Am J Cancer Res 2017;7(12):2491-2502
www.ajcr.us /ISSN:2156-6976/ajcr0063160

Original Article

EGFRwt/vIII-PKM2-β-catenin cascade affects proliferation and chemo-sensitivity in head and neck squamous cell carcinoma

Chao Jing[1,2,3*], Xin Qu[4*], Zhaoqing Li[1,2,3*], Chuanqiang Wu[1,2,3], Minghui Zhao[1,2,3], Yu Wang[1,2,3], Shanshan Sun[1,2,3], Shengchi Zhang[1,2,3], Jinliang Chen[1,2,3], Yu Qiao[1,2,3], Xiaomeng Hu[1,2,3], Xiaofeng Yao[1,2,3], Rui Jin[1,2,3], Xudong Wang[1,2,3], Lun Zhang[1,2,3], Xuan Zhou[1,2,3]

[1]Department of Maxillofacial and Otorhinolaryngological Oncology, Tianjin Medical University Cancer Institute and Hospital, Tianjin 300060, China; [2]Key Laboratory of Cancer Prevention and Therapy, Tianjin Cancer Institute, Tianjin 300060, China; [3]National Clinical Research Center of Cancer, Tianjin 300060, China; [4]Department of Breast and Thyroid Surgery, Tianjin 4th Centre Hospital, Tianjin 300140, China. [*]Equal contributors.

Received August 7, 2017; Accepted October 11, 2017; Epub December 1, 2017; Published December 15, 2017

Abstract: Patients suffered from head and neck squamous cell carcinoma (HNSCC) have an overall poor prognosis owing to proliferation and resistance to treatment. Hence, mining the underlying mechanism of malignancies above and translating the bench outcomes to clinical practice are in urgent need. Previous studies found that the epidermal growth factor receptor (EGFR) increases and co-expresses with EGFRvIII in HNSCC tissues, which indicates poor prognosis of HNSCC patients. Here, we clarify that compared with EGFRwt, EGFRwt/vIII enhances the capability of proliferation and colony formation in HNSCC cells *in vitro*, and reduces the sensitivity to cisplatin. Furthermore, EGFRwt/vIII induces nuclear translocation of the M2 isoform of pyruvate kinase (PKM2) in a time-dependent manner. The aberrant expression of PKM2 in HNSCC suggests unfavorable outcome. Especially, nuclear PKM2 determines the activation of β-catenin signaling and regulates the proliferation and chemo-sensitivity of HNSCC cells. Together, our findings demonstrate that EGFRwt/vIII-PKM2-β-catenin cascade controls the proliferation and chemo-sensitivity of HNSCC, thereby providing a promising strategy for diagnosis and therapy of HNSCC.

Keywords: HNSCC, EGFRvIII, PKM2, β-catenin, proliferation, chemo-sensitivity

Introduction

As one of human malignancies, head and neck cancer ranks the 6th common cancer around the world [1]. Furthermore, head and neck squamous cell carcinoma (HNSCC) accounts for most of head and neck cancer diagnosed worldwide, whose overall 5-year survival rate is about 53% [2]. Malignant proliferation and chemo-resistance are the main clinical symptoms in patients suffered from HNSCC, which leads to relapse and poor outcomes [3]. Cisplatin (CDDP) still acts as a core drug for patients with advanced and metastatic HNSCC, thus improving the sensitivity to CDDP is greatly critical. Although previous studies have shed a light on initiation and progression of HNSCC [4, 5], our understanding of proliferation and chemo-sensitivity of HNSCC still remains far from thorough because these are complicated processes, in which multiple molecules are involved.

As one of crucial genes, the epidermal growth factor receptor (EGFR), a member of ErbB family, is amplified in many tumors including HNSCC [6], and its overexpression correlates with poor prognosis [7, 8]. A recent work accomplished by The Cancer Genome Atlas (TCGA) has identified that EGFR is aberrant activated in either human papillomavirus-positive or -negative HNSCC patients [9]. EGFR plays a critical role in multiple aspect of tumor biology including cell proliferation, motility and invasiveness, angiogenesis and resistance to treatment [10], activated by various ligands such as EGF [11]. As one of most common EGFR mutant, EGFR variant III (EGFRvIII) lacks components of the ligand-bind-

ing domain in the extracellular portion, incapable of binding EGF as well as other ligands [12]. However, EGFRvIII is a constitutively active form, although at a low level of tyrosine phosphorylation [13]. Co-expression of EGFRwt (wild-type) and EGFRvIII has been uncovered in HNSCC, implicated in cell proliferation and CDDP sensitivity [14], but the underlying molecular mechanism is still unclear. In this study, we aimed to clarify specific signaling mediated by EGFRwt/vIII in the proliferation and chemo-sensitivity of HNSCC.

Reprogrammed metabolism is a key characteristic of many cancers in order to support rapid growth [15]. Aerobic glycolysis, also known as the Warburg effect, is the best-characterized metabolic phenotype observed in tumor cells [16]. As a rate-limiting enzyme of aerobic glycolysis, the M2 splice isoform of pyruvate kinase (PKM2) catalyzes the final step of glycolysis in cancer cells [17]. Besides, PKM2 has recently been recognized to translocate into the nucleus to function as a transcriptional co-activator and a protein kinase to regulate gene transcription and cell cycle progression [18, 19]. In human glioblastoma (GBM), PKM2 located into nucleus regulates β-catenin transactivation upon the activation of EGFR [20], and enhances c-Myc expression, which subsequently increases the expression of glycolytic enzymes, thereby promoting aerobic glycolysis in a positive feedback loop [21]. In the nucleus, PKM2 phosphorylates histones to function in the epigenetic regulation and promote gene transcription and tumorigenesis [18]. However, its non-catalytic functions remain elusive in HNSCC.

Here, we demonstrated that EGFRwt/vIII dramatically affected proliferation and chemo-sensitivity of HNSCC cells *in vitro*, partially mediated by β-catenin signal. PKM2 was highly expressed in HNSCC specimens and correlated with poor prognosis. In comparison to wild-type EGFR, EGFRwt/vIII induced elevated accumulation of nuclear PKM2 upon the treatment of EGF. Moreover, R399/400 was two predominant amino acid residues in nuclear localization signal (NLS) of PKM2, while the mutation of them would impede nuclear translocation. Nuclear PKM2 mediated the activation of β-catenin to heighten proliferation and weaken chemo-sensitivity of HNSCC cells *in vitro*. Together, these data confirm that EGFRwt/vIII-PKM2-β-catenin cascade serves as a key regulator of HNSCC proliferation and chemo-sensitivity.

Materials and methods

Cell culture and reagents

Human HNSCC cell lines CAL27, SCC25 and UM1 were purchased from ATCC (Manassas, VA, USA), while TB3.1 and TSCCA were obtained from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. The cell lines were maintained in DMEM, DMEM/Ham's F12, RPMI1640 or MEM (all from Invitrogen, Camarillo, CA, USA) supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml), under a humidified atmosphere (37°C, 5% CO_2). All HNSCC cells were authorized by STR analysis.

Lentivirus were purchased from Genechem (Shanghai, China) to establish stable SCC25 or UM1 clones expressing EGFRvIII. EGF was purchased from R&D Systems (Minnesota, USA) and dissolved in sterile PBS for storage and use. A Wnt/β-catenin pathway inhibitor, FH535 (Selleck, Shanghai, China) was added into cells at a final concentration of 10 μM for MTT assay.

Database analysis

The online software cBioPortal (www.cbioportal.org) [22, 23] and GEPIA (gepia.cancer-pku.cn) were provided to analyze TCGA (The Cancer Genome Atlas) database. HNSCC GEO dataset GSE37991 was used to compare the expression of EGFR, β-catenin and PKM2 between cancerous tissues and the adjacent normal counterparts as well as figure out the correlation among three of them.

Plasmid construction

The cDNA for wild-type PKM2 was amplified by PCR and cloned into pLVX-IRES-puro, while the PKM2 R399/400A mutant was generated by site-directed mutagenesis. The primers are provided in Supplementary Table 1. All constructions were verified by sequencing in AuGCT (Beijing, China).

Lentivirus packaging and transduction

Lentivirus was prepared in HEK293T cells using Lipofectamine 2000 (Invitrogen) according to

the manufacturer's instructions. In brief, the transfection was performed until the cultured cells reach to 60-70% confluence in a 10 cm-plate. 7.5 μg of expression plasmid with 12.5 μg of package plasmids were mixed in 1.5 ml of Opti-MEM, while 60 μl of Lipofectamine 2000 was mixed in another 1.5 ml of Opti-MEM for 5 min. The plasmids and Lipofectamine 2000 mixture was combined at room temperature for 20 min and then dropped randomly into cultured HEK293T cells. The transfection was incubated overnight before the medium was replaced with fresh DMEM with 10% FBS. Around 24 hours, the viral supernatant was harvested and filtered (0.45 μm filter) for use.

SCC25 or UM1 cells were seeded into a 6-well plate and infected by lentivirus with polybrene, further screened in complete medium containing 1 μg/ml of puromycin. Stable SCC25 or UM1 clones expressing EGFRvIII were labeled as SCC25vIII or UM1vIII, respectively. Moreover, cells with stably overexpression of PKM2 or PKM2 mutant were labeled as PKM2 wt or PKM2 R399/400A respectively, and EV (Empty Vector) was used as negative control.

Immunoblotting assay

Cells were lysed in RIPA buffer and performed for immunoblotting assay according to standard procedure. The PVDF membranes were incubated with specific primary antibodies at 4°C overnight: EGFR (1:1000), p-EGFR (Y1068, 1:1000), β-catenin (1:1000), p-β-catenin (S675, 1:1000), Cyclin D1 (1:1000, all purchased from Cell Signaling Technology, Danvers, MA, USA), PKM2 (1:1000), H3 (1:1000, Proteintech, Rosemont, USA) and GAPDH (1:5000, Sigma-Aldrich, Missouri, USA). Antibodies used in immunoblotting assay are listed in Supplementary Table 2. All images were acquired by ImageQuant LAS-4000 System (GE, Fairfield, Connecticut, USA).

Flow cytometry

Apoptosis assay was carried out according to the manufacturer instruction of Annexin V/PI Apoptosis Detection kit (BD, Franklin Lakes, NJ, USA). All experiments were manipulated on the same FACS Canto II (BD).

Clonogenic assay

HNSCC cells were counted and seeded into a 6-well plate (1000 cells/well). Cells were cultured for 10 days, then washed twice with PBS and stained with a dilute crystal violet (0.1%, w/v) solution.

Immunofluorescence staining

HNSCC cells were plated on 18 mm glass plate and then treated with EGF (100 ng/ml) for 12 hours. Then, cells were stained as previously described [24]. All images were acquired by Iamger.Z2 (Zeiss, Oberkochen, Germany).

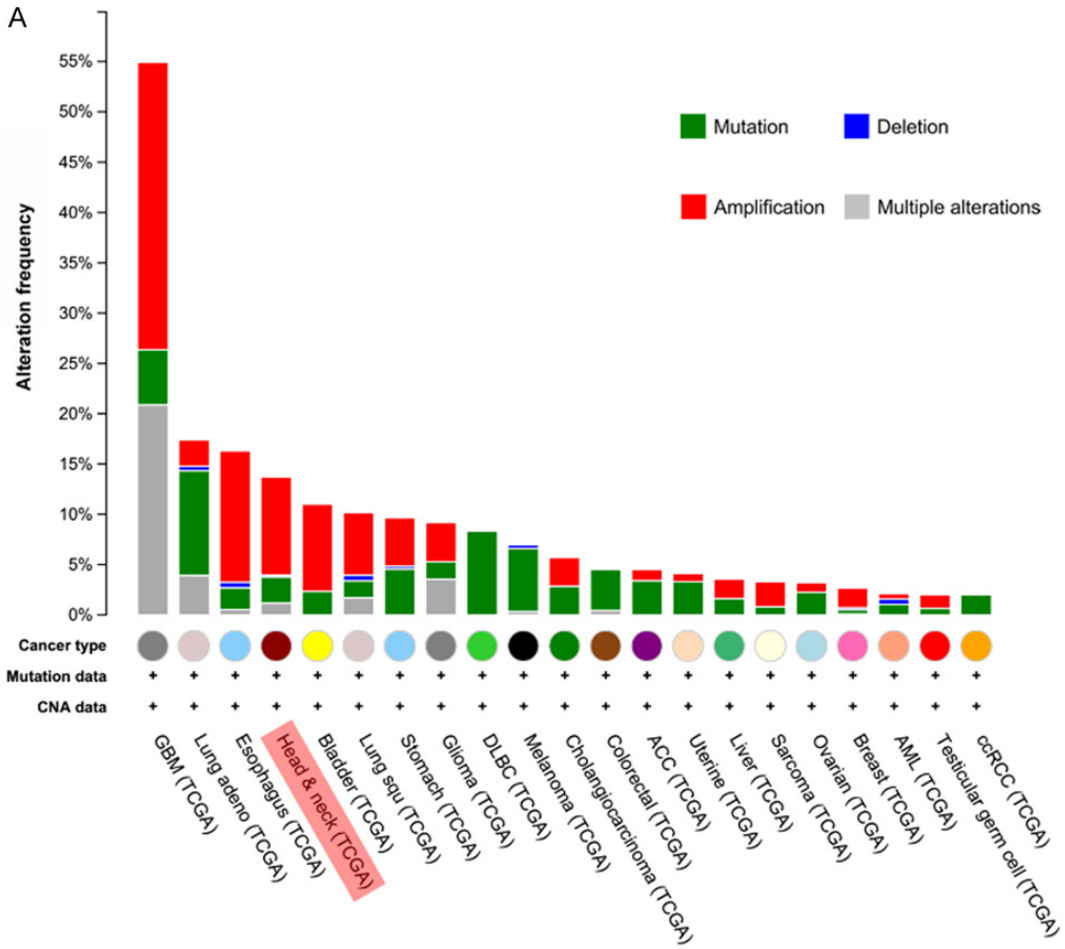
Statistical analysis

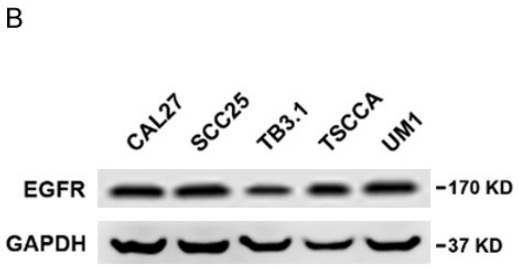
All experiments other were repeated at least three times. All data were presented as mean ± SD and analyzed by using two-tailed or paired Student's t-test. Graphs were illustrated by GraphPad Prism 6 (La Jolla, USA), in which *, **, *** and **** indicated $P<0.05$, $P<0.01$, $P<0.001$, $P<0.0001$, respectively.

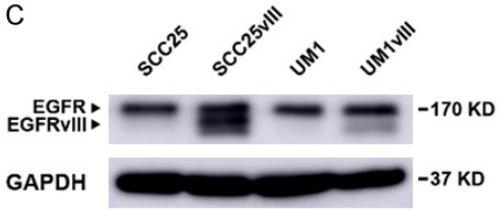
Results

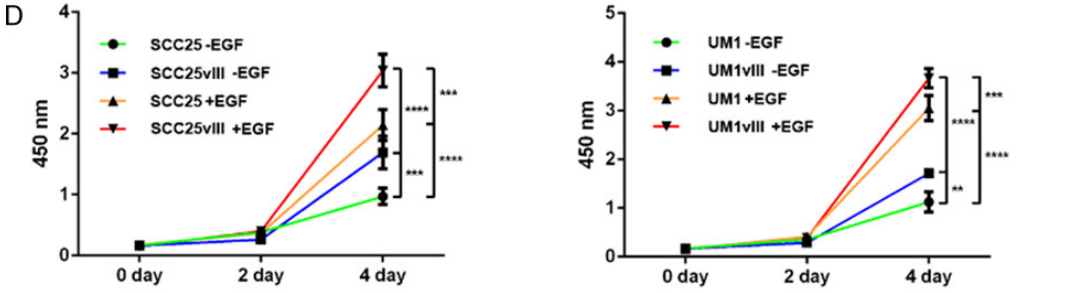
EGFRwt/vIII affects proliferation and chemo-sensitivity of HNSCC cells in vitro

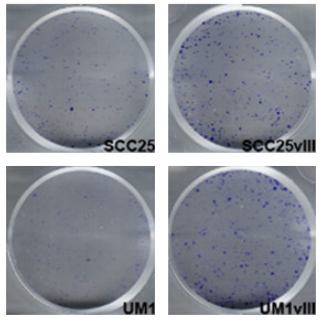
EGFR plays a prominent role in many tumors, whose increased activation and overexpression is strongly related with tumorigenesis and cancer progression [25]. Therefore, we first explored the frequency of EGFR gene alteration across multiple cancers using the online software cBioPortal. The results showed that there is significant copy number amplification of EGFR gene based on published data of TCGA in a variety of cancers including HNSCC, as well as gene mutation (**Figure 1A**). Meanwhile, immunoblotting assay indicated that EGFR was overexpressed in a panel of HNSCC cell lines (**Figure 1B**). As a most common mutant of EGFR, EGFRvIII is also detected to be expressed in HNSCC tissues [26], so we established stable SCC25 or UM1 clones expressing EGFRvIII (SCC25vIII or UM1vIII) by transduction to verify its function in HNSCC cells (**Figure 1C**). In comparison to parental cells (SCC25/UM1), SCC25vIII and UM1vIII cells both possessed stronger capacity of proliferation with or without EGF stimulation (**Figure 1D**). Both the size and number of colonies were improved in SCC25vIII and UM1vIII cells (**Figure 1E**). Moreover, with the treatment of CDDP, the apoptosis rate of SCC25vIII and UM1vIII cells was obviously decreased and the number of survival


A
Alteration frequency
55%
50%
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
Mutation
Deletion
Amplification
Multiple alterations
Cancer type
Mutation data
CNA data
GBM (TCGA)
Lung adeno (TCGA)
Esophagus (TCGA)
Head & neck (TCGA)
Bladder (TCGA)
Lung squ (TCGA)
Stomach (TCGA)
Glioma (TCGA)
DLBC (TCGA)
Melanoma (TCGA)
Cholangiocarcinoma (TCGA)
Colorectal (TCGA)
ACC (TCGA)
Uterine (TCGA)
Liver (TCGA)
Sarcoma (TCGA)
Ovarian (TCGA)
Breast (TCGA)
AML (TCGA)
Testicular germ cell (TCGA)
ccRCC (TCGA)


B
CAL27
SCC25
TB3.1
TSCCA
UM1
EGFR
GAPDH
~170 KD
~37 KD


C
SCC25
SCC25vIII
UM1
UM1vIII
EGFR
EGFRvIII
GAPDH
~170 KD
~37 KD


D
SCC25 -EGF
SCC25vIII -EGF
SCC25 +EGF
SCC25vIII +EGF
450 nm
0 day
2 day
4 day
UM1 -EGF
UM1vIII -EGF
UM1 +EGF
UM1vIII +EGF


E
SCC25
SCC25vIII
UM1
UM1vIII

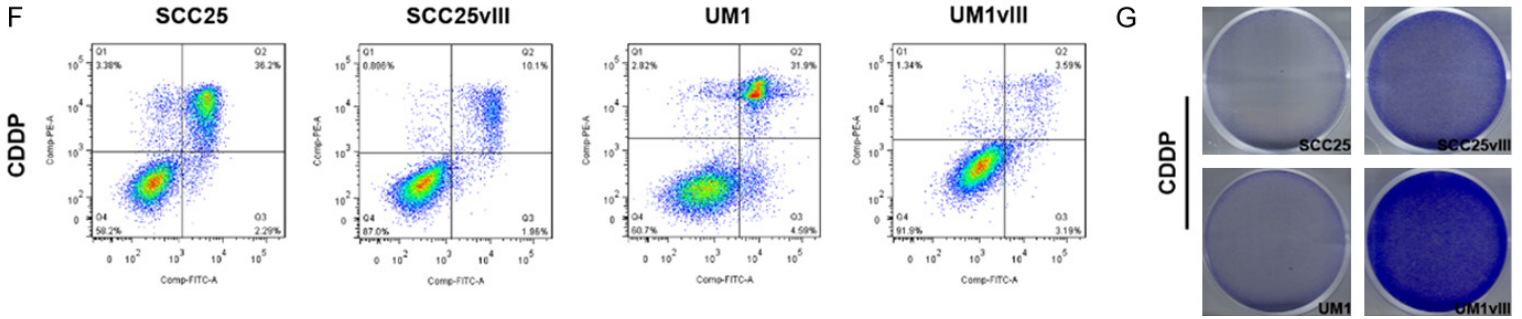


Figure 1. EGFRwt/vIII affects the proliferation and chemo-sensitivity of HNSCC cells. (A) The frequency of EGFR somatic alteration across multiple cancers including HNSCC. (B) The protein level of EGFR was measured in a panel of HNSCC cells by immunoblots. (C) The expression of EGFR and EGFRvIII in SCC25vIII and UM1vIII cells infected by lentivirus. (D) Growth curve showed that SCC25vIII and UM1vIII grew faster with or without EGF treatment. Data, mean ± SD, **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. (E) Colony formation assay indicated that the colony formation abilities of SCC25vIII and UM1vIII cells were significantly improved compared with parental cells. (F and G) SCC25vIII/UM1vIII exhibited de-sensitization to cisplatin treatment and decreased apoptosis using flow cytometry (F) and colony formation assay (G). CDDP, cisplatin.

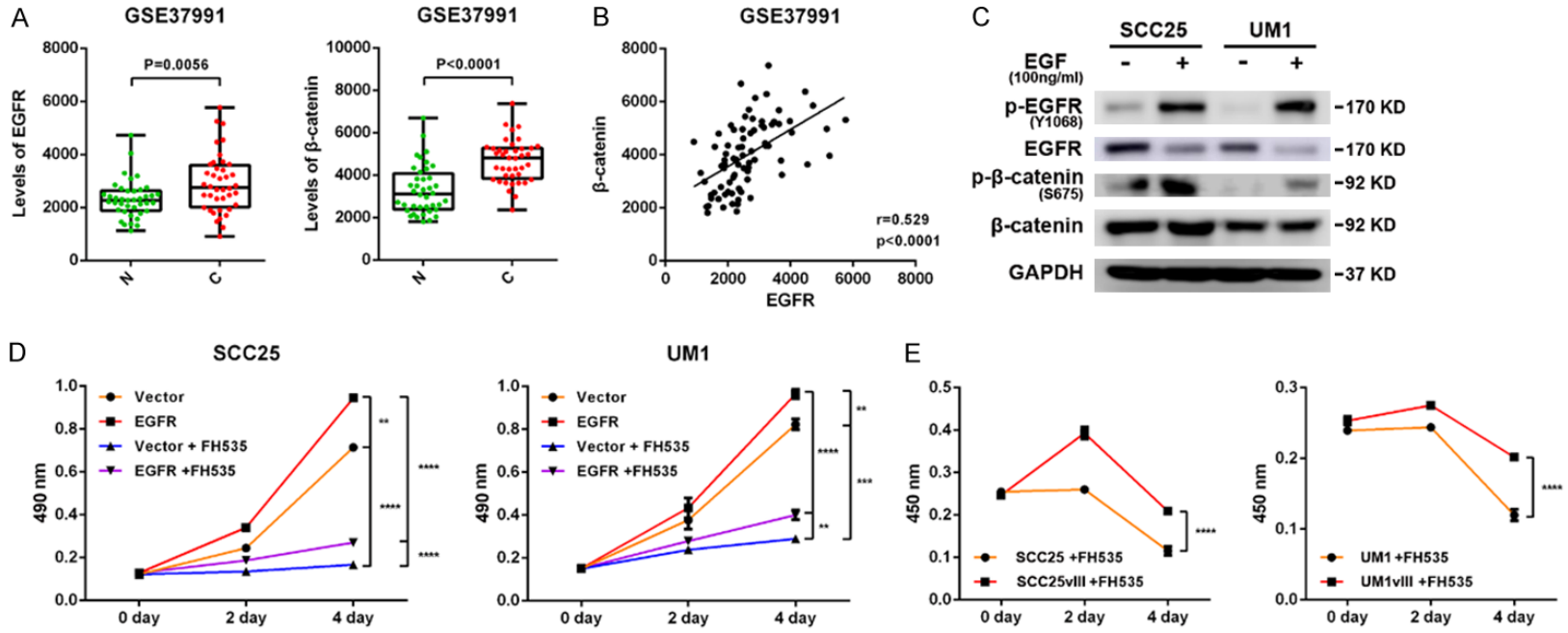


Figure 2. β-catenin pathway is involved in EGFRwt/vIII mediated aggressiveness in HNSCC cells. A. The analysis of GEO database (GSE37991) indicated that the expression of EGFR and β-catenin were both increased in HNSCC tissues. N, adjacent normal tissues, C, HNSCC tissues. B. GEO database revealed that EGFR correlated positively with β-catenin. C. EGF induced the activation of EGFR and the phosphorylation of β-catenin at S675 in HNSCC cells. D. MTT assay showed that the inhibition of β-catenin pathway by using FH535 attenuated cell proliferation induced by EGFR in HNSCC cells. E. MTS assay to detect the inhibition efficacy of FH535 in SCC25/UM1 or SCC25vIII/UM1vIII cells. Data in this figure, mean ± SD, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$.

increased significantly (**Figure 1F**, **1G**), which showed that EGFRwt/vIII weakened CDDP-mediated apoptosis.

β-catenin is implicated in aggressiveness mediated by EGFRwt/vIII in HNSCC cells

Previous studies have uncovered the cross-talk between Wnt/β-catenin and EGFR signaling [27]. β-catenin pathway is also reported to be associated with proliferation and CDDP sensitivity in HNSCC [28, 29]. Mining one available expression datasets (GSE37991) in Gene Expression Omnibus (GEO) validated that the expression of EGFR and β-catenin were both elevated in malignant head and neck specimens (**Figure 2A**), and revealed the positive correlation between them (**Figure 2B**). The activation of EGFR by EGF led to the phosphorylation of β-catenin in SCC25 and UM1 cells (**Figure 2C**). As a specific β-catenin inhibitor, FH535 represses recruitment of β-catenin and then antagonizes the transcription mediated by β-catenin/Tcf [30]. The treatment of FH535 hampered the cell growth induced by EGFR in HNSCC cells (**Figure 2D**). It's worth noting that the efficacy of pharmaceutical inhibition seemed to be declined in SCC25vIII/UM1vIII cells compared to SCC25/UM1 cells (**Figure 2E**), indicating that the β-catenin signaling of EGFRwt/vIII cells was more active than that of EGFRwt cells. Collectively, these data indicated that β-catenin pathway was essential for the malignance of HNSCC cells mediated by EGFRwt/vIII.

PKM2 positively correlates with the expression of β-catenin and indicates poor prognosis of HNSCC patients

In view of the importance of EGFRwt/vIII-β-catenin pathway in HNSCC cells, it is necessary to make clear the underlying mechanism between them. A key glycolysis enzyme, PKM2 is proved to be a bridge between EGFR and β-catenin in glioblastoma [20]. We assessed clinical properties of PKM2 expression in human HNSCC tissues firstly. Here, analysis of GEO data (GSE37991) demonstrated that PKM2 increased in HNSCC relative to adjacent normal tissues (**Figure 3A**), and the expression of PKM2 was positively correlated with that of β-catenin in both specimens and HNSCC cell lines (**Figure 3A**, **3B**), which was in line with the outcome of HNSCC dataset in TCGA analyzed by GEPIA (**Figure 3C**). In addition, higher expression of PKM2 indicated unfavorable prognosis of HNSCC patients based on the TCGA database (**Figure 3D**).

EGFRwt/vIII promotes the nuclear translocation of PKM2 in HNSCC cells

In addition to its glycolytic function in cytoplasm, PKM2 is induced translocation into the nucleus to mediate non-metabolic function [31]. Immunoblotting analysis showed that EGF treatment resulted in the nuclear accumulation of PKM2 in SCC25 and UM1 cells in a time-dependent manner (**Figure 4A**), which was reconfirmed by Immunofluorescence assay (**Figure 4B**). Besides, expression of EGFRvIII in SCC25 and UM1 cells resulted in a higher amount of nuclear PKM2 than was observed in parental cells, as well as an increased level of phosphorylated β-catenin (S675) (**Figure 4C**). Collectively, these results suggested that EGFRwt/vIII enhanced PKM2 nuclear translocation in HNSCC cells.

PKM2 R399/400 is crucial for its nuclear translocation and the activation of β-catenin in HNSCC cells

Yang W *et al.* demonstrated that NLS containing R399/400 in PKM2 is responsible for its nuclear translocation [21]. We established stable SCC25 and UM1 clones expressing wild-type PKM2 or R399/400A mutant, then verified the influence of these two amino acids towards subcellular location of PKM2. The results showed that PKM2 could accelerated phosphorylation of β-catenin at S675 in the nucleus than EV (Empty Vector) negative control in SCC25

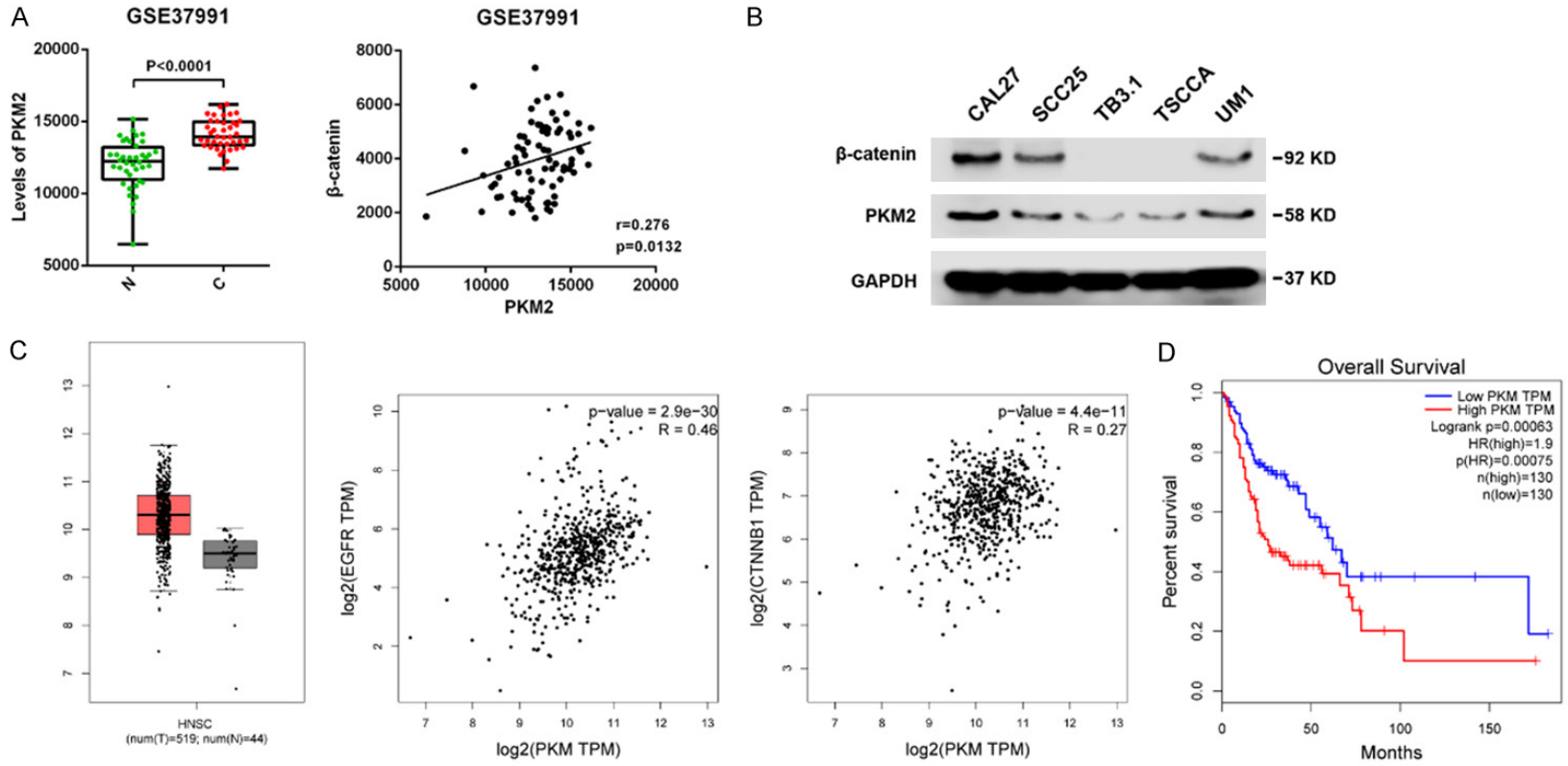


Figure 3. PKM2 correlates positively with β-catenin and indicates poor prognosis of HNSCC. A. The analysis of GEO database (GSE37991) indicated that PKM2 elevated in HNSCC tissues and correlated with β-catenin positively. N, adjacent normal tissues, C, HNSCC tissues. B. The protein levels of β-catenin and PKM2 were measured in a panel of HNSCC cells by immunoblots. C. TCGA data reconfirmed that PKM2 increased in HNSCC tissues and correlated with EGFR and β-catenin positively, which was analyzed by GEPIA. T, tumor samples, N, paired normal tissues, TPM, transcripts per million. D. Kaplan-Meier survival curve showed that higher PKM2 expression indicated worse prognosis.

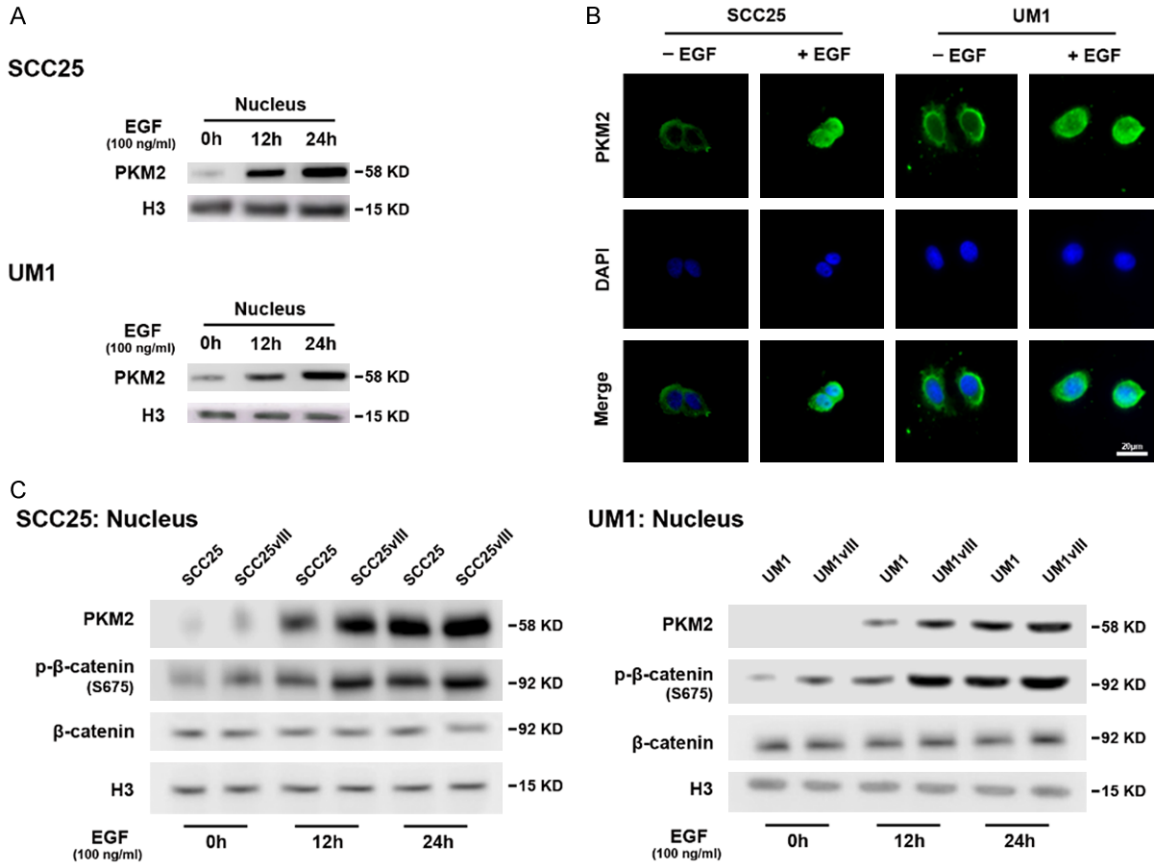


Figure 4. EGFRwt/vIII promotes the nuclear translocation of PKM2 in HNSCC cells. A. The analysis of cell fractionation indicated that EGF induced PKM2 nuclear translocation in a time-dependent manner. B. Immunofluorescence staining suggested that EGF treatment led to the accumulation of nuclear PKM2. Scale bars, 20 μm. C. The results of immunoblots showed that the nuclear translocation of PKM2 was more efficient in SCC25vIII/UM1vIII cells compared to SCC25/UM1 cells with EGF, as well as the activation of β-catenin.

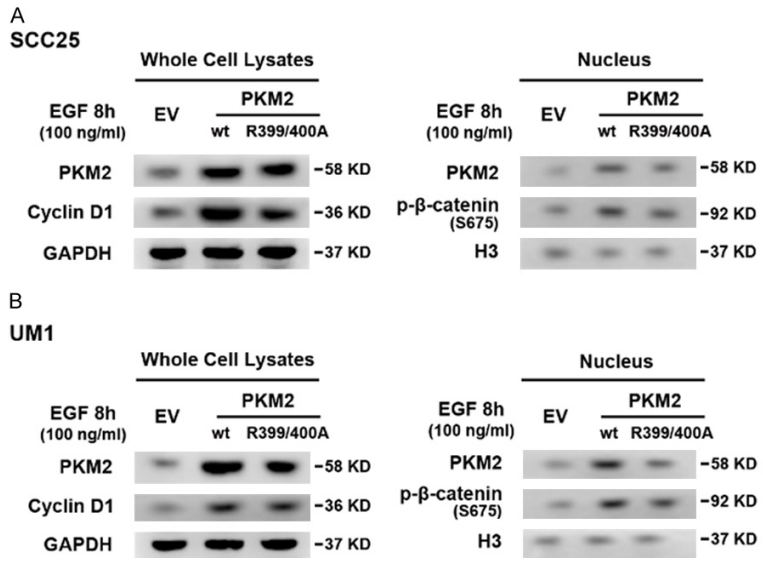


Figure 5. PKM2 R399/400 is essential for its nuclear translocation and the activation of β-catenin in HNSCC cells. (A and B) Immunoblots results of enforced expression of PKM2 and PKM2 R399/400A and the change of Cyclin D1 in stable SCC25 (A) and UM1 (B) cells (Left). Cell fractionation verified that nuclear PKM2 promoted the phosphorylation of β-catenin at S675, while the level of activated β-catenin was restrained in PKM2 R399/400A cells (Right). EV, empty vector.

cells (**Figure 5A**). The replacement of arginine with alanine at amino acids 399 and 400 affected little on the expression of PKM2, however cell fractionation illuminated that PKM2 R399/400A was failed to translocate into the nucleus upon EGF treatment (**Figure 5A**). In the meanwhile, R399/400A mutant reduced β-catenin phosphorylation at S675 in the nucleus compared with wild-type positive control, as well as the expression of Cyclin D1, which is one of downstream targets of β-catenin (**Figure 5A**). The resu-

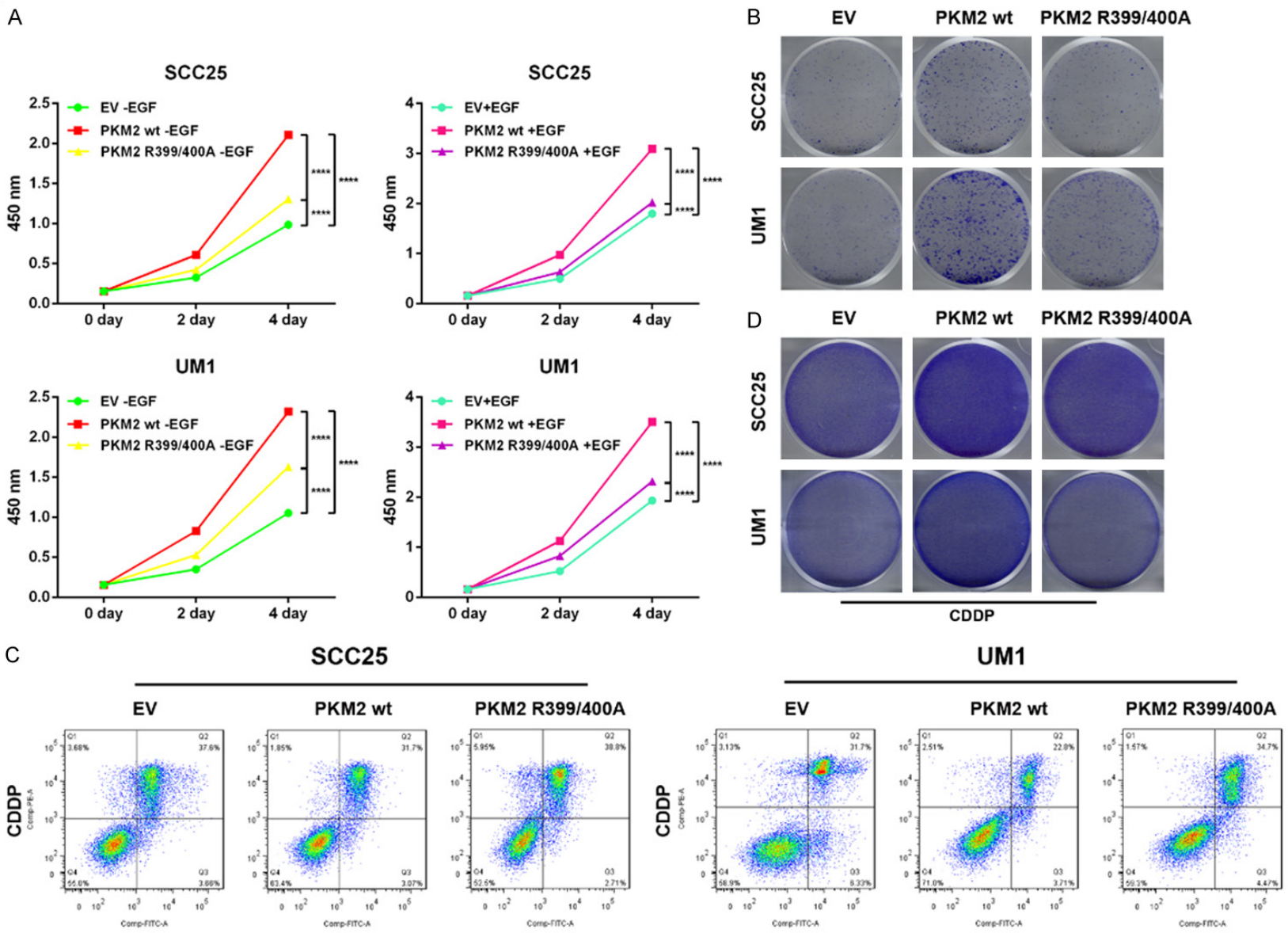


Figure 6. Nuclear PKM2 regulates the capacity of proliferation and chemo-sensitivity of HNSCC cells. (A) The growth curves of PKM2, PKM2 R399/400A stable clones and negative control with or without the treatment of EGF. Data, mean ± SD, ****P<0.0001. (B) Colony formation assay indicated that PKM2 facilitated colony formation ability of SCC25 and UM1 cells compared with EV and PKM2 R399/400A. (C and D) PKM2 de-sensitized HNSCC cells to cisplatin treatment and decreased apoptosis using flow cytometry (C) and colony formation assay (D) in relation to EV and PKM2 R399/400A. CDDP, cisplatin.

Its above were reconfirmed in UM1 cells by immunoblotting (**Figure 5B**). Taken together, these data revealed that R399/400 located in NLS of PKM2, was essential for EGF-induced nuclear translocation of PKM2 and activation of β-catenin in HNSCC cells.

Nuclear PKM2 regulates the capacity of proliferation and chemo-sensitivity of HNSCC cells

Next, we further explored whether PKM2 translocated into the nucleus held important implications for proliferation and chemo-sensitivity of HNSCC cells. When wild-type PKM2 expressed highly, the capacity of proliferation in SCC25 and UM1 cells was elevated notably with or without EGF treatment (**Figure 6A**). Furthermore, the cells above possessed stronger abilities of colony formation and CDDP-resistance than EV negative control (**Figure 6B-D**). Although stronger than EV, the proliferation and colony formation abilities of PKM2 R399/400A cells were decreased compared with PKM2 wt cells (**Figure 6A**, **6B**). As for chemo-sensitivity, there was no difference between PKM2 R399/400A mutant and EV group (**Figure 6C**, **6D**). These results together demonstrated that nuclear PKM2 regulated the proliferation and chemo-sensitivity of HNSCC cells.

Discussion

The appearance of targeted biologic products has revolutionized therapy for multiple cancers by focusing treatment on specific components implicated in aberrant signaling pathway. As one such target, EGFR is highly expressed in multiple tumors including HNSCC, and is reported to function as an oncogene and indicate poor prognosis [7]. Targeting the EGFR, with agents such as cetuximab, has achieved remarkable effect. However, the activation of alternative pathway and the presence of EGFR innate or acquired mutation lead to the resistance to targeted therapy. A truncated and constitutively active mutant, EGFRvIII signals upon self-phosphorylation and EGFR-dependent activation [13], commonly found in GBM. In HNSCC, co-expression of EGFR and EGFRvIII is also detected to contribute to growth and resistance to treatment [14], which was reconfirmed in this work. Even without the treatment of EGF, EGFRwt/vIII still promoted cell growth due to the self-activation of EGFRvIII. Once cultured with EGF, EGFRwt/vIII cells grew fastest among the groups, suggesting that co-expression of EGFR and EGFRvIII led to the increased activation of them, which is in line with previous study [32, 33]. However, the mechanism of EGFRwt/vIII mediated proliferation and chemo-sensitivity still needs to be further explored.

As a kind of receptor tyrosine kinase (RTK), EGFR initiates complex signaling cascades, once combined with its ligands. Except for some well-characterized pathways such as AKT and ERK signaling, the activation of STAT3 and β-catenin could also be induced by EGFR [34-36]. β-catenin is a multiple function protein depending on subcellular localization. As a key hub of canonical Wnt pathway, β-catenin is translocated to the nucleus where it binds TCF/LEF and recruits coactivators to initiate transcription of multiple genes and tumorigenesis [37]. β-catenin signaling is associated with cell proliferation, CDDP resistance and even poor prognosis in HNSCC. Interestingly, activated EGFR could also mediate the nuclear translocation of PKM2 to regulate β-catenin transactivation [20]. In our study, EGF/EGFR axis phosphorylated and activated β-catenin, while the level of p-β-catenin (S675) was higher in SCC-25vIII/UM1vIII cells. These results implied that β-catenin is essential for EGFRwt/vIII-dependent aggressiveness in HNSCC cells.

Pyruvate kinase (PK) catalyzes the final step in aerobic glycolysis to generate pyruvate and ATP. As one of the PK isoforms, PKM2 is implicated in tumorigenesis and progression of most cancers. One hand, PKM2 promotes metabolism to provide sufficient energy for cell rapid growth. On the other, the non-metabolic functions of PKM2 also seem to be indispensable. For instance, induced by EGF, PKM2 translocates into the nucleus and then interacts with β-catenin to regulate gene transcription. In view of the unknown character of PKM2 in HNSCC, we firstly analyzed GEO and TCGA databases to assess clinical properties of PKM2. Like in other tumors, significantly elevated PKM2 was detected in HNSCC tissues. Furthermore, Kaplan-Meier survival curve showed that higher PKM2 expression indicated worse outcome. Then, we found that EGF mediated activation of EGFR induced the nuclear translocation of PKM2. Specially, the accumulation of PKM2 and activated β-catenin in nucleus of SCC25vIII/UM1vIII cells was much more than that of SCC25/UM1 cells, on account

of the higher activity of EGFRwt/vIII. Moreover, although R399/400 in NLS of PKM2 were particularly critical, the mutation of them failed to switch off the nuclear translocation of PKM2 completely. But, once the level of nuclear PKM2 became lower, the phosphorylation of β-catenin at S675 was restrained and the augment of proliferation and chemo-desensitivity was decreased.

In conclusion, we demonstrate that EGFRwt/vIII affects cell proliferation and chemo-sensitivity of HNSCC via inducing PKM2 nuclear translocation and then promoting the activation of β-catenin pathway. Furthermore, the expression of PKM2 is aberrantly increased in HNSCC and indicates poor prognosis. These findings reveal the critical role of EGFRwt/vIII-PKM2-β-catenin cascade in HNSCC proliferation and chemo-sensitivity and provide PKM2 as a candidate for diagnosis and therapy of HNSCC.

Acknowledgements

This work was supported by National Natural Science Foundation of China (81702420, 81-572492) and CSCO-Merck Serono Oncology Research Fund, SCORE (Y-MT2015-017).

Disclosure of conflict of interest

None.

Address correspondence to: Xuan Zhou, Department of Maxillofacial and Otorhinolaryngological Oncology, Tianjin Medical University Cancer Institute and Hospital, Tianjin 300060, China; Key Laboratory of Cancer Prevention and Therapy, Tianjin Cancer Institute, Tianjin 300060, China; National Clinical Research Center of Cancer, Tianjin 300060, China. Tel: (86) 22-23340123 Ext. 3137; Fax: (86) 22-23359984; E-mail: byron2000zhou@sina.com

References

[1] Posner M and Vermorken JB. Induction therapy in the modern era of combined-modality therapy for locally advanced head and neck cancer. Semin Oncol 2008; 35: 221-8.

[2] Lam L, Logan RM and Luke C. Epidemiological analysis of tongue cancer in South Australia for the 24-year period, 1977-2001. Aust Dent J 2006; 51: 16-22.

[3] Shen DW, Pouliot LM, Hall MD and Gottesman MM. Cisplatin resistance: a cellular self-defense mechanism resulting from multiple epigenetic and genetic changes. Pharmacol Rev 2012; 64: 706-21.

[4] Belbin TJ, Singh B, Barber I, Socci N, Wenig B, Smith R, Prystowsky MB and Childs G. Molecular classification of head and neck squamous cell carcinoma using cDNA microarrays. Cancer Res 2002; 62: 1184-90.

[5] Chung CH, Parker JS, Karaca G, Wu J, Funkhouser WK, Moore D, Butterfoss D, Xiang D, Zanation A, Yin X, Shockley WW, Weissler MC, Dressler LG, Shores CG, Yarbrough WG and Perou CM. Molecular classification of head and neck squamous cell carcinomas using patterns of gene expression. Cancer Cell 2004; 5: 489-500.

[6] Kalyankrishna S and Grandis JR. Epidermal growth factor receptor biology in head and neck cancer. J Clin Oncol 2006; 24: 2666-72.

[7] Ang KK, Berkey BA, Tu X, Zhang HZ, Katz R, Hammond EH, Fu KK and Milas L. Impact of epidermal growth factor receptor expression on survival and pattern of relapse in patients with advanced head and neck carcinoma. Cancer Res 2002; 62: 7350-6.

[8] Chung CH, Ely K, McGavran L, Varella-Garcia M, Parker J, Parker N, Jarrett C, Carter J, Murphy BA, Netterville J, Burkey BB, Sinard R, Cmelak A, Levy S, Yarbrough WG, Slebos RJ and Hirsch FR. Increased epidermal growth factor receptor gene copy number is associated with poor prognosis in head and neck squamous cell carcinomas. J Clin Oncol 2006; 24: 4170-6.

[9] Cancer Genome Atlas Network. Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature 2015; 517: 576-82.

[10] Hatanpaa KJ, Burma S, Zhao D and Habib AA. Epidermal growth factor receptor in glioma: signal transduction, neuropathology, imaging, and radioresistance. Neoplasia 2010; 12: 675-84.

[11] Hynes NE and Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. Nat Rev Cancer 2005; 5: 341-54.

[12] Pedersen MW, Meltorn M, Damstrup L and Poulsen HS. The type III epidermal growth factor receptor mutation. Biological significance and potential target for anti-cancer therapy. Ann Oncol 2001; 12: 745-60.

[13] Huang HS, Nagane M, Klingbeil CK, Lin H, Nishikawa R, Ji XD, Huang CM, Gill GN, Wiley HS and Cavenee WK. The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling. J Biol Chem 1997; 272: 2927-35.

[14] Sok JC, Coppelli FM, Thomas SM, Lango MN, Xi S, Hunt JL, Freilino ML, Graner MW, Wikstrand CJ, Bigner DD, Gooding WE, Furnari FB and

Grandis JR. Mutant epidermal growth factor receptor (EGFRvIII) contributes to head and neck cancer growth and resistance to EGFR targeting. Clin Cancer Res 2006; 12: 5064-73.
[15] Kim JW and Dang CV. Cancer's molecular sweet tooth and the Warburg effect. Cancer Res 2006; 66: 8927-30.
[16] Gatenby RA and Gillies RJ. Why do cancers have high aerobic glycolysis? Nat Rev Cancer 2004; 4: 891-9.
[17] Altenberg B and Greulich KO. Genes of glycolysis are ubiquitously overexpressed in 24 cancer classes. Genomics 2004; 84: 1014-20.
[18] Yang W, Xia Y, Hawke D, Li X, Liang J, Xing D, Aldape K, Hunter T, Alfred Yung WK and Lu Z. PKM2 phosphorylates histone H3 and promotes gene transcription and tumorigenesis. Cell 2012; 150: 685-96.
[19] Yang W and Lu Z. Regulation and function of pyruvate kinase M2 in cancer. Cancer Lett 2013; 339: 153-8.
[20] Yang W, Xia Y, Ji H, Zheng Y, Liang J, Huang W, Gao X, Aldape K and Lu Z. Nuclear PKM2 regulates beta-catenin transactivation upon EGFR activation. Nature 2011; 480: 118-22.
[21] Yang W, Zheng Y, Xia Y, Ji H, Chen X, Guo F, Lyssiotis CA, Aldape K, Cantley LC and Lu Z. ERK1/2-dependent phosphorylation and nuclear translocation of PKM2 promotes the Warburg effect. Nat Cell Biol 2012; 14: 1295-304.
[22] Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E, Cerami E, Sander C and Schultz N. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal 2013; 6: pl1.
[23] Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, Antipin Y, Reva B, Goldberg AP, Sander C and Schultz N. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2012; 2: 401-4.
[24] Zhou X, Ren Y, Liu A, Jin R, Jiang Q, Huang Y, Kong L, Wang X and Zhang L. WP1066 sensitizes oral squamous cell carcinoma cells to cisplatin by targeting STAT3/miR-21 axis. Sci Rep 2014; 4: 7461.
[25] Nyati MK, Morgan MA, Feng FY and Lawrence TS. Integration of EGFR inhibitors with radiochemotherapy. Nat Rev Cancer 2006; 6: 876-85.
[26] Tinhofer I, Klinghammer K, Weichert W, Knodler M, Stenzinger A, Gauler T, Budach V and Keilholz U. Expression of amphiregulin and EGFRvIII affect outcome of patients with squamous cell carcinoma of the head and neck receiving cetuximab-docetaxel treatment. Clin Cancer Res 2011; 17: 5197-204.
[27] Hu T and Li C. Convergence between Wnt-beta-catenin and EGFR signaling in cancer. Mol Cancer 2010; 9: 236.
[28] Lee SH, Koo BS, Kim JM, Huang S, Rho YS, Bae WJ, Kang HJ, Kim YS, Moon JH and Lim YC. Wnt/beta-catenin signalling maintains self-renewal and tumourigenicity of head and neck squamous cell carcinoma stem-like cells by activating Oct4. J Pathol 2014; 234: 99-107.
[29] Li L, Liu HC, Wang C, Liu X, Hu FC, Xie N, Lu L, Chen X and Huang HZ. Overexpression of beta-catenin induces cisplatin resistance in oral squamous cell carcinoma. Biomed Res Int 2016; 2016: 5378567.
[30] Handeli S and Simon JA. A small-molecule inhibitor of Tcf/beta-catenin signaling down-regulates PPARgamma and PPARdelta activities. Mol Cancer Ther 2008; 7: 521-9.
[31] Tamada M, Suematsu M and Saya H. Pyruvate kinase M2: multiple faces for conferring benefits on cancer cells. Clin Cancer Res 2012; 18: 5554-61.
[32] Fan QW, Cheng CK, Gustafson WC, Charron E, Zipper P, Wong RA, Chen J, Lau J, Knobbe-Thomsen C, Weller M, Jura N, Reifenberger G, Shokat KM and Weiss WA. EGFR phosphorylates tumor-derived EGFRvIII driving STAT3/5 and progression in glioblastoma. Cancer Cell 2013; 24: 438-49.
[33] Li L, Chakraborty S, Yang CR, Hatanpaa KJ, Cipher DJ, Puliyappadamba VT, Rehman A, Jiwani AJ, Mickey B, Madden C, Raisanen J, Burma S, Saha D, Wang Z, Pingle SC, Kesari S, Boothman DA and Habib AA. An EGFR wild type-EGFRvIII-HB-EGF feed-forward loop regulates the activation of EGFRvIII. Oncogene 2014; 33: 4253-64.
[34] Chakraborty S, Li L, Puliyappadamba VT, Guo G, Hatanpaa KJ, Mickey B, Souza RF, Vo P, Herz J, Chen MR, Boothman DA, Pandita TK, Wang DH, Sen GC and Habib AA. Constitutive and ligand-induced EGFR signalling triggers distinct and mutually exclusive downstream signalling networks. Nat Commun 2014; 5: 5811.
[35] Chan KS, Carbajal S, Kiguchi K, Clifford J, Sano S and DiGiovanni J. Epidermal growth factor receptor-mediated activation of Stat3 during multistage skin carcinogenesis. Cancer Res 2004; 64: 2382-9.
[36] Ma L, Zhang G, Miao XB, Deng XB, Wu Y, Liu Y, Jin ZR, Li XQ, Liu QZ, Sun DX, Testa JR, Yao KT and Xiao GH. Cancer stem-like cell properties are regulated by EGFR/AKT/beta-catenin signaling and preferentially inhibited by gefitinib in nasopharyngeal carcinoma. FEBS J 2013; 280: 2027-41.
[37] Clevers H and Nusse R. Wnt/beta-catenin signaling and disease. Cell 2012; 149: 1192-205.

Supplementary Table 1. List of primers used in this investigation

Application	Sequence
Clones	
PKM2	F: TTGGATCCGCCACCATGTCGAAGCCCCATAGTGAAGCC
	R: TTCTCGAGCGGCACAGGAACAACACGCA
Mutations	
PKM2 R399/400A mut	F: CAATTATTTGAGGAACTCGCCGCCCTGGCGCCCATTACCAGC
	R: GCTGGTAATGGGCGCCAGGGCGGCGAGTTCCTCAAATAATTG

Supplementary Table 2. Primary antibodies used in this investigation

Primary antibody	Catalog	Vendor	Application
EGFR	2232S	Cell Signaling	WB
p-EGFR (Y1068)	3777S	Cell Signaling	WB
β-catenin	8480S	Cell Signaling	WB
p-β-catenin (S675)	4176S	Cell Signaling	WB
Cyclin D1	2922S	Cell Signaling	WB
PKM2	15822-1-AP	Proteintech	WB/IF
H3	17168-1-AP	Proteintech	WB
GAPDH	G8795	Sigma-Aldrich	WB