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Inflammatory response or oxidative stress induces upregulation of PTPN2 and thus promotes the progression of laryngocarcinoma

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Abstract. – OBJECTIVE: This study aims to investigate whether inflammatory response or oxidative stress could induce upregulation of PTPN2, thus promoting the progression of laryngocarcinoma.

PATIENTS AND METHODS: PTPN2 levels in laryngocarcinoma tissues and normal tissues were detected. In addition, PTPN2 levels in laryngocarcinoma tissues with stage 1/2 or stage 3/4 were determined as well. *In vitro* abundance of PTPN2 was measured in laryngocarcinoma cells and immortalized human nasopharyngeal epithelial cells. Survival analysis was conducted in laryngocarcinoma patients with high or low expression level of PTPN2. Subsequently, M4E cells were stimulated with inflammation (IFN-γ or TNF-α treatment) or oxidative stress (H_2O_2 stimulation), followed by determination of the protein level of PTPN2. In M4E cells stimulated with different concentrations of H_2O_2, the clonality and Ki-67 positive cell ratio were detected. Finally, clonality and Ki-67 positive cell ratio in M4E cells transfected with negative control or sh-PTPN2, regardless of H_2O_2 stimulation, were assessed.

RESULTS: PTPN2 was upregulated in laryngocarcinoma tissues, especially those in stage 3/4. Similarly, *in vitro* abundance of PTPN2 was higher in laryngocarcinoma cell lines. The high level of PTPN2 predicted poor prognosis in laryngocarcinoma patients. IFN-γ or TNF-α treatment upregulated the protein level of PTPN2. Meanwhile, H_2O_2 stimulation upregulated the protein level of PTPN2, dose-dependently increased clonality, and Ki-67 positive cell ratio in M4E cells. The knockdown of PTPN2 suppressed clonality and Ki-67 positive cell ratio in M4E cells stimulated by H_2O_2 or not.

CONCLUSIONS: Inflammatory response or oxidative stress could induce upregulation of PTPN2, thus promoting the proliferative ability of laryngocarcinoma.

Key Words:
Laryngocarcinoma, PTPN2, Inflammatory response, Oxidative stress.

Introduction

Laryngocarcinoma is one of the most common head and neck tumors characterized by local invasion and cervical lymphatic metastasis[1]. During the past two decades, the overall survival of laryngocarcinoma has not been greatly improved even though great strides have been made on tumor diagnosis and treatment. Yu et al[2] showed that 1-year and 2-year overall survival for untreated laryngocarcinoma is 56.4% and 26.5%, respectively. A systematic review reported that surgery combined with radiotherapy or chemotherapy is the most preferred therapy for laryngocarcinoma. Nevertheless, the 5-year survival after active treatment is only about 64.2%. The majority of laryngocarcinoma patients will suffer from postoperative dysphagia and speech dysfunction. However, effective therapeutic approaches for local recurrence and metastasis of laryngocarcinoma are lacked and urgently needed to be developed[3].

PTPN2 is a member of the tyrosine phosphatase (PTP) family[4]. In hematopoietic cells, it acts as an important negative regulator of T cell receptors (TCR) and cytokines. PTPN2 dephosphorylates the SRC family kinases Lck, Fyn, JAK1, and JAK3, thus exerting its biological functions[5,6]. PTPN2 deficiency activates $CD8^+$ T cells, thereafter, it induces autoimmune syndrome[7]. Secretion of IFN-γ and IL-6 can be accelerated by PTPN2 deficiency as well[8,9]. PTPN2 has been identified as a target for tumor immunotherapy. It is markedly upregulated in tumors beyond the control of immunotherapy[10]. Silence of PTPN2 is believed to enhance the immunotherapy-sensitivity in melanoma and colon cancer[10,11].

It is generally considered that PTPN2 is of significance in metabolic diseases and inflammatory diseases[12]. Our study focused on the changes of

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PTPN2 following the stimulation of inflammatory response or oxidative stress, and its biological functions in the progression of laryngocarcinoma were examined as well.

Patients and Methods

Sample Collection

Laryngocarcinoma tissues (n=54) and paracancerous tissues (n=54) were collected from First Affiliated Hospital of China Medical University. None of laryngocarcinoma patients received preoperative chemotherapy or radiotherapy. Tissue samples were immediately frozen in liquid nitrogen and preserved at -80°C. Patients and their families in this study have been fully informed. This study was approved by Ethics Committee of the First Affiliated Hospital of China Medical University.

Cell Culture

Laryngocarcinoma cells (TU212, Hep-2, M4E, M2E) and immortalized human nasopharyngeal epithelial cells (NP69) purchased from Cell Bank (Shanghai, China) were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 1% penicillin-streptomycin. Medium was regularly replaced every 1-2 days. Cell passage was conducted until 80-90% confluence. Fourth-fifth-generation cells with good condition were collected for treatment with IFN-γ, TNF-α, H_2O_2 or NAC.

Cell Transfection

The cells were inoculated in a 6-well plate and cultured to 70% confluence. Transfection was conducted using Poly plus. Six hours later, fresh medium was replaced. PTPN2 shRNA sequence: (Sense, 5′-3′): CACAAAGAAGTTACATCTT and (Antisense, 5′-3′): AAGATGTAACTTCTTTGTG.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Cellular RNA was isolated using TRIzol method (Invitrogen, Carlsbad, CA, USA) and reversely transcribed into complementary deoxyribose nucleic acid (cDNA), followed by PCR by SYBR Green method (TaKaRa, Otsu, Shiga, Japan). The primer sequences were listed as follows: PTPN2: forward: 5′-ATCGAGCGGGAGTTCGA-3′ and reverse: 5′-TCTGGAAACTTGGCCACTC-3′; GAPDH, forward: 5′-CGAGATCCCTCCAAAATCAA-3′, reverse: 5′-TTCACACCCATGACGAACAT-3′.

Western Blot

Cellular protein was isolated using radioimmunoprecipitation assay (RIPA) and electrophoresed (Beyotime, Shanghai, China). The protein sample was loaded on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), immersed in phosphate-buffered saline (PBS) containing 5% skim milk for 2 hours, and reacted with primary antibodies at 4°C overnight and secondary antibodies for 2 h. Band exposure was achieved by enhanced chemiluminescence (ECL) and processed by Image Software (NIH, Bethesda, MD, USA).

Colony Formation Assay

The cells were prepared for suspension with $1\times\mathbf{10}^6$/L. 2×10^3 cells/well were inoculated in a 6-well plate and cultured for 2 weeks. Afterwards, the cells were incubated with 4% polyformaldehyde for 30 min, dyed with 0.1% crystal violet for 30 min, and captured for visible colonies.

Ki-67 Staining

The cells were stained with Ki-67 PE (BioLegend, San Diego, CA, USA) at 4°C for 30 min. Before cell staining, cell viability should be confirmed to be higher than 99%. At least 50,000 living cells per sample should be analyzed and Ki-67 positive cell ratio was calculated using the FlowJo software. PTPN2 shRNA sequence: (Sense, 5′-3′): CACAAAGAAGTTACATCTT and (Antisense, 5′-3′): AAGATGTAACTTCTTTGTG.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp., Armonk, NY, USA) was used for statistical analyses. Data were expressed as mean ± SD (standard deviation). The *t*-test was used for analyzing the differences between the two groups. Survival analysis was assessed by Kaplan-Meier method, followed by Log-rank test for the comparison between the two curves. $p<0.05$ indicated the significant difference.

Results

Upregulated PTPN2 in Laryngocarcinoma

PTPN2 was found to be upregulated in laryngocarcinoma tissues than that in paracancerous

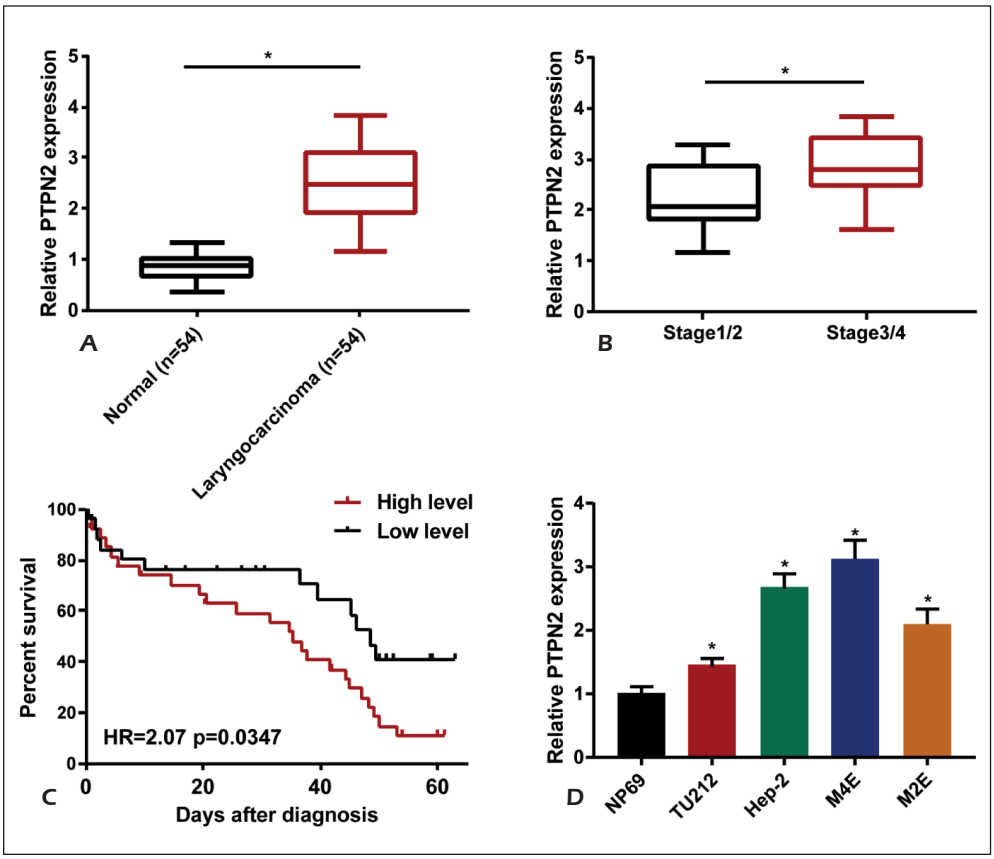

Figure 1. Upregulated PTPN2 in laryngocarcinoma. **A**, PTPN2 level in laryngocarcinoma tissues and paracancerous tissues. **B**, PTPN2 level in laryngocarcinoma patients with stage1/2 or stage3/4. **C**, Overall survival in laryngocarcinoma patients expressing high or low level of PTPN2. D, PTPN2 level in laryngocarcinoma cells (TU212, Hep-2, M4E, M2E) and immortalized human nasopharyngeal epithelial cells (NP69).

tissues (Figure 1A). Particularly, laryngocarcinoma patients in stage 3/4 expressed higher abundance of PTPN2 than those in stage 1/2 (Figure 1B). The Kaplan-Meier curves revealed worse survival in laryngocarcinoma patients expressing high level of PTPN2, suggesting that PTPN2 was unfavorable to the prognosis of laryngocarcinoma (HR=2.07, p=0.0347, Figure 1C). Meanwhile, PTPN2 was highly expressed in laryngocarcinoma cell lines as well (Figure 1D). In the following experiments, M4E cell line was selected because of its highest abundance of PTPN2 among the five tested laryngocarcinoma cell lines.

PTPN2 Was Activated by Inflammatory Response

M4E cells were treated with IFN-γ or TNF-α. Interestingly, the protein level of PTPN2 was remarkably upregulated following inflammatory response. Particularly, the treatment of both IFN-γ and TNF-α resulted in a pronounced upregulation of PTPN2 (Figure 2).

PTPN2 Was Activated by Oxidative Stress

After H_2O_2 stimulation, the protein level of PTPN2 was upregulated in M4E cells, which was further reversed by NAC, an antioxidant (Figure 3A). Moreover, the protein level of PTPN2 was dose-dependently upregulated in M4E cells stimulated with 0, 10, 20 or 30 μM H_2O_2 (Figure 3B). Both clonality and Ki-67 positive cell ratio were dose-dependently elevated in M4E cells, suggesting that oxidative stress promotes proliferative ability in laryngocarcinoma (Figure 3C, 3D).

Knockdown of PTPN2 Suppressed Proliferative Ability in Laryngocarcinoma

To validate the influence of PTPN2 on proliferative ability in laryngocarcinoma, M4E cells were transfected with negative control or sh-PTPN2, respectively. Clonality was attenuated after the knockdown of PTPN2 in M4E cells stimulated by H_2O_2 or not (Figure 4A). Similarly, Ki-67 positive cell ratio decreased after the transfection of

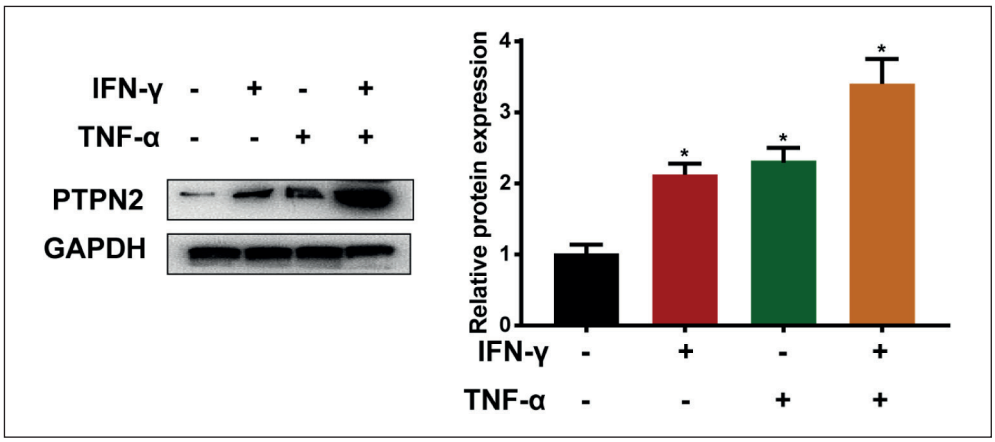

Figure 2. PTPN2 was activated by inflammatory response. Protein level of PTPN2 in M4E cells treated with IFN-γ, TNF-α or IFN-γ+TNF-α.

sh-PTPN2 regardless of H_2O_2 stimulation (Figure 4B). It is believed that PTPN2 is able to promote proliferative ability in laryngocarcinoma.

Discussion

Laryngocarcinoma is a common malignant tumor of the head and neck, and its incidence is about 5% of systemic malignant tumors[13]. More than 95% of laryngocarcinoma cases belong to laryngeal squamous cell carcinoma[14]. In recent years, the incidence of laryngocarcinoma is on the rise in our country. It is estimated that laryngocarcinoma has been the leading malignancy in head and neck cancers in northern China, and second in the southern China following nasopharyngeal cancer. Currently, the diagnosis and prognosis of laryngocarcinoma mainly rely on endoscopy and tissue biopsy, which are complex

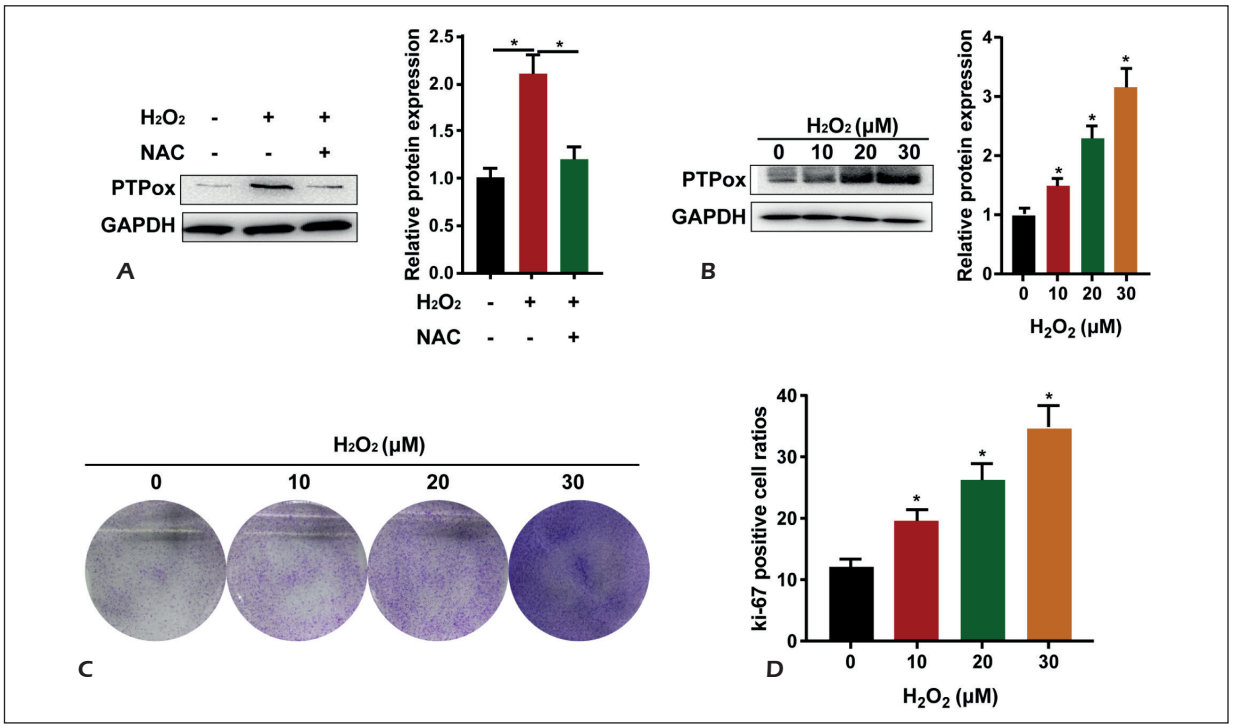

Figure 3. PTPN2 was activated by oxidative stress. **A**, Protein level of PTPN2 in M4E cells treated with H2O2 or H2O2+NAC. **B**, Protein level of PTPN2 in M4E cells treated with 0, 10, 20 or 30 μM H_2O_2. **C**, Clonality in M4E cells treated with 0, 10, 20 or 30 μM H_2O_2 (magnification: 10×). **D**, Ki-67 positive cell ratio in M4E cells treated with 0, 10, 20 or 30 μM H_2O_2.

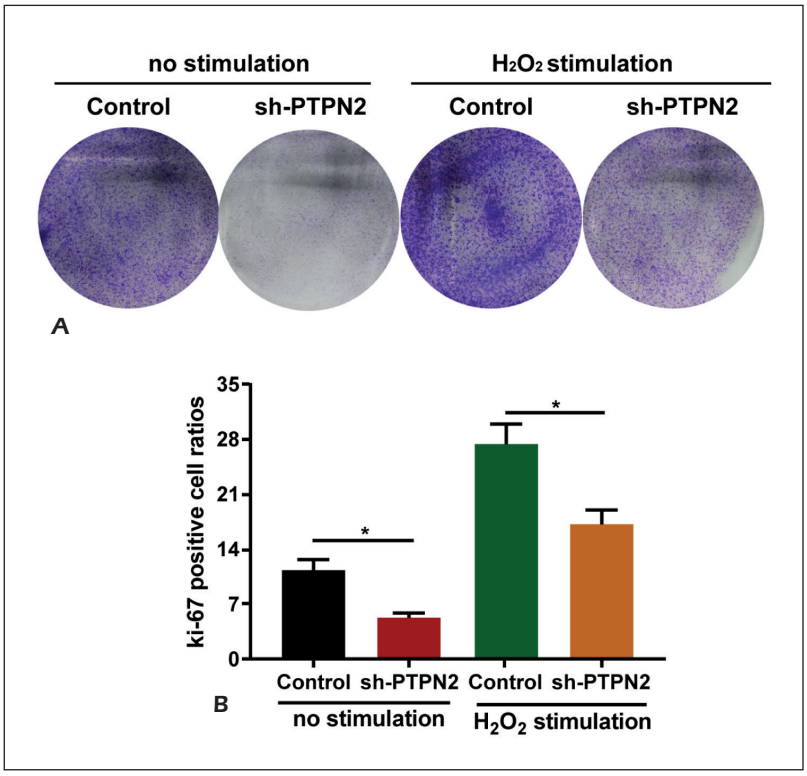

Figure 4. Knockdown of PTPN2 suppressed proliferative ability in laryngocarcinoma. **A**, Clonality in M4E cells transfected with negative control or sh-PTPN2 regardless of H_2O_2 stimulation (magnification: 10×). **B**, Ki-67 positive cell ratio in M4E cells transfected with negative control or sh-PTPN2 regardless of H_2O_2 stimulation.

and invasive. In the meantime, sensitivity of imaging examination is not satisfactory. Over 50% of laryngocarcinoma patients experience local infiltration or lymphatic metastasis at the initial diagnosis[15]. Therefore, researches on laryngocarcinoma are of clinical significance.

PTPN2 is an important member of the protein tyrosine phosphatase family. PTPN2 encodes two mRNAs, namely TC48 and TC45. TC48 is a 48-kDa molecule located in endoplasmic reticulum and Golgi complex and TC45 is a 45-kDa molecule located in the nucleus. External stimuli trigger nuclear translocation of TC45, which further leads to dephosphorylation of cytoplasmic proteins. As a result, blocked downstream pathways contribute to regulation on inflammatory response and immune reaction[16]. In PTPN2$^{-/-}$ animal model, serum levels of inflammatory mediators are elevated[17], accompanied by extensive and severe inflammatory response. Spalinger et al[11] have shown that PTPN2 is responsible for inflammasome activation, and thus controls intestinal inflammation and tumorigenesis of colon cancer. In glioma, PTPN2 aggravates the occurrence of tumor[12]. Consistently, PTPN2 was upregulated in laryngocarcinoma, and we confirmed that PTPN2 was unfavorable for the prognosis of laryngocarcinoma patients.

Inflammation is the basic innate immune response that damages tissue homeostasis. Chronic inflammatory process affects every stage of tumorigenesis, progression, metastasis, and treatment[18]. Cytokines are important molecules triggering and maintaining immune responses during anti-tumor processes[19]. In recent years, they have been identified in the regulation of laryngocarcinoma[20,21]. Oxidative stress occurs when the redox balance is broken in the body. Excessive production of free radicals results in decreased antioxidant ability. Subsequently, reactive oxygen and nitrogen are largely produced, which further form hydroxyl radicals and cause tissue or cell toxicity[22]. Kang et al[23] demonstrated the critical role of the oxidative stress in promoting the progression of laryngocarcinoma. In our investigation, IFN-γ or TNF-α treatment upregulated the protein level of PTPN2, suggesting that PTPN2 is activated following the inflammatory response. Meanwhile, H_2O_2 stimulation upregulated the protein level of PTPN2, dose-dependently increased clonality, and Ki-67 positive cell ratio in M4E cells. Knockdown of PTPN2 suppressed clonality and Ki-67 positive cell ratio in M4E cells either with H_2O_2 stimulation or not. Therefore, PTPN2 was verified to be activated by the inflammatory response or oxidative stress. Upregulated PTPN2 further influenced proliferative ability in laryngocarcinoma.

Conclusions

Above all, inflammatory response or oxidative stress could induce upregulation of PTPN2, thus promoting the proliferative ability of laryngocarcinoma.

Conflict of Interests

The authors declare that they have no conflict of interest.

References

1) Chu EA, Kim YJ. Laryngeal cancer: diagnosis and preoperative work-up. Otolaryngol Clin North Am 2008; 41: 673-695.
2) Yu Q, Zhang X, Ji C, Yang H, Gao M, Hong S, Hu G. Survival analysis of laryngeal carcinoma without laryngectomy, radiotherapy, or chemotherapy. Eur Arch Otorhinolaryngol 2012; 269: 2103-2109.
3) Rudolph E, Dyckhoff G, Becher H, Dietz A, Ramroth H. Effects of tumour stage, comorbidity and therapy on survival of laryngeal cancer patients: a systematic review and a meta-analysis. Eur Arch Otorhinolaryngol 2011; 268: 165-179.
4) van Vliet C, Bukczynska PE, Puryer MA, Sadek CM, Shields BJ, Tremblay ML, Tiganis T. Selective regulation of tumor necrosis factor-induced Erk signaling by Src family kinases and the T cell protein tyrosine phosphatase. Nat Immunol 2005; 6: 253-260.
5) Pike KA, Tremblay ML. TC-PTP and PTP1B: Regulating JAK-STAT signaling, controlling lymphoid malignancies. Cytokine 2016; 82: 52-57.
6) Doody KM, Bourdeau A, Tremblay ML. T-cell protein tyrosine phosphatase is a key regulator in immune cell signaling: lessons from the knockout mouse model and implications in human disease. Immunol Rev 2009; 228: 325-341.
7) Wiede F, Shields BJ, Chew SH, Kyparissoudis K, van Vliet C, Galic S, Tremblay ML, Russell SM, Godfrey DI, Tiganis T. T cell protein tyrosine phosphatase attenuates T cell signaling to maintain tolerance in mice. J Clin Invest 2011; 121: 4758-4774.
8) Aradi B, Kato M, Filkova M, Karouzakis E, Klein K, Scharl M, Kolling C, Michel BA, Gay RE, Buzas EI, Gay S, Jungel A. Protein tyrosine phosphatase nonreceptor type 2: an important regulator of Interleukin-6 production in rheumatoid arthritis synovial fibroblasts. Arthritis Rheumatol 2015; 67: 2624-2633.
9) Scharl M, Hruz P, McCole DF. Protein tyrosine phosphatase non-receptor Type 2 regulates IFN-γ-induced cytokine signaling in THP-1 monocytes. Inflamm Bowel Dis 2010; 16: 2055-2064.
10) Manguso RT, Pope HW, Zimmer MD, Brown FD, Yates KB, Miller BC, Collins NB, Bi K, LaFleur MW, Juneja VR, Weiss SA, Lo J, Fisher DE, Miao D, Van Allen E, Root DE, Sharpe AH, Doench JG, Haining WN. In vivo CRISPR screening identifies Ptpn2 as a cancer immunotherapy target. Nature 2017; 547: 413-418.
11) Spalinger MR, Manzini R, Hering L, Riggs JB, Gottier C, Lang S, Atrott K, Fettelschoss A, Olomski F, Kundig TM, Fried M, McCole DF, Rogler G, Scharl M. PTPN2 Regulates inflammasome activation and controls onset of intestinal inflammation and colon cancer. Cell Rep 2018; 22: 1835-1848.
12) Wu L, Wang F, Xu J, Chen Z. PTPN2 induced by inflammatory response and oxidative stress contributed to glioma progression. J Cell Biochem 2019: 120: 19044-19051.
13) Sanderson RJ, Ironside JA. Squamous cell carcinomas of the head and neck. BMJ 2002; 325: 822-827.
14) Almadori G, Bussu F, Cadoni G, Galli J, Paludetti G, Maurizi M. Molecular markers in laryngeal squamous cell carcinoma: towards an integrated clinicobiological approach. Eur J Cancer 2005; 41: 683-693.
15) Al-Ibraheem A, Buck A, Krause BJ, Scheidhauer K, Schwaiger M. Clinical applications of FDG PET and PET/CT in head and neck cancer. J Oncol 2009; 2009: 208725.
16) Bourdeau A, Dube N, Tremblay ML. Cytoplasmic protein tyrosine phosphatases, regulation and function: the roles of PTP1B and TC-PTP. Curr Opin Cell Biol 2005; 17: 203-209.
17) Heinonen KM, Bourdeau A, Doody KM, Tremblay ML. Protein tyrosine phosphatases PTP-1B and TC-PTP play nonredundant roles in macrophage development and IFN-gamma signaling. Proc Natl Acad Sci U S A 2009; 106: 9368-9372.
18) Elinav E, Nowarski R, Thaiss CA, Hu B, Jin C, Flavell RA. Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms. Nat Rev Cancer 2013; 13: 759-771.
19) Muller-Hubenthal B, Azemar M, Lorenzen D, Huber M, Freudenberg MA, Galanos C, Unger C, Hildenbrand B. Tumour biology: tumour-associated inflammation versus antitumor immunity. Anticancer Res 2009; 29: 4795-4805.
20) Tian L, Chen X, Sun Y, Liu M, Zhu D, Ren J. Growth suppression of human laryngeal squamous cell carcinoma by adenoviral-mediated interleukin-12. J Int Med Res 2010; 38: 994-1004.
21) Gunaydin RO, Kesikli SA, Kansu E, Hosal AS. Identification of the peripheral blood levels of interleukin-12, interleukin-10, and transforming growth factor-beta in patients with laryngeal squamous cell carcinoma. Head Neck 2012; 34: 393-397.
22) de Sa JP, Camara D, Porcacchia AS, Fonseca P, Jorge SD, Araldi RP, Ferreira AK. The roles of ROS in cancer heterogeneity and therapy. Oxid Med Cell Longev 2017; 2017: 2467940.
23) Kang N, Zhang JH, Qiu F, Tashiro S, Onodera S, Ikejima T. Inhibition of EGFR signaling augments oridonin-induced apoptosis in human laryngeal cancer cells via enhancing oxidative stress coincident with activation of both the intrinsic and extrinsic apoptotic pathways. Cancer Lett 2010; 294: 147-158.