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Role of integrin β1 as a biomarker of stemness in head and neck squamous cell carcinoma

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ABSTRACT

Objectives: Signaling between cancer stem cells (CSC) and their extracellular matrix has a crucial role in CSC progression and maintenance. However, mediators of this signaling pathway in head and neck squamous cell carcinoma (HNSCC) are largely unknown. Here, we explored whether integrin β1, which is one of the key regulators of the communication between cells and their microenvironment, affected the stemness of HNSCC cells.

Materials and methods: We examined self-renewal capacity, chemoresistance, and xenograft tumorigenicity after knockdown of integrin β1 in primary HNSCC cells. In addition, we studied the role of focal adhesion kinase (FAK), an intracellular downstream molecule of integrin signaling, in influencing stemness of HNSCC. The relevance of Notch1 and integrin β1 interactions in HNSCC cells was also examined. Finally, immunohistochemical analysis was carried out to test whether the coexpression of integrin β1 and Notch1 in the samples from HNSCC patients correlated with their survival.

Results: Targeting integrin β1 in HNSCC cells inhibited self-renewal, chemoresistance, and *in vivo* tumor-forming capacity. Treatment with an inhibitor of FAK decreased self-renewal capacities and expression of various putative stem cell markers (Oct4, Sox2, and Nanog) in a dose-dependent manner. Moreover, knockdown of integrin β1 decreased the expression of Notch1 and its target genes (Hey1 and Hes1). Notably, HNSCC patients demonstrating simultaneous expression of integrin β1 and Notch1 in their tissue samples had significantly worse survival rate.

Conclusion: Integrin β1/Notch1 axis has a significant role in the regulation of stemness in HNSCC.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most prevalent cancer worldwide, with an annual incidence of more than 50,000 cases in the United States [1]. Despite diagnostic and therapeutic developments for the cure of HNSCC, overall survival rate in the patients has shown only a marginal improvement over the past two decades [2]. Therefore, a deeper understanding of the tumorigenesis of HNSCC, which will facilitate the development of novel therapeutic targets for eradication of this dreadful cancer, is urgently needed.

HNSCC encompasses diverse and heterogeneous cells [3]. Cancer stem cells (CSCs) or cancer initiating cells, functionally defined by profound self-renewal and differentiation abilities and potential *in vivo* tumorigenicity, have been suggested as a critical modulator of the heterogeneity among HNSCC cells [4]. In a clinical setting, this concept has greater importance in that these CSCs show increased treatment resistance, resulting in tumor recurrence and metastasis, which are the major causes of death in HNSCC patients [3]. Therefore, identification

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of novel biomarkers that regulate the oncogenic capacity of CSCs in HNSCC may provide new therapeutic targets for improved management of HNSCC.

Integrins constitute a family of cell surface receptors involved in mediating interactions of a cell with the surrounding cells and extracellular matrix [5]. Each integrin is a heterodimeric protein complex consisting of an α and a β subunit, both of which are transmembrane glycoproteins with a single membrane-spanning segment and a short cytoplasmic domain [6]. Eighteen α subunits and eight β subunits are found in the human genome [7].

Integrins activate the intracellular signaling pathway in response to the diverse oncogenic stimuli, such as activated cytokine receptors or growth factor receptors. Consequently, tumor proliferation and aggravation may rely on integrin crosstalk with growth factor receptors or oncogenes in both tumor cells and their microenvironment [8]. Of the integrin subunits, tumor cell expression of β1, which interacts with various α subunits (αv, α5, α6, and α4), is correlated with disease progression in various tumors [8]. In addition, inactivation of integrin β1 significantly attenuates self-renewal ability of mammalian stem cells. The integrin β1 signaling pathway has been suggested as an essential pathway for metastasis and therapeutic resistance, which is a clinical feature of cancer stem cells, in HNSCC. Accordingly, Lim et al reported that the expression of β1 in oral cancer cells increased their cancer stemness-associated features, including the formation of holoclonal cell colonies and tumor spheres [9]. Koukourakis et al also suggested that the most potent stem cell marker in HNSCCs that affected both local control and survival, independently of all histopathological variables, was integrin β1. Therefore, we hypothesize that integrin β1 is more involved in cancer stemness phenotype of HNSCC than any other subunits of integrin [10]. However, the biological role of integrin β1 affecting stemness of HNSCC remains unclear. In the present study, we tried to examine whether integrin β1 increased the stemness phenotype of HNSCC.

Materials and methods

Cells and reagents

We used two human primary HNSCC-derived spheroid cells (KU-SCC1 and KU-SCC3) and have previously reported on the characteristics of these cell lines in view of CSC phenotype [4]. Briefly, these cells have self-renewal capacities, chemoresistance, and xenograft tumorigenicity as traits seen in CSC [4]. These cells were cultured in serum-free DMEM/F12 medium supplemented with N2 (GIBCO BRL, Grand Island, NY, USA), B27 (GIBCO), human recombinant epidermal growth factor (20 ng/ml; R&D Systems, Minneapolis, MN, USA), and human basic fibroblast growth factor (20 ng/ml; R&D Systems).

Knockdown of integrin β1 gene

Lentiviral construct expressing integrin β1-shRNA or scramble was obtained commercially (Sigma-Aldrich). Viral particles were produced in 293 T cells with packaging plasmid. HNSCC cells were transduced with integrin β1 shRNA lentiviruses. We also verified the knockdown of integrin β1 protein expression by western blotting.

Sphere forming assay

Primary HNSCC cells were dissociated into single cells and seeded in a 12-well plate at a density of 500 cells/well. Two weeks after plating, spheres with a diameter over 10 μm were counted.

CD44 expression by FACS

HNSCC cells were dissociated into single cells, washed, and suspended in phosphate buffered saline (PBS). Cells were labeled with anti-CD44 and fluorescein isothiocyanate-labeled secondary antibodies and then subjected to flow cytometry using a FACSCalibur machine (BD Biosciences, Franklin Lakes, NJ, USA).

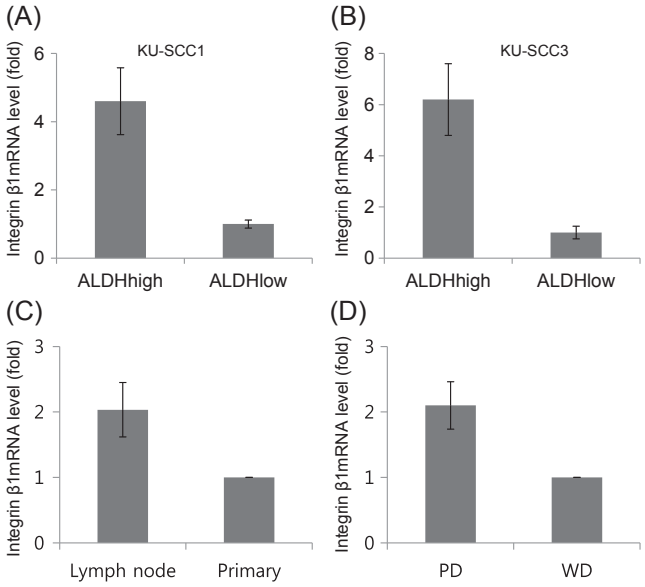


Fig. 1. Levels of integrin β1 expression are associated with aggressive phenotype of HNSCC cells. (A and B) mRNA levels of integrin β1 in primary ALDH^{high} or ALDH^{low} HNSCC cells. Data represent the means ± SD (N = 3). (C) mRNA levels of integrin β1 in tissue originated from metastatic lymph node or primary tumor lesion. Data represent the means ± SD (N = 3). Lymph node, metastatic lymph node tissues; Primary, primary tumor tissues. (D) mRNA levels of integrin β1 in poorly-differentiated or well-differentiated HNSCC tissue. PD, poorly-differentiated tumor tissue; WD, well-differentiated tumor tissue. Data represent the means ± SD (N = 3).

Evaluation of ALDH activity and isolation of ALDH^{high} and ALDH^{low} cells from HNSCC cells

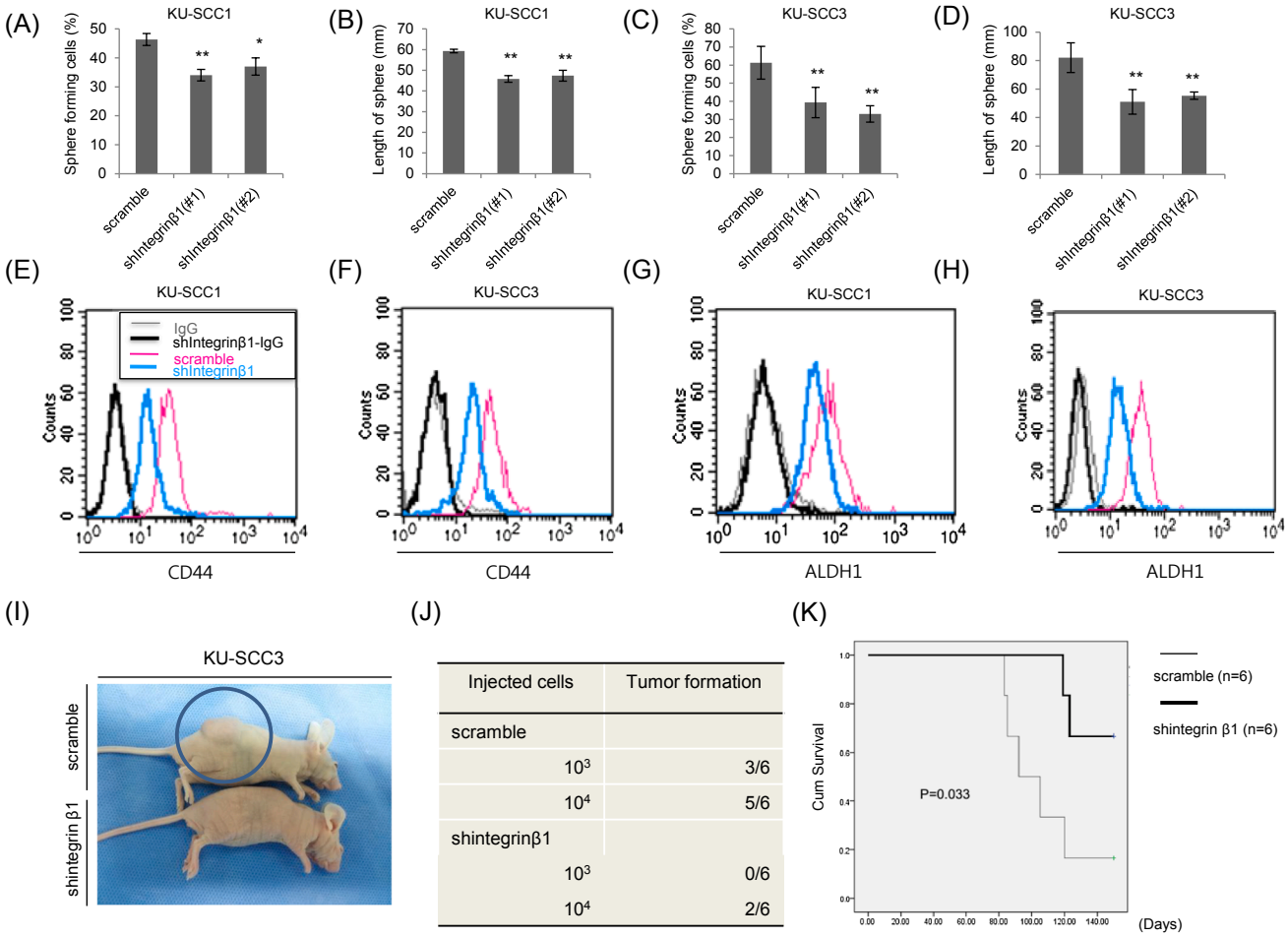
Cells from freshly dissociated HNSCC cells were analyzed using the Aldefluor assay kit (Stem Cell Technologies, Durham, NC, USA). Cells were suspended in Aldefluor assay-containing ALDH substrate (BAAA, 1 M per 1×10^6 cells). A sample of cells was stained under specific ALDH inhibitor, diethylaminobenzaldehyde, as a negative control. Flow cytometry-based sorting was conducted using a FACS Aria (Becton Dickinson, CA). The sorting gates were established using as negative controls the cells stained with PI only, for viability, the antibody alone.

Chemosensitivity assay

HNSCC cells were plated in a 96-well plate at a density of 7×10^3 cells per well and then treated with either cisplatin at various concentrations or DMSO. Two days later, 20 μl of 3-(4,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml in PBS) was added to each well. Plates were placed at room temperature for 3 h. Absorbance at 570 nm was measured using SpectraMax 190 (Molecular Devices, Sunnyvale, CA, USA).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA (3 μg) was reverse transcribed using the Reverse Transcriptase (RT) kit (Fermentas, Glen Burnie, MD, USA), according to the manufacturer's instructions. For semi-quantitative RT-PCR, the synthesized cDNA was added to a mixture of 1 U of Taq DNA polymerase (Roche Diagnostics, Indianapolis, IN, USA) and specific primers, and was amplified on the MJ Research Minicycler™ (Bio-Rad Laboratories, Waltham, MA, USA). PCR products were separated by



Injected cells	Tumor formation
scramble	
10^3	3/6
10^4	5/6
shintegrinβ1	
10^3	0/6
10^4	2/6

Fig. 2. Knockdown of integrin β1 decreases stemness of primary HNSCC cells. (A–D) Sphere-forming capacity of primary HNSCC cells transduced with shintegrin β1 or control vector. Data represent the means ± SD (N = 3). (*P < 0.05, **P < 0.01). (E and F) Expression levels of CD44 (a putative HNSCC marker) in primary HNSCC cells transduced with shintegrin β1 or control vector. (G and H) ALDH activity levels in primary HNSCC cells transduced with shintegrin β1 or control vector. (I and J) *In vivo* propagation of HNSCC cells in nude mice by xenografts of HNSCC cells transduced with either shScramble or shintegrin β1 construct. Representative pictures showing a tumor in a mouse at the cell injection site. (K) Kaplan-Meier survival curves for mice implanted with HNSCC cells transduced with either shScramble or shintegrin β1 construct (N = 6).

1.5% agarose gel electrophoresis and detected under ultraviolet light (Bio-Rad, Hercules, CA, USA). In addition, real-time reverse transcription-PCR (RT-PCR) analysis was subsequently carried out on the iCycler IQ real-time detection system (Bio-Rad, Hercules, CA, USA), using IQ Supermix with SYBR-Green (Bio-Rad). The sequences of human gene specific primers were depicted in Supplementary Table S1.

Western blotting

Cells were lysed in RIPA buffer (50 mM Tris-HCl of pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, mixture of protease inhibitors, and mixture of phosphatase inhibitors) on ice for 20 min. The lysates were centrifuged at 14,000 rpm and 4 °C for 20 min, and the protein concentration of the clear lysates was determined using the Coomassie (Bradford) protein assay kit (Pierce, Rockford, IL, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to resolve the proteins, which were transferred to the polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) at room temperature for 1 h, and then incubated with primary antibodies overnight in a cold room at 4 °C. Next day, the membrane was washed with TBST and incubated with the corresponding horseradish peroxidase-conjugated secondary antibody for 1 h. Finally, the immunoreactive bands were visualized by enhanced chemiluminescence detection.

Side population assay

The side population assay was performed as described previously [11]. Briefly, primary HNSCC cells were dissociated into single cells, and resuspended at a density of 1×10^6 cells per ml in a pre-warmed serum-free DMEM/F12 medium. Cells were treated with Hoechst 33,342 dye at a final concentration of 5 μg/ml. After mixing, cells were placed in 37 °C water bath for 90 min. Suspended cells were treated with propidium iodide (2 μg/ml) to exclude dead cells, and were analyzed using flow cytometry.

Xenograft tumorigenicity

All procedures involving animals were performed according to our institution's guidelines for the use of laboratory animals. The indicated number of dissociated HNSCC-control cells (10^3 and 10^4 cells) or HNSCC-shIntegrin β1 cells (10^3 and 10^4 cells) were suspended in serum-free DMEM/F12 with growth factor and Matrigel (Sigma-Aldrich) mixture (1:1 volume), or in DMEM/F12 with 10% FBS and Matrigel mixture (1:1 volume). Subsequently, each cell type was injected into the flank of 8-week-old female BALB/c nude mice using a

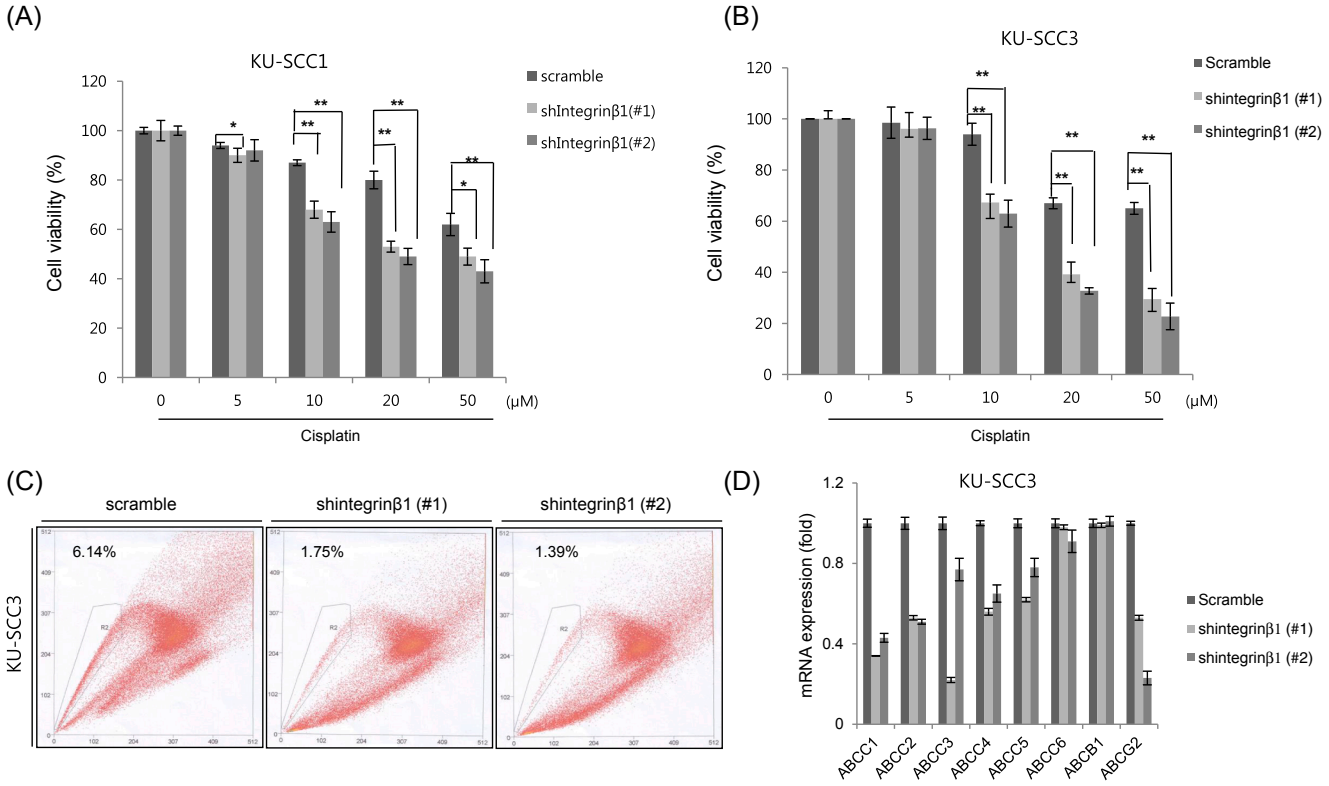


Fig. 3. Knockdown of integrin β1 increases cisplatin susceptibility of primary HNSCC cells. (A and B) MTT assay after administration of cisplatin at the indicated concentrations in primary HNSCC cells transduced with shintegrin β1 or control vector. Data represent the means ± SD (N = 3). (*P < 0.05, **P < 0.01). (C) Percentage of side population cells in primary HNSCC cells transduced with shintegrin β1 or control vector. (D) mRNA levels of various ABC transporter genes in primary HNSCC cells transfected with shintegrin β1 or control vector. Data represent the means ± SD (N = 3).

22-gauge needle. Engrafted mice were inspected weekly for tumor appearance by visual observation and palpation until post injection 8 weeks. All animal studies were approved by the Institutional Animal Care and Use Committee of Konkuk University.

Luciferase assay

Notch1 transcriptional activity in HNSCC cells treated with integrin β1 overexpression or control vector was determined by analyzing the relative luciferase activities of pGL3-CSL construct containing Notch1 promoter sequence (1.5 kb upstream the transcription start site) using the Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA). Transfection efficiency was normalized with the activity of Renilla luciferase, according to the instructions of the manufacturer.

Immunohistochemistry

Immunostaining for integrin β1 and Notch1 was carried out on tissue microarray slides consisting of 71 HNSCC tissue samples using antibodies against integrin β1 (dilution 1/400; ab3167: Abcam, MA, USA) and Notch1 (dilution 1/100; ab52627: Abcam, MA, USA). Considering the limitations of the representative areas of the tumor, we used duplicate 2-mm-diameter tissue cores from each donor block. Immunostaining was performed using a compact polymer method (Bond Intense detection kit, Leica Biosystems, Newcastle, UK). The results of the immunohistochemistry were interpreted in an unbiased way by independent 2 pathologists. Integrin β1 and Notch1 staining intensity was scored on a scale of 0–3 (0: negative; 1: weak; 2: moderate; 3: strong). When a discrepancy occurred between duplicate cores, higher score from the two tissue cores was used as the final score. For the statistical analysis, integrin β1 and Notch1 expression was scored as low (negative and weak) or high (moderate and strong).

Statistical analyses

All analyses were performed using SPSS 12.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was assessed using a two-tailed Student's *t*-test or ANOVA test. The association between integrin β1 and Notch1 expression and corresponding clinical parameters was analyzed by the Fisher's exact test. The survival analysis in human and mice samples was performed using the Kaplan-Meier survival curve and log-rank test. A p value less than 0.05 was considered statistically significant.

Results

Integrin β1 expression is increased in HNSCC stem-like cells

ALDH activity has been successfully used as a surrogate marker to enrich CSC populations in a variety of cancers including HNSCC [12]. Thus, we isolated $ALDH^{high}$ and $ALDH^{low}$ cells from primary HNSCC cells using FACS sorting via the ALDEFLUOR assay, and then evaluated integrin β1 mRNA levels in these cells. As shown in Fig. 1A & B, a significant increase in integrin β1 mRNA levels was observed in $ALDH^{high}$ HNSCC cells. CSC is known to critical activator of tumor metastasis [13]. Thus, we screened expression level of integrin β1 in either metastatic lymph node or primary tumor lesion in HNSCC patients sample, suggesting that integrin β1 mRNA levels in metastatic lymph node tissues were elevated compared to those in primary cancer tissues of same patients (Fig. 1C). It indicates that integrin β1 expression level is linked to tumor metastasis. In addition, we found that aggressive and poorly differentiated tumor tissues display a higher expression of integrin β1 mRNA than that of well-differentiated tumor tissues (Fig. 1D). Altogether, integrin β1 expression may be associated with the stemness phenotype of HNSCC cells.

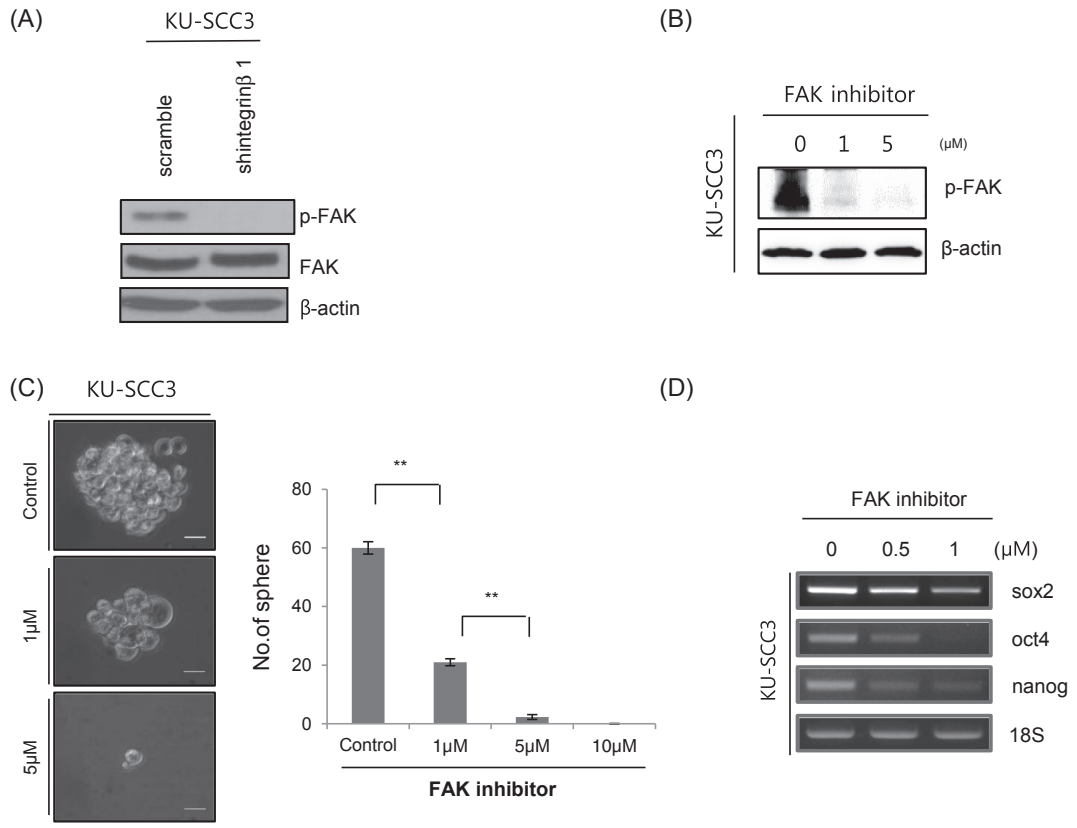


Fig. 4. Effect of suppression of integrin β1 on HNSCC stemness is mediated by suppression of FAK. (A) Protein level of p-FAK in primary HNSCC cells transduced with either shScramble or shintegrin β1 construct. (B) Protein level of p-FAK in primary HNSCC cells after treatment with FAK inhibitor. (C) Sphere-forming capacity of primary HNSCC cells after treatment with FAK inhibitor. Data represent the means ± SD (N = 3). (**P < 0.01). (D) mRNA levels of putative stemness markers (Oct4, Sox2, and Nanog) after treatment with FAK inhibitor.

Targeting integrin β1 attenuates the stemness phenotype of HNSCC cells

To elucidate the effect of integrin β1 on stemness of HNSCC cells, we established stable integrin β1 knockdown-HNSCC cells using lentiviral system (Supplementary Fig. 1). We observed a significant decrease in self-renewal capacity of HNSCC cells, compared with that of a non-targeted control, when integrin β1 was targeted with shRNA (Fig. 2A–D & Supplementary Fig. 2). Targeting integrin β1 also decreased the expression of Oct4 and SOX2 proteins, which are putative embryonic stem cell markers (Supplementary Fig. 3). Furthermore, both CD44 expression (Fig. 2E & F) and ALDH activity (Fig. 2G & H) were significantly decreased in HNSCC cells with shintegrin β1 construct. In addition, the most important property of CSCs is their potential to propagate tumors *in vivo* [4]. To address this issue, we examined the effects of integrin β1 downregulation on tumor-propagating capacity of HNSCC cells. HNSCC cells transfected with shintegrin β1 or control shRNA were transplanted into the flank of immunocompromised mice. Mice bearing HNSCC cells that express shintegrin β1 displayed reduced tumor formation and increased survival relative to those bearing HNSCC cells that express control shRNA (Fig. 2I–K). Taken together, these results indicated that the suppression of integrin β1 decreased stemness of HNSCC cells.

Knockdown of integrin β1 increases susceptibility of HNSCC cells to cisplatin therapy

The accumulating evidence suggested that CSC might contribute to drug resistance in cancer [14]. Therefore, we evaluated cisplatin susceptibility in response to the knockdown of integrin β1 in HNSCC cells. Knockdown of integrin β1 increased cisplatin susceptibility of HNSCC cells (Fig. 3A & B). Side population cells are a subset of cells that can expel the DNA fluorescent dye Hoechst 33,342 and are rich in CSCs [15]. We measured the percentage of side population cells after knockdown of integrin β1 in HNSCC cells. A significant reduction in these cells was observed in HNSCC cells with suppressed integrin β1 (Fig. 3C). Next, we studied expression level of various ABC transporters in these cells because one of mechanisms of CSC-related chemoresistance is the drug efflux by ABC transporters. We have demonstrated that suppression of integrin β1 decreases mRNA levels of various ABC transporters (Fig. 3D). All these results indicated that integrin β1 expression was linked to cisplatin resistance.

Focal adhesion kinase (FAK) is the main mediator of the integrin β1 signaling pathway affecting stemness of HNSCC cells

It was interesting to identify the intracellular molecule that is the major effector in the integrin β1-mediated stemness pathway in HNSCC cells. FAK is a cytoplasmic tyrosine kinase identified as the key mediator of intracellular signaling by integrins, which belong to a major family of cell surface receptors and facilitate communication with the extracellular matrix, in the regulation of different cellular functions [16]. FAK have also been found to play crucial roles in the maintenance of mammalian stem cells in studies using mouse models, suggesting that integrin signaling through FAK may serve as a functional marker for mammalian stem cells [9]. Therefore, we hypothesized that FAK may have a critical role in maintaining stemness of HNSCC cells as a downstream molecule of integrin β1. Integrin β1 knockdown suppressed phosphorylation of FAK in HNSCC cells (Fig. 4A). Importantly, treatment with FAK inhibitor (Fig. 4B) decreased self-renewal capacity (Fig. 4C) and expression of various putative stem cell markers (Oct4, Sox2, and Nanog) in a dose-dependent manner (Fig. 4D). Altogether, these results suggested that integrin β1 affected stemness of HNSCC cells via FAK activation.

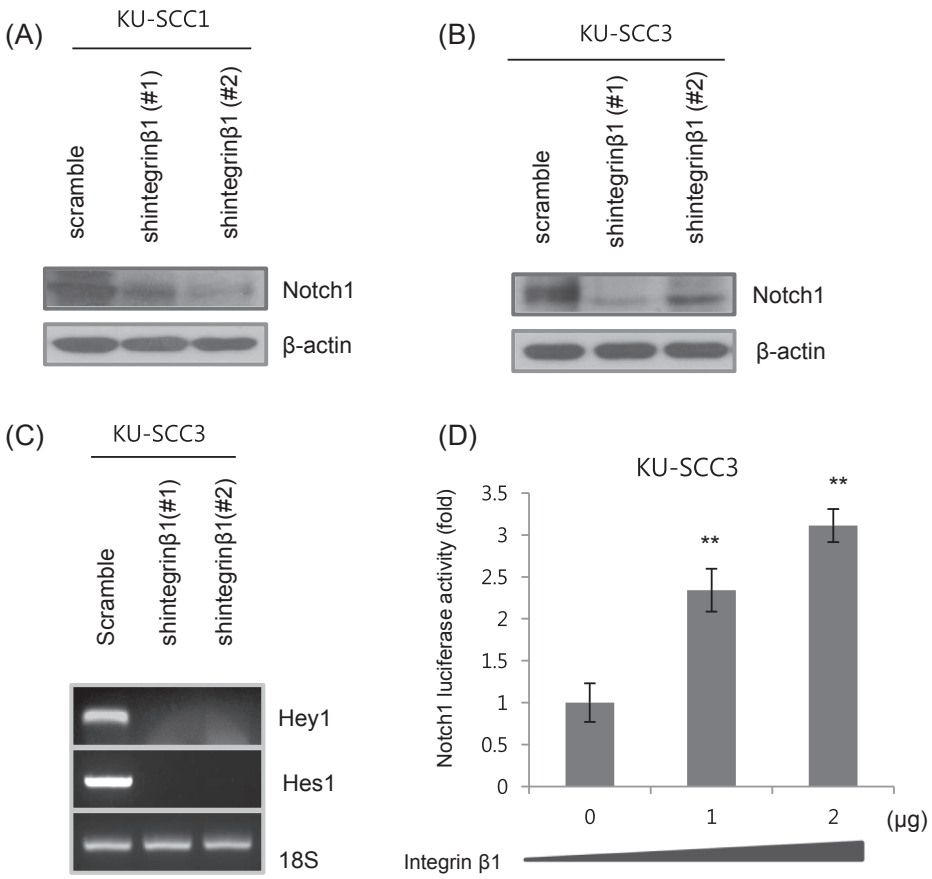


Fig. 5. Effect of suppression of integrin β1 on stemness of HNSCC cells mediates by down-regulation of Notch1. (A and B) Protein level of Notch1 in primary HNSCC cells transduced with either shScramble or shintegrin β1 construct. (C) mRNA levels of Hey1 and Hes1 in primary HNSCC cells transduced with either shScramble or shintegrin β1 construct. (D) Luciferase activity of CSL in primary HNSCC cells according to integrin β1 expression. Data represent the means ± SD (N = 3). (**P < 0.01).

The effect of integrin β1 on stemness of HNSCC cells may be associated with Notch1 expression

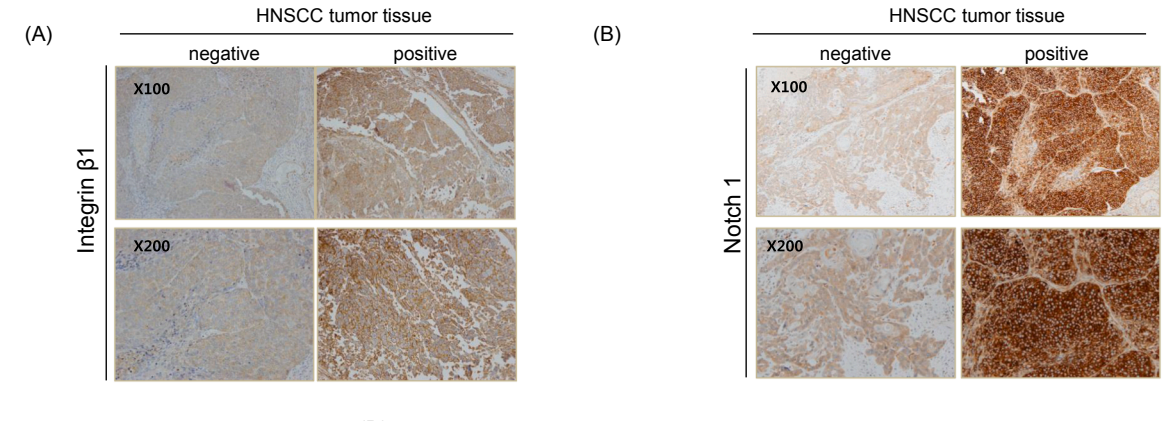
There are three major pathways (Wnt, Notch, and Sonic hedgehog) regulating the phenotype of embryonic stem cells [17]. Campos et al observed that integrin β1 and Notch1 are coordinated in neural stem cells [18]. We have previously reported that Notch1 preserves stemness of HNSCC cells [19]. Also, we identified that integrin β1 knockdown in HNSCC cells did not change mRNA level of Wnt target genes (cyclinD1 and c-Myc), and Sonig hedgehog target genes (Gli1 and Gli 2), respectively (Supplementary Fig. 4). Therefore, it was interesting to check whether the Notch1 pathway worked downstream to the integrin β1 pathway and affected stemness of HNSCC cells. Knockdown of integrin β1 decreased Notch1 protein levels (Fig. 5A & B) and mRNA levels of Hey1 and Hes1, which are the target genes of NICD (Fig. 5C). In addition, the reporter gene assay revealed that integrin β1 regulated the transcriptional activity of Notch1 (Fig. 5D). Furthermore, the expression level of notcu1 was reduced in tumor tissue generated from HNSCC cells with shintegrin β1 vector compared to with scramble vector (Supplementary Fig. 5). Our results suggested that integrin β1 might affect Notch1 expression, resulting in an increase in stemness of HNSCC cells.

Coexpression of integrin β1 and Notch1 is post-treatment prognosis indicator in patients with HNSCC

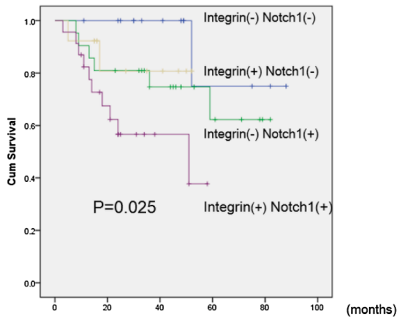
To investigate whether integrin β1 expression is clinically relevant in prognosis of HNSCC, 71 samples from patients with primary HNSCC were evaluated using antibody against integrin β1 and Notch1 (Fig. 6A & B). A positive correlation between integrin β1 expression and poor prognosis was observed; however, it failed to reach the statistical significance owing to the small samples size (data not shown). Thus, we further examined whether simultaneous expression of integrin β1 and Notch1 affected survival of the patients post-treatment. As shown in Fig. 6C, the survival of patients with coexpression of integrin β1 and Notch1 was the worst. It indicates that the coexpression of these two proteins can be a predictor of post-treatment prognosis in patients with HNSCC. Next, we examined the association of coexpression of the two proteins (integrin β1 and Notch1) with various clinico-pathological factors influencing the prognosis of HNSCC. Univariate analysis showed a statistically significant increase in the coexpression of integrin β1 and Notch1 in cases of poor histological grades and tumor recurrence and in the presence of lymph node metastasis (Supplementary Table 2). Logistic regression analysis showed that tumor recurrence were linked with high expression of both integrin β1 and Notch1 ($p < 0.01$) (Fig. 6D). Altogether, our data suggest that co-expression of integrin β1 and Notch1 is a valuable prognostic indicator for patients of HNSCC.

Discussion

Epithelial stem cells reside in niches with specialized microenvironments that control their fate by providing cues in the form of both cell-cell contacts and secreted factors [20]. Niches have been identified in various epithelial tissues, such as the breast, intestine, as well as in neural, epidermal, and hematopoietic systems [21]. Epithelial stem cells maintain their stemness through continuous interactions with niche. As integrins are the primary receptors involved in the communication between cells and their niche, they have recently been suggested as important markers and functional regulators of stem cells [22]. Especially, integrin β1 is highly expressed in normal stem cells, maintains stem cell niche, preserves a stable stem cell population, and controls the balance between stem cell renewal and differentiation [22]. In addition, integrin β1 signaling regulates diverse functions in tumor cells, including migration, invasion, proliferation, and survival, contributing to tumor progression [23]. Therefore, dysfunction of integrin β1 signaling can generate various CSC of solid tumor, such as



(C)



(D)

	Crude OR		Age-sex adjusted OR	
	OR (95% CI)	p-value	OR (95% CI)	p-value
N stage				
Integrin(-) Notch1(-)	1 (reference)		1 (reference)	
Integrin(-) Notch1(+)	7.33 (1.53-35.11)	0.013	9.41 (1.83-48.39)	0.007
Integrin(+) Notch1(-)	1.63 (0.29-9.26)	0.582	1.81 (0.3-10.94)	0.518
Integrin(+) Notch1(+)	3.36 (0.74-15.31)	0.117	3.4 (0.72-15.93)	0.121
Recurrence				
Integrin(-) Notch1(-)	1 (reference)		1 (reference)	
Integrin(-) Notch1(+)	1.41 (0.22-8.99)	0.715	1.48 (0.23-9.63)	0.684
Integrin(+) Notch1(-)	0.5 (0.04-6.28)	0.591	0.47 (0.04-6.06)	0.561
Integrin(+) Notch1(+)	6.55 (1.19-36.04)	0.031	6.52 (1.17-36.37)	0.032
Tumor grade				
Integrin(-) Notch1(-)	1 (reference)		1 (reference)	
Integrin(-) Notch1(+)	4.5 (1.06-19.11)	0.042	5.69 (1.24-26.14)	0.026
Integrin(+) Notch1(-)	2.1 (0.45-9.84)	0.346	2.36 (0.45-12.39)	0.311
Integrin(+) Notch1(+)	6.48 (1.48-28.34)	0.013	7.02 (1.51-32.63)	0.013

Fig. 6. Coexpression of integrin β1 and Notch1 correlates with survival rates of human HNSCC patients (N = 71). (A) Immunochemical expression of integrin β1 in primary HNSCC tissue. (B) Immunochemical expression of Notch1in primary HNSCC tissue. (C) Overall survival rates based on coexpression of integrin β1 and Notch1 expression. (D) Logistic regression analysis of clinico-pathological factors associated with coexpression of integrin β1 and Notch1 expression.

CSC from breast, prostate, and brain [8]. However, there are limited studies examining the role of integrin β1 in regulation of the CSC phenotype of HNSCC [9,10].

In this study, we have identified integrin β1, as being highly expressed in the CSC population of HNSCC. In addition, we observed that integrin β1 affected self-renewal capacity, expression of stemness markers, cisplatin resistance, and xenograft tumorigenicity. These findings indicate that integrin β1 controls the stemness phenotype of HNSCC cells.

Among different downstream molecules of the integrin signaling pathway, we focused on FAK functions affecting stemness of HNSCC cells. Integrin signaling through FAK activation promotes tumorigenesis because it maintains the pool of mammalian stem cells as target of oncogenic transformation [9]. Begum et al found that the inhibition of FAK activity through overexpression of FAK Y397F decreased tumor initiating potential in vitro and in vivo in pancreatic ductal adenocarcinoma [24]. The present study showed that the inhibition of FAK decreased self-renewal capacity and the expression of stemness marker in HNSCC cells. Therefore, we suggested a potential role of FAK in the maintenance of HNSCC stemness.

Cancer stem cells, like tissue specific stem cells in normal organs, maintain their stemness by interacting with specific microenvironment called stem cell niche [25]. Thus, it may be plausible that signaling pathway interconnecting between extracellular matrix, one of the microenvironments, has a critical role of CSC progression and maintenance, and a number of articles reported this relationship. For example, Hyaluronan, an important glycosaminoglycan in the extracellular matrix and its major cell surface receptor, CD44, have been suggested to be important cellular mediators influencing CSC progression in head and neck cancer [26]. Our study showed that integrin β1 expression might influence Notch1 expression as a downstream mediator. The crosstalk between integrin α1β1 and Notch1 suppresses the differentiation of cardiomyocytes [27]. Sarkar et al showed that glioma stem cells through integrin β1-mediated mechanism of tenacin-C/integrin β1/JAG1/Notch1 signaling axis [28]. Therefore, the integrin β1 signaling pathway may increase stemness of HNSCC cells through Notch1 activation; however, further studies will be required in future for better understanding of the mechanism.

Recently, Koshizuka et al showed that high expression of integrin β1 are linked to poor survival rate of patients with HNSCC [29], unlike our study in which expression of integrin β1 has no statistical significance in survival of HNSCC patients. However, our study also has a close tendency of high expression of integrin β1 and worse prognosis, but this difference is not statistically significant probably due to small patient number (N = 71). In addition, it should be considered that Koshizuka's work was based on TCGA data composed of heterogenous patient population with diverse treatment modalities and our data was generated from of immunohistochemical method using homogenous HNSCC patient tissues of a single institution. Interestingly, our study results showed that one half of the 71 HNSCC patients are either integrin (+)/Notch1 (−) or integrin (−)/Notch1 (+). It is difficult to know the exact reason for this, but the following explanation is possible. First, HNSCCs are known to be highly heterogeneous [30]. Second tumorigenesis mechanism are considerably different according to HPV (+) or HPV (−) cancer [31]. Furthermore, the case of the *Notch1* gene is still highly contradictory to its function (tumor suppressor gene vs

oncogene) in HNSCC [32]. Because the total 71 HNSCC patient specimens regardless of HPV status, notch1 mutation and to the characteristics of highly heterogeneous head and neck cancer, it is presumed that the above results can be obtained.

In conclusions, our study expands the current understanding on the biological role of integrin β1 in stemness of HNSCC cells. Based on the findings in the present study, we suggest that integrin β1 can be used a therapeutic target in the treatment of HNSCC. In addition to the integrin β1, further research on other integrin subunits, including alpha6 and β3, also will be needed in the near future.

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Declaration of Competing Interest

The authors declare no potential conflicts of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.oraloncology.2019.07.001.

References

[1] Leemans CR, Braakhuis BJ, Brakenhoff RH. The molecular biology of head and neck cancer. Nat Rev Cancer 2011;11:9–22.
[2] Sacco AG, Cohen EE. Current treatment options for recurrent or metastatic head and neck squamous cell carcinoma. J Clin Oncol 2015;33:3305–13.
[3] Reid PA, Wilson P, Li Y, Marcu LG, Bezak E. Current understanding of cancer stem cells: review of their radiobiology and role in head and neck cancers. Head Neck 2017;39:1920–32.
[4] Lim YC, Oh SY, Cha YY, Kim SH, Jin X, Kim H. Cancer stem cell traits in squamospheres derived from primary head and neck squamous cell carcinomas. Oral Oncol 2011;47:83–91.
[5] Raab-Westphal S, Marshall JF, Goodman SL. Integrins as therapeutic targets: successes and cancers. Cancers (Basel) 2017;9:110.
[6] Deb M, Sengupta D, Patra SK. Integrin-epigenetics: a system with imperative impact on cancer. Cancer Metastasis Rev 2012;31:221–34.
[7] Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell 2002;110:673–87.
[8] Desgrosellier JS, Cheresh DA. Integrins in cancer: biological implications and therapeutic opportunities. Nat Rev Cancer 2010;10:9–22.
[9] Lin HC, Wu CL, Chen YL, Huang JS, Wong TY, Yuan K. High-level β1-integrin expression in a subpopulation of highly tumorigenic oral cancer cells. Clin Oral Investig 2014;18:1277–84.
[10] Koukourakis MI, Giatromanolaki A, Tsakmaki V, Danielidis V, Sivridis E. Cancer stem cell phenotype relates to radio-chemotherapy outcome in locally advanced squamous cell head-neck cancer. Br J Cancer 2012;106:846–53.
[11] Wu CP, Zhou L, Xie M, et al. Identification of cancer stem-like side population cells in purified primary cultured human laryngeal squamous cell carcinoma epithelia. PLoS ONE 2013;8:e65750.
[12] Kurth I, Hein L, Mäbert K, et al. Cancer stem cell related markers of radioresistance in head and necksquamous cell carcinoma. Oncotarget 2015;6:34494–509.
[13] Wakamatsu Y, Sakamoto N, Oo HZ, et al. Expression of cancer stem cell markers ALDH1, CD44 and CD133 in primary tumor and lymph node metastasis of gastric cancer. Pathol Int 2012;62:112–9.
[14] Chikamatsu K, Ishii H, Takahashi G, et al. Resistance to apoptosis-inducing stimuli in CD44+ head and neck squamous cell carcinoma cells. Head Neck 2012;34:336–43.
[15] Liu Y, Cui P, Chen J, Li W. Isolation and phenotypic characterization of side population cells in oral squamous cell carcinoma. Mol Med Rep 2015;11:3642–6.
[16] McLean GW, Carragher NO, Avizienyte E, Evans J, Brunton VG, Frame MC. The role of focal-adhesion kinase in cancer - a new therapeutic opportunity. Nat Rev Cancer 2005;5:505–15.
[17] Matsui WH. Cancer stem cell signaling pathways. Medicine (Baltimore) 2016;95:S8–19.
[18] Campos LS, Decker L, Taylor V, Skarnes W. Notch, epidermal growth factor receptor, and beta1-integrin pathways are coordinated in neural stem cells. J Biol Chem 2006;281:5300–9.
[19] Lee SH, Do SI, Lee HJ, Kang HJ, Koo BS, Lim YC. Notch1 signaling contributes to stemness in head and neck squamous cell carcinoma. Lab Invest 2016;96:508–16.
[20] Cabarcas SM, Mathews LA, Farrar WL. The cancer stem cell niche–there goes the neighborhood? Int J Cancer 2011;129:2315–27.
[21] Voog J, Jones DL. Stem cells and the niche: a dynamic duo. Cell Stem Cell 2010;6:103–15.
[22] Seguin L, Desgrosellier JS, Weis SM, Cheresh DA. Integrins and cancer: regulators of cancer stemness, metastasis, and drug resistance. Trends Cell Biol 2015;25:234–40.
[23] Avraamides CJ, Garmy-Susini B, Varner JA. Integrins in angiogenesis and lymphangiogenesis. Nat Rev Cancer 2008;8:604–17.
[24] Begum A, Ewachiw T, Jung C, et al. The extracellular matrix and focal adhesion kinase signaling regulate cancer stem cell function in pancreatic ductal adenocarcinoma. PLoS ONE 2017;12:e0180181.
[25] Plaks V, Kong N, Werb Z. The cancer stem cell niche: how essential is the niche in regulating stemness of tumor cells? Cell Stem Cell 2015;16:225–38.
[26] Bourguignon LYW, Earle C, Shiina M. Activation of matrix hyaluronan-mediated CD44 signaling, epigenetic regulation and chemoresistance in head and neck cancer stem cells. Int J Mol Sci 2017;18:1849.
[27] Tian Y, Wang W, Lu Q, et al. Cross-talk of SFRP4, integrin α1β1, and Notch1 inhibits cardiac differentiation of P19CL6 cells. Cell Signal 2016;28:1806–15.
[28] Sarkar S, Mirzaei R, Zemp FJ, et al. Activation of NOTCH signaling by tenascin-C promotes growth of human brain tumor-initiating cells. Cancer Res 2017;77:3231–43.
[29] Koshizuka K, Kikkawa N, Hanazawa T, Yamada Y, Okato A, Arai T, et al. Inhibition of integrin β1-mediated oncogenic signalling by the antitumor *microRNA-29* family in head and neck squamous cell carcinoma. Oncotarget 2017;9:3663–76.
[30] Leemans CR, Snijders PJF, Brakenhoff RH. The molecular landscape of head and neck cancer. Nat Rev Cancer 2018;18:269–82.
[31] Canning M, Guo G, Yu M, Myint C, Groves MW, Byrd JK, et al. Heterogeneity of the head and neck squamous cell carcinoma immune landscape and its impact on immunotherapy. Front Cell Dev Biol 2019;7:52.
[32] Fukusumi T, Califano JA. The Notch pathway in head and neck squamous cell carcinoma. J Dent Res 2018;97:645–53.