RAPID COMMUNICATION

Ribonucleotide reductase subunit M1 blocks glucose catabolism via phosphorylation of pyruvate kinase M2 in human esophageal squamous cell cancer

Esophageal squamous cell cancer (ESCC) is one of the malignant tumors with high morbidity and mortality all over the world.[1] In recent years, combined chemotherapy has gradually been the effective treatment method for ESCC patients. Therefore, it is imperative to explore potential therapeutic targets for the treatment of ESCC. We previously reported a high-frequency amplification of pyrimidine metabolic pathway-related genes in ESCC tissues,[2] and ribonucleotide reductase subunit M1 (RRM1) was one of many abnormal genes. RRM1 is one of the important subunits of ribonucleoside reductase (RR) and plays a key role in cell DNA replication as a binding site of nucleotides to regulate substrate specificity and RR activity. RRM1 is expressed in almost all tumor cells, but its expression levels and functions are differently affected by tissue origin and cell location. The expression of RRM1 in lung cancer tissues was generally lower than that in adjacent tissues, while an opposite trend was observed in breast cancer. Reports about RRM1 in ESCC are less, and the mechanism(s) of carcinogenesis is still poorly understood. The present study aimed to understand the role of RRM1 on the malignant proliferation process, and further clarify the molecular mechanism in ESCC development, which would be helpful in anti-cancer therapy of ESCC patients.

Data obtained from the GEPIA database and immunohistochemical results showed that RRM1 expression in most ESCC tissues was abnormally higher than that in adjacent tissues (Fig. S1; Fig. 1A, B), and a significantly negative correlation between the RRM1 expression and survival time was observed, and patients with high RRM1 expression exhibited a worse prognosis (Fig. 1C), suggesting that RRM1 could be used as an indicator for early diagnosis, a potential predictor to estimate patients' survival or a potential target of antitumor drugs. RRM1 is often considered as a tumor suppressor gene inhibiting tumor cell proliferation, metastasis, and invasion, and is used as the main target of antitumor drugs. However, clinical trials showed the high sensitivity of lung cancer patients with low RRM1 expression to gemcitabine, and the chemotherapy scheme with gemcitabine is often used to treat the patients with low RRM1.[3] The response to clinical chemotherapy targeting RRM1 is closely related to the expression levels of RRM1 in cancer tissue. Different sensitivity of RRM1 on antitumor drugs was close to the carcinogenic mechanism of RRM1, but the mechanism has remained unclear.

To study the regulatory mechanism of RRM1, we first verified its biological effect in human ESCC cells. By siRNA interfering with RRM1 in ESCC cell lines, it was found that the S phase of the cell cycle was significantly increased, but the G2 phase was significantly decreased, indicating that the cell cycle was blocked in the S/G2 phase so that cell proliferation was inhibited (Fig. S2, 3). Results from RRM1-overexpressing ESCC cells further proved the key role of RRM1 on the cell cycle. Intracellular up-regulated RRM1 expression promoted the mass production of DNA synthetic substances providing material support for cell division, which were confirmed by targeted metabonomics analysis and HPLC-electrochemical detection identified more 2′-Deoxy-guanosine 5′-monophosphate (dGMP), deoxycytidine monophosphate (dAMP), deoxyadenosine monophosphate (dCMP), and deoxyinosine in RRM1-overexpressing cells (Fig. S4). The nucleic acid metabolism is closely related to the pentose phosphate pathway (PPP),[4] and the increased D-Ribose 5-phosphate (R5P) as substrates of nucleic acid synthesis from PPP indicated that the up-regulation of nucleic acid anabolism may be partly promoted by the enhancement of PPP. Moreover, the up-regulation of intermediate metabolites of

---

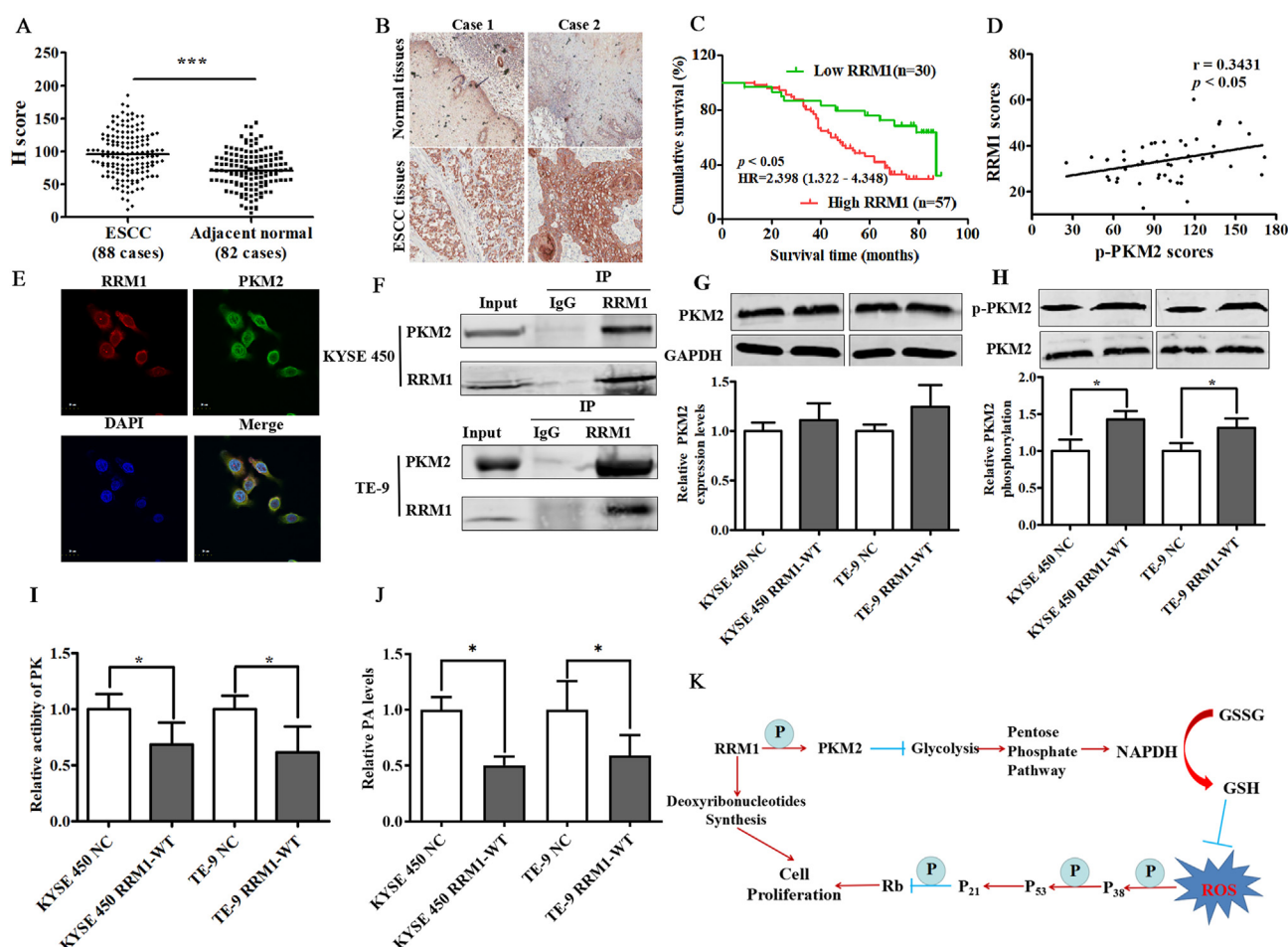Peer review under responsibility of Chongqing Medical University.

Figure 1 RRM1 induces tumorigenesis via phosphorylation of pyruvate kinase M2 in human esophageal squamous cell cancer (ESCC). **(A)** Histochemical scoring of RRM1 expression in ESCC and matched paracancerous tissue samples (*$P < 0.05$). **(B)** Representative images of RRM1 protein expression in ESCC tissue and normal tissue vis immunohistochemical analysis (Scale bar = 100 μm). **(C)** Kaplan−Meier survival analysis of overall survival of ESCC patients. **(D)** The correlation analysis between RRM1 and p-PKM2 expression was carried out by Pearson correlation coefficient. **(E)** Expression of RRM1 (red) and PKM2 (green) in cells was detected by immunofluorescence. **(F)** The interaction between RRM1 and PKM2 was confirmed by IP experiment. **(G, H)** PKM2 and phosphorylated PKM2 (p-PKM2) were detected by Western blot assays. **(I, J)** PK activities and PA levels were shown by bar graphs. **(K)** Regulation model of RRM1 on cell proliferation in ESCC. Arrows (→) and truncated lines (—|) indicated promoting and inhibiting effects, respectively. Data were representative of three independent experiments and are presented as mean ± standard deviation, and P values < 0.05 were considered statistically significant.

glycolysis, including D-glucose 6-phosphate (G6P), phospho-enolpyruvate (PEP), reduced nicotinamide adenine dinucle-otide (NADH), and adenosine triphosphate (ATP), was observed, but the levels of L-lactic acid and malic acid involved in the tricarboxylic acid cycle were down-regu-lated. These indicated that glucose catabolism in RRM1-overexpressing cells might be blocked, which could promote more carbon flowing into PPP and nucleic acid anabolism.

The enhanced PPP not only promotes nucleic acid anab-olism, but also significantly increases the intracellular co-enzyme II, and the up-regulated reduced nicotinamide adenine dinucleotide phosphate (NADPH) and nicotinamide adenine dinucleotide phosphate (NADP$^+$) in RRM1-over-expressing cells was observed. NADPH is an important in-termediate metabolite in PPP and provides a necessary co-factor that plays an essential role in lipogenesis and the entire antioxidant system. In the defense against intracel-lular oxidative stress, one of the major antioxidants is

glutathione (GSH) which neutralizes reactive oxygen species by the synthesis of oxidized glutathione disulfide (GSSG), and the cycling of GSSG to GSH depends on NADPH. The detec-tion of GSH and GSSG confirmed the enhanced glutathione system and relieved oxidative stress (Fig. S5). The reactive oxygen species (ROS) levels in cells, unexpectedly, were down-regulated. Lower ROS down-regulated three key tumor suppressor proteins (p-p38, p-p53, and p21; Fig. S5F), which up-regulated Rb phosphorylation and promoted cell cycles. Taken together, the enhanced glutathione system was pro-moted by a large number of NADPH provided from PPP, and relieved oxidative stress promoted cell proliferation of human ESCC by activating cell division via ROS-mediated p-p38/p-p53/p21/p-Rb pathway.

Undoubtedly, the blocked glucose catabolism may directly or indirectly result from RRM1. To explore the po-tential regulation mechanism, protein mass spectrographic analysis was performed. Pyruvate kinase M2 (PKM2) was

found to be the potential protein that interacted with RRM1, and the interaction relationship between both proteins was confirmed by immunocoprecipition (Fig. 1E, F). In the glycolytic pathway, pyruvate kinase (PK) is a key enzyme that catalyzes the transfer of a phosphoryl group from phosphoenolpyruvate to ADP to form pyruvic acid (PA) and ATP, and the PK activity is closely related to the phosphorylation of PKM2.[5] In the present study, the up-regulated phosphorylation levels of PKM2 were observed in both RRM1-overexpressing cells (Fig. 1G, H), and the inhibited PK activity and reduced PA were also found correspondingly (Fig. 1I, J), indicating that hyper-phosphorylation of PKM2 might be the main reason causing the decreased PK activity and impaired glucose decomposition. The immunohistochemical analysis further revealed a significant positive correlation between the expression levels of RRM1 and p-PKM2 (Fig. 1D).

Carcinogenesis of RRM1 may work by controlling the phosphorylation of PKM2 in human ESCC. To further clarify the regulatory mechanism, an inhibitor (gemcitabine) of RRM1 activity was used in RRM1-overexpressing cells. Results showed that the PKM2 phosphorylation level significantly decreased compared to that of control groups (Fig. S6A), but the PK activity and PA levels significantly increased (Fig. S6B, C). The enhanced PK activity may promote more intermediates into the tricarboxylic acid cycle, and reduce the carbon flux to PPP, inhibiting the synthesis of antioxidants and re-increasing the oxidative stress levels (Fig. S6D). Increased oxidative stress resulted in slowed cell cycle progression, and less deoxyribonucleotide caused by reduced RRM1 activity inhibited DNA production, finally slowing down cancer cell proliferation (Fig. S6E). Similar results have been achieved by the applied activator of PKM2 activity (TEPP-46) in RRM1-overexpressing cells, indicating a better clinical efficacy in the treatment of gemcitabine and TEPP-46 on ESCC.

The present study has identified RRM1 as a cancer-promoting gene by promoting cell cycle progression in human ESCC. We propose a potential prevention strategy of RRM1 through shunting glucose-derived carbon between glucose catabolism and pentose phosphate pathway and reveal the blocking mechanism of RRM1 on glucose catabolism works by a phosphorylation-independent mechanism on PKM2 as shown in Fig. 1K. The multifaceted role of RRM1 in cell metabolism and cell cycle progression makes it a valuable target to improve the clinical efficacy of ESCC chemotherapy.

Author contributions

J.Y., L.W., and H.S. developed the ideas and drafted the manuscript. J.Y., L.W., H.S, Z.Z., and Y.M. conducted the experiments. Z.H., L.Z., and Y.W. contributed to the data analysis. J.Y. revised the manuscript. All authors read and approved the final version of the manuscript for publication.

Conflict of interests

All authors declare that they have no conflict of interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2023.04.031.

References

1. Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2021;71(3):209−249.
2. Zhang L, Zhou Y, Cheng C, et al. Genomic analyses reveal mutational signatures and frequently altered genes in esophageal squamous cell carcinoma. *Am J Hum Genet*. 2015;96(4):597−611.
3. Lee JJ, Maeng CH, Baek SK, et al. The immunohistochemical overexpression of ribonucleotide reductase regulatory subunit M1 (RRM1) protein is a predictor of shorter survival to gemcitabine-based chemotherapy in advanced non-small cell lung cancer (NSCLC). *Lung Cancer*. 2010;70(2):205−210.
4. Polat IH, Tarrado-Castellarnau M, Bharat R, et al. Oxidative pentose phosphate pathway enzyme 6-phosphogluconate dehydrogenase plays a key role in breast cancer metabolism. *Biology (Basel)*. 2021;10(2):85.
5. Wang H, Nicolay BN, Chick JM, et al. The metabolic function of cyclin D3-CDK6 kinase in cancer cell survival. *Nature*. 2017; 546(7658):426−430.

Jian Yang [a,b,c,*], Lingxiao Wang [d], Hui Sun [e], Zhenxiang Zhao [a], Yanchuan Ma [a], Zhen Hu [f], Liqin Zhai [g], Yonggang Wang [d]
[a] Translational Medicine Research Center, Shanxi Medical University, Taiyuan, Shanxi 030001, China
[b] Department of Cell Biology and Genetics, Shanxi Medical University, Taiyuan, Shanxi 030001, China
[c] Key Laboratory of Cellular Physiology of the Ministry of Education, Shanxi Medical University, Taiyuan, Shanxi 030001, China
[d] Department of Colorectal Surgery, The Fifth Clinical Medical College of Shanxi Medical University, Taiyuan, Shanxi 030012, China
[e] Science & Technology Information and Strategy Research Center of Shanxi, Taiyuan, Shanxi 030006, China
[f] Department of Pathology, Shanxi Provincial People's Hospital, Taiyuan, Shanxi 030012, China
[g] Department of Anatomy, College of Basic Medicine, Shanxi Medical University, Taiyuan, Shanxi 030001, China

*Corresponding author. Translational Medicine Research Center, Shanxi Medical University, Taiyuan, Shanxi 030001, China.
E-mail address: jiany@sxmu.edu.cn (J. Yang)

5 January 2023
Available online 24 June 2023