Supplementary information

**Low-dose metformin reprograms the tumor immune microenvironment in human esophageal cancer**

Shuhong Wang1,2†, Yusheng Lin1,2,3†, Xiao Xiong1, Lu Wang1, Yi Guo4, Yuping Chen5, Shaobin Chen5, Geng Wang5, Peng Lin1, Hongcai Chen6, Sai-Ching Jim Yeung7,8\*, Edwin Bremer3, Hao Zhang1\*

**Authors affiliations**

1Department of General Surgery, The First Affiliated Hospital of Jinan University, and Institute of Precision Cancer Medicine and Pathology, Jinan University Medical College, Guangzhou, Guangdong, China.

2Shantou University Medical College, Shantou, Guangdong, China.

3Department of Hematology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands.

4Endoscopy Center, Affiliated Cancer Hospital of Shantou University Medical College, Shantou, Guangdong, China.

5Department of Thoracic Surgery, Affiliated Cancer Hospital of Shantou University Medical College, Shantou, Guangdong, China.

6Department of Immunotherapy and Gastrointestinal Oncology, Affiliated Cancer Hospital of Shantou University Medical College, Shantou, Guangdong, China.

7Department of Emergency Medicine, University of Texas MD Anderson Cancer Center, Houston, Texas, USA.

8Department of Endocrine Neoplasia and Hormonal Disorders, University of Texas MD Anderson Cancer Center, Houston, Texas, USA.

**\*Correspondence to:** Professor Hao Zhang, Institute of Precision Cancer Medicine and Pathology, Jinan University Medical College, 601 Huangpu Avenue West, Guangzhou, Guangdong 510632, China, Tel./Fax: +86 20 85224770, E-mail address: haozhang@jnu.edu.cn; Professor Sai-Ching Jim Yeung, Department of Emergency Medicine, University of Texas MD Anderson Cancer Center, Houston, Texas, USA, E-mail address: syeung@mdanderson.org.

†Shuhong Wang and Yusheng Lin share co-first authorship.

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**Running title:** Low-dose metformin turns TIME against cancer

**Supplementary materials and methods**

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**Immunohistochemical analysis**

We performed immunohistochemistry (IHC) staining as previously described (1-4). Briefly, 4-µm sections were cut from formalin-fixed paraffin-embedded specimens and underwent deparaffinization, rehydration, followed by endogenous peroxidase blocking and antigen retrieval. The following primary antibodies were used: Ki67 (Cat. ab16667; Abcam), cleaved Caspase-3 (Cat. 9664; Cell signaling), CD68 (Cat. ab213363; Abcam), CD11c (Cat. ab52632; Abcam), CD163 (Cat. ab182422; Abcam), CD4 (Cat. ab133616; Abcam), CD8 (Cat. ab101500; Abcam), FoxP3 (Cat. ab99964; Abcam), CD20 (Cat. ab78237; Abcam) and PD-L1 ([Cat. 66248-1-Ig](http://www.ptgcn.com/products/PD-L1-CD274-Antibody-66248-1-Ig.htm); ProteinTech). After incubation overnight at 4 °C with primary antibodies, sections were then incubated with horse radish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1h, followed by color development with 3, 3’-diaminobenzidine (DAB) substrate. Nuclei were counterstained with hematoxylin. The percentage of stained cells was calculated by dividing the number of stained cells by the total number of hematoxylin-stained nuclei, derived from ten random high-power ﬁelds (×400). All sections were evaluated by two independent observers.

**Immunofluorescence Microscopy**

Immunofluorescence (IF) assay for single marker staining were performed as previously described (3). The primary antibody against human PD-L1 ([Cat. 66248-1-Ig](http://www.ptgcn.com/products/PD-L1-CD274-Antibody-66248-1-Ig.htm); ProteinTech), murine PD-L1 ([Cat. 66248-1-Ig](http://www.ptgcn.com/products/PD-L1-CD274-Antibody-66248-1-Ig.htm); ProteinTech) and CD19 (Cat. ab25232; Abcam) were used. Alexa-Fluor 488-conjugated (Green) secondary antibody was used, and the nuclei were counterstained with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride). Images were obtained using Zeiss Imager A2a fluorescence microscope.

Multiplex Immunofluorescence (mIF) was performed for multiple markers staining using PerkinElmer Opal kit (Perkin-Elmer, Waltham, MA, USA) following the work flow outlined by the manufacturer. The following primary antibodies were used: CD4 (Cat. ab133616; Abcam), CD20 (Cat. ab78237; Abcam), CD8 (Cat. ab101500; Abcam), p-AMPK (Cat. ab23875; Abcam), p-STAT3 (Cat. #9145; Cell Signaling Technology), TNF-α (Cat. ab9739; Abcam), IFN-γ (Cat. ab9657; Abcam), CD11c (Cat. ab52632; Abcam) and IL-10 (Cat. ab34843; Abcam). For murine tissues, the following antibodies were used: CD4 (Cat. ab183685; Abcam), CD8α (Cat. #98941; Cell Signaling Technology), FoxP3 (Cat. ab99964; Abcam), F4/80 (Cat. ab240946; Abcam), CD206 (Cat. ab64693; Abcam), CD11c (Cat. ab52632; Abcam), p-AMPK (Cat. ab23875; Abcam), p-STAT3 (Cat. #9145; Cell Signaling Technology), TNF-α (Cat. ab9739; Abcam), IFN-γ (Cat. ab9657; Abcam) and IL-10 (Cat. ab9969; Abcam). Nuclei were stained with DAPI. Images were acquired using a Vectra 3 pathology imaging system microscope (PerkinElmer, Waltham, MA, USA). Colocalization between markers was quantified by the Manders’ coefficient using ImageJ software colocalization plugin JACoP (5).

**Analysis of apoptosis by Annexin V-FITC**

Apoptosis was quantified with an Annexin V-FITC apoptosis detection kit (Beyotime Institute of Biotechnology) as described by the manufacturer’s instructions. After exposure to metformin (1 mM, 3 mM or 5 mM) for 48 h, tumor cells were collected and washed with PBS, gently resuspended in Annexin V binding buffer and incubated with Annexin V-FITC/Propidium Iodide. Flow cytometry was performed using Cellquest software (BD Biosciences, San Jose, CA, USA).

**Preparation of primary human macrophages**

Monocytes were enriched from peripheral blood mononuclear cells (PBMCs) (obtained from healthy donors after informed consent) by magnetic-activated cell sorting using CD14 magnetic beads (Miltenyi Biotec). Monocytes were differentiated into macrophages (M0) in RPMI 1640 culture medium supplemented with fetal bovine serum (10%), granulocyte-macrophage colony-stimulating factor (50 ng/ml) and macrophage colony-stimulating factor (50 ng/ml) for 7 days. To generate type 1 macrophages, M0 cells were primed by lipopolysaccharide and IFN-γ for additional 24 h.

***In vitro* macrophage phagocytosis assay**

KYSE 140 and KYSE 150, the ESCC cells, were cultured in RPMI 1640 culture medium (Gibco) supplemented with 10% fetal bovine serum (Systembio), 10 mmol/L glutamine, 100 units/ml penicillin (Sigma, St. Louis, MO), and 100 μg/ml streptomycin (Sigma). These cell lines authenticated using short tandem repeat validation analysis by the Cell Culture Service, Beijing Microread Genetics Co., Ltd. (Beijing, China). Cells were immediately amplified after the authentication, and frozen in liquid nitrogen serve as the stock for our experiments.

KYSE 140 cells or KYSE 150 cells were incubated with or without metformin (3 mM) for 48 h at 37 °C. Subsequently, macrophages incubated with or without metformin (3 mM) for 48 h at 37 °C were harvested and pre-seeded at 6 × 104 cells/well in 12-well plates. Tumor cells were labeled with cell proliferation dye CFSE (Thermofisher) or pHrodo green pH indicator (Thermofisher) according to manufacturer’s instructions. Tumor cells were washed and added to pre-seeded macrophages (effector to target ratio =1:5) and incubated for 3 h at 37 °C. Tumor cells were gently removed by washing with PBS 2–3 times and phagocytosis was analyzed by confocal microscopy (LSM 880) or flow cytometry (BD Bioscience, San Jose, CA, USA). Cells were stained with anti-human CD11b-Alexa Flour 594 (1 μg/ml) to visualize macrophages. Each condition was quantified by evaluating three randomly chosen fields of view.

For flow cytometry (6), dissociated cells were stained with anti-human CD11b-Alexa Flour 594 (1 μg/ml). The percentage of phagocytosis was calculated by counting the number of macrophages containing pHrodo-labeled tumor cells per 100 macrophages.

**References**

1. Dong H, Xu J, Li W, Gan J, Lin W, Ke J*, et al.* Reciprocal androgen receptor/interleukin-6 crosstalk drives oesophageal carcinoma progression and contributes to patient prognosis. J Pathol **2017**;241(4):448-62 doi 10.1002/path.4839.

2. Dong H, Ma L, Gan J, Lin W, Chen C, Yao Z*, et al.* PTPRO represses ERBB2-driven breast oncogenesis by dephosphorylation and endosomal internalization of ERBB2. Oncogene **2017**;36(3):410-22 doi 10.1038/onc.2016.213.

3. Gan J, Ke X, Jiang J, Dong H, Yao Z, Lin Y*, et al.* Growth hormone-releasing hormone receptor antagonists inhibit human gastric cancer through downregulation of PAK1-STAT3/NF-kappaB signaling. Proc Natl Acad Sci U S A **2016**;113(51):14745-50 doi 10.1073/pnas.1618582114.

4. Xiong X, Ke X, Wang L, Yao Z, Guo Y, Zhang X*, et al.* Splice variant of growth hormone-releasing hormone receptor drives esophageal squamous cell carcinoma conferring a therapeutic target. Proc Natl Acad Sci U S A **2020**;117(12):6726-32 doi 10.1073/pnas.1913433117.

5. Bolte S, Cordelieres FP. A guided tour into subcellular colocalization analysis in light microscopy. J Microsc **2006**;224(Pt 3):213-32 doi 10.1111/j.1365-2818.2006.01706.x.

6. Du L, Wang L, Gan J, Yao Z, Lin W, Li J*, et al.* MTA3 Represses Cancer Stemness by Targeting the SOX2OT/SOX2 Axis. iScience **2019**;22:353-68 doi 10.1016/j.isci.2019.11.009.