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RAPID COMMUNICATION

Bilirubin inhibits lung carcinogenesis by up-regulating cystatin A expression in tumor-infiltrating macrophages

As one of the leading causes of cancer deaths worldwide, the pathogenesis of lung cancer is still not completely understood. Bilirubin, a product of heme metabolism, has long been considered a waste product of the body. Increasing evidence suggests that bilirubin has additional antioxidant, anti-inflammatory, and proteasome inhibitory activities. However, the specific role of bilirubin in the formation and development of lung cancer has not been elucidated.

To investigate the effect of bilirubin on lung carcinogenesis, we used a mouse model of lung tumors induced by urethane[1] and assessed the effect of bilirubin treatment for 4 weeks (Fig. S1A). The number of tumors and total tumor volume in the urethane plus bilirubin treatment group (4 weeks) were smaller than those in the urethane group (Fig. S1B, C). However, there was no significant difference in the mean radius of lung tumors and body weight between the two groups (Fig. S1D, E). Bilirubin also reduced the number and total tumor volume of urethane-induced tumors in mice following treatment with bilirubin for 16 weeks (Fig. S1F–J). Collectively, these mouse studies suggest that bilirubin inhibits urethane-induced lung carcinogenesis, but not tumor growth.

Numerous studies have shown that bilirubin has antioxidant properties.[2] Furthermore, we previously reported that bilirubin inhibits proteasome function by targeting the deubiquitinase activity of the 19S proteasome in the nervous system.[3] Therefore, we used the antioxidant N-acetyl-L-cysteine (NAC) and the 19S proteasome inhibitor b-AP15 to explore whether the effect of bilirubin on lung tumorigenesis is related to its antioxidant activity or proteasome inhibitory activity (Fig. 1A). We found that bilirubin treatment (20 mg/kg or 30 mg/kg) decreased the lung coefficient of mice with lung cancer (Fig. 1B). Meanwhile, neither NAC nor b-AP15 treatment caused changes in lung coefficient (Fig. 1B), indicating that bilirubin may have superiority in tumor suppression compared to NAC or b-AP15. We also found that bilirubin and b-AP15 decreased the number and total volume of urethane-induced lung tumors, while NAC intervention failed to inhibit lung tumors (Fig. 1C, D). Consistent with the above results, there was no statistically significant difference in the tumor radius between the different groups (Fig. S2A). Hematoxylin and eosin staining of mouse lung tissue showed that the urethane-induced lung tumor was mainly lung adenocarcinoma (Fig. S2B). To test whether bilirubin exhibits the proteasome inhibition function, we examined the expression of ubiquitinated proteins in lung tissues by immunohistochemistry. Unexpectedly, we found no significant differences in ubiquitinated protein levels between groups at 28 weeks (Fig. S2C). However, we found that short-term treatment of bilirubin for 4 weeks increased the accumulation of ubiquitinated proteins in mouse lungs (Fig. S2D–F), suggesting that bilirubin may exhibit proteasome inhibition function in the early stage of lung carcinogenesis and the balance disturbance may recover in the late stage.

To further explore the underlying molecular mechanisms of bilirubin-mediated tumor suppression, we performed proteomics and transcriptomics of lung tissues from mice treated with bilirubin for 4 weeks (Fig. 1E). Differentially expressed genes analysis in proteomics (Fig. S3A, B) and transcriptomics (Fig. S3C, D) was performed by the Limma package. The differential genes obtained from the union set intersection of two omics were enriched in pathways, including inflammatory response, regulation of catalytic activity, regulation of hydrolase activity, regulation of endopeptidase activity, and neutrophil aggregation (Fig. 1F). Notably, CSTA (the Stfa2l1 ortholog in mice) was the only common variant gene/protein that was up-regulated in the two omics crossovers (Fig. 1G). To validate the proteomic and transcriptomic results, we examined the

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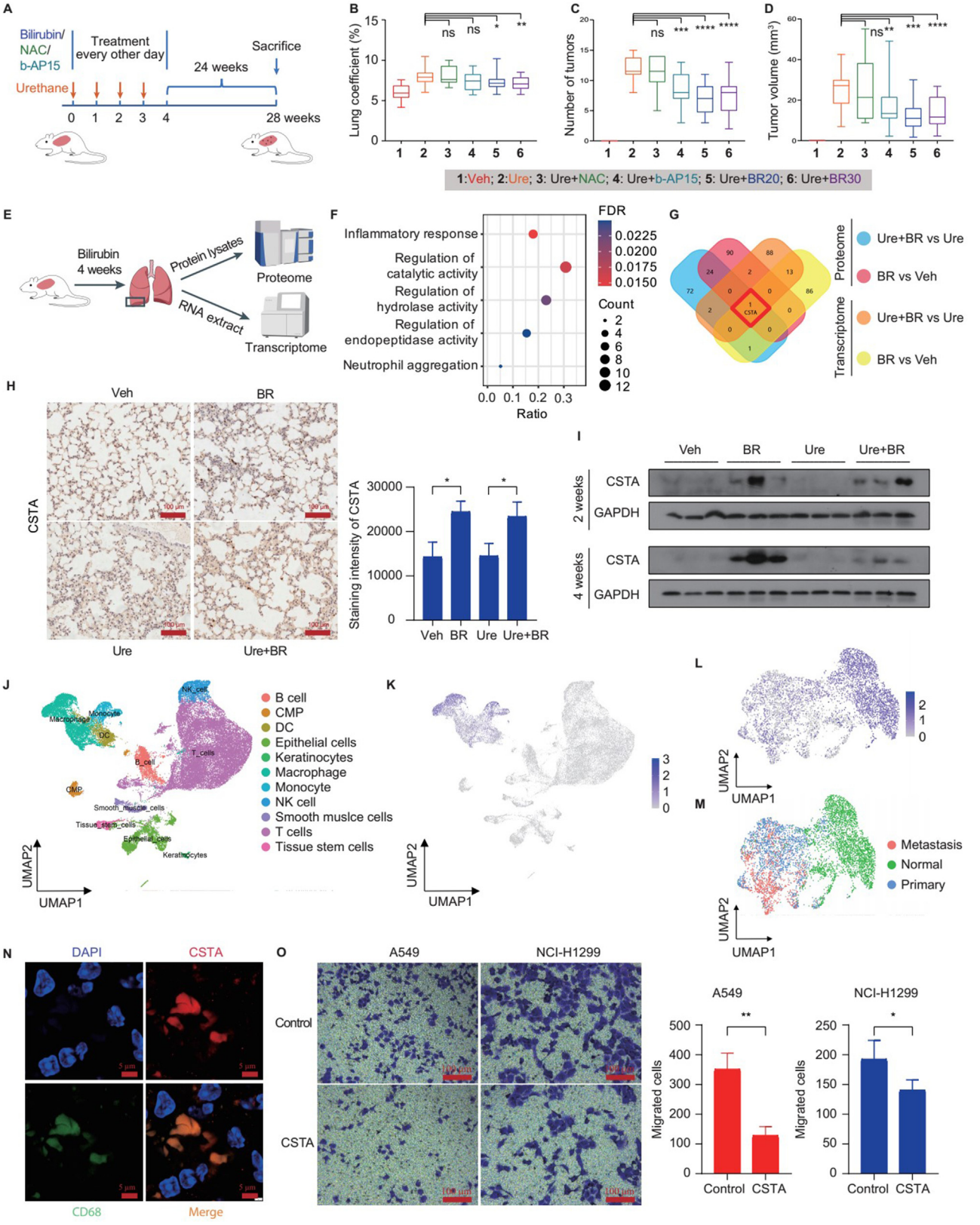

Figure 1 Bilirubin suppresses lung carcinogenesis through up-regulating cystatin A expression in tumor-infiltrating macrophages. **(A–D)** Bilirubin-induced proteasome inhibition may contribute to its effect on urethane-induced lung tumors in mice. Scheme of bilirubin (BR, 20 or 30 mg/kg), 100 mg/kg NAC, or 5 mg/kg b-AP15 intervention in urethane-induced lung tumors in mice **(A)**. The lung coefficient **(B)**, the ratio of lung weight to total body weight), number **(C)**, and total tumor volume **(D)** of lung dissection in the indicated group. Number of mice in each group: Veh ($n = 23$); Ure ($n = 17$); Ure + NAC ($n = 9$); Ure + b-AP15 ($n = 25$); Ure + BR20

changes in the expression of CSTA in the lung and liver of mice after short-term treatment with bilirubin. After bilirubin treatment, the increase in CSTA expression was more pronounced in the lung than in the liver (Fig. S3E), indicating that bilirubin may function in an organ-specific manner. However, bilirubin had little effect on the expression of other cysteine cathepsin family proteins, including cathepsin C (CTSC), cathepsin D (CTSD), and cathepsin S (CTSS) (Fig. S3F). Immunohistochemistry and Western blot analysis of mice lung tissues also confirmed up-regulation of CSTA by bilirubin treatment for 2 weeks or 4 weeks (Fig. 1H, I). Next, we analyzed mRNA expression of *CSTA* from TCGA datasets through UALCAN (http://ualcan.path.uab.edu/) and found that *CSTA* was lower in tumor samples from patients with lung adenocarcinoma compared to the normal tissues (Fig. S4A, B). In addition, using the KMplot database (www.kmplot.com), we found that high levels of CSTA were associated with survival benefits in lung adenocarcinoma patients (Fig. S4C). These findings support that CSTA down-regulation contributes to the progression of patients with lung adenocarcinoma.

Unexpectedly, bilirubin did not up-regulate the expression of CSTA in lung epithelial cell lines (Fig. S5), indicating that bilirubin may not act directly on lung epithelial cells. To determine which cell type(s) express (es) CSTA in the tumor of lung cancer patients, we performed an analysis of the lung adenocarcinoma single-cell sequencing dataset (GSE123902).[4] We examined the expression of *CSTA* across cell populations and found that *CSTA* expression was specifically enriched in macrophages and monocytes (Fig. 1J, K). In addition, the macrophage population with high *CSTA* expression is likely to act as a non-malignant macrophage population that was deficient in the process of lung adenocarcinoma (Fig. 1L, M; Fig. S6A–E, S7A–D). These results were also validated with another independent lung cancer single-cell sequencing dataset (GSE131907)[5] (Fig. S8, S9). Through pseudo-temporal analysis, we found that *CSTA*-enriched cell populations (cluster0, cluster4) were mainly in the initial stage of the trajectory (Fig. S7E, F). Thus, we speculated that the *CSTA*-enriched macrophage population might suppress malignant transformation during the pathological process of lung adenocarcinoma. Furthermore, we found that CSTA was mainly expressed in CD68-positive macrophages in the lungs of mice injected with short-term bilirubin (Fig. 1N). Similarly, the expression of CSTA in mouse peritoneal macrophages was up-regulated by intraperitoneal injection of bilirubin in mice (Fig. S10A). Human recombinant CSTA protein inhibits migration, but not cell viability, of A549 and NCI-H1299 lung cancer cell lines (Fig. 1O; Fig. S10B). Therefore, bilirubin might inhibit the occurrence of lung cancer by promoting the up-regulation of CSTA in macrophages. Given the important role of macrophage differentiation in tumorigenesis, future work will need to analyze whether the up-regulation of CSTA affects macrophage polarization towards M1 or M2 types.

Ethics declaration

The study was approved by the Ethics Committee of Guangzhou Medical University.

Author contributions

Qingtian Huang discussed the design, analyzed the data, and wrote the manuscript. Xin Chen hypothesized, designed, and wrote the manuscript. Na Zhang and Jiangtuan Wang participated in the experiment and worked on the protocol. Qingtian Huang and Na Zhang equally contributed as the first author. Xu Hua assisted in data interpretation. Leyi Yao, Qianqian Yang, Ding Yan, Xi Chen, Qian Xue, Jiawen Wu, and Min Xiao participated in the animal experiment. Xueheng Wu and Qin Liu provided biostatistics services. Xiaofen Li wrote the application for animal ethics review, and hypothesized and discussed the design. Jinbao Liu hypothesized, designed, and discussed the design. Daolin Tang assisted in data interpretation and edited the manuscript.

Conflict of interests

The authors declare no conflicting interests.

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($n = 23$); Ure + BR30 ($n = 20$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. ns, no significance. **(E–G)** Proteomics and transcriptomics analysis identifies CSTA up-regulation induced by bilirubin treatment. Scheme of bilirubin intervention in urethane-induced lung tumors in mice and subsequent transcriptomics and proteomics analysis **(E)**. The bubble plot shows the gene-enriched GO pathways after the intersection and union of the differential genes of BR *vs*. Veh and Ure + BR *vs*. Ure for the two omics **(F)**. Venn plot showing the number of differential genes between groups in transcriptome and proteomics **(G)**. BR, bilirubin; Ure, urethane; Veh, vehicle. **(H)** Immunohistochemical staining and quantification of CSTA proteins in the lungs of urethane-induced mouse lung cancer model with bilirubin intervention for 4 weeks ($n = 5$). $^{*}P < 0.05$. **(I)** Western blot analysis of the expression of CSTA in the lungs of urethane-induced mouse lung cancer model following bilirubin intervention for 2 or 4 weeks. **(J–M)** Single-cell sequencing analysis of CSTA expressed cell types in patients with lung adenocarcinoma. UMAP displays cell types annotated by SingleR **(J)**. Differential expression of CSTA at the single-cell level **(K)**. Tissue information of macrophage origin **(L)**. Expression of CSTA in macrophages **(M)**. **(N)** The expression of CSTA and CD68 in the lungs of mice following intraperitoneal injection of bilirubin detected by confocal fluorescence microscopy. **(O)** The migration ability of A549 and NCI-H1299 lung cancer cells treated with 4 μg/mL human recombinant CSTA proteins ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$.

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Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2023.02.024.

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