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Preliminary study on Xiaoliusan-induced inhibition of hepatocellular carcinoma using gene chip analysis

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Abstract

Objective: We explored inhibiting effects of Xiaoliusan (XLS) on hepatocellular carcinoma (HCC) cell growth by examining differential gene expression in HepG2 cells after XLS treatments.

Methods: HepG2 cells were treated for 24 h with either XLS ('treatment') or with PBS ('control') for 24 h to assess the effects of XLS on HepG2 proliferation. Differential gene expression was examined using a gene chip, and GO enrichment, KEGG pathway enrichment, and protein interaction network analyses were performed.

Results: XLS inhibited HepG2 proliferation in a dose-dependent manner, with an IC_{50} of 1.45 mg/mL. The whole-genome ChIP test of XLS-treated cells showed differential expression of 802 genes, 283 of which were up-regulated and 519 were down-regulated, compared with control cells. Using inductive analysis, we found that differentially expressed genes were mainly genes associated with the extracellular matrix (*COL1A1*, *COL1A2*, and ECM-receptor interaction pathway, among others) and with cell differentiation and proliferation (RAC1, MYC, and PI3K-Akt signaling pathway, among others).

Conclusions: XLS inhibited HCC cell growth by targeting extracellular matrix genes in the tumor microenvironment and tumor growth, however, further research would be needed to elucidate the underlying mechanisms.

Keywords: Xiaoliusan, Hepatocellular carcinoma, Gene chip, HepG2

Background

Primary hepatocellular carcinoma (HCC) is one of the most common malignancies in the world and is associated with the poorest prognoses [1]. Approximately 788,000 HCC-related deaths were estimated to have occurred in 2015, representing the second-highest cancer mortality rate [2]. Clinical characteristics of HCC onset are inconspicuous, and most patients show no symptoms during the early phase. Thus, at the time of diagnosis, in many cases no radical treatment such as surgery is performed due to tumor progression, metastasis, and invasion in blood vessels. Treatment strategies combining traditional Chinese and western medicine may be an effective way to improve the quality of life and survival rates of HCC patients.

Xiaoliusan (XLS), also referred to as Wu's Xiaoliusan, is a traditional Chinese medicine advertised by Professor Wu Zhengxiang as a treatment of malignant tumors. Administration of only XLS or of XLS in combination with transarterial chemoembolization prolonged survival and apparently improved quality of life in patients with middle- and late-stage HCC [3–5]. In previous studies, we found that XLS inhibited tumor growth and proliferation and regulated immunity responses to tumor cell invasion; furthermore, it appeared to promote T-cell proliferation and NK cell activation in tumor-bearing mice [3–6]. However, due to the complex composition of XLS, the underlying mechanisms were not unambiguously identified. In the current study, we used human HCC cells (HepG2 cell line) to explore potential effects of XLS

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treatments. Furthermore, a preliminary investigation on the underlying mechanism and potential targets was performed using gene chip analyses. Our results show the need for further research on XLS.

Methods

HCC cell treatments

HepG2 cells were obtained from the Institute of Hepatology, Shanghai University of Traditional Chinese Medicine, Shanghai, China. XLS was obtained from the Shanghai Medicine Green Valley Pharmaceutical Co., Ltd. (patent number ZL200810043770.9). The main ingredients were Radix Pseudostellariae, *Atractylodes macrocephala* Koidz, *Coix lacryma-jobi* seeds, Fructus aurantii immaturus, Rhizoma Amorphophalli, Radix acutus, *Commelina appendiculata*, *Eupolyphaga steleophaga*, semen impatients, roasted tortoise shell, roasted turtle shell, Chinese sage herb, *Salvia chinensis*, *Selaginella doederleinii* Hieron.

A gene chip was obtained from Affymetrix (Japan; PrimeView Human Gene Expression Array 901838, Clariom S Array, human 902926, Clariom D Assay, human 902922; Thermo Fisher). The following instruments were used: a GeneChip Scanner 3000 7G, a GeneChip Fluidics Station 450, and a GeneChip Hybridzation Oven 645.

HepG2 cells were inoculated in RPMI1640 culture medium containing 10% fetal bovine serum and were maintained at 37 °C under 5% CO_2. Culture medium was renewed every three to four days, and cells in the logarithmic growth phase were used for experiments. Cells were treated for 24 h with either with PBS as a control or with XLS dissolved in the culture medium at a concentration of 1.45 mg/mL. A cell counting kit 8 was used to assess inhibitory effects of XLS on HepG2 proliferation.

HepG2 cells were digested using 0.25% trypsin and were re-suspended in the culture medium. A total of 6 × 103 cells per well (100 μL) were seeded in 96-well plates and were cultured for 24 h. Growth medium was exchanged when cells entered the logarithmic growth phase. Cells were then treated with XLS at concentrations of 0, 1.25, 1.5, 1.75, 2.0, 2.25, 2.5, or 5.0 mg/mL for 24 h, and the cell survival rate was evaluated. Additionally, the IC_{50} was calculated. The cell growth inhibition rate was calculated according to the following formula: Growth inhibition rate (%) = (1-average OD value of the XLS group/average OD value of the control group) × 100%.

Affymetrix GeneChip Human Transcriptome Array 2.0

TRIzol was used to extract total RNA from HepG2 cells, which was then purified using an RNeasy MiniKit. RNA quality and integrity was assessed using 1%-agarose gel electrophoresis and spectrophotometry. Three samples of each treatment were subjected to gene chip analyses according to the manufacturer's instructions. Single-stranded and double-stranded cDNA was synthesized from RNA and was then purified. Double-stranded cDNA was used as a template. An RNA transcription labeling kit was used for in vitro transcription and synthesis of biotin-labeled cDNA. The Human Transcriptome Array 2.0 gene chip was pre-hybridized at 45 °C for 10 min in a hybridization oven at 640 °C. Hybridization buffer was added, and equal volumes of the treated hybridization solution was used for hybridization at 45 °C for 16 h, followed by washing and staining using an automatic washing station.

Differentially expressed genes were analyzed using Gene Ontology (GO) enrichment analysis, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, and protein-protein interaction (PPI) network analysis.

Bioinformatics and statistical analysis

Chip data processing and bioinformatic analyses

A chip scanner was used to scan the gene chip, and Image Viewer software was used to produce a hybridization signal overlay map. Raw data were standardized and corrected using Partek GS 6.5 software. Data of treated and untreated cells were compared using the chip. GO enrichment and KEGG pathway enrichment analyses were applied to identify differentially expressed genes using the DAVID tool (https://david.ncifcrf.gov/home.jsp). In a protein interaction network analysis of differentially expressed genes, we used a string tool to detect PPI, and a combined score of > 0.7 was used as a threshold to produce PPI relationship pairs of differentially expressed genes. Statistical analyses were performed using SPSS 17.0 statistical software. Statistical significance is reported as $P < 0.05$.

Results

Inhibition of HepG2 proliferation

XLS treatment of HepG2 cells produced a significant dose-dependent decrease in cell survival. Using linear regression analysis, the IC_{50} of XLS in HepG2 cells was found to be 1.45 mg/mL (Figure 1).

Gene chip results

A total of 802 differentially expressed genes was detected in the XLS group, 283 of which were up-regulated, and 519 were down-regulated. These 802 differentially expressed genes were evaluated using GO bio-annotation, which revealed that genes associated with molecular functions, biological processes, and cellular components were included. We produced a bar chart for the most significant TOP10 functions, in which the abscissa indicates the *P*-value (-log10-transformed). Larger values indicated higher enrichment significance Differentially expressed genes associated with biological processes comprised predominantly those coding for extracellular matrix proteins (47 genes), extracellular structural composition (47 genes), and cell matrix adhesion (38 genes; Figure 2).

According to the KEGG pathway enrichment analysis, differentially expressed genes were predominantly associated with the following three signaling pathways: the extracellular matrix receptor interaction pathway (16 genes), the PI3K-Akt signaling pathway (34 genes), and the focal adhesion pathway (22 genes; Figure 3). The abscissa indicates *P*-values (-log10-transformed). Larger values indicated higher the enrichment significance.

PPI analysis produced 307 nodes and 699 edges in a present scale-free network. The most important nodes in the network showed a high degree of connectivity (Table 1), such as RAC1, MYC, and COL1A2 (Figure 4). Red nodes indicate differentially up-regulated genes, green nodes indicate differentially down-regulated genes. Edges denote protein interactions.

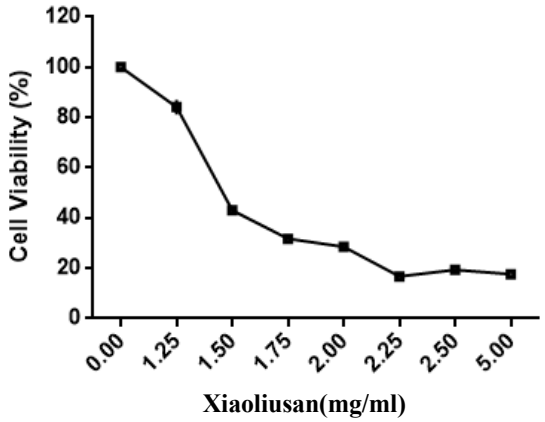

Figure 1 Xiaoliusan inhibits HepG2 cell proliferation

Discussion

Effect on the extracellular matrix (ECM) in the tumor microenvironment

Combining our experimental results with gene database analyses, we found that differentially expressed genes after XLS treatment were closely associated with the extracellular matrix. For example, in the PPI network analysis, we found that nodes with higher connectivity included genes encoding collagen/protease, including *COL1A2*, *COL1A1*, *MMP2*, and *COL3A1*. In addition, GO enrichment analysis indicated that differentially expressed genes were mainly associated with extracellular matrix organization (molecular functions), extracellular matrix (biological processes), and collagen binding (cellular components). Furthermore, KEGG pathway enrichment analysis results suggested that XLS may affect ECM-receptor interactions.

Recent studies found that the reconstruction of ECM in the tumor microenvironment, including ECM synthesis, distribution, and degradation, is closely associated with differentiation, proliferation, invasion, and metastasis of malignant tumors [7]. Reconstructed ECM was found to affect tumor progression through interactions between structure and signaling pathways [8]. Type-I collagen is the main component of ECM and is encoded by the genes *COL1A1* and *COL1A2*. Type-I collagen represents the main fibrous component of solid malignant tumors which is critically involved in tumor growth. However, evidence suggests that tumor cells that are able to produce type-I collagen have lower tumorigenic potential [9] but are highly aggressive [10]. Moreover, Yu et al. found that over-expression of *COL1A2* in colon cancer cells mitigated cell proliferation, migration, and invasion, and inhibited tumorigenesis [11]. In the present study, we found that XLS led to up-regulated gene expression of *COL1A2* and *COL1A1*, which affected the extracellular matrix-related signaling pathways. We suggest that XLS may influence HCC differentiation, proliferation, and invasion by affecting type-I collagen, thereby inhibiting HCC progression.

Effects of XLS on HCC development

Our PPI network analysis showed that XLS down-regulated genes associated with tumor growth, proliferation, and migration, and other HepG2 cell genes such as RAC1, MYC, PRKACA, and PLK1. Additionally, KEGG pathway enrichment analysis results suggested that the PI3K-Akt signaling

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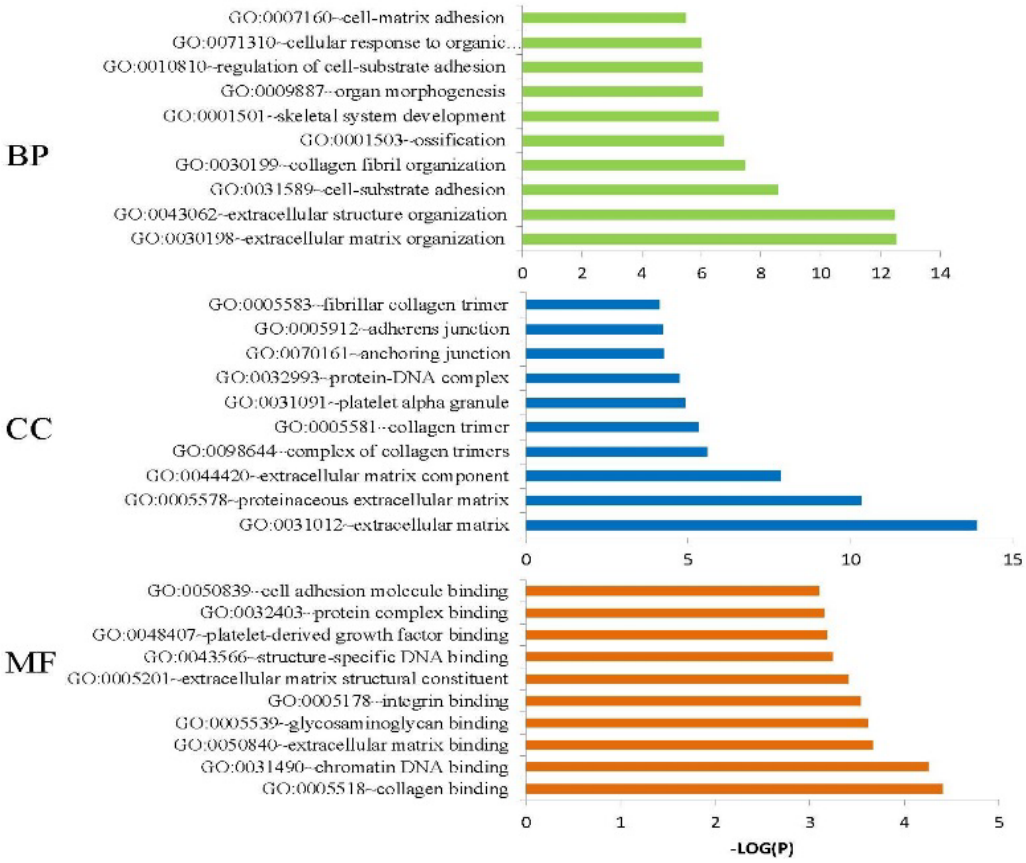

Figure 2 Bar graph of GO enrichment analysis of the differentially expressed genes (TOP10)

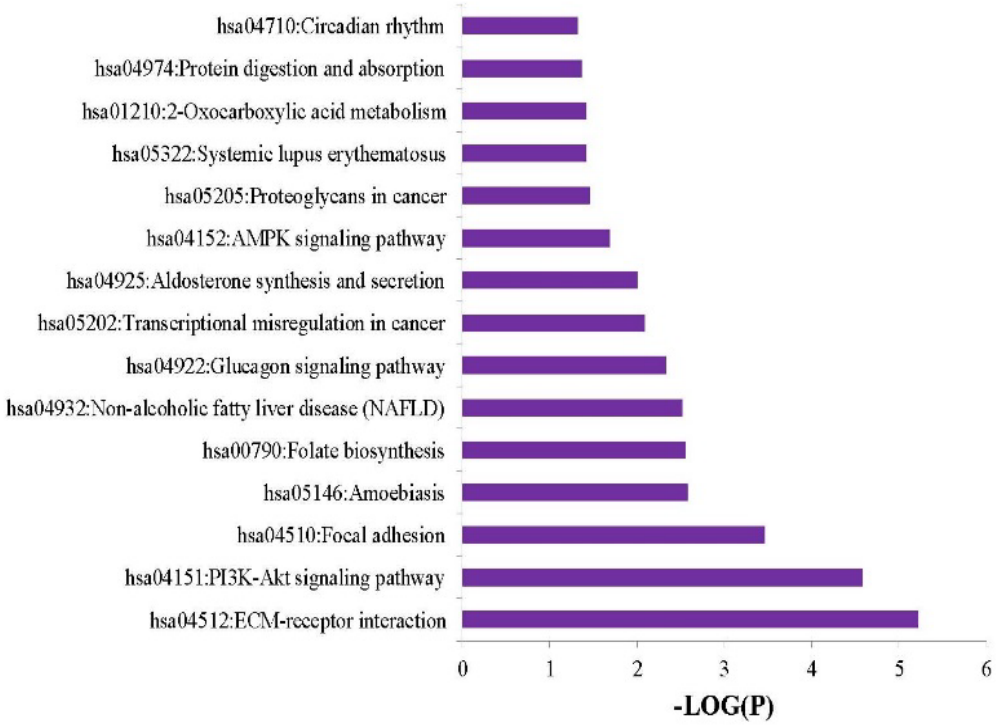

Figure 3 Bar graph for KEGG pathway enrichment analysis of differentially expressed genes

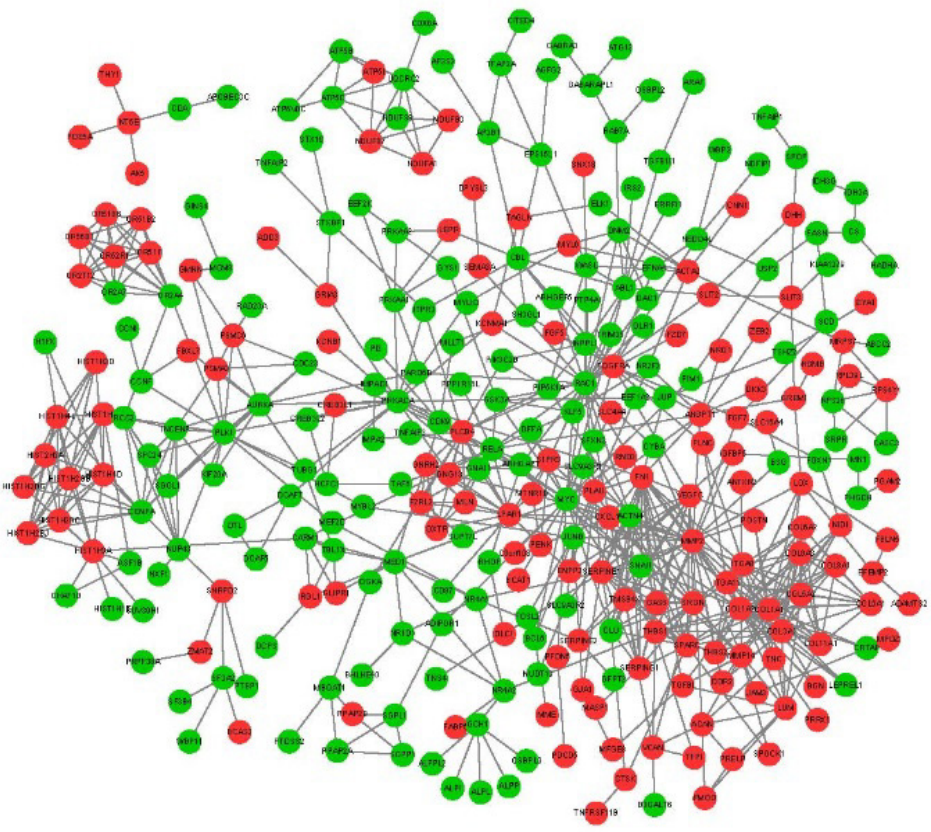

Figure 4 Protein-protein interaction network diagram of differentially expressed genes

Table 1 Connectivity of differentially expressed genes in the protein-protein interaction networks (TOP 10)

Gene	type	degree
RAC1	down	23
MYC	down	23
COL1A2	up	23
FN1	up	23
COL1A1	up	23
MMP2	up	20
PRKACA	down	18
COL3A1	up	18
PLK1	down	17
SERPINE1	up	16

pathway and the focal adhesion pathway (adhesion spot pathway) were also affected by XLS. Among these, Rac1 is an important intracellular signal transduction molecule which regulates a variety of cellular mechanisms and gene expression. It is involved in many biological, processes including phagocytosis, adhesion, cell movement, and cell proliferation. Furthermore, Rac1 is closely associated with tumor cell migration and invasion, angiogenesis, proliferation, and apoptosis, and with cell-cycle regulation. Rac protein was found to be over-activated and over-expressed in most human invasive tumors [12]. Yang et al. reported up-regulation of Rac1 expression in HCC, which was correlated with poor prognosis [13]. Rac1 can affect occurrence and development of tumors by regulating multiple signaling pathways. Previous studies showed that Rac1 can control cell growth and movement by activating effectors of its downstream pathways such as p21 activated kinase (PAK), mitogen-activated protein kinase (MAPK), and phosphoinositide 3 kinase (PI3K) [14, 15]. In addition, breast cancer [16], prostate cancer [17], bladder cancer [18], and other tumors are closely

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associated with activation of the Rac1-PI3K/Akt pathway during tumor development. In the current study, we found that besides Rac1, also genes related to cell cycle and proliferation, such as MYC, PRKACA, and PLK1 were down-regulated by XLS, and multiple signaling pathways such as PI3K-Akt were affected by the treatment. Therefore, we speculate that XLS may affect the HCC growth.

Furthermore, XLS treatments also affected genes related to tumor microenvironments, including *TGFB1*, *ICAM*, and *VCAM*. Transforming growth factor β (TGF-β) 1, which is encoded by the *TGFB1* gene, plays an important role in tumor development processes. TGF-β regulates expression and functional status of cyclin, cyclin-dependent kinase (CDK), and c-myc in the early stage of tumors [19-20], which leads to cell cycle arrest in the G1 phase and to tumor growth inhibition. Conversely, in advanced tumors, most tumor cells produce TGF-β through autocrine or paracrine mechanisms, which results in abnormally high TGF-β concentrations in tumor tissues. TGF-β frequently promotes epithelial-mesenchymal cell transformation of tumor cells, inhibits immune cell functions, and enhances tumor invasion and metastasis [21]. In previous studies, we also found that XLS treatments significantly decreased serum TGF-β1 levels of HCC patients, whereas proportions and activity of NK cells were increased. Therefore, XLS may promote immune cell activation and affects tumor cells by regulating TGF-β1.

Multiple genes are involved in the occurrence and development of HCC, and its pathogenesis is not comprehensively understood. Traditional Chinese medicine attempts to target multiple effectors in tumor therapy. HCC can be classified into "accumulation," "rock," and "tumor," according to its clinical characteristics. According to traditional Chinese medical theory, tumors are characterized by "positive deficiency and evil truth." Therefore, the basic principles of traditional Chinese medicine for treating malignant tumors are to promote blood circulation and eliminate pathogenic factors. XLS has been used previously in order to treat malignant tumors [3]. Radix Pseudostellariae, *Atractylodes macrocephala* Koidz, *Coix lacryma-jobi* seeds and fructus aurantii immaturus are supposed to invigorate the spleen and remove dampness and phlegm. *Rhizoma Amorphophalli*, *Radix acutus* and *Commelina appendiculata* diminished tumor burdens; roasted tortoise and turtle shells are supposed to nourish "Yin", invigorate the kidneys, remove blood stasis, strengthen the spleen and invigorate qi, deplete scatters, and eliminating cancers [3–5]. Based on previous studies, we preliminarily explored potential effects of XLS through classification of differentially expressed genes in HepG2 cells, including genes of the extracellular matrix in the tumor microenvironment, tumor growth, and tumor-associated cytokines. Our findings suggest further investigation on this topic.

References

1. Heimbach JK, Kulik LM, Finn R, et al. AASLD guidelines for the treatment of hepatocellular carcinoma. Hepatol 2018, 67: 358–380.
2. World Health Organaization [Internet]. Cancer [cited 2020 January 15]. Available from: http://www.who.int/mediacentre/factsheets/fs297/en/
3. Wu M, Wu KL, Zhang T. Examples of "Wu's Xiaoliusan" in treating hepatocellular carcinoma. Shanghai J Tradit Chin Med 2017, 51: 21–23. (Chinese)
4. Wu M, Gao YQ, Zhang B, et al. Clinical observation of Xiaoliusan in treating patients with advanced primary hepatocellular carcinoma. Shanghai J Tradit Chin Med 2017, 51: 77–79. (Chinese)
5. Wu M, Gao YQ, Zhang B, et al. Short-term efficacy of Xiaoliusan combined with TACE in the treatment of advanced hepatocellular carcinoma. Shanghai J Tradit Chin Med 2018, 52: 56–58. (Chinese)
6. Wu ZX, Wu KL, Zhang XT. Differentiation and treatment experience of traditional Chinese medicine in malignant lymphoma treatment. J Shanghai Univ Tradit Chin Med 2009, 23: 1–3. (Chinese)
7. Filipe EC,Chitty JL,Cox TR,et al.Charting the unexplored extracellular matrix in cancer. Int J Exp Pathol 2018, 99: 58–76.
8. Yamada KM, Collins JW, Cruz Walma DA, et al. Extracellular matrix dynamics in cell migration, invasion and tissue morphogenesis. Int J Exp Pathol 2019, 6: 100
9. Sengupta PK, Smith EM, Kim K, et al. DNA Hypermethylation Near the Transcription Start Site of Collagen 2(I) Gene Occurs in Both Cancer Cell Lines and Primary Colorectal Cancers. Cancer Res 2003, 63: 1789–1797.
10. Liang Y, Diehn M, Bollen AW, et al. Gupta N. Type I collagen is overexpressed in medulloblastoma as a component of tumor microenvironment. J Neuro-oncol 2008, 86: 133–141.

11. Yu YF. The effect of Col1A2 on colorectal cancer cell proliferation, migration and invasion. China Medical University, 2018.
12. Hernández E, De La Mota-Peynado A, Dharmaw ardhane S, et al. Novel inhibitors of Rac1 in metastatic breast cancer. P R Health Sci J, 2010, 29: 348–356.
13. Yang W, Lv S, Liu X, et al. Upregulation of Tiam1 and Rac1 correlates with poor prognosis in hepatocellular carcinoma. Jpn J Clin Oncol 2010, 40: 1053–1059.
14. Cotteret S, Chernoff J. The evolutionary history of effectors downstream of Cdc42 and Rac. Genome Biol 2002, 3: REVIEWS0002.
15. Fritsch R, de Krijger I, Fritsch K, et al. RAS and RHO families of GTPases directly regulate distinct phosphoinositide 3-kinase isoforms. Cell 2013, 153: 1050–1063.
16. Yang Y, Du J, Hu ZZ, et al. Activation of Rac1-PI3K/Akt is required for epidermal growth factor induced PAK1 activation and cell migration in MDA-MB-231 breast. J Biomed Res 2011, 25: 237–245.
17. Veronica Henderson, Basil Smith, Liza J Burton, et al. Snail promotes cell migration through PI3K/AKT-dependent Rac1 activation as well as PI3K/AKT-independent pathways during. Cell Adh Migr 2015, 9: 255–264.
18. Zhang YJ, Liu SB, Wang L, et al. A novel PI3K/AKT signaling axis mediates Nectin-4-induced gallbladder cancer cell proliferation, metastasis and tumor growth. Cancer Letters 2016, 375: 179–189.
19. Lecarpentier Y, Schussler O, Hébert JL, e t al. Multiple Targets of the Canonical WNT/β-Catenin Signaling in Cancers. Front Oncol 2019, 9: 1248.
20. Tiwari A, Swamynathan S, Alexander,et al. NKLF4 Regulates Corneal Epithelial Cell Cycle Progression by Suppressing Canonical TGF-βSignaling and Upregulating CDK Inhibitors P16 and P27. Invest Ophthalmol Vis Sci 2019, 60.
21. Ashraf TS, Obaid A, Saeed TM, et al. Formal model of the interplay between TGF-l Cycle Progression by Suppressing Canonical ular carcinoma. Math Biosci Eng 2019.

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