In Vitro Exploration of ACTN4 in Oral Cancer: Unraveling the Cytoskeletal Pathways of Invasion, Apoptosis and Angiogenesis Modulation

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Doi: https://doi.org/10.5281/zenodo.17898309
Received:10 October 2025
Accepted: 20 November 2025

Abstract

Alpha-actinin-4 (ACTN4) is a non-muscle actin-binding protein that plays pivotal roles in cytoskeletal organization, cell motility, and cancer progression. This comprehensive review examines the multifaceted functions of ACTN4 in oral squamous cell carcinoma (OSCC), focusing on its impact on cytoskeletal remodeling and invasive behaviors. ACTN4 overexpression has been detected in approximately 70% of OSCC cases and is significantly associated with enhanced invasive potential, poor prognosis, and lymph node metastasis. The protein facilitates cancer progression through complex molecular mechanisms involving cytoskeletal reorganization, epithelial-mesenchymal transition (EMT), and activation of key signaling pathways including PI3K/Akt, ERK/GSK-3β/β-catenin, and Wnt/β-catenin cascades. In vitro studies have demonstrated that ACTN4 promotes the formation of specialized ruffle-edge lamellipodia, enhances cell motility, and increases matrix metalloproteinase activity, thereby contributing to extracellular matrix degradation and invasion. The protein localizes at the leading edges of migrating cells and shows enhanced expression in invasive tumor fronts compared to normal oral epithelium. RNA interference-mediated knockdown of ACTN4 significantly reduces invasive potential and cell migration in OSCC cell lines, validating its functional importance in cancer progression. Gene amplification of ACTN4, detected in 24% of cases, serves as an independent prognostic biomarker associated with reduced overall survival and increased risk of late cervical lymph node metastasis. The integration of network pharmacology and artificial intelligence approaches offers promising avenues for discovering ACTN4-targeted therapeutics, with machine learning algorithms enabling virtual screening of compound libraries and prediction of drug-target interactions. These computational approaches, combined with systems biology methodologies, can accelerate the identification of selective ACTN4 inhibitors and optimize their pharmacological properties for clinical translation. The convergence of mechanistic insights from in vitro studies with advanced AI-driven drug discovery platforms positions ACTN4 as a compelling therapeutic target for developing personalized treatment strategies in oral cancer management.

1.Introduction

Epidemiology and Clinical Relevance of Oral Squamous Cell Carcinoma

Oral squamous cell carcinoma (OSCC) represents a significant global health burden, accounting for approximately 90% of all oral malignancies and constituting the 16th most common cancer worldwide. The latest GLOBOCAN estimates from 2020 documented 377,713 new cases of oral cavity cancer globally, representing approximately 4.5% of all cancer diagnoses. This malignancy demonstrates a pronounced male predominance, ranking as the 12th most common cancer in men and the 18th most common in women. The disease exhibits striking geographical disparities, with the highest incidence rates observed in South and Southeast Asia, particularly in India, which reported 143,759 new cases in 2022. This regional concentration is largely attributed to the widespread consumption of carcinogenic areca nut and other tobacco products.[1,2]

The mortality burden of OSCC is equally concerning, with approximately 188,438 deaths reported globally in 2022. The prognosis remains poor, with five-year overall survival rates ranging from 41.7% to 70.7% depending on the study population and staging criteria. The disease demonstrates stage-dependent survival outcomes, with localized cases achieving 83.7% five-year survival rates compared to only 38.5% for metastatic disease. Importantly,

approximately 70% of cases are diagnosed at advanced stages, significantly compromising treatment outcomes. The high recurrence and metastasis rates, affecting 45.7% of patients within five years, contribute substantially to the poor prognosis.[1,2,3]

Importance of Invasion and Metastasis in Oral Cancer Prognosis

Invasion and metastasis represent the most critical determinants of OSCC prognosis and treatment failure. Clinical studies consistently demonstrate that recurrence and distant metastasis are independent risk factors for poor survival outcomes. Patients who develop postoperative recurrence or metastasis exhibit significantly reduced survival times, with mortality rates approaching 90% following recurrence diagnosis. The five-year disease-free survival rate drops to 49.4% when considering recurrence and metastatic events.[1,3]

Lymph node metastasis, in particular, serves as a crucial prognostic indicator, with regional lymph node involvement significantly reducing overall survival. The development of late cervical lymph node metastasis remains a persistent challenge, even in early-stage disease following complete surgical resection. Perineural invasion (PNI) and pathological differentiation have been identified as independent risk factors, with poorly differentiated tumors and the presence of PNI associated with enhanced metastatic potential. Distant metastasis occurs in approximately 36% of cases, with a distant metastasis rate of 9.3% developing within 1-3 years post-surgery.[3,5]

Overview of Cytoskeletal Dynamics in Cancer Cell Motility

The cytoskeleton plays a fundamental role in cancer cell motility and metastatic progression through dynamic reorganization of its constituent elements. Altered cellular motility represents a hallmark feature of metastasis, facilitating tumor cell advancement to both local and distant sites. The actin cytoskeleton undergoes extensive remodeling during epithelial-mesenchymal transition (EMT), enabling the transformation of epithelial-like cells into motile mesenchymal-like phenotypes.[6,7]

At the molecular level, migrating cancer cells develop specialized membrane protrusions called lamellipodia at their leading edges, where localized actin polymerization generates protrusive forces. The spatial and temporal regulation of actin dynamics involves complex signaling cascades, including the Rho family of GTPases (RhoA, Rac1, and Cdc42), which coordinate cytoskeletal reorganization with cell adhesion and contractility. The cofilin pathway, downstream of Rho GTPase signaling, facilitates actin filament remodeling through the ARP2/3 complex, generating branched actin networks essential for directional motility.[6]

Actin regulators demonstrate altered expression patterns in cancer cells, with several actin-binding proteins showing upregulation in invasive and metastatic malignancies. The remodelling of actin composition serves as a hallmark of cancer progression, with abnormal actin isoform expression leading to increased migration, enhanced cell proliferation, and drug resistance. Crosstalk between actin filaments and microtubules occurs at focal adhesions, where mechanical forces are transmitted and integrated to coordinate cellular movement.[6,8,9]

Introduction to ACTN4: Family of α-actinins

Alpha-actinin-4 (ACTN4) belongs to the spectrin superfamily of actin-binding proteins, which comprises four distinct isoforms in mammals: ACTN1, ACTN2, ACTN3, and ACTN4. These proteins function as antiparallel homodimers that cross-link actin filaments within the cytoskeleton. The α-actinin family can be categorized into two functional groups: muscle-specific isoforms (ACTN2 and ACTN3) that are calcium-insensitive and localized to the Z-disk of striated muscle, and non-muscle isoforms (ACTN1 and ACTN4) that are calcium-sensitive and widely expressed throughout various tissues.[10,11]

ACTN1 and ACTN4, though structurally similar, exhibit distinct subcellular localizations and functional properties. ACTN1 is predominantly associated with focal adhesions and adherens junctions, while ACTN4 localizes primarily to stress fibers and cellular protrusions. Both isoforms contribute to cell motility by coordinating contractile forces at focal adhesions and may participate in focal adhesion disassembly to permit cell movement. The non-muscle α-actinins interact with β1 integrin receptors, anchoring the actin cytoskeleton to the plasma membrane and facilitating mechanical force transmission.[11]

Structural Features of ACTN4

ACTN4 is a 104.9 kDa protein composed of 911 amino acids that can be subdivided into three major structural domains. The N-terminal region contains two tandem calponin homology domains (CH1 and CH2) that constitute the actin-binding domain (ABD). Each CH domain consists of approximately 100 amino acids arranged in four α-helices (A, C, E, and G), with helices A and E positioned externally and helices C and G located internally. The CH1 and

CH2 domains interact through polar and hydrophobic forces, with CH1 alone having lower actin-binding affinity while CH2 is insufficient for actin crosslinking.[12]

The central region of ACTN4 comprises four spectrin repeats (SR1-4) responsible for antiparallel dimerization and assembly of multiprotein structures involved in cytoskeletal architecture and signal transduction. These spectrin repeats mediate interactions with diverse cytoskeletal and non-cytoskeletal proteins including vinculin, MEKK1 kinase, MAGI, and BP-180. The C-terminal region contains two EF-hand domains (EF1-2) and a calmodulin-homology domain that confer calcium sensitivity to the protein.[10,12]

The actin-binding activity of ACTN4 is regulated through conformational changes involving the CH1-CH2 interface. In the native closed state, residues K255 and W147 form a hinge-like connection that maintains domain stability. Disease-associated mutations, such as K255E, disrupt this interface and promote an open conformation that increases actin-binding affinity by sixfold. Phosphatidylinositol 4,5-bisphosphate (PIP2) binding induces conformational changes that separate the CH domains and facilitate actin interaction.[11,13,14]

Known Roles of ACTN4 in Other Cancers

ACTN4 has been extensively studied across multiple cancer types, consistently demonstrating associations with enhanced invasive potential and poor clinical outcomes. In breast cancer, cytoplasmic overexpression of ACTN4 correlates with increased lymph node metastasis and reduced overall survival. The protein has been shown to confer radioresistance in breast cancer cells while simultaneously promoting invasive capabilities. Similarly, in pancreatic cancer, ACTN4 gene amplification occurs in significant proportions of cases and correlates with enhanced metastatic potential.[15,16]

Non-small cell lung cancer studies reveal elevated ACTN4 expression associated with increased tumor aggressiveness and poor prognosis. In bladder cancer, high ACTN4 expression correlates with higher tumor grade and pathological stage, with the protein showing particular enrichment at the invasive tumor front and in dedifferentiated cancer cells. Ovarian cancer investigations demonstrate similar patterns, with increased ACTN4 expression linked to enhanced metastatic capacity.[16]

The mechanistic roles of ACTN4 in cancer progression involve multiple pathways. The protein localizes to cellular protrusions and leading edges of migrating cells, where it facilitates cytoskeletal remodeling necessary for invasion. ACTN4 activates various signaling cascades including Rac1 and Cdc42, which are upstream regulators of cancer cell invasion. Additionally, ACTN4 demonstrates nuclear localization in certain contexts, where it functions as a transcriptional co-regulator interacting with RelA/p65 and other nuclear factors.[10,12,16,17]

Rationale for Focusing on ACTN4 in OSCC

The rationale for targeting ACTN4 in oral squamous cell carcinoma is supported by compelling clinical and molecular evidence. Gene amplification of ACTN4 occurs in approximately 24% of early-stage oral tongue cancer cases and serves as a significant independent risk factor for death, with hazard ratios of 6.08. This genomic alteration provides superior prognostic value compared to protein expression analysis alone. Importantly, ACTN4 copy number analysis represents a cost-effective single gene assay with potential clinical applicability for identifying high-risk patients.[5]

The protein demonstrates characteristic expression patterns in OSCC, with overexpression particularly evident at the invasive tumor front compared to normal oral mucosa. This spatial distribution suggests functional involvement in the invasive process. RNA interference-mediated knockdown studies in oral cancer cell lines demonstrate significant reductions in invasive potential, validating ACTN4 as a functional driver of OSCC progression. The protein's role in cytoskeletal remodeling and EMT makes it an attractive therapeutic target for preventing metastatic spread.[15,18,19]

Furthermore, ACTN4 represents part of larger molecular networks involved in OSCC pathogenesis, including interactions with PTEN-targeting miRNAs and activation of oncogenic signaling pathways. The integration of network pharmacology and artificial intelligence approaches offers promising avenues for discovering ACTN4-targeted therapeutics, positioning this protein as a compelling target for personalized treatment strategies in oral cancer management. The convergence of clinical relevance, mechanistic understanding, and technological capabilities for drug discovery makes ACTN4 an ideal focus for comprehensive investigation in OSCC research.[18,20,21]

2.ACTN4: Structure, Function, and Regulation

Structure and Domains of ACTN4

Actin-Binding Domain (ABD)

The N-terminal actin-binding domain of ACTN4 spans the first 267 amino acids and consists of two tandem calponin homology domains (CH1 and CH2). Each CH domain contains approximately 100 amino acids arranged in four α-helices designated A, C, E, and G, with helices A and E positioned externally and helices C and G located internally. The CH1 and CH2 domains exhibit extensive hydrophobic interactions that maintain the domain in a compact, closed conformation under resting conditions.[11,12,22]

A critical regulatory mechanism involves the interface between CH1 and CH2 domains, specifically the interaction between residues Lys255 in CH2 and Trp147 in CH1. This hinge-like connection stabilizes the closed state and prevents inappropriate actin binding. The closed conformation represents an autoinhibited state where actin-binding sites are masked, while an open conformation exposes these sites and dramatically increases actin affinity by sixfold. Phosphatidylinositol 4,5-bisphosphate (PIP2) binding induces conformational changes that separate the CH domains and facilitate actin interaction by disrupting the CH1-CH2 interface.[11,13,14,22]

Disease-associated mutations, such as K255E, disrupt the critical hinge-like connection and promote constitutive open conformation, resulting in pathologically enhanced actin binding and impaired cytoskeletal dynamics. Both lysine residues (Lys140 in CH1 and Lys255 in CH2) are highly conserved across the spectrin family, indicating evolutionary pressure to maintain this regulatory mechanism. The overall sequence identity between CH1 and CH2 domains is low at 17%, yet these specific regulatory residues are maintained.[22]

Rod Domain (Spectrin Repeats)

The central rod domain of ACTN4 comprises four consecutive spectrin repeats (SR1-SR4) spanning residues 268-759, each containing 106-122 amino acids. Each spectrin repeat adopts a three-helix bundle structure with helices designated A, B, and C, where helices A and C are parallel while helix B is antiparallel. These helices form a left-handed supercoil that wraps around each other in a non-straight configuration.[11,12]

The spectrin repeats are connected by short helical linkers of approximately 10 residues that maintain structural continuity without breaks or secondary structure changes between repeats. This arrangement creates a rigid connector spanning 240 Å in length and 40-50 Å in width when assembled into antiparallel homodimers. The antiparallel assembly involves approximately 38 residues per monomer distributed across different spectrin repeats, forming direct polar interactions at the dimer interface.

The rod domain exhibits exceptional mechanical stability with rupture forces exceeding 60 pN required for dimer dissociation, significantly higher than other actin crosslinkers like filamin (14 pN). This high mechanical strength is essential for ACTN4's function as a stable actin crosslinker capable of withstanding cellular forces. The 90° twist along the long axis during dimer formation creates a curved interface that stabilizes the entire rod structure.[11]

Beyond structural integrity, spectrin repeats serve as docking platforms for numerous cytoskeletal and signaling proteins, including vinculin, MEKK1 kinase, MAGI, BP-180, NMDA receptor subunits, α-catenin, integrins, L-selectin, and intercellular adhesion molecules. These interactions are crucial for generating multiprotein assemblies that integrate cytoskeletal architecture with signal transduction pathways.[12]

EF-Hand Calcium-Binding Domain

The C-terminal region of ACTN4 contains a calmodulin-like domain (CaM) composed of four EF-hand motifs arranged as two pairs (EF1-2 and EF3-4). Each EF-hand features two short α-helices connected by a loop region in a helix-loop-helix configuration capable of coordinating calcium ions. However, functional calcium binding is restricted to EF1 of the N-terminal lobe, with the other EF-hands rendered non-functional through evolutionary mutations.[11,12]

Calcium binding to EF1 occurs with a dissociation constant of approximately 104 μM through an endothermic and entropically driven process. This binding induces conformational changes from a closed to an open state, altering the interaction between the CaM domain and the adjacent neck region. The conformational shift affects the orientation of actin-binding domains within the ACTN4 dimer, ultimately modulating F-actin crosslinking capacity.[24]

The calcium-induced structural changes are relatively moderate compared to other EF-hand proteins like calmodulin, involving primarily clockwise rotation about the entering helix with minimal opening between helices. Binding of calcium stabilizes the loop region connecting EF-hand helices, reducing internal mobility and exposing hydrophobic residues for interactions with regulatory partners. This mechanism allows calcium to function as an allosteric regulator of ACTN4's actin-bundling activity.[24]

Physiological Role in Actin Filament Organization and Focal Adhesion

ACTN4 functions as a critical organizer of the actin cytoskeleton through its ability to crosslink actin filaments into parallel bundles and anchor them to focal adhesions. The protein localizes primarily to stress fibers and cellular protrusions, where it coordinates contractile forces and facilitates mechanical force transmission. Unlike ACTN1, which promotes focal adhesion maturation, ACTN4 creates immature focal adhesions characterized by rapid turnover and enhanced cell motility.[25,26]

The functional distinction between ACTN4 and ACTN1 involves differential recruitment of focal adhesion proteins. While both isoforms recruit paxillin normally, ACTN4 fails to recruit zyxin to focal adhesions, preventing maturation into stable adhesion structures. This defective zyxin binding results from structural differences in the rod domain that impair protein-protein interactions. The formation of immature focal adhesions by ACTN4 facilitates rapid assembly and disassembly cycles essential for cell migration and invasion.[25]

ACTN4 demonstrates a complex relationship with stress fiber formation, functioning as a suppressor rather than promoter of these structures. Depletion of ACTN4 results in more prominent actin stress fibers with increased actin polymer incorporation, suggesting that ACTN4 normally limits stress fiber stability. This regulatory function involves promoting rapid actin turnover dynamics and preventing excessive stabilization of contractile structures. The protein's role in wound healing demonstrates its importance in collective cell migration, where coordinated dissolution of stress fibers is required for proper cellular advance.[26]

Post-Translational Modifications and Subcellular Localization

ACTN4 undergoes various post-translational modifications that regulate its function and localization. Phosphorylation represents a major regulatory mechanism, with rapid phosphorylation occurring within seconds following EGF receptor activation. This phosphorylation is mediated through direct interaction between ACTN4 and the EGF receptor, enabling rapid cytoskeletal remodeling in response to growth factor signaling. The protein contains multiple phosphorylation sites that can be modified by kinases including Akt, IκB kinase (IKK), and various serine/threonine kinases.[27,28]

Acetylation at lysine residues provides another layer of regulation, with specific modifications affecting protein stability and interactions. Acetylation can compete with ubiquitination at the same lysine residues, thereby stabilizing ACTN4 and preventing proteasomal degradation. The balance between acetylation and deacetylation, mediated by acetyltransferases and histone deacetylases, fine-tunes ACTN4 function in response to cellular conditions.[27,28]

ACTN4 exhibits dynamic subcellular localization patterns that reflect its functional states. Under normal conditions, the protein localizes to focal adhesions and along actin stress fibers, where it maintains cytoskeletal organization. During cell migration, ACTN4 concentrates at cellular protrusions and leading edges of migrating cells, facilitating cytoskeletal remodeling necessary for motility. Disease-associated mutations can disrupt normal localization, causing protein aggregation and loss of functional distribution.[12,13]

Nuclear localization of ACTN4 has been observed under specific conditions, where it functions as a transcriptional co-regulator. In the nucleus, ACTN4 interacts with transcription factors including RelA/p65 and serves as an atypical coactivator controlling gene expression networks related to cell growth. This nuclear function represents an additional layer of ACTN4's regulatory capacity beyond its cytoskeletal roles.

Regulation at Transcriptional and Post-Transcriptional Levels

Transcriptional Regulation

ACTN4 expression is controlled by multiple transcriptional regulatory mechanisms involving various signaling pathways. The NF-κB signaling pathway represents a major transcriptional regulator of ACTN4, with enhanced expression observed during inflammatory responses and pathological conditions. HDAC7 functions as a key transcriptional regulator of ACTN4, with the HDAC7/ACTN4 regulatory axis playing crucial roles in disease progression. Upregulation of this pathway is associated with inflammatory responses, apoptosis modulation, and various pathological processes.[27]

The protein's expression is also regulated by developmental signaling pathways and tissue-specific transcription factors that control its spatial and temporal expression patterns. During cancer progression, various oncogenic transcription factors can enhance ACTN4 expression, contributing to the protein's overexpression observed in multiple malignancies. Epigenetic modifications, including DNA methylation and histone modifications, provide additional layers of transcriptional control.[15,16,29]

MicroRNA-Mediated Regulation

ACTN4 is subject to extensive post-transcriptional regulation through microRNA-mediated mechanisms. Multiple miRNAs target ACTN4 mRNA, including hsa-miR-23a-3p, hsa-miR-3175, and hsa-miR-23b-3p, which regulate protein levels in response to cellular conditions. These miRNAs demonstrate tissue-specific and condition-dependent expression patterns that fine-tune ACTN4 levels.[27]

The miRNA regulatory network surrounding ACTN4 involves complex feedback loops and cross-regulatory mechanisms. Some miRNAs exhibit context-dependent effects, either suppressing or enhancing ACTN4 expression depending on cellular conditions and co-regulatory factors. The dysregulation of ACTN4-targeting miRNAs is implicated in various pathological processes, including cancer progression and cardiovascular diseases.[27,30]

Signaling Pathway Integration

ACTN4 regulation integrates multiple signaling pathways including PI3K/Akt, MAPK, Wnt/β-catenin, and calcium signaling cascades. These pathways converge to control ACTN4 expression, post-translational modifications, and subcellular localization in response to environmental cues. Growth factor signaling, particularly through EGF receptor activation, rapidly modulates ACTN4 phosphorylation and function.[15]

Mechanical signaling represents another important regulatory mechanism, with ACTN4 responding to cellular tension and substrate stiffness through mechanosensitive pathways. The protein's calcium sensitivity allows integration of calcium signaling with cytoskeletal dynamics, enabling rapid responses to changes in intracellular calcium concentrations. This multi-layered regulation ensures that ACTN4 function is precisely controlled and appropriately responsive to cellular needs and environmental conditions.[31]

3.ACTN4 and Cytoskeletal Remodeling in Cancer

Mechanisms of Actin Filament Crosslinking

ACTN4 functions as a sophisticated actin filament crosslinker that fundamentally alters cytoskeletal organization through multiple mechanisms. The protein forms antiparallel homodimers that span approximately 240 Å in length, creating rigid connectors between actin filaments separated by distances of 40-50 Å. Unlike simple bundling proteins, ACTN4 crosslinks actin filaments into parallel arrays while maintaining the flexibility necessary for dynamic reorganization.[15,25]

The crosslinking mechanism involves the dual actin-binding domains at the N-terminus of each ACTN4 monomer, which can simultaneously engage two separate actin filaments. This configuration allows ACTN4 to organize actin networks with specific geometric arrangements that optimize force transmission and contractile efficiency. The protein exhibits remarkable mechanical stability, with rupture forces exceeding 60 pN required for dimer dissociation, significantly higher than other actin crosslinkers like filamin (14 pN).[22,32]

Paradoxically, recent studies have revealed that ACTN4 actually suppresses the formation of massive stress fibers rather than promoting them. ACTN4 depletion results in more prominent and stable stress fibers due to increased tropomyosin loading onto actin filaments. This occurs because ACTN4 competes with tropomyosin for F-actin binding sites, and in its absence, tropomyosin stabilizes actin filaments by shielding them from cofilin-mediated disassembly. The competition between ACTN4, tropomyosin, and cofilin creates a regulatory network that fine-tunes actin turnover dynamics essential for cell motility.[26]

The crosslinking activity of ACTN4 facilitates buckling-mediated contractility in disordered actin networks, which is distinct from the transport-mediated contractility observed in organized sarcomeres. By increasing network connectivity while preventing excessive filament stabilization, ACTN4 enables the rapid cytoskeletal remodeling necessary for cancer cell invasion and migration. This mechanism explains why ACTN4 overexpression enhances cancer cell motility despite appearing to suppress stress fiber formation.[25,26]

Role in Lamellipodia and Filopodia Formation

ACTN4 plays a critical role in the formation of specialized membrane protrusions, particularly ruffle-edge lamellipodia that are distinct from canonical flat lamellipodia. Live-cell imaging studies demonstrate that ACTN4 is most abundant at the leading edges of extending lamellipodia, where it facilitates the formation of multilayered membrane ruffles characteristic of highly invasive cancer cells. These ACTN4-enriched ruffle-edge lamellipodia exhibit unique morphological features with multilayered membrane folds that differ significantly from conventional flat protrusions.[33]

The formation of ACTN4-enriched lamellipodia is highly dependent on PI3K activity, with PI3K inhibition by LY294002 markedly diminishing ACTN4 localization in lamellipodia while leaving its distribution in stress fibers and

focal adhesions unaffected. This selective dependence suggests that ACTN4 recruitment to lamellipodia involves specific signaling pathways distinct from those regulating its cytoskeletal functions. The protein's enrichment in lamellipodia correlates with enhanced migratory capacity, as ACTN4-enriched lamellipodia extend forward at average speeds of 0.37 ± 0.15 μm/min compared to only 0.07 ± 0.04 μm/min for ACTN4-modest flat lamellipodia.[33]

ACTN4 also contributes to filopodia formation and maintenance in cancer cells. The protein localizes to invadopodia, finger-like protrusions that are particularly prominent in pancreatic cancer cells and are associated with matrix degradation capabilities. Knockdown of ACTN4 reduces the formation of cellular protrusions associated with invasion and migration, confirming its functional importance in membrane dynamics. The protein's involvement in filopodia formation involves interactions with actin-regulatory proteins and signaling molecules that coordinate protrusion dynamics with directional migration.[17,34]

Importantly, ACTN4 knockdown suppresses ruffle-edge lamellipodia formation and cell migration during wound healing assays in multiple cancer cell lines including A549 lung cancer and MDA-MB-231 breast cancer cells. This effect is specific to ACTN4, as ACTN1 knockdown does not produce similar defects in lamellipodia formation. The specificity suggests that ACTN4 has evolved unique functions in cancer cell motility that cannot be compensated by other α-actinin isoforms.[25,34]

ACTN4's Interaction with Integrins, Vinculin, and Focal Adhesion Kinase (FAK)

ACTN4 participates in complex protein networks at focal adhesions that integrate cytoskeletal dynamics with cell-matrix adhesion signaling. The protein interacts directly with β1 integrin receptors, anchoring the actin cytoskeleton to the plasma membrane and facilitating mechanical force transmission. This interaction is crucial for mechanotransduction, as it allows cells to sense and respond to substrate stiffness and external mechanical forces.[35,36]

The relationship between ACTN4 and vinculin represents a key regulatory mechanism in focal adhesion dynamics. Unlike ACTN1, which promotes focal adhesion maturation by recruiting both paxillin and zyxin, ACTN4 creates immature focal adhesions characterized by defective zyxin recruitment. While both ACTN4 and ACTN1 can recruit paxillin normally, ACTN4 fails to bind zyxin due to structural differences in its rod domain. This defective zyxin binding prevents focal adhesion maturation and promotes rapid turnover cycles essential for cell migration.[35]

Vinculin's interaction with ACTN4 involves the vinculin head domain, which regulates integrin dynamics and clustering. The vinculin-talin interaction leads to clustering of activated integrins and increases integrin residency time in focal adhesions. However, when ACTN4 is present, this stabilization is countered by the protein's tendency to promote focal adhesion disassembly, creating a dynamic equilibrium that facilitates cell motility.[25,35]

Focal Adhesion Kinase (FAK) modulates ACTN4 function through several mechanisms involving integrin activation and mechanotransduction signaling. FAK acts upstream of talin in regulating integrin activation, and this pathway influences ACTN4's ability to organize cytoskeletal structures. FAK-mediated phosphorylation events can modify ACTN4 activity and localization, particularly in response to growth factor signaling such as EGF receptor activation. The rapid phosphorylation of ACTN4 following EGF stimulation enables quick cytoskeletal remodeling necessary for cell migration and invasion.[36]

The integration of FAK, vinculin, and ACTN4 signaling creates a mechanosensitive system that responds to both chemical and physical cues. This system allows cancer cells to adapt their cytoskeletal organization and adhesive properties based on the mechanical properties of their environment. The dysregulation of these interactions in cancer cells contributes to enhanced migratory capacity and metastatic potential.[36]

How Cytoskeletal Changes Drive Migration and Invasion

ACTN4-mediated cytoskeletal remodeling drives cancer cell migration and invasion through multiple coordinated mechanisms that fundamentally alter cellular mechanics and behavior. The protein's ability to organize actin networks into dynamic, contractile structures enables cells to generate the forces necessary for movement through tissue barriers. This process involves the temporal and spatial coordination of protrusion formation, adhesion dynamics, and contractile force generation.[37]

The enhanced contractility generated by ACTN4-organized actin networks facilitates cell body translocation and trailing edge retraction during migration. ACTN4 promotes buckling-mediated contractility by increasing actin network connectivity while maintaining filament flexibility. This type of contractility is particularly suited for mesenchymal migration, where cells must navigate through complex three-dimensional tissue environments. The protein's influence on stress fiber organization allows cells to establish front-to-back polarity and generate the traction forces necessary for directional movement.[26]

ACTN4's role in invadopodia formation provides cancer cells with the ability to degrade extracellular matrix barriers. Invadopodia are specialized membrane protrusions that concentrate matrix metalloproteinases (MMPs) at their tips, enabling localized ECM degradation. ACTN4 localizes to these structures and facilitates their formation and stability. The protein's interaction with membrane-type 1 matrix metalloproteinase (MT1-MMP) at ruffle-edge lamellipodia suggests direct involvement in matrix degradation processes.[33,38]

The coordinated action of multiple MMPs, including MMP2, MMP9, and MMP14, at ACTN4-enriched invadopodia enables cancer cells to breach basement membranes and invade surrounding tissues. MMP14 (MT1-MMP) is particularly important as it functions both as a direct matrix-degrading enzyme and as an activator of MMP2. The temporal regulation of MMP targeting to invadopodia ensures that matrix degradation occurs precisely when and where it is needed for invasion.[38]

ACTN4 also promotes epithelial-mesenchymal transition (EMT), a process that transforms epithelial cells into motile mesenchymal-like cells capable of invasion and metastasis. During EMT, ACTN4 overexpression is associated with loss of epithelial markers like E-cadherin and acquisition of mesenchymal characteristics. The protein facilitates the cytoskeletal reorganization necessary for this transition, including the dissolution of cell-cell junctions and the formation of migratory structures.[15,17]

The enhancement of cell migration by ACTN4 involves the coordination of multiple cellular processes including mechanotransduction, adhesion dynamics, and force generation. The protein's ability to sense and respond to mechanical forces allows cancer cells to adapt their behavior based on tissue stiffness and other physical properties of their environment. This mechanosensitive response enables cancer cells to optimize their migratory strategies for different tissue contexts encountered during metastasis.[39]

4.ACTN4 in Oral Squamous Cell Carcinoma (OSCC)

Expression Levels in OSCC Tissues and Cell Lines

Tissue Expression Patterns

ACTN4 demonstrates significant overexpression in oral squamous cell carcinoma tissues compared to normal oral mucosa. Comprehensive immunohistochemical analysis of 64 primary OSCC specimens revealed ACTN4 overexpression in 38 cases (59.4%), significantly more frequent than in normal oral mucosal specimens. This overexpression pattern has been consistently validated across multiple independent studies, with expression rates ranging from 59% to 77% depending on the study cohort and detection methodology.[15,19]

The spatial distribution of ACTN4 expression in OSCC tissues reveals critical functional insights. The protein shows characteristic enrichment at the invasive tumor front compared to the central tumor mass and normal epithelium. This spatial pattern suggests direct involvement in the invasive process, as ACTN4 localizes precisely where tumor cells encounter and breach tissue barriers. The invasive front expression pattern has been observed consistently across different studies and correlates with enhanced invasive potential.[5]

Fluorescence in situ hybridization (FISH) analysis demonstrates that ACTN4 gene amplification occurs in approximately 24% of early-stage oral tongue cancers. Importantly, gene amplification and protein overexpression represent independent phenomena, with only 30-40% of high-expressing ACTN4 tumors also displaying increased gene copy numbers. This discordance suggests that multiple regulatory mechanisms beyond gene amplification contribute to ACTN4 overexpression in OSCC.[15]

Cell Line Expression Studies

Seven human OSCC cell lines demonstrate variable ACTN4 expression levels that correlate directly with their invasive potential. Cell lines with higher ACTN4 expression consistently exhibit greater invasive capacity in Matrigel invasion assays. This correlation provides functional validation for the clinical observations linking ACTN4 expression to tumor aggressiveness.[19]

RNA interference-mediated knockdown studies in OSCC cell lines confirm the functional significance of ACTN4 expression. siRNA-mediated reduction of ACTN4 expression significantly decreases invasion potential across multiple OSCC cell lines. These experiments demonstrate that ACTN4 is not merely a biomarker but actively contributes to the invasive phenotype of oral cancer cells.[19]

Correlation with Tumor Grade and Stage

Histopathological Correlations

ACTN4 expression in OSCC does not correlate significantly with conventional histopathological parameters including tumor differentiation, vascular invasion, lymphatic invasion, or mode of invasion. This lack of correlation with traditional prognostic factors suggests that ACTN4 represents an independent biological mechanism of tumor progression. The absence of correlation with histological grade indicates that ACTN4-mediated invasion can occur across all differentiation levels.[40]

Despite the lack of correlation with individual histopathological factors, ACTN4 expression demonstrates significant associations with invasive behavior when assessed functionally. The protein's enrichment at invasive tumor fronts, regardless of overall tumor grade, supports its role as a driver of local invasion independent of differentiation status. This pattern suggests that ACTN4 confers invasive capabilities that transcend traditional histological classifications.[15]

Stage-Dependent Expression

Advanced-stage OSCC demonstrates higher ACTN4 expression frequencies compared to early-stage disease. Studies of ovarian carcinoma, which shares similar invasive characteristics with OSCC, show that 77% of advanced-stage tumors express ACTN4 compared to only 33% of early-stage cases. Similar stage-dependent expression patterns are likely present in OSCC, though specific data for oral cancers requires further investigation.[42]

The relationship between ACTN4 and tumor staging appears more complex than simple stage-dependent expression. Gene amplification of ACTN4, detected by FISH, occurs even in early-stage (I/II) oral tongue cancers and serves as a powerful independent prognostic factor. This suggests that ACTN4 alterations may be early events in OSCC progression that confer metastatic potential independent of conventional staging criteria.[5]

Correlation with Metastatic Potential

Lymph Node Metastasis

ACTN4 expression demonstrates a strong positive correlation with delayed cervical lymph node metastasis in OSCC patients. Disease-free survival analysis reveals significantly shorter intervals to lymph node metastasis in ACTN4-positive patients compared to ACTN4-negative cases ($p=0.010$). The sensitivity and specificity of ACTN4 expression for predicting delayed cervical lymph node metastasis are 73% and 68%, respectively.[40]

The correlation with lymph node metastasis is particularly evident in patients who undergo curative surgery for early-stage disease. Among 12 patients who developed late cervical lymph node metastasis following partial glossectomy, 10 (83.3%) were ACTN4-positive. This high proportion suggests that ACTN4 expression can identify patients at risk for delayed metastasis even after apparently complete surgical resection.

Gene amplification of ACTN4 provides superior prognostic value for lymph node metastasis compared to protein expression alone. Among patients who developed late cervical lymph node metastasis after surgery, those with ACTN4 gene amplification had significantly worse outcomes, with 5-year survival rates of 0% compared to 88.9% for patients without gene amplification.[5]

Distant Metastasis

ACTN4 expression correlates with enhanced metastatic potential beyond regional lymph nodes. Studies in multiple cancer types, including those relevant to OSCC biology, demonstrate associations between ACTN4 expression and distant organ metastasis. In ovarian carcinoma, which shares similar metastatic patterns with oral cancers, ACTN4 expression is detected in 100% of metastatic lesions compared to 76% of primary tumors.[42]

The mechanism underlying ACTN4's role in metastasis involves the formation of specialized membrane protrusions called ruffle-edge lamellipodia. These structures, enriched in ACTN4, exhibit enhanced matrix degradation capabilities through co-localization with membrane-type 1 matrix metalloproteinase. This mechanism provides cancer cells with the ability to breach tissue barriers and establish distant metastases.[33]

Patient Survival Outcomes

Overall Survival

Gene amplification of ACTN4 serves as a powerful independent prognostic factor for overall survival in OSCC patients. Kaplan-Meier analysis demonstrates that patients with ACTN4 gene amplification have significantly shorter overall survival times compared to those without amplification ($p=0.0010$). The median survival time for patients with gene amplification is 826 days, while patients without amplification have not reached 50% mortality in long-term follow-up. Multivariate Cox regression analysis confirms that ACTN4 gene amplification is an independent risk factor for death in stage I/II oral tongue cancer patients, with a hazard ratio of 6.08 (95% CI: 1.66-22.27, $p=0.0064$). This

hazard ratio indicates that patients with ACTN4 gene amplification have more than six times the risk of death compared to those without amplification.[5]

Interestingly, protein expression of ACTN4 by immunohistochemistry does not correlate significantly with overall survival in OSCC patients. This discordance between gene amplification and protein expression suggests that gene dosage effects may be more critical for prognosis than absolute protein levels. The superior prognostic value of gene amplification analysis makes it a more clinically relevant biomarker than protein expression assessment.[40]

Disease-Free Survival

ACTN4 expression significantly impacts disease-free survival in OSCC patients. Patients positive for ACTN4 expression demonstrate significantly shorter disease-free survival intervals compared to ACTN4-negative patients ($p=0.010$). This correlation suggests that ACTN4 promotes early recurrence and metastatic spread following initial treatment.

The impact on disease-free survival is particularly evident in the context of delayed cervical lymph node metastasis. ACTN4-positive patients show higher rates of late cervical lymph node involvement, which significantly impacts long-term disease control. The protein's ability to predict delayed metastasis makes it valuable for identifying patients who may benefit from more aggressive surveillance or adjuvant therapy.

Univariate analysis reveals that ACTN4 expression is an independent prognostic factor for disease-free survival in OSCC. This independence from other clinical and pathological factors suggests that ACTN4 represents a distinct biological mechanism of tumor progression that complements traditional prognostic indicators.[40]

Mechanistic Studies Linking ACTN4 to Cell Motility, Invasion, and EMT

Cell Motility Mechanisms

Live-cell imaging studies reveal that ACTN4 drives cell motility through the formation of specialized ruffle-edge lamellipodia that differ morphologically and functionally from canonical flat lamellipodia. ACTN4-enriched lamellipodia extend forward at average speeds of 0.37 ± 0.15 μm/min compared to only 0.07 ± 0.04 μm/min for ACTN4-modest flat protrusions. This enhanced motility results from the unique multilayered membrane fold structure characteristic of ACTN4-enriched protrusions.[33]

The formation of ACTN4-enriched lamellipodia is highly dependent on PI3K activity, with PI3K inhibition by LY294002 markedly diminishing ACTN4 localization in lamellipodia while leaving its distribution in stress fibers and focal adhesions unaffected. This selective dependence indicates that ACTN4 recruitment to lamellipodia involves specific signaling pathways distinct from its other cytoskeletal functions.

Correlative light and electron microscopy (CLEM) demonstrates that ACTN4-enriched lamellipodia exhibit characteristic morphology of multilayered ruffle-edges with tightly stacked membrane folds. Similar ruffle-edge lamellipodia are observed in highly invasive A549 lung cancer and MDA-MB-231 breast cancer cells, suggesting this mechanism is conserved across cancer types.[33]

Invasion Mechanisms

ACTN4 promotes cancer cell invasion through multiple coordinated mechanisms involving matrix degradation and cytoskeletal remodeling. Matrigel invasion assays demonstrate direct correlations between ACTN4 expression levels and invasive potential in OSCC cell lines. RNAi-mediated knockdown of ACTN4 significantly reduces invasion potential, confirming its functional role in this process.[41]

The invasive capability conferred by ACTN4 involves co-localization with membrane-type 1 matrix metalloproteinase (MT1-MMP) at ruffle-edge lamellipodia. This co-localization suggests that ACTN4-enriched protrusions have enhanced matrix degradation capabilities, enabling cancer cells to breach extracellular matrix barriers. The combination of enhanced motility and matrix degradation provides cancer cells with the tools necessary for tissue invasion.

Wound healing assays confirm that ACTN4 knockdown suppresses both ruffle-edge lamellipodia formation and cell migration in cancer cell monolayers. This dual effect on both protrusion formation and overall migration demonstrates that ACTN4 coordinates multiple aspects of the invasive phenotype.[33]

Epithelial-Mesenchymal Transition (EMT)

ACTN4 promotes epithelial-mesenchymal transition through activation of key signaling pathways, particularly the ERK/GSK-3β/β-catenin/Slug cascade. Overexpression of ACTN4 enhances phosphorylation of ERK and GSK-3β (ser9), leading to stabilization of β-catenin and increased expression of the EMT transcription factor Slug. This pathway activation results in loss of epithelial characteristics and acquisition of mesenchymal properties.[43]

The β-catenin/Slug pathway represents a critical mechanism by which ACTN4 promotes malignant transformation. ERK inhibition with PD98059 suppresses GSK-3β phosphorylation, reduces active β-catenin levels, and suppresses Slug expression in ACTN4-overexpressing cells. This pathway dependence demonstrates that ACTN4's effects on EMT require specific signaling cascade activation.

Functional studies demonstrate that ACTN4-mediated EMT enhances both proliferative and invasive capacities of cancer cells. Cancer tissue-origin spheroids (CTOS) established from patient specimens show identical pathway activation patterns, confirming the clinical relevance of these mechanistic findings. The combination of EMT promotion and enhanced motility makes ACTN4 a key driver of cancer progression and metastasis.[43]

5. ACTN4 and Epithelial–Mesenchymal Transition (EMT)

Role of EMT in Oral Cancer Progression

Epithelial–mesenchymal transition (EMT) is a phenotypic program whereby stationary epithelial cells acquire mesenchymal traits, including motility and invasiveness. In OSCC, EMT contributes critically to tumor progression by enabling local invasion, intravasation, and metastatic dissemination. EMT correlates with poor prognosis, treatment resistance, and recurrence in oral cancer patients, as cells undergoing EMT lose epithelial markers (e.g., E-cadherin) and gain mesenchymal markers (e.g., N-cadherin, vimentin), facilitating dissemination through the extracellular matrix.

ACTN4-Mediated Regulation of EMT Markers

- E-cadherin: ACTN4 overexpression induces downregulation of E-cadherin, weakening cell–cell adhesion and enabling detachment of tumor cells from the primary epithelial layer.
- N-cadherin: Concomitant upregulation of N-cadherin (“cadherin switch”) by ACTN4 promotes heterotypic adhesions conducive to motility and interaction with stromal cells.
- Vimentin: ACTN4 drives vimentin expression, reorganizing intermediate filaments to support mesenchymal morphology and increased cellular plasticity.
- Snail and Twist: ACTN4 activates transcription factors Snail and Twist, which directly repress epithelial genes and activate mesenchymal programs, amplifying the EMT cascade.

Integration with Signaling Pathways

1. PI3K/Akt:
   ACTN4 enhances PI3K/Akt signaling, leading to phosphorylation and inactivation of GSK-3β. Inactive GSK-3β fails to degrade Snail, resulting in Snail accumulation, E-cadherin repression, and EMT induction.
2. MAPK/ERK:
   ACTN4 overexpression increases ERK phosphorylation. Activated ERK phosphorylates GSK-3β at Ser9, stabilizing β-catenin and promoting nuclear translocation. Nuclear β-catenin cooperates with TCF/LEF to upregulate Snail and Twist, reinforcing EMT.
3. Wnt/β-catenin:
   By stabilizing β-catenin via ERK-mediated GSK-3β inhibition, ACTN4 potentiates canonical Wnt signaling. Nuclear β-catenin drives transcription of EMT effectors including Twist, further consolidating mesenchymal phenotypes.

Proposed Mechanistic Model of ACTN4-Driven EMT in OSCC

1. ACTN4 Overexpression: Genetic amplification or transcriptional upregulation of ACTN4 increases cytoplasmic ACTN4 levels.
2. Signal Activation: Elevated ACTN4 recruits and activates PI3K/Akt and MAPK/ERK pathways at focal adhesions.

3. GSK-3β Inhibition: Akt and ERK phosphorylate GSK-3β (Ser9), preventing β-catenin degradation and Snail ubiquitination.
4. EMT Transcriptional Activation: Stabilized β-catenin and Snail accumulate in the nucleus, upregulating Snail, Twist, and mesenchymal markers while repressing E-cadherin.
5. Cytoskeletal Remodeling: ACTN4 crosslinks actin into dynamic networks supporting lamellipodia and stress fiber disassembly, synergizing with EMT to promote cell motility and invasion.
6. Phenotypic Transition: OSCC cells undergo loss of polarity, gain mesenchymal morphology, and acquire invasive capabilities.

This integrated model positions ACTN4 as a central orchestrator of EMT and cytoskeletal dynamics in OSCC, linking extracellular signals to transcriptional programs and structural reorganization that drive oral cancer progression.

6. In Vitro Experimental Exploration

Cell Line Models

Human OSCC cell lines commonly employed for ACTN4 studies include HSC-3, SCC-9, SCC-25, and CAL-27. These lines exhibit variable basal ACTN4 expression and invasive potentials, enabling comparative analyses.

- ACTN4 Overexpression: Full-length human ACTN4 cDNA is cloned into mammalian expression vectors (e.g., pcDNA3.1) under a CMV promoter. Transient or stable transfections are performed using lipofection reagents (e.g., Lipofectamine 3000) with antibiotic selection (e.g., G418) for stable clones.
- ACTN4 Knockdown: Small interfering RNAs (siRNAs) targeting ACTN4 transcripts are transfected using RNAi-optimized reagents. Knockdown can be confirmed by qPCR and immunoblotting 48–72 hours post-transfection. Alternatively, CRISPR/Cas9 editing with single guide RNAs directed at ACTN4 exons enables permanent gene disruption; clones are isolated by limiting dilution and validated by sequencing and immunoblot.

Functional Assays

- Wound Healing Assay: Confluent monolayers are scratched with a pipette tip. Time-lapse images at 0, 12, and 24 hours quantify wound closure. Migration rate is calculated as percent gap closure over time.
- Transwell Migration and Invasion Assays:
    - *Migration:* Cells are seeded in serum-free medium in the upper chamber of Transwell inserts. After 12–24 hours, migrated cells on the lower membrane face are fixed, stained, and counted.
    - *Invasion:* Inserts are precoated with Matrigel to simulate extracellular matrix. Invaded cells are quantified similarly to migration assays, providing a measure of invasive capacity.
- Western Blotting and Immunofluorescence: Protein lysates from treated cells are probed for EMT markers (E-cadherin, N-cadherin, vimentin), cytoskeletal regulators (FAK, p-FAK, cofilin), and ACTN4. Immunofluorescence staining of fixed cells with phalloidin (F-actin) and antibodies against focal adhesion proteins visualizes cytoskeletal architecture and ACTN4 localization.
- Immunocytochemistry: Cells grown on coverslips are stained with fluorescent phalloidin and anti-ACTN4, anti-vimentin, or anti-E-cadherin antibodies. Confocal microscopy captures changes in actin stress fibers, lamellipodia, and EMT marker distribution.

Quantitative Analysis

- Image Analysis: Migration and invasion assays are quantified using ImageJ with plugins such as “MRI Wound Healing Tool” for scratch assays and “Cell Counter” for Transwell assays. For immunofluorescence, AI-based segmentation (e.g., CellProfiler, Ilastik) automates measurement of fluorescence intensity and protrusion metrics (lamellipodia area, filopodia number).
- Statistical Methods: All experiments are performed in triplicate. Data are expressed as mean ± SEM. Statistical significance is assessed using unpaired two-tailed t-tests for two-group comparisons or one-way ANOVA with Tukey’s post hoc test for multiple comparisons. A p-value < 0.05 is considered significant.

7. Integrating Network Pharmacology and AI for ACTN4 Inhibitor Discovery

Network Pharmacology Approach

Network pharmacology provides a systems-level framework to identify candidate ACTN4 inhibitors by elucidating the broader interactome and pathway architecture in which ACTN4 operates. Mapping the ACTN4 interactome using databases such as STRING and GeneMANIA reveals direct and indirect protein–protein interactions, including focal adhesion kinase (FAK), integrin β1, vinculin, cofilin, and members of the Rho GTPase family. Integration with TCGA transcriptomic data identifies co-expressed hub genes, such as VCL, PXN, and FAK, that coordinate cytoskeletal remodeling and EMT programs alongside ACTN4. Pathway enrichment analyses highlight key signaling modules—PI3K/Akt, MAPK/ERK, and Wnt/β-catenin—as potential axes for therapeutic intervention. DrugBank screening of approved compounds targeting these hubs can prioritize drugs with polypharmacological potential, enabling rapid repurposing opportunities and identification of synergistic combinations that modulate ACTN4-driven networks.

AI and Computational Tools

Artificial intelligence accelerates inhibitor discovery by predicting target–ligand interactions and narrowing down candidate libraries. Supervised machine learning models, such as random forests and deep neural networks trained on known actin-binding protein inhibitors, can predict the binding likelihood of small molecules to the ACTN4 ABD or rod domain interfaces. Virtual screening of extensive natural compound libraries (e.g., ZINC, NPASS) using AI-guided docking protocols prioritizes hits with favorable binding affinities and drug-like properties. Top candidates undergo molecular docking into high-resolution structures of ACTN4 domains (e.g., PDB 2R0O for ABD, PDB 6O31 for spectrin repeats) to refine binding poses. Molecular dynamics simulations assess the stability of docked complexes under physiological conditions, providing insight into binding kinetics and conformational adaptability. Combined AI and simulation workflows enable iterative optimization of lead compounds targeting ACTN4's actin-binding interface or its regulatory PIP2-binding sites.

Potential Natural Compounds

Natural products offer a rich source of bioactive scaffolds with cytoskeletal and EMT-modulating properties. Phytochemicals such as curcumin, resveratrol, and quercetin have demonstrated inhibition of PI3K/Akt and Wnt/β-catenin signaling, leading to reduced ACTN4 expression and impaired EMT in various cancer models. In silico docking studies reveal that curcumin can occupy the CH1–CH2 interdomain pocket of ACTN4's ABD, potentially stabilizing the closed autoinhibited conformation and reducing actin crosslinking activity. Resveratrol derivatives preferentially dock to the EF-hand lobe, modulating calcium-dependent conformational shifts. Quercetin and related flavonoids exhibit high binding affinities at the PIP2-binding cleft, disrupting lipid-mediated ACTN4 activation at membrane protrusions. These computational predictions warrant experimental validation through binding assays, cell-based functional tests, and structure–activity relationship (SAR) studies to advance natural compound leads toward selective ACTN4 inhibition in OSCC.

8. Therapeutic and Translational Perspectives

Targeting ACTN4 as a Therapeutic Strategy in OSCC

Given its central role in cytoskeletal remodeling, invasion, and EMT, ACTN4 represents a promising therapeutic target in oral squamous cell carcinoma (OSCC). Direct inhibition of ACTN4's actin-binding activity could impair cancer cell motility and metastatic dissemination. Strategies include small molecules that stabilize the closed conformation of the CH1–CH2 interface, preventing actin crosslinking, or compounds that disrupt PIP_2-mediated activation at the plasma membrane. Gene amplification and overexpression of ACTN4 in OSCC underscore its validity as a target, with amplification serving as both a biomarker and predictor of therapeutic response.

Combination with Existing Chemotherapeutics or Immunotherapies

Integration of ACTN4 inhibitors into current treatment regimens may enhance efficacy and overcome resistance. Combining ACTN4-targeted agents with platinum-based chemotherapy (e.g., cisplatin) could sensitize tumor cells by impairing repair-associated cytoskeletal dynamics necessary for survival after DNA damage. Preclinical studies suggest that cytoskeletal disruption enhances chemosensitivity by promoting mitotic defects and apoptosis. Furthermore, ACTN4 inhibition may potentiate immunotherapy by altering tumor–stroma interactions and enhancing

immune cell infiltration; disruption of focal adhesion signaling can increase tumor immunogenicity and improve responses to PD-1/PD-L1 blockade. Biomarker-driven patient selection based on ACTN4 gene amplification or expression can identify subgroups most likely to benefit from these combination strategies.

Potential of ACTN4 Inhibitors in Precision Oncology

Precision oncology approaches can leverage ACTN4 status for personalized therapy. Patients with ACTN4 gene amplification exhibit poor prognosis and high metastatic risk; these individuals represent ideal candidates for ACTN4-directed interventions. Companion diagnostics using FISH or digital PCR assays for ACTN4 amplification can guide therapeutic decision-making. Additionally, integration of transcriptomic and proteomic data from patient biopsies can inform combination regimens tailored to each tumor's signaling landscape, such as concomitant targeting of PI3K/Akt or MAPK/ERK pathways alongside ACTN4 inhibition. Ultimately, the development of selective ACTN4 inhibitors—validated through in vitro and in vivo models of OSCC—holds promise for reducing metastasis, improving survival outcomes, and advancing precision medicine in oral cancer care.

9. Challenges and Future Directions

Limitations of Current In Vitro Approaches

Traditional two-dimensional (2D) monolayer cultures of OSCC cell lines, while instrumental for dissecting ACTN4 functions, fail to recapitulate the complex tumor microenvironment, cell–cell interactions, and extracellular matrix (ECM) architecture present in vivo. These simplified models often underestimate biomechanical constraints and do not capture gradients of nutrients, oxygen, or therapeutic agents, potentially leading to overestimation of drug efficacy. Furthermore, monolayer systems obscure the influence of stromal and immune components on ACTN4-driven invasion and EMT, limiting translational relevance.

Need for Three-Dimensional Models and Organoids

Three-dimensional (3D) culture systems—including spheroids, organotypic raft cultures, and patient-derived organoids—offer improved physiological fidelity by more accurately modeling tissue architecture, ECM composition, and mechanical forces. Incorporation of fibroblasts, endothelial cells, and immune cells into 3D co-cultures can elucidate how ACTN4 mediates tumor–stroma cross talk and immune evasion. Organoids derived from OSCC patient tumors retain genetic heterogeneity and tumor-specific phenotypes, providing platforms for high-content screening of ACTN4 inhibitors under more clinically relevant conditions. Advanced microfluidic "tumor-on-a-chip" technologies further enable controlled perfusion, real-time imaging of invasion, and incorporation of immune components to assess combinatorial therapies.

Translational Gap between In Vitro Findings and Clinical Application

Bridging the gap between bench and bedside requires rigorous validation of ACTN4-targeted strategies in preclinical animal models that faithfully mimic human OSCC progression and metastasis. Genetically engineered mouse models (GEMMs) with conditional ACTN4 overexpression or knockout in the oral epithelium can demonstrate in vivo relevance of in vitro discoveries. Patient-derived xenografts (PDXs) preserve tumor heterogeneity and microenvironmental factors, enabling evaluation of drug pharmacodynamics and pharmacokinetics. Early-phase clinical trials incorporating biomarker-driven patient selection—based on ACTN4 gene amplification or expression—are essential to establish drug safety, optimal dosing, and preliminary efficacy.

Integration of AI-Driven Drug Discovery Pipelines with Experimental Validation

While artificial intelligence and network pharmacology accelerate the identification of candidate ACTN4 inhibitors, iterative experimental validation remains critical. Incorporating high-throughput screening of AI-predicted compounds in 3D organoid and PDX models can refine lead selection based on efficacy, toxicity, and mechanistic biomarkers. Integration of multi-omics profiling—including transcriptomics, proteomics, and metabolomics—before and after treatment will inform adaptive AI models, improving predictive accuracy and enabling dynamic optimization of compound libraries. Establishing collaborative platforms that merge computational predictions with real-world experimental data will expedite the translation of ACTN4-targeted therapies into clinical practice, ultimately improving outcomes for patients with OSCC.

Conclusion

Alpha-actinin-4 emerges as a pivotal regulator of cytoskeletal remodeling, cell motility, and epithelial–mesenchymal transition in oral squamous cell carcinoma. Overexpression and gene amplification of ACTN4 in OSCC correlate with enhanced invasion, metastatic potential, and poor clinical outcomes. Mechanistic studies reveal how ACTN4

orchestrates actin filament crosslinking, lamellipodia formation, and focal adhesion dynamics to empower tumor cells to breach tissue barriers. By integrating network pharmacology with artificial intelligence and molecular simulations, novel ACTN4 inhibitors can be rapidly identified from natural and synthetic libraries, enabling targeted disruption of its actin-binding activity and regulatory interactions. The combination of high-fidelity in vitro models, such as 3D organoids and tumor-on-a-chip systems, with preclinical animal studies will bridge translational gaps and validate computational predictions. Ultimately, targeting cytoskeletal regulators like ACTN4 offers a transformative strategy to impede metastasis and improve survival for OSCC patients. The synergy between wet-lab experimentation and AI-driven drug discovery heralds a new era of precision oncology focused on the dynamic scaffold of cancer cell invasion.

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