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Master Thesis

探討微小核醣核酸 17-92 群簇對人類口腔鱗狀上皮細胞癌移行能力之影響

The Effect of miR-17-92 cluster on Tumor Migration in Human Oral Squamous Cell Carcinoma

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中文摘要

口腔癌之普及率在全球常見癌症中排名第八位，且經行政院衛生署公布之統計資料，民國九十八年與九十九年的十大癌症死因中，口腔癌是唯一排名上升的惡性腫瘤（第六升至第五），足以顯現口腔癌在台灣的盛行率與研究潛力。約有九成的癌症病患最後死於癌症轉移的發生，為了提升癌症病患的存活機率，關於癌症轉移的研究已經成為刻不容緩的議題。本實驗室為了釐清口腔癌發生轉移的相關分子機制，利用了 miRNA 的微陣列對 TW2.6 與 TW2.6 MS-10 進行分析，發現一個 miRNA 群簇－miR-17-92 cluster，在移行能力較高的 TW2.6 MS-10 細胞株中有下降的現象。於是我們進一步分析常見口腔癌細胞株的 miR-17-92 cluster 表現量，發現其表現量與各細胞間的移行能力呈現負相關。為了確認 miR-17-92 cluster 是否能影響細胞移行能力，我們將 miR-17-92 cluster 在 TW2.6 MS-10 與 SAS 中過量表現，發現其確實能降低此兩細胞株的移行能力。而為了瞭在此 cluster 中，何者扮演主要調控者的角色，我們又分別在 TW2.6 MS-10 與 SAS 中分別過量表現 miR-17、miR-19b、miR-20a 與 miR-92a，結果發現只有 miR-17 與 miR-20a 對兩株癌細胞具有明顯的移行抑制能力。而從病人的數據也顯示，在高期數與發生淋巴轉移的病人，miR-17 與 miR-20a 有比較低的表現量。從臨床檢體與細胞實驗中顯示，在口腔癌中 miR-17 與 miR-20a 似乎扮演著重要角色。為了釐清 miR-17 與 miR-20a 抑制細胞移行能力的分子機制，我們先以生物資訊軟體 TargetScan 與 Microcosm 進行下游標的的預測，並且利用 Ingenuity Pathway

Analysis (IPA) 對預測標的進行功能性的分析，篩選出同時是 miR-17 與 miR-20a 的下游標的，且對細胞移行能力有影響的目標基因－ITGβ8。經由 RT-PCR 發現，在 TW2.6 MS-10 細胞中 ITGβ8 比 TW2.6 有較高的表現量，但由暫時轉染各個 miRNA 的數據顯示，ITGβ8 才是 miR-17 與 miR-20a 可能的下游基因。而為了確定 ITGβ8 在口腔癌中的功能，我們利用 shRNA 的方式抑制 TW2.6 MS-10 細胞中 ITGβ8 的表現，發現細胞移行能力隨著 ITGβ8 表現量下降而降低。接下來我們構築了野生型與突變型的 ITGβ8 3'UTR 來證明 ITGβ8 真的為 miR-17 與 miR-20a 的直接下游。實驗結果顯示，ITGβ8 的確是 miR-17 與 miR-20a 的下游調控基因。綜合以上實驗結果顯示，miR-17-92 cluster 確實有抑制口腔癌移行能力的功能，而其中又以 miR-17 與 miR-20a 為主要調控者，乃藉由種源序列與 ITGβ8 的 3'UTR 結合，使 ITGβ8 的 mRNA 進行降解而達到抑制細胞移行能力的效果。

CONTENTS

Introduction

Oral squamous cell carcinoma (OSCC) has become the 10 most frequent cancers worldwide, according to the statistical data, up to 500000 new patients being diagnosed each year[1.] Smoking , alcohol abuse[2] and betel quid chewing are major risk factors in this malignancy disease, which primarily affects the oropharynx, oral cavity, hypopharynx, and larynx[3]. Epidemiologic studies have shown a wide variation of incidence between worldwide areas of OSCC, which been reported to be increasing in the betel quid chewing area such as South-east Asia and Taiwan in recent years[4]. OSCC has become the sixth leading cause of death from cancer in Taiwan, accompanying with the increased incidence rate in the past decade[1]. Despite the improved treatment for OSCC patients, the overall 5-year survival rate of OSCC patients is still one of the lowest among common malignant neoplasms and has not significantly improved during the last two decades[5]. Survival rates for OSCC are significantly influenced by tumor stage, including tumor size, lymph node involvement and distant metastasis[6]. Although tumor size could be minimized by surgical treatment, the recurrence of OSCC at primary site or regional recurrence at peripheral lymph node always happened due to the migration and invasion ability of the invisible OSCC cells[7]. Hence metastasis has become the major reason for poor prognosis of OSCC. However, the molecular mechanisms about the migration and

metastasis ability of OSCC are poorly understood, so it is urgent to identify a possible mechanism involved in the process of invasion and metastasis.

MicroRNAs (miRNAs) are small non-coding, single-strand regulatory RNAs ranging from 17 to 25 nucleotides that influence gene expression at the post-transcriptional level by targeting to the 3' untranslated region (3'UTR) of downstream mRNAs with its seed sequence, leading to translational repression or degradation[8,9]. Up to the present, over 1000 different human miRNAs have been identified and uploaded to the miRBase database[10]. Bioinformatics analysis reveals that miRNAs can target at least 30 % of all human genes which play crucial roles in regulating fundamental cellular biological processes such as cell cycle, differentiation and apoptosis[11]. Therefore, deregulations of miRNAs may disrupt the balance of gene regulating networks that determine the cells' fate which could lead to cancer progression. Under transcriptional control by oncogenes, tumor suppressor genes, epigenetic mechanisms and genomic abnormalities, deregulating of miRNAs seems to be complex mechanisms during cancer development[9]. Abnormal expression levels of miRNAs in tumors have important pathogenetic consequences: miRNAs that are overexpressed in tumours contribute to oncogenesis (oncomiRs) by downregulating tumour suppressors, whereas miRNAs lost by tumors generally participate in oncogene overexpression are viewed as tumor suppressor miRNAs[12,13]. Several studies now have reported that

miRNAs affect the expression of genes and pathways involved in cancer pathogenesis from initiation to metastasis disease[14,15] including OSCC[16,17]. Since metastasis is crucial for prognosis of OSCC patients, it is needed to figure out the role of miRNAs which participate in OSCC metastasis.

In human and other vertebrate, some miRNAs are usually transcribed together as polycistronic primary transcripts which are processed into multiple individual mature miRNAs[9]. One of the polycistronic miRNA clusters is miR-17-92 that comprises six mature miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92-1) which are regularly organized within an 800 base-pair intron region of *C13orf25* on human chromosome 13[18,19].miR-17-92 cluster are highly conserved between different species, but the exonic sequences of *C13orf25* are not[20,] suggesting that the importantly biological role of this transcript is produce these miRNAs[21]. The role of miR-17-92 cluster played in cancer pathogenesis was first described by Ota et al. in 2004[18].They described an amplification of human genomic locus-13q31.3 which encoding these miRNAs in B cell lymphoma. The gene *C13orf25* was located within this interval and the expression level of *C13orf25* correlated with the amplification status of 13q31.3.According to these findings, miR-17-92 cluster was first defined as an oncomiR. Through expression profiling studies, overexpression of these miRNAs was observed not only in hematopoietic malignancies, but also an universal phenomenon in solid tumors

such as those derived from breast, colon, lung, pancreas, prostate, and stomach[22]. Although most data support a major role of miR-17-92 cluster in tumor progression, there are some proofs suggesting that in some situations, loss-of-function of these miRNAs might be advantageous for cancer cells. In certain caner types such as ovarian cancers, breast cancers and melanomas, loss-of-heterozygosity at the 13q31.3 locus has been reported [23].Since miR-17-92 cluster located on 13q31.3, loss-of-heterozygosity at this locus lead to down-regulation of miR-17-92 cluster. According to our miRNA microarray data between TW2.6 and TW 2.6 MS-10, a more aggressive cell line selected from TW2.6 parental cell, we observed that miR-17-92 cluster was down-regulated in TW2.6 MS-10.So we hypothesized that miR-17-92 cluster as a tumor suppressor miRNAs may involve in OSCC tumor progression and metastasis. Therefore, we sought to identify the functional roles of miR-17-92 cluster and the downstream target genes involved in migration abilities in OSCC.

Materials & Methods

Cell lines, reagent, and culture CA9-22, CAL-27, HSC-3, SAS were cultured in DMEM medium .TW2.6 and TW2.6 MS-10 were cultured in F12/DMEM medium. Both DMEM and F12/DMEM medium were supplemented with 10 % fetal bovine serum (FBS), 1 % antibiotics, and 2 mM L-glutamine (Biological Industries Ltd., Israel) at 37 ℃ in a humidified atmosphere of 5 % CO2 and 95 % air. OSCC cell line TW2.6 MS-10 was a more aggressive cell line selected by transwell from low-migration ability oral cancer cell line: TW2.6. 0.05 % trypsin/EDTA (Biological Industries) was used to detach adherent cells from culture dishes for routine culture.

Taqman-based qRT-PCR assays of miRNA expression A TaqMan miRNA assay was used to determine the mature miR-NA expression level. Total RNA was extracted with the TRIzol re-agent (Invitrogen, Carlsbad, CA) then reverse-transcr ibed into complemen tary DNA using a TaqMan MicroRNA Reverse Tran-scription Kit (Applied Biosystems, Foster City, CA). Clear and sharp 28S and 18S rRNA bands were detected in RNA extracts by agarose gels. PCR reactions were first incubate d at 16℃ for 30 min and then at 42℃ for 30 min followed by inactivation at 85℃ for 5 min. Reactions were then incubated in a 96-well plate at 50 ℃ for 2 min and 95℃ for 10 min, followed by 40 cycles of 95℃ for 15 s and 60℃ for 1 min using the ABI Prism 7500 Sequence Detec-tion System (Applied Biosystems, Foster City, CA). The

relative expression of each miRNA was normalized against RNU-6B. The relative expression was calculated using the compara tive threshold (CT) method. In clinical sample analysis, a calibrator sample (case 46th) was used to ensure all reactions occurred in the similar man-ner. The sample of 46th was an OSCC patient with stage II and ex-pressed moderate amount of miR17-92 cluster. **All the experiments performed were carried out at least in triplicate .**

Reverse transcription–polymerase chain reaction. Reverse transcription of total RNA isolated from cells was quantified using T3000 thermocycler .5 μg of total RNA mixed with 5X reverse transcriptase buffer, 0.1 M dithiothreitol, all four deoxynucleoside 5´-triphosphates (dNTPs; each at 6.25 m*M*), 1 μg of (dT)12–18 primer,and 50 U of MultiScribeTM reverse transcriptase (Invitrogen Corporation, Calsbad,CA) in a final reaction volume of 25 μL. The reaction mixture was incubated at 65 ℃ for 5 min and followed by heating at 42 ℃ for 1 hour. Polymerase chain reaction (PCR) was performed in a final reaction volume of 20 μl containing 1 μl of the cDNA product with 10X reaction buffer containing 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, all four dNTPs (each at 167 μM), 2.5 U of Taq DNA polymerase, and 0.1 μ*M* each primer.PCR programs were set in Biometra Thermoblock as denaturing for 3 seconds at 95 ℃, annealing for 30 seconds at a specific annealing temperature, and elongating for 30 seconds at 72 ℃ for a total of

specific cycles, followed by final extension at 72 ℃ for 5 minutes. Electrophoresis was performed in a 2 % agarose gel with Equal volumes of each PCR sample, then stained with ethidium romide (EtBr) and photographed under UV exposure. **All the experiments performed were carried out at least in triplicate .**

Plasmids and transient transfection. Lipofectamine 2000 reagent (Invitrogen Corporation,Calsbad, CA) was used for plasmid transfection with Opti-MEM medium (Invitrogen Corporation, Calsbad, CA) for 4 - 6hours and then replaced the hole reagent with fresh complete medium. Transfected cells were harvested after 24 hours for wound healing migration assay and for RNA isolation after 48 hours.

Wound healing migration assay. Wound-produced culture insert (400 μl ± 50 μl; ibidi Gmbh, Germany) loaded with 75-80 uL of 5 x 10^5 cells per 1 mL (approximately 40000 cells per insert) and incubated overnight for attachment. Culture inserts were removed next day and cells were washed twice with PBS and supplied with serum free medium. Cell migration ability was measured by photographing the wounded area and quantified by ImageJ. **All the experiments performed were carried out at least in triplicate.**

Luciferase reporter assay. The 30UTR of human ITG b8 was PCR-amplified and cloned into a pMIR-Rep ort vector. These constructs (1 lg) were individually co-transfected with 1 lg of control plasmid or plasmids expressing the miR-17-92

cluster or a single miRNA expression vector and the TK plasmid (0.2 lg) into SAS and TW2.6 MS-10 cells. Luciferase activity was measured 48 h after transfection using the Dual-luci f-erase reporter assay system (Promega, Madison, WI). **All the exper-iments performed were carried out at least in six times.**

Statistical analyses. Data are presented as the mean ± standard error of the mean (SEM). Statistical analyses were performed with unpaired Stu-dent's two-taile d t-test. A p value of <0.05 was considered signifi-cant. The background data of the patients with OSCC were compare d using the Mann–Whitney test for scale variables (ex-pressed as the mean ± standard deviation (SD)). Survival data were analyzed using the Kaplan–Meier method. Kaplan–Meier curves were compared using the log-rank test.

Result

miR-17-92 cluster expression negatively correlated with migration ability in oral squamous cell carcinoma

To study the possible role of miR-17-92 of migration regulation in oral squamous cell carcinoma, we established a more aggressive cell line- TW2.6 MS-10 by transwell selection model from low-migration cells, TW2.6, and further confirmed the migration ability in TW2.6 MS-10 cells (Fig. sup). To understand the effect of microRNAs (miRNAs) profile in OSCC metastasis, the miRNA microarray analysis between TW2.6 and TW2.6 MS-10 was performed. According to the array data (Fig. 1A), a miRNA cluster miR-17-92, including miR-17, miR19b, miR-20a, miR-92a, was significantly down-regulated in TW2.6 MS-10 compared to the parental cells. In order to confirm the array data, we performed the Q-PCR analysis. Sportingly, the results showed that approximately 2.5 fold of these four MicroRNAs were down-regulated in TW2.6 MS-10 compared to TW2.6 (Fig. 1B). To further confirm the relationship between miR-17-92 cluster and OSCC migration ability, we analyzed miR-17-92 expression level in five different wild-type OSCC cell lines, including CAL-27, TW2.6, SAS, Ca922 and HSC-3. The data demonstrated that miR-17-92 cluster expression level was inversely associated with the migration phenotype in OSCC lines (Fig. 1C). Thus, we hypothesized that miR-17-92 cluster may play a

critical role in oral cancer metastatic progression.

miR-17-92 cluster down-regulated migration ability in OSCC cell lines

Since expression levels of miR-17-92 cluster were down-regulated in TW2.6 MS-10, we restored the expression level of this cluster by dosage transfection of miR-17-92 over-expression plasmid. Migration ability of TW2.6 MS-10 cells was down-regulated in a dose–and-time dependent manners (Fig. 2A).To further check the effect of miR-17-92 cluster in cell migration, we selected another OSCC cell line SAS to confirm the former result. In figure 2B, we showed that dosage expressions of miR-17-92 cluster in SAS also down-regulate its migration ability at 6 hr and 12 hr (Fig. 2B). After transient transfection, miR-17-92 cluster expression level of TW2.6 MS-10 and SAS increased 2-3.5 folds and 3-6 folds separately compared to scrambled control. According to those data, we suggested that miR-17-92 cluster could inhibit OSCC migration ability.

Migration ability was predominantly suppressed by miR-17 and miR-20a

Since a single miRNA could have a lot of downstream targets, it is complex to figure out the whole mechanism under the regulation of miR-17-92 cluster. Moreover, in order to reveal which miRNA(s) in this cluster was (were) the dominant regulator(s) in OSCC migration instruction, it is necessary to simplify the question to a single miRNA scale. Thus we cloned the four miRNAs: miR-17, miR-19b, miR-20a, and

miR-92a separately. Through the transient expression of the four different miRNAs in TW2.6 MS-10 cells, we found that miR-17 and miR-20a played a key role in migration regulation (Fig. 3A).Only the cells transfected with miR-17 or miR-20a had observed significant change in migration ability but no alterations between control vector, miR-19b and miR-92a. Migration ability of TW2.6 MS-10 decreased about 0.5-0.6 fold at **6 hr** and about 0.7 fold at **12 hr**. For the sake of proving precisely whether miR-17 and miR-20a are the functional miRNAs involving in migration regulation, we used SAS cell line to do the foregoing experiments. The same results were found in SAS that miR-17 and miR-20a predominantly participated in migration regulation (Fig. 3B). Migration ability of SAS was down-regulated approximately 0.5 fold at 6 hr and about 0.6-0.7 fold at 12 hr. Those data highlighted miR-17 and miR-20a as main miRNAs of miR-17-92 cluster to regulate migration ability in OSCC.

miR-17 and miR-20a expression level negatively correlated with TNM stage in oral cancer patients

Through the fore-mentioned experiments, we had demonstrated that miR-17 and miR-20a of miR-17-92 cluster were important in regulating migration ability in OSCC *in vitro*. In order to correlate this phenomenon to clinical signatures, Q-PCR was performed to determine the expression level of miR-17 and miR-20a in patient

samples. To analyze the correlation between the expression level of the two miRNAs and the progression status of OSCC patients, patient's data were grouped by TNM stage. According to the organized data, we found a negative correlation between mir-20a expression level and OSCC patient's progression stage (Fig. 4A). Furthermore, lymph node metastasis of OSCC patients also reversely correlated with miR-20a expression level (Fig. 4A). Both two correlations were statistically significant ($P < 0.05$). RT-PCR analysis of miR-17 in OSCC patient tumor samples also demonstrated that low miR-17 expression was significantly associated with progression stage and lymph node metastasis (Fig. 4B).These data indicated that miR-17 and miR-20a were clinically important in OSCC patients. We also found that patients with highly expressed miR-20a had better survival probability than those low-miR-20a expressed patients. (Fig. 4C)

miR-17 and miR-20a regulated OSCC migration ability through inhibited their downstream target ITGβ8

To identify the mechanism of miR-17/20a-involved OSCC cancer progression, we searched for possible downstream genes using bioinformative screening analysis of miRNA target databank: TargetScan, and Microcosm, which compute optimal sequence complementarily between a set of mature miRNAs and a given mRNA using a weighted dynamic programming algorithm. From these two databanks overlapping

between the predicted targets of miR-17 and miR-20a, many putative downstream target genes were identified. To narrow down the suspicious targets, Ingenuity pathway analysis (IPA) program was performed. Through functional analysis by IPA, we focused on those genes involving in migration and cell mobility regulation.ITGβ8 and LAMA3 were ranked as the most probable targets of miR-17 and miR-20a. We then proceeded to determine their mRNA levels in previously established TW2.6 MS-10 and its parental cells. Fig. 5A showed that ITGβ8 and LAMA3 were both with a corresponding change to endogenous miR-17 and miR-20a expression level (Fig. 1B) in OSCC cells. To further verify the direct effect of miR-17 and miR-20a on ITGβ8 and LAMA3 regulation, we transiently transfected miR-17, miR-19b,miR-20a and miR-92a expression plasmid separately into TW2.6 MS-10 cells. The results showed that ITGβ8 expression were markedly down-regulated by miR-17 and miR-20a , while LAMA3 had no significant change (Fig. 5B).To evaluate the migration ability and the mechanistic link between miR-17, miR-20a and ITGβ8, we knock-downed ITGβ8 expression by transfected short-hairpin RNA (shITGβ8) in TW2.6 MS-10 cells. Fig. 5C demonstrated that loss of ITGβ8 could down-regulate the migration phenotype in TW2.6 MS-10 cells. Although miR-17-92 cluster had no effect on regulating LAMA3 expression level, there was still a concern that the highly expressed LAMA3 in TW2.6 MS-10(Fig. 5A) could influence its own migration

ability. For further elucidated this question, we knock-downed *LAMA3* expression by transfected short-hairpin RNA (shLAMA3) in TW2.6 MS-10 cells. Results showed that knock-downed the expression level of LAMA3 had no effect on migration ability in TW2.6 MS-10 cells (Fig. 5D). Collectively, these data supported that ITGβ8 was a crucial target down-regulated by miR-17 and miR-20a and hence suppressed migration activities in OSCC cell lines.

ITGβ8 was a direct downstream target of miR-17 and miR-20a

To further elucidated that ITGβ8 was a direct downstream target of miR-17 and miR-20a, we constructed a wild type ITGβ8 3'UTR and a mutated ITGβ8 3'UTR to pMIR-reporter plasmid (Fig. 6A). Wild type ITGβ8 reporter plasmid was co-transfected with EmGFP, miR-17, miR-19b, miR-20a, miR-92a and miR-17-92 cluster into TW2.6 MS-10 separately and TK plasmid was used as internal control. The luciferase activity was down-regulated approximately 25 % in the group transfected with miR-17,miR-20a,and miR-17-92 cluster in TW2.6 MS-10 cells (Fig. 6B).For advanced confirmed that it was a common regulation mechanism in OSCC, we used another OSCC cell line SAS to perform the same experiments. A much more significant down-regulation of luciferase activity was found in SAS cells, approximately 50 % decreased compared to control vector (Fig. 6C). We found no obvious alteration of luciferase activity between each group when the mutated ITGβ8

3’UTR was used. (Fig. 6A, Fig. 6B). Here we didn’t find synergistic regulation of miR-17 and miR-20a in both SAS and TW2.6 MS-10 cells. Since the direct regulation of ITGβ8 by miR-17 and miR-20a had been proved, we further evaluated the correlation between miR-20a and ITGβ8 expression level within OSCC patients. A negative correlation between miR-20a and ITGβ8 was found (Fig 6 D), but there was no significant negative correlation between miR-17 and ITGβ8 (Data not shown). According to the result, we proved that miR-17 and miR-20a, most probably the miR-20a, could down-regulate ITGβ8 mRNA expression level through direct target its 3’UTR, leading to mRNA degradation and resulted in inhibiting cell migration ability in OSCC.

Discussion

The double-sided sword effect of miR-17-92 cluster has been reported widely in different kind of cancers: Expression profiling studies have indicated that widespread over-expression of these miRNAs in diverse tumor subtypes including both hematopoietic malignancies and solid tumors such as those derived from breast, colon, lung, pancreas, prostate, and stomach[22,24] ; in contrast, some evidence suggesting that down-reglation of these miRNAs might be advantageous for cancer cells in certain settings. Loss-of-heterozygosity at the 13q31.3 locus has been observed in multiple tumor types, leading to down-regulation of the whole cluster. Through a series of genome-wide analysis about copy number alterations in different cancers, the data revealed that the miR-17-92 cluster was deleted in 16.5 % of ovarian cancers, 21.9 % of breast cancers, and 20 % of melanomas[22]. miR-17-92 cluster was first reported as tumor suppressor miRNA in breast cancer through inhibits cellular invasion and tumor metastasis[25], especially the miR-17 and miR-20a. Although the role of miRNAs participated in OSCC progression has been reported[16,17], however, the role of miR-17-92 cluster in OSCC has been addressed limitedly. Here we demonstrated that in human OSCC, miR-17-92 cluster attenuates cellular migration ability. Down-regulation of ITGβ8 expression is a key mechanism by which miR-17-92 cluster inhibits migration competence. This is the first report to disclose the

migrational inhibitory effect by miR-17-92 cluster in OSCC.

miRNAs are small regulatory RNAs that act by blocking the translation and increasing the degradation of target transcripts. miRNAs play a critical role in many biological processes including development and differentiation and many studies have shown that major changes in miRNA levels occur in different type of cancers. They regulate diverse biological processes, and bioinformatic data indicates that each miRNA can control hundreds of gene targets, underscoring the potential influence of miRNAs on almost every genetic pathway. Recent evidence has shown that mutation and miss-regulation of miRNAs correlate with various human cancers and indicates that miRNAs can function as tumour suppressor miRNAs or oncomirs. In our study, we also demonstrated the "tumor suppressor miRNA" activity of miR-17-92 cluster in oral cancer. This result was supported by Richard G. Pestell's group that they reported miR-17 and miR-20a could inhibit cellular invasion and tumor metastasis in breast cancer[25]. We showed that miR-17-92 cluster significantly decreased migration abilities of oral cancer cells. miR-17, miR-19b, miR-20a and miR-92a were separately cloned to further validate the effect on migration regulation. Here we illustrated that miR-17 and miR-20a, the miRNAs with the same seed sequence, dominantly participated in migration regulation of OSCC.

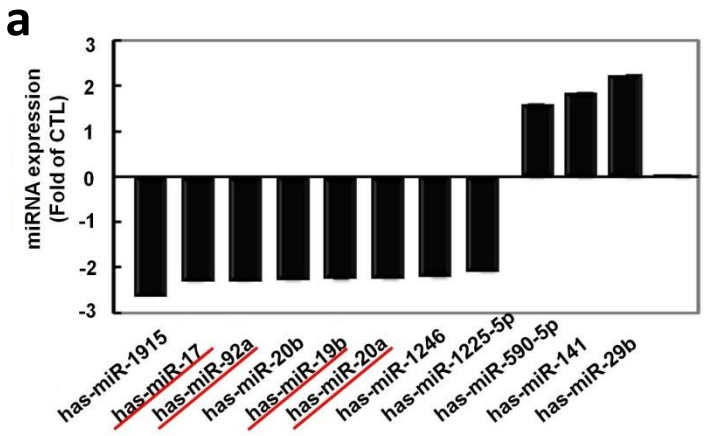
During the mining process for candidate target genes of miR-17 and miR-20a, we

selected the most probable two targets (ITGβ8 and LAMA3) through bioinformatic analysis. However, the validation analysis showed that ITGβ8 was the most probable target of miR-17 and miR-20a. Through 3'-UTR reporter assay, we further demonstrated that ITGβ8 was the direct target of miR-17 and miR-20a.We also found a non-synergistic effect of miR-17 and miR-20a in the regulation of ITGβ8, probably because the two miRNAs recognized the same sequence on ITGβ8's 3'UTR. Integrins are a large family of cell surface molecules mediating diverse biologic roles such as angiogenesis, vasculogenesis, lymphoid trafficking, immune cell function, and cancer cell growth and metastasis[26,27]. Integrins consist of a single α and a single β subunit, forming 24 known heterodimers[28]. The αv subunit-containing subfamily consists of five members:αvβ1, αvβ3, αvβ5, αvβ6, and αvβ8.The integrin β8 subunit is expressed in various epithelial, mesenchymal , and select immune cell types. In the field of cancer researches, ITGβ8 has been found to be increased in BRCA-1 positive breast cancers and has been identified as part of a six-gene expression signature predicting lung metastasis from breast cancer, revealing its oncogenic activity[29]. However, the role of ITGβ8 in OSCC migration has never been addressed. We here viewed ITGβ8 as not only an oncogene to up-regulate migration ability but also the downstream target of miR-17 and miR-20a in OSCC. Together with our results, we suggested that miR-17-92 cluster, especially miR-17 and miR-20a, carried out their

“tumor suppressor miRNA” function through targeting various oncogenic genes such as ITGβ8.

In summary (Fig.7A), our study proposes a model that miR-17-92 cluster attenuates OSCC migration ability through inhibited its downstream target ITGβ8, and we also highlight the critical role of miR-17 and miR-20a, most probably the miR-20a, from the whole cluster as the main regulator through binding to ITGβ8’s 3’UTR.

Figures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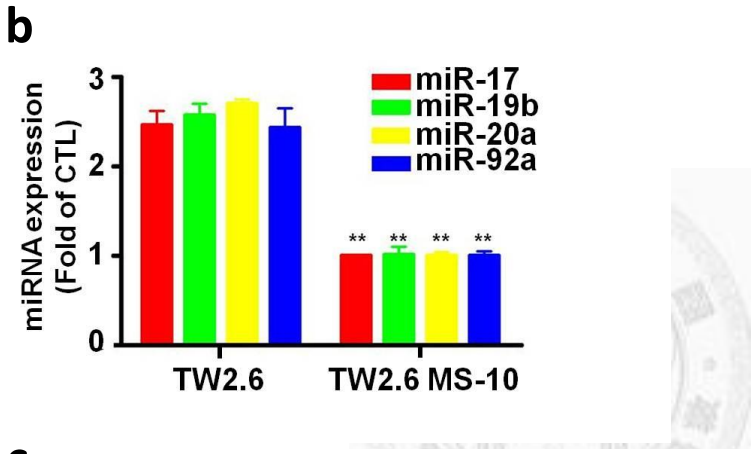
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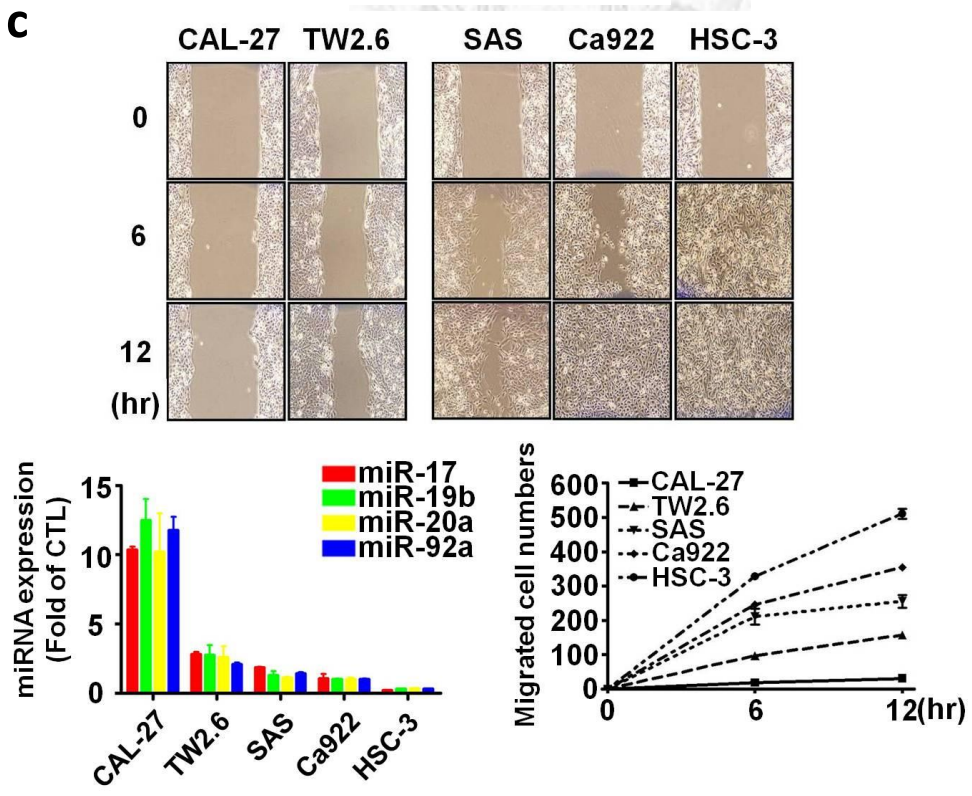
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CAL-27
TW2.6
SAS
Ca922
HSC-3
Migrated cell numbers
CAL-27
TW2.6
SAS
Ca922
HSC-3
0
6
12(hr)

Figure.1 miR-17-92 cluster expression negatively correlated with migration ability in oral squamous cell carcinoma.

(a)miRNA microarray was used to screen miRNA expression profile between TW2.6 and TW2.6 MS-10. miRNAs with fold change over 2 times were selected and categorized into fig 1A. (b) Quantitative real-time PCR analysis was performed to confirm the miRNA array data , miR-17-92 cluster were approximately 2.5 fold down-regulated in TW2.6 MS-10 cells(**$P < 0.01$). Migration ability between TW2.6 and TW2.6 MS-10 was showed in supplement figure. (c) Wound healing assays. Culture insert was used to determine migration ability of the five WT OSCC cell lines. The wound healing was determined at the time points as indicated. Migration ability negatively correlated with miR-17-92 cluster expression in different oral cancer cell lines. The less migrated cell lines such as CAL-27 and TW2.6 expressed higher level of miR-17-92.

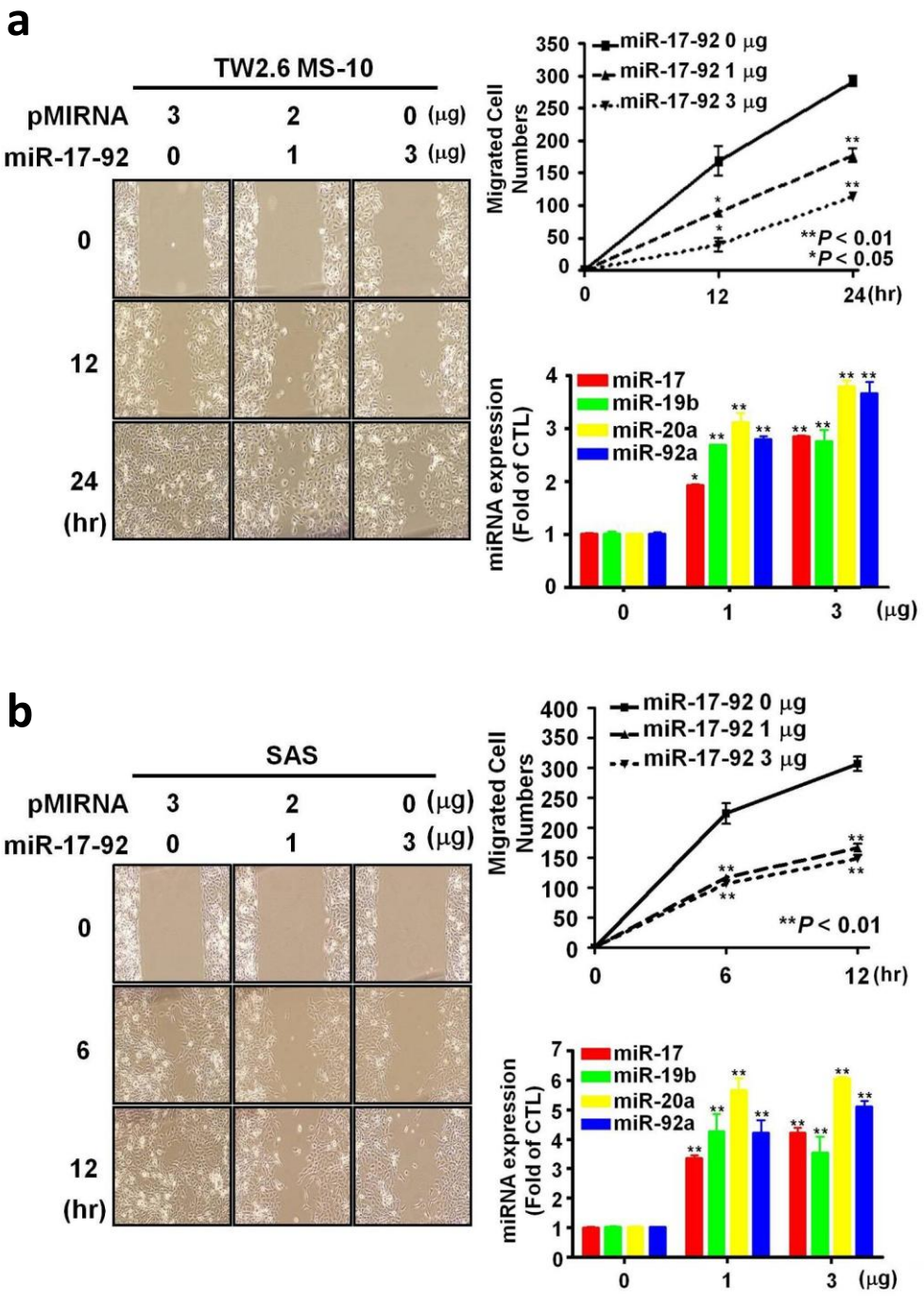


Figure.2 Migration ability down-regulated by transient expression of miR-17-92 cluster.

(a) Transiently transfected of miR-17-92 cluster decreased migration ability of TW2.6 MS-10. A significant suppression of migration ability was observed at 12 hour and 24 hour (*P < 0.05 and **P < 0.01). Migration ability was down-regulated in a

time and dose dependent manner in TW2.6 MS-10 cells. (b) Transiently transfected of miR-17-92 cluster decreased migration ability of SAS. Migration ability of SAS was significantly down-regulated at both 6 hr and 12 hr (**$P < 0.01$) .Migration ability was determined by wound healing assay and transfection efficiency was measured by quantitative real-time PCR analysis.

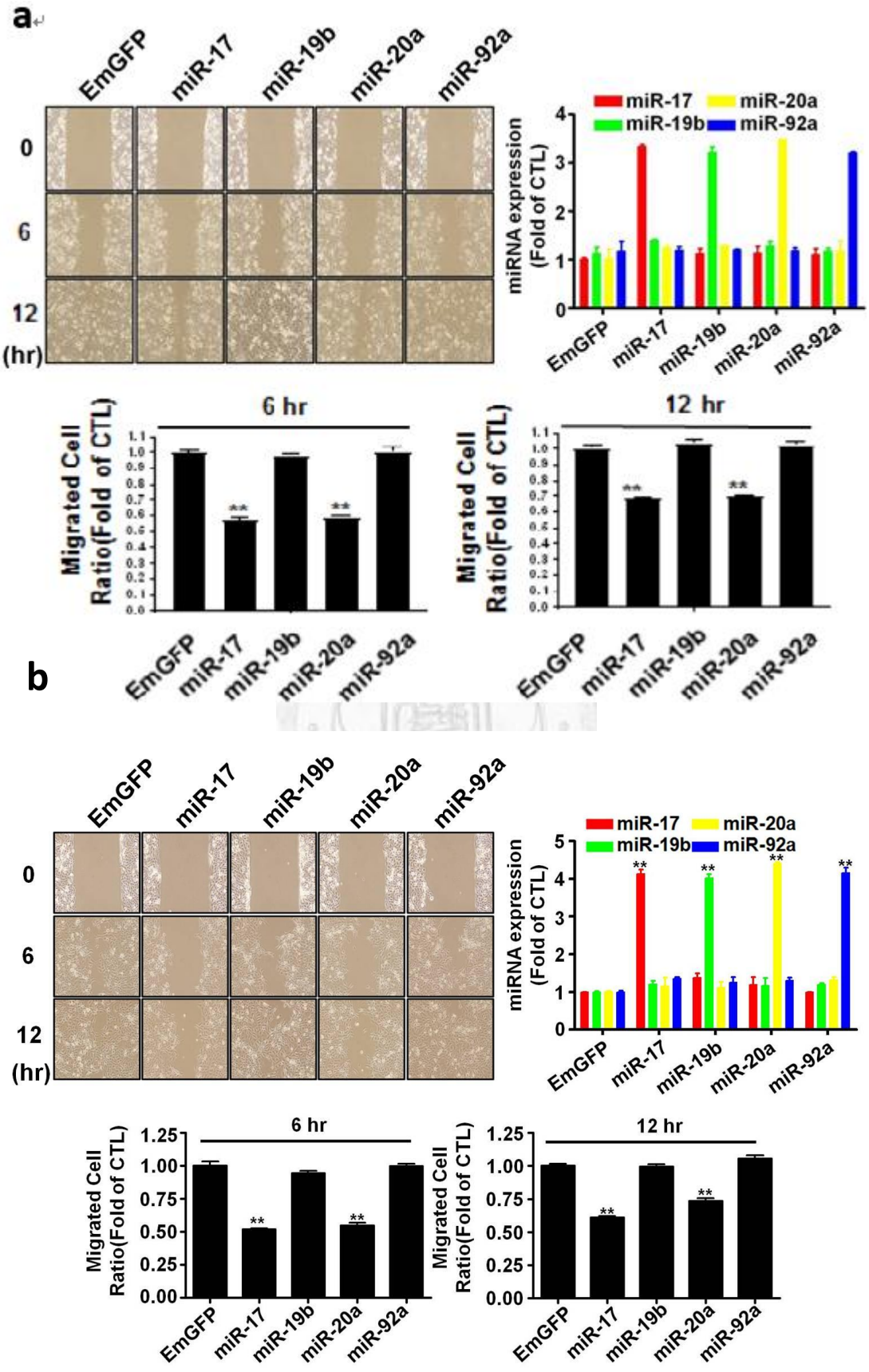

a
EmGFP
miR-17
miR-19b
miR-20a
miR-92a
0
6
12
(hr)
miR-17
miR-20a
miR-19b
miR-92a
miRNA expression
(Fold of CTL)
6 hr
12 hr
Migrated Cell
Ratio(Fold of CTL)
b

Figure.3 Migration ability was predominantly suppressed by miR-17 and miR-20a.

(a) miR-17 and miR-20a dominantly suppressed migration ability of TW2.6 MS-10.The suppression ability of miR-17 and miR-20a were most significant at 6 hour, approximately 0.5-0.6 fold down-regulation compared to miR-19b, miR-92a and vector control(**P < 0.01). (b) miR-17 and miR-20a dominantly suppressed migration ability of another OSCC cell line SAS. The suppression ability of miR-17 and miR-20a were most significant at 6 hour, approximately 0.5 fold down-regulating compared to miR-19b, miR-92a and vector control. Q-PCR was performed to determine the expression level of miRNAs. Migration ability was measured by culture inserts and quantitated by counting through Image J software.(**P < 0.01)

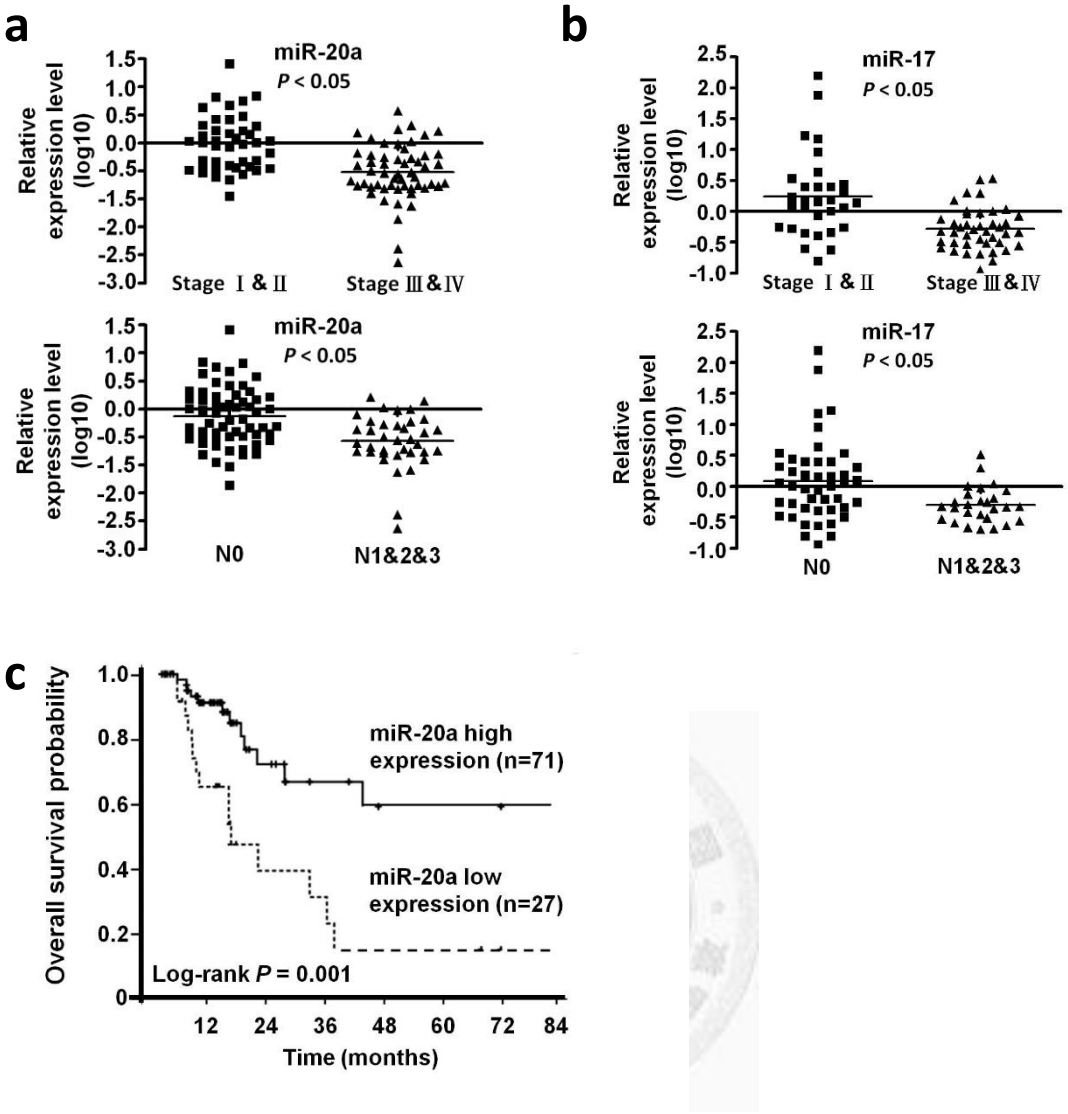


Figure.4 miR-17 and miR-20a expression level inversely correlated with progression stage and lymph node metastasis status in OSCC patients.

(a) miR-20a and (b) miR-17 expression level in OSCC patients. We grouped OSCC patients with progression stage (upper) and lymph node metastasis status (lower).miR-20a and miR-17 expression level decreased in not only the advanced stage patients but also in patients with lymph node metastasis ($P < 0.05$).

(b) Kaplan-Meier curve depicting overall survival probability of miR-20a.

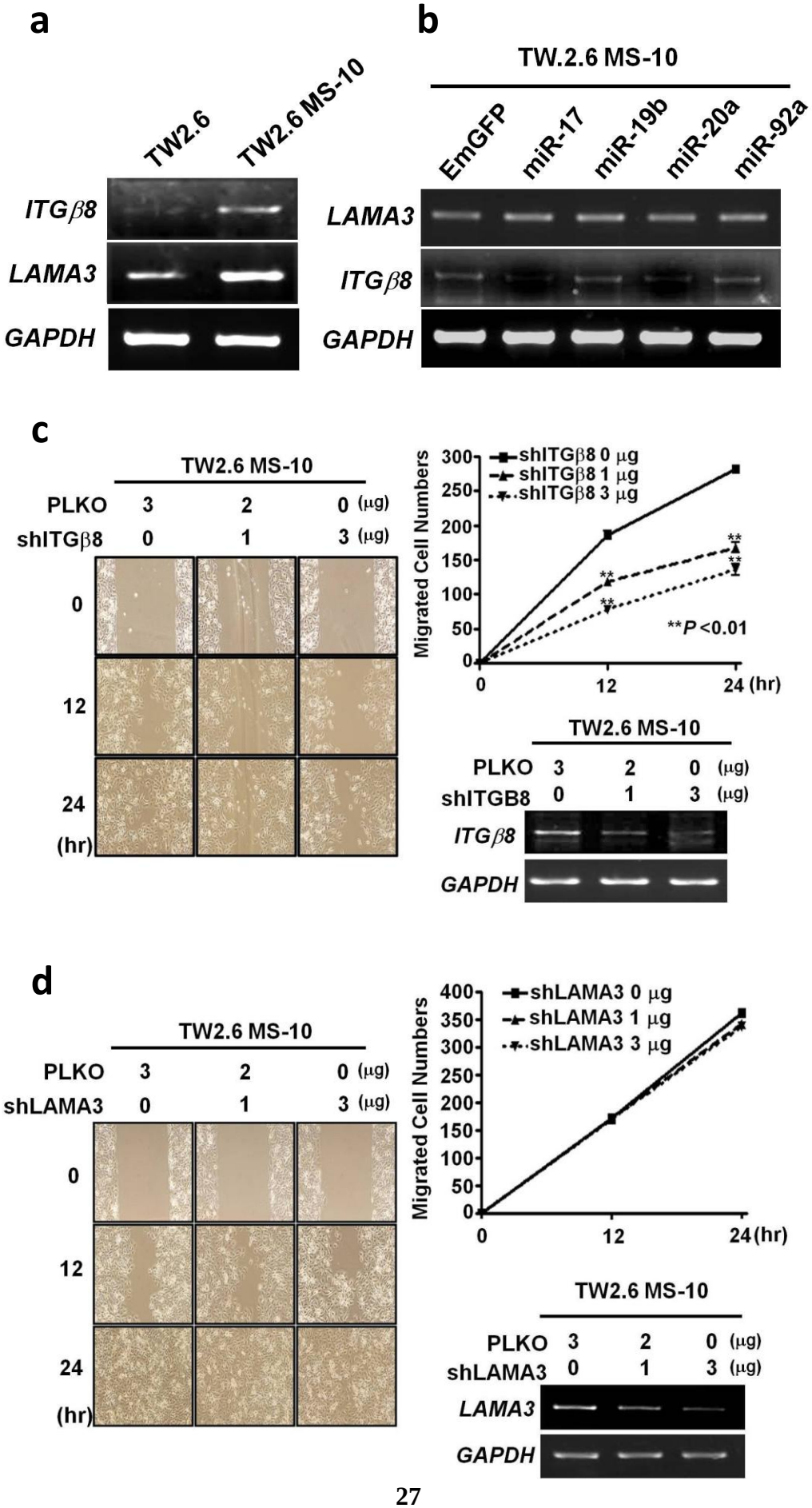
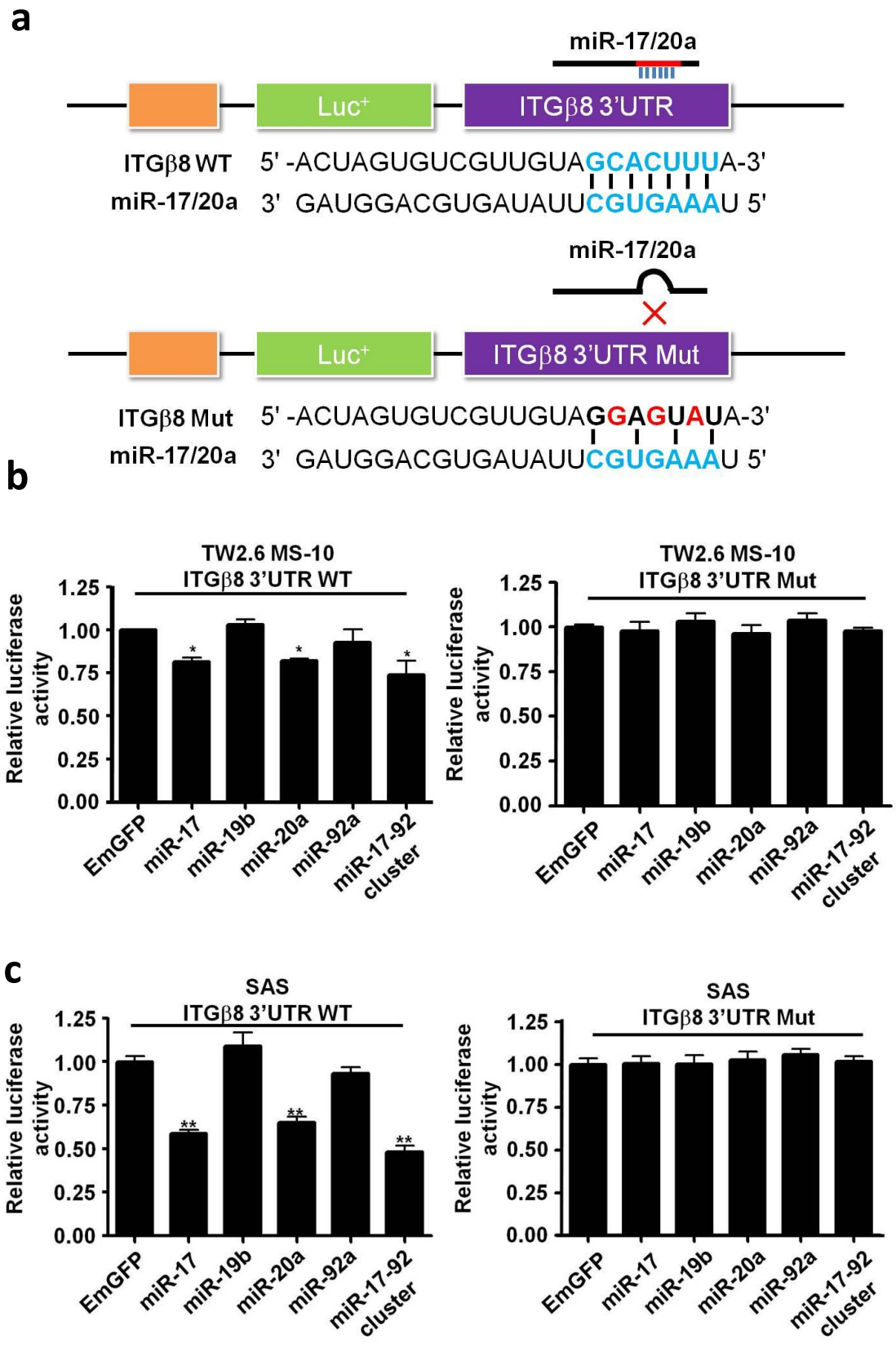

a
TW2.6
TW2.6 MS-10
ITGβ8
LAMA3
GAPDH
b
TW.2.6 MS-10
EmGFP
miR-17
miR-19b
miR-20a
miR-92a
LAMA3
ITGβ8
GAPDH
c
TW2.6 MS-10
PLKO 3 2 0 (μg)
shITGβ8 0 1 3 (μg)
0
12
24
(hr)
shITGβ8 0 μg
shITGβ8 1 μg
shITGβ8 3 μg
Migrated Cell Numbers
**P <0.01
0 12 24 (hr)
TW2.6 MS-10
PLKO 3 2 0 (μg)
shITGB8 0 1 3 (μg)
ITGβ8
GAPDH
d
TW2.6 MS-10
PLKO 3 2 0 (μg)
shLAMA3 0 1 3 (μg)
0
12
24
(hr)
shLAMA3 0 μg
shLAMA3 1 μg
shLAMA3 3 μg
Migrated Cell Numbers
0 12 24 (hr)
TW2.6 MS-10
PLKO 3 2 0 (μg)
shLAMA3 0 1 3 (μg)
LAMA3
GAPDH

Figure.5 miR-17 and miR-20a regulated OSSS migration ability through inhibited their downstream target ITGβ8.

(a) Screening of the predicted downstream target in TW2.6 and TW2.6 MS-10. Expression level of ITGβ8 and LAMA3 were significantly up-regulated in TW2.6 MS-10 cells. (b) ITGβ8 was down-regulated by miR-17 and miR-20a in TW2.6 MS-10 cells. RT-PCR was performed to determine mRNA expression level. (c) ITGβ8 indeed regulated migration ability in TW2.6 MS-10 cells (left). Migration ability of TW2.6 MS-10 cells was down-regulated by transiently transfected short-hairpin RNA (shITGβ8)(**$P < 0.01$). Migration ability was measured by wound healing assay. The wound healing was determined at the time points as indicated. Transfection efficiency was determined by RT-PCR (right lower). (d) LAMA3 was not a main regulator of migration ability in TW2.6 MS-10 cells. There was no obvious change of migration ability in TW2.6 MS-10 cells (left) after transiently transfected short-hairpin RNA (shLAMA3). Migration ability was measured by wound healing assay. The wound healing was determined at the time points as indicated. Transfection efficiency was determined by RT-PCR (right lower). Image J software was used to quantitate migrated cells.


a
miR-17/20a
Luc+
ITGβ8 3'UTR
ITGβ8 WT 5' -ACUAGUGUCGUUGUAGCACUUUA-3'
miR-17/20a 3' GAUGGACGUGAUAUUCGUGAAAU 5'
miR-17/20a
Luc+
ITGβ8 3'UTR Mut
ITGβ8 Mut 5' -ACUAGUGUCGUUGUAGGAGUAUA-3'
miR-17/20a 3' GAUGGACGUGAUAUUCGUGAAAU 5'
b
TW2.6 MS-10
ITGβ8 3'UTR WT
Relative luciferase activity
1.25
1.00
0.75
0.50
0.25
0.00
EmGFP
miR-17
miR-19b
miR-20a
miR-92a
miR-17-92 cluster
TW2.6 MS-10
ITGβ8 3'UTR Mut
c
SAS
ITGβ8 3'UTR WT
SAS
ITGβ8 3'UTR Mut

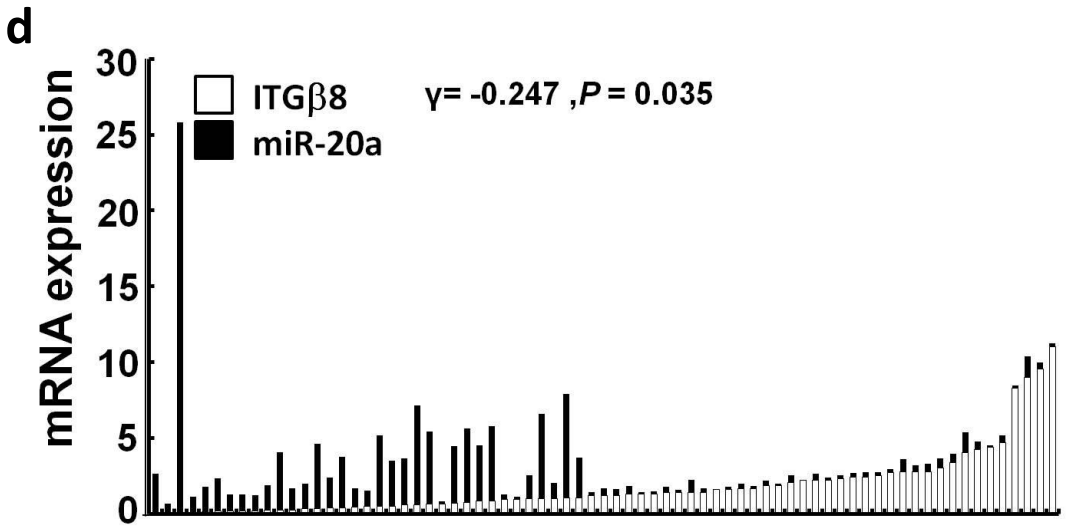


Figure.6 ITGβ8 was a direct downstream target of miR-17 and miR-20a.

(a) Diagram depicting the 3'UTR reporter constructs. Wild type (WT) and mutated (Mut)ITGβ8 3'UTR were cloned into pMIR-reporter vector. (b) Luciferase reporter assay analysis of pMIR-report-ITGβ8 WT/MUT 3'UTR in TW2.6 MS-10 cells which separately transfected of miR-17, miR-19b, miR-20a ,miR-92a and control vector. The luciferase activity was down-regulated approximately 25 % in the group transfected with miR-17,miR-20a,and miR-17-92 cluster in TW2.6 MS-10 cells(*$P < 0.05$). (c) The luciferase activity was down-regulated approximately 50 % in the group transfected with miR-17,miR-20a,and miR-17-92 cluster in TW2.6 MS-10 cells(**$P < 0.01$). In both TW2.6 MS-10 and SAS cells, we did not find synergistic regulation of miR-17 and miR-20a,due to the two miRNAs used the same binding site on ITGβ8 3'UTR. (d) The expression level of ITGβ8 in OSCC patients was

determined by Q-PCR, which negatively correlated with miR-20a expression level in OSCC patients.

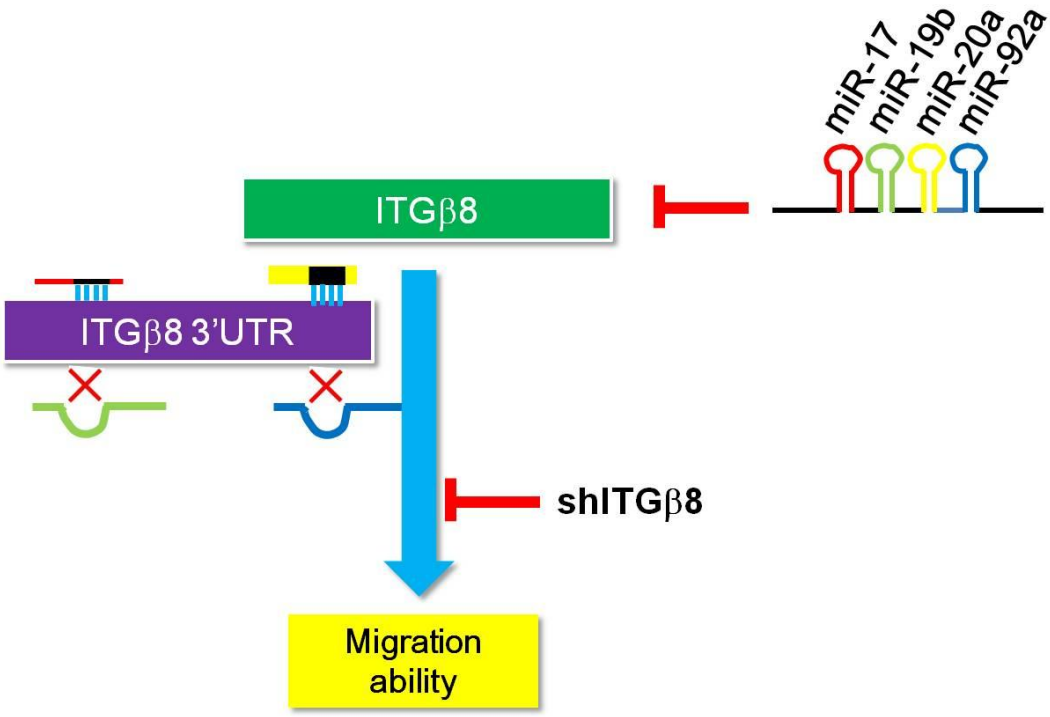


Figure.7 Flow chart of miR-17-92-inhibited cell migration ability through down-regulated ITGβ8 in OSCC.

SUPPLEMENTAL DATA

1

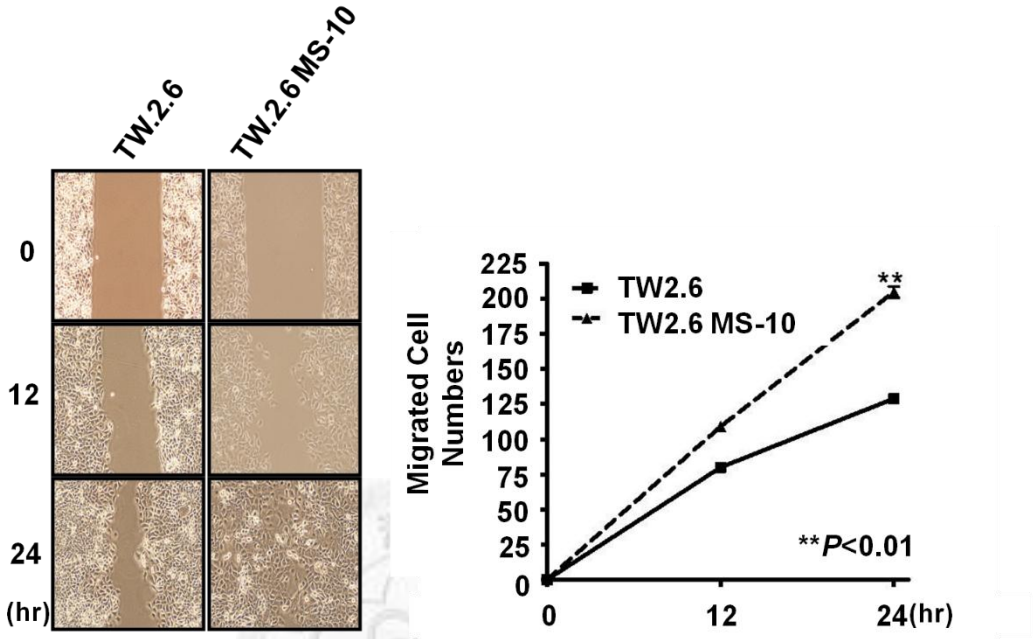


Fig.S1 Migration ability between TW2.6 and TW2.6 MS-10

2

TW2.6 MS-10	Fig. 3A				
	1(GFP4)	2(Mir-17)	3(Mir-19b)	4(Mir-20a)	5(Mir-92a)
20101224(n=1)	0	17	19	20	92
6hr	130	74	132	80	126
12hr	200	135	205	147	195
20110108(n=2)	0	17	19	20	92
6hr	155	77	150	102	155
12hr	211	136	210	149	217
20110122(n=3)	0	17	19	20	92
6hr	150	85	128	92	140
12hr	220	122	211	148	218
Treatment	0	17	19	20	92
6 hr	130	74	132	80	126
	155	77	150	102	155
	150	85	128	92	140
ave	145	78.66666667	136.6666667	91.33333333	140.3333333
error bar	13.22875656	5.686240703	11.71893055	11.01514109	14.50287328
fold of ave	1	0.542528736	0.942528736	0.629885057	0.967816092
fold of error bar	0.091232804	0.039215453	0.080820211	0.07596649	0.100019816
P value		0.001337086	0.459939644	0.005693091	0.701620616
		**		**	
	0	17	19	20	92
12 hr	200	135	191	141	191
	211	136	220	155	218
	220	122	200	148	221
ave	210.3333333	131	203.6666667	148	210
error bar	10.0166528	7.810249676	14.84362939	7	16.52271164
fold of ave	1.000015848	0.62283079	0.968319625	0.703656159	0.998431037
fold of error bar	0.04762283	0.037132783	0.070572042	0.03328056	0.078555013
p value		0.000414182	0.554159876	0.000906003	0.977593631

Fig.S2 Detailed statistical analyses of Fig 3A

Reference

1. Chen YJ, Chang JT, Liao CT, Wang HM, Yen TC, Chiu CC, et al.Head and neck cancer in the betel quid chewing area: recent advances in molecular carcinogenesis. Cancer Sci(2008);**99**(8): 1507-1514.
2. Zygogianni AG, Kyrgias G, Karakitsos P, Psyrri A, Kouvaris J, Kelekis N, et al.Oral squamous cell cancer: early detection and the role of alcohol and smoking. Head Neck Oncol(2011);**3**(2.
3. Haddad RI, Shin DM.Recent advances in head and neck cancer. N Engl J Med(2008);**359**(11): 1143-1154.
4. Warnakulasuriya S.Global epidemiology of oral and oropharyngeal cancer. Oral Oncol(2009);**45**(4-5): 309-316.
5. Bagan JV, Scully C.Recent advances in Oral Oncology 2007: epidemiology, aetiopathogenesis, diagnosis and prognostication. Oral Oncol(2008);**44**(2): 103-108.
6. Bagan J, Sarrion G, Jimenez Y.Oral cancer: clinical features. Oral Oncol(2010);**46**(6): 414-417.
7. Kowalski LP, Sanabria A.Elective neck dissection in oral carcinoma: a critical review of the evidence. Acta Otorhinolaryngol Ital(2007);**27**(3): 113-117.
8. Huntzinger E, Izaurralde E.Gene silencing by microRNAs: contributions of

translational repression and mRNA decay. Nat Rev Genet(2011);**12**(2): 99-110.

9. Farazi TA, Spitzer JI, Morozov P, Tuschl T.miRNAs in human cancer. J Pathol(2011);**223**(2): 102-115.
10. Griffiths-Jones S.miRBase: the microRNA sequence database. Methods Mol Biol(2006);**342**(129-138.
11. Inui M, Martello G, Piccolo S.MicroRNA control of signal transduction. Nat Rev Mol Cell Biol(2010);**11**(4): 252-263.
12. Esquela-Kerscher A, Slack FJ.Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer(2006);**6**(4): 259-269.
13. Krutovskikh VA, Herceg Z.Oncogenic microRNAs (OncomiRs) as a new class of cancer biomarkers. Bioessays(2010);**32**(10): 894-904.
14. Baranwal S, Alahari SK.miRNA control of tumor cell invasion and metastasis. Int J Cancer(2010);**126**(6): 1283-1290.
15. Dykxhoorn DM.MicroRNAs and metastasis: little RNAs go a long way. Cancer Res(2010);**70**(16): 6401-6406.
16. Wu B-h, Xiong X-p, Jia J, Zhang W-f.MicroRNAs: New actors in the oral cancer scene. Oral Oncol(**In Press, Corrected Proof**(
17. Gomes CC, Gomez RS.MicroRNA and oral cancer: future perspectives. Oral Oncol(2008);**44**(10): 910-914.

18. Ota A, Tagawa H, Karnan S, Tsuzuki S, Karpas A, Kira S, et al.Identification and characterization of a novel gene, C13orf25, as a target for 13q31-q32 amplification in malignant lymphoma. Cancer Res(2004);**64**(9): 3087-3095.

19. Hayashita Y, Osada H, Tatematsu Y, Yamada H, Yanagisawa K, Tomida S, et al.A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. Cancer Res(2005);**65**(21): 9628-9632.

20. Tanzer A, Stadler PF.Molecular evolution of a microRNA cluster. J Mol Biol(2004);**339**(2): 327-335.

21. Mendell JT.miRiad roles for the miR-17-92 cluster in development and disease. Cell(2008);**133**(2): 217-222.

22. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, et al.A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A(2006);**103**(7): 2257-2261.

23. Zhang L, Huang J, Yang N, Greshock J, Megraw MS, Giannakakis A, et al.microRNAs exhibit high frequency genomic alterations in human cancer. Proc Natl Acad Sci U S A(2006);**103**(24): 9136-9141.

24. Petrocca F, Visone R, Onelli MR, Shah MH, Nicoloso MS, de Martino I, et al.E2F1-regulated microRNAs impair TGFbeta-dependent cell-cycle arrest and apoptosis in gastric cancer. Cancer Cell(2008);**13**(3): 272-286.

25. Yu Z, Willmarth NE, Zhou J, Katiyar S, Wang M, Liu Y, et al.microRNA 17/20 inhibits cellular invasion and tumor metastasis in breast cancer by heterotypic signaling. Proc Natl Acad Sci U S A(2010);**107**(18): 8231-8236.

26. Danen EHJ, Sonnenberg A.Integrins in regulation of tissue development and function.

27. Hynes RO.The emergence of integrins: a personal and historical perspective. Matrix Biol(2004);**23**(6): 333-340.

28. Takada Y, Ye X, Simon S.The integrins. Genome Biol(2007);**8**(5): 215.

29. Culhane AC, Quackenbush J.Confounding effects in "A six-gene signature predicting breast cancer lung metastasis". Cancer Res(2009);**69**(18): 7480-7485.