm and then transduce the signal to the nucleus by SMADs proteins.[14] The receptor SMAD or R-SMAD (e.g., SMAD2 or SMAD3) gets directly phosphorylated by type 1 receptor, hence forming an activated complex with co-SMAD (SMAD4) protein.[14] These complexes then translocate to the nucleus, where the regulation and transcription of the target genes occur. The TGF-β/SMAD pathway is regulated by ubiquitinating/deubiquitinating and the phosphorylation and dephosphorylation process. Ubiquitinating refers to the post-translational modifications that modify or degrade a variety of SMAD components of the TGF-β/SMAD signaling pathway. Ubiquitination antagonized the activity of substrate protein (i.e., monoubiquitination). An ubiquitinating enzyme (which ubiquitinates substrate protein) acts as a repressor and is a reversible process. Deubiqutinating removes the covalently attached ubiquitin molecules from the substrate protein and aids in the regulation of the substrate protein.[15]

SMAD4 act as a central receptor of TGF-β signaling and is crucial for most TGF-β biological effects, including embryonic development, tumor suppression, and metastasis, and the reduction or absence of SMAD4 expression promotes the carcinogenesis of OSCC.[16] Inactivated SMAD4 deplete the TGF-β/SMAD tumor suppressor response and promotes the metastasis (Fig. 1). In the present study, we performed the interactions and the possible inhibition of the target (TRIM33/TIF1-γ) in the TGF-β/SMAD pathway by using metastasis inhibitors via docking analysis.

Methods

Functional Information About Mutated Genes and Selection of the Target

Uniprot database (http://www.uniprot.org) was opened, and the name of the gene was typed, and the functional information (i.e., about the molecular function and biological pathways) were retrieved and recorded. Among all USP9X that encoded deubiquitinating enzyme for smad4 were significantly altered in OSCC-GB. The losses of USP9X promotes the ubiquitination of smad4 and the expression level of Trim33 protein. Tif1g/Trim33 inactivates Smad4 via ubiquitination, resulting in the loss of Smad4 in the TGF-β/SMAD signaling pathway and thereby promoting the metastasis. Ectodermin/Trim33a transcriptional cofactor restricts the transcriptional activity of SMAD4 through its PHD-domain overexpression of TIF1γ, which causes inactivation of SMAD4. The knockdown of Tif1γ inhibits tumor cell invasion. For the ubiquitination of Smad4, Trim33 requires its PHD-domain; therefore, for docking analysis, only chain A and chain B were extracted from the complex of Trim33 PHD-domain and Histone peptide. Therefore, in silico interaction by docking analysis of Tif1g/Trim33 was considered as the target molecule (Fig. 2).

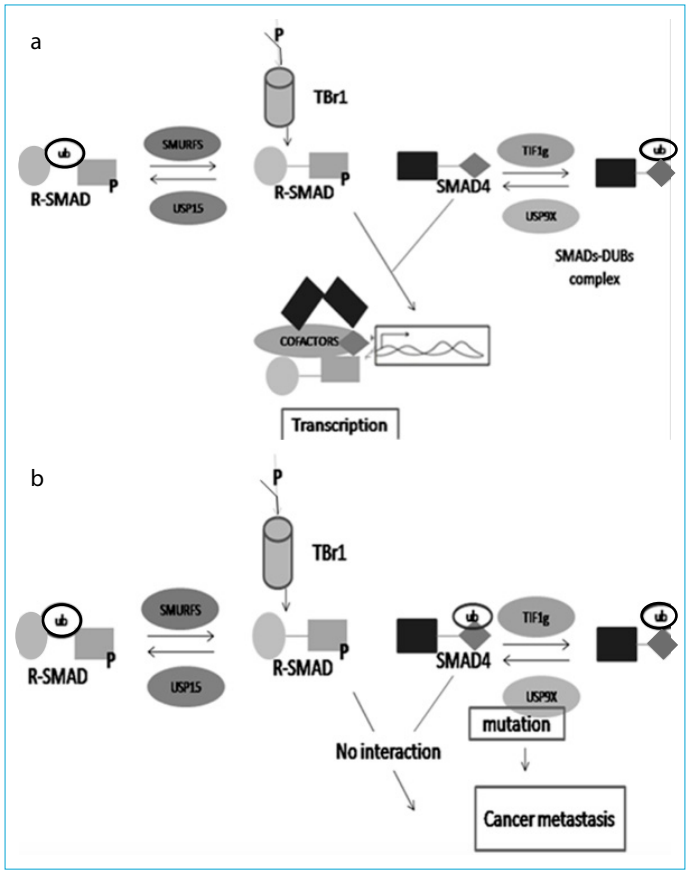

Figure 1. TGF-β/SMAD signaling pathway in normal and OSCC-GB. **(a)** TGF-β family of cytokines bind to the serine/threonine kinase domain of typeI and typeII receptor in the cytoplasm transduced signals to the nucleus by SMADs proteins, R-SMAD phosphorylated by type1 receptor forming activated complex with SMAD4 complex are then translocate to the nucleus, where regulation and transcription of target genes take place TGF-β/SMAD pathway is regulated by ubiquitinating /deubiquitinating and phosphorylation and dephosphorylation process. **(b)** In Gingivo buccal oral squamous cell carcinoma Ubiquitination antagonized the activity or localization of substrate protein stops the interaction of R-SMAD and SMAD4 leads to cancer metastasis.

Active Site Prediction of the Target Protein

The 3D coordinates of the active site of target protein is one of the important requirements for in silico docking analysis. The Ligsite software was used to predict the active sites of Trim33 protein. This software uses the Connolly surface and defines the surface-solvent-surface events instead of the protein-solvent-protein event for the prediction of active sites. Three active sites (x = −14.254, −25.255, 1.085, y = −2.469, −33.756, −13.597, z = −17.392, −53.977, 2.883) have been predicted by the Ligsite software (http://projects.biotec.tu-dresden.de/pocket/).

Protein Preparation

The structure of Tif1g/Trim33 proteins with pdb id 3u5n (Fig. 3) was opened in the Autodock window. Polar hydrogens were added, followed by the addition of Kollman charges and saved in the 3u5n.pdbqt format.

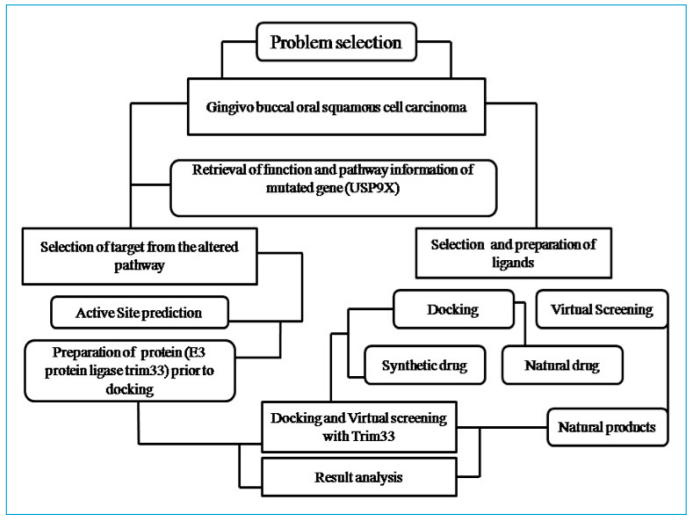

Figure 2. Flowchart of Methodology. Showing different steps involved in for in-silico interactions study with TRIM33 protein.

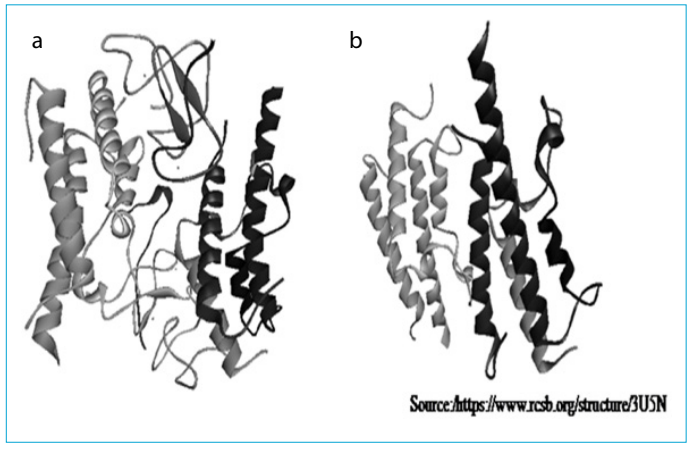

Figure 3. Crystal structure of TRIM33 **(a)** Complex of TRIM33 PHD-Bromo and H3 (1-20) k9me3k14ac Histone peptide PDB ID- 3U5n. Method: X-RAY DIFFRACTION Resolution: 1.95 A **(b)** Structure of Trim33 PHD- domain chain A and B after omitting Histone.

Ligand Selection and Preparation

Ligands selection is based on stage-specific metastasis of known inhibitors in various types of cancer that can be tested against the target of OSCC-GB. An interaction study was performed by selecting 10 natural inhibitors and 12 synthetic cancer inhibitors, as specified in Table 1 and Table 2. For molecular docking ligands, the files were opened in the Autodock window. The root and torsion numbers were detected and saved in the —.pdbqt format.

Grid Preparation

The grid box was prepared by giving X-Y-Z coordinates, and

Table 1. Common synthetic compounds reported against various types of cancers

S.No	Inhibitors	Pubchem ID	Cancer types	References
1 .	Lapatinib (Tykerb/Tyverb, GW572016)	208908	HNSCC	Agulnik, Mark, et al. "Phase II study of Lapatinib in recurrent or metastatic epidermal growth factor receptor and/or erbB2 expressing adenoid cystic carcinoma and non–adenoid cystic carcinoma malignant tumors of the salivary glands." Journal of Clinical Oncology 25.25 (2007): 3978-3984.
2.	Foretinib (GSK1363089)	42642645	HNSCC	Seiwert, Tanguy, et al. "Phase II trial of single-agent foretinib (GSK1363089) in patients with recurrent or metastatic squamous cell carcinoma of the head and neck." Investigational new drugs31.2 (2013): 417-424.
3.	Dasatinib(BMS-354825)	3062316	HNSCC	Brooks, H. D., et al. "Phase II study of dasatinib in the treatment of head and neck squamous cell carcinoma (HNSCC)." Journal of Clinical Oncology 27.15S (2009): 6022-6022.
4.	Erlotinib (Terceva, OSI-774)	176870	HNSCC	Cohen, Ezra EW, et al. "Erlotinib and bevacizumab in patients with recurrent or metastatic squamous cell carcinoma of the head and neck: a phase I/II study." The lancet oncology 10.3 (2009): 247-257.
5.	Gemcitabine	60750	Pancreatic cancer	Giroux, Valentin, et al. "p8 is a new target of gemcitabine in pancreatic cancer cells." Clinical cancer research 12.1 (2006): 235-241.
6.	Letrozole	3902	Breast cancer	Geisler, Jürgen, et al. "Influence of letrozole and anastrozole on total body aromatization and plasma estrogen levels in postmenopausal breast cancer patients evaluated in a randomized, cross-over study." Journal of Clinical Oncology 20.3 (2002): 751-757
7.	Etoposide	36462	Breast cancer	Bockbrader, Katrina M., Mingjia Tan, and Yi Sun. "A small molecule Smac-mimic compound induces apoptosis and sensitizes TRAIL-and etoposide-induced apoptosis in breast cancer cells." Oncogene 24.49 (2005): 7381.
8.	Palbociclib	5330286	Breast cancer	Qin, Ge, et al. "Palbociclib inhibits epithelial-mesenchymal transition and metastasis in breast cancer via c-Jun/COX-2 signaling pathway." Oncotarget 6.39 (2015): 41794.
9.	Ifosfamide	3690	Cervical cancer	Buda, A., et al. "Role of ifosfamide in cervical cancer: an overview." Oncology 65.Suppl. 2 (2003): 63-66.
10 .	Aspirin	2244	Colon Cancer	Ying, Jun, et al. "Aspirin inhibited the metastasis of colon cancer cells by inhibiting the expression of toll-like receptor 4." Cell & bioscience 8.1 (2018): 1
11 .	Carboplatin	10339178	Lung cancer	Cohen, Martin H., et al. "FDA drug approval summary: bevacizumab (Avastin®) plus carboplatin and paclitaxel as first-line treatment of advanced/metastatic recurrent nonsquamous non-small cell lung cancer." The oncologist 12.6 (2007): 713-718.
12.	Topotecan	60700	Lung cancer	O'brien, Mary Er, et al. "Phase III trial comparing supportive care alone with supportive care with oral topotecan in patients with relapsed small-cell lung cancer." Journal of Clinical Oncology 24.34 (2006): 5441-5447

the dimensions of the box were set to be 60-60-60 units for both the docking and virtual screening analysis. The grid file was saved as —sample_grid.gpf in the Autodock4.2 and conf.txt file in the Autodock Vina.

Docking Studies

Molecular docking software Autodock 4.2 (http://autodock.scripps.edu/) and Virtual screening software Autodock Vina (http://vina.scripps.edu) were used for the protein-ligand

Table 2. Sources of natural compounds reported against various types of cancer

S.No	Natural compounds	Pubchem ID	Source	Cancer types	References
1.	Circumin	969516	Turmeric	Cervical cancer	Zaman, Mohd S., et al. "Curcumin nano formulation for cervical cancer treatment." Scientific reports 6 (2016): 20051.
2.	Genistein	5280961	Soyabean, Lupin	Breast cancer	Chen, Jun, et al. "Genistein induces apoptosis by the inactivation of the IGF-1R/p-Akt signaling pathway in MCF-7 human breast cancer cells." Food & function 6.3 (2015): 995-1000
3.	Isoquercitinin	5280804	Apple, Onion	Colon cancer	Amado, Nathália G., et al. "Isoquercitrin suppresses colon cancer cell growth in vitro by targeting the Wnt/β-catenin signaling pathway." Journal of Biological Chemistry 289.51 (2014): 35456-35467
4.	Resveratrol	445154	Peanuts, Grapes	Pancreatic cancer	Bonucci, Massimo, et al. "Integrated cancer treatment in the course of metastatic pancreatic cancer: complete resolution in 2 cases." Integrative cancer therapies 17.3 (2018): 994-999
5.	Sulforaphane	5350	Cabbage, Broccoli, Sprouts	Oral Cancer	Liu, Chia-Ming, et al. "Sulforaphane targets cancer stemness and tumor initiating properties in oral squamous cell carcinomas via miR-200c induction." Journal of the Formosan Medical Association116.1 (2017): 41-48
6.	Gingerols	3473	Ginger root	Breast cancer	Lee, Hyun Sook, et al. "[6]-Gingerol inhibits metastasis of MDA-MB-231 human breast cancer cells." The Journal of nutritional biochemistry19.5 (2008): 313-319
7.	Aloe-emodin	10207	Aloe Vera	Colon cancer	Suboj, Priya, et al. "Aloe emodin inhibits colon cancer cell migration/angiogenesis by downregulating MMP-2/9, RhoB and VEGF via reduced DNA binding activity of NF-κB." European journal of pharmaceutical sciences 45.5 (2012): 581-591
8.	Epigallocatechin gallete	65064	Green Tea	Skin cancer	Siddiqui, Imtiaz Ahmad, Rohinton S. Tarapore, and HasanMukhtar. "Prevention of skin cancer by green tea: past, present and future." Cancer biology & therapy 8.13 (2009): 1288 1291
9.	Honokiol	72303	Magnolia tree	Lung cancer	Singh, Tripti, and Santosh K. Katiyar. "Honokiol inhibits non-small cell lung cancer cell migration by targeting PGE2-mediated activation of β-catenin signaling." PloS one 8.4 (2013): e60749.
10.	Daurinol	14704582	Arylnapthalene lignin plant	Lung and Breast cancer	Woo, Jong Kyu, et al. "Daurinol blocks breast and lung cancer metastasis and development by inhibition of focal adhesion kinase (FAK)." Oncotarget 8.34 (2017): 57058

interaction study. All compounds were docked by keeping the protein rigid. The searching algorithm for docking was set as the Genetic Algorithm, and the input file was saved as —sample_dock.dpf.

Virtual Screening

Virtual screening is a high-throughput docking in which a large numbers of ligands are docked simultaneously at the target. In our study, we employed this method to discover new inhibitors for OSCC-GB cancer. Natural products were downloaded from the African database of Zinc Library (http://zinc15.docking.org/catalogs/afronp/). The drug likeness property or the ADMET properties of these compounds were checked using the DruLito tool (http://www.niper.gov.in/pi_dev_tools/DruLiToWeb). Compounds that did not follow the Lipinski rule of 5 were discarded. Out of 880 compounds, only 608 compounds followed the Lipinski rule. Hence, the virtual screening of 608 compounds was performed using the Autodock vina, and further top 10 compounds with the lowest binding energy and rmsd values were selected for the study.

Analysis of the Interaction Studies

Docking and virtual screening results were analyzed with the lowest binding energy, and the rmsd values of each were taken as the criteria of analysis. The best ligands with

Table 3. List of newly identified genes in OSCC-GB and their functions

Genes	Function	Involvement in Pathways
USP9X	Deubiquitination of protein (e.g., SMAD4), crucial for regulation of protein at the level of protein turnover by preventing degradation of protein through inhibition of ubiquitin molecules. Also involved in axonal growth, neuronal cell migration and tumor suppressor.	TGFβ/SMAD signaling, BMP signaling pathway mTor pathway, axon guidance pathway
MLL4	Histone methyl transferase, DNA binding, chromatin regulator and act as a co-activator of p53 (tumor suppressor gene).	P53 signaling pathway
ARID2	Chromatin regulator, DNA binding.	Hippo signaling pathway
UNC13C	Calcium ion binding, phospholipids binding, neurotransmitter.	
TRPM3	Calcium channel activity, cation channel activity, and neurotransmitter and also has a tumor suppressor activity.	

the least energy of binding with the protein and the rmsd value were selected.

The LIG-PLOT (https://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/) was used to study the H-Bond interaction interactions of the protein-ligand complex.

Results

Functional Analysis of Newly Identified or Significantly Mutated Genes

The functional and pathways' information of mutated genes were retrieved from uniprot (as mentioned in Table 3). Our focus was mainly on USP9X considering that its mutation rate is extremely common in gingivobuccal cancer. The loss of USP9X lead to the inactivation of SMAD4 protein, and both are crucial for the TGFβ/SMAD signaling. Based on the literature, this pathway is altered in several cancer types, such as HNSCC, OSCC, and breast cancer. The alteration in this pathway can be attributed to smad4 reduction and post-transcriptional changes.

Molecular Docking and Virtual Screening

The Autodock 4.2 predicted the best conformation of known ligand (synthetic and natural) with TRIM33. The lowest binding energy and the rmsd values were used as the criteria to analyze the docking result. The binding energy of Trim33 docked with known natural and synthetic inhibitors are listed in Table 4 and Table 5. Out of 3 active sites, the second active site coordinates were best in interaction with ligand based on binding affinity (Fig. 4).

Based on the abovementioned criteria, lapatanib and palbociclib (synthetic inhibitors) and aloe-emodin and resaveratrol (natural inhibitors) were found to be the best interacting molecules (Fig. 5) that are useful for further H-Bond interaction analysis through ligplot (https://www.ebi.

Table 4. Molecular docking analysis of synthetic drugs against Trim33

S.No	Compound Name	Pocket 1			Pocket 2			Pocket 3		
		Pose	Binding Energy	RMSD	Pose	Binding Energy	RMSD	Pose	Binding Energy	RMSD
1.	Lapatinib*	2	-7.43	0.00	10	-9.22	0.00	5	-5.42	0.00
2.	Foretinib	6	-6.73	0.00	1	-6.34	0.00	3	-3.92	0.00
3.	Dasatinib	5	-6.79	0.00	2	-7.63	0.00	3	-5.71	0.00
4.	Erlotinib	8	-6.10	0.00	4	-7.19	0.00	1	-5.40	0.00
5.	Letrozole	6	-6.12	0.00	1	-8.12	0.00	10	-6.14	0.00
6.	Palbociclib*	4	-8.55	0.00	2	-8.33	0.00	3	-5.71	0.00
7.	Aspirin	4	-4.73	0.00	1	-6.29	0.00	7	-5.44	0.00
8.	Etoposide	1	-7.05	0.00	3	-8.62	0.00	4	-6.54	0.00
9.	Isoflamide	9	-4.43	0.00	8	-5.00	0.00	10	-4.52	0.00
10.	Carboplatin	9	-4.46	0.00	9	-4.62	0.00	2	-4.40	0.00
11.	Topotecan	4	-7.43	0.00	8	-8.77	0.00	1	-6.96	0.00
12.	Gemcitabine	1	-4.68	34.99	6	-5.67	0.00	4	-5.40	0.00

*Best docked inhibitor

Table 5. Molecular docking analysis of natural drugs against TRIM33.

S.No.	Compound Name	Pocket 1			Pocket 2			Pocket 3		
		Pose	Binding Energy	RMSD	Pose	Binding Energy	RMSD	Pose	Binding Energy	RMSD
1.	Curcumin	2	-5.93	0.00	2	-7.35	0.00	1	-6.12	0.00
2.	Genistein	8	-6.33	0.00	10	-7.53	0.00	9	-6.54	0.00
3.	Isoquercitinin	10	-4.75	0.00	2	-5.42	0.00	5	-4.66	0.00
4.	Resveratrol*	7	-6.77	0.00	1	-8.22	0.00	10	-6.05	0.00
5.	Sulforaphane	2	-5.56	0.00	5	-5.51	0.00	5	-4.40	0.00
6.	Gingerols	1	-5.13	0.00	1	-5.70	0.00	7	-4.52	0.00
7.	Aloe-emodin*	4	-5.99	0.00	3	-8.09	0.00	6	-6.45	0.00
8.	Epigallocatechin gallete	4	-5.78	0.00	2	-4.26	0.00	8	-7.27	0.00
9.	Honokiol	3	-6.69	0.00	10	-8.05	0.00	9	-6.28	0.00
10.	Daurinol	6	-7.66	0.00	1	-8.00	0.00	7	-8.08	0.00

* Best docked inhibitor.

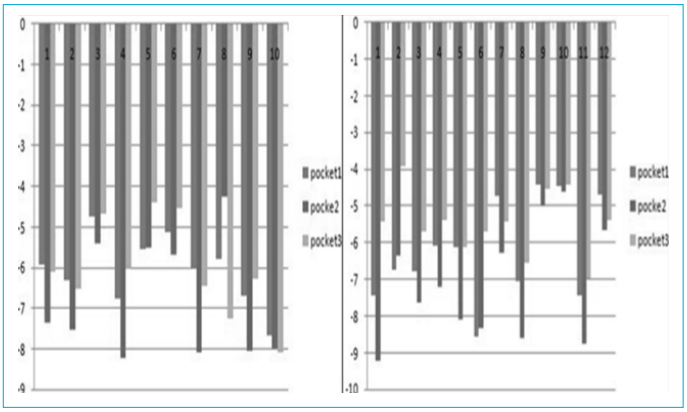

Figure 4. Binding energy of Trim33 with natural and synthetic compounds in all the three pockets Graph showing the binding energy of target and inhibitors interactions among all the pockets, pocket 2 have best binding energy in both natural and synthetic compounds docking. **(a)** Binding energy of Trim33 with natural inhibitors in all the three pockets. **(b)** Binding energy of Trim33 with synthetic inhibitors in all the three pockets.

ac.uk/thornton-srv/software/LIGPLOT/).

The criteria for evaluating virtual screening results were similar to that of molecular docking. Since the binding affinity of known drugs was better in the second active site, virtual screening was performed on the second active site.

By using the above paradigm, top 10 compounds were listed in Table 6, and the interaction studies were performed for the compounds whose binding energy was greater than that of known drugs.

Ligplot Interaction Analyses

TRIM33 and inhibitors interaction are displayed in the Table 7 and Table 8 in the form of H-Bond. E3 ubiquitin-protein ligase TRIM33 shows the best interaction with the natural

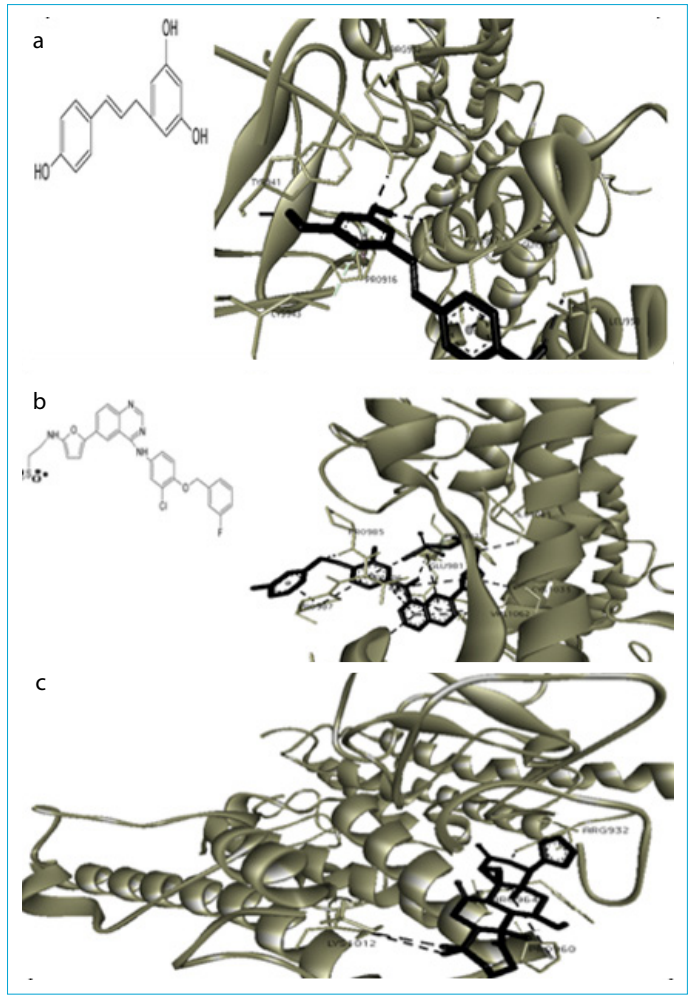

Figure 5. Binding mode of natural and synthetic inhibitors with Trim33 protein **(a)** Structure and Binding mode of Resveratrol with different residues of Trim33. **(b)** Structure and Binding mode of Lapatanib with different residues of Trim33 protein. **(c)** Binding mode of natural lead compound (croman-4-one) with Trim33 protein.

Table 6. Lead molecules screened through Virtual screening

Zinc ID	Popular Name	Binding Energy
ZINC95485943*	(3-furyl-dihydroxy-tetramethyl-BLAHtrione)	-9.3
ZINC35941652*	(2S)-2-(2,2-dimethylchroman-6-yl)-7-hydroxy-chroman-4-one	-9.2
ZINC00898806	Xylopine	-9.1
ZINC95482	N-[4-[(2,6-dimethoxypyrimidin-4-yl)sulfamoyl]phenyl]-2,3-dihydro-1,4-benzodioxine-6-carboxamide	-9.0
ZINC14825190	5-hydroxy-7-(7-hydroxy-2,2-dimethyl-chromen-6-yl)-2,2-dimethyl-pyrano[3,2-g]chromen-6-one	-9.1
ZINC95485971	1,3,6,7-tetrahydroxy-2-(4-methylpent-3-enyl)xanthen-9-one	-9.1
ZINC31168265	(3S)-3-hydroxy-3,11-dimethyl-1,2-dihydrocyclopenta[b]anthracene-5,10-dione	9.0
ZINC43121107	(E)-1-(2,4-dihydroxyphenyl)-3-(2,2-dimethylchroman-6-yl)prop-2-en-1-one	-8.9
ZINC95486025	1-[3-[(1R,4aS,9aS)-2-ethyl-1,3,4,4a,9,9a-hexahydropyrido[3,4-b]indol-1-yl]propyl]-9-methyl-pyrido[3	-8.9
ZINC03645832	Senegalensin	-8.8

* Represents the best result than docking of known inhibitors

Table 7. Interacting residues of natural and synthetic drugs against Trim33

Target	Ligands							
TRIM33	Resveratrol		Aloe-emodin		Lapatinib		Palbociclib	
	Residues	H-Bond Distance (Å)	Residues	H-Bond Distance (Å)	Residues	H-Bond Distance (Å)	Residues	H-Bond Distance (Å)
	GLU967, Arg932, Tyr941, Leu958, His 1017	3.10, 3.74, 3.75, 3.14, 3.33	Glu981, Met1001, ASN1034	2.53, 3.10, 2.63	Glu981, Met1001	3.02, 3.00	Glu981	2.90

Table 8. Interacting residues of lead molecules against Trim33

Target	Ligands			
TRIM33	3-furyl-dihydroxy-tetramethyl-BLAHtrione		(2S)-2-(2,2-dimethylchroman-6-yl)-7-hydroxy-chroman-4-one	
	Residues	H-Bond Distance (Å)	Residues	H-Bond Distance(Å)
	Arg932	3.00	GLU98	3.12

drug resveratrol that forms 5 H-Bond with GLU 967 at a distance of 3.10 Å, Arg932 forms the H-Bond at a distance of 3.74 Å, Leu958 forms H-Bond at 3.75 Å distance, Tyr941 forms H-Bond at 3.75 Å, and His 1017 forms H-Bond at a distance of 3.33 Å (Fig. 6a).

Among the synthetic drugs, lapatinib showed the best interaction with Trim33. The residues involved in the formation of H-Bond include GLU981 and MET1001, which were at a distance of 3.02 Å and 3.00 Å (Fig. 6b). The interaction of E3 ubiquitin-protein ligase with the lead compound was not as good as that of known drugs, although the binding energy of lead compounds was higher than that of known inhibitors. The lead compounds screened by virtual screening had lesser H-Bond than the known drugs. The top two were lead residues.

Discussion

Our analysis revealed the involvement of the TGFβ/SMAD signaling pathway and its associated protein E3 ubiquitin ligase TRIM33 in cervical lymph node metastasis. The TGF-β signaling pathways promote cancer metastasis when both smad4 and USP9X functionality are lost. USP9X loss and E3 ubiquitin-protein ligase TRIM33 overexpression is an important factor involved in the metastasis of OSCC-GB. In

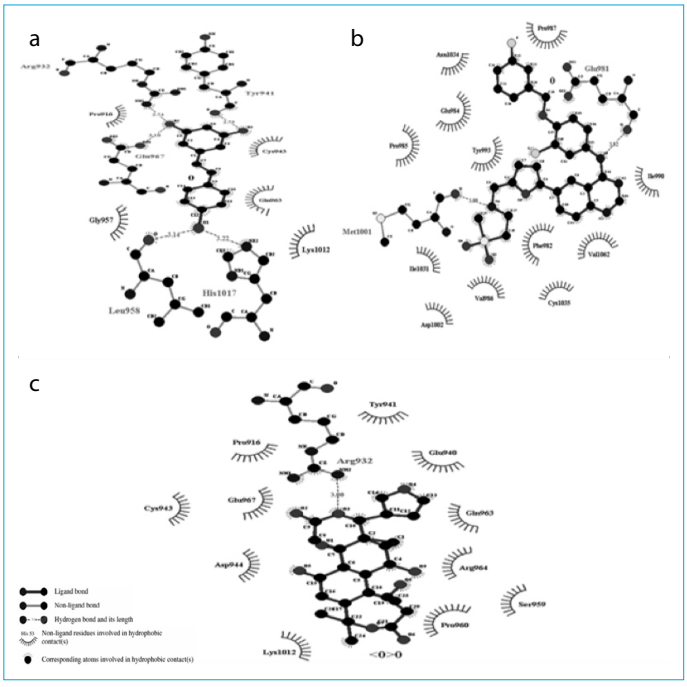

Figure 6. Interaction result of E3 ubiquitin-protein ligase TRIM33 with synthetic, natural and lead molecules using Ligplot. **(a)** Interaction with Resveratrol forming five H-Bond with GLU967, Arg932, Leu958 and His 1017. **(b)** Interaction with Lapatinib forming two H-bond withGLU981 and MET1001. **(c)** Interaction with lead compound forming H-bond with ARG 932 residue.

fact, it may act as a new therapeutic target in OSCC-GB. A list of 12 well-known synthetic drugs along with 10 natural drugs are used for molecular docking. In silico docking studies on the current work provides evidence that, among all screened inhibitors, lapatanib (synthetic) and resaveratrol (natural), have been proved to have a good potential in targeting metastasis in OSCC-GB. The cessation of metastasis may decrease the loco regional failure as well as mortality due to OSCC-GB, which is one of the crucial issues in India. Although the provided analysis and methodologies are adequate and constitute a set of powerful tools to guarantee the real-time requirements of the in silico approach, there is some scope for improvements. To determine the exact strength and duration of H-Bond, MD (Molecular Dynamics) Simulation can be performed with the docked molecules. Our results need to be validated through wet lab experiments in the future.

Disclosures

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

Authorship Contributions: Concept – S.S.; Design – S.S.; Supervision – A.K.S.; Materials – S.S.; Data collection &/or processing – S.S.; Analysis and/or interpretation – S.S., A.K.S.; Literature search – S.S.; Writing – S.S.; Critical review – A.K.S.

References

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. CA: a cancer journal for clinicians 2010;60:277–300.
2. Szafarowski T, Szczepanski MJ. Cancer stem cells in head and neck squamous cell carcinoma. Otolaryngologia Polska 2014;68:105–11.
3. Dikshit R, Gupta PC, Ramasundarahettige C, Gajalakshmi V, Aleksandrowicz L, Badwe R, et al; Million Death Study Collaborators. Cancer mortality in India: a nationally representative survey. Lancet 2012;379:1807–16.
4. Ghantous Y, Abu I.E. Global incidence and risk factors of oral cancer. Harefuah 2017;156:645–9.
5. Ahmed S.Q, Junaid M, Awan S, Choudhary M.M, Kazi M, Masoom, et.al., Relationship of tumor thickness with neck node metastasis in buccal squamous cell carcinoma: An experience at a tertiary care hospital. International archives of otorhinolaryngology 2017;21:265–9.
6. Mamelle G, Pampurik J, Luboinski B, Lancar R, Lusinchi A, Bosq. Lymph node prognostic factors in head and neck squamous cell carcinomas. The American journal of surgery 1994;168:494–8.
7. Walvekar RR, Chaukar DA, Deshpande MS, Pai PS, Chaturvedi P, Kakade A, et al. Squamous cell carcinoma of the gingivobuccal complex: predictors of locoregional failure in stage III-IV cancers. Oral Oncol 2009;45:135–40.
8. India Project Team of the International Cancer Genome Consortium. Mutational landscape of gingivo-buccal oral squamous cell carcinoma reveals new recurrently-mutated genes and molecular subgroups. Nat Commun 2013;4:2873.
9. Dupont S, Mamidi A, Cordenonsi M, Montagner M, Zacchigna L, Adorno M, et al. FAM/USP9x, a deubiquitinating enzyme essential for TGFbeta signaling, controls Smad4 monoubiquitination. Cell 2009;136:123–35.
10. Gallo LH, Ko J, Donoghue DJ. The importance of regulatory ubiquitination in cancer and metastasis. Cell Cycle 2017;16:634–48.
11. Massagué J, Wotton D. Transcriptional control by the TGF-beta/Smad signaling system. EMBO J 2000;19:1745–54.
12. Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. N Engl J Med 2000;342:1350–8.
13. Akhurst RJ, Derynck R. TGF-beta signaling in cancer--a double-edged sword. Trends Cell Biol 2001;11:S44–51.
14. Massagué J. TGF-beta signal transduction. Annu Rev Biochem 1998;67:753–91.
15. Nijman SM, Luna-Vargas MP, Velds A, Brummelkamp TR, Dirac AM, Sixma TK, et al. A genomic and functional inventory of deubiquitinating enzymes Cell 2005;123:773–86.
16. Iamaroon A, Pattamapun K, Piboonniyom S.O. Aberrant expression of Smad4, a TGF-β signaling molecule, in oral squa-

mous cell carcinoma Journal of oral science 2006;48:105-109.
17. Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, et al. Effect of high intensity intermittent training on heart rate variability in prepubescent children. European journal of applied physiology 2009;105:731–8.
18. Pathak KA, Gupta S, Talole S, Khanna V, Chaturvedi P, Deshpande MS, et al. Advanced squamous cell carcinoma of lower gingivobuccal complex: patterns of spread and failure. Head & Neck: Journal for the Sciences and Specialties of the Head and Neck 2005;27:597–602.
19. Sadaie M, Salama R, Carroll T, Tomimatsu K, Chandra T, Young AR, et al. Redistribution of the Lamin B1 genomic binding profile affects rearrangement of heterochromatic domains and SAHF formation during senescence. Genes & development 2013;27:1800–8.
20. Hussain S, Zhang Y, Galardy P. DUBs and cancer: the role of deubiquitinating enzymes as oncogenes, non-oncogenes and tumor suppressors. Cell cycle 2009;8:1688–97.
21. Hoge CW, McGurk D, Thomas JL, Cox AL, Engel CC, Castro CA. Mild traumatic brain injury in US soldiers returning from Iraq. New England journal of medicine 2008;358:453–63.
22. Liu T, Ghosal G, Yua J, Chen J, Huang J. FAN1 acts with FANCI-FANCD2 to promote DNA interstrand cross-link repair. Science 2010;329:693–6.
23. Bornstein S, White R, Malkoski S, Oka M, Han G, Cleaver T, et al. Smad4 loss in mice causes spontaneous head and neck cancer with increased genomic instability and inflammation. The Journal of clinical investigation 2009;119:3408–19.
24. Zhao M, Mishra L, Deng CX. The role of TGF-β/SMAD4 signaling in cancer. International journal of biological sciences 2018;14:111–23.
25. Fadlullah MZH, Chiang IKN, Dionne KR, San Yee P, Gan CP, Sam KK, et al. Genetically-defined novel oral squamous cell carcinoma cell lines for the development of molecular therapies. Oncotarget 2016;7:27802–18.
26. Ligr M, Wu X, Daniels G, Zhang D, Wang H, Hajdu C, et al. Imbalanced expression of Tif1γ inhibits pancreatic ductal epithelial cell growth. Am J Cancer Res 2014;4:196–210.
27. Chen XF, Zhang HJ, Wang HB, Zhu J, Zhou WY, Zhang H, et al. Transforming growth factor-β1 induces epithelial-to-mesenchymal transition in human lung cancer cells via PI3K/Akt and MEK/Erk1/2 signaling pathways. Mol Biol Rep 2012;39:3549–56.

Web servers

- http://www.rcsb.org/pdb/home/home.do
- http://projects.biotec.tu-dresden.de/pocket/
- http://autodock.scripps.edu/
- www.ebi.ac.uk/thornton-srv/software/LigPlus/
- https://www.pymol.org
- http://www.niper.gov.in/pi_dev_tools/DruLiToWeb