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Metabolismo energético e sensibilidade a drogas antimetabólicas em linhagens de câncer de pulmão de não-pequenas células

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ÍNDICE

RESUMO 5

1. INTRODUÇÃO 7

1.1 Câncer de pulmão 7

1.2 Reprogramação metabólica 8

1.2.1 Efeito Warburg 8

1.2.2 Outras vias alteradas 9

1.3 Antimetabólitos 11

2 ARTIGO CIENTÍFICO 14

3 CONCLUSÃO E PERSPECTIVAS 38

REFERÊNCIAS 39

Regras de Formatação da Revista Molecular Oncology 42

RESUMO

O câncer de pulmão é a neoplasia maligna mais insidiosa da oncologia, sendo responsável pelo maior número de mortes relacionadas ao câncer no mundo. Oitenta e cinco por cento dos casos de câncer de pulmão são de não-pequenas células (CPNPC), e sua terapêutica consiste em ressecção cirúrgica para pacientes em estágios iniciais e quimioterapia/radioterapia para pacientes em estágios avançados. Apesar dos progressos nas pesquisas em câncer, o prognóstico de pacientes em estágios avançados permanece ruim, portanto faz-se necessária o desenvolvimento de novas abordagens terapêuticas. Sabe-se que tumores sólidos apresentam metabolismo energético aberrante, com alto consumo de glicose e liberação de lactato mesmo na presença de oxigênio, o chamado Efeito Warburg. Além disso, esse processo envolve diversas rotas metabólicas, e é conhecido como reprogramação do metabolismo. A superexpressão de diferentes isoformas de enzimas-chave é o principal mecanismo regulatório, e esse fenômeno, por ser único em tumores, parece ser um alvo mais estável e seletivo para terapia. Dessa forma, o objetivo desse trabalho é estabelecer o perfil metabólico das linhagens de CPNPC, A549 e H460, assim como de uma linhagem de epitélio pulmonar BEAS-2B, relacionando com a sensibilidade a antimetabólitos e sua expressão gênica. Foi observado que ambas as linhagens tumorais apresentam alta liberação de lactato e acidificação do meio, em especial a H460. Também foi analisado o perfil de respiração dessas linhagens, onde ambas apresentaram uma alta reserva respiratória quando comparadas com a BEAS-2B, principalmente a A549. Ainda, as células tumorais foram tratadas com 4 antimetabólitos, 2-deoxiglicose, 3-bromopiruvato, floretina e alfa-ciano-4-hidroxicinamato, e se observou uma seletividade dessas drogas para a linhagem H460. A análise da expressão gênica demonstrou um enriquecimento das vias glicolíticas nas linhagens tumorais quando comparadas com a BEAS-2B, assim como um enriquecimento de isoformas Warburg. Por fim, foi validada a relevância clínica desses resultados com a observação do enriquecimento

de isoformas Warburg em biópsias de CPNPC em comparação com biópsias de tecidos sadios e em biópsias de adenocarcinoma de pacientes mortos em comparação com biópsias de adenocarcinoma de pacientes vivos. Nossos dados demonstram a relevância clínica das isoformas Warburg e essas análises podem, portanto, estabelecer novos alvos moleculares para futuros estudos na terapia de CPNPC.

1. INTRODUÇÃO

1.1 Câncer de pulmão

O câncer é um sério problema de saúde pública da atualidade. Devido ao crescente envelhecimento da população, apesar dos notáveis avanços das pesquisa e do progresso em relação a sua incidência, hoje estima-se que uma em cada três mulheres e um em cada dois homens nos Estados Unidos irão desenvolver a doença durante suas vidas (Siegel et al., 2012). O câncer de pulmão é o tipo mais comum, de forma a apresentar altos números de casos estimados e ser a causa mais frequente de morte por câncer, representando 18,2% das mortes totais. Ainda, 55% dos casos de câncer de pulmão ocorrem em países em desenvolvimento (Ferlay et al., 2010), visto que a diferença de incidência entre países apresenta uma relação direta com a proporção de fumantes entre a população. O tabagismo é a causa de 80% dos casos de câncer de pulmão em homens e de pelo menos 50% dos casos em mulheres (Jemal et al., 2011). Aproximadamente 85% dos cânceres de pulmão são de não-pequenas células (CPNPC) e apesar dos avanços nos tratamentos quimioterápicos, o prognóstico permanece ruim, já que a maioria dos casos é diagnosticado em estágios avançados, em vista dos estágios iniciais serem assintomáticos (Siegel et al., 2012). O tratamento utilizado em pacientes de CPNPC é baseado no estadiamento clínico da doença, de forma que 71% dos pacientes em estágio inicial sofrem rescisão cirúrgica, enquanto que pacientes em estágio avançado utilizam quimioterapia, radioterapia ou uma combinação destes dois tratamentos (Siegel et al., 2012). Entretanto, a quimioterapia consiste em drogas que agem sistemicamente, de forma pouco seletiva e, assim, agem tanto no tumor como nas células sadias. Dessa forma, as drogas devem ser administradas em doses subótimas, de modo a tentar minimizar os efeitos colaterais nos pacientes (López-Lázaro, 2010).

Desde o começo do século XX, o câncer é visto como uma doença genética, sendo resultado de alterações cromossômicas. Somente nos anos 1980 foram descritos os primeiros oncogenes e genes supressores de tumor, e a partir disso surgiu a hipótese de que mutações nesses genes poderiam ser a causa do câncer (López-Lázaro, 2010). Baseado nisso, por muito tempo, o foco de muitos estudos foi a terapia gênica como tratamento para o câncer. Entretanto, as alterações genéticas envolvidas nesse processo são muito numerosas e instáveis, além de abrangerem não só mutações gênicas, mas também alterações epigenéticas (Folkman et al., 2000). Dessa forma, faz-se necessária uma nova abordagem no tratamento do câncer, tendo em vista um alvo mais estável e mais confiável.

1.2 Reprogramação metabólica

1.2.1 Efeito Warburg

Em tecidos diferenciados, na presença de oxigênio, a maioria das células metaboliza glicose formando piruvato, o qual é transportado para mitocôndria e oxidado, de forma a gerar dióxido de carbono e, ao alimentar a cadeia respiratória, maximizar a produção de ATP. Já na ausência de oxigênio, o piruvato proveniente da glicose é convertido em lactato (Vander Heiden et al., 2009). Esse é o chamado Efeito Pasteur, no qual Louis Pasteur observou, em 1861, que as leveduras utilizavam glicose muito mais rapidamente na ausência de oxigênio (Racker, 1974). Entretanto, em 1926, Otto Warburg observou que células cancerígenas apresentavam metabolismo energético aberrante, diferente do que observado por Pasteur em tecidos sadios. Segundo Warburg, células tumorais tendem a converter glicose em lactato mesmo na presença de oxigênio, fenômeno o qual chamou de “glicólise aeróbica” (Warburg, 1956).

Embora Otto Warburg estivesse errado em sua hipótese de que o motivo pelo qual tumores utilizam glicólise aeróbica ser devido a células cancerígenas apresentarem disfunção

mitocondrial, seus achados são de extrema importância, e hoje apresentam alta relevância clínica. Uma importante aplicação prática dos estudos de Warburg é o uso da tomografia por emissão de pósitrons (PET) utilizando o análogo de glicose 2-deoxi-2-(^{18}F)flúor-D-glicose (FdG). Essa técnica permite a detecção de cânceres primários e metastáticos devido ao aumento de captação de glicose em células tumorais (Gatenby and Gillies, 2004). Além disso, o uso de FdG/PET permite a quantificação da captação de glicose, e foi observada uma relação de pior prognóstico e maior agressividade tumoral com o aumento da captação de glicose (Mochiki et al., 2004). Esse resultado também foi observado em linhagens celulares de câncer de mama (Gatenby and Gillies, 2004).

Por muito tempo, este fenômeno não foi compreendido, visto que não se via uma verdadeira vantagem em produzir apenas 4 mol de ATP por mol de glicose pela glicólise aeróbica, enquanto que a fosforilação oxidativa gera aproximadamente 36 mol de ATP por mol de glicose. Entretanto, células tumorais adquirem uma vantagem evolutiva ao reprogramar seu metabolismo para responder ao estresse ambiental. Ainda, esse processo permite um aumento na proliferação celular, visto que tumores necessitam do esqueleto de carbono proveniente da glicose e outros intermediários para suprir sua alta taxa biossintética (Gatenby and Gillies, 2004; López-Lázaro, 2010; Tennant et al., 2010).

1.2.2 Outras vias alteradas

O destino da glicose dentro da célula pode variar em células tumorais em comparação ao tecido normal, assim como pode variar em diferentes estágios do câncer (Herling et al., 2011), de forma que a reprogramação metabólica envolve não só a glicólise, mas diversas outras vias. Esse processo é regulado por alterações de enzimas-chave de cada via, principalmente por substituições por diferentes isoformas (Tennant et al., 2010).

Na glicólise, dentre as isoformas importantes superexpressas em câncer, destacam-se os transportadores de glicose GLUT1 (Kunkel et al., 2003) e GLUT3. As evidências da importância da regulação desses transportadores corroboram com o fato de que essas isoformas são expressas fisiologicamente em tecidos que necessitam mais de glicose, como o cérebro (Scheepers et al., 2004). Além disso, já está bem descrita a superexpressão da enzima hexoquinase 2 (HK-2) em tecidos cancerígenos (Mathupala et al., 2010), assim como a preferencia desses tecidos pelas isoformas M2 da piruvato quinase (PKM-2), frutose-2,6-bifosfatase 3 (PFKFB-3), aldolase-C (ALDC) e fosfoglicerato cinase-1 (PGK-1) (Herling et al., 2011)

A via das pentoses é uma rota importante na reprogramação metabólica, visto que ocorre produção de NADPH, necessário na síntese de lipídeos e nucleotídeos, assim como no balanço redox intracelular; e ribose-5-fosfato (R5P), importante na síntese de nucleotídeos. Dessa forma, a enzima glicose-6-fosfato desidrogenase tem um papel significativo por desviar a glicose-6-fosfato da glicólise para a via das pentoses. Ainda, foi observado que a isoforma transcetolase-like 1 (TKTL-1) está superexpressa em diversos tumores (Langbein et al., 2006), assim como a isoforma da enzima transaldolase-1 (TALDO-1) (Herling et al., 2011).

Desde 1951, sabe-se que o metabolismo de lipídeos está aumentado em tumores (Tennant et al., 2010). Na síntese de ácidos graxos, o citrato proveniente do ciclo de Krebs, ao ser exportado da mitocôndria, é convertido à acetil-CoA pela enzima ATP citrato liase (ACLY). O acetil-CoA é então convertido a malonil-CoA pela acetil-CoA carboxilase (ACC), e este é o substrato para a enzima-chave ácido graxo sintase (FASN). Esta enzima apresenta-se de forma importante na reprogramação metabólica, visto que já foi observado aumento de sua expressão em câncer colorretal (Rashid et al., 1997).

Igualmente importante é o metabolismo do piruvato, visto que seu destino determina a utilização pela mitocôndria ou a produção de lactato. Pela ação da pirtuvato desidrogenase

(PDH), ocorre sua conversão em acetil-CoA. Este processo é controlado pela enzima piruvato desidrogenase quinase 1 (PDK1), a qual bloqueia a atividade da PDH. Além disso, outra enzima essencial nesse processo é a lactato desidrogenase A (LDHA), a qual converte piruvato em lactato (Koukourakis et al., 2005). Ainda, com a alta produção de lactato, para manter o fluxo glicolítico e o pH fisiológico intracelular, células tumorais apresentam superexpressão de reguladores de pH, como os transportadores de monocarboxilatos (MCT), em especial a isoforma MCT-4 (Izumi et al., 2003).

O ciclo de Krebs em células cancerígenas pode favorecer a carboxilação redutiva em detrimento do metabolismo oxidativo. Isso ocorre pois, com a entrada de glutamina, esta pode entrar no ciclo de Krebs como α-cetoglutarato e sofrer carboxilação redutiva, formando isocitrato pela ação da enzima isocitrato desidrogenase (IDH), que atuará no sentido reverso do ciclo de Krebs (Yoo et al., 2008). Isto pode ser uma forma de produção de citrato e ácidos graxos através da glutamina, favorecendo a biossíntese (Ward and Thompson, 2013). Entretanto, não se sabe se esse processo ocorre através da isoforma citosólica IDH1 ou mitocondrial IDH2. Ainda, foram observadas formas alteradas dessas enzimas capazes de reduzir α-cetoglutarato a 2-hidroxiglutarato (2-HG), cuja expressão está relacionada com a carcinogênese (Ward and Thompson, 2013).

Como o metabolismo energético aberrante é uma característica única dos tumores, o foco em vias metabólicas alteradas em células cancerígenas e, ainda mais importante, nas alterações enzimáticas que ocorrem durante a reprogramação metabólica, apresentam-se como novas e potenciais estratégias terapêuticas (López-Lázaro, 2010).

1.3 Antimetabólitos

Por dependerem da via da glicólise para gerar ATP, células tumorais necessitam da manutenção dessa via para sobreviver e proliferar. Dessa forma, a inibição da glicólise vem

sendo estudada como um alvo terapêutico (Figura 1). A 2-desoxiglicose (2-DG), um análogo de glicose, é um composto que age como inibidor competitivo na via glicolítica. Ao ser absorvida, a 2-DG é fosforilada pela hexocinase e, ao contrário da glicose-6-fosfato, não pode mais ser metabolizada, acumulando dentro da célula e inibindo a glicólise (Pelicano et al., 2006). Este composto não apresentou efeito significante no crescimento tumoral como monoterapia, mas pode sensibilizar os tumores a terapias convencionais por reduzir os níveis de ATP (Tennant et al., 2010). O 3-bromopiruvato (3BP) é um outro inibidor da hexocinase, sendo muito estudado como um possível tratamento anticâncer. Entretanto, o composto também inibe a hexocinase mitocondrial, bloqueando a respiração pela mitocôndria (Y H Ko et al., 2001). Ainda, a captação de glicose é um possível alvo terapêutico por inibição do GLUT através do uso da floretina, a qual já foi demonstrado ser capaz de induzir apoptose *in vivo* e *in vitro* em hepatomas humano (C.-H. Wu et al., 2009). Por fim, com a superexpressão dos transportadores de monocarboxilatos e a dependência da secreção de lactato apresentados em células tumorais, um possível composto terapêutico é o alfa-ciano-4-hidroxicinamato (CHC), inibidor de MCTs 1, 2 e 4 (Kumar et al., 2013), que já foi demonstrado ser capaz de diminuir o tamanho de tumores (Sonveaux et al., 2008b).

Dessa forma, faz-se necessário o estabelecimento da relação entre a expressão de determinadas isoformas com a sensibilidade a antimetabólitos. O objetivo desse trabalho é, portanto, estabelecer um perfil metabólico de diferentes tipos de câncer através de um painel de dados bioquímicos e de expressão gênica, buscando uma assinatura metabólica que justifique a sensibilidade de cada linhagem celular às drogas testadas. Ainda, a partir de dados de expressão gênica, é possível sugerir alvos específicos superexpressos durante a carcinogênese, podendo ser associados a uma nova estratégia terapêutica mais seletiva e eficaz.

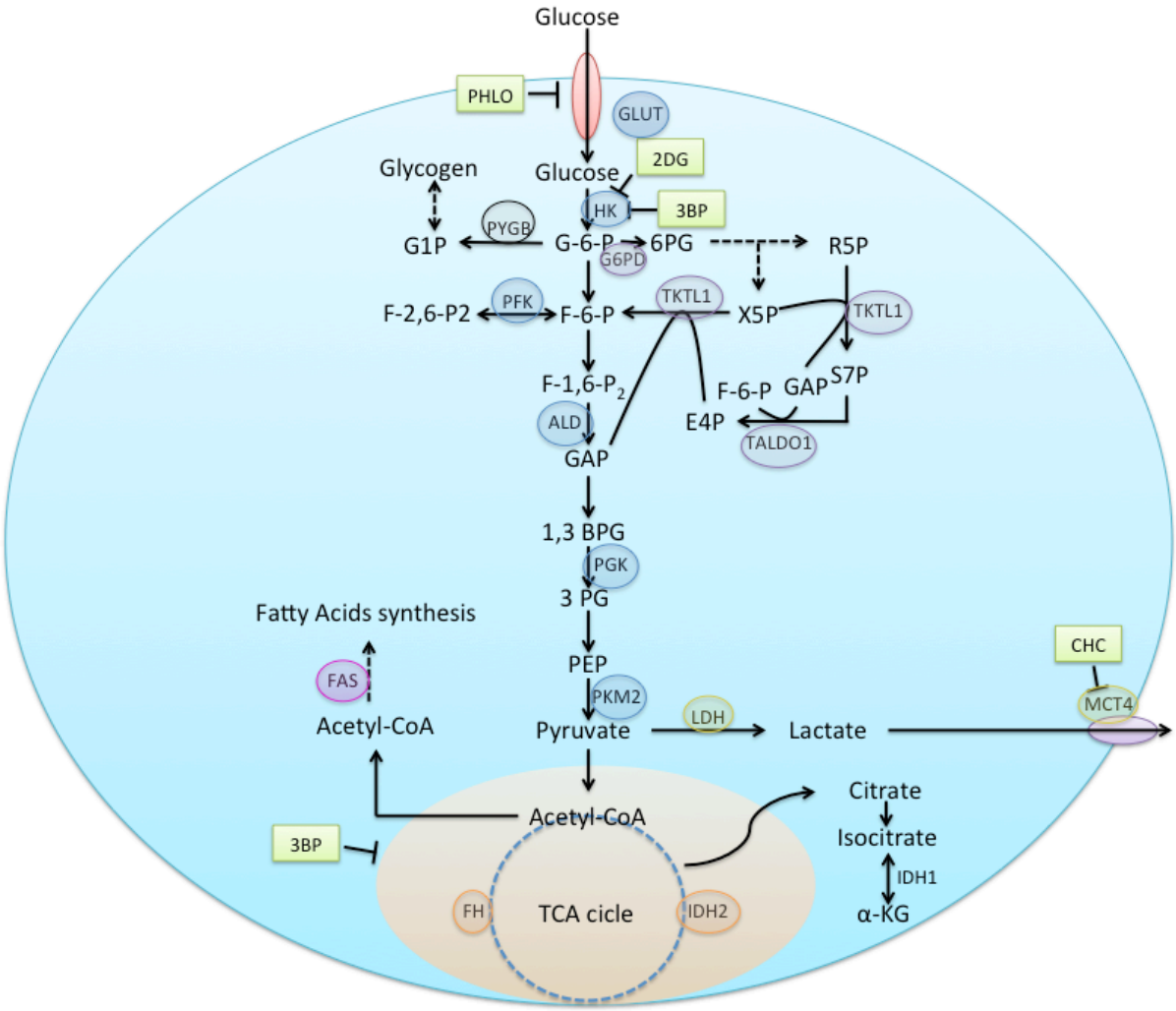

Figura 1: Rotas bioenergéticas envolvidas na reprogramação metabólica. Enzimas-chave estão representadas em círculos de diferentes cores, onde: azul = glicólise; preto = metabolismo do glicogênio; roxo = via das pentoses; amarelo = metabolismo do piruvato; rosa = síntese de ácidos graxos; laranja = ciclo de Krebs. Quadrados verdes representam anti-metabólitos em testes clínicos. Abreviações: Phlo = floretina; 2-DG = 2-desoxiglicose; 3BP = 3-bromopiruvato; CHC = alfa-ciano-4-hidroxicinamato; GLUT = transportador de glicose; HK = hexocinase; PFK = fosfofrutocinase; ALD = aldolase; PGK = fosfoglicerato cinase; PKM2 = piruvato cinase-M2; PYGB = glicogênio fosforilase; G6PD = glicose-6-fosfato desidrogenase; TKTL1 = transcetolase-like 1; TALDO1 = Transaldolase-1; LDH = lactato desidrogenase; MCT4 = transportador de monocarboxilatos 4; FH = fumarato hidratase; IDH = isocitrato desidrogenase; FAS = ácido graxo sintetase.

2 ARTIGO CIENTÍFICO

Energetic metabolism and sensibility to anti-metabolites in non-small cell lung cancer cell lines

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Abstract

Lung cancer is the most common and most lethal type of tumor, and 80% of those are non-small cell lung cancer (NSCLC). Despite the progress on cancer research, the prognosis remains poor for patients with advanced-stage of disease, whose treatment is mostly based on chemotherapy. Current studies and proposed therapies rely on the identification of specific genetic alterations in tumor specimens, the so-called pharmacogenetic approach. In addition, solid tumors present an aberrant metabolism, with high glucose uptake and lactate production even in the presence of oxygen, which is called Warburg Effect. This phenomenon is

controlled by altered enzymes expression, mostly by replacement of different isoforms. Therefore, the gene expression analysis of NSCLC cell lines can reveal new therapeutic targets.

This study aims to associate the gene expression pattern related to energetic metabolic pathways on two NSCLC cell lines, A549 and H460, as well as a non-tumoral cell line, BEAS-2B, with their metabolic profile and sensibility to antimetabolites. In order to establish the metabolic profile of the cell lines, we evaluated the medium acidification, lactate production and respiratory rate (basal, oligomycin-independent and maximum respiration rate). The cancer cell lines were also analyzed by their sensibility to antimetabolites such as 2-deoxyglucose, 3-bromopyruvate, phloretin and α-cyano-4-hydroxycinnamate. In addition, by a bioinformatic approach, we evaluated the expression of glycolytic and oxidative pathways on cell line microarray, as well as the expression of Warburg-related enzymes isoforms. These analyses were also applied to tumor tissue microarray. We observed that the tumoral cell lines present a more glycolytic profile when compared to non-tumoral, and also observed that H460 seems to have a more glycolytic phenotype and A549 a more oxidative phenotype. We also confirmed the clinicopathological significance of these data, since it was observed that tumor tissues upregulate Warburg isoforms, reflecting a poor patient outcome.

Keywords: Non-small cell lung cancer; Antimetabolites; Warburg Effect; Gene Enrichment Analysis

Highlights

A549 and H460 present a glycolytic metabolism when compared to BEAS-2B.

H460 is more glycolytic than A549, and shows specific sensitivity to antimetabolites.

Glycolytic pathways and Warburg isoforms are enriched in cancer cell lines when compared to BEAS-2B.

Warburg isoforms are enriched in tumor tissues when compared to normal lung tissue.

Enrichment in Warburg isoforms is also related to poor-prognosis adenocarcinoma.

1. Introduction

Lung cancer is the most common malignant tumor, representing 12.7% of all new cases, and responsible for 18.2% of total deaths (Ferlay et al., 2010). Cigarette smoking is the cause of 80% of worldwide lung cancer in men, and at least 50% in women, and 55% of the disease occur in developing countries (Jemal et al., 2011). Approximately 85% of all lung cancers are non-small cell lung cancer (NSCLC) (Siegel et al., 2012) that can be divided according to the histological type in squamous cell carcinoma, adenocarcinoma, and large-cell carcinoma. For these patients, treatment is determined by disease stage, and surgery remains the main treatment for early-stage. For advanced-stage patients, chemotherapy forms the foundation of their treatment and is critical in determining their survival and quality of life (Ramalingam and Belani, 2008). However, most of the drugs used on chemotherapy is toxic to both tumor and normal cell, and to minimize side effects, they must be administrated in suboptimal levels (López-Lázaro, 2010). This reflects on the poor prognosis for those with distant stage disease, where the 5 years survival rate is 3.6% (Siegel et al., 2012).

Since 1980, when oncogenes and the tumor suppressors genes were discovered, the main focus of several studies and clinical trials has been the cancer treatment by reversing mutations on those genes. Nevertheless, recent analysis carried out in the last decade have shown that those genetic alterations are too numerous and too unstable (López-Lázaro, 2010), and cancer alterations include not only mutations, but epigenetics changes as well (Folkman et al., 2000).

In 1926, Otto Warburg demonstrated that tumor cells have an aberrant energetic metabolism, with high glucose consumption and lactate production, even in the presence of oxygen, and hence this phenomena is referred to as "aerobic glycolysis" (Warburg, 1956). This process can be understood as a selective growth advantage, considering that, in order to proliferate, cells need the carbon building blocks provided by glucose to increase their biosynthetic capacity (Gatenby and Gillies, 2004). As others alterations in cancer cells metabolism have been discovered, the whole process is known to be a reprogramming of metabolism, where tumor cells go through a metabolic shift, enhancing glycolysis to produce energy and others anabolic pathways to synthesize proteins and lipids independently of oxygen availability (Ward and Thompson, 2013). This process is regulated by alterations in key enzymes of several pathways, mainly by overexpression of specific enzyme's isoform (Herling et al., 2011).

Considering that the aberrant energetic metabolism is a unique feature of tumor cells, the focus on altered metabolic pathways and its regulation during the reprogramming of metabolism seems to be a good therapeutic approach. There are currently several drugs under development and trials targeting those alterations in tumors. One of them, 2-deoxy-D-glucose (2-DG), acts as a glycolysis inhibitor. Once inside the cell, the compound is phosphorylated by hexokinase to 2-DG-P, which cannot be further metabolized by phosphohexose isomerase, and then accumulates in the cell, inhibiting glycolysis (Pelicano et al., 2006). Another glycolysis inhibitor, 3-bromopyruvate (3-BP), inhibits hexokinase, but also inhibits its mitochondrial isoform, causing ATP depletion both by glycolytic metabolism inhibition and mitochondrial respiration inhibition (Y H Ko et al., 2001) (Pereira da Silva et al., 2009). The glucose uptake can also be a potential target, and the anti-metabolite phloretin, a glucose transporter (GLUT) inhibitor, have been shown to induce apoptosis *in vivo* and *in vitro* in human liver cancer cells (C.-H. Wu et al., 2009).

Finally, as several pH regulators are up regulated in tumor cells, such as the monocarboxylate transporters (MCTs), a possible therapeutic compound is α-cyano-4-hydroxycinnamate (CHC), that is known to inhibit MCTs 1, 2 and 4 (Kumar et al., 2013) and has been shown to decrease tumor sizes (Sonveaux et al., 2008a).

2. Materials and Methods

a. Cell culture

The human NSCLC cell lines of adenocarcinoma A549 and large-cell carcinoma H460 (NCI-Frederick Cancer DCTD tumor/cell line repository), as well as the lung epithelium immortalized cell line BEAS-2B (ATCC®) were cultured in RPMI-1640 medium containing 2 mM of L-glutamine, 10% of fetal bovine serum (FBS) and antibiotics, buffered at pH 7.4. Cells were maintained in exponential growth in a humidified incubator (5% CO_2, 37°C).

b. Cytotoxicity assay

Cells were seeded in a 96-well plate and, 24 h later, cells were treated with 2-DG, 3-BP, Phloretin, CHC and Tris-Buffer (Sigma- Aldrich) at different concentrations. At the end of incubation time (72 h), the percentage of proliferation was evaluated by the sulforhodamine B assay (SRB) as described previously (Skehan et al., 1990). Briefly, cells were fixed with trichloroacetic acid 10% and incubated at 4°C for 1 h. The plates were washed five times with distilled water and air-dried. Cell growth was measured by staining with sulforhodamine B dye (0.2% w/v in 1% acetic acid). The unbound dye was washed 3–5 times with 1% acetic acid and plates were air-dried. The adsorbed dye was dissolved in Tris-Buffer (0.01 M, pH 10.4) and plates were gently shaken for 20 min on a mechanical shaker. The optical

density was recorded using a 96 well plate reader at 490 nm.

c. Oxygen consumption

The baseline oxygen consumption was assessed using high-resolution respirometry (Oroboros Oxygraph-O2k, Austria). Cells were removed from culture dishes through trypsinization and approximately 2 x 10^6 cells were added to RPMI-1640 FBS-free medium and transferred into the oxygraph. Basal consumption, oligomycin-independent respiration (proton leak) and FCCP-stimulated respiration (maximum respiration) were assessed.

d. Medium acidification

Cells were seeded in a 12-well plate with fresh medium with pH set at 7.4. The medium from each well was removed for pH measurement with a pH meter (Marte) in different times (24, 48, 72 and 96 h).

e. Lactate production

Cell medium was collected at times 0, 1, 2 and 3 h and transferred to a 96-well plate. The medium lactate was assessed using the Lactate assay kit from Labtest.

f. Microarray dataset

The microarray data was extracted from the Gene Expression Omnibus (GEO) repository (http://www.ncbi.nlm.nih.gov/geo/). Four datasets of interest were chosen and analyzed: GEO ID: GSE4824; GSE6013; GSE11117; and GSE3141.

g. Enrichment analysis

In order to evaluate enriched pathways in each group of the datasets, we used the Gene

Set Enrichment Analysis (GSEA) (Subramanian et al., 2005). Gene sets were created based on pathways provided by Kyoto Encyclopedia of Genes and Genome (KEGG) (http://www.genome.jp/kegg/) and on classical enzyme isoforms up regulated on Warburg-like cancer phenotype.

3. Results

a. Metabolic characterization of cell lines

The culture media have phenol red, a pH indicator, and its color exhibits a gradual transition from red to yellow over the pH range 8.2 and 6.8. We observed that our cell lines presented different patterns in this color shift, where non-tumoral BEAS-2B showed a red medium even after 96 h, whereas A549 medium had an orange shade and H460 presented a fast shift to yellow. Extracellular pH measurement confirmed that tumor cells have a higher medium acidification, especially H460 (Figure 1).

In order to evaluate whether the medium acidification was caused by lactate production, we measured the extracellular lactate. A549 and H460 showed a very similar pattern, with a much higher lactate production comparing with BEAS-2B, where extracellular lactate had a very small range over time (Figure 2).

It was also evaluated how the cells responded to medium buffering, therefore cells were treated with different concentrations of Tris-buffer. Interestingly, the treatment showed a very cytotoxic effect on H460, but not on A549 or BEAS-2B (Figure 3). This represents that H460 depends on the extracellular acidification in order to proliferate.

Analyzing basal oxygen consumption, it is clear that A549 cells have higher oxygen consumption comparing with normal cell line BEAS-2B and with H460 (Figure 4A). Oligomycin-independent respiration appears to be similar between tumor cell lines (Figure 4B). Adding FCCP, maximum respiration is provided. Both tumor cell lines have higher

maximum respiration than BEAS-2B, and A549 have higher maximum respiration when compared with H460. The difference between maximum respiration and basal respiration provides the respiratory reserve capacity. Hence, it is observed that tumor cell lines have higher respiratory capacity than BEAS-2B (Figure 4C). This means that tumor cells use very little of their capacity, which can confirm that they prefer to use a glycolytic pathway rather than a oxidative one, despite being able to use both, while BEAS-2B uses nearly all of its capacity.

b. Cytotoxic effects of anti-metabolites

After establishing a metabolic profile for each cell line, we tested the effects of four anti-metabolic drugs, 2-DG, 3BP, Phloretin and CHC on tumor cell lines and it was established the drug concentration that required to inhibit 50% of cell growth (GI_{50}). It has been observed that H460 presented a more glycolytic metabolism when compared to A549 (M. Wu et al., 2007). Based on that, we established a selectivity index, which shows if the compound is more selective to a Warburg phenotype (H460) or a non-Warburg phenotype (A549) (Table 1). 2-desoxyglucose is the only drug that inhibited both cell lines equally, while the other compounds seem to be more selective to the Warburg cancer cell line. 3-bromopyruvate appears to be nearly 70% more specific to H460, showing significant selectivity to Warburg-phenotype.

c. Gene expression analysis

In order to confirm the data obtained on experimental results, we decided to analyze the gene expression profile of metabolic pathways on our cell lines. Based on microarray data, it was observed that when comparing A549 and BEAS-2B, pyruvate metabolism, glycolysis and pentose phosphate pathways were enriched on A549. Furthermore, comparing H460 with

BEAS-2B, the same pathways were enriched on H460, in addition to the TCA cycle. There were no significant differences when comparing A549 with H460. We then compared the cell lines on the expression of enzymes isoforms presented on Warburg-like phenotype. In both tumor cell lines, this gene set was enriched when compared to BEAS-2B separately, and also when comparing tumor cell lines versus BEAS-2B together (Table 2). These results corroborate with the data found on experimental results, showing both tumor cell lines with similar metabolic profile and BEAS-2B being more oxidative.

The clinicopathological significance of these parameters was investigated using metadata analysis of tumor tissue microarray. We used microarray clinical datasets of NSCLC patients with different histological types. Analysis failed to present significant results on pathway enrichment when comparing different outcomes on the same histological type (data not shown). However, when comparing NSCLC biopsies with control lung biopsies, we observed enrichment on Warburg-like enzymes isoforms on the cancer biopsies. Also, in a different dataset, results presented significant differences on enrichment of Warburg-like isozymes on poor-outcome adenocarcinoma when compared with good-outcome adenocarcinoma (Table 3). These data provided possible targets in therapy, where it is possible to observe that some isoforms analyzed are enriched on most cancer cell lines and tumor tissues (Table 4).

4. Discussion

Despite the increasing progress on science, cancer is still a very lethal disease, and lung cancer is responsible for most of the death on oncology. Current therapies are still inefficient, since the disease mechanisms remain obscure. However, recently the Warburg Effect theory has been brought to light again, and studies on this field are providing a new view on carcinogenesis. In this study, we analyzed the metabolic profile of three cell lines,

where two of them are tumoral. Based on our results, it is observed that H460 has the highest extracellular acidification, and also a dependence on it to proliferate. On the other hand, A549 showed almost the same rate of lactate production than H460, even though it has lower medium acidification. Also, A549 presents higher basal and maximum respiration, as well as higher reserve capacity. Both cell lines, when compared to non-tumoral BEAS-2B, showed a more glycolytic metabolism, accordingly to the Warburg Effect. The gene enrichment from glycolytic pathways and Warburg isoforms on both cell lines when compared to BEAS-2B confirmed these hypothesis, showing that the reprogramming of metabolism can be a selective target for therapy. There are several studies demonstrating intratumor heterogeneity (Endesfelder et al., 2012), and the evidence that there are differences in metabolism even among different cancer cells supports the idea of personalized therapy, where anti-metabolites will be more effective on tumor cells that present a more glycolytic metabolism. Our results corroborate with this idea, since when the cancer cell lines were tested against anti-metabolites, most of them were selective to H460. This also confirms the hypothesis that H460 presents a more glycolytic metabolism than A549.

Our results have also shown to have clinical relevance, as it was observed that lung tumor biopsies presented gene enrichment of Warburg isoforms when compared to normal lung biopsies. In addition, lung tumor biopsies of low survival rate patients presented enrichment of Warburg isoforms when compared to high survival rate patients' biopsies. The compilation of enriched isoforms in all analyses presented on Table 4 provides evidence on isoforms related to poor-prognosis, for those enriched on dead adenocarcinoma patients, and carcinogenesis, for those enriched on NSCLC when compared to normal tissue, as well as on the tumoral cell lines when compared to BEAS-2B. Also, IDH1, LDHA and PYGB were enriched in all analyses, showing that there is a strong relation between their expression with carcinogenesis and poor-outcome, therefore being good therapeutic targets. Indeed, inhibition

of LDHA has been shown to inhibit tumor progression (Le et al., 2010), whereas there are studies on PYGB as a cancer biomarker (Tashima et al., 2000).

More studies are necessary to reveal targets that can really be used on clinics. However, identifying these potential targets is already a big step on cancer therapy, since the altered metabolism in tumor appears to be a more stable therapeutic approach.

5. Conclusion

In summary, our results point out metabolic differences in cancer cell lines, suggesting a personalized therapy with drugs that have shown to be more selective for glycolytic tumors. Furthermore, we demonstrated the enrichment of cancer cell lines of glycolytic pathways when compared with non-tumoral cell line, as well as enrichment of Warburg isoforms. Finally, these data together with clinicopathological analysis on Warburg isoforms enrichment, it is observed isoforms related both to carcinogenesis and poor-prognosis. Therefore, metabolic isoforms are revealed as new therapeutic targets.

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Figures/Tables and legends

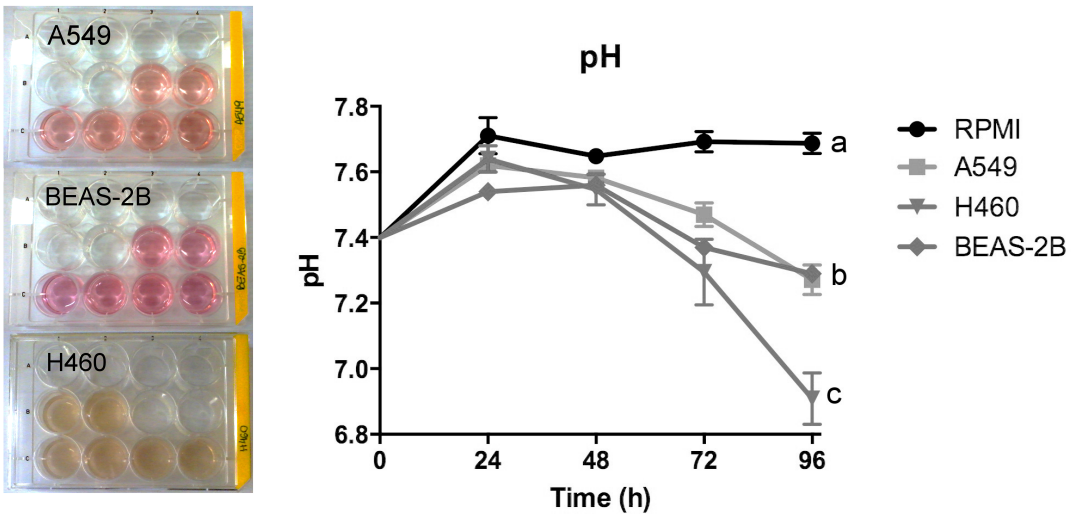


Figure 1: Medium acidification by A549, H460 and BEAS-2B human cell lines. pH of cell culture media was measured with a pH meter after 24, 48, 72 and 96h. Analyses showed significant differences ($p < 0.05$) between the control and the cell lines, as well as between H460 and A549 and BEAS-2B (One-way ANOVA; n = 3-5). Inset shows representative A549, BEAS-2B and H460 cultures after 96h.

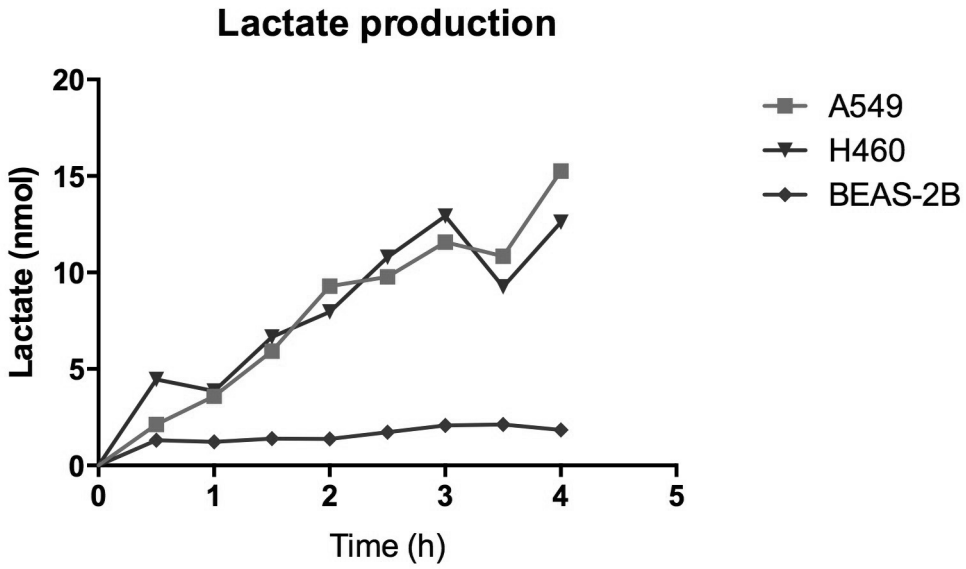


Figure 2: Lactate production by A549, H460 and BEAS-2B. Lactate concentration was enzymatically measured in cultures supernatant for 4h. (n = 1).

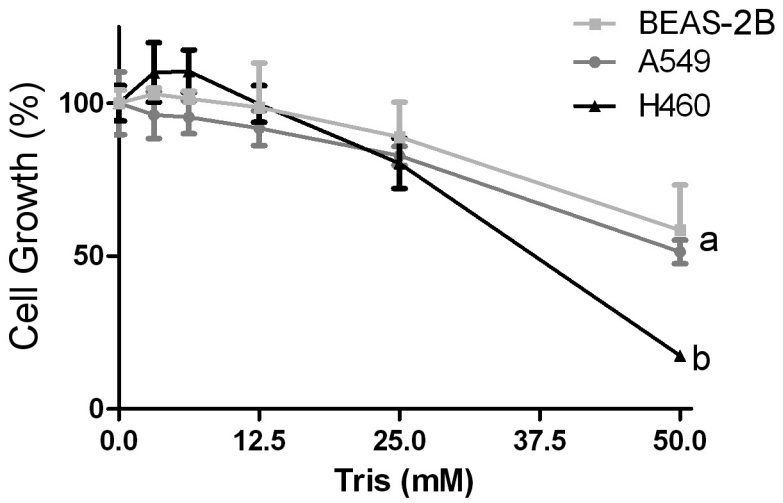


Figure 3: Effect of medium buffering by Tris on cell growth. Cells were cultivated in Tris buffer for 72h and cell growth was evaluated by SRB method. It was observed significant differences between H460 and the other cell lines (One-way ANOVA; n = 3).

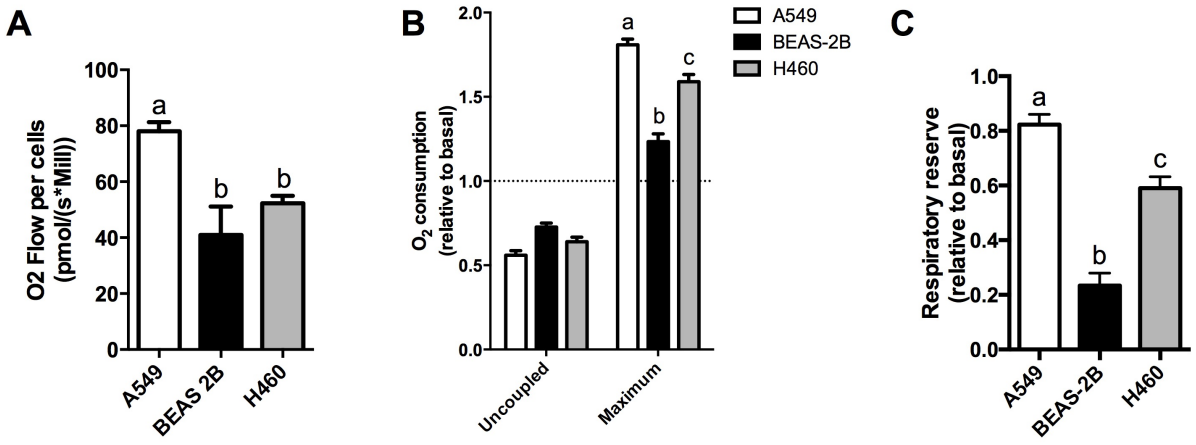
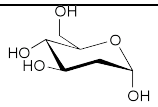
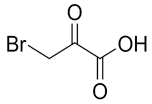
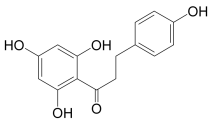
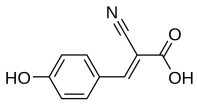


Figure 4: Respiration profile of the cell lines A549, BEAS-2B and H460. An equal amount ($2x10^6$ cells) of A549, BEAS-2B and H460 cells were placed into the oxygraph on RPMI FBS-free medium. (A) Basal oxygen consumption measurement revealed significant differences ($p < 0.005$) between A549 and BEAS-2B, as well as between A549 and H460 ($p < 0.0001$) (Student's *t* test; $n = 3$-8). (B) Uncoupled and maximum respiration were assessed by addition of oligomycin and FCCP, respectively. It was observed significant differences ($p < 0.0001$) between A549, BEAS-2B and H460 maximum respiration (One-way ANOVA; $n = 3$-8). (C) Respiratory reserve capacity was calculated relatively to basal by the difference between maximum respiration and basal respiration. It was observed significant differences ($p < 0.0001$) between A549, BEAS-2B and H460 (One-way ANOVA; $n = 3$-8).

Table 1: *In vitro* cytotoxicity (Growth Inhibition dose 50) of metabolic inhibitors on human non-small cell lung cancer cell lines.

Metabolic Inhibitor		GI_{50} (μM)		
Structure	Name	H460 cells	A549 cells	Selectivity Index
	2-Deoxy-D-glucose	3,130 ± 540	3,160 ± 457	1.01
	3-Bromopyruvate	116.0 ± 1.58	196.2 ± 26.22	1.69*
	Phloretin	96.88 ± 3.87	127.6 ± 16.42	1.32
	α-cyano-4-OH-cinnamate	3,190 ± 270	4,950 ± 830	1.55

Drug GI_{50} were determined in exponentially growing cells by the Sulforhodamine B (SRB) method, following NCI60 protocol, as described in materials and methods section. Selectivity index (SI) were calculated according to SI = GI_{50} of compound in the non-Warburg cancer cell line / GI_{50} in Warburg cancer cell line, where GI_{50} is the drug concentration that required to inhibit 50% of cell growth.

Table 2: Enrichment analysis of metabolic pathways and Warburg isoforms on human NSCLC and lung epithelium cell lines with respective *p* values.

Analysis	Gly	Pyr	PPP	TCA	OXPHOS	War
A549 *vs.* BEAS-2B	<0.001	<0.001	<0.001	N.S	N.S	<0.005
H460 *vs.* BEAS-2B	<0.001	<0.001	<0.001	<0.005	N.S	<0.001
Tumor *vs.* BEAS-2B	-	-	-	-	-	<0.001

Enrichment analyses were made via GSEA. Gene sets were created based on the metabolic pathways glycolysis (Gly), pyruvate metabolism (Pyr), pentose phosphate pathway (PPP), tricarboxylic acid cycle (TCA) and oxidative phosphorylation (OXPHOS), provided by Kyoto Encyclopedia of Genes and Genome (KEGG) (http://www.genome.jp/kegg/). Also, another gene set was created based on Warburg isoforms (War). All analyses were made on microarray data provided by GEO. GEO ID: GSE4824. Significance is shown by the representation of the *p* value for each analysis. N.S stands for not significant.

Table 3: Gene Set Enrichment Analysis (GSEA) of Warburg isoforms on human NSCLC and lung tissue.

Datasets	Analysis	Enrichment	*P* value
GSE11117	Tumor *vs.* Normal tissue	Warburg isoforms	<0.005
GSE3141	Dead *vs.* Alive adenocarcinoma patients	Warburg isoforms	<0.005

Enrichment analyses were made by GSEA on tumor tissue microarray. Gene set analyzed was based on Warburg isoforms. Microarray datasets were extracted from GEO. Significance is shown by its nominal *P*-value.

Table 4: Enriched Warburg isoforms on each analysis

Gene symbol	Gene name	NSCLC *vs* Control	ADC dead *vs* ADC alive	A549 *vs* BEAS-2B	H460 *vs* BEAS-2B	Tumor *vs* BEAS-2B
FH	Fumarate hydratase			✓	✓	✓
G6PD	Glucose-6-phosphate dehydrogenase		✓	✓	✓	✓
HK2	Hexokinase 2	✓	✓			
IDH1	isocitrate dehydrogenase 1	✓	✓	✓	✓	✓
LDHA	Lactate dehydrogenase A	✓	✓	✓	✓	✓
PFKFB3	Fructose-2,6-biphosphatase 3		✓	✓	✓	✓
PGK1	phosphoglycerate kinase 1	✓	✓		✓	✓
PYGB	Glycogen phosphorylase,; brain	✓	✓	✓	✓	✓
SLC16A3	Monocarboxylic acid transporter 4	✓	✓			
SLC2A1	Facilitated glucose transporter 1	✓				✓
TALDO1	transaldolase 1			✓	✓	✓
TKTL1	transketolase-like 1	✓		✓		

Enriched Warburg isoforms based on GSEA analysis for each group. NSCLC = Non-small cell lung cancer; ADC dead = adenocarcinoma dead patients; ADC alive = adenocarcinoma alive patients.

Appendix A

Genes included on each gene set

Pentose Phosphate Pathway	Pyruvate Metabolism	TCA cicle	Warburg isoforms
FBP1	ME3	CS	SLC2A1
PRPS1L1	ACOT12	DLAT	HK2
ALDOA	LDHAL6A	DLD	PFKFB3
ALDOB	DLAT	DLST	ALDOC
RPIA	DLD	FH	PGK1
ALDOC	LDHD	IDH1	PGAM2
G6PD	ALDH2	IDH2	PKM
PGLS	ALDH1B1	IDH3A	LDHA
GPI	ALDH9A1	IDH3B	SLC16A3
DERA	ALDH3A2	IDH3G	FH
PFKL	AKR1B1	MDH1	IDH1
PFKM	GLO1	MDH2	FASN
PFKP	HAGH	ACLY	G6PD
PGD	ACACA	ACO1	TKTL1
PGM1	ACACB	OGDH	TALDO1
PGM2;	ACAT1	ACO2	PYGB
PRPS1	ACAT2;	PC	
PRPS2	LDHA	PCK1	
RPE	LDHB	PCK2	
RBKS	LDHC	PDHA1	
TALDO1	MDH1	PDHA2	
TKT	MDH2	PDHB	
RPE	ME1	OGDHL	
TKTL1	ME2	SDHA	
TKTL2	ALDH7A1	SDHB	
FBP2	PC	SDHC	
RGN	PCK1	SDHD	
H6PD	PCK2	PDHX	
	PDHA1	SUCLG2	
	PDHA2	SUCLG1	
	PDHB	SUCLA2	
	PKLR		
	PKM		
	ACSS2		
	AKR1B10		
	PDHX		
	HAGHL		
	ACSS1		
	LDHAL6B		
	GRHPR		
	ACYP1		
	ACYP2		

Glycolysis			
AKR1A1	ALDH1B1	LDHA	PKLR
ADH1A	ALDH1A3	LDHB	PKM
ADH1B	FBP1	LDHC	PGM2
ADH1C	ALDH3B1	PGAM4	ACSS2
ADH4	ALDH3B2	ALDH7A1	G6PC2
ADH5	ALDH9A1	PCK1	BPGM
ADH6	ALDH3A2	PCK2	TPI1
GALM	ALDOA	PDHA1	HKDC1
ADH7	ALDOB	PDHA2	PDHX
LDHAL6A	ALDOC	PDHB	ADPGK
DLAT	G6PC	PFKL	ACSS1
DLD	GAPDH	PFKM	FBP2
ENO1	GAPDHS	PFKP	LDHAL6B
ENO2	GCK	PGAM1	
ENO3	GPI	PGAM2	
ALDH2	HK1	PGK1	
ALDH3A1	HK2	PGK2	
ALDH1B1	HK3	PGM1	

Oxidative phosphorilation					
NDUFC2-KCTD14	COX15	MT-ND4L	NDUFB9	ATP5D	PPA1
COX17	CYC1	MT-ND5	NDUFB10	ATP6V1D	NDUFA12
TCIRG1	ATP6V0E2	MT-ND6	NDUFC1	ATP5E	NDUFA4L2
ATP5H	COX7B2	NDUFA1	NDUFC2	ATP5F1	SDHA
ATP5L	ATP6V0A2	NDUFA2	NDUFS1	ATP5G1	SDHB
UQCR11	ATP6V0D2	NDUFA3	NDUFS2	ATP6V1H	SDHC
COX6B2	ATP6V1C2	NDUFA4	NDUFS3	ATP5G2	SDHD
NDUFA11	PPA2	NDUFA5	NDUFV1	ATP5G3	LHPP
ATP6V1G3	UQCRQ	NDUFA6	NDUFS4	ATP5I	UQCRB
COX4I1	UQCR10	NDUFA7	NDUFS5	ATP5J	UQCRC1
COX5B	COX8C	NDUFA8	NDUFS6	ATP6V1A	UQCRC2
COX6A1	NDUFS7	NDUFA9	NDUFS8	ATP6V1B1	UQCRFS1
COX6A2	MT-ATP6	NDUFA10	NDUFV2	ATP6V1B2	UQCRH
COX6B1	MT-ATP8	NDUFAB1	NDUFV3	ATP6V0C	COX4I2
COX6C	MT-CO1	NDUFB1	ATP12A	ATP6V1C1	ATP6V0E1
COX7A1	MT-CO2	NDUFB2	ATP4A	ATP6V1E1	ATP6V1E2
COX7A2	MT-CO3	NDUFB3	ATP4B	ATP6V0B	ATP6V0D1
COX7B	MT-CYTB	NDUFB4	ATP5A1	ATP6V1G2	COX7A2L
COX7C	MT-ND1	NDUFB5	ATP5B	ATP6V0A1	ATP6V1F
COX8A	MT-ND2	NDUFB6	ATP6V0A4	ATP6AP1	COX5A
COX10	MT-ND3	NDUFB7	ATP5C1	ATP5O	ATP6V1G1
COX11	MT-ND4	NDUFB8	NDUFA13	NDUFB11	ATP5J2

3 CONCLUSÃO E PERSPECTIVAS

Nesse trabalho, demonstramos que linhagens de células tumorais apresentam um perfil metabólico glicolítico, com alta liberação de lactato e grande reserva respiratória. A linhagem celular não-tumoral, por outro lado, apresentou-se oxidativa, e esses dados estão de acordo com o conhecido Efeito Warburg. Ainda, foram testadas diferentes drogas anti-metabólitas nas linhagens tumorais, e foi observado que há uma seletividade desses compostos pela linhagem H460, confirmando a hipótese de que essa linhagem é mais glicolítica do que A549. Esses resultados corroboram com a ideia de terapia personalizada, uma vez que há uma grande heterogeneidade entre tumores, e até mesmo dentro de um mesmo tumor. Dessa forma, o uso de drogas específicas para cada tipo de tumor pode ser uma boa estratégia terapêutica, de forma a ser seletiva e eficaz. Também, foram analisados os perfis de expressão gênica quanto ao metabolismo energético de cada linhagem, onde foi observado que as linhagens tumorais apresentam um enriquecimento para genes de vias glicolíticas, como glicólise, metabolismo do piruvato e via das pentoses. Também foi visto nas linhagens tumorais o enriquecimento de isoformas Warburg, responsáveis pela reprogramação metabólica. Por fim, foi observada a importância desses resultados na clínica, onde se viu que biópsias de câncer de pulmão também apresentam um enriquecimento para isoformas Warburg. Este dado também foi observado para pacientes mortos de adenocarcinoma quando comparados com pacientes vivos do mesmo tipo histológico, demonstrando uma relação da expressão dessas enzimas com o desfecho do paciente. Analisando as isoformas enriquecidas em todas as análises, é possível sugerir novos alvos para estudos na terapia do câncer. Dessa forma, as perspectivas desse trabalho envolvem mais estudos em relação a esses alvos, para que possivelmente se consiga sugerir um novo biomarcador para o câncer.

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TABLE OF CONTENTS

- **Description** p.1
- **Audience** p.2
- **Impact Factor** p.2
- **Abstracting and Indexing** p.2
- **Editorial Board** p.2
- **Guide for Authors** p.4

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