Hypothesis

Mutant p53 and mTOR/PKM2 Regulation in Cancer Cells

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Abstract

Mutations of *TP53* gene are the most common feature in aggressive malignant cells. In addition to the loss of the tumor suppressive role of wild-type p53, hotspot mutant p53 isoforms display oncogenic proprieties notoriously referred as gain of functions (GOFs) which result in chemoresistance to therapies, genomic instability, aberrant deregulation of cell cycle progression, invasiveness and enhanced metastatic potential, and finally, in patient poor survival rate. The identification of novel functional oncogenic pathways regulated by mutant p53 represent and intriguing topic for emerging therapies against a broad spectrum of cancer types bearing mutant *TP53* gene. Mammalian target of rapamycin (mTOR), as well as pyruvate kinase isoform M2 (PKM2) are master regulators of cancer growth, metabolism, and cell proliferation. Herein, we report that GOF mutant R175H and R273H p53 proteins trigger PKM2 phosphorylation on Tyr 105 through the involvement of mTOR signaling. Our data, together with the newly discovered connection between mutant p53 and mTOR stimulation, raise important implications for the potential therapeutic use of synthetic drugs inhibiting mTOR/PKM2 axis in cancer cells bearing mutant *TP53* gene. We further hypothesize that mTOR/PKM2 pathway stimulation serves to sustain the oncogenic activity of mutant p53 through both the enhancement of chemoresistance and of aerobic glycolysis of cancer cells. © 2016 IUBMB Life, 68(9):722–726, 2016

Keywords: *mutant p53; cancer; chemoresistance; mTOR; PKM2*

Introduction

TP53 is the most frequently mutated gene among all human cancers. Most mutant forms of p53 are caused by single amino acid substitutions mapping to the DNA binding domain (1). The high prevalence of missense substitutions, particularly certain "hotspot" mutations, suggests a selective advantage during cancer progression driving chemoresistance, invasion, and metastasis (2). Many researchers have investigated the underlying mechanisms of these gain-of-functions (GOFs) of mutant p53 proteins and it has become evident that many of these functions are the result of mutant p53 interaction with other proteins or transcription factors (3). Indeed, in addition to the loss of WTp53 tumor suppression functions, mutant p53 variants determine marked alterations of several cellular pathways deeply influencing cell homeostasis and metabolism (4).

The mammalian target of rapamycin (mTOR), a serine/threonine protein kinase, is a downstream effector of PI3K and Akt signaling. It comprises two different complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), which are structurally similar but functionally different. In particular, mTORC1 is the direct target of rapamycin or its analogues, such as everolimus. mTORC1 leads to cell anabolic growth by

Abbreviations: *Gain of Functions, (GOFs); Mammalian Target of Rapamycin, (mTOR); Pyruvate Kinase isoform M2, (PKM2); mTOR Complex 1, (mTORC1); mTOR Complex 2, (mTORC2); PhosphoEnolPyruvate, (PEP); AMP-activated protein Kinase, (AMPK); Epidermal Growth Factor Receptor, (EGFR); Hepatocyte Growth Factor Receptor, (HGFR); Transforming Growth Factor beta, (TGF-β); PhosphoInositide 3-Kinase, (PI3K); Protein Kinase B, (PKB/Akt); Death-Associated Protein Kinase 1, (DAPK1); Tuberous SClerosis 1, (TSC1); Tuberous SClerosis 2, (TSC2); Hypoxia Inducible Factor-1α, (HIF-1α); Histone Deacetylase 3, (HDAC3); Cyclin D1, (CCD1); Signal Transducer and Activator of Transcription 3, (STAT3); Cancer Upregulated Drug Resistant, (CUDR); Proto-oncogene serine/threonine-protein kinase, (Pim-1); Telomerase Reverse Transcriptase, (TERT)*

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promoting mRNA translocation and protein synthesis and also it has roles in glucose metabolism and lipid synthesis (5).

Pyruvate kinase is an enzyme that catalyzes the conversion of phosphoenolpyruvate (PEP) and ADP to pyruvate and ATP in glycolysis and plays a role in regulating cell metabolism. The M2 isoform of pyruvate kinase (PKM2) supports anabolic metabolism and is mainly expressed in cancer tissues. The enzymatic activity of PKM2 is allosterically regulated by both intracellular pathways, including mTOR signaling and metabolites. Preferential expression of PKM2 in tumor cells is implicated in cancer cell survival and drug resistance, for instance, by increasing the level of the anti-apoptotic protein Bcl-xL via enhancing NF-κB p65 stabilization (6). *In vivo* delivery of siRNAs specifically targeting PKM2, but not PKM1, causes substantial tumor regression of established xenografts (7), further providing interest for PKM2 as an anticancer target.

Despite recent studies demonstrate the fundamental biologic role of the mTOR/PKM2 signaling pathway, its involvement in chemoresistance and metabolic alterations driven by mutant p53 isoforms remains still unknown. In this article, we provide evidence of a direct connection between mutant p53 and PKM2 regulation and, in particular, P(Tyr105)PKM2 enhancement by mTOR signaling. We further hypothesize that mTOR/PKM2 pathway might largely contribute to both chemoresistance and metabolic alterations in cancer cells bearing mutant *TP53* gene.

Material and Methods

Cell Culture and Chemicals

PaCa3 (WTp53), Panc1 (mutant p53-R273H), and AsPC1 (p53-null) human pancreatic adenocarcinoma cell lines were grown in RPMI medium (Thermo Fisher Scientific, Milan, Italy), supplemented with 10% FBS, and 50 μg/mL gentamicin sulfate (BioWhittaker, Lonza, Bergamo, Italy). Cell lines were incubated at 37 °C with 5% CO_2. Everolimus (EVE; RAD-001) was obtained from Sigma (Milan, Italy).

Transient Transfection Assays

Exponentially growing cells were seeded at 5×10^3 cells/well in 96-well plates or at 2.5×10^5 cells in 60 mm cell culture plates. The ectopic expression of mutant p53 in p53-null cancer cells was carried out transfecting pcDNA3-mutp53R273H or pcDNA3-mutp53R175H expression vectors, or their relative negative control (pcDNA3). Wild-type and mutant p53 protein expression was transiently knocked-down by transfection with pRSUPER-p53 vector or its negative control (pRSUPER), kindly provided by Dr. Agami (The Netherlands Cancer Institute, Amsterdam) (8). Transfections were carried out for 48 h using Lipofectamine 3000 (Thermo Fisher Scientific), according to the manufacturer's instructions.

Immunoblot Analysis

Cells were harvested, washed in PBS, and resuspended in lysis buffer in the presence of phosphatase and protease inhibitors (50 mM Tris–HCl pH 8, 150 mM NaCl, 1% Igepal CA-630, 0.5% Na-Doc, 0.1% SDS, 1 mM Na_3VO_4, 1 mM NaF, 2.5 mM EDTA, 1 mM PMSF, and 1× protease inhibitor cocktail). After incubation on ice for 30 min, the lysates were centrifuged at 14,000*g* for 10 min at 4 °C and the supernatant fractions were used for Western blot analysis. Protein concentration was measured by Bradford reagent (Pierce, Milan, Italy) using bovine serum albumin as a standard. Protein extracts (50 μg/lane) were resolved on a 12% SDS-polyacrylamide gel and electro-blotted onto PVDF membranes (Millipore, Milan, Italy). Membranes were blocked in 5% low-fat milk in TBST (50 mM Tris pH 7.5, 0.9% NaCl, 0.1% Tween 20) for 1 h at room temperature and probed overnight at 4 °C with a mouse polyclonal anti-p53 (1:500) (Santa Cruz, #sc-263), rabbit polyclonal anti-phospho (Tyr105)PKM2 (1:1,000) (Cell Signaling #3827), mouse polyclonal anti-PKM2 (1:1,000) (Sigma #WH0005315M1), mouse monoclonal anti-alpha-tubulin (1:2,500) (Oncogene #CP06), rabbit monoclonal anti-phospho (Tyr705)STAT3 (1:1,000) (Cell Signaling #9131), rabbit monoclonal anti-STAT3 (1:1,000) (Cell Signaling #9132), or mouse monoclonal anti-α-tubulin (1:2,500) (Oncogene #CP06) antibodies. Horseradish peroxidase conjugated antimouse or antirabbit IgGs (1:8,000 in blocking solution) (Upstate Biotechnology, Milan, Italy) were used as secondary antibodies. Immunodetection was carried out using chemiluminescent substrates (Amersham Pharmacia Biotech, Milan, Italy) and recorded using a HyperfilmECL (Amersham Pharmacia Biotech). ECL results were scanned and the amount of each protein band quantitated using NIH Image J software (http://rsb.info.nih.gov/nihimage/).

Statistical Analysis

ANOVA (*post hoc* Bonferroni) analysis and graphical presentations were performed by GraphPad Prism version 5. Statistically significant results were referred with a P value <0.05. Values are the means of three independent experiments ($\pm$SD).

Mutant p53 Triggers mTOR Pathway

Our research group recently published that mutant p53 stimulates mTOR signaling and that this event enhances the sensitivity of cancer cells bearing mutant *TP53* gene to the mTOR inhibitor everolimus as compared with WTp53 counterpart (9). Mechanistically, mTOR signaling stimulation by mutant p53 might be mainly due by two events: (i) the blockage of the mTOR-antagonist pathway of AMP-activated protein kinase (AMPK) by mutant p53 (9,10); (ii) the mutant p53-mediated stimulation of growth factor receptors' signaling, as epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (HGFR), or transforming growth factor (TGF)-β receptor, thus promoting PI3K/Akt pathway and in turn activating many downstream effectors including mTORC1 (11,12). In addition, Zhao *et al.* recently identified a further mechanism

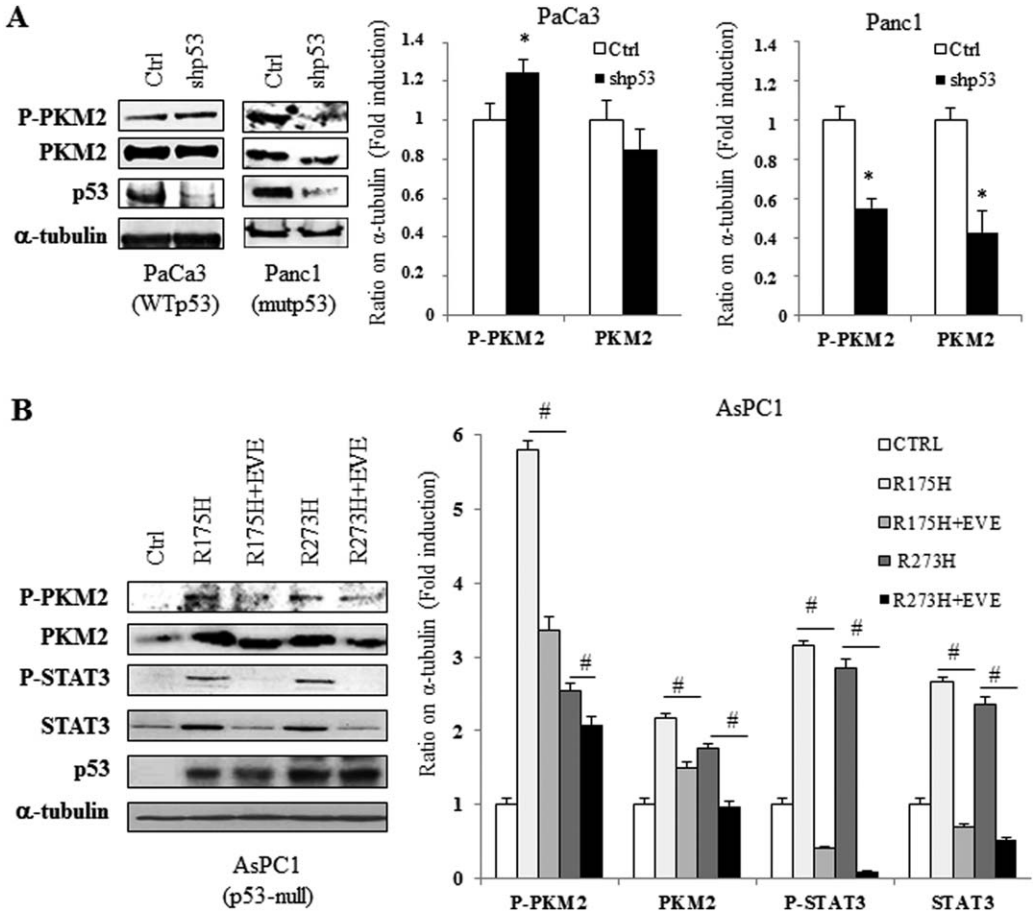

FIG 1

*(A) PaCa3 and Panc1 pancreas cancer cells were seeded in 100-mm diameter culture dishes and transfected for 48 h with pRSuper-p53 vector (shp53) or with the empty vector as negative control (Ctrl). Whole-cell extracts were used for Western blot analysis using the indicated antibodies and bands were quantified as described in Material and Methods. Values are the means of three independent experiments (±SD). Statistical analysis: *P < 0.05 shp53* versus *Ctrl. (B) AsPC1 pancreas cancer cells were transfected for 48 h with the plasmids coding for mutant p53 proteins (R175H or R273H), or with empty pCDNA3 as negative control (Ctrl), in the absence or presence of 5 μM everolimus. Whole-cell extracts were used for Western blot analysis using the indicated antibodies and bands were quantified. Values are the means of three independent experiments (±SD). Statistical analysis: #P < 0.05 R175H + EVE* versus *R175H and R273H + EVE* versus *R273H.*

by which mutant p53 might trigger mTOR signaling. They discovered that death-associated protein kinase 1 (DAPK1), found highly expressed in p53-mutant cells, mediated the disruption of the TSC1/TSC2 complex, resulting in the activation of mTOR pathway. They showed that high DAPK1 expression causes increased cancer cell growth and it is associated with worse outcomes in patients with p53-mutant cancers (13).

Mutant p53 Induces PKM2 and STAT3 Phosphorylation in a mTOR-Dependent Manner

PKM2 is a limiting glycolytic enzyme that catalyzes the final step in glycolysis, a fundamental metabolic pathway frequently enhanced in cancer. However, the involvement of PKM2 in cancer is not constrained to the regulation of glycolysis. Indeed, beyond its dominant role in glycolysis to achieve the nutrient demands of cancer cell proliferation, PKM2 contributes to tumorigenesis through crucial nonmetabolic functions. It has been reported that PKM2 can translocate into the nucleus of the cells and directly interact with the hypoxia inducible factor (HIF)−1α subunit promoting transactivation of HIF-1 target genes by enhancing its binding and recruitment with p300, a transcriptional coactivator, which regulates HIF-1 activity (14). PKM2 can further interact with β-catenin phosphorylated by EGFR signaling and this interaction is required for the two proteins to be recruited to the cyclin D1 (CCND1) promoter (15). This results in the removal of histone deacetylase 3 (HDAC3) from the promoter, histone H3 acetylation, and CCND1 expression (16). Thus, PKM2-dependent β-catenin transactivation is described to be required for EGFR-promoted tumor cell proliferation and brain tumor development (17). Intriguingly, PKM2 in addition to play a role as a transcriptional coactivator, may function as a protein kinase that uses PEP as a phosphate donor and phosphorylates substrates involved in metabolic reprogramming. Nuclear PKM2 directly

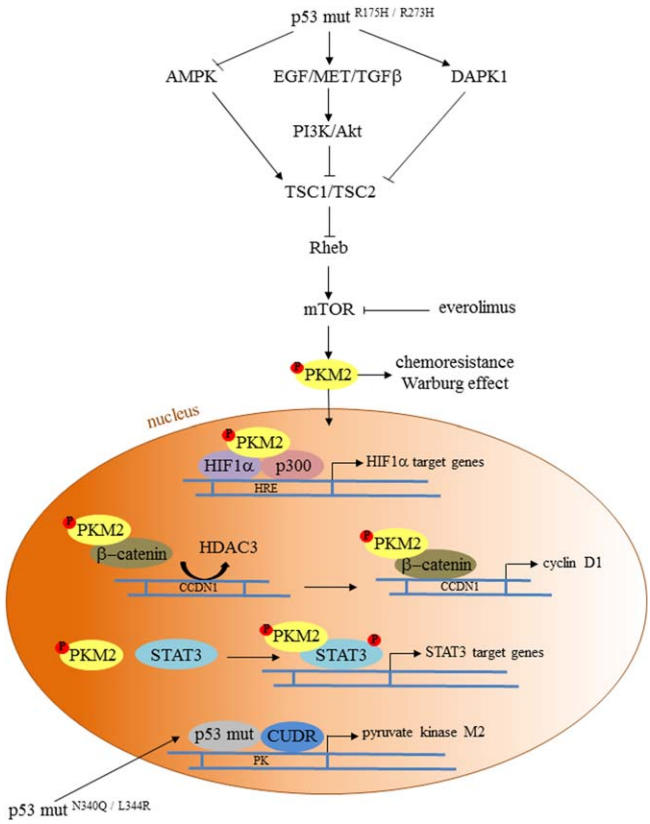

FIG 2 *Schematic representation of the regulation of the mTOR/PKM2 axis in p53 mutated cancer cells.*

phosphorylates signal transducer and activator of transcription 3 (STAT3) stabilizing its nuclear localization (18). The activation of STAT3 in malignant cells is possibly one of the most important molecular signatures for promoting the progression of cancer (19).

Concerning mutant p53 isoforms, Wu *et al.* very recently demonstrated that the double N340Q/L344R mutant p53 promotes hepatocarcinogenesis through upregulation of PKM2 (20). Mechanistically, mutant p53 (N340Q/L344R) forms a complex with cancer up-regulated drug resistant (CUDR), a novel noncoding RNA, and the complex binds and stimulates the promoter regions of PKM2 gene. Increased PKM2 binds to histone H3 favoring its phosphorylation onto the promoter region of proto-oncogene serine/threonine-protein kinase (Pim1), which in turn enhances the expression of telomerase reverse transcriptase (TERT) and other related genes thus prolonging telomeres in cancer cells (20). In this study, we report for the first time that R175H and R273H hotspot mutant p53 isoforms significantly enhance expression of PKM2 and STAT3 and their mTOR-mediated phosphorylation levels (Fig. 1). Indeed, the addition of the mTOR inhibitor everolimus significantly reverted both P(Tyr105)PKM2 and P(Tyr705)STAT3 induced by mutant p53 ectopic expression, suggesting a key role for mTOR on mutant p53-driven stimulation of PKM2 and its downstream targets. On the contrary, PKM2 expression failed to be significantly modulated by WTp53 and its phosphorylated form on Tyr105 is significantly increased after WTp53 knockdown (Fig. 1). Overall, these data let us to hypothesize that the stimulation of mTOR/PKM2 pathway is aberrantly induced by GOF mutant isoforms of p53. Intriguingly, it has been demonstrated that PKM2 phosphorylation on Tyr105 stabilizes the homodimeric conformation of PKM2, which induces a bottleneck in the glycolytic pathway favoring a high rate of biosynthesis of macromolecules as nucleotides, phospholipids, and amino acids (21) but also indirectly sustaining aerobic glycolysis (also named Warburg effect) by regulating gene expression (18).

Involvement of mTOR/PKM2 Axis in Cancer Chemoresistance and Metabolism Alteration

During the last year it has been finally clarified that signaling pathways triggered by GOF mutant p53 isoforms play a critical role in tumor development, metastasis, and resistance to therapy (22–24). In addition, since mTOR signaling is involved in modulating cancer metabolism, growth, survival, invasion, and chemoresistance, it has been recognized as a therapeutic target for anticancer drug discovery (25). Moreover, Sun *et al.* demonstrated that mTOR is a key activator of the Warbug effect, revealing PKM2 as a crucial metabolic enzyme in the oncogenic mTOR-induced glycolysis (26). Furthermore, PKM2 has been also found involved in chemoresistance and cancer progression in various tumor types (27,28). Altogether these findings support a crucial oncogenic role for mTOR/PKM2 axis in cancer.

Conclusions

Overall these data allow us to hypothesize that the oncogenic activity of mutant p53 proteins is, at least partially, due by the stimulation of mTOR/PKM2 axis leading to chemoresistance, cancer-related metabolic alterations, such as the Warburg effect, and the nuclear translocation of P(Tyr105)PKM2 itself, which in turn can transcriptionally activate several oncogenic target genes or directly phosphorylate signaling mediators, as STAT3 (Fig. 2). In this sense, it may be strongly desirable to assess the effect of potential drug candidates for specific inhibition of PKM2 activity in a broad spectrum of cancer cells bearing GOF mutant *TP53* gene *in vitro* culture systems and confirm their effects *in vivo* xenograft models.

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