


Review

# Role of p53 Family Proteins in Metformin Anti-Cancer Activities

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## Abstract

Metformin has been used as therapy for type 2 diabetes for many years. Clinical and basic evidence as indicated that metformin has anti-cancer activities. It has been well-established that metformin activates AMP-activated protein kinase (AMPK), which in turn regulates energy homeostasis. However, the mechanistic aspects of metformin anti-cancer activity remain elusive. p53 family proteins, including p53, p63 and p73, have diverse biological functions, including regulation of cell growth, survival, development, senescence and aging. In this review, we highlight the evidence and mechanisms by which metformin inhibits cancer cell survival and tumor growth. We also aimed to discuss the role of p53 family proteins in metformin-mediated suppression of cancer growth and survival.

Key words: metformin, AMPK, p53, p63, p73, cancer

## Introduction

Metformin is widely used to treat type 2 diabetes. Metformin enhances cell sensitivity to insulin[1], inhibits hepatic glucose production and promotes peripheral glucose uptake and, consequently, lowers blood glucose levels[2-4]. In addition, metformin can inhibit glucagon-induced elevation of cyclic adenosine monophosphate (cAMP) to decrease blood glucose[5]. In recent years, accumulating evidence indicates that metformin has biological activities beyond the treatment of diabetes, including anti-aging[6, 7] and anti-obesity effects[8, 9]. In fact, metformin was the first FDA-approved drug in a clinical anti-aging trial[10]. Moreover, numerous clinical and basic studies have implicated metformin in tumor suppression[11]. Currently, the FDA has approved more than 300 clinical trials to investigate the effects of metformin on cancer treatment ([www.clinicaltrials.gov](http://www.clinicaltrials.gov)).

p53 is a critical tumor suppressor gene frequently mutated in human cancers[12] and is the most studied gene in the human genome[13]. p53 protein contains five distinct domains, including an N-terminal transactivation domain (TAD), a proline-rich domain (PRD), a DNA binding domain (DBD), an oligomeri-

zation domain (OD), and a C-terminal regulatory domain (CTD)[14] (Figure 1A). p53 family proteins, p63 and p73, contain either a p53-homologous transactivation domain (TAp63 and TAp73) or lack of this domain ( $\Delta$ Np63 and  $\Delta$ Np73). Together with alternative splicing variants at C-termini, there are multiple p63 and p73 protein isoforms (Figure 1A). Due to similar structures of DNA binding domains and oligomerization domains, p53 family proteins share a subset of down-stream transcription targets, and they can mutually form oligomers [15]. Indeed, p63 and p73 have biological functions similar to that of p53, including regulation of cell growth, survival, development and senescence[14]. However, p63 and p73 have distinct biological functions. For instance, p63 has a critical role in embryonic development and is a pivotal regulator of cell-cell adhesion maintenance [16]. On the other hand, p73 is important for immunological functions and neurological development [17]. With regard to tumorigenesis, unlike the p53 gene that is frequently mutated in human tumors and cancers, there are rare mutations in the p63 or p73 genes although expression of p63 or p73 are often altered. Interestingly, p63 seems to be involved in

both tumorigenesis and tumor suppression. On the one hand, p63 is often overexpressed in human squamous cell carcinoma and is critical for cell proliferation and growth[18-21]. On the other hand, down-regulation of p63 plays a pivotal role in cancer metastasis[22-25]. Notably, p63 and p73 are required for p53-dependent apoptosis in response to DNA damage[26] (Figure 1B). Mutant p53 can promote cell invasion via the inhibition of TAp63[27].

Deregulated energetics is a hallmark of cancer cells. Most cancer cells exhibit elevated aerobic glycolysis, known as the Warburg effect[28]. Accumulating evidence reveals that p53 and p53 family members are important in regulation of metabolism. Activation of p53 can transcriptionally inhibit GLUT1 or GLUT4 expression to suppress glucose uptake[29]. In addition, p53 induces expression of TIGAR (TP53-induced glycolysis and apoptosis regulator), a transcription factor involved in suppression of glycolysis and pentose phosphate pathway[30]. Contrary to wild type p53, mutant p53 promotes aerobic glycolysis via increased GLUT1 plasma membrane association[31]. On the other hand,  $\Delta$ Np63 can directly transactivate HK2 expression in facilitating aerobic glycolysis[32]. TAp63 transactivates Sirt1, AMPK $\alpha$ 2 or LKB1 in regulation of lipid and glucose metabolism[33]. p73 can also regulate cancer cell metabolism. p73 activates expression of glutaminase-2 (GLS-2) and promotes serine biosynthesis[34]. Metformin has been well established in modulating several master metabolic regulators, such as AMPK, mTOR or HIF1A[35-37]. Therefore, both metformin and p53 family proteins are critically involved in regulation of cancer metabolism, suggesting that function of p53 family proteins and metformin anti-cancer activities are intrinsically connected, which will be our major focus in this review.

## Metformin anti-cancer activity in various human cancers

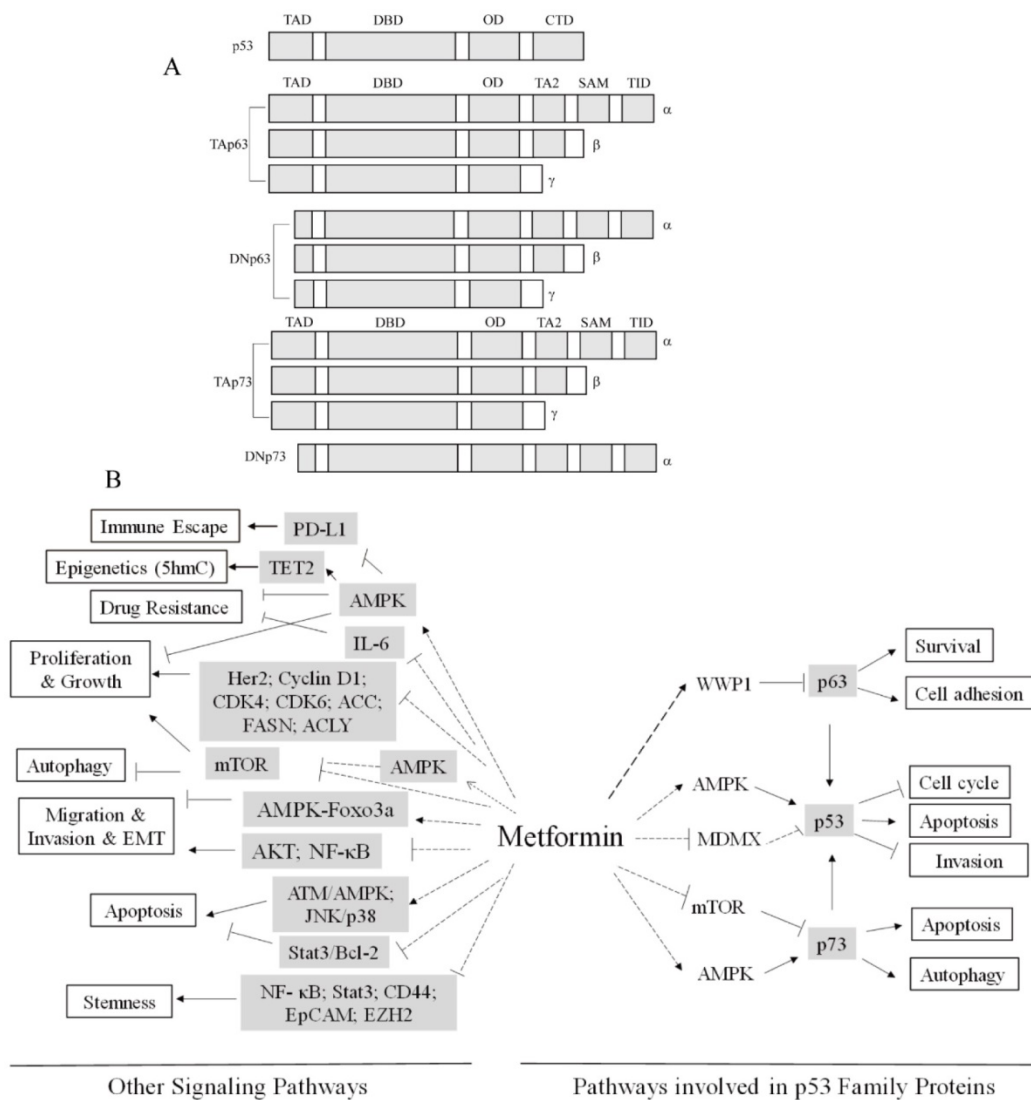
Metformin has been used to treat type 2 diabetes

for many years. Accumulating evidence has indicated that metformin can dramatically reduce tumor incidence in cancer patients with type 2 diabetes[38, 39]. Metformin inhibits growth, survival and metastasis of various cancer cells derived from breast, lung, esophagus, pancreas, liver and blood[40, 41] (Table 1). In addition, it has been recently shown that metformin has anti-tumor activities via an improved immune response[42-44] and modulation of cancer-related epigenetic modification[45]. The current literature indicates that metformin inhibits tumorigenesis via distinct signaling in different cancer types (Table 1). Importantly, metformin can regulate expression and activities of p53 family proteins in the suppression of tumorigenesis, which are discussed in detail in a later section. Here, we will focus on the effects of metformin on different human cancers.

Breast cancer is the most common cancer in women, especially in 30-59 years old females[46]. It has been reported that long-term use of metformin can dramatically reduce the risk of breast cancer in patients with type 2 diabetes[47]. In addition, breast cancer patients receiving metformin and neoadjuvant chemotherapeutic drugs (Table 1) have a higher pathological complete response rate than patients not receiving metformin[48]. At the molecular levels, metformin can inhibit mTOR signaling or suppress expression of cyclin D1 and ErbB2 (Her2) to inhibit breast cancer cell proliferation and growth[49-51] (Figure 1B). In addition, metformin can selectively inhibit breast cancer stem cells via suppressing nuclear translocation of NF- $\kappa$ B and phosphorylation of STAT3[43, 52] (Figure 1B). Furthermore, recent evidence demonstrated that high expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 $\alpha$ ) can enhance breast cancer resistance to metformin[53]. Currently, there are 42 FDA-approved clinical trials that are investigating the effect of metformin on breast cancer treatment (Table 1).

**Table 1.** List of selected clinical trials involving in metformin.

Cancer Type	Effectors	In Combination with Clinical Drugs	Clinical Trials (Approved by FDA)
Breast Cancer	AMPK/mTOR/NF- $\kappa$ B; STAT3; cyclin D1; Her2	5-FU+Doxorubicin+ Cyclophosphamide; Doxorubicin+Cyclophosphamide; 5-FU+Epirubicin+Cyclophosphamide; Paclitaxel; Docetaxel	42
Lung Cancer	ATM/ AMPK; JNK/p38; IL-6	Afatinib; Crizotinib; Alectinib; Ceritinib; Nivolumab	15
Esophagus Cancer	Stat3/Bcl-2; Clycin D1; CDK4; CDK6; AMPK; NF- $\kappa$ B; AKT	5-FU; Cisplatin	1
Liver Cancer	NF- $\kappa$ B; AMPK; AMPK / Foxo3a; ACC; FASN; ACLY	Sorafenib; Rapamycin; Aspirin	7
Pancreatic Cancer	Sp transcription factors; AMPK; GPCR; IR; CD44; EpCAM; EZH2	Rapamycin; Aspirin; Gemcitabine	16
Leukemia	mTOR; AMPK	Vincristine; Bortezomib; Paclitaxel	7



**Figure 1. (A)** Schematic representation of the structures of p53, p63 and p73. Using the alternative transcription starting sites, it generates TA or ΔN isoforms. Using the alternative splicing at the C-termini, it generates α, β or γ isoforms. The transactivation domains (TAD), the DNA-binding domains (DBD), the oligomerization domains (OD), the C-terminal regulatory domains (CTD), the second transactivation domains (TA2), the sterile alpha motif (SAM) and the transactivation inhibitory domains (TID) are indicated. The ΔNp63 and ΔNp73 variants lack the N-terminal TAD homologues to that of p53 but their N-termini possess transactivation activities. **(B)** Pathways Involved in Metformin Anti-Cancer Activities.

Use of metformin is associated with lower risk of lung cancer[54]. Metformin improves outcomes of chemotherapy and/or radiotherapy and survival for non-small cell lung cancer (NSCLC) patients with type 2 diabetes[55]. Metformin can induce apoptosis in lung cancer cells via activating ATM-AMPK and JNK/p38 MAPK pathways[56, 57] (Figure 1B). In addition, metformin can sensitize EGFR-TKI-resistant lung cancer cells through inhibition of IL-6 signaling [58] (Figure 1B). In recent years, several studies have demonstrated that a combination of metformin and other clinically proven drugs are beneficial in lung cancer treatment. For instance, metformin can significantly increase the efficacy of afatinib (EGFR inhibitor) to treat non-small cell lung cancers bearing mutant EGFR[59] (Table 1). In addition, a combination

of metformin and ALK inhibitors, including crizotinib, alectinib or ceritinib, exhibits a synthetic effect on ALK<sup>+</sup> NSCLC cells[60] (Table 1). Most recently, a phase II clinical trial was undertaken to evaluate the effects of a combination of nivolumab (PD-1 monoclonal antibody) and metformin on advanced NSCLC[61] (Table 1). Currently, there are 15 FDA-approved clinical trials to examine metformin anti-lung cancer activity (Table 1). Notably, several clinical studies have shown no clear correlation between metformin use and risk of lung cancer in patients with type 2 diabetes[62, 63]. Thus, more studies are needed to clarify whether metformin is beneficial for lung cancer patients.

Esophageal squamous cell carcinoma (ESCC) is the third most common carcinoma in digestive tracts

and the sixth leading cause of cancer death in the world[64]. Clinical analyses have indicated that metformin can reduce the risk of esophagus cancer, especially after 2 years of metformin use[65]. Metformin can induce ESCC cell autophagy and apoptosis via inactivation of the Stat3-Bcl-2 pathway[64] (Figure 1B). In addition, metformin can suppress the expression of cyclin D1, CDK4 and CDK6, resulting in ESCC cell growth retardation[66] (Figure 1B). Furthermore, metformin can inhibit migration and invasion of ESCC cells via inhibiting NF- $\kappa$ B nuclear localization[67] or AKT signaling[68] (Figure 1B). Moreover, metformin can enhance esophagus cancer cell sensitivity to chemotherapeutic drugs, 5-FU[69] or cisplatin[70, 71]. Currently, there is one clinical trial approved by the FDA that is exploring the effect of metformin on ESCC (Table 1).

Accumulating evidence has indicated that metformin can inhibit the development of hepatocellular carcinoma (HCC) and improve survival of HCC patients [72-74]. Metformin inhibits NF- $\kappa$ B signaling to suppress HCC cell growth in an AMPK-dependent manner[75]. In addition, metformin inhibits HCC cell migration and down-regulates the epithelial to mesenchymal transition (EMT) via the AMPK-Foxo3a pathway[76] (Figure 1B). Interestingly, metformin inhibits liver tumor growth via suppressing expression of several lipogenic enzymes such as Acetyl-CoA carboxylase (ACC), fatty acid synthase (FASN) and ATP citrate lyase (ACLY), independent of AMPK[77]. Therefore, it seems that metformin inhibits HCC development in both AMPK-dependent and AMPK-independent pathways (Figure 1B). Notably, a combination of metformin and sorafenib, the first-line therapeutic drug for HCC treatment, can significantly inhibit HCC cell proliferation and induce HCC cell autophagy via suppressing mTOR signaling[78] (Figure 1B). However, several clinical studies have shown that chronic treatment with metformin can increase HCC tumor aggressiveness and resistance to sorafenib[79] or have shown no superior efficacy when adding metformin to sorafenib in the treatment of advanced HCC[80]. Furthermore, combined treatment with metformin and rapamycin or aspirin is beneficial to HCC patients[81, 82]. Most recently, it was shown that silencing of hexokinase 2 (HK2) can sensitize HCC cells to metformin[83]. Therefore, a combination of metformin and glycolysis inhibition may be a new strategy for HCC treatment. Currently, there are 7 FDA-approved critical trials that are investigating metformin anti-HCC activity (Table 1).

With regard to pancreatic cancers, several clinical studies have indicated that use of metformin is associated with improved outcomes of pancreatic

cancer patients with diabetes[84, 85]. Metformin down-regulates expression of Sp transcription factors, activates AMPK kinase activity, and disrupts crosstalk between G protein-coupled receptors and insulin receptor signaling, leading to inhibition of pancreatic cancer cell proliferation[41, 86, 87]. Metformin can also inhibit stemness of pancreatic cancer stem cells, via re-expression of miRNAs to reduce the expression of cancer stem cell markers, such as CD44, EpCAM and EZH2[88, 89] (Figure 1B). A combination of metformin and rapamycin, aspirin or gemcitabine exhibits better effects in suppressing pancreatic cancer cell proliferation and tumor growth[90-93]. Currently, there are 16 FDA-approved clinical trials that are investigating the effect of metformin on pancreatic cancer (Table 1).

Metformin can inhibit proliferation of T-cell acute lymphoblastic leukemia (T-ALL) cells by inducing autophagy and apoptosis, likely via inhibition of mTOR signaling[94]. Our results showed that metformin can enhance leukemia cell sensitivity to the chemotherapeutic drug, vincristine, via activating AMPK[95] (Figure 1B). In addition, several studies have shown that bortezomib[96] or paclitaxel[97] can enhance metformin anti-leukemia activity. Currently, there are 7 FDA-approved clinical studies that are investigating the effect of metformin on leukemia treatment (Table 1).

Taken together, metformin anti-cancer activities are evident, likely via different mechanisms in various human cancers. Combinational therapy using metformin and other therapeutic drugs may be an effective strategy for cancer treatment.

## Metformin, AMPK and p53 family proteins

It is now clear that metformin can inhibit tumorigenesis in various human cancers and tumors via different signaling pathways. The most demonstrated downstream effector of metformin is AMPK. Different mechanisms by which metformin activates AMPK and the role of AMPK in metformin-mediated biological outcomes have been extensively studied. On the other hand, the p53 family proteins are core components in the regulation of growth and survival upon various stresses. The interplay between metformin, AMPK and p53 family proteins seems critical in metformin-mediated anticancer activities.

AMPK plays a critical role in energy homeostasis via targeting and phosphorylation of mTOR, acetyl-coA carboxylase 1 (ACC1) or modulation of GLUT activity, among others, to regulate protein metabolism, lipid metabolism or glucose metabolism, respectively[98]. Metformin is known to indirectly



activate AMPK in two different ways. Metformin at a high dose (~mM) inhibits the mitochondrial respiratory chain complex I, resulting in an increased AMP/ATP ratio, which in turn promotes AMP binding to AMPK  $\gamma$  subunit, AMPK conformational changes and exposure of AMPK $\alpha$  T172 to be phosphorylated and activated by its upstream kinase LKB1 [2]. On the other hand, it was recently shown that metformin at a low dose (~ $\mu$ M) can activate AMPK through the lysosomal pathway independent of the AMP/ATP ratio[35].

Mounting evidence has indicated that AMPK is involved in cancer cell growth and survival [99]. Clinical studies have demonstrated that AMPK activity is decreased in cancer [75, 95, 100]. Lack of LKB1, a direct AMPK upstream kinase, leads to inhibited AMPK activity[101]. In fact, knockout of LKB1 promotes K-Ras<sup>G12D</sup>-induced lung cancer metastasis[102]. In addition, activated AKT can directly phosphorylate AMPK $\alpha$  on Serine 485, resulting in AMPK conformational changes and blockage of AMPK $\alpha$  T172 from phosphorylation[103, 104]. Recent studies demonstrated that ubiquitin ligases UBE2O or MAGE-A3/6-TRIM28 can promote ubiquitination of AMPK $\alpha$ , resulting in proteasome-dependent degradation and an increase in cell proliferation and tumor growth[105, 106]. Furthermore, knockout of AMPK $\alpha$  can enhance c-Myc-induced lymphoma progression[107]. Notably, single AMPK $\alpha$ 1 or AMPK $\alpha$ 2 knockout mice do not exhibit accelerated tumor formation[108-110], suggesting that lack of AMPK $\alpha$ , alone, is not sufficient in driving tumorigenesis.

Numerous studies have indicated that activation of AMPK inhibits cell proliferation and tumor growth[111]. Glucose deprivation can induce cell death via activation of AMPK[112]. Activation of AMPK can also affect multiple signaling, including mTOR, p53 and NF- $\kappa$ B, to regulate cell growth and survival[113-115]. Currently, small molecules, such as berberine, AICAR and OSU-53[116-118], have been shown to activate AMPK in suppression of cell proliferation and tumor growth. Currently, the role of AMPK in the metformin anti-cancer activities has been extensively studied. Metformin inhibits cell proliferation and tumor growth in AMPK-dependent or AMPK-independent pathways[36, 119]. It has been reported that metformin can reverse multidrug resistance via activating AMPK[120]. Interestingly, several clinical drugs, including paclitaxel and aspirin, can enhance metformin-mediated activation of AMPK to inhibit tumor growth [93, 121]. Most recently, it has been shown that metformin is involved in cancer immune response via activating AMPK, which directly phosphorylates PD-L1 on S195

resulting in ER-associated degradation (ERAD)[44]. In addition, Metformin can also regulate epigenetic modifications by promoting AMPK-mediated phosphorylation of TET2 on S99, resulting in TET2 protein stabilization and accumulation of 5-hydroxymethylcytosine (5hmC)[45].

Activated AMPK can induce phosphorylation of p53 on serine 15, leading to cell-cycle arrest[113]. In addition, AMPK can phosphorylate SIRT1 on Thr344 and inhibit its deacetylase activity toward p53, resulting in enhanced p53 transactivation activity to promote apoptosis of HCC cells[115]. AMPK can also directly phosphorylate p73 on S426, leading to p73 nuclear accumulation, which in turn promotes apoptosis[122]. In contrast, the AMPK catalytic subunit, AMPK $\alpha$ , can interact with p73 $\alpha$ , promoting p73 $\alpha$  degradation independent of AMPK kinase activity[123]. Hence, the precise effects of AMPK on p73 and its biological outcomes need to be further investigated. With regard to the effects of AMPK on p63, much less is known. Recently, it was reported that lovastatin, a statin drug used for lowering cholesterol, can induce phosphorylation of p63 via activating AMPK-p38MAPK signaling[124].

## The role of p53 in metformin anti-cancer action

Several reports have shown that p53 is involved in metformin anti-cancer action[125-127]. Metformin activates AMPK, which induces p53 phosphorylation and activation to inhibit melanoma invasion and metastasis[125]. Metformin can also inhibit MDMX expression and reduce MDMX-p53 interactions, leading to p53 activation and cell death in lymphoma cells[126] (Figure 1B). A combination of metformin and 2-deoxyglucose induces apoptosis in prostate cancer cells in a p53-dependent manner[127]. Several studies have shown that metformin inhibits the viability of cancer cells lacking p53[128, 129]. Moreover, metformin can sensitize p53-deficient colorectal cancer cells to radiotherapy[129]. Therefore, these results indicate that metformin inhibits cancer cell growth and survival in both p53-dependent and p53-independent ways.

Reactive oxygen species (ROS) play a critical role in regulation of tumorigenesis and cellular response to anticancer therapies[130]. Metformin can reduce cancer risk via inhibiting cellular ROS production[131]. Mechanistically, metformin suppresses ROS production via inhibiting NAD(P)H oxidase activity or activating AMPK/FOXO3a, which facilitates antioxidant thioredoxin[132, 133]. Notably, ROS can act as an upstream factor that activates p53 and a downstream effector of p53 that mediates apoptosis (reference). However, whether ROS-p53 or

p53-ROS axis plays a role in metformin-mediated anticancer activity need to be further investigated.

### Role of p63 in metformin anti-cancer action

$\Delta$ Np63 $\alpha$  is the predominant p63 protein isoform expressed in epithelial cells[134].  $\Delta$ Np63 $\alpha$  is a critical transcriptional regulator of the cell adhesion program, through regulation of a subset of adhesion molecules, including integrin  $\beta$ 4, integrin  $\alpha$ 6 and fibronectin 1[16].  $\Delta$ Np63 $\alpha$  expression is significantly reduced in advanced human cancers, including breast cancer, lung cancer and bladder cancer[22, 135, 136]. Work from our lab and others showed that oncogenic PI3K/HER2/Ras commonly inhibits expression of  $\Delta$ Np63 $\alpha$ , facilitating cell migration and cancer metastasis[22, 25]. Furthermore, we found that MKP3 and CD82 are the downstream targets of  $\Delta$ Np63 $\alpha$  in regulating cancer metastasis[23, 24].

Notably,  $\Delta$ Np63 $\alpha$  has been shown to be overexpressed in squamous cell carcinoma (SCC) and to promote SCC cell proliferation and survival[21]. Our study indicated that protein isomerase Pin1 directly binds to and stabilizes  $\Delta$ Np63 $\alpha$  to promote proliferation of head and neck squamous cell carcinoma cells (HNSCC)[19].  $\Delta$ Np63 $\alpha$  can also suppress p73-induced apoptosis to promote survival of HNSCC cells[21]. Our recent study demonstrated that metformin dramatically inhibits  $\Delta$ Np63 $\alpha$  protein expression to inhibit SCC cell viability under glucose starvation or in combination with 2-deoxyglucose (2-DG)[18]. At the molecular level, metformin upregulates WWP1 expression to destabilize  $\Delta$ Np63 $\alpha$  protein independent of AMPK[18], indicating that  $\Delta$ Np63 $\alpha$  is an important downstream target of metformin (Figure 1B).

### Role of p73 in metformin anti-cancer activity

Like p53, p73 has anti-proliferative and pro-apoptotic activities[137]. p73 plays a critical role in cancer cell response to chemotherapy[138]. Unlike p53, the p73 gene is rarely mutated in human tumors. Metformin can stabilize p73 via activation of AMPK to induce cell apoptosis[122] (Figure 1B). Metformin can also increase p73 protein expression to promote cell autophagy[139] (Figure 1B). These results indicate that p73 is important in metformin anti-cancer action. Further *in vivo* studies are needed to verify whether metformin targets p73 in the regulation of cancer cell growth and survival.

### Conclusion

There is no doubt that metformin possesses

anti-cancer activities. The effects of metformin in suppression of tumorigenesis have been demonstrated in many human cancers derived from breast, lung, esophagus, liver, pancreas and blood. In the past decade, many signaling pathways impacted by metformin have been identified. AMPK is the most studied down-stream effector of metformin. Metformin can activate AMPK in mitochondria-dependent and lysosome-dependent pathways. Activated AMPK inhibits tumor growth and survival via regulating its downstream targets including p53/p63/p73, mTOR, ACC, and MAPK. p53 family proteins are intimately involved in metformin-mediated inhibition of cell growth and survival. In particular, p63 emerges as a novel metformin target in the inhibition of cell survival and tumor growth. However, whether p63 is a critical target of metformin in cancer patients needs to be further investigated.

Metformin is speculated to be a promising candidate drug for cancer treatment in combination with therapeutic drugs. Currently, more than 300 FDA-approved clinical trials are undergoing investigation of metformin anti-cancer activity, including numerous studies that use a combination of metformin with other drugs such as afatinib [59], sorafenib [79] and chemotherapeutic drugs[48, 140]. It would be interesting to examine a combination of metformin with p53/p63/p73-targeted drugs, if available, in cancer treatment.

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### Author Contributions

YY and XZX idealized the general structure of the text, YY, ZWH and XZX wrote the paper, idealized and designed the figures.

### Competing Interests

The authors have declared that no competing interest exists.

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