Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.jzus.zju.edu.cn; www.springerlink.com
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Comparative analysis of a panel of biomarkers related to protein phosphatase 2A between laryngeal squamous cell carcinoma tissues and adjacent normal tissues*#

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https://doi.org/10.1631/jzus.B1900179

Laryngeal squamous cell carcinoma (LSCC) is the most common type of head and neck squamous cell carcinoma (HNSCC) worldwide. Protein phosphatase 2A (PP2A) dysfunction has been widely reported in a broad range of malignancies due to its distinctive role in miscellaneous cellular processes. However, it is poorly understood whether aberrant alterations of PP2A are involved in the network of oncogenic events in LSCC. Here, we detected a panel of PP2A-associated proteins using western blot in both laryngeal squamous cell carcinoma tissues and paired adjacent normal tissues from patients (Data S1). We found that phospho-PP2A/C (Y307), α4, cancerous inhibitor of protein phosphatase 2A (CIP2A), Akt, ezrin, phospho-ezrin (T567), 14-3-3, and focal adhesion kinase (FAK) showed increased expression levels in carcinoma tissues relative to normal tissues, while phospho-Akt (T308) showed decreased levels. Our study, thus, provides a rationale for targeting PP2A to develop novel therapies and proposes a combination of interrelated biomarkers for the diagnostic evaluation and prognosis prediction in LSCC.

Despite the advanced understanding of molecular mechanisms underlying LSCC oncogenesis (Kozakiewicz and Grzybowska-Szatkowska, 2018), the treatment outcome has not yet been fundamentally improved, with an overall survival rate of less than 50% for advanced stages after salvage surgery. There is a pressing need to identify novel molecular prognostic biomarkers in this malignancy that can contribute to early diagnosis and precise prediction.

PP2A, one of the major serine/threonine phosphatases in mammalian cells, is a heterotrimeric complex consisting of a catalytic subunit (C), a structural subunit (A), and a regulatory subunit (B). It was initially characterized to be a tumor suppressor owing to its negative role as an "off" switch for multiple oncogenic kinase-driven signaling pathways. Aberrations in PP2A holoenzyme assembly and functions have long been implicated in cancer, including cases of HNSCC (Ouchida et al., 2019). Therefore, re-activating the enzymatic activity of PP2A provides a promising strategy for anti-cancer

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* Project supported by the Medical Scientific Research of Zhejiang Province (Nos. 2009A044 and 2015KYA055), the Natural Science Foundation of Zhejiang Province (No. LY13H130001), the Program of Science Technology Department (No. 2013C33208), and the Program of Traditional Chinese Medicine Administration of Zhejiang Province (No. 2012ZA085), China
# Electronic supplementary materials: The online version of this article (https://doi.org/10.1631/jzus.B1900179) contains supplementary materials, which are available to authorized users
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therapy (Kauko et al., 2018), for example, by blocking endogenous inhibitor proteins from binding with PP2A catalytic domain. However, emerging studies focusing on PP2A as a therapeutic target shift the old paradigm (Kiely and Kiely, 2015) and suggest that pharmacologic inhibition of PP2A could conversely enhance antitumor response. These contradictory facts highlight the need for a better explanation of the complicated regulatory role of PP2A in the context of a broad range of malignancies.

Our previous studies have shown that PP2A activity in various cancer cell lines could be strongly inhibited by a cyanobacteria-derived toxin called microcystin-LR (MC-LR) (Wang H et al., 2014; Wang BL et al., 2017; Wang HY et al., 2017), including in the human laryngeal epithelial derived cancer cell line Hep-2 (Wang BL et al., 2017). We demonstrated that the phosphorylation of PP2A/C at Y307, which is negatively related with PP2A activity, and the phosphorylation of PP2A substrates, including ezrin and Akt, increased in MC-LR-treated cancer cells. We also found different interactions between PP2A/C and α4 protein upon PP2A inhibition among different cancer cells. Based on these in vitro data acquired using a xenobiotic potent PP2A inhibitor, we tried to explain the endogenous expression differences of these PP2A-related proteins between carcinoma tissues and adjacent normal tissues in patient samples. By depicting a PP2A-centered molecular signature, we aim to provide a cross-sectional investigation on a panel of biomarkers that may be of clinical significance and prognostic value in LSCC.

The endogenous PP2A activity is tightly controlled through many regulatory mechanisms (Wang HY et al., 2017). Reversible post-translational modifications of PP2A/C to modulate its catalytic activity have been well documented. For example, the phosphorylation of PP2A/C at Tyr307 decreases PP2A activity by inhibiting the association of PP2A/C with the PR55/PR61 subunits. A highly conserved non-catalytic regulatory subunit of PP2A, α4, plays a key role in PP2A core enzyme assembly and stability (Wang HY et al., 2017). The binding of PP2A/C to α4 decreases its catalytic activity, while under cellular stress, release of PP2A/C from α4 is essential for the adaptive assembly of PP2A holoenzyme. This subunit is also reported to possess an oncogenic function owing to its ubiquitously high expression in primary human cancers (Chen et al., 2011). CIP2A, a PP2A inhibitor protein, has also been widely addressed in different cancers owing to its oncogenic role. Notably, overexpression of CIP2A has been linked to poor differentiation level, radioresistance, and low survival of patients with HNSCC (Ventelä et al., 2015). Consistent with the results of previous studies (Wang BL et al., 2017), our results show that the protein levels of phosphorylated PP2A/C, α4, and CIP2A were all increased in laryngeal squamous cell carcinoma tissues compared to paired adjacent normal tissues (Fig. 1), indicating that PP2A activity is probably strongly inhibited in LSCC as a result of multiple disrupted mechanisms.

Akt, one of the most important substrates of PP2A, is a significant survival kinase and is frequently hyperactivated in cancer (Liu et al., 2016a). The phosphorylation at both Thr308 site and Ser473 site is required for its full activation (Liu et al., 2016b). Based on the assumption of impaired PP2A activity, it is conceivable that Akt might be hyperphosphorylated in carcinoma tissues versus normal tissues. However, our results show that despite elevated total protein level, its phosphorylation at Thr308 was surprisingly lower in laryngeal carcinoma tissues relative to normal tissues (Fig. 2). To further confirm whether Akt signaling pathway is indeed activated, it is necessary to detect the phosphorylation of Akt at Ser473 as well as to assess that of downstream molecules such as S6K1.

Ezrin, another substrate subject to PP2A-mediated dephosphorylation, serves as a linker and signal transducer connecting the actin cytoskeleton and the plasma membrane (McClatchey, 2014). The phosphorylation of ezrin at Thr567 is fundamental for its activation, which is known to confer metastatic advantage in cancer. Our data indicate that both total ezrin and its phosphorylated form may act as predictors of progression in laryngeal cancer (Fig. 2). However, their specific mechanism in metastasis, such as cytoskeletal rearrangement (Wang BL et al., 2017), is yet to be determined.

14-3-3 protein family are phospho-serine/phospho-threonine binding proteins involved in many pathological cellular processes through binding a multitude of functionally diverse ligands (Cau et al., 2018). PP2A is an important regulator of 14-3-3 binding interactions, as exemplified in the case of

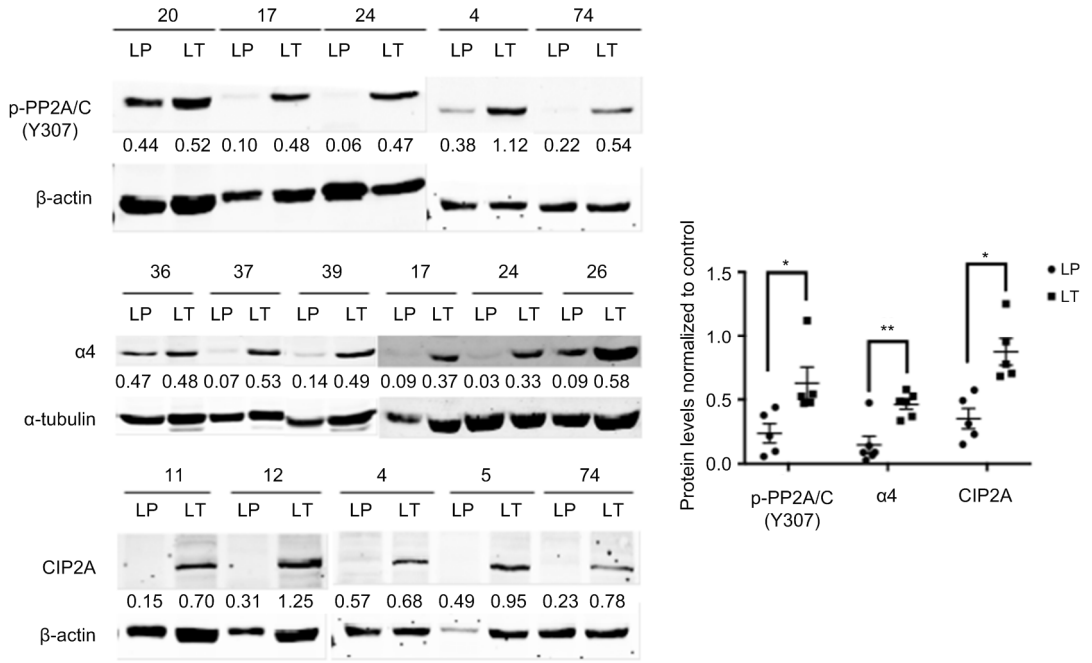

Fig. 1 Different expression levels of p-PP2A/C (Y307), α4, and CIP2A

Western blot of whole cell extracts from 28 pairs of patient samples. Representative figures for five or six patients are shown. Either β-actin or α-tubulin was used as the loading control. The corresponding densitometric analysis data are presented as the fold changes in protein levels relative to the control (n=5 or 6). * P<0.05; ** P<0.01. p-PP2A/C: phospho-protein phosphatase 2A/C; CIP2A: cancerous inhibitor of protein phosphatase 2A; LT: laryngeal squamous cell carcinoma tissues; LP: adjacent normal tissues

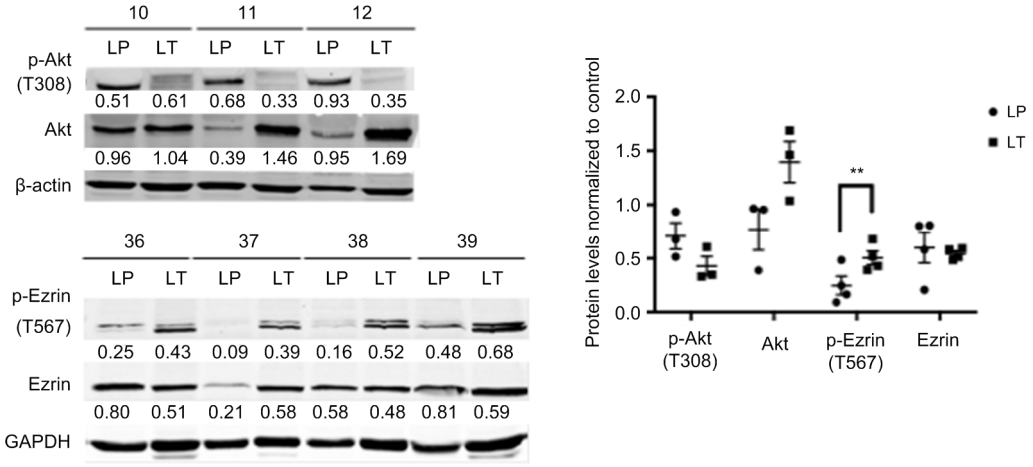

Fig. 2 Different expression levels of p-Akt (T308), Akt, p-ezrin (T567), and ezrin

Western blot of whole cell extracts from 28 pairs of patient samples. Representative figures for three or four patients are shown. Either β-actin or GAPDH was used as the loading control. The corresponding densitometric analysis data are presented as the fold changes in protein levels relative to the control (n=3 or 4). ** P<0.01. p-Akt: phospho-Akt; p-ezrin: phospho-ezrin; LT: laryngeal squamous cell carcinoma tissues; LP: adjacent normal tissues; GAPDH: glyceraldehyde-3-phosphate dehydrogenase

Cdc25, where B56δ-PP2A holoenzyme-mediated dephosphorylation of Cdc25 at Thr138 controls 14-3-3 release and Cdc25 activation for mitotic entry. Regarding its role in cancer, the aberrant expression of 14-3-3 proteins is variably dependent on its isoforms and tissue of origin (Morrison, 2009). We showed here that total 14-3-3 proteins are upregulated in laryngeal cancer (Fig. 3), but it remains unknown which particular family member, such as 14-3-3ζ, contributes to tumorigenesis in LSCC. FAK, one of

the key members of focal adhesion complex, is overexpressed and activated in several advanced-stage solid cancers (Sulzmaier et al., 2014). PP2A-mediated FAK/Src/paxillin dephosphorylation controls cytoskeleton dynamics, cell adhesion and migration. Our data indicate a relatively high expression level of FAK in laryngeal cancer compared to adjacent normal tissues (Fig. 3), although whether this increase results from assumed PP2A disruption or results in enhanced cell motility and metastasis requires to be further elucidated.

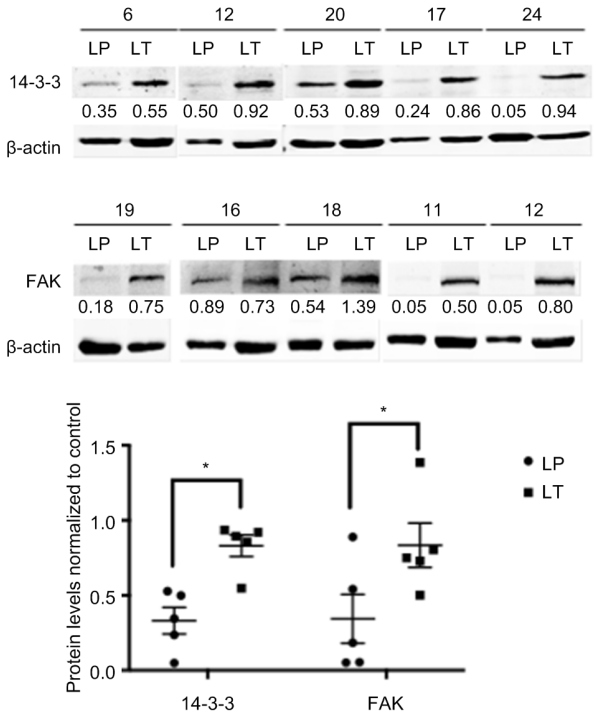

Fig. 3 Different expression levels of 14-3-3 and FAK
Western blot of whole cell extracts from 28 pairs of patient samples. Representative figures for five patients are shown. β-Actin was used as the loading control. The corresponding densitometric analysis data are presented as the fold changes in protein levels relative to the control (n=5). * P<0.05. FAK: focal adhesion kinase; LT: laryngeal squamous cell carcinoma tissues; LP: adjacent normal tissues

To our knowledge, this is the first study analyzing a network of PP2A-oriented proteins in primary laryngeal carcinoma samples. Our findings suggest that diagnostic evaluation of laryngeal carcinoma for a panel of PP2A-related molecular biomarkers might help to stratify patients with more malignant subtypes for more aggressive therapies. Nevertheless, the causal relationships between these biomarkers are yet to be identified. It is also worth pointing out that with the complete information on patients' perioperative clinicopathological characteristics and corresponding imaging data during subsequent follow-up, we would have been able to establish a multivariate analysis model for clinical prediction of metastasis, invasion, prognosis, or survival of LSCC.

Contributors

Han-ying WANG wrote the manuscript after integrative data analyses. Hui YUAN collected clinical specimens. Jing-hui LIU, Bei-lei WANG, Kai-lun XU, and Pu HUANG performed western blots and statistical analysis. Zhi-hong LIN conducted and guided the surgical laryngectomy. Li-hong XU designed this research and supervised the whole study. All authors read and approved the final manuscript.

Acknowledgments

We thank Shu-yan LIN, Yu-sheng TONG, Cong-hui WANG, and Tao CHEN of Zhejiang University (Hangzhou, China) for performing western blot analysis.

Compliance with ethics guidelines

Han-ying WANG, Hui YUAN, Jing-hui LIU, Bei-lei WANG, Kai-lun XU, Pu HUANG, Zhi-hong LIN, and Li-hong XU declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study.

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List of electronic supplementary materials

Data S1 Materials and methods

中文概要

题　目：喉癌患者癌组织与癌旁正常组织之间蛋白磷酸酶 2A 及其相关分子标志物表达水平的比较研究

目　的：探讨喉癌患者组织样本中蛋白磷酸酶 2A 及其相关蛋白在癌组织与癌旁组织之间的表达差异，建立可用于临床进行喉癌患者预后分层的多指标预测体系。

创新点：以蛋白磷酸酶 2A 为核心，探讨系列肿瘤转移相关指标参与喉癌发生发展的分子机制。

方　法：收集 2012~2015 年间浙江大学医学院附属第二医院耳鼻咽喉科 28 例喉鳞状细胞癌患者的临床组织样本，提取总蛋白后进行免疫印迹分析。

结　论：与癌旁正常组织相比，喉癌组织中 phospho-PP2A/C（Y307）、α4、CIP2A、Akt、ezrin、phospho-ezrin（T567）、14-3-3 以及 FAK 蛋白水平均显著升高，而 phospho-Akt（T308）蛋白水平则显著降低。

关键词：喉癌；蛋白磷酸酶 2A；生物标志物