Fibulin-3 sponges Tiam1 to manipulate MMP-7 activity through β-catenin signaling in oral squamous cell carcinoma

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Research Article

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Abstract

Purpose: Oral squamous cell carcinoma (named OSCC) is considered as the most frequent malignancy in oral cavity, which has became a rapid increasing problem for the global public health with unclear underling molecular mechanism. Previously, we reported Tiam1 (T-lymphoma invasion and metastasis inducing factor 1) as a potential oncogene for OSCC. Here, we in-depth explored its signaling mechanism.

Methods: The mRNA and protein expression levels of primary differentially expressed genes (Tiam1, Fibulin-3 and MMP-7) were measured in different TNM stages of OSCC patients using RT-PCR and ELISA respectively. Using human OSCC cell line CAL27 cell line, the relationships between these factors have been analyzed and the direct interaction has been also examined. The luciferase reporter assay was established for the promoter region of MMP-7. Both the epithelial (E-cadherin) and mesenchymal protein markers (Vimentin and Snail) have been investigated using western blotting.

Results: The mRNA and protein activities of Fibulin-3 declined as the increase of TNM stage. Inversely, the mRNA and protein levels of Tiam1 and MMP-7 elevated significantly as OSCC progressed. Tiam1 transfection in CAL27 cells stimulated the expression of MMP-7 by accelerating the nuclear translocation of β-catenin, which was opposite to the functions of Fibulin-3. Moreover, Tiam1 interacted directly with Fibulin-3. The Tiam1 induced OSCC epithelial-mesenchymal transition (EMT) via MMP-7 activation, which was dependent of β-catenin direct binding on the promoter region.

Conclusions: Collectively, these results indicated that Tiam1 competed with Fibulin-3 for nuclear β-catenin translocation, which subsequently stimulated MMP-7 expression by TCF-4 domain interaction following EMT initiation in OSCC development. Our systematical work here hypothesized an innovative signaling cassette for OSCC progression, which provided beneficial references for future clinical study.

Introduction

Oral squamous cell carcinoma (named OSCC) represents the most frequent malignancy in oral cavity, which has been considered as an increasing problem for the global public health. OSCC is ranked as the sixth leading malignancy worldwide, which comprises for more than 90% of all oral malignancies and approximately 38% of head and neck tumors[1]. Based on the most recent GLOBOCAN estimate in Europe between 2012 and 2015, the overall incidence and mortality for OSCC kept a dramatic increasing tendency[2]. The OSCC is considered as an aggressive tumor, the clinical managements of which are still relied on the classical histologic parameters such as TNM and tumor grading[3]. The treatments for OSCC are limited at this moment, due to the facts of controversial molecular mechanism behind the disorder. With decades of efforts, the understanding of OSCC has exhibited great improvement. Researcher have reached agreement that the multiple genetic alterations caused by chronic exposure to carcinogens should play critical roles in formation as well as development of OSCC. While, in 2011, it was suggested the top 10 primary key alterations fundamental to OSCC development, which are: sustaining proliferative signaling, evading growth suppressors, avoiding immune destruction, activating invasion and metastasis,

tumor-promoting inflammation, enabling replicative immortality, inducing angiogenesis, genome instability and mutation, resisting cell death, and deregulating energetics[4]. It is worthy noting that the activating invasion and metastasis from lymphomas is considered as a critical procedure, which also explain the truths of high percentage of OSCC patients followed with lymphomas metastasis.

Previously, we performed a differentially expressed genes (DEGs) analysis for OSCC patients followed with lymphomas metastasis (article in parallel preparation). Among these DEGs, Tiam1, also called T-lymphoma invasion and metastasis inducing factor 1, displayed a significant increased expression level in patients with lymphomas metastasis. Moreover, Tiam1 manipulated tumor cell proliferation, migration and invasion. With further investigation, another DEG attracted our attention, which was Fibulin-3 (also called EGF-containing fibulin extracellular matrix protein 1 (EFEMP1)). The Fibulin-3 represents a secreted extracellular matrix glycoprotein, encoded by the EFEMP1 gene which belongs to the eight-membered fibulin protein family[5]. Emerging as a functionally unique member of this family, Fibulin-3 has demonstrated close connections with multiple cancers, which is also suggested as a potential biomarker of malignant mesothelioma[6–8]. Moreover, the several alternations of Fibulin-3 have been implicated in the formation and progression of cancers, in which Fibulin-3 has displayed a significance as a therapeutic target. Our colleagues previously have shown that the Fibulin-3 was functional as a suppressor of lung cancer invasion and metastasis, which regulated matrix metalloproteinases-7 (MMP-7) activity[9]. Moreover, the central target of this signaling cassette was Wnt/β-catenin factor. Meanwhile, the Tiam1 gene is considered as a Wnt signaling associated gene which drives cancerous cell self-renewal and metastasis. In numerous cancer, Tiam1 has been demonstrated as a key antagonist of Wnt signaling through suppressing TAZ and YAP[10].

Based on the previous findings, we speculated that Tiam1 might cooperated with Fibulin-3 to modulate downstream signaling axis (Wnt/β-catenin signaling). The Wnt signaling inversely regulated MMP7 activity in OSCC cells. Overall, the innovative signaling network interacted with tumor cell epithelial–mesenchymal transition, which finally affect OSCC progression. The collaborative *in vivo* and *in vitro* analysis here, provided a beneficial reference for future OSCC study.

Material And Methods

Study subjects and gene expression analysis

The tissue specimens of 20 OSCC patients were prepared previously (article in parallel preparation). Using the traditional WHO classification, the OSCC patients were categorized into TNM I-IV, 5 participants in each group. All procedure in this study was approved by the institutional review committee. There was no significant difference for clinical characteristics (age, gender etc.) between two groups.

The TRIzol reagent was used to extract total RNA from the tissues. At the same time, the high-capacity RNA to cDNA kit (Applied Biosytems) was established to reversely transcribe RNA (1000 ng) to cDNA, and

the qPCR amplification was performed with SYBR Green (Qiagen). The final result value was normalized according to the internal reference gene GAPDH of each parallel sample.

ELISA analysis

The concentration of Tiam1, Fibulin-3 as well as MMP-7 protein was measured using ELISA double antibody sandwich method in peripheral blood of OSCC patients. The specific operation was carried out in strict accordance with the instructions of the kit (Abcam company, US). The experimental results were conducted triplicate.

Cell culture

The human OSCC cell line CAL27 cell line was purchased from ATCC Co. The base medium for this cell line was DMEM＋10% FBS＋1% P/S. The transfection was obtained using Lipofectamine™ 2000 (Invitrogen) following the manufacturer's instructions.

Western blotting analysis

The NPC cells were harvested and washed twice with phosphatebuffered saline (PBS). The protein concentration of cell lysate was determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific, China). 30 mg of proteins were loaded on premade 8–15% SDS polyacrylamide gel for separation and then electrotransferred onto a nitrocellulose membrane. The blots were incubated in PBS buffer with 5% defat milk and 0.02% Teween-20 at room temperature for 1 h before primary antibody incubation overnight at 4C.

The antibodies including β-catenin, β-actin, MMP-7, Fibulin-3, Tiam1, E-cadherin, Snail, Slug, Vimentin were obtained from Santa Cruz Biotechnology Inc., US and applied with 1:1000 dilution. The SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, China) was used for visualization of immunoreactive proteins.

The luciferase reporter assay

The luciferase reporter assay was established as previously described[9]. The wild-type (WT) MMP-7 luciferase reporter was constructed by cloning a 264 bp fragment in the promoter region of MMP-7 into pBV-Luc plasmid and mutant MMP-7 reporter was generated by site-directed mutagenesis using QuickChange XL site-directed mutagenesis kit (Agilent Technologies, SantaClara, CA).

Functional analysis of CAL27 cells

The cell proliferation was examined using CCK-8 kit (Fisher, China). At the same time, the cell apoptosis was measured based on flow cytometry analysis after Annexin V FITC/PI double staining. The colony formation assay, wound healing assay and migration assay were followed the instructions previously reported[11].

Statistics

All the experiments were performed three times independently. The SAS 9.4 software was developed for data analysis. The continuous variables were tested for normal distribution and the student t-test in SAS 9.4 was used to analyze the difference between two groups, with $P < 0.05$ was considered as a significant difference.

Results

The expression of Tiam1, MMP-7 and Fibulin-3 were closely associated with OCSS progression

Our previous work suggested that Tiam1, MMP-7 and Fibulin-3 were all differentially expressed in OSCC patients. By categorizing all OSCC samples into TNM stage I-IV using the traditional WHO classification, it could be shown that the RNA activity of Fibulin-3 declined as the increase of TNM stage (Fig. 1A). Inversely, the RNA expression level of Tiam1 and MMP-7 elevated significantly as the increase of TNM stage.

Furthermore, the concentration of Tiam1, MMP-7 and Fibulin-3 was explored using ELISA assay for peripheral blood of OSCC patients. As shown in Fig. 1B, the concentrations of Tiam1 and MMP-7 protein ascended while the concentration of Fibulin-3 protein decreased as TNM stage went up ($P < 0.05$). These results suggested that Tiam1 and MMP-7 were functional as hazard factors for poor prognosis of OSCC patients (Fig. 1B).

Tiam1 antagonized Fibulin-3 for MMP-7 regulation

As previously demonstrated, transfection of Fibulin-3 in CAL27 cells markedly reduced the expression of MMP-7. Moreover, Tiam1 transfection upregulated the expression level of MMP-7. Treatment of Tiam1 could abolish the inhibition of Fibulin-3 for MMP-7 manipulation (Fig. 2A and 2B).

It has been approved that the MMP-7 was a direct target for Wnt/β-catenin signaling cassette[12]. Here, Fibulin-3 expression could efficiently suppress the level of nuclear β-catenin in CAL27 cells. While, Tiam1 expression could antagonized the functions of Fibulin-3 and recovered the activity of nuclear β-catenin (Fig. 2C). These outcomes suggested that Tiam1 upregulated the expression of MMP-7 by accelerating the nuclear translocation of β-catenin, which was opposite to the functions of Fibulin-3. At the same time, Tiam1 interacted directly with Fibulin-3 as a complex in OSCC cells, supported by CO-IP results (Fig. 2D).

Tiam1 stimulated MMP-7 expression directly via Wnt/β-catenin signaling axis

There are two TCF-4 binding elements (TBEs) in the promoter region of MMP-7, which are bound and activated directly by nuclear β-catenin. The wild-type (WT) MMP-7 luciferase reporter was consisted of the two TBEs. Fibulin-3 transfection highly reduced the luciferase activity of WT reporter, which could be compensated by Tiam1 co-transfection in CAL27 cells (($P < 0.05$, Fig. 3A). While, for the mutant MMP-7

reporter without two TBEs, the luciferase activity of mutant reporter could not abolished by Fibulin-3 transfection. At the same time, the tranfection of Tiam1 could not elevate the luciferase activity of mutant reporter either. Overall, these results indicated that Tiam1 competed with Fibulin-3 for nuclear β-catenin translocation, which subsequently stimulated MMP-7 expression by TCF-4 domain interaction.

Tiam1 Induced Oscc Epithelial-mesenchymal Transition (Emt) By Activating Mmp-7

The EMT process has been shown to be closely associated with numerous types of tumors. In this study, both the epithelial (E-cadherin) and mesenchymal protein markers (Vimentin and Snail) have been investigated using western blotting. Shown as Fig. 4, silencing of Tiam1 in CAL27 cells increased the expression of E-cadherin while suppressed the expression of mesenchymal markers, such as Vimentin as well as Snail. While, Fibulin-3 transfection reversed the effects. More importantly, the effects of Tiam siRNA could be attenuated by co-transfection with Fibulin-3 siRNA. These results suggested that Tiam1 mediated the EMT processes in OSCC cells via sponging with Fibulin-3.

Discussion

The OSCC represents a heterogeneous classification of cancers arising from the mucosal lining of the oral cavity. The major risk factors include excessive alcohol consumption, smoking and betel quid chewing[13]. Even with decades of efforts in the prevention, early screening, diagnosis and treatments of OSCC, the disorder has brought many obstacles to clinical treatment due to the controversial molecular mechanisms[14]. Previously, using High-throughput sequencing on OSCC patients tissues, our work demonstrated that Tiam1 was a primary specific expressed DEG for OSCC. Tiam1 was first discovered by Habets and his colleagues based on a proviral tagging in combination with *in vitro* selection for invasiveness[15]. Cell clones with Tiam1 transfection could successively generate experimental metastases in nude mice, and transfection of truncated Tiam-1 cDNAs into noninvasive cells provided the ability of cells invasive, which supported the invasion functions of the gene. As every coin has two sides, Tiam1 has been shown to function as both an oncogene and a tumor repressor based on the types of tumor. However, researchers have reached a agreement that Tiam1 is closer to a potential oncogene. At this moment, Tiam1 at least was reported to be closely associated with numerous cancers, including esophageal[16], renal carcinoma[17], thyroid[18] and nasopharyngeal[19]. However, based on our search, the connection between Tiam1 and OSCC has not been fully addressed yet.

Previous, our colleagues suggested that inhibition of Fibulin-3 had significant functional effects in non-small-cell lung cancer (NSCLC) patients[9], which suppressed the growth and invasion of NSCLC cells. The Fibulin-3 acts as an inhibitor for MMP-7 expression through aberrant activation of Wnt/β-catenin signaling. Actually, the high levels of nuclear β-catenin as well as MMP-7 promote the activity of TCF-4 and cyclin D1 to enhance lung cancer cell proliferation[20, 21]. Examined the differentially expressed gene

profile for OSCC, we also found the Fibulin-3 and MMP-7 as two DEGs. Moreover, further investigations could confirmed the inverse correlation between Tiam1 and Fibulin-3 in the OSCC progression (Fig. 1). With in-depth exploration, our data suggested that Tiam1 and Fibulin-3 competed with each other, collectively they manipulated β-catenin nuclear translocation and sequentially maintained the expression level of MMP-7 in the OSCC cells. This initiated a potential underling molecular mechanism for OSCC development and formation as well as allowed us to hypothesize a signaling model (Fig. 5). Based on this model, without stimulation, the Fibulin-3 forms a complex with Tiam1 and inhibits the function of Tiam1 (shown as a in the model). On the other hand, the destruction of the complex allows the Tiam1 to promote β-catenin nuclear translocation which inversely binds to the promoter region of MMP-7 for activation. The alleviated MMP-7 could elevated the level of EMT in OSCC (shown as b in the model).

Previously, Ding etc. examined the expression level of Tiam1 in PDAC pancreatic cancer[11]. In the work, they approved that Tiam1 was upregulated significantly in pancreatic cancer tissues compared with that in non-cancerous tissues. At the same time, increased level of Tiam1 was positively correlated with lymph node metastasis and pathological status in pancreatic cancer patients. Similar to the findings here, they also claimed that Tiam1 overexpression promoted EMT by decreasing the expression of the mesenchymal markers Vimentin and Snail and by elevating the expression of the epithelial marker E-cadherin (Fig. 4).

EMT plays a critical role in several tumor progression and there are multiple steps involved in the process, which results in a phenomenon as epithelial cells to behave more like mesenchymal cells. During this transition, cells could undergo a switch from a polarized epithelial phenotype to a highly mobile mesenchymal phenotype, with losing of epithelial markers such as E-cadherin, claudin, and zona occludens 1 (ZO-1) and gaining of expression of mesenchymal markers such as vimentin and N-cadherin[22]. Almost every tumor type is unexceptional correlated with EMT[23, 24]. Meanwhile, MMP-7, as a key cellular structural protein, is involved in several aspects of this complicated mechanism. For instance, using MMP-7 knocking-out mice, it could be demonstrated that MMP-7 stimulated prostate carcinogenesis through induction of EMT via regulating of IL-17[25].

Collectively, in this integrate work, we systematically elaborated a potential signaling model for OSCC development and progression. Our results supported Tiam1 as an oncogene for OSCC, antagonizing with Fibulin-3 for β-catenin nuclear translocation. Finally, the innovative signaling cassette manipulated OSCC EMT transition via targeting MMP-7. All the work here provided new insights for developing biological or pharmacological agents to target OSCC.

Declarations

Conflict of interest statement

The authors declare no competing interests.

Funding Sources

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Figures

Figure 1.

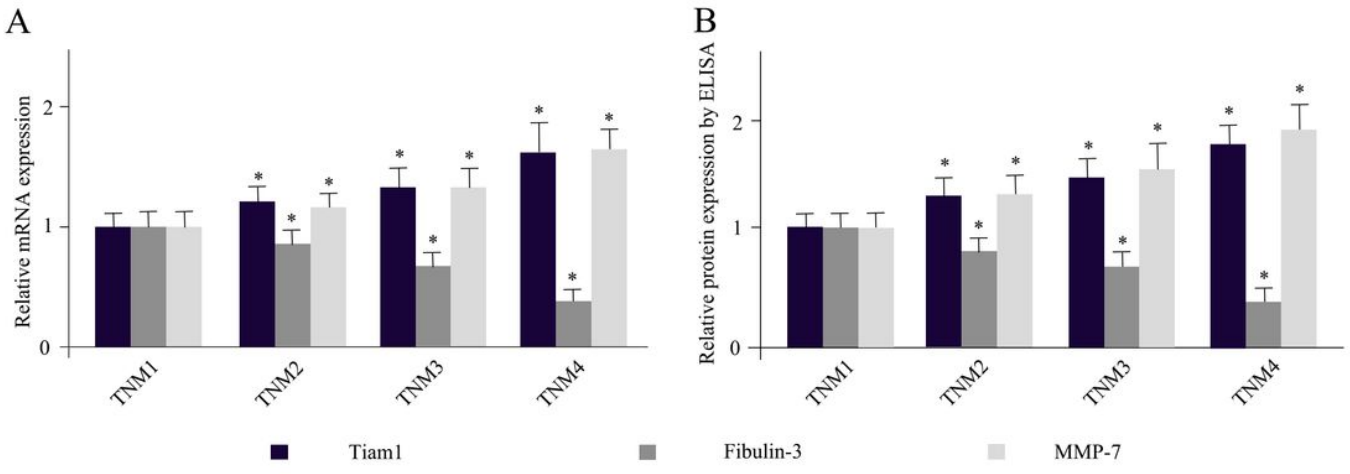

Figure 1

The expression of Tiam1, MMP-7 and Fibulin-3 were closely associated with OCSS progression. (A) The RT-PCR analysis of Tiam1, MMP-7 and Fibulin-3 between different TNM stages for OSCC patient and normalized to that of OSCC patients at TNM1 stage. * indicated $P<0.05$ compared with corresponding OSCC patients of TNM1 stage. (B) The ELISA measurement of Tiam1, MMP-7 and Fibulin-3 protein from peripheral blood of OSCC patients (TNM1-4 respectively). * indicated $P<0.05$ compared with corresponding OSCC patients of TNM1 stage.

Figure 2.

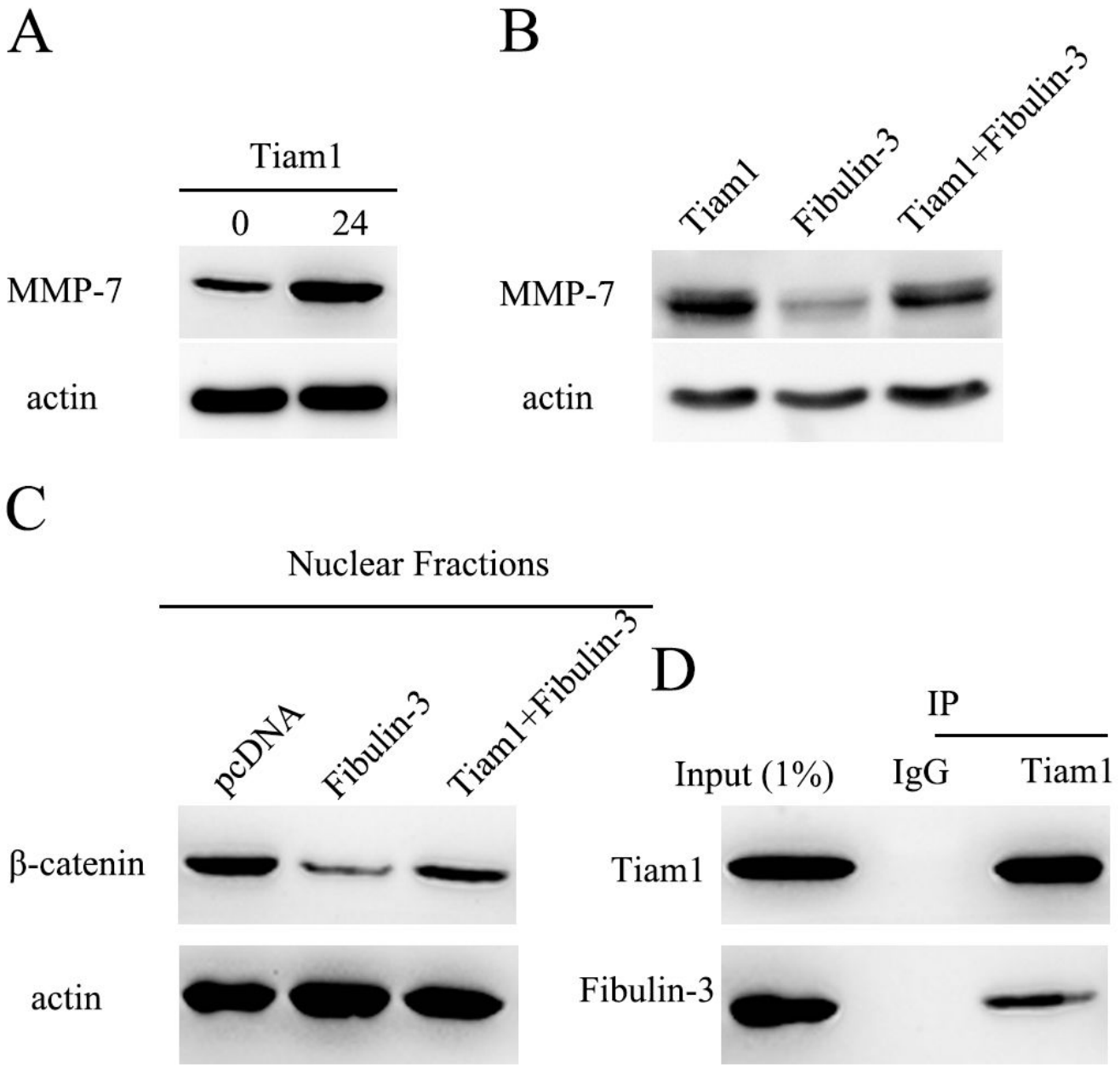

Figure 2

Tiam1 antagonized Fibulin-3 for MMP-7 regulation. (A) Western blot analysis of MMP-7 expression in CAL27 cells at 24h after transfection with Tiam1 or the control empty vector. (B) Western blot assessment of MMP-7 expression in CAL27 cells at 24h after transfection with Fibulin-3 or Tiam1 individually or two factors together. (C) Western blot examination of β-catenin in nuclear fractions

isolated from CAL27 cells at 24h after transfection with the control empty vector, Fibulin-3 or Fibulin-3 plus Tiam1. (D) The CO-IP for the interaction between Tiam1 and Fibulin-3.

Figure 3.

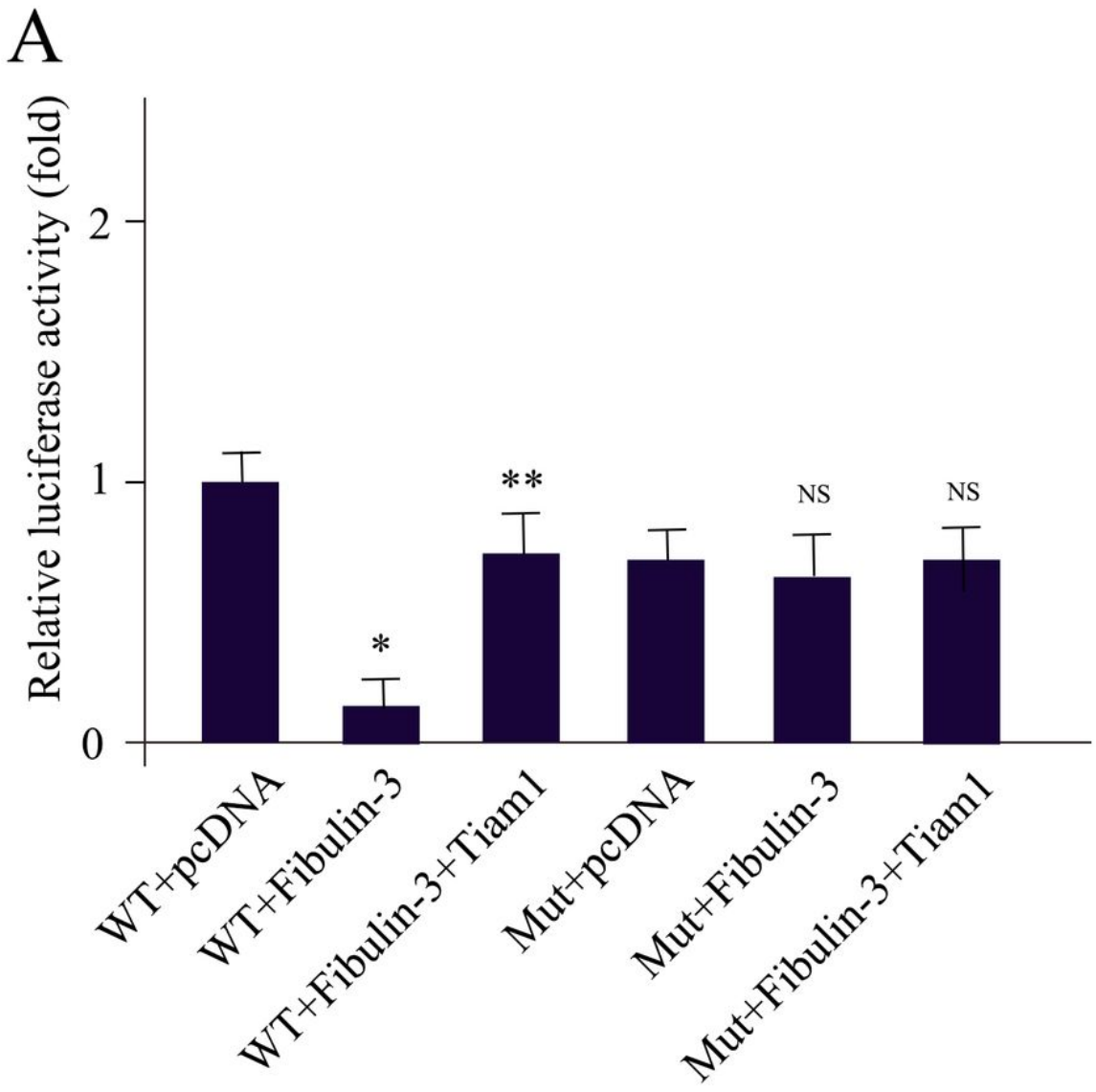

Figure 3

Tiam1 stimulated MMP-7 expression directly via Wnt/β-catenin signaling axis. The CAL27 cells were transfected with Fibulin-3 or Tiam1 individually or two factors together along with WT or mutant MMP-7

reporter construct. Luciferase activities were measured 24h after transfection and normalized to that of empty reporter pcDNA. * indicated $P<0.05$ compared with pcDNA empty vector reported transfection; ** indicated $P<0.05$ compared with Fibulin-3 transfected along with WT MMP-7 reporter; NS mean non significant.

Figure 4.

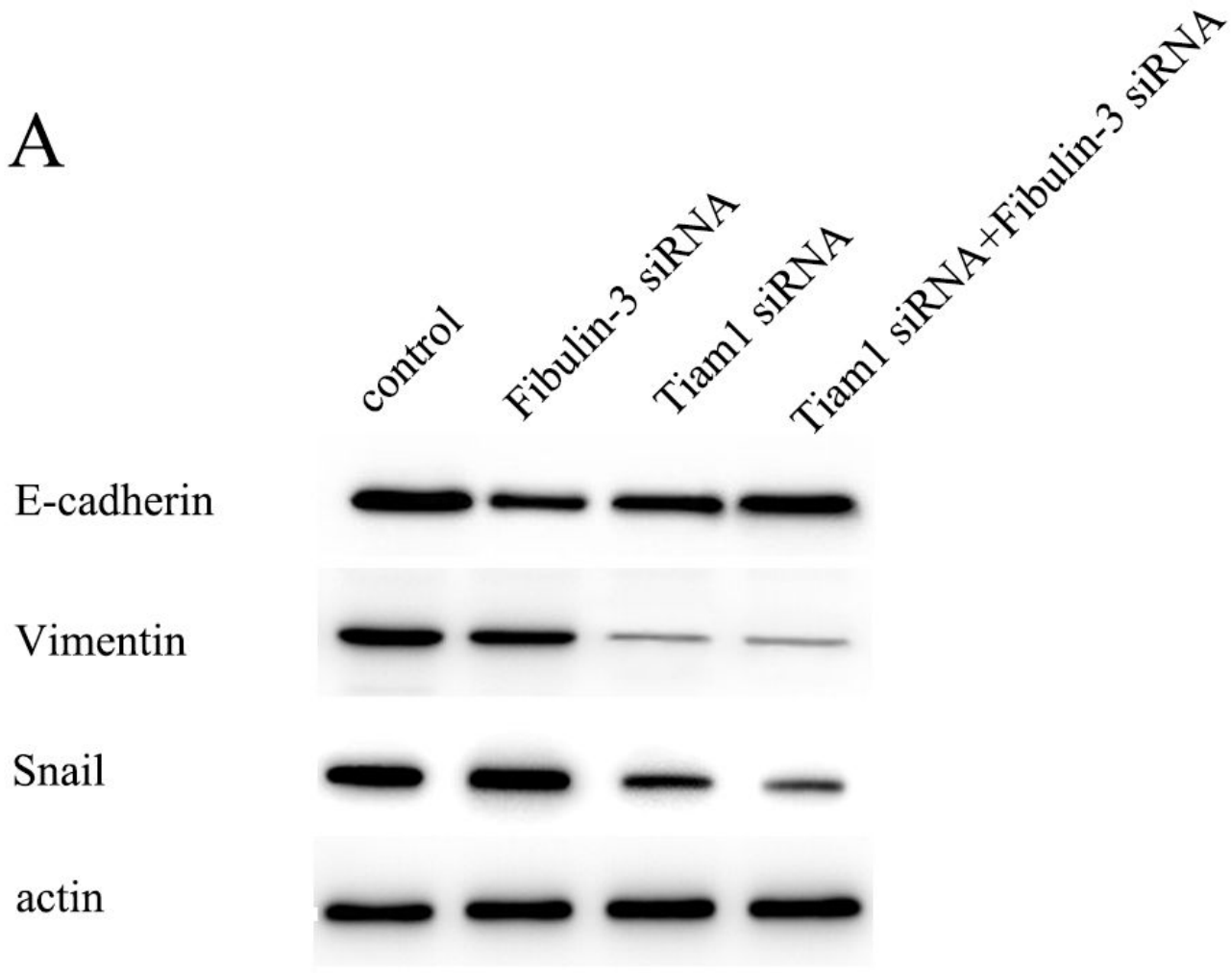

Figure 4

Tiam1 induced OSCC epithelial-mesenchymal transition (EMT) by activating MMP-7. Western blot analysis of E-cadherin, Vimentin and Snail for CAL27 cells transfected with Tiam1 siRNA, Fibulin-3 siRNA as well as co-transfection with Tiam1 and Fibulin-3 siRNA respectively.

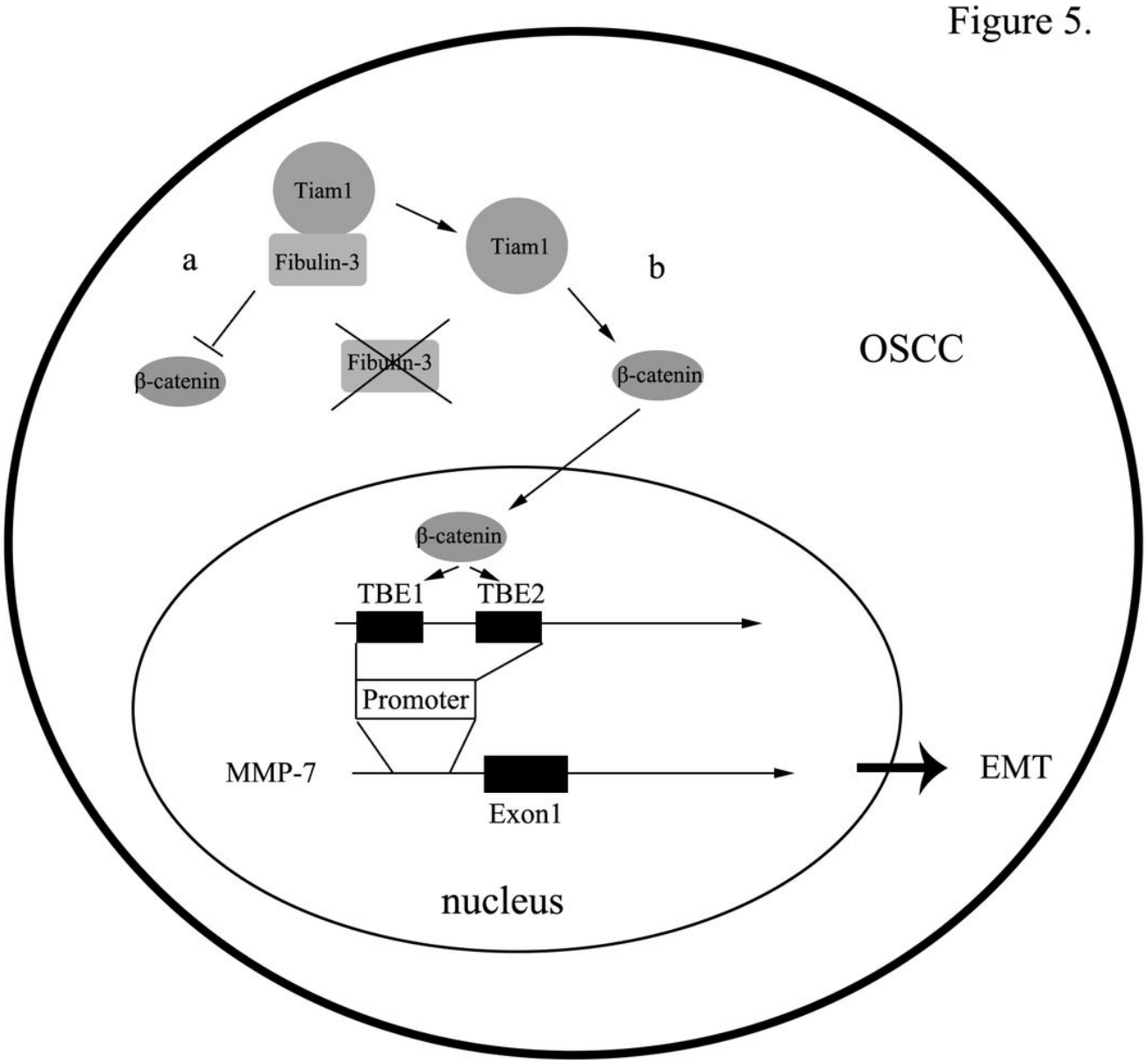

Figure 5

The scheme diaphragm of the hypothesized signaling model.