

## Original Article

# Reduced serum miR-138 is associated with poor prognosis of head and neck squamous cell carcinoma

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**Abstract:** Circulating microRNAs (miRNAs) have been demonstrated to be potential biomarkers in various cancers including head and neck squamous cell carcinoma (HNSCC). The aim of this study was to assess the diagnostic and prognostic significance of serum miR-138 in HNSCC. Quantitative reverse-transcription PCR (qRT-PCR) was used to measure serum miR-138 levels in 113 HNSCC cases and 60 controls. The results showed that serum miR-138 expression was remarkably down-regulated in HNSCC patients compared to healthy volunteers. Moreover, serum miR-138 levels in 32 patients after receiving surgical treatment were significantly increased. Also, the receiver operating characteristic (ROC) curve analysis revealed that serum miR-138 could discriminate HNSCC patients from controls with high accuracy. Furthermore, a positive correlation was observed between decreased serum miR-138 and worse clinical outcome, as well as shorter survival. Then serum miR-138 was confirmed to be an independent prognostic indicator for HNSCC. Our results demonstrated that serum miR-138 might be a potential biomarker for detection and prognosis prediction of HNSCC.

**Keywords:** Head and neck squamous cell carcinoma, serum mir-138, prognosis, biomarker

## Introduction

Head and neck squamous cell carcinoma (HNSCC), which mainly occurs in the oral cavity, the pharynx and the larynx, is the sixth most common cause of cancer mortality around the world [1, 2]. Annually, an estimated 600,000 emerging cases and 350,000 deaths with HNSCC are reported worldwide. Despite of considerable advances in the surgical and medical treatments for HNSCC, the survival rate in patients with HNSCC has not improved throughout the last three decades [3, 4]. Therefore, identifying new effective indicators for early detection of this malignancy will be of great value.

MicroRNAs (miRNAs) are a group of non-coding small (19-25 nucleotides in length) RNAs that post-transcriptionally regulate the expression of target messenger RNAs (mRNAs), resulting in mRNA degradation or translational repression [5]. miRNAs have been demonstrated to regulate multiple cellular processes such as differentiation, proliferation, migration and apoptosis. MiRNAs may function as either oncogenes

or tumor suppressors under certain conditions [6]. MiRNAs are very stable in blood, and circulating miRNAs are used as potential biomarkers for HNSCC. For instance, increased serum miR-186-5p levels were significantly correlated with shorter overall survival and progression free survival, indicating that miR-186-5p played an oncogenic role in HNSCC [7]. Similarly, serum miR-99a levels were greatly elevated in HNSCC patients after receiving surgery, suggesting that serum miR-99a could serve as biomarker for monitoring the therapeutic responses of HNSCC [8].

MiR-138, a family of microRNA precursors, has been extensively studied and found to act as a tumor suppressor in various tumor types, such as HNSCC [9], non-small cell lung cancer [11], colorectal cancer [12], ovarian cancer [13], osteosarcoma [14], hepatocellular carcinoma [15], gallbladder carcinoma [16], cervical cancer [17] and bladder cancer [18]. However, the expression level and clinical value of serum miR-138, especially with respect to the diagnosis and prognosis of HNSCC remains poorly known. Therefore, the aim of this study was to

## Diagnostic and prognostic value of miR-138 in HNSCC

**Table 1.** Association between serum miR-138 levels and clinical features in 113 HNSCC patients

Clinical parameters	Cases (N=113)	MiR-138 levels		P value
		High (n=57)	Low (n=56)	
Age				0.7838
≤61	62	32	30	
>61	51	25	26	
Gender				0.5201
Men	101	52	49	
Women	12	5	7	
Tobacco consumption				0.2667
Yes	40	23	17	
No	73	34	39	
Alcohol consumption				0.1420
Yes	44	26	18	
No	69	31	38	
Primary tumor sites				0.2983
Oral cavity/oropharynx	59	27	32	
Others	54	30	24	
pT stage				0.1091
T0-2	55	32	23	
T3-4	58	25	33	
Differentiation				0.0467*
Well	34	22	12	
Moderate/poor	79	35	44	
pN stage				0.0994
N0-1	30	19	11	
N2-3	83	38	45	
Clinical stage				0.0046*
I/II	43	29	14	
III/IV	70	28	42	

\*P<0.05 was considered to be statistically significant.

investigate the potential clinical significance of serum miR-138 in HNSCC.

### Materials and methods

#### Ethics statement

This study was conducted with the approval of, and all participants provided written informed consents.

#### HNSCC patients and blood samples

In this study, a total of 113 subjects diagnosed with HNSCC were enrolled. Patients were excluded if they had received chemotherapy, radiotherapy or other treatments before surgery. Tumors were staged according to the criteria of the Union for International Cancer

Control (UICC) Staging System. Detailed clinical variables, including age, gender, tobacco consumption, alcohol consumption, primary tumor sites, pT stage, pN stage, differentiation and clinical stage, were listed in **Table 1**. Overall survival (OAS) was defined as the time from diagnosis to the date of death or last follow-up. Disease-free survival (DFS) was defined as the time from diagnosis to the date of recurrence or death or last follow-up. Sixty healthy individuals were recruited as controls.

Venous blood (~5 mL) was taken from each participant prior to any treatment. Thirty-two cases among all 113 patients underwent tumor resection, and 32 plasma samples from these patients six months after surgical resection were collected. All blood specimens were centrifuged and the serum was separated, and the separated supernatant was stored at -80°C until further analysis.

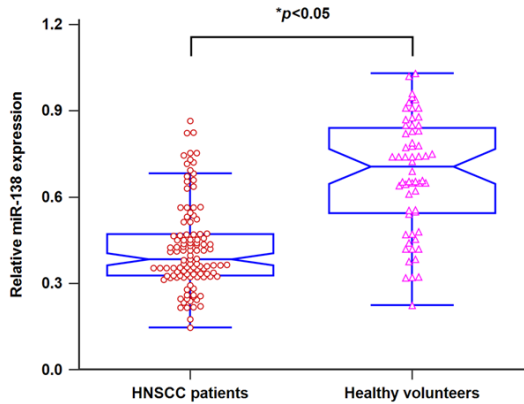
#### Isolation of total RNA and quantitative reverse-transcription PCR (qRT-PCR)

Total RNA was isolated from serum samples using a miR-Neasy RNA isolation Kits (Qiagen, Venlo, Netherlands) following the manufacturer's protocol. Reverse transcription was performed using the TaqMan miRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). PCR reactions for quantification of miR-138 were carried out in triplicate using TaqMan 2×Universal PCR Master Mix, and run on a 7500 Real-Time PCR System (Applied Biosystems, CA, USA). The relative expression of miR-138 in serum was normalized to endogenous reference gene RNU6, and calculated using the  $2^{-\Delta\Delta Ct}$  method.

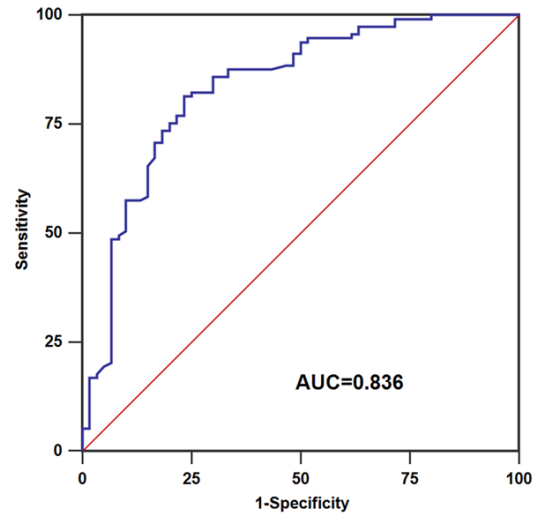
#### Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc.,

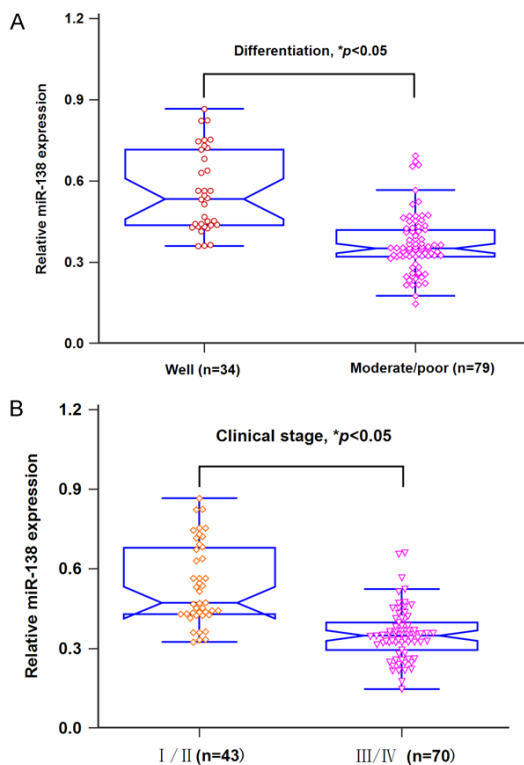
## Diagnostic and prognostic value of miR-138 in HNSCC



**Figure 1.** Serum miR-138 levels was significantly down-regulated in HNSCC patients.



**Figure 3.** Serum miR-138 yielded an AUC value of 0.836 in differentiating HNSCC patients from controls.



**Figure 2.** A. Patients with well differentiation had higher miR-138 expression than those with moderate/poor differentiation. B. Patients in I/II stage had higher miR-138 expression than those in III/IV stage.

San Diego, California, USA) and MedCalc 16.4.3 (MedCalc, Ostend, Belgium). Difference of serum miR-138 expression levels between groups was analyzed using Mann-Whitney U test. The correlation between serum miR-138 expression and clinical parameters was assessed by the Pearson Chi-square test. Receiver operat-

ing characteristic (ROC) curves and the area under the ROC curve (AUC) were constructed to evaluate the predictive power of serum miR-138 for HNSCC. OAS and DFS curves were plotted using Kaplan-Meier method, and the log-rank test was used to determine *P* values. Multivariate Cox proportional hazards model was used to estimate the effect of serum miR-138 expression and clinical characteristics on OAS/DFS. A *p* value less than 0.05 was defined statistically significant.

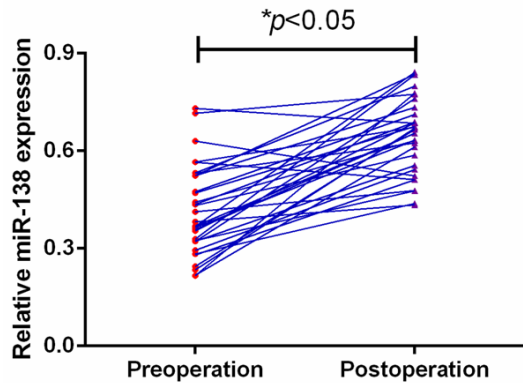
### Results

#### *Serum miR-138 was markedly decreased in HNSCC patients and its diagnostic value*

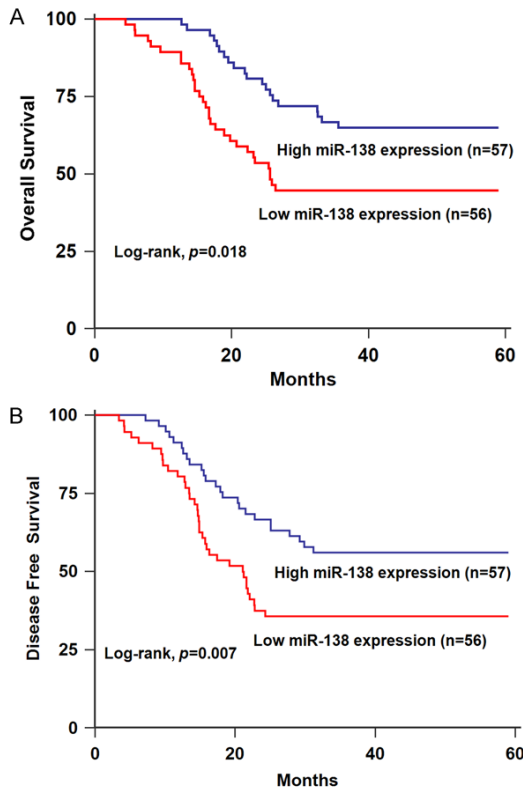
The expression levels of serum miR-138 in 113 HNSCC cases and 60 healthy volunteers were detected by qRT-PCR. As shown in **Figure 1**, serum miR-138 expression levels were greatly down-regulated in HNSCC patients compared to those in controls ( $P<0.05$ ). Moreover, a significant increase in serum miR-138 levels was found in subjects ( $n=34$ ) with well differentiation in comparison with those ( $n=79$ ) with moderate/poor differentiation ( $P<0.05$ , **Figure 2A**). In addition, serum miR-138 expression in stage III/IV subjects ( $n=70$ ) was much lower than that in stage I/II patients ( $n=43$ ,  $P<0.05$ , **Figure 2B**).

A ROC curve analysis was used to evaluate the diagnostic accuracy of miR-138 in serum

## Diagnostic and prognostic value of miR-138 in HNSCC



**Figure 4.** Serum miR-138 expression in post-operative blood samples was greatly elevated compared with paired pre-operative samples.



**Figure 5.** A. Patients in low miR-138 expression group had shorter overall survival. B. Patients in low miR-138 expression group had worse disease free survival.

between HNSCC patients and normal controls. As shown in **Figure 3**, serum miR-138 levels could discriminate patients with HNSCC from healthy subjects with an AUC value of 0.836, and the specificity and sensitivity were 76.67% and 81.42%, respectively. These findings im-

plied that serum miR-138 could be a potential indicator for HNSCC monitoring.

### *Pre-operative and post-operative serum miR-138 in HNSCC patients*

Circulating miR-138 levels in 32 paired blood specimens of HNSCC patients with tumor resection were measured, and serum miR-138 levels in the post-operative samples were dramatically elevated compared to those in the pre-operative samples ( $P < 0.05$ , **Figure 4**).

### *Down-regulation of serum miR-138 correlated with poor clinical variables of HNSCC patients*

To analyze the correlation of serum miR-138 with clinicopathological factors, we divided all the HNSCC patients into a low ( $n=56$ ) and a high ( $n=57$ ) expression group according to the median serum miR-138 level. As presented in **Table 1**, serum miR-138 expression was strongly associated with differentiation ( $P=0.0467$ ) and clinical stage ( $P=0.0046$ ), but not associated with other characteristics such as age, gender, tobacco consumption, alcohol consumption, primary tumor sites, pT stage and pN stage (all  $P > 0.05$ ).

### *Down-regulation of miR-138 correlated with worse survival in HNSCC patients*

The Kaplan-Meier analysis showed that HNSCC patients with lower serum miR-138 levels had significantly worse OAS ( $P=0.018$ , **Figure 5A**) and DFS ( $P=0.007$ , **Figure 5B**) than those with higher expression of this miRNA.

### *Serum miR-138 was an independent prognostic indicator for HNSCC patients*

Multivariate Cox proportional hazard regression model was employed and showed that serum miR-138 expression (OAS: HR=3.55, 95% CI=1.39-5.68,  $P=0.023$ ; DFS: HR=4.13, 95% CI=1.46-6.97,  $P=0.019$ ), clinical stage (OAS: HR=4.81, 95% CI=1.68-8.06,  $P=0.012$ ; DFS: HR=5.31, 95% CI=1.87-8.92,  $P=0.005$ ) and differentiation (OAS: HR=2.56, 95% CI=1.21-3.95,  $P=0.032$ ; DFS: HR=2.82, 95% CI=1.34-4.45,  $P=0.028$ ) were independent prognostic biomarkers for predicting poorer OAS/DFS in HNSCC patients (**Table 2**).

## Diagnostic and prognostic value of miR-138 in HNSCC

**Table 2.** Multivariate Cox regression analysis for OAS and DFS

Factors	Multivariate analysis	
	HR (95% CI)	P value
Overall survival (OAS)		
Differentiation (Moderate/poor vs. well)	2.56 (1.21-3.95)	0.032*
Clinical stage (III/IV vs. I/II)	4.81 (1.68-8.06)	0.012*
MiR-138 expression (low vs. high)	3.55 (1.39-5.68)	0.023*
Disease free survival (DFS)		
Differentiation (Moderate/poor vs. well)	2.82 (1.34-4.45)	0.028*
Clinical stage (III/IV vs. I/II)	5.31 (1.87-8.92)	0.005*
MiR-138 expression (low vs. high)	4.13 (1.46-6.97)	0.019*

Footnote: CI: confidence interval; HR: hazard ratio. \* $P < 0.05$  was considered to be statistically significant.

### Discussion

In this study, we found that serum miR-138 expression was remarkably reduced in HNSCC patients compared to healthy subjects. Also, serum miR-138 levels were significantly elevated in post-operative blood samples taken from patients with tumor resection. Furthermore, ROC curve analysis showed that miR-138 in serum could distinguish HNSCC patients from controls with relatively high accuracy. Subsequently, a positive correlation was observed between down-regulated serum miR-138 expression and aggressive clinical factors, as well as shorter OAS/DFS. Finally, serum miR-138 level was verified to be an independent prognostic indicator for HNSCC. Taken together, our results suggested that miR-138 might act as an anti-oncogenic miRNA in HNSCC.

In accordance with our findings, some previous studies demonstrated that miR-138 exerted a tumor suppressive function in HNSCC. For example, Liu and colleagues showed that miR-138 overexpression repressed cell invasion and induced cell cycle arrest, apoptosis in HNSCC cell lines. Moreover, knockdown of miR-138 stimulated cell invasion and restrained apoptosis [9]. MiR-138 was reduced in both primary HNSCC tumor and cell lines, and up-regulated miR-138 expression suppressed cell motility, invasion colony and fiber formation. RhoC was proved to be a downstream target of miR-138 [10].

Besides HNSCC, miR-138 was reported to act as a tumor suppressor in some other types of cancers. In non-small cell lung cancer, miR-138 expression was markedly reduced in cancerous

tissues and miR-138 underexpression was associated with poor clinical outcome. Additionally, ectopic miR-138 expression significantly suppressed cancer cell growth, migration and invasion by targeting yes-associated protein 1 [11]. In colorectal cancer, Long et al provided *in vitro* and *in vivo* evidence to reveal that decreased miR-138 expression greatly stimulated cancer cell migration and invasion through inversely regulating twist basic helix-loop-helix transcription factor 2 gene [12]. In ovarian cancer, miR-138 overexpression repressed cancer metastasis with *in vivo* evidence via targeting

SOX4 and HIF-1 $\alpha$ . Also, down-regulation of miR-138 was closely associated with aggressive clinical features [13]. In osteosarcoma, Jiang and colleagues found that miR-138 levels were decreased both in cancer tissues and cell lines. Furthermore, loss of miR-138 promoted cancer cell proliferation, invasion and inhibited cell apoptosis *in vitro* by regulating differentiated embryonic chondrocyte gene 2 [14]. Similarly, miR-138 expression was remarkably reduced both in hepatocellular carcinoma tissues and cell lines. In addition, up-regulation of miR-138 inhibited tumorigenicity of cancer cells by degrading SOX9 [15]. In gallbladder carcinoma, Ma et al showed that miR-138 expression was underexpressed in cancerous samples. Enforced miR-138 expression greatly restrained cancer cell proliferation via directly targeting Bag-1 [16]. Also, Sun et al confirmed that miR-138 levels were significantly decreased in bladder cancer tissues. Reduced miR-138 expression promoted cancer cell invasion and metastases through up-regulating ZEB2 [17]. In cervical cancer, miR-138 expression was dramatically downregulated in cancer tissues and cell lines. Moreover, restoration of miR-138 suppressed tumorigenicity *in vitro* and tumor growth *in vivo* by negatively regulating hTERT [18].

In conclusion, we showed that serum miR-138 expression was significantly downregulated in HNSCC patients. ROC curve analysis revealed that serum miR-138 was an accurate indicator for differentiating HNSCC patients from controls. Moreover, miR-138 underexpression was closely correlated with worse clinical outcome and shorter survival. Therefore, serum miR-138

could serve as a promising marker for the diagnosis and prognosis of HNSCC.

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#### Disclosure of conflict of interest

None.

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#### References

- [1] Cui L, Cheng S, Liu X, Messadi D, Yang Y, Hu S. Syntenin-1 is a promoter and prognostic marker of head and neck squamous cell carcinoma invasion and metastasis. *Oncotarget* 2016; 7: 82634-82647.
- [2] Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; 61: 69-90.
- [3] Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010; 127: 2893-2917.
- [4] Xu S, Ma D, Zhuang R, Sun W, Liu Y, Wen J, Cui L. DJ-1 is upregulated in oral squamous cell carcinoma and promotes oral cancer cell proliferation and invasion. *J Cancer* 2016; 7: 1020-1028.
- [5] Ambros V. The functions of animal microRNAs. *Nature* 2004; 431: 350-355.
- [6] Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell* 2009; 136: 215-233.
- [7] Summerer I, Unger K, Braselmann H, Schuettrumpf L, Maihoefer C, Baumeister P, Kirchner T, Niyazi M, Sage E, Specht HM, Multhoff G, Moertl S, Belka C and Zitzelsberger H. Circulating microRNAs as prognostic therapy biomarkers in head and neck cancer patients. *Br J Cancer* 2015; 113: 76-82.
- [8] Hou B, Ishinaga H, Midorikawa K, Shah SA, Nakamura S, Hiraku Y, Oikawa S, Murata M, Takeuchi K. Circulating microRNAs as novel prognosis biomarkers for head and neck squamous cell carcinoma. *Cancer Biol Ther* 2015; 16: 1042-1046.
- [9] Liu X, Jiang L, Wang A, Yu J, Shi F, Zhou X. MicroRNA-138 suppresses invasion and promotes apoptosis in head and neck squamous cell carcinoma cell lines. *Cancer Lett* 2009; 286: 217-222.
- [10] Islam M, Datta J, Lang JC, Teknos TN. Down regulation of RhoC by microRNA-138 results in de-activation of FAK, Src and Erk1/2 signaling pathway in head and neck squamous cell carcinoma. *Oral Oncol* 2014; 50: 448-456.
- [11] Xiao L, Zhou H, Li XP, Chen J, Fang C, Mao CX, Cui JJ, Zhang W, Zhou HH, Yin JY, Liu ZQ. MicroRNA-138 acts as a tumor suppressor in non small cell lung cancer via targeting YAP1. *Oncotarget* 2016; 7: 40038-40046.
- [12] Long L, Huang G, Zhu H, Guo Y, Liu Y, Huo J. Down-regulation of miR-138 promotes colorectal cancer metastasis via directly targeting TWIST2. *J Transl Med* 2013; 11: 275.
- [13] Yeh YM, Chuang CM, Chao KC, Wang LH. MicroRNA-138 suppresses ovarian cancer cell invasion and metastasis by targeting SOX4 and HIF-1 $\alpha$ . *Int J Cancer* 2013; 133: 867-878.
- [14] Jiang B, Mu W, Wang J, Lu J, Jiang S, Li L, Xu H, Tian H. MicroRNA-138 functions as a tumor suppressor in osteosarcoma by targeting differentiated embryonic chondrocyte gene 2. *J Exp Clin Cancer Res* 2016; 35: 69.
- [15] Liu Y, Zhang W, Liu K, Liu S, Ji B, Wang Y. MiR-138 suppresses cell proliferation and invasion by inhibiting SOX9 in hepatocellular carcinoma. *Am J Transl Res* 2016; 8: 2159-2168.
- [16] Ma F, Zhang M, Gong W, Weng M, Quan Z. MiR-138 suppresses cell proliferation by targeting Bag-1 in gallbladder carcinoma. *PLoS One* 2015; 10: e0126499.
- [17] Sun DK, Wang JM, Zhang P, Wang YQ. MicroRNA-138 regulates metastatic potential of bladder cancer through ZEB2. *Cell Physiol Biochem* 2015; 37: 2366-2374.
- [18] Zhou N, Fei D, Zong S, Zhang M, Yue Y. MicroRNA-138 inhibits proliferation, migration and invasion through targeting hTERT in cervical cancer. *Oncol Lett* 2016; 12: 3633-3639.