

Resveratrol -Induced Demethylation of *LATS1* and *LATS2* Genes in Oral Squamous Cell Carcinoma

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Abstract

Objective: This study aimed to investigate the potential of Resveratrol in reversing promoter hypermethylation of *LATS1/2* genes, key components of the Hippo Signaling Pathway, in OSCC cell lines. **Materials and Methods:** In vitro investigation was conducted after getting encouraging outcomes from consensus molecular docking & molecular dynamics (MD) simulations upto 100 nanoseconds. *In vitro* studies were conducted using CAL33 and SCC84 OSCC cell lines. Cell viability of the cells was assessed by MTT assay method to determine IC₅₀ values. Cellular morphological changes induced by treatment were observed under microscope in comparison to control cells. Migration ability of cells was examined by wound healing assay, whereas apoptotic induction was confirmed through DNA fragmentation analysis. and To assess methylation reversal, extracted DNA is subjected to sodium bisulfite treatment followed by methylation-specific PCR (MS-PCR). **Results:** Resveratrol exhibited IC₅₀ values of 130.14 μ M and 133.94 μ M in CAL33 and SCC84 cells respectively, induced apoptotic body formation, significantly inhibited cell migration, and caused DNA fragmentation, while crucially, MS-PCR analysis revealed progressive demethylation of *LATS1/2* genes over six days at 25 μ M concentration. This is shown by a reduction in methylation-specific bands intensity. **Conclusion:** These findings demonstrate that resveratrol effectively reverses promoter hypermethylation of *LATS1/2* genes in OSCC cells through DNMT inhibitory activity at sub-cytotoxic doses, representing a promising non-toxic epigenetic therapeutic agent for oral cancer treatment with potential for clinical translation.

Keywords: Promoter hypermethylation, Tumor Suppressor Genes (TSGs), Epigenetic modifications, Resveratrol, *LATS1/2*

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Introduction

OSCC predominantly manifests in the oral cavity, which includes the gums, tongue, cheeks, lips, and hard palate. It is commonly referred to as cancer of the lip, tongue, base of the mouth, cheeks, and palate, characterized by uncontrolled malignant tissue growth [1]. OSCC typically arises in areas such as the buccal mucosa, lower lip, tongue, palate, gingiva, vestibule, and alveolus, with a higher prevalence in males compared to females [2]. This type of cancer accounts for over 95% of oral cancer cases [3]. According to Globocan, 2022, oral cancer constitutes 38% of all head and neck malignancies globally, with an estimated 389,485 new cases & 188,230 deaths annually. In 2022 alone, approximately twenty million new cancer cases were reported worldwide, with

projections suggesting this number could rise to thirty-five million by 2050 [4]. The 5 year survival rate for OSCC patients has remained low over the years (Davaatsend et al. 2023). A number of risk factors contribute to this complex disease, including human papillomavirus (HPV) infection, dietary influences, genetic predisposition, and tobacco and alcohol consumption. Smoking and drinking often have synergistic effects on carcinogenesis since tobacco smoke contains more than 4,000 harmful chemicals including carcinogens like nicotine, methanol, arsenic, and methoxymethylfurfural which exacerbate the risk of developing OSCC [5]. Epigenetic modifications, like promoter hypermethylation (PH), which are influenced by environmental and lifestyle factors are the contributors

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in carcinogenesis. Several genes like *p16*, *BRCA1*, *MGMT*, *RASSF1A*, *DAPK1*, *GSTP1*, *PTEN* and many more have been identified as losing their tumor suppressive functions in different cancer types due to PH [6-10] and restoring these functions will be a promising therapeutic approach. *LATS1* (Large Tumor Suppressor 1) and *LATS2* (Large Tumor Suppressor 2) PH is also linked with cancer progression in several cancer types [11-12]. *LATS1/2* genes are important regulator of Hippo signal transmission pathway that is crucial machinery for cell growth and apoptosis [13]. Inactivation of these genes, frequently mediated by epigenetic silencing such as PH, restrains their tumour suppressor functions [14,15]. This consequently causes stabilization and activation of oncoproteins that favour oncogenic phenotypes and play a role in the development of cancer. Activated *LATS1/2* promotes YAP/TAZ phosphorylation, resulting in their ubiquitination and degradation, thereby limiting cellular proliferation. Additionally, *LATS1* interacts with Cdc2 during the mitotic phase of the cell cycle, negatively regulating its activity and ensuring proper cell cycle progression. It also regulates the tumor-suppressive transcriptional factor p53, cell cycle checkpoint regulators, and the centrosomal protein phosphatase CDC25B, further demonstrating its multifaceted role in maintaining cellular integrity [16]. Research on OSCC has revealed significant associations between *LATS1/2* PH and disease development [17]. A study examining 70 OSCC tissue samples and 70 normal oral samples found that both *LATS1/2* PH showed significant relationships between cases and controls [18]. PH of *LATS1/2* has been extensively studied in breast cancer. Research has shown that *LATS1* mRNA levels in breast cancer patients with hypermethylation were significantly lower than in cancer samples without hypermethylation. The same pattern was observed for *LATS2*, with significantly lower mRNA expression in hypermethylated tumors. These findings suggest that PH serves as a critical mechanism for silencing both genes in breast cancer [19,20]. PH is a predominant epigenetic mechanism leading to inactivation of tumor suppressor genes in cancer. This process is characterized by the increased methylation of cytosine residues in the CpG islands located in the promoter region of the gene, leading to chromatin compaction and repression of transcription. DNA methyltransferase 1 (DNMT1) is an enzyme that selectively interacts with hemimethylated CpG sites and maintain the methylation patterns during development. For *LATS1/2*, PH has been reported as a common mechanism of silencing in several tumours. The close correlations between *LATS1/2* PH, reduced gene expression and poor clinicopathological characteristics suggest that they may be one of the new potential candidates for diagnostic and prognostic biomarkers [19,21]. Moreover, the functional recovery of *LATS1/2* by demethylating drugs offers potential therapeutic implications. With the increasing comprehension of the epigenetic regulation of *LATS1/2*, our findings suggest that exploration of these genes can provide promising targets for the prediction of cancer diagnosis/prognosis and therapeutic intervention, facilitating the precision medicine to cancer patients. PH is

influenced by environmental and lifestyle factors which do not alter the DNA sequence itself, unlike mutations. Since these changes do not involve a permanent alteration of the genetic code, they are reversible. This reversibility makes them ideal targets for therapeutic approaches, offering potential strategies to restore normal gene function without modifying the DNA sequence. To date, 5-aza-2'-deoxycytidine has been used to restore the expression of the demethylated gene [22, 23]. Natural Compounds with chemopreventive properties are preferred over chemical demethylating agents due to the cytotoxic effects in epigenetic reversal with the potential to be used in cancer therapy [24, 25]. Resveratrol (Rsv) which is a natural polyphenol found in grapes, berries is known for its anti-inflammatory, cardioprotective, antioxidant properties. Direct evidence that resveratrol reverse PH of specific tumor suppressor genes is sparse. In silico study of targeting DNMT1 by Rsv has been performed to exhibit a combination of hydrogen bonds and hydrophobic interactions, with its aromatic rings positioned favourably within the binding pocket. The consensus molecular docking analysis revealed strong binding affinity of Rsv with DNMT1. 3D and 2D interaction further elucidate distinct binding modes and specific molecular interaction with DNMT1. Further to this, molecular dynamics (MD) simulations of 100 nanosecond show the impact of Rsv binding on DNMT1 interaction dynamics, stability, and flexibility. After getting promise results in MD simulation study, in vitro study for the reversal of hypermethylation in the OSCC cell lines using Rsv. have been performed.

Materials and Methods

Methodology

Consensus Molecular Docking: The crystallographic structure of DNMT1 (PDB: 4wxx) was acquired from RCSB-PDB [26] and optimized using UCSF Chimera's Dockprep, removing extraneous elements and adding polar hydrogens. The DNMT1 catalytic domain and binding pocket were characterized via literature, PDB 4wxx, CASTp, and AADS. Resveratrol was retrieved from PubChem and converted to PDBQT format using Open Babel [27]. A consensus molecular docking strategy was implemented using Qvina, Smina, and AutoDock Vina 1.2.0 to enhance binding mode and affinity prediction robustness [28].

Molecular Dynamics Simulation: All atom molecular dynamics (MD) simulations were performed with GROMACS for 100 nanoseconds [29, 30]. The DNMT1-ligand complex was parameterized using the CHARMM36 force field [31] and CGenFF for the ligand, then solvated in a TIP3P water model under periodic boundary conditions [32, 33]. System stability and dynamics were assessed through RMSD, RMSF, Rg, SASA, hydrogen bond persistence, PCA, and protein-ligand interaction trajectories. Binding free energies were computed using MM/PBSA calculations, with trajectory analyses done via GROMACS utilities and MD Analysis [34].

Cell Culture

CAL33 (obtained from Cancer Biology Laboratory,

School of Life Sciences, JNU) and UPCI: SCC84 cells (a gift from Dr. Susanne M. Gollin, University of Pittsburgh US) were cultured in DMEM (GIBCO), and DMEM–HAM'sF12 medium (GIBCO), respectively. The culture medium contained 10% fetal bovine serum (FBS, GIBCO) and 1% penicillin-streptomycin (GIBCO). The cultures were maintained in a humidified, 5% CO₂ condition at 37°C. A 100mM stock of resveratrol (Rsv) was made in DMSO (≤0.1%v/v) and kept at –20°C until used. All the experiments were performed with logarithmically growing cells.

Cytotoxicity Assay

The cytotoxicity of resveratrol on CAL33 and SCC84 cell lines was tested by MTT assay. The cells were seeded into 96-well plates at a density of 1×10⁴ cells per well and allowed to attach for 24h, then treated with different concentrations of resveratrol (serial dilution) for 48h, which was followed by aspiration of the medium in wells and adding MTT solution (0.5mg/ml in phosphate buffer; 100μL per well). Following 2 h incubation at 37°C, plates were decanted and formed formazan crystals were solubilized in 100 μl dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm in a plate reader. The percentage of cell viability was calculated relative to control untreated cells, and IC₅₀ values were derived from dose response curves. Each experiment was performed in triplicate.

Morphological Assessment

Morphological alterations in CAL33 and SCC84 cells following resveratrol exposure at the determined IC₅₀ concentration were examined under an inverted microscope after 24 h of treatment, with untreated cells serving as controls.

Wound Healing Assay

For migration assay, cells were plated in six-well plates until they grew to 90–100% confluence. The cell monolayer was scratched linearly with a sterile 200μL pipette tip. Detached cells were washed away by 1X phosphate buffer (pH7.0), and fresh medium with or without Rsv. was added afterwards. Closure of wound was viewed by the inverted microscope at 0h, 24h and 48h. All assays were repeated three times.

DNA Isolation

Genomic DNA was isolated by the standard phenol-chloroform procedure. Cells were then lysed in a digestion buffer (Tris-HCl, NaCl and EDTA) plus proteinase K (0.2mg/ml) and RNase (1mg/ml). The pellet of purified DNA was dissolved in TE buffer (10Mm Tris-HCL, 1Mm EDTA, pH8.0) stored at –20 °C until further analysis [15].

DNA Fragmentation Assay

To assess apoptosis, DNA from control and Rsv treated cells (after 48 h exposure) was extracted and analysed by agarose gel electrophoresis using a 100 bp DNA ladder as a molecular size marker. The appearance of DNA

laddering indicated apoptotic fragmentation.

Sodium Bisulfite Treatment and Methylation-Specific PCR (MSP)

20μl of genomic DNA resuspended in TE buffer underwent bisulfite modification using the commercial conversion kit from Zymo Research according to the supplied instructions. Subsequently, methylation-specific polymerase chain reaction was conducted as shown in Table 1 employing primer sets designed to distinguish between methylated and unmethylated CpG sites within the *LATS1* and *LATS2* gene regions. (Primer details provided in Table 2). The 25 μM concentration was selected based on prior literature supporting sub-cytotoxic concentrations for epigenetic modulation, and is approximately five-fold below the determined IC₅₀ values (~130 μM), ensuring epigenetic specificity without confounding cytotoxic effects.

Results

Molecular docking: The molecular docking analysis revealed binding affinity of Rsv with DNMT1, shown in Table 3.

Rsv demonstrated strong binding energy in with all the various software. 3D interaction analysis shown in Figure 1 revealed distinct binding modes for Rsv within the DNMT1 binding pocket. The 2D shown in Figure 1 interaction diagrams further elucidated the specific molecular interactions. Rsv exhibited a combination of hydrogen bonds and hydrophobic interactions, with its aromatic rings positioned favourably within the binding pocket.

MD Simulation

RMSD

The structural stability and conformational dynamics of the DNMT1-Resveratrol complex were comprehensively evaluated through RMSD analysis over the 100-nanosecond simulation trajectory (Figure 2A). While the 100-nanosecond trajectory demonstrated consistent stabilization of the DNMT1-resveratrol complex, extension to 500 ns would provide more robust insights into long-term binding dynamics and is planned as future work. The RMSD calculations reveal distinct stability profiles between the apo protein

Table 1. Methylation-specific PCR Conditions and Expected Amplicon sizes for *LATS1* and *LATS2* Gene pQromoter Analysis

Parameter	<i>LATS1</i>	<i>LATS2</i>
Initial Denaturation	94°C for 10 min	95°C for 10 min
Number of Cycles	35	35
Cycle Conditions		
Denaturation	94°C, 40 s	95°C, 45 s
Annealing	55°C, 40 s	55°C, 40 s
Extension	72°C, 40 s	72°C, 40 s
Final Extension	72°C for 10 min	72°C for 10 min

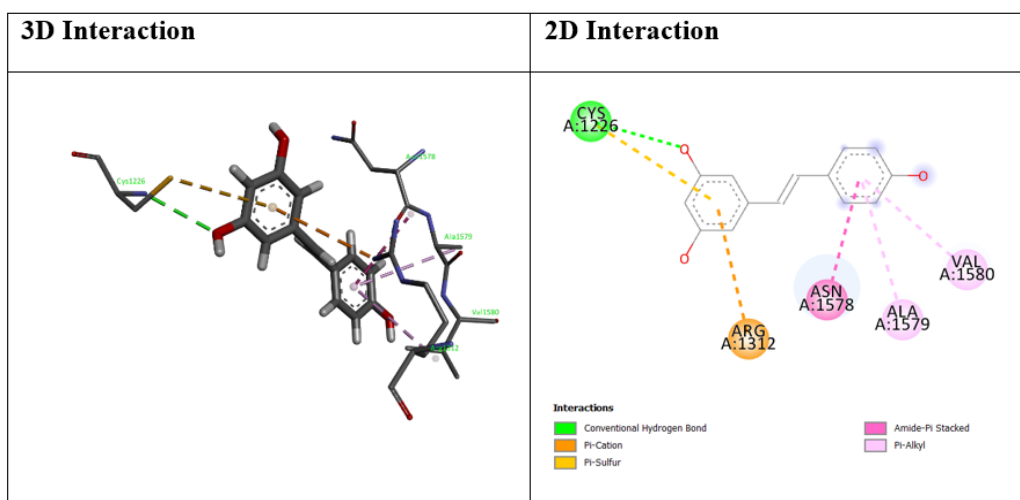


Figure 1. Molecular Docking Analysis Showing Binding Interactions between DNMT1 and Resveratrol. (A) Three-dimensional (3D) representation of resveratrol (shown in stick representation) bound within the active site pocket of DNMT1. The ligand adopts a favorable orientation with aromatic rings positioned optimally within the binding cavity. (B) Two-dimensional (2D) interaction diagram illustrating the specific molecular interactions between resveratrol and DNMT1. The diagram depicts hydrogen bonds and hydrophobic interactions with key amino acid residues in the binding pocket.

Table 2. Primer Sequences, Amplicon Sizes, Annealing Temperature for PCR Analysis

Gene	Primer sequence	Amplicon size
<i>LATS1</i>	MF5'- GGAGTTT CGTTTTGTC-3'	
	MR5'- CGACGTAATAACG AACGCCTA-3'	138bp
	UF5'- TAGGTTGGAGTGTGGTGGT-3'	
	UR5'- CCCAACATAATAACAAACACCT-3'	121bp
<i>LATS2</i>	MF5'- ATTTCGGTTTATTGTAATTTTC-3'	
	MR5'- AACCAACATAATAAAACCCCG-3'	148bp
	UF5'- TTTGTTTTTTT GGGTTTAAGT-3'	
	UR5'- CCAACATAATA AAACCCCA -3'	130bp

Table 3. Results of Consensus Molecular Docking

Compound name	Smina binding energy in kcal/mol	Qvina	AutoDock Vina
Resveratrol	-7.90672827	-7.3	-7.313

(DNMT1) and the ligand-bound complex, providing insights into binding-induced structural changes and conformational equilibration patterns. The apo DNMT1 protein demonstrated higher structural variability with an average RMSD of 17.809 ± 2.156 Å, indicating significant conformational fluctuations and structural drift throughout the simulation period. The elevated standard deviation suggests extensive structural sampling and reduced conformational stability in the absence of ligand binding. In contrast, the DNMT1, Rsv complex exhibited superior structural stability with a markedly lower average RMSD of 14.518 ± 1.330 Å, representing an 18.5% reduction in structural deviation compared to the apo form. The substantial reduction in both average RMSD and standard deviation upon Rsv binding demonstrates that ligand association significantly stabilizes the protein structure and restricts conformational mobility.

The 38.3% decrease in standard deviation (from 2.156 Å to 1.330 Å) indicates that Rsv binding not only reduces overall structural fluctuations but also promotes more consistent conformational behaviour throughout the simulation. This stabilization effect suggests that Rsv forms optimal interactions within the DNMT1 binding pocket, effectively constraining protein dynamics and promoting a more thermodynamically stable complex.

RMSF

The residue-specific flexibility patterns were analysed to understand local protein dynamics and ligand-induced conformational changes in both apo and Resveratrol-bound DNMT1 states (Figure 2B). The RMSF analysis reveals distinct patterns of residue mobility that provide insights into the mechanism of ligand-induced protein stabilization. The apo DNMT1 protein exhibited higher average residue fluctuations of 4.272 ± 3.927 Å, indicating significant local flexibility across multiple protein regions. The elevated standard deviation suggests highly variable residue mobility with distinct regions showing markedly different flexibility patterns. Upon Rsv binding, the protein demonstrated reduced average fluctuations of $3.628 \pm$

2.906 Å, representing a 15.1% decrease in overall residue mobility. The 26.0% reduction in standard deviation (from 3.927 Å to 2.906 Å) indicates that Rsv binding not only reduces overall protein flexibility but also promotes more uniform residue behaviour across the protein structure. This homogenization of residue flexibility suggests that ligand binding has a global stabilizing effect, reducing the extreme flexibility variations observed in the apo

state. The RMSF profile analysis reveals that Rsv binding particularly affects loop regions and binding site periphery, where the most significant flexibility reductions are observed, while maintaining necessary functional motions in catalytic critical areas.

Radius of Gyration

The global structural compactness and conformational

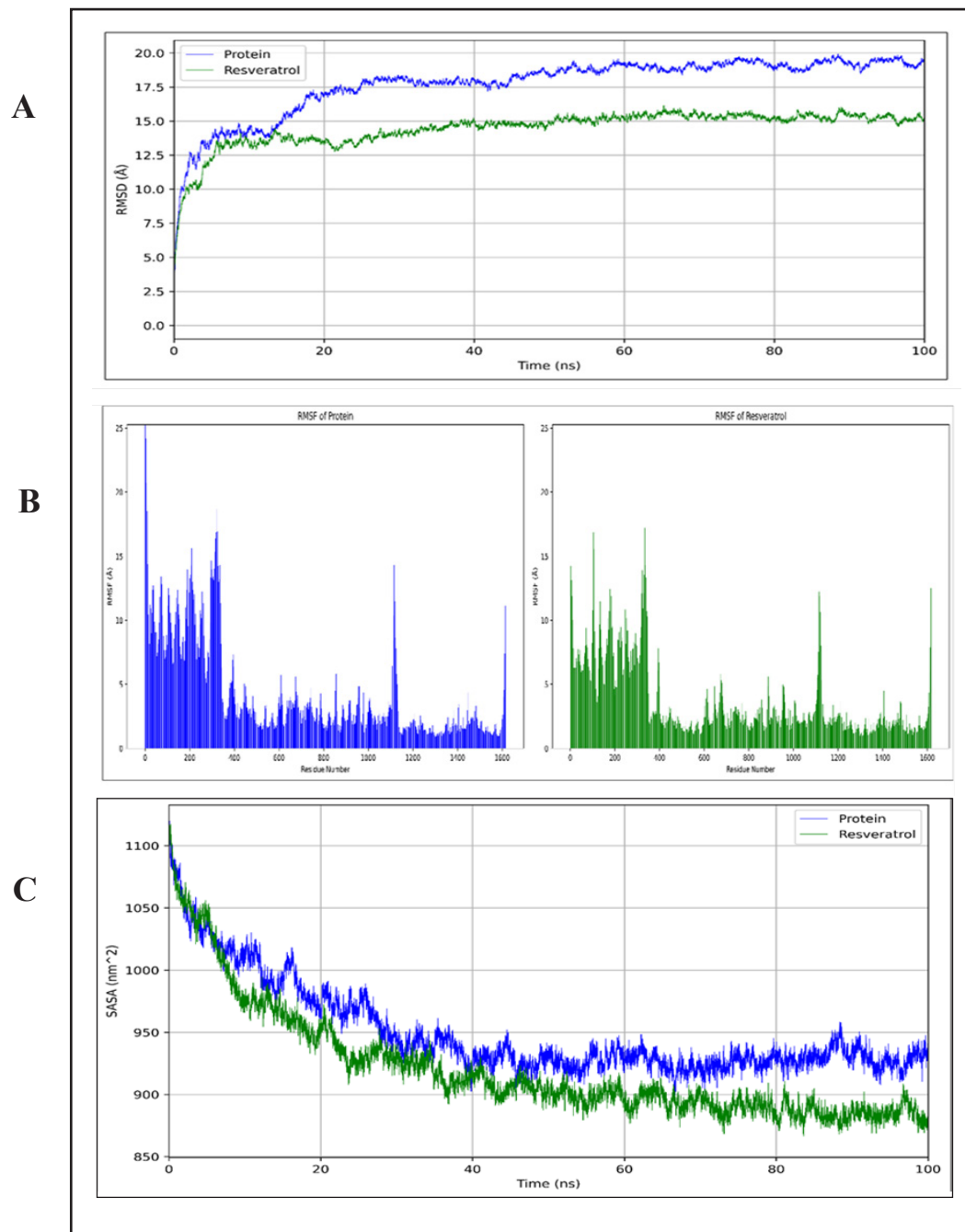
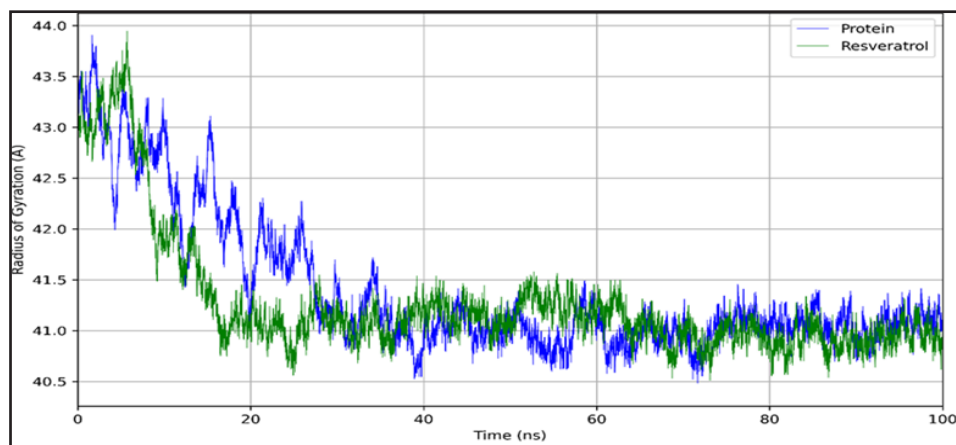
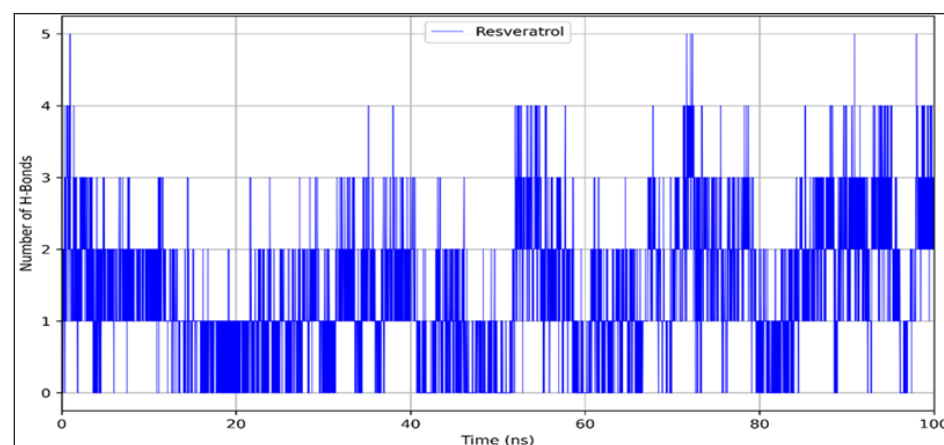


Figure 2. Molecular Dynamics Simulation Analysis of DNMT1-Resveratrol Complex Over 100 Nanoseconds A. Root Mean Square Deviation (RMSD) trajectories comparing apo DNMT1 protein and resveratrol-bound DNMT1 complex over the simulation period. The resveratrol-bound complex exhibits lower RMSD values compared to apo protein, indicating enhanced structural stability upon ligand binding, with an 18.5% reduction in structural deviation B. Root Mean Square Fluctuation (RMSF) analysis of individual residues in apo DNMT1 and resveratrol-bound protein. The ligand-bound state shows reduced average fluctuations compared to apo form, representing decrease in residue mobility, with particularly significant reductions observed in loop regions and binding site periphery C. Radius of gyration (Rg) profiles over simulation time demonstrating the compactness of protein structure. Resveratrol binding results in slightly more compact protein conformation with a reduced standard deviation compared to apo protein, indicating enhanced structural consistency.

D



E



Continued Figure 2. Molecular Dynamics Simulation Analysis of DNMT1-Resveratrol Complex Over 100 Nanoseconds. D. Solvent Accessible Surface Area (SASA) analysis showing protein-solvent interaction dynamics. The resveratrol-bound complex demonstrates reduced surface exposure compared to apo protein, representing a reduction in solvent accessible area due to interface formation upon ligand binding E. Hydrogen bond formation analysis depicting temporal evolution of polar interactions between DNMT1 and resveratrol throughout the simulation trajectory. The persistent hydrogen bonding pattern demonstrates stable polar interactions with key binding site residues, contributing to overall complex stability and optimal binding energy maintenance.

stability of the DNMT1 protein were evaluated through Radius of Gyration (Rg) analysis over the 100-nanosecond simulation trajectory (Figure 2C). The Rg measurements provide insights into overall protein dimensions and the impact of ligand binding on protein structural organization. The apo DNMT1 protein maintained an average Rg of 41.372 ± 0.670 Å, indicating a relatively compact protein conformation with moderate dimensional fluctuations. Upon Rsv binding, the protein complex demonstrated

a slightly more compact average structure with an Rg of 41.249 ± 0.591 Å, representing a 0.30% reduction in protein dimensions. Although the change in average Rg is modest, the 11.8% decrease in standard deviation (from 0.670 Å to 0.591 Å) indicates enhanced structural consistency and reduced dimensional variability. The reduced standard deviation upon ligand binding suggests that Rsv promotes more stable maintenance of protein compactness throughout the simulation.

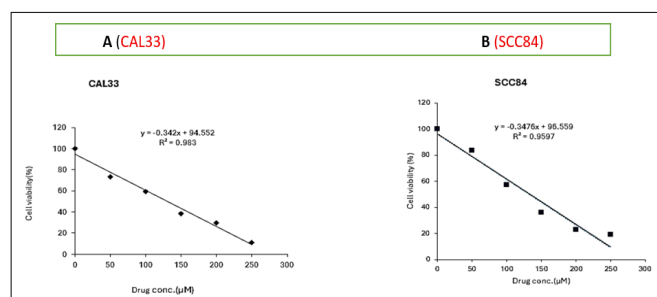


Figure 3. Dose- Dependent Curve of Resveratrol on Cell Viability in OSCC Cell Lines. A, CAL33 cells treated with Rsv (0-250μM) and B, SCC84 cells treated with Rsv (0-250μM). Cell viability (%) was assessed using MTT assay after treatment with increasing concentration of Rsv.

Table 4. Relative mRNA Expression (Fold Change) of *LATS-1* and *LATS-2* Following Treatment with 5-Aza-2'-deoxycytidine and Resveratrol (n=3 replicates; mean $\pm$ SEM; normalised to 18S rRNA).

Gene	Treatment	18S rRNA Ct (mean)	Gene Ct (mean)	Δ Ct (mean $\pm$ SD)	$\Delta\Delta$ Ct	Fold Change ($2^{-\Delta\Delta Ct}$) mean $\pm$ SEM	Significance vs. Control
		Reference gene	Ct (gene) – Ct (18S)		$2^{-\Delta\Delta Ct}$	Student's t-test	
<i>LATS-1</i>	Control	19.03	26.79	7.76 $\pm$ 0.74	0	1.000 $\pm$ 0.30	(Reference)
	5-Aza-2'-Deoxycytidine	20.33	23.44	3.11 $\pm$ 0.65	-4.64	26.74 $\pm$ 0.658	** (p<0.01)
	Resveratrol	14.94	22.04	7.10 $\pm$ 3.20	-0.66	7.01 $\pm$ 0.618	* (p<0.05)
<i>LATS-2</i>	Control	19.03	25.37	6.34 $\pm$ 0.36	0	1.000 $\pm$ 0.15	(Reference)
	5-Aza-2'-Deoxycytidine	20.33	22.14	1.82 $\pm$ 1.66	-4.53	34.35 $\pm$ 1.906	*** (p<0.001)
	Resveratrol	14.94	20.58	5.64 $\pm$ 1.10	-0.70	2.42 $\pm$ 0.89	* (p<0.05)

Δ Ct = Ct(gene) – Ct (18S rRNA); $\Delta\Delta$ Ct = Δ Ct(sample) – Δ Ct(control); Fold change = $2^{-\Delta\Delta Ct}$. Values expressed as mean $\pm$ SEM (n=3 replicates). Statistical significance by Student's t-test vs. untreated control. * p<0.05; ** p<0.01; *** p<0.001. 5-Aza-dCyd = 5-Aza-2'-deoxycytidine. Bold values indicate statistically significant fold changes.

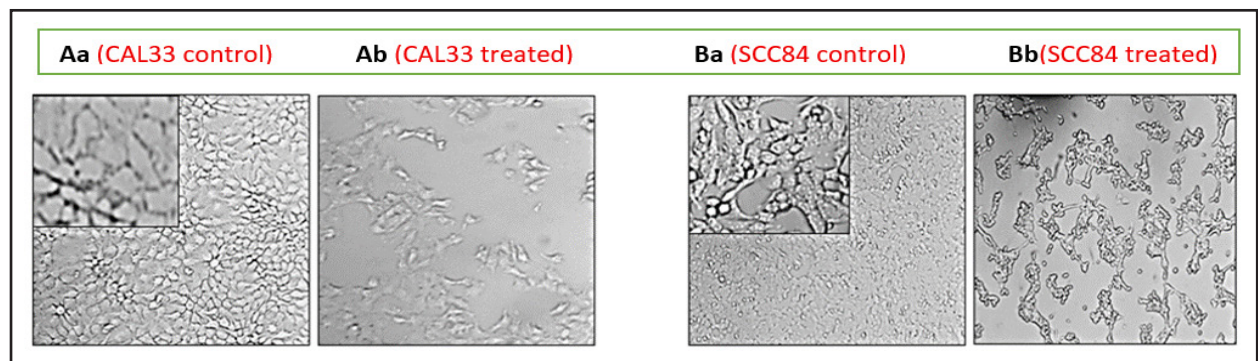


Figure 4. Morphological Changes in OSCC Cell Lines after Treatment with $1C_{50}$ Concentration of Rsv for 24 hours Aa. Untreated CAL33 control cell (20X) showing normal cellular morphology Ab. Rsv-treated CAL33 cells (20X) showing reduced cell density and altered morphology Ba. Untreated SCC84 control cell (20X) showing normal cellular morphology Bb. Rsv-treated CAL33 cells (20X) showing reduced cell density and altered morphology.

The subtle compaction effect combined with enhanced dimensional stability indicates that Rsv. binding optimizes protein structure organization without causing dramatic conformational changes, while significantly improving structural coherence and reducing breathing motions that could compromise binding stability.

Solvent Accessible Surface Area

The protein surface exposure and hydrophobic burial characteristics were evaluated through solvent accessible surface area analysis over the 100-nanosecond simulation trajectory (Figure 2D). The SASA measurements provide critical insights into protein-ligand interface formation

and surface reorganization upon ligand binding. The apo DNMT1 protein exhibited an average SASA of $948.048 \pm 37.155 \text{ nm}^2$, indicating substantial solvent exposure across the protein surface. Upon Rsv binding, the complex demonstrated reduced surface exposure with an average SASA of $917.452 \pm 44.436 \text{ nm}^2$, representing a 3.23% reduction in solvent accessible surface area. This surface burial effect suggests that Rsv binding promotes interface formation and reduces overall protein-solvent contact. Interestingly, while the average SASA decreased upon ligand binding, the standard deviation increased by 19.6% (from 37.155 nm^2 to 44.436 nm^2), indicating greater surface exposure variability in the ligand-bound state.

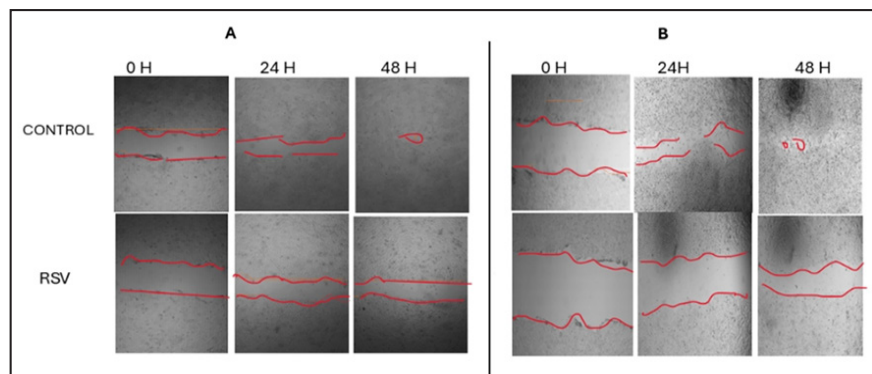


Figure 5. Representative Images Showing Open Wound Area at 0h, 24h and 48h in Control and Rsv Treated Cell Lines. A clear reduction in open wound area in control depicts elevated cell migration in comparison with Rsv treated cell lines A. CAL33 control: CAL33 Rsv B. SCC84 control: SCC84 Rsv

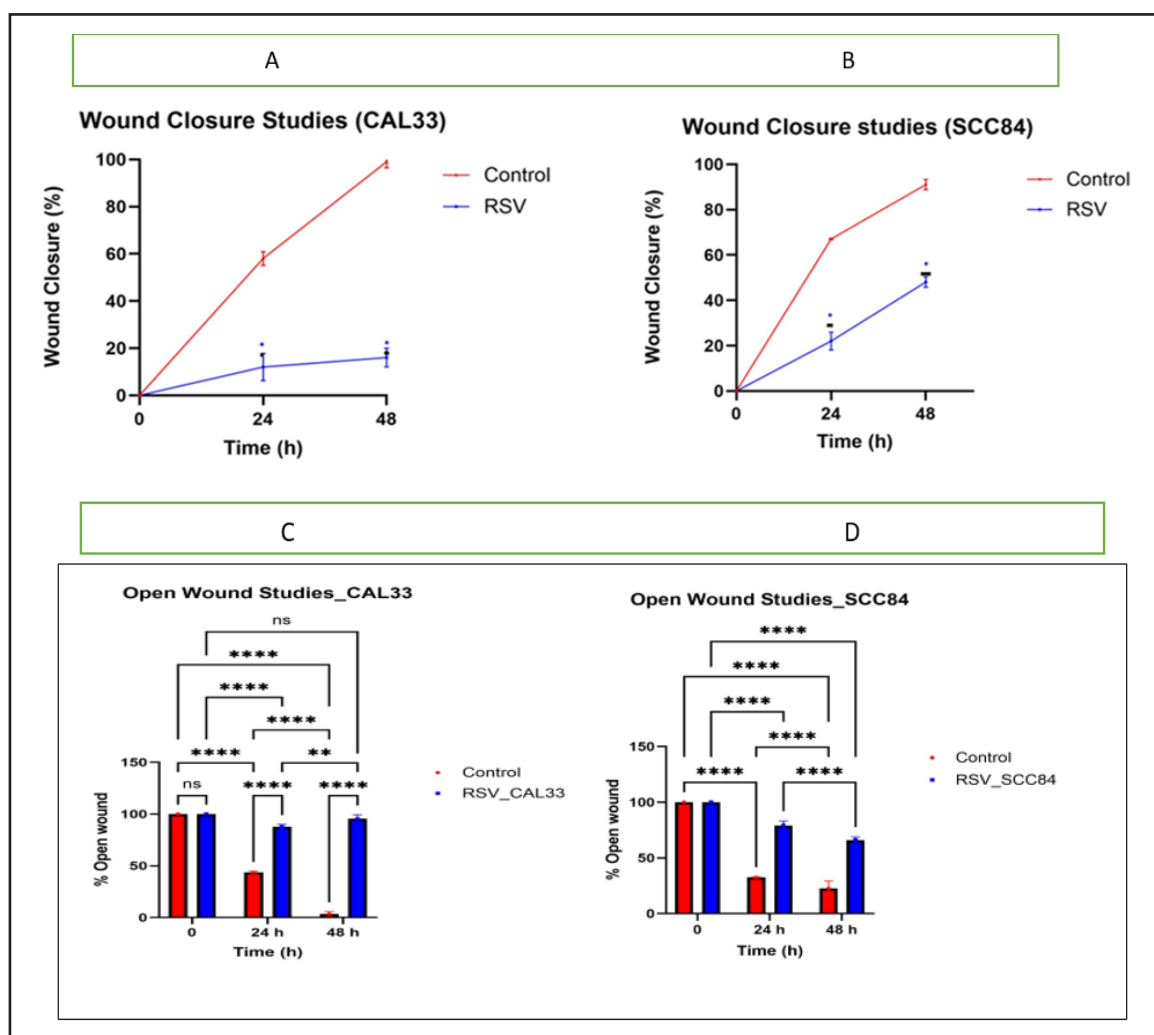


Figure 6. The Percentage of Wound Closure and Open Wound Area. Quantitative analysis of wound closure (%) and open wound area (%) was calculated by measuring the open wound area using manual quantification. Statistical significance was calculated by two-way ANOVA (p-value <0.05) A. wound closure (%) in CAL33 B. wound closure (%) in SCC84 C. open wound (%) in CAL33 D. open wound (%) in SCC84

This increased variability may reflect dynamic surface breathing motions associated with ligand accommodation or conformational adjustments that optimize protein-ligand interactions. The overall reduction in SASA, combined with increased variability, suggests that Rsv binding creates a more buried binding interface while allowing dynamic surface adjustments that may facilitate optimal interaction formation.

Hydrogen bond formation Analysis

The intermolecular hydrogen bonding patterns and their temporal stability were analysed throughout the

100-nanosecond simulation to assess the strength and persistence of polar interactions between DNMT1 and Resveratrol (Figure 2E). Hydrogen bond analysis provides critical insights into the molecular basis of binding affinity and complex stability. The hydrogen bond formation analysis reveals dynamic interaction patterns between DNMT1 and Rsv throughout the simulation trajectory. The temporal evolution of hydrogen bonding demonstrates periods of stable interaction formation alternating with transient disruption and reformation events. The analysis indicates that Rsv maintains consistent polar interactions with key binding site residues, with hydrogen

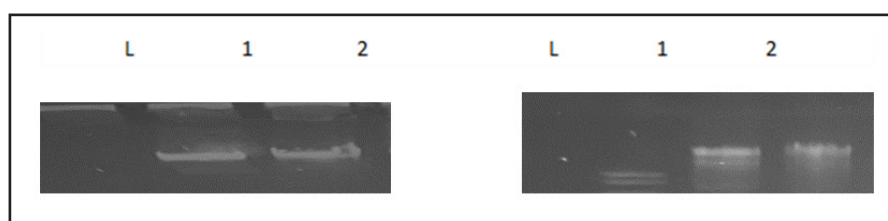


Figure 7. Inter-Nucleosomal Fragmented DNA Resveratrol -Treated CAL33 and SCC84 cells for 48 hours A (1. 100 bp ladder, 2. Control CAL33 DNA,3 Control SCC84 DNA), B. (1.100 bp ladder 2. CAL33 RSV treated DNA 3. SCC84 RSV- treated DNA)

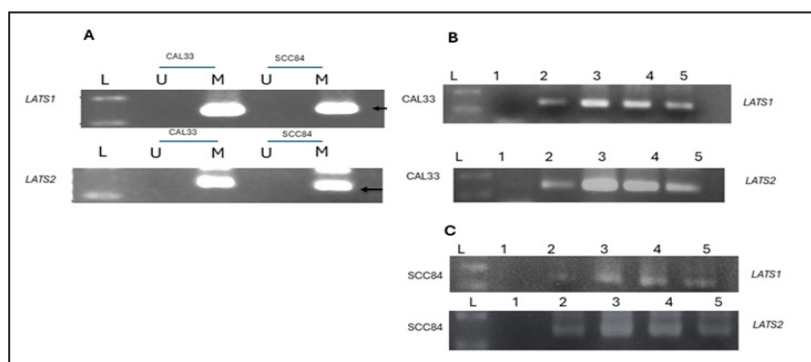


Figure 8. MS-PCR Product of *LATS1* and *LATS2* A. Control (L- Ladder 100bp, U-Unmethylated specific band, M-Methylated specific band) B. Treatment CAL33 (L- Ladder 100bp) 1. Lane 1: Negative control 2. Lane 2: Positive control(5-azadeoxycytidine) 3. Lane 3: 48h Rsv 4. Lane 4 :72h Rsv 5. RSV-6 days C. Treatment SCC84 (L- Ladder 100bp)1. Lane 1: Negative control 2. Lane 2: Positive control (5-azadeoxycytidine) 3. Lane 3: 48h Rsv 4. Lane 4: 72h Rsv 5. Lane 5: 6 days

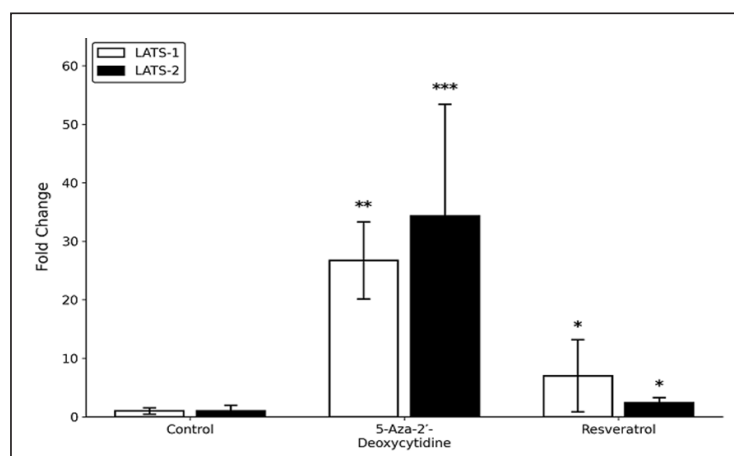


Figure 9. RT-qPCR Analysis of *LATS-1* and *LATS-2* mRNA Expression. *LATS-1* white bars, *LATS-2* black bars. mean $\pm$ SEM (n=3) * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

bond formation showing characteristic patterns of binding site optimization. The persistence of hydrogen bonding throughout the simulation, combined with the observed structural stabilization effects, suggests that polar interactions play a crucial role in maintaining the DNMT1-Rsv complex stability. The dynamic nature of hydrogen bond formation and reformation indicates that the binding interface undergoes continuous optimization to maintain optimal polar interactions, contributing to the overall binding energy and complex stability observed in the molecular dynamics simulation.

Cytotoxicity Assay

The IC_{50} value of Rsv in CAL33 and SCC84 cells was observed to be 130.14 μ M and 133.94 μ M at 48h using MTT assay (Figure 3). Cell viability (%) was calculated. Results were expressed as mean $\pm$ SD.

Morphological Assay

The morphological studies showed that treatment of CAL33 and SCC84 cells with Rsv. induce visible morphological alterations like nuclear condensation, loss in cell-cell junction and formation of apoptotic bodies (Figure 4).

Wound healing assay

This assay evaluates how cells migrate following mechanical disruption of the monolayer. Scratches were introduced into both untreated and Rsv.exposed cultures of each cell line.

Photomicrographs captured at 0h, 24h, and 48h post-wounding are presented in Figure 5. In control cultures (Figures 5A and 5B), the scratch gap closed approximately halfway by 24 hours and achieved complete closure by 48 hours. Conversely, Rsv-treated cells displayed markedly impaired wound closure, with less than half of the gap sealed even after 48 hours. Quantitative analysis of the remaining open wound area over time was performed to determine resveratrol's effect on cellular motility (Figure 6). These results clearly indicate that resveratrol treatment substantially inhibited cell migration in a manner that increased with exposure duration.

DNA Fragmentation analysis

Agarose gel analysis revealed distinct DNA fragmentation in both CAL33 and SCC84 cell lines following treatment with resveratrol at IC_{50} doses for 48h. The ladder-like banding pattern observed in treated samples, compared against intact DNA from control cells and a 100 bp size standard, indicated internucleosomal DNA breakdown consistent with prog (Figure 7).

Methylation Reversal Analysis

Treatment with 25 μ M Rsv. resulted in *LATS1/2* demethylation. It was concluded after observing decrease in width and intensity of methylation band as compared with control in both CAL33 and SCC84 cell line. Rsv treatment has been given at 48h, 72h and 6 days. Noticeable decrease in methylation specific band intensity has been seen after 6 days of treatment with Rsv indicating demethylation. 5-azadeoxycytidine is taken as positive control for promoter demethylation. It is noteworthy that the current assessment of methylation reversal is qualitative in nature, based on the relative intensity of MSP bands; sodium bisulfite sequencing experiments are presently underway in our laboratory to provide precise quantitative confirmation of the extent of promoter demethylation. Both the cell lines were treated with 5-azadeoxycytidine having concentration of 5 μ M for 72 hrs (Figure 8).

To further validate the demethylation findings, 5-Aza-2'-deoxycytidine (5-Aza-dCyd) and the natural polyphenolic compound resveratrol on the mRNA expression of tumour suppressor genes *LATS-1* and *LATS-2*, RT-qPCR analysis was performed using 18S rRNA as the reference gene, and relative expression was calculated by the $2^{-\Delta\Delta C_t}$ method (n=3 replicates; results expressed as mean $\pm$ SEM). Treatment with 5-Aza-dCyd resulted in a marked and statistically significant upregulation of both genes compared to untreated controls. Specifically, *LATS-1* expression was elevated to 26.74 ± 0.658 -fold ($p < 0.01$, **), while *LATS-2* expression was elevated to 34.35 ± 1.906 -fold ($p < 0.001$, ***) relative to the control, indicating that 5-Aza-dCyd effectively reversed promoter hypermethylation-mediated silencing of both genes. Treatment with resveratrol also produced a statistically significant increase in *LATS-1* expression (7.01 ± 0.618 -fold; $p < 0.05$, *) compared to control. Interestingly, resveratrol treatment resulted in a modest but statistically significant upregulation of *LATS-2* expression (2.42 ± 0.89 -fold; $p < 0.05$, *), which, although lower in magnitude than the 5-Aza-dCyd response, nonetheless represents a biologically meaningful restoration of expression relative to the silenced baseline in untreated controls. These findings collectively suggest that both 5-Aza-2'-deoxycytidine and resveratrol are capable of reactivating *LATS-1* and *LATS-2* tumour suppressor gene expression, consistent with their known roles as demethylating and epigenetically modulatory agents, respectively. The results are illustrated in Figure 9 and summarised in Table 4

Discussion

Silencing of tumor suppressor genes (TSGs) through promoter hypermethylation (PH) represents a critical early event in oral squamous cell carcinoma (OSCC) pathogenesis. This results in the inactivation of the hypermethylated TSGs in OSCC. Among these, *LATS1* and *LATS2* gene, core components of the Hippo signaling pathway exhibit frequent epigenetic inactivation across

multiple malignancies, including OSCC. The Hippo pathway functions as a central regulator of organ size, tissue homeostasis, and cellular proliferation through its downstream effectors YAP (Yes-Associated Protein) and TAZ (Transcriptional co-activator with PDZ-binding motif) [35, 36]. When *LATS1/2* are functional, they phosphorylate YAP/TAZ at critical serine residues, leading to cytoplasmic sequestration and proteasomal degradation. However, hypermethylation-mediated silencing of *LATS1/2* disrupts this regulatory mechanism due to the downregulation of these genes, permitting unphosphorylated YAP/TAZ to accumulate in the nucleus where they interact with TEAD transcription factors to drive expression of proliferative and anti-apoptotic genes [37, 38]. This pathway dysregulation has been implicated in OSCC progression, with studies demonstrating that nuclear YAP/TAZ localization correlates with aggressive tumor phenotypes, lymph node metastasis, and poor clinical outcomes [39, 40]. Therefore, therapeutic strategies aimed at restoring *LATS1/2* expression through epigenetic reprogramming represent a promising approach for OSCC management.

The present study employed an integrated computational and experimental approach to investigate resveratrol's (Rsv) potential as an epigenetic therapeutic agent. Our molecular docking analysis revealed strong binding affinity between Rsv and DNMT1 (binding energies: -7.3 to -7.9 kcal/mol), characterized by hydrogen bonding and hydrophobic interactions within the enzyme's catalytic pocket. These findings align with recent computational studies demonstrating that dietary polyphenols, including Rsv, competitively inhibit DNMT1 by occupying the catalytic site and disrupting S-adenosylmethionine (SAM) cofactor binding [41, 42]. The 100-nanosecond molecular dynamics simulation provided critical insights into binding stability, revealing an 18.5% reduction in RMSD and 15.1% decrease in RMSF for the DNMT1-Rsv complex compared to apo-DNMT1. These structural stabilization effects suggest that Rsv binding induces conformational constraints that impair DNMT1's enzymatic activity. Recent crystallographic and computational studies have similarly demonstrated that polyphenolic compounds stabilize DNMT1 in catalytically incompetent conformations, thereby preventing methyl transfer to DNA substrates [43, 44].

While the in silico data strongly supports DNMT1 inhibitory activity of resveratrol, direct experimental validation via DNMT1 activity assays (e.g., ELISA-based) has not yet been performed and remains a limitation of the current study. The computational evidence is supportive but not conclusive. Guided by these computational predictions, our in vitro experiments demonstrated that Rsv exerts dose-dependent cytotoxicity in CAL33 and SCC84 OSCC cell lines, with IC_{50} values of 130.14 μ M and 133.94 μ M, respectively. These concentrations are consistent with recent reports documenting Rsv's anti-proliferative effects in oral cancer models [45, 46]. Importantly, morphological analysis revealed characteristic apoptotic features including membrane blebbing, chromatin condensation, and apoptotic body formation following

Rsv treatment. These observations were corroborated by DNA fragmentation assays showing the characteristic internucleosomal DNA laddering pattern indicative of programmed cell death. Recent mechanistic studies have elucidated that Rsv induces apoptosis in OSCC cells through multiple converging pathways, including mitochondrial membrane depolarization, caspase cascade activation, and modulation of Bcl-2 family proteins [47, 48]. Wound healing assay revealed profound inhibition of cell migration in Rsv-treated cultures, with less than 50% wound closure after 48 hours compared to near-complete closure in controls. This anti-migratory effect reflects Rsv's ability to suppress epithelial-mesenchymal transition (EMT), a critical process in cancer metastasis. Previous studies have demonstrated that Rsv downregulates EMT transcription factors including SNAIL, SLUG, and TWIST while restoring E-cadherin expression in oral cancer cells [49]. Furthermore, Rsv inhibits matrix metalloproteinases (MMP-2 and MMP-9) through NF- κ B pathway suppression, thereby preventing extracellular matrix degradation required for invasive phenotypes [50, 51]. These findings position Rsv as a potential anti-metastatic agent targeting one of the most lethal aspects of OSCC progression.

The most significant finding of this study is the demonstration of time-dependent demethylation of *LATS1/2* promoters following Rsv treatment at sub-cytotoxic concentrations (25 μ M). Methylation-specific PCR analysis revealed progressive reduction in methylation-specific band intensity over a six-day treatment period, with maximal demethylation observed at day 6 (Figure 8). This temporal pattern is consistent with the mechanism of passive demethylation, whereby DNMT inhibition during DNA replication leads to progressive loss of methylation marks through successive cell divisions [52]. The use of 5-aza-2'-deoxycytidine as a positive control validates our methodology and confirms that Rsv achieves demethylation comparable to established clinical agents, but critically, at concentrations well below its cytotoxic threshold. Recent studies have validated the clinical relevance of *LATS1/2* methylation in OSCC. A 2020 investigation of North Indian OSCC patients documented *LATS1* and *LATS2* promoter hypermethylation frequencies of 38% and 55%, respectively, with strong correlations to advanced tumor stage and poor prognosis [14, 15]. Similar findings have been reported in other OSCC cohorts globally, establishing *LATS1/2* hypermethylation as a nearly universal epigenetic alteration in oral cancer pathogenesis [53, 54]. Moreover, functional studies have demonstrated that enforced *LATS2* re-expression in OSCC cells suppresses proliferation, induces apoptosis, and inhibits xenograft tumor growth, validating the therapeutic potential of *LATS1/2* restoration strategies [55, 56]. RT-qPCR analysis confirmed statistically significant reactivation of both *LATS-1* and *LATS-2* following treatment with 5-Aza-2'-deoxycytidine and resveratrol, normalised to 18S rRNA ($2^{-\Delta\Delta C_t}$ method; n=3). 5-Aza-dCyd produced robust upregulation of *LATS-1* (26.74 ± 0.658 -fold; $p < 0.01$) and *LATS-2* (34.35 ± 1.906 -fold; $p < 0.001$), confirming pharmacological

reversibility of promoter hypermethylation-mediated silencing. Resveratrol similarly restored *LATS-1* (7.01 ± 0.618 -fold; $p < 0.05$) and *LATS-2* (2.42 ± 0.89 -fold; $p < 0.05$) expression significantly above baseline, supporting its role as a candidate DNMT1 inhibitor and potential epigenetic therapeutic agent.

Multifaceted mechanisms of resveratrol beyond DNMT inhibition

While this study focused on Rsv's demethylating activity, it is important to recognize that Rsv exerts pleiotropic anti-cancer effects through multiple complementary mechanisms. Recent research has revealed that Rsv activates SIRT1 (sirtuin-1), a NAD⁺-dependent deacetylase that enhances p53 tumor suppressor function through deacetylation, thereby promoting apoptosis and cell cycle arrest [57, 58]. Additionally, Rsv modulates the NF- κ B inflammatory signaling pathway, reducing expression of pro-inflammatory cytokines and survival factors that contribute to the tumor-promoting microenvironment. Rsv also activates the Nrf2 antioxidant response pathway, enhancing cellular detoxification capacity and protecting against carcinogen-induced DNA damage [59, 60].

A critical observation from our study is the five-fold separation between Rsv's demethylating concentration (25 μ M) and its cytotoxic IC₅₀ values (>130 μ M), establishing a substantial therapeutic window. This contrasts sharply with conventional demethylating agents such as 5-aza-2'-deoxycytidine and 5-azacitidine, which exhibit dose-limiting hematological toxicities and narrow therapeutic indices [61, 62]. These synthetic agents function as nucleoside analogs that incorporate into DNA, forming irreversible covalent complexes with DNMTs and causing DNA damage, replication fork stalling, and off-target cytotoxicity [63, 64]. In contrast, Rsv's non-nucleoside mechanism permits sustained epigenetic modulation without genomic integration or catastrophic DNA damage.

Moreover, Rsv's excellent safety profile, established through extensive human consumption history and clinical trials, positions it favorably for long-term chemoprevention applications [65, 66].

Clinical translation potential and future directions

Our findings demonstrate proof-of-concept that Rsv can reverse *LATS1/2* hypermethylation in OSCC cells, *LATS1/2* methylation status holds significant promise as a multifunctional clinical biomarker: (1) early detection in high-risk populations through saliva-based methylation screening [65] (2) prognostic stratification, as methylation correlates with aggressive features and poor outcomes [14, 15] (3) predictive marker for identifying patients likely to respond to epigenetic therapies [67] and (4) monitoring therapeutic efficacy through serial ctDNA methylation analysis [68].

Limitations and Future Perspectives

Despite the promising findings of the present study, certain limitations must be acknowledged. First,

methylation reversal was assessed qualitatively through MSP band intensity; sodium bisulfite sequencing experiments currently underway will provide quantitative confirmation of CpG methylation changes at *LATS1/2* promoters. Second, while molecular docking and MD simulations strongly support DNMT1 inhibitory activity of resveratrol, direct experimental validation through DNMT1 activity assays remains to be performed. Third, the specificity of resveratrol's demethylating effects towards *LATS1/2*, as opposed to broader global DNMT inhibition, has not yet been fully established; analysis of additional tumor suppressor genes including p16 and MGMT is ongoing. Fourth, the MD simulation trajectory was limited to 100 nanoseconds, and extension to 500 ns in future studies would provide more robust insights into long-term protein-ligand binding dynamics. These limitations notwithstanding, the current study provides valuable proof-of-concept evidence for resveratrol as a demethylating agent in OSCC, warranting further investigation.

In conclusion, this study demonstrates that resveratrol reverses *LATS1/2* promoter hypermethylation in OSCC cell lines through DNMT1 inhibitory activity at sub-cytotoxic concentrations, restoring Hippo pathway tumor suppressive function. Rsv's favourable safety profile, multi-targeted mechanisms, and substantial therapeutic window, position it as a promising epigenetic therapeutic agent for OSCC. Future research priorities include advanced formulation development to enhance bioavailability, validation in patient-derived models, establishment of pharmacodynamic biomarkers, and initiation of early-phase clinical trials. Integration of natural epigenetic modulators like Rsv into precision oncology paradigms represents a paradigm shift toward safer, mechanism-based therapeutic strategies that exploit the reversible nature of epigenetic alterations while minimizing toxicities associated with conventional cytotoxic agents.

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Competing interests

The authors report no conflict of interest.

Authors contribution

MJ conducted the majority of the literature review and was primarily responsible for drafting the manuscript. AD contributed to the composition and substantive editing of key sections. SC and AS were involved in the initial conceptualization and design of the study and provided substantial critical feedback throughout manuscript development. All authors reviewed and approved the final version of the manuscript.

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