NRF2 activation by antioxidant anti-diabetic agents accelerates tumor metastasis

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Abstract

Cancer is a common comorbidity of diabetic patients; however, little is known about the effects that anti-diabetic drugs have on tumors. We discovered that common classes of drugs used in type 2 diabetes mellitus, the hypoglycemic dipeptidyl peptidase-4 inhibitors (DPP-4i) saxagliptin and sitagliptin, as well as the antineuropathic alpha-lipoic acid (ALA), do not increase tumor incidence but increase the risk of metastasis of existing tumors. Specifically, these drugs induce prolonged activation of the nuclear factor-E2-related factor 2 (NRF2)-mediated antioxidant response via inhibition of KEAP1-C151-dependent ubiquitination and subsequent degradation of NRF2, resulting in up-regulated expression of metastasis-associated proteins, increased cancer cell migration, and promotion of metastasis in xenograft mouse models. Accordingly, knockdown of *NRF2* attenuated naturally-occurring and DPP-4i-induced tumor metastasis, whereas NRF2 activation accelerated metastasis. Furthermore, in human liver cancer tissue samples, increased NRF2 expression correlated with metastasis. Our findings suggest that antioxidants that activate NRF2 signaling may need to be administered with caution in cancer patients, such as diabetic patients with cancer. Moreover, NRF2 may be a potential biomarker and therapeutic target for tumor metastasis.

Introduction

Accumulating epidemiological evidence suggests that diabetes increases the risk of multiple cancers, including colon, liver, and breast cancers (1). Therefore, the increased prevalence of diabetes suggests that the incidence of individuals with both diabetes and cancer is also rising. Anti-diabetic drugs modulate glucose metabolism, the insulin-like growth factor-1 axis, or other factors associated with tumor initiation and progression, and therefore they may affect tumor behavior (1, 2). Moreover, because diabetic patients are chronically exposed to anti-diabetic drugs, this long-term exposure as well as drug accumulation resulting from compromised renal and hepatic functions commonly seen in these patients may amplify toxic effects of anti-diabetic drugs (3). Therefore, understanding the effects of anti-diabetic agents on tumor biology is indispensable for the development of specialized drug therapy that is safe to treat diabetic patients with cancer.

Few studies have investigated the effects that anti-diabetic drugs have on tumors; some have suggested that anti-diabetic agents affect the incidence of cancer. Lewis and Piccinni reported a positive correlation between the dose and time of pioglitazone administration and bladder cancer risk (4, 5). Whether insulin analogs (especially glargine) increase cancer risk is currently controversial (6). On the other hand, metformin was found to reduce the risk of multiple cancers (such as breast, colon, and pancreatic cancers) and to inhibit the proliferation of cancer cells by activating the LKB1/AMPK pathway, indicating its potential as a cancer chemoprevention agent (7, 8). However, little is known about the effect of anti-diabetic agents on comorbid tumors in diabetic patients. To study the effects of anti-diabetic drugs on the biological behavior of existing tumors, we screened common clinical anti-diabetic agents such as metformin, various insulin analogs, and dipeptidyl peptidase-4 inhibitors (DPP-4i) *in vitro*.

Specifically, we assessed the proliferation and migration of different cancer cells after treatment with these drugs. We discovered that the DPP-4i saxagliptin (Sax), which is currently recommended by the American Association of Clinical Endocrinologists (AACE) as first-line hypoglycemic treatment in type 2 diabetes mellitus (T2DM) (9), potentially promoted migration of cancer cells. To clarify the correlation between DPP-4i and tumor metastasis and to reveal the mechanism underlying our preliminary observations, we further performed a series of *in vitro* and *in vivo* experiments. Here we report that DPP-4i Sax and sitagliptin (Sit), as well as alpha-lipoic acid (ALA, an antioxidant used to treat diabetic neuropathy), promote tumor metastasis through activation of the nuclear factor-E2-related factor 2 (NRF2)-mediated antioxidant response, though they don't increase tumor incidence. Additionally, NRF2 pathway activation correlated with tumor metastasis and may thus serve as a potential biomarker and therapeutic target.

Results

DPP-4i anti-diabetic treatment does not increase cancer risk

Previous studies have suggested that anti-diabetic treatments may modify neoplastic risk factors or modulate existing tumors' biological behavior. To examine the effect of DPP-4i on cancer risk, we performed a meta-analysis of randomized clinical trials on DPP-4i up to July 2015. We did not find any correlation between DPP-4i monotherapy (saxagliptin, sitagliptin, vildagliptin, linagliptin, or alogliptin; $n=29$, $OR=0.80$, 95% $CI=0.52-1.24$) or combinations with other anti-diabetic drugs and total cancer incidence ($n=88$, $OR=1.07$, 95% $CI=0.91-1.26$). These results were consistent irrespective of the anti-diabetic drug used alone (**table S1**) or in combination (**table S2**). Specifically, DPP-4i treatment did not increase the risk of digestive ($n=18$, $OR=0.89$, 95% $CI=0.42-1.85$), dermal ($n=9$, $OR=1.05$, 95% $CI=0.40-2.71$), or urological cancers ($n=11$, $OR=0.94$, 95% $CI=0.38-2.37$) (**table S3**), although previous studies reported that DPP-4i may be associated with increased incidence in these tumor types (*10, 11*). This meta-analysis suggests that DPP-4i alone or in combination with other anti-diabetic drugs do not increase cancer risks.

DPP-4i (Sax and Sit) enhance tumor metastasis

Currently, it remains unclear whether DPP-4i treatment is detrimental to diabetic patients with existing tumors. To address this, we tested two DPP-4i compounds, Sax and Sit, with SW480, HCT116, and HuH-7 cancer cells and determined that within the therapeutic range (0.1 μM or 0.6 μM , respectively), DPP-4 activity was inhibited (**fig. S1**). Sax and Sit did not affect cell proliferation (**fig. S2A**) or alter cancer cell sensitivity to cisplatin (**fig. S2B**). However, Sax and Sit markedly increased cell migration and invasion of multiple cancer cell lines (colon SW480 and HCT116, hepatic HuH-7, breast MDA-MB-231, lung A549, ovary SKOV-3, and melanoma

A-375) (**Fig. 1A**) that represent common diabetic comorbidities (12). Similar results were obtained when various concentrations of Sax, Sit, and vildagliptin (Vil, another DPP-4i) were used (**fig. S3**). Next, we measured the expression of metastasis-associated proteins (HIF-1, COX-2, and VEGF) (13, 14) and cancer type-specific metastasis proteins (BAMBI and APRIL for colon cancer, CORTACTIN for hepatocellular carcinoma) (15-17) in SW480, HCT116, and HuH-7 cell lines and found that Sax and Sit increased expression of these proteins (**Fig. 1B**). We also tracked HuH-7 cell migration with time-lapse microscopy (**Fig. 1C**) and observed greater persistence and forward migration index (FMI) after Sax and Sit treatment (**Fig. 1D**). These results indicate that Sax and Sit promote migration and invasion of various cancer cell lines.

We further studied the effect of Sax and Sit on tumor metastasis in nude mice inoculated with HCT116-LUC⁺ or HuH-7-LUC⁺ cells (spontaneous and experimental metastatic models) (18). Sax- and Sit-treated mice showed obvious body weight losses during the course of the experiment (**Fig. 1E**) at doses that have been proven to not affect normal mouse weight (19). Bioluminescent imaging indicated that 6-week Sax and Sit treatment promoted metastatic capacity in both models (**Fig. 1F**). Histopathologic analyses revealed that livers and lungs of Sax- and Sit-treated mice had more visible metastatic nodules and micro-metastases detected by H&E and anti-VIMENTIN staining (**Fig. 1G**). We also observed higher expression of metastasis-associated proteins in the livers and lungs after 6-week Sax and Sit treatments (**Fig. 1H**). Taken together, these results show that Sax and Sit enhance metastasis of existing tumors without increasing cancer risk.

DPP-4i activate NRF2 via KEAP1-C151-dependent inhibition of ubiquitination

To understand how Sax and Sit promote tumor metastasis, we measured tumor progression-associated factors such as lactate production, ATP metabolism, and oxidative stress (20-22). Neither intracellular nor secreted lactate (**fig. S4A**), nor ATP production (**fig. S4B**) were affected by treatment, but Sax and Sit greatly reduced reactive oxygen species (ROS) production (**Fig. 2A**), increased GSH/GSSG ratios (**Fig. 2B** and **fig. S5**), and decreased 8-oxo-dG in cancer cells (**Fig. 2C**), suggesting that Sax and Sit may reduce oxidative stress in cancer cells. Furthermore, Sax- and Sit-induced cell migration and invasion were prevented by administration of buthionine sulfoximine (BSO), an inhibitor of new glutathione synthesis, indicating that glutathione synthesis is required for the effects of Sax and Sit on tumor metastasis (**fig. S6**). Commonly, ROS concentrations are suppressed by activation of the transcription factor NRF2 and subsequent expression of antioxidant systems (22); therefore, we measured the protein expression of NRF2 and its downstream targets NQO1 and GCLM. We observed that the NRF2 pathway was markedly activated by Sax and Sit treatment *in vitro* (**Fig. 2D**) and *in vivo* (**Fig. 2, E and F**). Similar results were obtained by treating cancer cells with commercial human drugs Sax or Sit (**fig. S7**). Thus, these results suggest that Sax and Sit activate NRF2 to increase intracellular antioxidants and neutralize ROS in cancer cells.

DPP-4i are structurally diverse drugs, so we next investigated if other members of this class activate NRF2. Our results showed that Sax, Sit, Vil, alogliptin (Alo), and linagliptin (Lin) induced NRF2 and its downstream targets (**fig. S8A**), as well as antioxidant response element (ARE)-driven luciferase reporter gene expression (**fig. S8B**). Furthermore, we performed a 2D similarity search in SYBYL-X 2.0 and found that although the similarity of Sax and Vil is 72.8%, the other 5 DPP-4i have less than 40% similarity, which means that these DPP-4i do not

share a common scaffold (**fig. S8C**). The fact that structurally diverse DPP-4i inhibited DPP-4 and induced NRF2 indicates that their ability to activate NRF2 may be a consequence of inhibiting DPP-4.

Next, we further explored the molecular mechanism of DPP-4i-induced NRF2 activation focusing on Sax. We measured mRNA and protein expression of NRF2 and its targets in HuH-7 cells treated with Sax. Both mRNA and protein expression of these targets increased after treatment; however, *NRF2* transcription did not apparently change, suggesting that Sax modulates NRF2 post-transcriptionally (**Fig. 2G**). Because expression of NRF2 is primarily controlled at the protein level by the KELCH-like ECH-associated protein-1 (KEAP1)-containing E3 ubiquitin ligase complex through ubiquitination and subsequent proteasomal degradation of NRF2 (23), we next assessed the ubiquitination and half-life of NRF2. We observed decreased NRF2 ubiquitination and a three-fold half-life increase (from 13.7 to 50.4 min) after Sax treatment (**Fig. 2, H and I**). Furthermore, because cysteine151 in KEAP1 is specifically required for NRF2 activation by canonical NRF2 inducers sulforaphane (SF) and tert-butylhydroquinone (tBHQ) (24), we examined the effect of this cysteine in NRF2 induction by Sax. We observed that NRF2 induction by Sax was inhibited in KEAP1-C151S mutant cells (**Fig. 2J**), indicating that Sax activates NRF2 in a KEAP1-C151 dependent manner.

NRF2 inhibition attenuates tumor metastases

Recent data indicate that persistent activation of NRF2, the “dark side of NRF2”, promotes tumorigenesis (22); however, whether persistent NRF2 activation promotes tumor metastasis is unclear. Because Sax and Sit promote tumor metastasis and also activate NRF2 signaling, we tested whether Sax and Sit promote tumor metastasis via activation of NRF2. We knocked down

NRF2 in SW480, HCT116, and Huh-7 cells and observed a marked decrease of Sax and Sit-induced metastasis-associated proteins (**Fig. 3A**). Moreover, *NRF2* silencing reduced Sax- and Sit-driven cancer cell migration (**Fig. 3B**) and inhibited persistence and FMI (**Fig. 3C**), as well as intrinsic migratory capacity (**Fig. 3D**). Similarly, knockdown of *NRF2* with lentiviral shRNA targeting *NRF2* in HuH-7-LUC⁺ greatly reduced the expression of *NRF2* and its downstream targets at the protein level in cells (**Fig. 3E**) and mRNA in metastatic nodules *in vivo* (**Fig. 3F**). It also decreased intrinsic (**Fig. 3G**) and Sax- and Sit-induced (**Fig. 3H**) metastases in mice inoculated with these cells. Together, these results suggest that *NRF2* inhibition attenuates tumor metastases.

Activation of *NRF2* promotes tumor metastasis

To further confirm the role of *NRF2* in tumor metastasis, we investigated the effects of *NRF2* activation on migration and metastasis. We noted that the expression of metastasis-associated proteins increased upon genetic (by *NRF2* overexpression) or pharmacologic [treatment with SF, a well-characterized *NRF2* activator (25)] *NRF2* activation (**Fig. 4, A and B**). Similarly, cell migration was also enhanced (**Fig. 4C**). Moreover, time-lapse microscopy confirmed increased directed and persistent migration of cells treated with SF (**Fig. 4D**). *In vivo*, SF treatment also activated the *NRF2* pathway (**Fig. 4, E and F**). We observed that the normally nontoxic low-dose SF treatment (25) (10 mg/kg, three times per week) caused animal weight loss (**Fig. 4G**), which might be related to an increased metastatic tumor burden (**Fig. 4H**), similar to what was observed with Sax and Sit treatments. Consistently, an increase in micro-metastasis was confirmed by H&E staining and IHC analysis of VIMENTIN in liver and lung tissues (**Fig. 4I**). These results

indicate that NRF2 activation increases the ability of cancer cells to migrate, which results in increased metastasis.

Pharmacological activation of NRF2 by ALA promotes tumor metastasis

Oxidative stress contributes to the onset of diabetes and its associated complications, making the prescription of antioxidants a regular clinical practice (26). Some antioxidants used for diabetic treatment, such as ALA, are known NRF2 activators (27). Because our results showed that NRF2 activation accelerates tumor metastasis, we investigated whether ALA also promotes tumor metastasis. Indeed, ALA treatment activated the NRF2 pathway, increased metastasis-associated protein expression (**Fig. 5A**), and promoted migration in SW480 and HCT116 cells (**Fig. 5B**). *In vivo*, low-dose ALA, which also does not affect normal mouse weight (28), increased NRF2 and downstream protein expression (**Fig. 5, C and D**) and caused weight loss in tumor bearing-mice (**Fig. 5E**), similar to what we previously observed for Sax, Sit, and SF treatments. Moreover, ALA increased total metastatic tumor burden (**Fig. 5F**) and increased micro-metastasis to the liver and lungs (**Fig. 5G**). Thus, these results indicate that pharmacological activation of NRF2 by ALA also promotes cancer cell migration and tumor metastasis.

NRF2 is a potential metastatic marker in human liver cancer

To further characterize the value of NRF2 expression in cancer metastasis, we analyzed human liver cancer tissue microarrays, including 176 primary tumor samples (105 non-metastatic, 69 lymph node metastases, 2 distant metastases) and 19 metastatic tumors, to correlate the expression of NRF2 and its downstream target GCLM with patient clinicopathological features (images of H&E staining and clinical information can be obtained from

<http://www.biomax.us/tissue-arrays/Liver/LV1504>, <http://www.biomax.us/tissue-arrays/Liver/LV1505>, and <http://www.biomax.us/tissue-arrays/Liver/LV1221>). Overall, compared to non-metastatic cancer, metastatic cancer (either primary tumor or metastatic lesion) had greater NRF2 and GCLM expression, $P < 0.0001$ (**Fig. 6, A and B**). NRF2 and GCLM expression were associated with lymph node metastasis and tumor stage ($P < 0.0001$), but were not correlated to sex, age, pathology type, tumor size, or grade (**Fig. 6, C and D; table S4 and S5**). Therefore, our data suggest that NRF2 activation contributes to enhanced metastasis in human liver cancer, and as such, may be a promising biomarker as well as a therapeutic target.

Discussion

Recent evidence has shown that diabetes may increase the risk of many cancers, and the incidence of diabetic patients with cancer has increased (1). In this subpopulation, long-term anti-diabetic therapy may potentially have an impact on existing tumors (1-3). Therefore, there is a public health concern to choose anti-diabetic drugs that maximize anti-diabetic benefits but have no tumor-promoting effects.

Here, we determined that the hypoglycemic drugs used in T2DM, DPP-4i Sax and Sit (9) and the anti-neuropathic antioxidant ALA (28), increased the risk of metastasis of multiple tumors which represent common comorbidities of diabetes. Our meta-analysis of the existing clinical evidence showed that DPP-4i used alone or in combination with other drugs, did not increase the risk of cancers, indicating that these drugs have no carcinogenic effects. However, the results we present here indicate that Sax and Sit enhance cancer cells' mobility and invasive capacity and may promote tumor metastasis. Consistent with our results, a recent case report showed that Sax aggravated the condition of a patient with metastatic carcinoid tumor (29). However, DPP-4 inhibition was also recently reported to improve anti-tumor immunity by regulating CXCL10-mediated lymphocyte trafficking, showing potential for tumor immunotherapy applications (30). Future studies that fully characterize the effects of DPP-4i on tumors will be required for a comprehensive evaluation of the administration of DPP-4i in cancer patients. Additionally, we showed that ALA also promoted tumor metastasis, whereas some reports showed that ALA can inhibit tumor cell proliferation and induce apoptosis (31), highlighting the need for a deep and comprehensive understanding of anti-diabetic drugs' effects on cancer biology.

Our findings suggest that antioxidants that activate NRF2 may accelerate tumor metastasis. By analyzing metabolic factors associated with tumor progression (20-22), we found that Sax and Sit did not affect lactate metabolism or ATP generation in cancer cells, but reduced ROS by activating the NRF2-mediated antioxidant response. Additionally, Sax- and Sit-promoted metastasis was markedly attenuated by *NRF2* knockdown, which suggested that Sax and Sit promote tumor metastasis mainly through activation of NRF2. Notably, Sax and Sit activated NRF2 at lower doses than SF (0.1 and 0.6 μ M vs. 5-10 μ M, respectively), indicating that these are more potent drugs. Sax inhibited NRF2 ubiquitination in a KEAP1-C151 dependent manner, but we do not rule out the possibility that it could also act through other KEAP1 cysteine sensors, such as C226, C434, or C613, as reported previously (32, 33). In addition, KEAP1-independent mechanisms, like glycogen synthase kinase-3 (GSK-3)-dependent Skp1-Cul1-F-box (SCF)/ β -TrCP-mediated degradation of NRF2, might also contribute to the effect of Sax (34). Therefore, further studies are needed to completely elucidate the mechanism of NRF2 induction by DPP-4i. Many antioxidants, such as ALA, have been confirmed to activate NRF2 (27). Here we showed that activation of NRF2, either by activators (DPP-4i, ALA, and SF) or by overexpression, decrease oxidative stress and promote tumor metastasis. Our results support the notion that ROS limit distant metastasis and antioxidants accelerate migration and invasion of cancer cells (35, 36). However, contradicting results have raised the possibility that oxidative stress might promote metastasis in some cell lines (37). We believe that specific characteristics of antioxidants and cell lines with different gene mutations may contribute to the contradictory results regarding the role of ROS and antioxidant supplementation in tumor metastasis. Further investigations will be necessary to dissect the role of oxidative stress in cancer metastasis in different tumor types and models.

Our findings also offer a potential therapeutic target for tumor metastasis. The dark side of NRF2 in tumor biology was recently discovered. Many studies, including ours, showed that NRF2 expression was increased in various human cancers (38), and this aberrant accumulation of NRF2 correlated with oncogene expression, cell proliferation, and cancer chemoresistance (22, 39). Here, we identified that persistent NRF2 activation also increased tumor metastasis. Consistent with our results, osteopontin induced glioma cell migration and invasion via NRF2 activation (40). Most importantly, *NRF2* knockdown decreased cancer cell migration and tumor metastasis, suggesting that NRF2 may serve as a promising therapeutic target for tumor metastasis. The detailed mechanism by which NRF2 regulates metastasis-associated proteins is currently unclear. Studies have shown that *NRF2* knockdown correlates with reduced HIF-1 α and low VEGF expression; however, NRF2 does not regulate *HIF-1 α* transcriptionally but through indirect mechanisms (41, 42). Moreover, a recent study identified ARE and ARE-like sequences in the promoter region of *COX-2*, suggesting that NRF2 may directly control *COX-2* transcription (43). Further investigation of how these metastasis-associated proteins are regulated by NRF2 is still needed.

Our human tissue microarray analysis provides clinicopathological insight into the association of NRF2 expression and metastasis. In liver cancer tissue samples, the expression of NRF2 and GCLM in metastasis-positive lesions (lymph nodes and distant metastases) was higher than in metastasis-negative lesions. The expression of NRF2 and GCLM also correlated with tumor stage. These results showed that NRF2 may not only be used to evaluate the sensitivity and response to chemo and radiotherapy as previously reported (44), but may also serve as a prognostic biomarker for tumor metastasis.

One limitation of our study is the weakness of using cell lines to study the metastatic process. Cancer cell lines are often derived from the primary site or distant metastases of cancer patients. Tumorigenicity of these cells mainly reflects their ability to form tumors at different sites if they are injected intravenously. Even if these cells are injected in the paw, they could still rapidly enter the circulation by mechanisms that are distinct from those that trigger normal metastasis. This is the inherent weakness of using cancer cell lines in xenograft models to study the metastasis process. Moreover, the variation among different cancer cell lines with regard to their origins and mutations (**table S6**) might influence the interpretation of the results. Future studies of tumor metastasis in diabetic mice will more adequately reflect the current clinical application of DPP-4i.

In summary, our study shows that the anti-diabetic DPP-4i (Sax and Sit) and the anti-neuropathic ALA promote tumor metastasis via NRF2 activation by inhibition of NRF2 ubiquitination in a KEAP1-C151-dependent manner, although they do not increase cancer incidence. In human cancer tissues, NRF2 expression positively correlated with tumor metastasis, suggesting that NRF2 may be a potential biomarker and therapeutic target. Our findings indicate that antioxidants that activate NRF2 signaling should be administered with caution in cancer patients. Our study also argues for a need to perform more comprehensive preclinical and clinical studies in cancer patients, such as the subpopulation of diabetic patients with cancer, to ensure the safety of antioxidants.

Supplementary materials

Materials and Methods

Fig. S1. Sax and Sit inhibit the activity of DPP-4.

Fig. S2. Sax and Sit do not affect tumor cell proliferation or sensitivity to chemotherapeutics.

Fig. S3. Sax, Sit and Vil at different dosages accelerate tumor cell migration.

Fig. S4. Sax and Sit do not change the levels of lactate and ATP production in tumor cells.

Fig. S5. The effect of Sax and Sit on GSH and GSSG levels.

Fig. S6. BSO prevents Sax- and Sit-induced cancer cell migration and invasion.

Fig. S7. Commercial forms of Sax and Sit activate NRF2 and promote tumor cell migration.

Fig. S8. Sax, Sit, Vil, Alo and Lin do not share a common scaffold, but they all activate the NRF2 pathway.

Table S1. DPP-4i monotherapy does not increase the overall risk of tumors.

Table S2. DPP-4i do not increase the overall risk of tumors.

Table S3. DPP-4i monotherapy does not increase the risk of digestive system tumors, skin tumors, or urinary system tumors.

Table S4. Clinical characteristics of NRF2 expression in liver cancer.

Table S5. Clinical characteristics of GCLM expression in liver cancer.

Table S6. The tissue origin and mutational profile of cancer cell lines used.

Table S7. Exact p values (provided as an Excel file).

Table S8. List of primers and siRNA/shRNA sequences.

Table S9. Original data (provided as an Excel file).

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Author contributions

H.W. and XF.L.: acquisition of data, analysis and interpretation of data, statistical analysis; M.L., Y.H., XY.L., ZL.T. and Q.T.: analysis and interpretation of data, drafting of the manuscript; LL.Z., R.Z., Y.Z., YR.W., Q.L., WJ.L., XC.W., F.F. and Q.OY.: acquisition of data; M.R.V.: manuscript editing; SC.Y. and DD.Z.: analysis and interpretation of data, critical revision of the manuscript for important intellectual content, obtained study funding, study supervision; HT.Z.: study concept and design, analysis and interpretation of data, drafting of the manuscript, critical revision of the manuscript for important intellectual content, obtained study funding, study supervision. All authors have read and approved the manuscript for publication.

Competing interests

The authors declare that they have no competing interests.

Data and materials availability

The information of GV298 expression plasmid can be obtained from <http://www.genechem.com.cn/Zaiti.aspx?zt=GV298>. The scanned H&E staining images and clinical information of human liver cancer tissue microarrays are accessible from <http://www.biomax.us/tissue-arrays/>

Liver/LV1504, <http://www.biomax.us/tissue-arrays/Liver/LV1505> and <http://www.biomax.us/tissue-arrays/Liver/LV1221>.

Figure Legends

Fig. 1. DPP-4i (Sax and Sit) promote tumor metastasis. **(A-D)** The indicated cancer cell lines were treated with 0.1 μ M saxagliptin (Sax) or 0.6 μ M sitagliptin (Sit) for 24 h. The quantification of migration assay **(A, left)** and matrigel invasion assay **(A, right)** are shown. n=10 fields, the experiments were performed in triplicates. **(B)** Metastasis-associated protein expression was detected by immunoblotting. HuH-7 cell movements were tracked by time-lapse microscopy **(C)**, and the persistence and forward migration index (FMI) of cell migration were analyzed using cell tracking software **(D, n=20)**. **(E-H)** Nude mice were foot pad (FP)-inoculated with 1×10^5 HCT116-LUC⁺ cells or tail vein (TV)-injected with HuH-7-LUC⁺ cells in a spontaneous or experimental metastasis model, respectively. One week later, mice (n=9) were treated with Sax (15 mg/kg), Sit (120 mg/kg), or normal saline (via oral gavage) every day for 6 weeks. **(E)** Mean mouse body weights; 0 w indicates Sax or Sit treatment initiation. **(F)** *In vivo* bioluminescence imaging of metastatic tumors after 6 weeks of DPP-4i treatment. **(G)** Representative images of liver and lung, and their corresponding tissue sections stained with H&E or antibody against VIMENTIN to confirm micro-metastasis. Scale bars: 1 cm for macroscopic view; 1 mm for liver H&E; 200 μ m for VIMENTIN and lung H&E. **(H)** Immunoblotting of metastasis-associated proteins from liver and lung. * $P < 0.01$ for A, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for other panels, as determined by ANOVA. Exact p values are given in **table S7**. Results are presented as means $\pm$ s.d.

Fig. 2. DPP-4i activate the NRF2 pathway via KEAP1-C151-dependent inhibition of ubiquitination. **(A-D)** Cells were treated with 0.1 μ M Sax or 0.6 μ M Sit for 24 h. **(A)** For ROS measurement, cells were incubated with 5 μ g/ml 2',7'-dichlorofluorescein diacetate (DCFH-DA)

for 1 hour, and fluorescent intensity (FL1-H) were measured by flow cytometry and quantified. **(B)** GSH/GSSG ratio was measured. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, as analyzed by ANOVA; exact p values are given in **table S7**. Results are presented as means, n=3. **(C)** Representative images of 8-oxo-dG staining (red, with DAPI in blue), scale bar: 20 μ m. **(D)** Immunoblotting of NRF2 and its downstream targets. **(E-F)** In the experiment described for Fig. **1E**, the liver and lungs of each mouse with colorectal or liver cancer were harvested after 6 weeks of Sax or Sit treatment. Representative images of NRF2 expression in metastatic nodules detected by IHC **(E)**, and the expression of NRF2 downstream targets assessed by immunoblotting **(F)** are shown; scale bar: 100 μ m. **(G)** HuH-7 cells were treated with Sax for 24 h, and cell lysates were subjected to qRT-PCR and immunoblotting for NRF2 and its targets. $*P < 0.05$, $**P < 0.01$, as determined by Student's *t* test. Exact p values are in **table S7**. Results are presented as means $\pm$ s.d, n=3. **(H)** NRF2 ubiquitination was detected by immunoprecipitation analysis. HuH-7 cells were co-transfected with plasmids encoding NRF2 and HA-Ub for 24 h, and then treated with sulforaphane (SF, 7.5 μ M) or Sax along with MG132 (10 μ M, a proteasome inhibitor used to block degradation of ubiquitinated proteins) for 4 h. **(I)** NRF2 protein half-life was determined by pulse-chase assay and immunoblotting. HuH-7 cells were treated with Sax for 4 h, and then cycloheximide (50 μ M) was administered to block protein synthesis. Total cell lysates were harvested at different time points and band intensities were quantified to determine half-life ($t_{1/2}$). **(J)** HuH-7 cells transfected with KEAP1-WT or KEAP1-C151S plasmids were treated with Sax for 24 h, and expression of NRF2 and KEAP1 was detected by immunoblotting. 0.1 μ M Sax was used for **G-J**.

Fig. 3. NRF2 inhibition attenuates tumor metastases. (A-C) Cells were transfected with NRF2 siRNA or control (Ctl) siRNA, and then treated with 0.1 μ M Sax or 0.6 μ M Sit. (A) The expression of NRF2, its downstream targets (NQO1 and GCLM), and metastasis-associated proteins (HIF-1, COX-2, BAMBI, APRIL, and CORTACTIN) was detected by immunoblotting. (B) Cell migration was measured and quantified (n=10). (C) HuH-7 cells were tracked by time-lapse microscopy, and the persistence and FMI were assessed (n=20). (D) Cells were transfected with NRF2 siRNA, NRF2 expression was detected by immunoblotting (**top**), and cell migration was assessed (**bottom**, n=10). (E) HuH-7-LUC⁺ cells were infected with lentivirus (LV) expressing Ctl or NRF2 shRNA. The expression of NRF2 and GCLM was assessed by immunoblotting at the indicated time points. (F-G) 1×10^5 Ctl shRNA- or NRF2 shRNA-transfected HuH-7-LUC⁺ cells were tail vein-injected into nude mice (n=9). Four weeks later, RT-PCR was used to detect the mRNA expression of *NRF2*, *GCLC*, and *GCLM* in metastatic nodules (F), and representative bioluminescence images were shown (G). (H) Nude mice were tail vein-injected with shRNA-transfected HuH-7-LUC⁺ cells. One week later, mice were treated with Sax (15 mg/kg) or Sit (120 mg/kg) every day for 3 weeks (n=9), and then bioluminescent images were acquired. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, as determined by Student's *t* test. Exact p values are in **table S7**. Results are presented as means $\pm$ s.d.

Fig. 4. Activation of NRF2 promotes tumor metastasis. (A-B) NRF2, GCLM, and metastasis-associated proteins' expression was detected in cancer cell lines. The indicated cancer cell lines were transfected with Ctl (pCI empty vector) or pCI-NRF2 expression plasmid (A), or treated with 0.1 μ M Sax, 0.6 μ M Sit, or 5-10 μ M SF for 24 h (B). (C) Quantification of cell migration after pCI-NRF2 transfection or 7.5 μ M SF treatment (n=10). (D) HuH-7 cells with or without 7.5

μ M SF treatment were tracked by time-lapse microscopy, and the relative persistence and FMI are shown (n=20). **(E-I)** Nude mice were tail vein-injected with 1×10^5 HuH-7-LUC⁺ cells in an experimental metastasis model. One week later, mice were started on treatment with 10 mg/kg SF i.p. three times per week for 3 weeks (n=7). Representative images show NRF2 expression in metastatic liver nodules assessed by IHC (scale bars: 100 μ m) **(E)**, and NRF2 downstream proteins' expression was assessed by immunoblotting **(F)**. **(G)** Mean mouse body weights, where 0 w indicates SF treatment initiation. **(H)** Representative bioluminescent images show total metastatic tumor burden. **(I)** H&E staining and IHC staining for VIMENTIN in the liver and lungs confirms micrometastasis. Scale bars: 1 mm for liver H&E; 200 μ m for VIMENTIN and lung H&E. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, as determined by Student's *t* test. Exact p values are in **table S7**. Results are presented as means $\pm$ s.d.

Fig. 5. Pharmacologic activation of NRF2 by ALA promotes tumor metastasis. **(A)** NRF2, GCLM, and metastasis-associated protein expression was detected in cancer cell lines treated with 0-0.6 mM ALA. **(B)** Quantification of cell migration after 0.4 mM ALA treatment (n=10). **(C-G)** Nude mice were tail vein-injected with 1×10^5 HuH-7-LUC⁺ cells in an experimental metastasis model, and one week later were treated with ALA (80 mg/kg, i.p.) every day for 3 weeks (n=6). **(C)** Representative images of NRF2 expression in metastatic liver nodules detected by IHC, scale bar: 100 μ m. **(D)** Expression of NRF2 downstream targets assessed by immunoblotting. **(E)** Mean mouse body weights; 0 w indicates ALA treatment initiation. **(F)** Representative bioluminescent images for total metastatic tumor burden. **(G)** H&E staining and IHC for VIMENTIN in liver and lung to confirm micro-metastasis. Scale bars: 1 mm for liver H&E, 200 μ m for VIMENTIN and lung H&E. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, as

determined by Student's *t* test. Exact *p* values are in **table S7**. Results are presented as means $\pm$ s.d.

Fig. 6. Expression of NRF2 and GCLM is associated with metastasis in human liver cancer. (**A-B**) Tissue microarrays with samples from 195 liver cancer patients were used to detect protein expression of NRF2 and GCLM by IHC. Representative images of primary tumors from patients with no metastasis (-, *n*=105), lymph node metastasis (N, *n*=69), distant metastasis (M, *n*=2), or metastatic tumors (metastatic lesion, *n*=19) stained for NRF2 (**A, left**) and GCLM (**B, left**) are shown. Scale bars: 1 mm and 100 μ m. All stained tissue sections were semi-quantitatively scored (**A-B, right**) as previously described (38). (**C-D**) NRF2 (**left**) and GCLM (**right**) expression in primary tumors (*n*=176) were correlated with lymph node metastasis (**C**) and tumor stage (**D**). Fisher's exact test was used to analyze the association between two categorical variables, $*P < 0.05$, $***P < 0.0001$. Exact *p* values are in **table S7**.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

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