

Manuscript Number:

Title: LINKING TP53 MUTATION TO ENERGY METABOLISM AND GLYCOLYTIC
EFFICIENCY IN BREAST CANCER

Article Type: Regular Paper

Keywords: glycolysis; gluconeogenesis; breast cancer; Warburg effect;
next generation sequencing

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Abstract: Promising new hallmarks of cancer include chromosomal instability, immune response, inflammation and alteration of energy metabolism. Of these, energy metabolism involves molecular mechanisms shifting cancer cells to aerobic glycolysis. Our goal was to evaluate the correlation between mutation in TP53, the most commonly mutated tumor suppressor gene and metabolism. We established a database comprising mutation and RNA-seq expression data of the TCGA repository and receiver operating characteristics (ROC) analysis was performed to compare expression of each gene between TP53 mutated and wild type samples. All together 762 breast cancer samples were evaluated of which 215 had TP53 mutation. Top up-regulated metabolic genes include glycolytic enzymes (SLC2A5, SLC2A6, HK3, GPI, PFKF, GAPDH, PGK1, and ENO1), glycolysis regulator (PDK1) and pentose phosphate pathway enzymes (PGD, TKT, and RPIA). Gluconeogenesis enzymes (G6PC3, FBP1) were down-regulated. Oxygen consumption (OCR) and extracellular acidification rates (ECAR) were measured in TP53 wild type MCF7 and TP53 mutant MDA-MB-231 and JIMT-1 cell lines with a microfluorimetric analyzer. Cells were treated with metabolic inhibitors including oligomycin, antimycin, and rotenone. Experiments were performed in the presence and absence of D-glucose and L-glutamine. Applying metabolic inhibitors in cell culture experiments resulted in higher glycolytic and mitochondrial activity in TP53 mutant breast cancer cell lines. In summary, TP53 mutation influences energy metabolism at multiple levels. Our results provide evidence for the synergistic activation of multiple hallmarks linking to these the mutation status of a key driver gene.

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Professor Fabrice Rappaport and Susanne Arnold

Editors of BBA Bioenergetics

Dear Professors Fabrice Rappaport and Susanne Arnold,

Please find enclosed our manuscript “**Linking TP53 mutation to energy metabolism and glycolytic efficiency in breast cancer**” for your consideration as a research paper in *BBA Bioenergetics*.

Chronic cell proliferation of cancer requires corresponding adjustment of energy metabolism for rapid cell growth and division. Here, we demonstrate that *TP53* mutations influence oxidative and glycolytic metabolism at several levels and thus we provide evidence for the simultaneous activation of multiple cancer hallmarks. We also show differential response of *TP53* mutant and wild-type breast cancer cell lines to metabolic inhibitors. Based on our findings we anticipate a more significant effect of agents influencing energy metabolism in patients harboring *TP53* mutation.

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The manuscript presents original research, it has not been previously published and it is not being considered for publication elsewhere. Thank you for considering our manuscript, we are looking forward to your comments.

Yours truly,

Balázs Györfy MD, PhD, DSc

LINKING TP53 MUTATION TO ENERGY METABOLISM AND GLYCOLYTIC EFFICIENCY IN BREAST CANCER

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Keywords: glycolysis, gluconeogenesis, breast cancer, Warburg effect, next generation sequencing

Highlights:

- In 762 patients, *TP53* mutation increased glycolysis and inhibited gluconeogenesis.
- Higher glycolytic and mitochondrial activity was observed in *TP53* mutant cell lines.
- Energy metabolism inhibitors had stronger effect in *TP53* mutant tumors.

ABSTRACT

Promising new hallmarks of cancer include chromosomal instability, immune response, inflammation and alteration of energy metabolism. Of these, energy metabolism involves molecular mechanisms shifting cancer cells to aerobic glycolysis. Our goal was to evaluate the correlation between mutation in *TP53*, the most commonly mutated tumor suppressor gene and metabolism. We established a database comprising mutation and RNA-seq expression data of the TCGA repository and receiver operating characteristics (ROC) analysis was performed to compare expression of each gene between *TP53* mutated and wild type samples. All together 762 breast cancer samples were evaluated of which 215 had *TP53* mutation. Top up-regulated metabolic genes include glycolytic enzymes (*SLC2A5*, *SCL2A6*, *HK3*, *GPI*, *PFKP*, *GAPDH*, *PGK1*, and *ENO1*), glycolysis regulator (*PDK1*) and pentose phosphate pathway enzymes (*PGD*, *TKT*, and *RPIA*). Gluconeogenesis enzymes (*G6PC3*, *FBP1*) were down-regulated. Oxygen consumption (OCR) and extracellular acidification rates (ECAR) were measured in *TP53* wild type MCF7 and *TP53* mutant MDA-MB-231 and JIMT-1 cell lines with a microfluorimetric analyzer. Cells were treated with metabolic inhibitors including oligomycin, antimycin, and rotenone. Experiments were performed in the presence and absence of D-glucose and L-glutamine. Applying metabolic inhibitors in cell culture experiments resulted in higher glycolytic and mitochondrial activity in *TP53* mutant breast cancer cell lines. In summary, *TP53* mutation influences energy metabolism at multiple levels. Our results provide evidence for the synergistic activation of multiple hallmarks linking to these the mutation status of a key driver gene.

1. INTRODUCTION

Chronic cell proliferation requires corresponding adjustment of energy metabolism in order to fuel rapid cell growth and division. Normal cells process glucose to pyruvate via glycolysis, and thereafter to CO₂ in the mitochondria via an affiliated ATP producer mechanism, the oxidative phosphorylation. A feature of cancer cell metabolism first observed by Otto Warburg in 1956 involves their capability to reprogram glucose metabolism (increase glycolysis rate) in aerobic conditions¹. By limiting their energy metabolism this leads to a state that has been termed “aerobic glycolysis” (also known as the “Warburg effect”). Increased rate of glycolysis allows the diversion of glycolytic intermediates into various biosynthetic pathways that are required for assembling new cells². The very same process can also be observed in normal, rapidly dividing embryonic cells³. In addition, high glycolysis in tumor cells correlates with the degree of tumor malignancy, its ability to form metastasis and has been showed to be related to resistance against chemo- and radiotherapy treatments^{4,5}.

Driver genes behind this process include *GLUT1*, which enables the uptake of glucose necessary for the process and the *HIF1* alpha and *HIF2* alpha transcription factors responsible for the initiation of glycolysis by triggering the expression of multiple target genes⁶. Altered energy metabolism is in close connection to the six established hallmarks of cancer (activation of oncogenes, loss of tumor suppressors, replicative immortality, evasion of apoptosis, induction of neoangiogenesis and of metastases). Moreover, transformed energy metabolism is proving to be so widespread in cancer cells that it has been recently accepted as a new hallmark of cancer⁷.

TP53 is frequently mutated in human cancers; somatic *TP53* mutations occur in almost every type of human tumors and in over 50% of all tumors⁸. As the “guardian of the genome”, *TP53* plays a key role in maintaining genomic stability and tumor prevention⁹. In breast cancer, genes correlated to *TP53* mutation predict the risk of tumor relapse¹⁰. *TP53* positively correlates to *MKI67* expression and thus to tumor proliferation¹¹. In other tumors with low *TP53* mutation rates, *TP53* is often inactivated by alternative mechanisms¹². *TP53* can also impact glycolysis through mechanisms including transcriptionally repressing the expression of glucose transporters, down-regulating rate limiting enzymes of glycolysis, decreasing the expression of transporters responsible for lactate extrusion and negatively regulating the AKT/mTOR and NF-κB signaling pathways¹³. *TP53* can also modulate expression of glycolytic enzymes like phosphoglycerate mutase¹⁴ and TIGAR, a stimulator of gluconeogenesis and the pentose phosphate pathway¹⁵. Furthermore it was found that *TP53*

has effect on the ATP level by influencing the mitochondrial energy production via disruption of the SCO2 gene¹⁶.

In our research our conceptual idea was that we assume that the mutations in *TP53* will not only influence genes because its tumor suppressor properties but will also directly impact enzymes of glycolysis and citrate cycle involved in the energy metabolism of the cells. To validate this hypothesis, we utilized a large breast cancer cohort where both mutation state and gene expression levels were available for each gene. To evaluate expression of genes involved in metabolic cycles we designated sub-cohorts based on *TP53* mutation state. Using these datasets we demonstrate that genes driven by *TP53* mutation status have a high relevance in establishing the Warburg effect. In our *in vitro* experiments we analyzed metabolic profiles in three breast cancer cell lines with different *TP53* status (wild type MCF7 and mutated *TP53* in MDA-MB-231 and JIMT-1) and demonstrate that glycolytic activity is concomitant with *TP53* status.

2. METHODS

2.1. *In silico* analyses

2.1.1. NGS data download

Genome-wide sequencing data and RNA-seq data for breast cancer patients were obtained from The Cancer Genome Atlas (TCGA) of the National Cancer Institute (<http://cancergenome.nih.gov/>)¹⁷. The aligned TCGA datasets were downloaded from the CGHub repository (website: <https://cghub.ucsc.edu/>) using the CGHub download client software GeneTorrent (version 3.8.5).

2.1.2. Mutation calling

Mutation calling and annotation was done with *MuTect* using default parameters. The human reference genomes GRCh37, GRCh37-lite and HG19 used for mutation calling were downloaded according to the CGHub website's "Reference Assemblies" guideline (available at <https://browser.cghub.ucsc.edu/help/assemblies>). To reduce the total number of mutations, we only accepted somatic mutations that were labeled with 'KEEP' according to the *MuTect* judgment algorithm, and were present in at least four reads with a minimum of 20 fold read coverage. The identified mutations were annotated with *MuTect* using the most recent versions of the dbSNP (build 139) and COSMIC (version 68) databases¹⁸. The identified

sequence variations were functionally annotated using SNPEff v3.5¹⁹. The reference databases used with SNPEff were downloaded with the SNPEff downloader.

2.1.3. Processing of RNA-seq data

RNA-seq data for breast cancer patients was obtained from the TCGA repository for those patients who also had mutation data as well. We downloaded pre-processed level 3 data generated using the Illumina HiSeq 2000 RNA Sequencing Version 2 platform. Expression levels for these samples were determined using a combination of MapSplice and RSEM. Individual patient files were merged in R using into a single database¹¹.

2.1.4. Statistical analysis

Separate ROC analysis was performed for the expression of each gene grouped by TP53 mutation status. ROC analysis was completed in the R statistical environment (<http://www.r-project.org>) using the ROC Bioconductor library (<http://www.bioconductor.org>) as described previously²⁰. Statistical significance was set at FDR adjusted p value<0.05.

2.2. *In vitro* experiments

2.2.1. Cell lines

Human breast cancer cell lines MCF7, MDA-MB-231 and JIMT-1 were obtained from the American Type Culture Collection (Manassas, VA, USA). Cell lines were cultured in RPMI medium supplemented with 10% fetal bovine serum with 1% penicillin-streptomycin and 0,1% amphotericin (Life Technologies). Cells were grown at 37°C under a humidified atmosphere of 95% air and 5% CO₂.

2.2.2. STR analysis

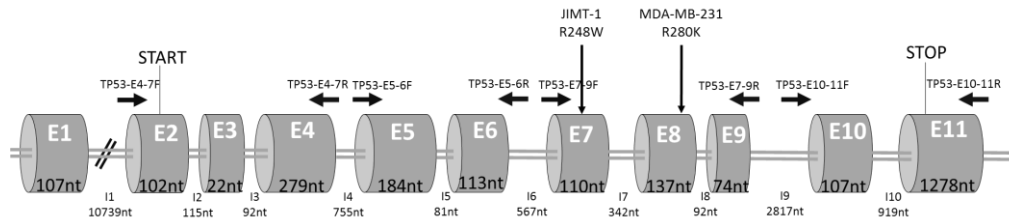
Cell line verification was completed by Short tandem repeat (STR) analysis on 9 exact loci of the human genome. Genomic DNA was extracted by DNeasy Kit (Qiagen) according to the protocol provided by the manufacturer. DNA concentration and clarity was confirmed by Nanodrop ND-1000. The STR analysis was accomplished by Fragment Analysis Facility at Johns Hopkins University (Baltimore, USA). Then, results were compared to the STR database of the German Collection of Microorganisms and Cell Cultures at the Leibniz Institute (<http://www.dsmz.de>). All the tested cell lines were contamination free.

2.2.3. TP53 mutation validation

Direct DNA sequencing was performed using genomic DNA amplified by polymerase chain reaction. In MDA-MB-231 and JIMT-1 mutation detection was performed in exons 7-8 of *TP53*, while in the case of MCF7 each of the 11 exons were sequenced in full length (**Figure 1A**). Primers were designed to be located in intronic sequences to allow amplification of genomic DNA only. The used primers and primer features are listed in **Table 1** and illustrated in **Figure 1**.

Figure 1. Mutations in the *TP53* gene. (A) Locations of primers and mutations in the gene structure. Grey boxes represents exons (E1-11), double grey lines between exons represent introns (I1-10). Numbers show the nucleotide length. Vertical arrows show positions of mutations in the two mutant cell lines (JIMT-1 and MDA-MB-231). Horizontal arrows show the primers used in the PCR amplification designed for product sequencing. (B) Result of the validation of *TP53* mutation in the mutant cell lines by comparison of sequence chromatograms to the wild type *TP53* cell line MCF7.

A)



B)

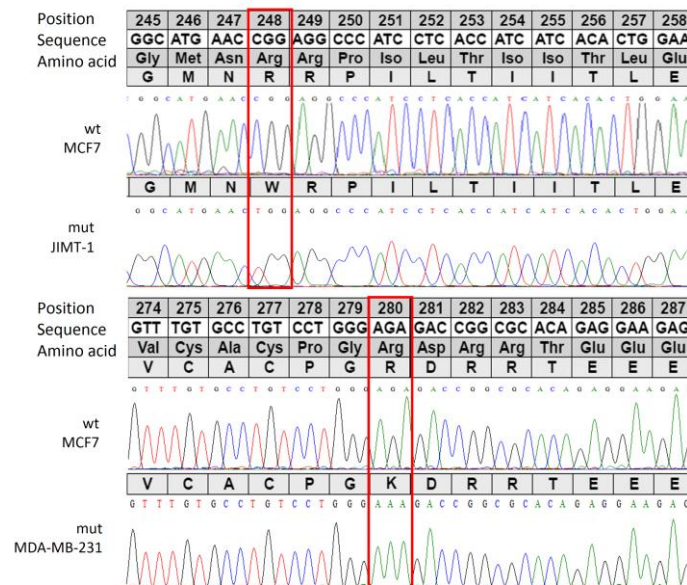


Table 1. Primers for mutation detection in the genomic *TP53* gene.

PRIMER	EXON	INTRON	SEQUENCE	PRODUCT
TP53-E2-4F	2-4	2, 3	5'-TCCTCTTGCAGCAGCCAGACTGC-3'	620bp
TP53-E2-4R	2-4	2, 3	5'-GGCATTGAAGTCTCATGGAAGC-3'	
TP53-E5-6F	5-6	5	5'-CCTGACTTTCAACTCTGTCTCC-3'	400bp
TP53-E5-6R	5-6	5	5'-CCAGAGACCCCAGTTGCAAACCAG-3'	
TP53-E7-9F	7-9	7, 8	5'-CTCATCTTGGGCCTGTGTTATCTCC-3'	760bp
TP53-E7-9R	7-9	7, 8	5'-CCACTTGATAAGAGGTCCCAAGACTTAG-3'	
TP53-E10-11F	10-11	10	5'-CCTCCTCTGTTGCTGCAGATCC-3'	1110bp
TP53-E10-11R	10-11	10	5'-GGCTGTCAGTGGGGAACAAGAAG-3'	

The PCR reaction was performed in 25µl final volume, containing 500ng of genomic DNA, 10mM of each dNTP (Invitrogen), 10µM of each of the eight primers, 5 units of Taq polymerase (Invitrogen), 2.5µl 10x buffer, completing to the final volume with nuclease free H₂O. The amplification reaction was carried out in thermocycler (Swift Maxi, ESCO) with an initial denaturation step of 3 min at 94°C, followed by 35 cycles consisting of three steps: 94°C for 30 sec, 53°C for 30 sec, and 72°C for 2 min. Annealing temperature was optimized to the primers melting temperature. The last cycle was followed by an extension step of 6 min at 72°C. The PCR product was purified, and DNA sequencing was performed at the Department of Genomic Medicine and Rare Disorders (Semmelweis University, Budapest, Hungary). The DNA sequence was analyzed by BioEdit and Genedoc programs (**Figure 2B**).

2.2.4. Measurement of cellular respiration and glycolytic activity

Mitochondrial and glycolytic functions of the wtTP53 MCF-7 and the mutTP53 MDA-MB-231 and JIMT-1 cells were monitored using Seahorse XF96 extracellular flux analyzer. (Seahorse Bioscience, North Billerica, MA, USA) Oxygen consumption rate (OCR), as a representation of cellular respiration, and extracellular acidification rate (ECAR), as a measure of glycolytic activity, were assessed in parallel in an assay based on microfluorimetric detection using an XF96 Analyzer, as previously described²¹. Breast cancer cells were plated one day before measurement on Seahorse XF96 cell culture microplates at a 2x10⁴ cells/well density. The growth medium beyond cells was replaced by either the DMEM-based or the EC assay medium 2.5 h before measurement. OCR and ECAR were assessed by the XF96 Analyzer based on determination of O₂ concentration and pH.

During the measurement, 20 to 26 μ l of substrates or inhibitors (at all times prepared freshly in the respective assay medium) were administered into each well to reach the desired final concentrations. Laboratory chemicals were obtained from Sigma (St Louis, MO, USA). L-Glutamine was from Merck (Merck KGaA, Darmstadt, Germany). All additive stock solutions were each time freshly prepared. The composition of the Extracellular assay medium (EC) was: 143 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 8.3 mM HEPES (pH 7.4). The DMEM-based glucose-free medium was purchased from Seahorse Bioscience (# 102365, North Billerica, MA, USA).

Through OCR and ECAR measurements the effects of adding D-glucose/ L-glutamine, oligomycin, 2,4-dinitrophenol (DNP), and antimycin A + rotenone were determined. The use of these metabolic modulators permits determination of several parameters of mitochondrial and glycolytic functions. Key parameters of mitochondrial function include basal respiration, ATP turnover, proton leak and maximal respiration. The difference between the maximal and the basal respiration creates a reserve respiratory capacity of a cell to create ATP via oxidative phosphorylation in response to increased demand of energy (for summary see **Figure 4B**). Key parameters of cellular glycolysis include glycolysis, and glycolytic capacity (**Figure 4D**). Glycolytic reserve is the difference between glycolytic capacity and glycolysis that reflects to the cellular capability to rise the glycolytic rate upon increased energy demand.

3. RESULTS

3.1. Database setup

Breast cancer samples from the TCGA database with genome-wide sequencing, gene expression and clinical data for 762 patients were used in the statistical analysis. We analyzed n=547 *TP53* wild type and n=215 *TP53* mutant BC patient samples. Clinical characteristics for all these patients are summarized in **Table 2**.

Table 2. Aggregate clinical characteristics of patient samples included in the study. *Clinical data for some parameters do not add up to 100% because not all data was available for each patient.*

TP53 MUTATIONAL STATUS	Wild type	Mutated
n	547	215
STAGE		
Stage I	95 (18.0%)	33 (16.2%)
Stage II	303 (57.4%)	115 (56.4%)
Stage III	111 (21.0%)	48 (23.5%)
Stage IV	9 (1.7%)	4 (1.9%)
T / N / M CLASSIFICATION		
T1	145 (27.4%)	47 (23.0%)
T2	302 (57.2%)	126 (61.8%)
T3	59 (11.2%)	22 (10.8%)
T4	20 (3.8%)	8 (3.9%)
N0	236 (44.7%)	92 (45.1%)
N1	195 (36.9%)	68 (33.3%)
N2	54 (10.2%)	25 (12.3%)
N3	32 (6.1%)	16 (7.8%)
M0	457 (86.5%)	179 (87.7%)
M1	11 (2.1%)	4 (2%)
MOLECULAR SUBTYPE		
ER+	433 (82.1%)	96 (47.1%)
ER-	63 (11.9%)	99 (48.5%)
HER2+	93 (17.6%)	50 (24.5%)
HER2-	435 (82.4%)	154 (75.5%)
PGR+	377 (71.4%)	85 (41.5%)
PGR-	117 (22.2%)	111 (54.4%)
Basal	50 (9.5%)	89 (43.7%)
Luminal A	58 (10.9%)	4 (1.9%)
Luminal B	398 (75.4%)	85 (41.7%)
HER2+ER-	22 (4.2%)	26 (12.7%)
RELAPSE		
RFS event	21 (3.9%)	10 (4.9%)
Death	51 (9.6%)	22 (10.8%)

3.2. Assessment of energy metabolism in wild type and mutated clinical samples

ROC analysis was performed to identify genes associated with *TP53* mutation. We identified 2,178 genes positively expressed and 1,584 genes negatively regulated genes in the mutant *TP53* cohort compared to the wild type *TP53* cohort at $p < 0.05$. This entire gene list was analyzed based on carbon metabolism pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG database)²². The up-regulated gene group had connection with two pathways: glucose degradation (glycolysis) and pentose phosphate pathway, plus contained a glycolysis regulator. The down-regulated genes were connected to two pathways, gluconeogenesis and fatty acid oxidation pathway (FAOP), and a mitochondrial regulator gene was also included.

3.3. Expression changes of the most important genes

ROC results for the 16 top ranking genes that had connection to cellular energy metabolisms are listed in **Table 1**. The glycolysis pathway included eight up-regulated enzymes, two glucose transporters and six carbohydrate modulator enzymes. The glucose transporters *GLUT5* and *GLUT6* mRNA expression were almost twofold higher in the mutTP53 breast cancer patients than in wtTP53 patients (**Figure 2A**). Hexokinases (HK) perform the first step in most glucose metabolism pathways. mRNA expression of one isozyme of HK (*HK3*) was twofold higher in the mutTP53 patient group (**Figure 2A**). Phosphofructokinase (PFKP) enzymes process the third step of glycolysis, and its isoform gene expression was twofold higher in the mutTP53 group (**Figure 2B**). Glucose-6-phosphate isomerase (*GPI*) gene product performs the second step of glycolysis, and its expression was one and a half times as much in the mutTP53 patient group than in the wtTP53 patient group. The protein product of *GAPDH* gene converts D-glyceraldehyde 3-phosphate into 3-phospho-D-glyceroyl phosphate via the fifth step of glycolysis, and its expression was one and a half times higher in the mutTP53 group. The phosphoglycerate kinase 1 (PGK1) converts 1,3-diphosphoglycerate to 3-phosphoglycerate and phosphorylates ADP to ATP (substrate level phosphorylation) during the sixth step, its expression was almost one and a half times higher. Enolase 1 (*ENO1*) catalyze the ninth step of glycolysis, its expression was one and a half higher in the mutTP53 group (**Figure 2C**). Pyruvate dehydrogenase kinase (*PDK1*) phosphorylates and inactivates pyruvate dehydrogenase complex (PDHc) thus decreases glycolytic flux to tricarboxylic acid cycle²³, its expression was more than one and a half times higher in the mutTP53 group. Pentose phosphate pathway (PPP) is a glycolysis parallel

metabolic pathway that generates materials for the reductive biosynthesis reactions. Three up-regulated genes participate in PPP including 6-phosphogluconate dehydrogenase (*PGD*), ribose 5-phosphate isomerase (*RPIA*), and transketolase (*TKT*) – each of these genes was up-regulated (**Figure 2E**).

Genes enclosed in the down-regulated groups included two out of three key enzymes of gluconeogenesis, glucose-6-phosphatase (G6P) and fructose-1,6-bisphosphatase (FBP1). Gluconeogenesis is a parallel pathway compared to glycolysis, its enzymes perform the reversible conversion of carbohydrate substrates to glucose²³. The *G6PC3* gene encodes the catalytic subunit of G6P. RNA expression of both of the two gluconeogenesis enzymes was almost half as much in the mutTP53 group as in the wtTP53 patient group (**Figure 2F**). The analysis showed other down-regulated genes, such as the *GLS2* and *GAMT* (**Figure 2D**). *GLS2* gene encodes a mitochondrial glutaminase that is an enzyme in glutaminolysis, and promotes mitochondrial respiration and increases ATP generation²⁴. RNA expression of *GLS2* was less than half in the mutTP53 group than in the wtTP53 patient group. The *GAMT* gene encoded guanidinoacetate N-methyltransferase that is part of the creatine synthesis, and may have a TP53-dependent regulator function of fatty acid oxidation²⁵.

In summary, mutant TP53 influenced genes in the glycolysis and other energy metabolic pathways at multiple levels. The expression changes for each of the involved genes are summarized in **Figure 3**.

Table 3. Energy metabolism genes with the highest AUC value identified in mutant TP53 breast cancer patients. Positive AUC values correspond to up regulation and negative AUC values to down regulation.

GENE	CODED PROTEIN	AUC	P-VALUE
GLYCOLYSIS ENZYMES			
<i>SLC2A5</i>	Solute carrier family 2, member 5 (<i>GLUT5</i>)	0.70	1.9e-17
<i>SLC2A6</i>	Solute carrier family 2, member6 (<i>GLUT6</i>)	0.73	4.1e-21
<i>HK3</i>	Hexokinase 3	0.73	6.8e-21
<i>GPI</i>	Glucose-6-phosphate isomerase	0.70	2.6e-16
<i>PFKP</i>	Phosphofructokinase P	0.70	1.6e-17
<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase	0.68	5.7e-14
<i>PGK1</i>	Phosphoglycerate kinase 1	0.72	1.1e-19
<i>ENO1</i>	Enolase 1	0.77	1.1e-27
GLUCONEOGENESIS ENZYMES			
<i>G6PC3</i>	Glucose-6-phosphatase catalytic 3	-0.70	-5.4e-17
<i>FBP1</i>	Fructose-1,6-biphosphatase 1	-0.70	-4.2e-17
REGULATORS OF ENERGY METABOLISMS			
GLYCOLYSIS REGULATORS			
<i>PDK1</i>	Pyruvate dehydrogenase kinase 1	0.70	4.3e-17
MITOCHONDRIAL REGULATORS			
<i>GLS2</i>	Mitochondrial glutaminase 2	-0.73	-7.0e-22
FATTY ACID OXIDATION PATHWAY REGULATORS			
<i>GAMT</i>	Guanidinoacetate N-methyltransferase	-0.71	-8.7e-19
PENTOSE PHOSPHATE PATHWAY ENZYMES			
<i>PGD</i>	6-phosphogluconate dehydrogenase	0.67	4.4e-13
<i>RPIA</i>	Ribose 5-phosphate isomerase A	0.69	2.9e-15
<i>TKT</i>	Transketolase	0.69	8.1e-16

Figure 2. Gene expression of glycolysis enzymes in mutant *TP53* cohort (mutTP53, n=215) and in wild type *TP53* cohort (wtTP53, n=547) breast cancer samples. White columns show the mutTP53 patient group gene expression level and grey columns show the wtTP53 patient group. Data are presented as mean \pm 95% confidence interval. (A) *SLC2A5*: Solute carrier family 2 (*GLUT5*); *SLC2A6*: Solute carrier family 2 (*GLUT6*); *HK3*: Hexokinase 3, (B) *PFKP*: Phosphofructokinase P, (C) *GPI*: Glucose-6-phosphate isomerase; *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase; *PGK1*: Phosphoglycerate kinase 1; *ENO1*: Enolase 1, (D) *GAMT*: guanidinoacetate N-methyltransferase, (E) *PGD*: 6-phosphogluconate dehydrogenase; *RPIA*: Ribose 5-phosphate isomerase; *TKT*: Transketolase, (F) *G6PC3*: Glucose-6-phosphatase catalytic 3, *FBP1*: Fructose-1,6-biphosphatase 1. All

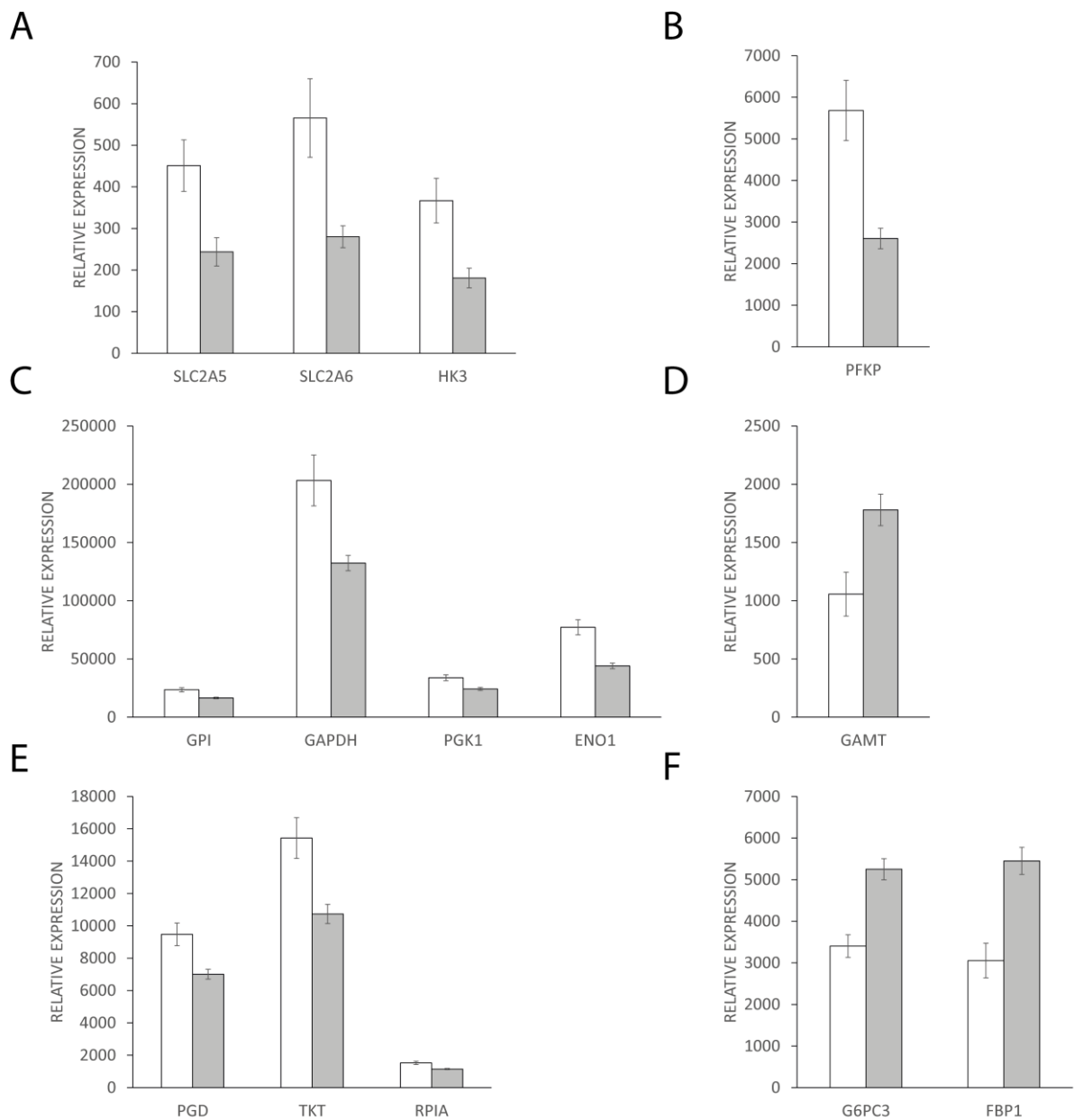
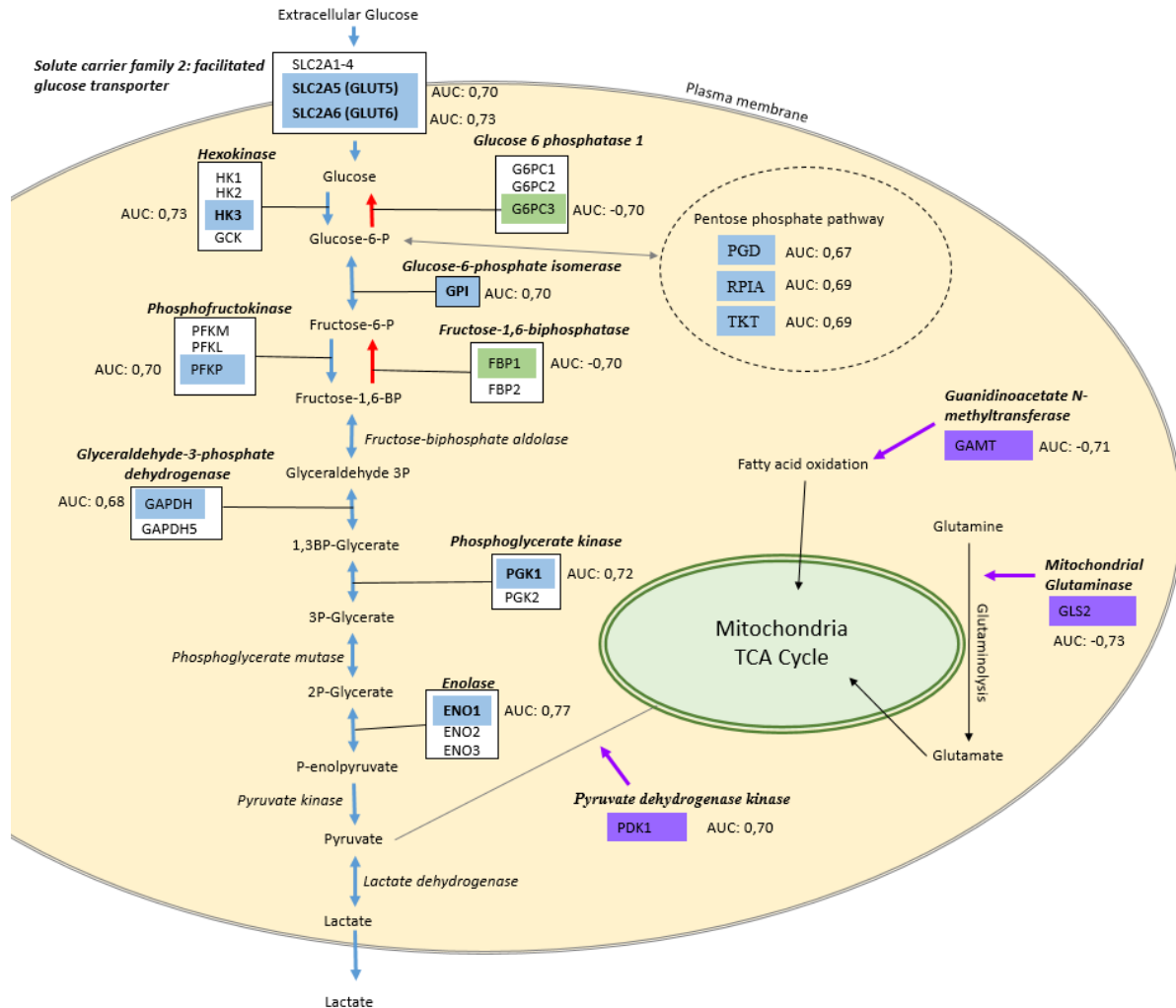


Figure 3. Summary of genes involved in the glycolytic processes affected by *TP53* mutation. Blue boxes indicate genes up regulated in *TP53* mutant samples, green boxes indicate down regulated genes, purple boxes indicates metabolic regulators affected by mut*TP53*. Blue arrows mark the glycolysis pathway, red arrows indicate the gluconeogenesis pathway and purple arrows indicate regulators of related metabolism pathways.



3.4. Cell culture setup

To investigate the effect of *TP53* mutation, we compared the metabolic activity between wt*TP53* and mut*TP53* breast tumor cell lines. First, *TP53* mutations were validated by sequencing the appropriate exon. Location of the mutations were from the *Catalogue of somatic mutations in cancer* (COSMIC) database. In case of JIMT-1 the amino acid exchange from arginine (R, CGG) to tryptophan (W, TGG) is located in the seventh exon at amino acid #248 (**Figure 1B**). In case of MDA-MB-231 the amino acid exchange from arginine (R, AGA) to lysine (K, AAA) is located in the eighth exon at amino acid #280. The *TP53* mutation in MDA-MB-231 cell line can explain the previously described resistance of the cell

line against antimetabolites²⁶. We have determined all 11 exons in MCF7 to validate the wild type sequence (data not shown).

3.5. Oxygen consumption in DMEM based medium

There were significant differences in absolute oxygen consumption among the investigated cell lines. In terms of oxygen consumption, MCF-7 cells exhibited the highest basal rate followed by MDA-MB-231 and JIMT-1 cells, in decreasing order. All the cell lines responded to oligomycin with a decrease of oxygen consumption rate (OCR) showing that oxidative phosphorylation had been active in all the cases (**Figure 4A**). If data were expressed as percentages of the basal oxygen consumption rate, then, in the presence of oligomycin, even in JIMT-1 cells, there was a 70% decrease of the oxidation rate (data not shown). Reserve respiratory capacity followed the same pattern as the baseline respiration; far the highest value was found for MCF-7 and the lowest for JIMT-1 (**Figure 4A**). Mitochondrial respiration was found responsible for at least 80% of the total oxygen consumption.

Administration of glucose to these cancer cell lines resulted in significant decrease in oxygen consumption in each case; the most pronounced effect was exhibited by JIMT-1 cells (32.9 ± 3.9 %) (**Figure 4B**). Addition of an uncoupler significantly augmented the rate of oxidation in both MDA-MB-231 and MCF-7 cells (without glucose) (**Figure 4A**). However, in JIMT-1 cells uncoupler dinitrophenol (DNP) stimulated respiration showed a higher rate relative to the one in the presence of glucose, although it was still beneath the baseline (**Figure 4B**).

3.6. Extracellular acidification in DMEM based medium

The basal rates of pH change showed remarkable parallelisms to basal rates of oxygen consumptions. The highest rate of acidification was measured in MCF-7 cells whereas the lowest rate was found in JIMT-1 cells (**Figure 4C**). In DMEM based medium the energy donor substrates are mainly those amino acids that are capable of being decomposed to TCA cycle intermediates. Consequently, acidification in this medium can principally be attributed to the CO₂ that is released during (oxidative) decarboxylation. Under these conditions oligomycin slows acidification down as it hampers mitochondrial oxidation involving the TCA cycle and reactions of oxidative decarboxylation.

When DNP alleviated the oligomycin mediated inhibition, TCA cycle could start working with maximal speed and pH changes could turn to be of the highest rates. Inhibition of mitochondrial metabolism drove the rate of acidification well below the baseline (**Figure**

4C). Glucose significantly enhanced the extracellular acidification rate (ECAR) in the DMEM based medium for all the cell lines (**Figure 4D**). The most pronounced elevation was observed in JIMT-1 cells where the rate of acidification was increased by a factor of twelve; in MCF-7 cells glucose stimulated acidification resulted only in a less than twofold increase. In the presence of glucose, MDA-MB-231 and MCF-7 cells responded to oligomycin with a significant further acidification, while in JIMT-1 cells the effect of oligomycin was not significant (**Figure 4D**).

3.7. Oxygen consumption in extracellular assay medium

Cells were kept in energy substrate-free (EC) medium for 2.5 hours before measurement. Interestingly this starvation period did not affect significantly the basal rate of respiration indicating that cells could fulfill their energy requirements by mobilizing their own energy stores; probably autophagic mechanisms have also been activated. The basal rate of O₂ consumption was thus the highest in MCF-7 cells, followed by MDA-MB-231 and JIMT-1 cells (**Figure 4E**) (similarly to that of experiments performed in DMEM-based media). All of the cell lines responded well to oligomycin; the magnitude of the decline of respiration was found to be approximately 60% indicating that mitochondria were well coupled (**Figure 4E**). Uncoupler mediated stimulation of respiration was of the smallest magnitude in JIMT-1 cells, even when oxygen consumption was normalized to the baseline.

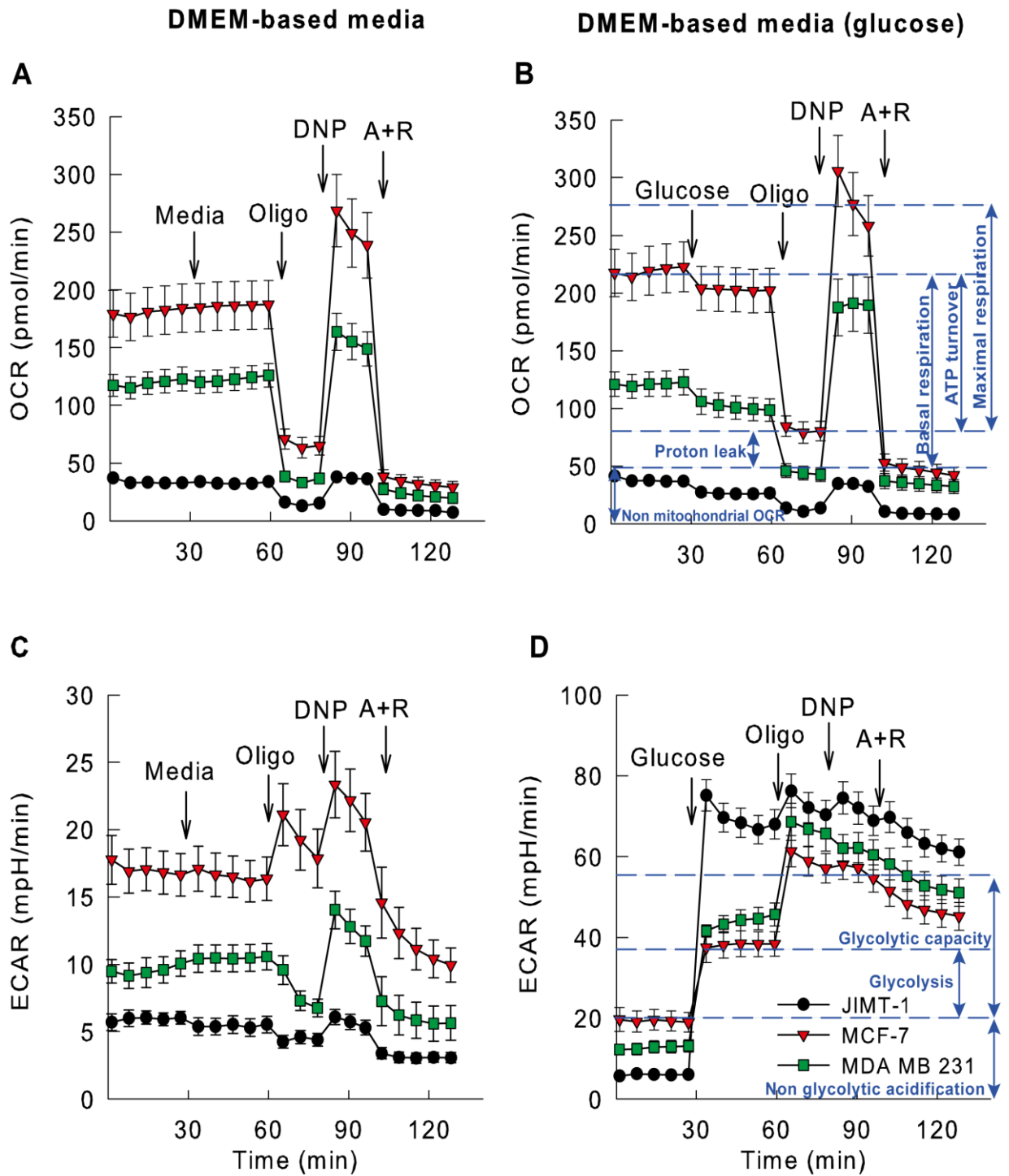
Glucose triggered a significant drop in O₂ consumption in all of the cell lines; the greatest effect was observed in JIMT-1 cells (48.5 ± 5.5 %) (**Figure 4E**). All of the cell lines responded to oligomycin in a similar fashion. The rate of reserve respiration measured in the presence of an uncoupler was the smallest in JIMT-1 cells; in the presence of glucose it has not even reached the basal respiratory rate.

Glutamine induced a small (<20%), but significant increase in O₂ consumption in JIMT-1 and MDA-MB-231 cells (**Figure 4F**). The uncoupler DNP enhanced the rate of respiration to higher than the baseline in all of the cell lines. Although, JIMT-1 cells exhibited the smallest elevation of the rate, but even their oxygen consumption was now more intense than the one represented by the baseline indicating that glutamine was indeed a good respiratory substrate in the absence of glucose for this mainly glycolytic cell line (**Figure 4F**).

3.8. Extracellular acidification in extracellular assay medium

In EC medium the smallest rate of acidification was measured for JIMT-1 cells. There was no significant difference between the acidification rates of MDA-MB-231 and MCF-7 cells. However, glucose augmented the acidification rate of JIMT-1 cells 15-fold (**Figure 4G**) indicating their extreme dependence on glycolysis. Similarly to data collected in DMEM based medium, to a lesser extent glycolysis also accelerated in MDA-MB-231 and MCF-7 cell lines upon addition of glucose and this acidification was further stimulated by oligomycin. The maximal acidification capacity was similar in the three cell lines investigated. Glutamine enhanced the rate of oxidation, but this elevation was not accompanied by an elevation of the rate of acidification (**Figure 4H**). This lack of acidification can potentially be explained by the release of ammonia due to glutaminase and glutamate dehydrogenase reactions.

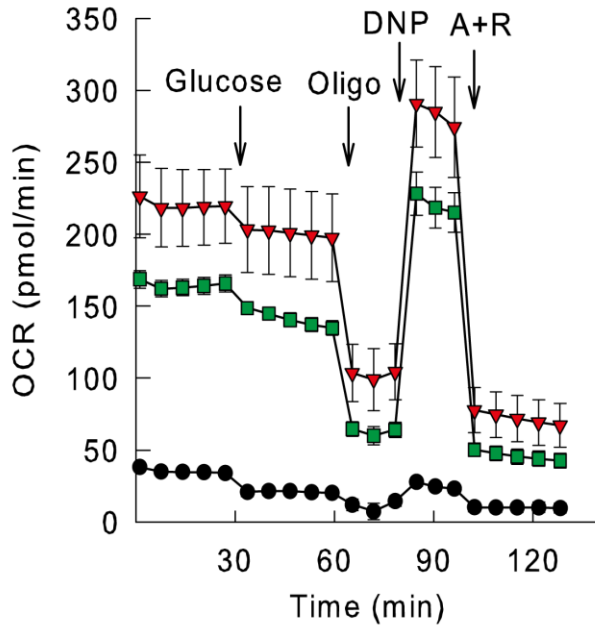
Figure 4. Higher glycolytic and mitochondrial activity observed in *TP53* mutant cell lines. Oxygen consumption (OCR) and extracellular acidification rates (ECAR) of JIMT-1, MCF-7 and MDA-MB-231 breast cancer cells (legends are displayed in panels F and I) measured in DMEM-based media (**A-D**) or Extracellular assay media (**E-H**). OCR and ECAR were determined using a microfluorimetric Seahorse XF96 Analyzer. Cells were measured i) in the absence (**A,C**) or presence (**B,D**) of D-glucose in DMEM-based media and ii) in the presence of D-glucose (**E,G**) or L-glutamine (**F,H**) as single substrates in Extracellular assay media. Bioenergetic and glycolytic functions were assessed upon addition of various metabolic inhibitors (e.g. oligomycin (“oligo”), an inhibitor of the mitochondrial FoF1 ATP-synthase or antimycin A + rotenone (“A+R”), inhibitors of the mitochondrial respiratory chain). The highest OCR was exhibited with the uncoupler 2,4-dinitrophenol (DNP) given. D-glucose (10 mM), L-glutamine (2.5 mM), oligomycin (2 μ M), 2,4-dinitrophenol (100 μ M), antimycin A + rotenone (1-1 μ M; all in final concentrations) were given as displayed on plots. Results are averages of measurements performed on three consecutive preparations of each individual cell culture (N=12-24, ~20,000 cells/well); individual data points are represented as mean \pm S.E.M. The blue dashed lines in **B** and **D** serve as an interpretation guideline – they represent a schematic average value of multiple time point measurements and the double arrow indicate the key oxygen consumption (**B**) and extracellular acidification (**D**).



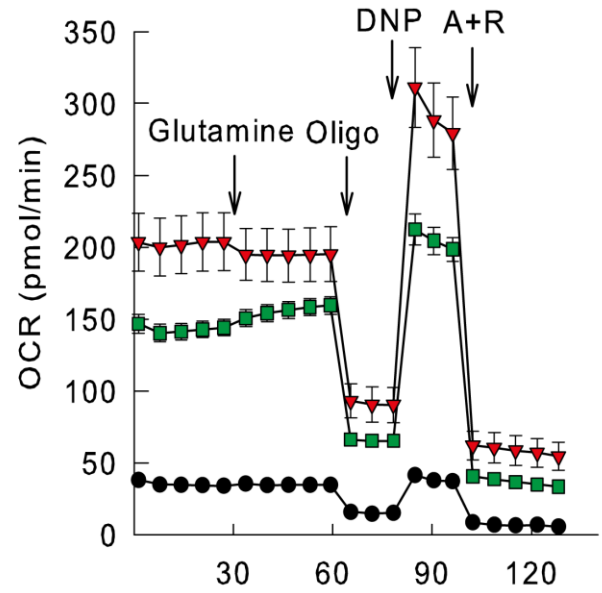
Extracellular assay media (glucose)

Extracellular assay media (glutamine)

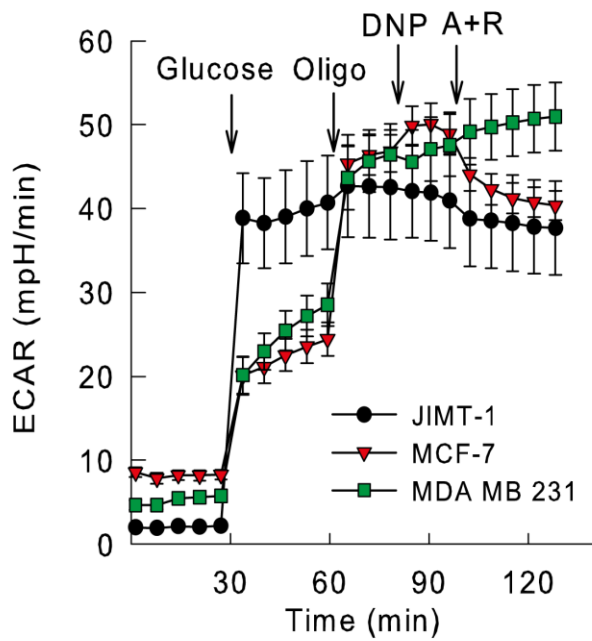
E



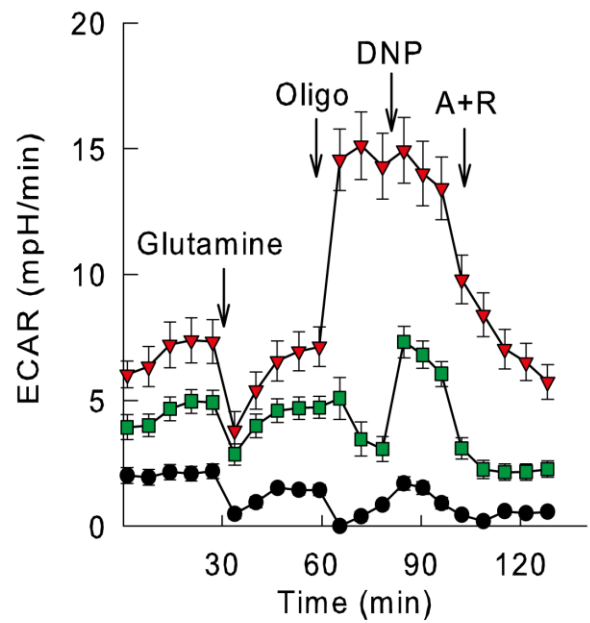
F



G



H



4. DISCUSSION

Cancer cell lines show many characteristic tumor-specific alterations in glycolysis. Cancer cells may consume more than 10 times more glucose than their normal counterparts²⁷. High rate of glucose uptake can be detected and used as a diagnosis of cancer. Accelerated glucose metabolism in cancer is associated with increased expression of glucose transporters, especially GLUT1. TP53 can repress at the transcriptional level GLUT1-4 gene expression that results in decreased glucose uptake²⁸. In our study, multiple genes in metabolic pathways were significantly linked to *TP53* mutation including Hexokinase 3, Phosphofructokinase, Platelet (PFKP) and Pyruvate dehydrogenase kinase. Under stress condition, TP53 can gear up glucose uptake by increasing hexokinase transcription²⁹. Glucose molecules have to be phosphorylated intracellularly by hexokinases (HK). HK II is upregulated in many cancer cell types³⁰. Phosphofructokinase 1 (PFK1) is the rate limiting step of glycolysis. In tumor cells a post-translationally modified form is expressed frequently³¹. One the major mechanisms responsible for the Warburg phenomenon is that HIF-1 α and TP53 have effect on the regulation of pyruvate dehydrogenase complex³².

TP53 can promote fatty acid oxidation when available glucose levels are low to generate ATP in the tricarboxylic acid (TCA) cycle using alternative energy sources. The TP53-dependent regulation of fatty acid oxidation pathway occurs by activation of GAMT³³. Gluconeogenesis, a reverse glycolytic pathway, generates glucose from small noncarbohydrate precursors. Zhang et al.³⁴ showed roles for TP53 in the down-regulation of gluconeogenesis both *in vitro* in colon and liver cancer cells and *in vivo*, by down regulation the gene expression of *PCK* and *G6PC*, the rate limiting enzymes of gluconeogenesis. Our analysis showed that the gene expression alterations between mutant and wild type *TP53* patients showed higher expression of glucose transporters that contributes to the higher uptake of glucose, and higher expression of key glycolysis enzymes and pentose phosphate pathway (PPP) enzymes.

Song and colleagues³⁵ published a proteomic comparison between normal and breast cancer tissues. Differentially expressed proteins in tumor tissues were related to glycolysis/gluconeogenesis among other pathways. Overexpressed proteins included GAPDH, ENO1, lactate dehydrogenase (LDH), pyruvate kinase (PKM2) and aldolase A (ALDOA). The fact that these proteins were up-regulated in the invasive carcinoma group supports the

general concept that rapid growing tumor cells show higher rate of glucose metabolism than normal cells³⁵. At the same time mutant *TP53* negatively affected the expression of gluconeogenesis, the glucose synthetic pathway, and the fatty acid oxidation pathway that contributes to the degradation of fatty acids. Recent studies suggest that some of the glucose metabolism enzymes – including GPI and PGK, genes identified in our analysis – are multi-functional proteins. The encoded protein of GPI is also referred to as autocrine motility factor based on an additional function as a tumor-secreted cytokine and angiogenic factor³⁶. The glycolysis enzyme PGK participates in angiogenesis by functioning to reduce disulfide bonds in the serine protease, plasmin by tumor cells³⁷.

Li and colleagues³⁸ exposed that MDAMB-231 and another mutTP53 cell line (T47D) consumed significantly more glucose and secreted more lactate than the non-tumorigenic and wtTP53 cell line, MCF10A. In the two mutTP53 cell lines (MDA-MB-231 and T47D) 80-94% of the glucose consumed was used to make lactate, while in the MCF10A only the 50% of glucose consumed was used to make lactate. In our study we show that wtTP53 MCF-7 cells display the lowest glycolytic activity in the presence of glucose in amino acid rich media. In DMEM based medium, MCF-7 cells were the most oxidative ones among the investigated cells. JIMT-1 cells exhibited the highest rate of glycolysis and the most pronounced inhibition of oxidative metabolism in the presence of glucose. MDA-MB-231 cells showed a moderate glycolytic activity and mitochondrial activity among these cell lines. None of the cell lines investigated here presented with a high glutamine dependence of oxidation.

During the past years significant efforts have been dedicated to the identification of mediators that selectively eliminate cancer cells based on their metabolic alterations, some of them now close to clinical evaluation³⁹. Multiple attempts have been executed to inhibit the rate limiting enzymes of glycolysis, first the unselective inhibitor of hexokinase 2-deoxy-p-glucose (2-DG) was added to patients with glioma⁴⁰. Promising preclinical results showed with other HK inhibitors, 3-bromopyruvate (3-BP)⁴¹ and methyl jasmonate⁴² with anticancer effects in mouse models. However, clinical development of 2-DP and 3-BP hexokinase inhibitors has been discontinued – most probably the similarities between malignant cell and normal cell (manly highly proliferative cells) metabolisms will make it difficult to select the appropriate patient cohort eligible for such treatments⁴³.

Lonidamine, a derivative of indazole-3-carboxylic acid delivered impressive results to inhibit aerobic glycolysis in cancer cells by inhibit HK in clinical trials⁴⁴. Preclinical data

showed that inhibition of glucose transporters (GLUT1 inhibition with WZB117 or with silybin) also have antineoplastic effects ^{45, 46}. Further studies have shown that koniginic acid, a potent and selective inhibitor of GAPDH, kills a broad range of highly glycolytic cell lines ⁴⁷. Imatinib treatment decreased the activity of both HK and GAPDH in leukemia and myeloid tumors ⁴⁸. Pyruvate dehydrogenase kinase (PDK) is often overexpressed in malignant cells, the dichloroacetate (DCA) that originally used in lactic acidosis therapy inhibits PDK, and results mitochondrial defects in cancer cells used by patients with glioblastoma ⁴⁹. Oxythiamine is a thiamine antagonist, inhibits both PDK and transketolase (TKT), and responsible for significant anticancer activity ⁵⁰.

In summary, our analysis extends previous results and we show that *TP53* mutation status simultaneously affect multiple metabolic pathways. Based on our findings one can anticipate a more significant effect of agents influencing energy metabolism in patients harboring *TP53* mutation.

ACKNOWLEDGEMENTS

The study was supported by the OTKA K108655 grant and the Medinprot program.

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