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TESE DE DOUTORADO

FENÓTIPOS AGRESSIVOS E PROPOSTAS TERAPÊUTICAS EM TUMORES SÓLIDOS

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Na evolução, na vida, na academia e na biologia tumoral:

"Não é o mais forte nem o mais inteligente que sobrevive,

mas aquele que melhor se adapta às mudanças."

dito popular inspirado no trabalho de

Charles Robert Darwin

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SUMÁRIO

PARTE I

RESUMO

Estima-se que 1 em 5 pessoas morrerão de câncer e, neste momento 1 em 200 vivem com a patologia. Classicamente, é descrita como uma doença genética, e por muito tempo as abordagens terapêuticas focaram em mutações. Apesar de casos de sucesso, a eficácia terapêutica ainda é insuficiente, estima-se que 2 em 3 pessoas diagnosticadas com câncer morrerão de causas relacionadas à doença. Apesar da imensa variabilidade genética, diferentes mutações convergem para um número limitado de fenótipos. Assim, o estudo do câncer sob uma ótica evolutiva focando em fenótipos agressivos é uma alternativa promissora.

Portanto, o objetivo do presente trabalho foi investigar fenótipos agressivos em tumores sólidos, buscando caracterizar alterações e propor alvos e terapias. Para isto, investigou-se câncer de pulmão e mama sob diferentes aspectos biológicos.

O capítulo I estudou metabolismo redox em câncer de pulmão de não-pequenas células (NSCLC). Aqui, demonstramos que o desbalanço redox oxidativo intracelular é um fenótipo agressivo. Posteriormente, sugerimos abordagens antioxidantes como uma boa proposta terapêutica.

O capítulo II estudou adenocarcinoma de pulmão sob o contexto da reprogramação metabólica. Neste, sugerimos que o metabolismo glicolítico dependente de transportadores de glicose (GLUT's) e lactato/piruvato (MCT's) é um fenótipo agressivo. Por fim, propomos que inibidores para isoformas específicas destes transportadores podem ter impacto clínico nesta malignidade.

O capítulo III investigou uma abordagem terapêutica para um fenótipo agressivo bem estabelecido, a resistência à hipóxia. Utilizando modelos *in vitro* e *in vivo* de câncer de mama triplo-negativo (TNBC), encontramos que o inibidor epigenético JQ1 é capaz de inibir genes e rotas importantes à sobrevivência da célula cancerosa em hipóxia. Deste modo, é proposto esta abordagem epigenética na terapêutica de TNBC.

Portanto, foi demonstrado aqui que a investigação de fenótipos agressivos é capaz de contribuir para a oncologia molecular, identificando padrões e apontando alvos. Foi dito no passado que "nada em biologia faz sentido senão à luz da evolução", e foi sob esta filosofia que o presente trabalho foi desenvolvido e buscou contribuir na busca da cura do câncer.

ABSTRACT

It is estimated that 1 in 5 people will die of cancer, and now 1 in 200 live with this pathology. Classically, cancer is described as a genetic disease, and for a long time therapeutic approaches focused on mutations. Despite some successes, treatment efficacy is still insufficient, it is estimated that 2 out of 3 people diagnosed with cancer will die from causes related to the disease. Despite the large genetic variability, different mutation converge to a limited number of phenotypes. Thus, the investigation of cancer under an evolutive perspective that focus on aggressive phenotypes is an promising alternative approach.

Therefore, this study aimed to investigate aggressive phenotypes in solid tumors, seeking to characterize changes and propose targets and therapies. For this, we investigated lung cancer and breast under different biological aspects.

Chapter I studied redox metabolism in non-small cell lung cancer (NSCLC). Here, we show that redox imbalance favouring intracellular oxidative stress is an aggressive phenotype. Later, we suggest antioxidants approaches as a promising therapeutic window.

Chapter II studied lung adenocarcinoma in the context of metabolic reprogramming. Herein, we suggest that the glycolytic metabolism dependent on transporters of glucose (GLUT's) and lactate / pyruvate (MCT's) is an aggressive phenotype. Then, we propose that inhibitors for specific isoforms of GLUT's and MCT's may have clinical impact in this malignancy.

Chapter III investigated a therapeutic approach for cells that are resistant to hypoxia, a well-established aggressive phenotype. Using *in vitro* and *in vivo* models of triple negative breast cancer (TNBC), we find that the epigenetic inhibitor JQ1 is able to inhibit important genes and pathways in hypoxic cancer cell survival. Thus, we propose this epigenetic approach in the management of TNBC.

Therefore, it was demonstrated here that investigation of aggressive phenotypes can contribute to the molecular oncology, identifying patterns and pointing targets. It was once said that "nothing in biology makes sense except in the light of evolution", this study was conducted under this philosophy to contribute in the search for the cure of cancer.

LISTA DE ABREVIATURAS

AdC – adenocarcinoma

LCC – carcinoma de células grandes, do inglês *large cell carcinoma*

ROS – espécies reativas do oxigênio, do inglês reactive oxygen species

NSCLC – câncer de pulmão de células não-pequenas, do inglês *non-small cell lung cancer*

OMS – Organização Mundial da Saúde

SQC – carcinoma escamoso, do inglês *squamous cell carcinoma*

TNBC – câncer de mama triplo-negativo, do inglês *triple negative breast cancer*

CT – tomografia computadorizada, do inglês *computed tomography*

GLUT – transportador de glicose, do inglês *glucose transporter*

MCT – transportador de monocarboxilato, do inglês *monocarboxylate transporter*

INTRODUÇÃO

Câncer

Definição

Segundo a Organização Mundial da Saúde (OMS), Câncer é a uma centena de doenças caracterizadas pelo crescimento desordenado de células anormais, podendo invadir tecidos adjacentes e espalharem-se para outros órgãos do corpo. Este último fenômeno é conhecido como metástase, e é a maior causa de morte por câncer (OMS, 2015).

O câncer é uma doença moderna. Por milênios, os seres humanos morriam principalmente por violência, acidentes e doenças infecciosas. Em 1900, as principais causas de morte eram pneumonia, gripe e tuberculose. Mas atualmente, as principais causas são doenças cardiovasculares e câncer (BRODY, 2014).

Epidemiologia

Sem dúvida, estamos diante de uma epidemia mundial, que segundo estimativas crescerá 70% nos próximos 20 anos. Mais de 32 milhões de pessoas vivem com câncer atualmente, ou 1 em 215*. O câncer hoje é responsável por aproximadamente 15% de todas as mortes no mundo e no Brasil. Anualmente, são previstos 14, 1 milhões de novos casos e 8, 2 milhões de mortes por câncer. Destes, 57% dos novos casos e 65% dos óbitos ocorrem nas

* Esses números não incluem câncer de pele não-melanoma, devido à dificuldade em coletar e contar tais tumores. Para constar, câncer de pele é o mais comum, o número de novos casos é superior à incidência anual de câncer de mama, próstata, pulmão e cólon combinados. Porém, é o mais fácil de curar quando diagnosticado e tratado precocemente (FUNDAÇÃO DO CÂNCER DE PELE, 2015).

regiões menos desenvolvidas do globo. Entretanto, a mortalidade é maior nos países mais desenvolvidos (BRODY, 2014; FERLAY et al., 2014). Em território brasileiro, estimam-se 576 mil novos casos de câncer por ano para o biênio 2014-2015 (INCA, 2014).

Assim como muitos países em desenvolvimento, o Brasil sofre um processo conhecido como "envelhecimento da população". Este, é consequência do aumento da urbanização e avanços técnico-científicos que traz consigo uma maior exposição a fatores de risco. Além disso, há uma maior adoção de dieta e estilo de vida ocidentais. Este processo alterou o perfil de morbimortalidade, reduzindo a ocorrência de doenças infectocontagiosas e colocando as doenças crônico-degenerativas no centro de atenção dos de saúde da população (INCA, 2014).

Origem

Classicamente, o câncer é definido como uma doença genética. Ou seja, a partir da alteração na expressão de um gene, uma ou mais células adquirem uma vantagem adaptativa sobre as demais. A partir daí, outras alterações se somam até que surge um grupo de células que proliferam independente do controle do organismo e são capazes de invadir tecidos adjacentes, ou seja, um tumor maligno (NOWELL, 1976). Hoje sabemos que os cânceres são heterogêneos, ou seja, mesmo que todas as células malignas possivelmente tenham um antecessor comum, existem subpopulações com diferentes alterações. Todo processo ocorre à semelhança da seleção Darwiniana, com mutações aleatórias inserindo variabilidade e pressões ambientais selecionando o mais bem adaptado (MCGRANAHAN; SWANTON, 2015).

As mutações no DNA são a forma mais direta de alterar a expressão gênica, mas não é a única. Portanto, talvez não devêssemos caracterizar o câncer como uma doença genética, mas sim da expressão gênica. Mecanismos como epigenética e microRNA's alteram a expressão gênica sem alterar a sequência do DNA. Assim, o estudo desses fenômenos acrescentou uma

camada de complexidade à tumorigênese e abriu novas possibilidades terapêuticas (AZAD et al., 2013; DAWSON; KOUZARIDES, 2012; HAYES; PERUZZI; LAWLER, 2014; JONES, 2014).

Fatores de Risco e Prevenção

Diversos fatores estão associados com maior incidência do câncer, mesmo que muitas vezes não compreendemos os mecanismos envolvidos. Assim, dizemos que o câncer é uma doença multifatorial, podendo os fatores serem endógenos ou exógenos (Tab.1).

Tabela 1 Fatores de Risco e Oportunidades de Prevenção de Câncer *(FUNDAÇAO DO CÂNCER DE PELE, 2015; OMS, 2015)*

Fatores de Risco	Exemplo	Prevenção	Observações
MUTAGÊNICOS			
Físicos	Luz UV Radiação	Reduzir exposição	
Químicos	Tabaco Asbestos Aflatoxina Arsênico	Reduzir exposição Campanhas anti-fumo Controle sanitário	O tabaco responde por 20% dos casos e 70% das mortes, especialmente em câncer de pulmão, bexiga e rim
Biológicos	HBC e HCV, vírus da hepatite B e C HPV, papilomarvírus humano HIV, vírus da imunodeficiência humano	Vacinação	Normalmente associados à inflamação no tecido Muito comum em países pobres Associado com câncer de fígado e cérvix
ESTILO DE VIDA			
Dieta	Pobre em frutas, vegetais e fibras Rica em carne vermelha processada, sais, fritura	Alimentação pode prevenir câncer de estômago e intestino, entre outros	Associado especialmente a câncer de estômago, esôfago, bexiga, pâncreas, prostate, mama, pulmão, colorectal, e fígado
Álcool	Consumo regular 2-3 doses/dia	Reduzir o consumo	Boca, garganta e mama
Sedentarismo e Sobrepeso		Prática de atividades física Controle do peso	Associado com diversos tipos de câncer
Bronzeamento	Luz solar em excesso Câmaras de bronzeamento artificial	Usar protetor solar Evitar exposição excessiva	São previstos mais casos de câncer de pele por bronzeamento do que câncer de pulmão por tabaco
Hormonal	Contraceptivo oral Terapia de reposição hormonal	Usar com indicação médica	Mama
Envelhecimento	Acúmulo de dano ao DNA Menor capacidade de mecanismos de reparo		A incidência de câncer aumenta exponencialmente com a idade

Recentemente, a mídia divulgou errônea e inconsequentemente um trabalho científico o qual afirmava que "65% dos casos de câncer ocorrem por azar". Na verdade, o trabalho

demonstra que 65% da probabilidade de desenvolver um câncer se deve às mutações aleatórias na replicação do DNA, ou seja, é aleatória (TOMASETTI; VOGELSTEIN, 2015). Os autores publicaram uma carta explicando o mal entendido com a seguinte metáfora "quanto mais longa a viagem, maior o risco de um acidente de carro, porém ninguém seria inconsequente de descartar fatores como a qualidade da estrada, o bom funcionamento do carro ou a destreza do motorista como fatores que podem determinar a ocorrência de um acidente".

Este estudo não foi validado experimentalmente e possui diversas limitações, mas atenta para nosso pouco conhecimento acerca dos mecanismos que governam a evolução cancerosa (WODARZ; ZAUBER, 2015). Nestes casos, a prevenção não é uma opção. Ainda, abre uma janela terapêutica em mecanismos que reduzam a taxa de mutação. Uma droga que já foi descrita para este fim é a aspirina (JACOBS, 2011; THUN; JACOBS; PATRONO, 2012).

Reside no poder público em profissionais da saúde a responsabilidade de lidar com esta epidemia. Estudos epidemiológicos, educação em saúde, formulação de legislação específica e de opinião pública são fundamentais para a prevenção. Estima-se que aproximadamente 50% dos casos seriam evitados apenas com alteração no estilo de vida: alimentação adequada, evitar sobrepeso, fazer exercícios regularmente, não fumar, proteger a pele do sol, e vacinação (OMS, 2015). Todas medidas simples e acessíveis que muitas vezes são deixadas de lado. Entretanto, novos casos devem seguir ocorrendo e, para estes, a pesquisa é fundamental.

Detecção Precoce E Métodos De Triagem

Muitos tipos de câncer apresentam taxas de cura altas quando são detectados precocemente. Isso ocorre principalmente porque em estágios iniciais eles podem ser removidos cirurgicamente ou atacados com radioterapia localizada. Infelizmente, a maioria dos tumores só apresentam sintomas em estágios avançados. Por isso, é fundamental que sejam feitos exames de triagem rotina (OMS, 2015).

Um teste de triagem é definido como uma aplicação sistemática de um teste assintomático em uma população-alvo que não define diagnósticos, mas levanta suspeitas. Assim, é necessária a confirmação por outros métodos, mas trata-se da melhor ferramenta para identificar indivíduos com alterações que sugerem uma lesão tumoral ou pré-tumoral para aplicação imediata de tratamento, evitando assim a progressão do câncer. Infelizmente, não existem ainda métodos de triagem eficientes para todos os tipos de câncer. Como exemplos podemos citar: inspeção visual com ácido acético (VIA) para câncer cervical; teste de HPV para câncer cervical; teste de Papanicolau para câncer cervical; exame de toque retal para câncer de próstata; e mamografia para câncer de mama.

Por fim, a pesquisa aqui também é fundamental para a identificação de novos biomarcadores e fatores diagnósticos. Assim, seremos capazes de identificar uma maior gama de tumores precocemente.

Tratamento

Atualmente dispomos de três tratamento que podem ser combinados, são eles: quimioterapia, radioterapia e cirurgia. O objetivo principal é curar o paciente ou prolongar sua vida, sem perda de qualidade de vida. É fundamental também que o paciente receba cuidados paliativos e suporte psicológico (OMS, 2015). Como estamos falando de uma centena de doenças, um diagnóstico correto é fundamental para um tratamento eficiente e adequado.

Tipos mais comuns

Quatro tipos de câncer são conjuntamente responsáveis por mais da metade dos casos existentes na população mundial, são eles (em ordem de representatividade): mama, próstata, cólon retal e pulmão (Fig. 1). Estes 4 tipos são também os mais frequentemente diagnosticados, porém com a ordem sendo alterada para: pulmão, mama, cólon retal e próstata (Fig. 2). Por fim,

câncer de pulmão é o que apresenta o pior prognóstico, sendo o maior responsável por mortes dentre todos os tipos e também o mais letal, dentre os 4 mais prevalentes (Fig. 2).

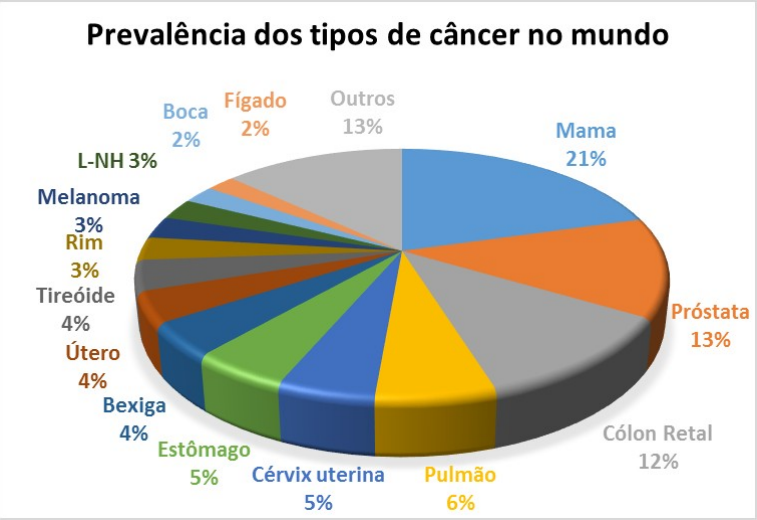


Figura 1 Prevalência (casos com até 5 anos de diagnóstico) dos tipos de câncer no mundo. Outros: ovário (1,7%), leucemias (1,5%), esôfago (1,4%), laringe (1,3%), sistema nervoso (1%), faringe (0,9%), mieloma múltiplo (0,7%), nasofaringe (0,7%), testículo (0,6%), pâncreas (0,6%), vesícula biliar (0,6%), linfoma Hodgkin (0,5%) e sarcoma (0,2%). L-NH – linfoma não-Hodgkins (FERLAY et al., 2014).

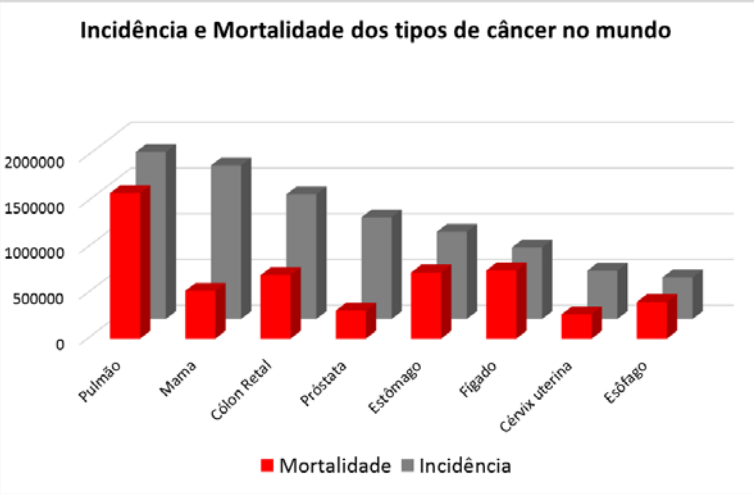


Figura 2 Incidência e mortalidade dos tipos de câncer no mundo (números absolutos do ano de 2012) (FERLAY et al., 2014).

No Brasil, os mesmos 4 são os de maior incidência (em ordem de representatividade): próstata, mama, cólon e pulmão (Fig. 3), excluindo-se câncer de pele mão-melanoma. Na população brasileira, câncer de próstata é mais comum. O câncer de mama é o maior responsável por mortes relacionadas ao câncer nas mulheres brasileiras, enquanto nos homens é o de pulmão (INCA, 2014).

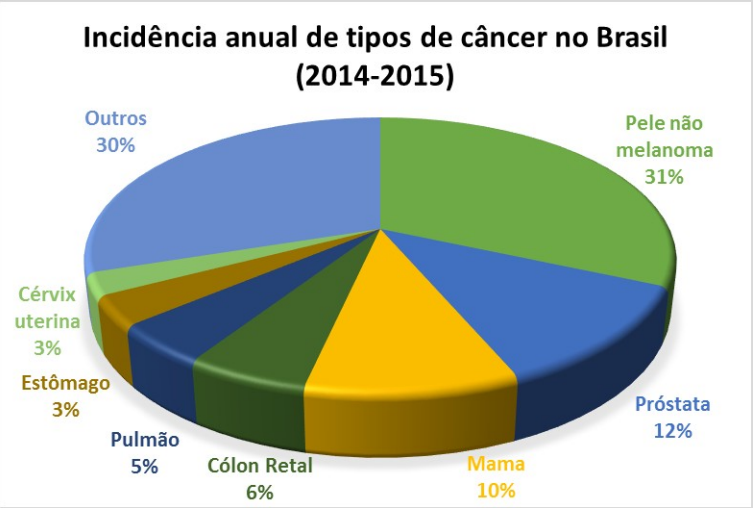


Figura 3 Incidência anual (número de novos casos previstos) de tipos de câncer no Brasil para o biênio (INCA, 2014).

Este trabalho estudou os dois tipos de câncer com maior incidência mundial: pulmão e mama. Por isso, será feita uma descrição mais detalhada destes dois tipos de câncer a seguir.

Câncer de Pulmão

Epidemiologia

Até o começo século XX, essa doença era muito rara, responsável por 0,7% das mortes por câncer nos EUA em 1914 comparado aos 27% de 2014 (DEWEERDT, 2014). Hoje, é o tipo mais comum e com maior número de morte. A sobrevida é baixa (5-10 % sobrevida em 5 anos) (INCA, 2014; OMS, 2015) (Fig.4). Somente no Brasil, estima-se que 20 mil pessoas morrerão de câncer de pulmão no ano de 2015 (INCA, 2014).

Sintomas e Detecção

A doença é assintomática nos estágios inicias, de modo que 75% dos casos são detectados tardiamente, quando os tratamentos mais eficazes apresentam menor sucesso. Os principais sintomas apresentados são: tosse seca duradoura (3 semanas), dispnéia (falta de ar) em esforços comum, dor torácica contínua, hemoptise (sangue no escarro), pneumonias repetitivas, inchaço no pescoço ou na face, perda de peso, rouquidão por mais de 1 semana.

Entretanto, quando detectada precocemente a sobrevida em 5 anos é superior a 70% (DEWEERDT, 2014). Métodos de triagem para detecção precoce ainda são pouco sensíveis para câncer de pulmão (FIELD, 2014).

Há pouco tempo foi demonstrado que o diagnóstico por tomografia computadorizada (CT, do inglês *computed tomography)* de baixa dose foi capaz de reduzir mortalidade em adultos entre 55 e 80 anos que fumaram 30 maços/ano e ainda fumam ou pararam há pelo menos 15 anos (ABERLE et al., 2013). Entretanto esta recomendação não é indicada para países subdesenvolvidos, devido à alta incidência de doenças pulmonares granulomatosas, o que aumenta a taxa de falso positivo. Com isso, intervenções e preocupações perigosas e desnecessárias a pacientes falso positivos se sobrepõe ao benefício de detecções precoces. Por isso, estão sendo desenvolvidos programas de computador capazes de analisar forma e textura do nódulo, melhorando assim a sensibilidade de detecção da CT (BOURZAC, 2014b).

Fatores de risco

O consumo de tabaco e derivados está associado a 90% dos casos diagnosticados. Isto ajuda a explicar a maior incidência na população masculina, embora o aumento da população tabagista feminina esteja equilibrando esta tendência. O fumo passivo também é um fator de risco. Entretanto, 10% dos casos diagnosticados são em não-fumantes (FERLAY et al., 2014). Estes casos, tratam-se de doenças molecularmente distintas, que apenas acontecem no mesmo órgão (DE BRUIN et al., 2014; HERBST; HEYMACH; LIPPMAN, 2008). Além disso, o tabagismo dificulta ainda mais a terapia deste tipo de câncer, uma vez que pacientes fumantes exibem maior depuração de quimioterápicos, resultando em menor eficácia (O'MALLEY et al., 2014).

Isoladamente, câncer de pulmão em não-fumantes seria a sétima maior neoplasia em números de morte (DEWEERDT, 2014). Portanto, devemos investigar outros fatores de risco.

A poluição ambiental está associada à ocorrência de câncer de pulmão. Não se encontrou um limiar inferior. Ou seja, a quantidade de poluição atmosférica encontrada em cidades grandes é motivo de preocupação pública (WATSON, 2014).

Outras causas incluem exposição ocupacional (metais pesados e asbestos respondem por 5-10% dos casos) e fatores ambientais como decaimento de materiais radioativos. Ainda, irmãos, irmãs e filhos de pessoas que tiveram câncer de pulmão apresentam risco levemente aumentado para o desenvolvimento desse câncer. Entretanto, não se conhece nenhum fator hereditário específico e a explicação pode ser o fumo passivo ou mesmo a transmissão do hábito tabagista. Ainda, outras doenças, como a tuberculose, bronquite crônica e enfisema, aumentam o risco de câncer de pulmão, provavelmente devido à inflamação crônica do órgão (FERLAY et al., 2014; INCA, 2014; OMS, 2008).

Cenário atual

Em alguns países ocidentais, as taxas de incidência e mortalidade estão diminuindo devido a políticas anti-tabagista, e avanços terapêuticos (OMS, 2008). Porém, esta evolução é observada quase que exclusivamente na população masculina. Dentre as mulheres, a incidência e a mortalidade segue aumentando, provavelmente porque a população feminina começou a fumar mais tarde (FERLAY et al., 2014).

Tratamento

A terapia depende do estágio em que a doença é diagnosticada (Fig.4). Como muitas vezes o câncer de pulmão é detectado em estágio avançado, a quimioterapia citotóxica é a única opção viável, com sucesso limitado. Assim, a identificação de fenótipos específicos e terapias dirigidas é a esperança nestes casos (HERBST; HEYMACH; LIPPMAN, 2008).

Por muito tempo o tipo histológico (Fig.5) e o estadiamento patológico foram a única maneira de direcionar a terapia. Hoje, conhecemos mais de 30 alterações frequentes em câncer de pulmão (Fig.6), porém somente 2 delas obtiveram sucesso com terapia dirigida. Conhecido

há mais tempo, tumores com mutação em EGFR podem se beneficiar do tratamento com erlotinib, gefitinib e afatinib. O outro caso, são pacientes com mutações em ALK que podem se beneficiar de terapia com crizotinib e ceritinib. Infelizmente, muitos casos desenvolvem resistência ao tratamento em 9-12 meses (GRIDELLI et al., 2014). Ainda, terapias imunossupressoras surgiram como uma promessa otimista para diversos tipos de câncer, incluindo pulmão (SUNDAR et al., 2014). Porém, esta terapia ainda deverá se mostrar eficaz para carcinomas pulmonares e, caso seja, apenas uma parcela destes pacientes será beneficiada.

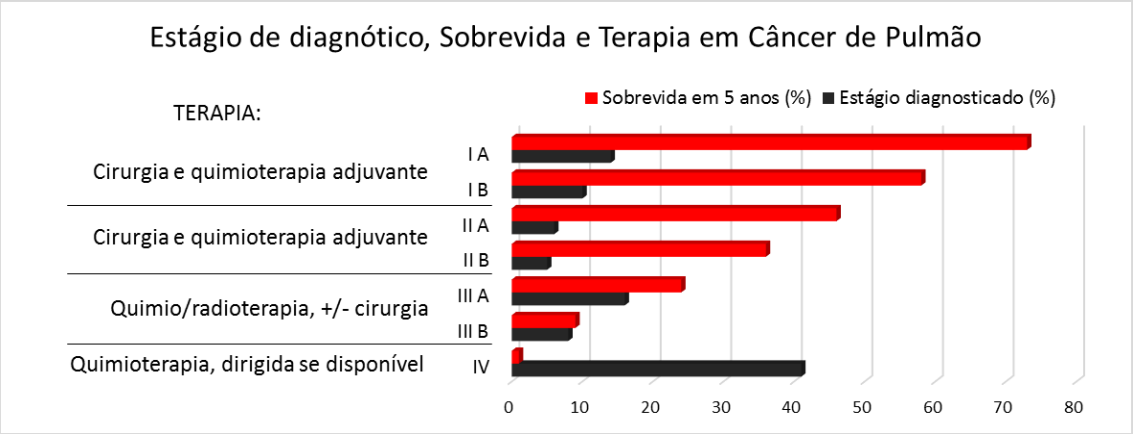


Figura 4 Estágio de diagnóstico, opções terapêuticas e sobrevida em câncer de pulmão (HEIST; ENGELMAN, 2012).

No entanto, a maior parte dos pacientes com câncer de pulmão recebem quimioterapias convencionais citotóxicas, muitas vezes pouco eficaz e com inúmeros efeitos colaterais. Portanto, a maioria dos pacientes de câncer de pulmão ainda carecem de uma melhor caracterização que possam indicar um terapia eficiente (CHEN et al., 2014; EISENSTEIN, 2014).

Tipos histológicos

Os casos de câncer de pulmão são classificados em: câncer de pulmão de pequenas-células, que abrange 15% dos casos e é o subtipo mais agressivo; sendo todos os outros coletivamente denominados câncer de pulmão de células não pequenas (NSCLC, do inglês *non-small cell lung cancer*) (Fig.5). O NSCLC, representa 85% dos casos e inclui diversos tipos histológicos, os três principais são: carcinoma escamoso (SQC, do inglês *squamous cell carcinoma*), Adenocarcinoma (AdC) e carcinoma de células grandes (LCC, do inglês *large cell*

carcinoma). Porém, NSCLC é um grupo de doenças distintas com heterogeneidades genéticas e celulares (Fig.6), cuja etiologia, tratamento e prognóstico difere muito (CHEN et al., 2014).

Os dois tipos histológicos predominantes são AdC (~50%) e SQC (~40%). Geralmente, AdC se originam nas vias aéreas mais distais. Por outro lado, os SQC se originam nas vias proximais e estão mais relacionados com o hábito de fumar. No entanto, o tabagismo está associado com todas as formas de câncer de pulmão (HERBST; HEYMACH; LIPPMAN, 2008). A incidência de adenocarcinomas têm aumentado devido à alteração na composição dos produtos contendo tabaco (como, a presença de filtros no cigarro e diferentes carcinógenos) (BURNS, 2014; HOFFMANN; DJORDJEVIC; HOFFMANN, 1997). LCC é diagnosticado por exclusão, ou seja, quando o tumor não apresenta diferenciação morfológica glandular nem escamosa e tampouco expressa marcadores moleculares dos outros dois subtipos (KERR, 2012). Entretanto, não se sabe ainda quão geneticamente diferente os LCC são dos outros subtipos (CHEN et al., 2014).

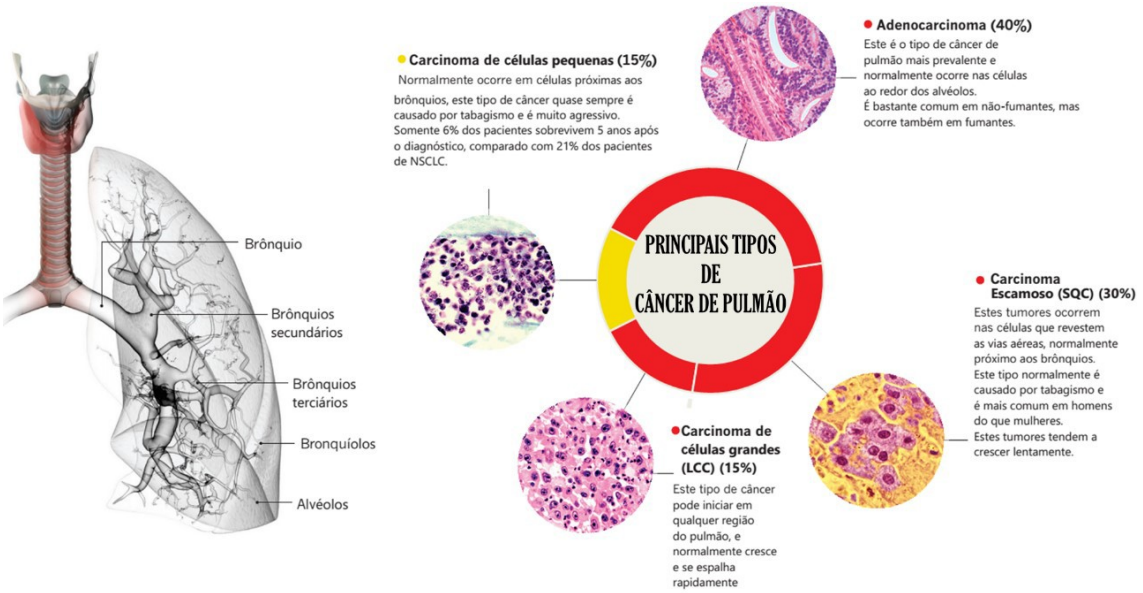
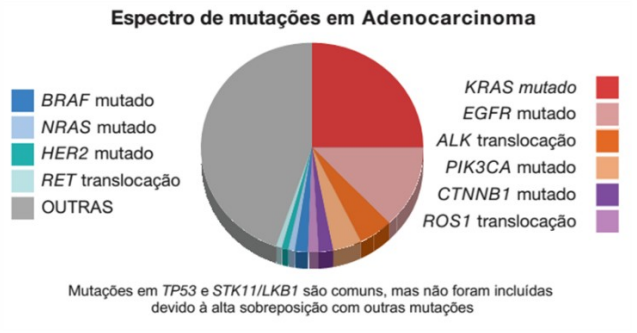
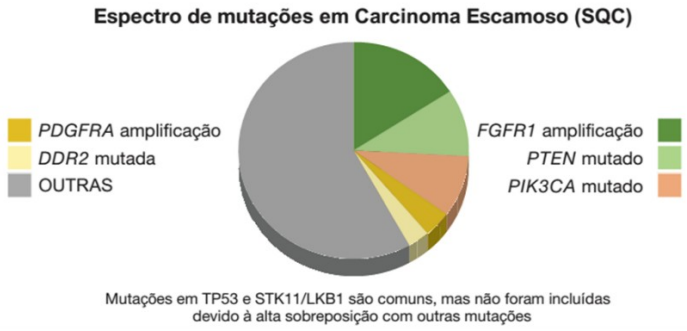


Figura 5 Principais características do tipos histológicos de câncer de pulmão (BENDER, 2014).





Alvo	Droga
EGFR	Erlotinib
	Gefitinib
	PF299804
	Afatinib (BIBW2992)
ALK	Crizotinib
	LDK378
	AP26113
	AF802
ROS1	Crizotinib
HER2	PF299804
	Afatinib (BIBW2992)
FGFR	BGJ398
	FP1039 (HGS1036)
	Ponatinib (AP24534)
FGFR/ PDGFRA/ VEGFR	BIBF1120
	Pazopanib
	Lenvatinib (E7080)
	Brivanib (BMS-582,664)
	Dovitinib (TKI258)
PDGFRA	MEDI575
	IMC-3G3
PI3K	BKM120
	PX-866
	GDC-0941
	SAR245408
PI3K/ MTOR	GDC-0980
	BEZ235
	SAR245409
MEK	MEK162
	GDC-0973
	GSK1120212
	MSC1936369B
STAT3	OPB51602
AKT	MK2206

Figura 6 Diferenças moleculares entre subtipos de câncer de pulmão e terapia dirigida para cada mutação (HEIST; ENGELMAN, 2012).

Câncer de Mama

Epidemiologia

Este é o tipo de câncer é o mais comum e o maior responsável por mortes na população feminina. Somente 1% dos pacientes são homens (MAXMEN, 2012). A sobrevida média após cinco anos é de 61%. Anualmente, mais de 1,3 milhões de mulheres são diagnosticadas com câncer de mama em todo mundo (GRAYSON, 2012). Acima dos 35 anos a incidência cresce exponencialmente e estima-se que 1 em 8 mulheres irão desenvolver câncer de mama ao longo da vida, nos EUA (MAXMEN, 2012). A incidência mais elevada é observada na América do Norte, regiões da América do Sul, incluindo Brasil e Argentina, na Europa ocidental e Austrália.

No Brasil, estima-se 57 mil casos e 13 mil mortes por câncer de mama por ano. A mortalidade no Brasil ainda é elevada devido ao diagnóstico tardio (INCA, 2014).

Sintomas e Detecção

Os sintomas mais comuns são: presença de nódulo no seio, calor, inchaço, rubor e escamação, alteração no tamanho, forma ou textura do mamilo, ocorrência de nódulo na axila, saída de secreção pelo mamilo ou inversão do mamilo para dentro da mama.

O único método de rastreio é a mamografia, a cada 2 anos em mulheres entre 50 e 74 anos ou em mulheres de qualquer idade com histórico familiar de câncer de mama. Antes desta idade, a taxa de falso positivo pode gerar intervenções e preocupações desnecessárias . Por fim, o auto-exame das mamas não é aconselhado, devido à ausência de benefício e maior ocorrência de falsos positivos em mulheres que adotam esta prática (BOURZAC, 2014b; OMS, 2008).

Fatores de Risco

Os principais fatores de risco associados com câncer de mama são*:

- Histórico familiar e mutação dos genes BRCA1/2
- Alta densidade da mama (em mamografia)
- Nuliparidade (nunca parir) e primeira gravidez tardia (> 30 anos)
- Menarca precoce (< 12 anos) e menopausa tardia (>50 anos)
- Contraceptivos orais aumenta proporcionalmente o risco (DOLLE et al., 2009)
- Ingestão de álcool regular (2-3 doses/dia)
- Reposição hormonal pós-menopausa, principalmente por mais de cinco anos
- Sedentarismo e dieta não-saudável

Por outro lado, alguns fatores estão associados com menor incidência, são eles:

- Amamentar
- Menopausa induzida

* Uma lista mais detalhada dos fatores de risco e prevenção associados com câncer de mama pode ser acessada em: http://www.pathophys.org/breast-cancer/

- Atividade física
- Para quem ingere álcool regularmente, o consumo de folato pode ser protetor (presente em grande quantidade em fígado de boi, espinafre e feijão)

Acredita-se que tanto obesidade quanto o consumo de bebidas alcoólicas predisponham ao câncer de mama através de mecanismos envolvendo controle hormonal e do metabolismo. Por fim, o consumo de tabaco e a exposição a pesticidas não apresentam relação com a incidência de câncer de mama (INCA, 2014; OMS, 2008).

Cenário atual

Nos últimos 50 anos, a taxa de sobrevida dobrou, apresentando valores em torno de 80% nos países desenvolvidos e 55% nos países em desenvolvimento (MAXMEN, 2012). O investimento na pesquisa em câncer de mama é um dos maiores, comparado com outras patologias. Atribui-se a isso a sua alta prevalência conjuntamente com campanhas públicas, como "outubro rosa". Entretanto, aproximadamente meio milhão de mulheres ainda morrem desta doença a cada ano (GRAYSON, 2012).

Tipos histológico & Terapia

O desfecho dos pacientes de câncer de mama varia muito de acordo com o subtipo. O câncer de mama é classificado quanto à expressão dos receptores de estrogênio (ER) e progesterona (PR) e superexpressão de HER2 (Fig.7). Quando o tumor expressa um ou mais destes marcadores existem terapias direcionadas (SHARP; HARPER-WYNNE, 2014). Infelizmente, 15-20% dos casos não apresentam expressão de nenhum deles. Estes últimos, são classificados como câncer de mama triplo-negativo (TNBC, do inglês *triple negative breast cancer*). Argumenta-se que "é cientificamente insano definir algo pelo que ele não é. Mas, clinicamente, é o que temos."(POWELL, 2012).

Aproximadamente 65% dos casos de câncer de mama expressam ER (ER+) e são chamados de luminal, devido à origem histológica (Fig.7). Estes casos, respondem à terapia

hormonal e apresentam o melhor prognóstico: sobrevida em 5 anos de até 85% (SANDHU et al., 2010). Outro tipo comum é o HER2+, respondendo 10-15% dos casos. HER2 é um receptor de fator de crescimento epidérmico (EGFR) envolvido na sinalização de proliferação e apoptose e é o alvo do anticorpo monoclonal trastuzumab (comercializado como herceptin®) (FIGUEROA-MAGALHÃES et al., 2014). Para ilustrar a importância desta droga, câncer de mama HER2+ não tratados era o pior prognóstico dentre todos os subtipos de câncer de mama (ROY; PEREZ, 2009).

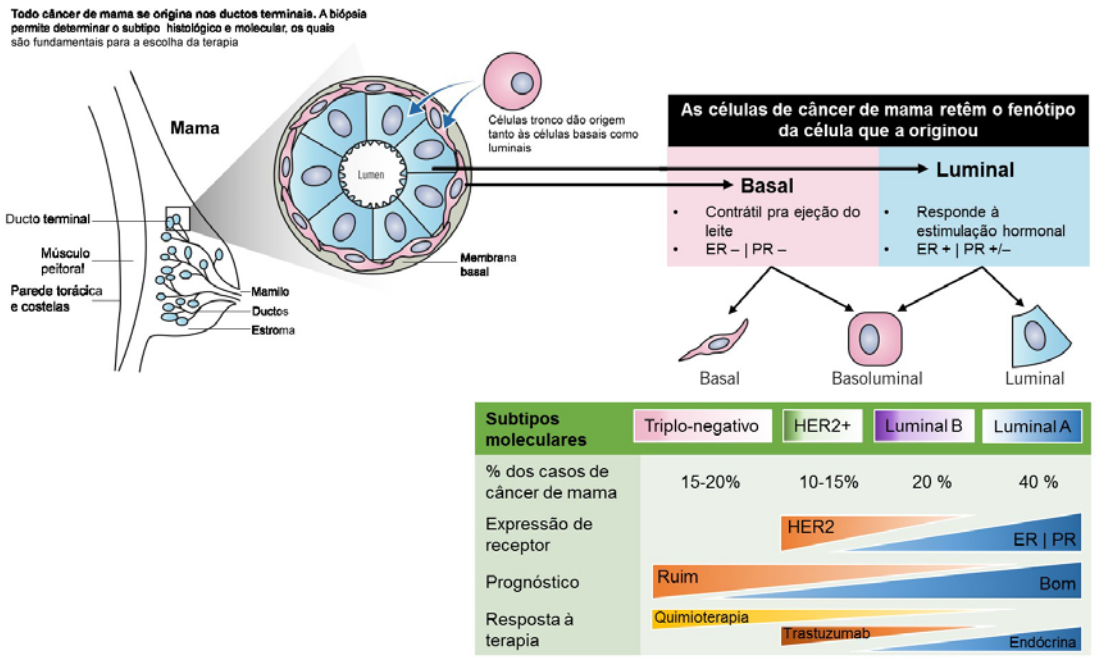


Figura 7 Representação da origem histológica dos tipos de câncer de mama e suas principais características (WONG; CHAUDHRY; ROSSI, 2015).

Por fim, temos o grupo TNBC, normalmente de origem basal (Fig.7) e que apresenta o pior prognóstico dentre os casos de câncer de mama, em decorrência da ausência de uma terapia dirigida (GRAYSON, 2012; SANDHU et al., 2010). A primeira menção a TNBC no PubMed é de 2005, desde então o número de publicações sobre o tema cresceu exponencialmente, evidenciando o reconhecimento da importância deste subtipo. TNBC abrange um grupo bastante diverso, e ainda estamos no início da caracterização molecular para identificação de subgrupos que sejam passíveis de terapia dirigida (LEHMANN et al., 2011).

Hoje sabemos que o TNBC é mais prevalente em jovens, negras e hispânicas e portadoras de mutação em BRCA. Para piorar, seu crescimento rápido e prevalência em jovens, dificulta a detecção por mamografia. Ainda, os casos de TNBC são mais propensos a metastasear para pulmões e cérebro, em oposição a outros tipos de câncer de mama que frequentemente metastaseiam para os ossos (FOULKES; SMITH; REIS-FILHO, 2010). Os primeiros ensaios clínicos em TNBC datam de 2006 e algumas alternativas promissoras começaram a aparecer.

Fármacos anti-EGFR (cetuximab) ou análogos de nucleosídeos (gemcitabina) apresentam pouca eficácia como agente único, mas a combinação com alquilantes leva a uma resposta melhor (BASELGA et al., 2013; WANG et al., 2010). O tratamento anti-angiogênico (bevacizumab) em combinação com a quimioterapia apresentou alguns efeitos benéficos, que não foram reproduzidos em estudos de fase III (ROBERT et al., 2011).

Os genes BRCA1/2 estão envolvidos em reparo de DNA, assim como a PARP (do inglês *poly ADP ribose polymerase*). Assim, o desenvolvimento de inibidores de PARP para portadoras de mutação em BRCA recebeu muito investimento. A esperança é que o nível basal de dano seria tóxico ao tumor, mas não às normais. Estas drogas foram promissores inicialmente, mas falharam em um ensaio clínico de fase III (GUHA, 2011). São aguardados estudos com outros inibidores, mais específicos *in vitro* (KELLY; BUZDAR, 2013). Além disso, estudos propondo co-tratamento com inibidor de PARP e diversos outros agentes quimioterápicos (inibidores de HDAC, cisplatina, inibidores de mTOR, inibidores de EGFR) e até mesmo radioterapia têm sido publicados (AL-EJEH et al., 2013; DE et al., 2014; HA et al., 2014; XIA et al., 2014).

Um novo composto chamado *glembatumumab vedotin (*CDX-011), apresentou resultados promissores em estudo de fase I/II. Trata-se de um composto conjugado da droga auristatina E com um anticorpo monoclonal anti-gpNMB, cujo alvo é uma glicoproteína de membrana recém descoberta, a NMB (gpNMB). Está, é super-expressa em 40-60% dos casos de

câncer de mama, promove metástase em modelos animais e é marcador prognóstico (BENDELL et al., 2014). Novos estudos estão sendo aguardados.

Outra alternativa é atacar o metabolismo do TNBC, pois este subtipo apresenta maior consumo de glicose comparado aos outros (BASU et al., 2008), indicando maior utilização da via glicolítica, provavelmente relacionado com agressividade (ver Capítulo II). O fármaco metformina é utilizado no controle da diabetes e vêm sendo investigado para tratamento de TNBC. Esta droga demonstrou afetar a proliferação, apoptose (LIU et al., 2009), angiogênese e metástase (ORECCHIONI et al., 2014) e atuar em sinergia com o quimioterápico paclitaxel (ROCHA et al., 2011). Ensaios clínicos com metformina em TNBC estão em andamento.

Apesar disto, a quimioterapia é a única alternativa na clínica, quando cirurgia e radiação não são possíveis. Porém, a mortalidade ainda é grande e não é uniforme, indicando que TNBC abrange múltiplas doenças. Portanto, é necessário uma melhor compreensão de diferentes fenótipos tumorais para uma classificação adequada e um direcionamento terapêutico correto.

Acredita-se que um mesmo subtipo pode conter diferentes perfis moleculares. Estudos encontraram pelo menos 6 assinaturas moleculares em TNBC (SORLIE et al., 2003), porém a relevância clínica destes dados é desconhecida. Assim, estudos de caracterização molecular poderão impactar profundamente a terapia de TNBC (TURNER; REIS-FILHO, 2013).

Caracterização Celular e Molecular

Hoje reconhece-se a importância de aspectos celulares e moleculares para a progressão do tumor e eficácia da terapia. Um artigo seminal definiu 6 *hallmarks*[*] do câncer (HANAHAN;

[*] Neste trabalho o termo em inglês *hallmark* será utilizado por não haver uma tradução correspondente que preserve o sentido e impacto do termo original.

WEINBERG, 2000) (Fig.8), os quais, segundo os autores resume a complexidade do câncer em princípios básicos que ajudam a entender a carcinogênese e direcionar a terapia.

São eles: (1) auto-suficiência em sinais proliferativos; (2) insensibilidade a sinais anti-proliferação; (3) evasão de apoptose; (4) potencial replicativo ilimitado; (5) indução de angiogênese; e (6) invasão e metástase. Apesar da vasta aceitação (é o artigo mais citado do periódico *Cell* com mais de 15 mil citações em uma década), a relevância terapêutica dos *hallmarks* é questionada, uma vez que apenas a invasividade é exclusiva de tumores malignos, sendo os outros 5 existentes também em tumores benignos (LAZEBNIK, 2010). Mais tarde, os autores revisaram o trabalho e incluíram mais 2 *hallmarks* e 2 fatores possibilitadores (HANAHAN; WEINBERG, 2011) (Fig.8), são eles: (1) reprogramação metabólica; (2), evasão ao sistema imune; (3) instabilidade genômica; e (4) inflamação crônica. Estes trabalhos permitiram uma melhor compreensão dos processos envolvidos na carcinogênese e permitiram direcionar o desenvolvimento de fármacos capazes. Hoje, podemos atacar farmacologicamente todos os *hallmarks* (Fig.8), muito embora isso não signifique que somos capazes de curar os pacientes.

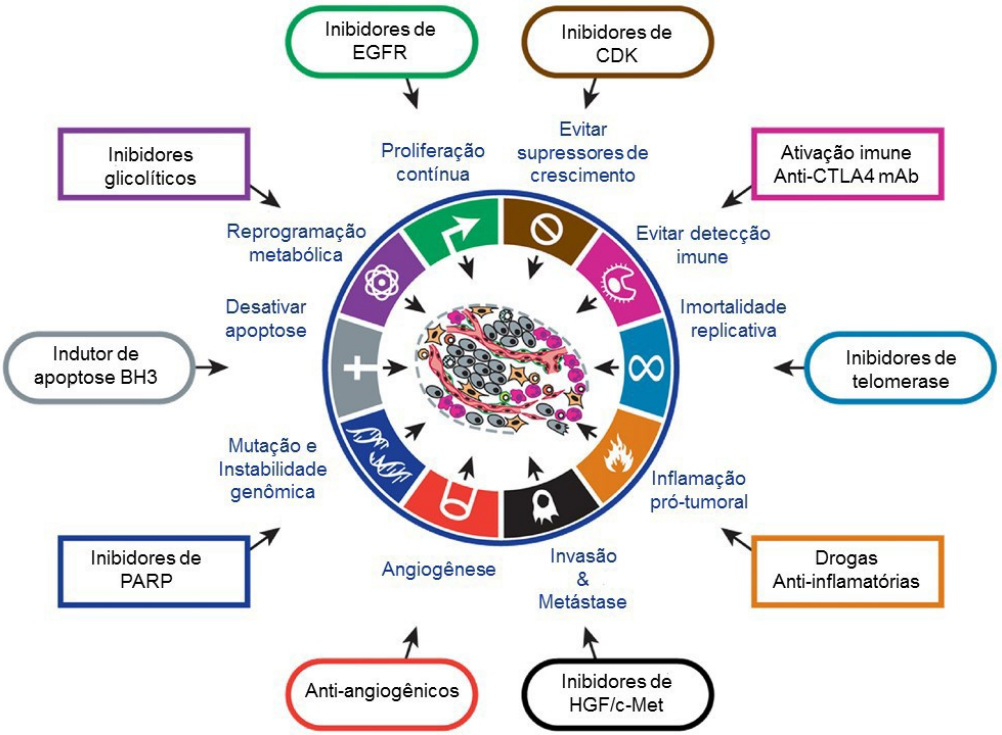


Figura 8 *Hallmarks* do câncer e possibilidades terapêuticas (HANAHAN; WEINBERG, 2011).

Dificuldade de Cura & Complexidade da doença

O conhecimento de causas e intervenções terapêuticas é extenso e não para de crescer sob financiamento multimilionários ao redor do globo. Estudos de *triagem* para descoberta de novas drogas capazes de tratar diferentes neoplasias são extremamente ineficientes (DAHLIN; INGLESE; WALTERS, 2015; PAUL et al., 2010), mas mesmo assim todo ano centenas de trabalhos com esse tipo de abordagem são publicados em todo o mundo (EDWARDS et al., 2009; HARVEY; EDRADA-EBEL; QUINN, 2015). Ainda, o câncer é a doença que tem maior mobilização popular e arrecadação de doações, mesmo não sendo a mais letal. Entretanto, como o câncer ainda é capaz de matar mais de 8 milhões de pessoas anualmente?

Ineficiência das terapias atuais

Obviamente estamos diante de um problema complexo. Primeiro, por muito tempo procuramos uma cura única para uma centena de doenças. Depois, o desenvolvimento de uma terapia dirigida enfrenta diversas barreiras como limitações farmacológicas ou fisiológicas. Por fim, o entendimento fisiopatológico do câncer ainda tem muitas lacunas (BOURZAC, 2014a). Mas esta lamentação não responde nossa questão.

Na verdade, a medicina atual é muito boa em curar cânceres quando eles são detectados cedo e classificados corretamente (OMS, 2015). Infelizmente, muitos casos de câncer são diagnosticados em estágios avançados. Dois fatores contribuem para este fenômeno: a natureza assintomática da doença; e a negligência do paciente aos sintomas iniciais. Para reverter este quadro, devemos investir em biomarcadores diagnósticos, sensibilidade das técnicas atuais e ampla divulgação de sintomatologia. Porém, temos que ter uma melhor alternativa para aqueles pacientes que mesmo assim sejam diagnosticados tardiamente.

Para estes casos, o foco deve ser na correta classificação e na compreensão do comportamento dos tumores agressivos. Quanto à correta classificação dos tumores, na prática

o estadiamento clínico se baseia majoritariamente na anatomia, histologia e morfologia do tumor ou mesmo no histórico do paciente (DEWEERDT, 2014), ignorando a heterogeneidade molecular, mesmo sabendo que este direciona diferentes comportamentos em tumores com a mesma classificação. Quanto à compreensão do comportamento de tumores agressivos, já conhecemos diversos fatores que impedem uma melhor resposta às terapias disponíveis:

1. **Os *hallmarks* são fisiológicos.** Estudos pré-clínicos prevêem uma especificidade não observada na prática. Isto não surpreende, pois todos os *hallmarks* são mecanismos fisiológicos. O problema reside no tipo celular e no momento fisiológico que estes comportamentos são expressos. Desta forma, as terapias dirigidas são limitadas pela toxicidade às células saudáveis (CHEN et al., 2014).

2. **Compensação.** Ao atacar uma rota de sinalização, a célula é capaz de induzir o mesmo comportamento por outra via (HANAHAN; WEINBERG, 2011). Assim, mesmo inibindo o alvo desejado, a terapia pode não ter a eficácia esperada.

3. **Heterogeneidade intratumoral.** O câncer não é uma massa de clones, mas uma população de diferentes células (GERLINGER et al., 2012). Ainda, existem células saudáveis que favorecem a agressividade (HANAHAN; WEINBERG, 2011). Fibroblastos, células endoteliais e mielóides controlam a composição da matriz extracelular, angiogênese, resposta imune, proliferação e morte tecidual e sua associação com tumores predizem pior prognóstico (CHEN et al., 2014). Assim, a heterogeneidade prediz não somente que nem todas as células responderão à terapia, mas também que pode haver uma seleção (ROBERTSON-TESSI et al., 2015), de modo a facilitar que o tumor reincida (Fig.9).

4. **Resistência a drogas.** Muitas vezes, os benefícios do quimioterápico são temporários, e os tumores desenvolvem resistência. Assim, quando o tumor

reincide, a terapia não funciona mais (DEWEERDT, 2014). Esta resistência pode surgir por adaptação ou por seleção, neste o tumor reincidente seria a "vitória evolutiva" de uma minoria resistente (EISENSTEIN, 2014) (Fig.9).

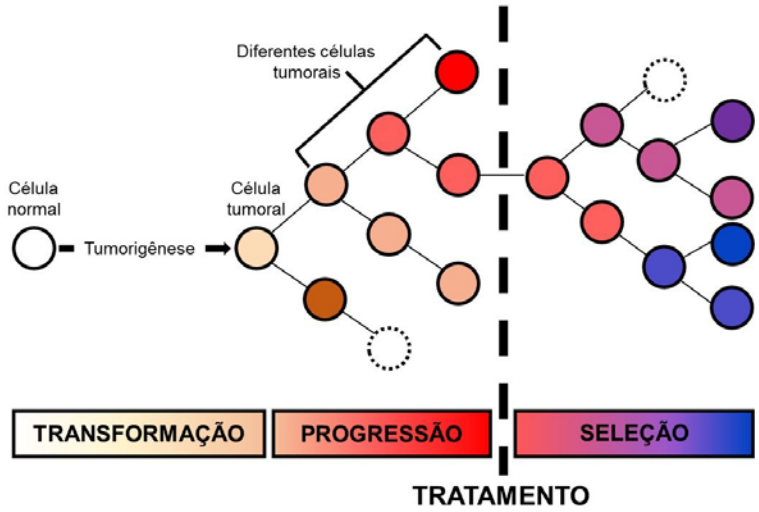


Figura 9 Representação da evolução tumoral, com acúmulo de alterações, heterogeneidade e seleção.

Aprendemos o suficiente sobre a biologia do câncer para saber que combatê-lo com coquetéis quimioterápicos que atacam indiscriminadamente células proliferativas é pouco eficiente. Os processos descritos acima ajudam a explicar porque falhamos tantas vezes até o momento na busca da cura do câncer.

Fenótipos agressivos

Hoje conhecemos muitos fatores associados a um fenótipo agressivo, desde alterações gênicas até comportamentos celulares. Porém, as mutações muitas vezes não têm valor terapêutico apreciável, apesar do seu inestimável acréscimo para a compreensão da biologia tumoral. Temos exemplos de sucesso, como o trastuzumab (Herceptin®), para câncer de mama HER2+, e o imatinib (Gleevec®), para leucemia mielóides crônica positiva para o cromossomo

Filadélfia. Por outro lado, temos mutações bem conhecidas que são não-drogáveis*, e, portanto, inúteis do ponto de vista terapêutico. Este é o caso da família Ras e da proteína p53: eles são, respectivamente, o oncogene e o supressor tumoral mais prevalente e bem estudado e, apesar do esforço investido na busca de terapias (KHOO et al., 2014; LEDFORD, 2015), até o momento nenhuma abordagem foi bem sucedida (EISENSTEIN, 2014).

No entanto, distintas alterações convergem a um mesmo fenótipo, o qual prediz progressão tumoral (SANDHU et al., 2010), mesmo que não se saiba qual mutação seja a responsável (GRAVITZ, 2014). Assim, a correta identificação dos comportamentos associados a um fenótipo agressivo, pode levar ao desenvolvimento de novas e melhores abordagens terapêuticas (GRAVITZ, 2014; LÓPEZ-LÁZARO, 2010) (Fig.10).

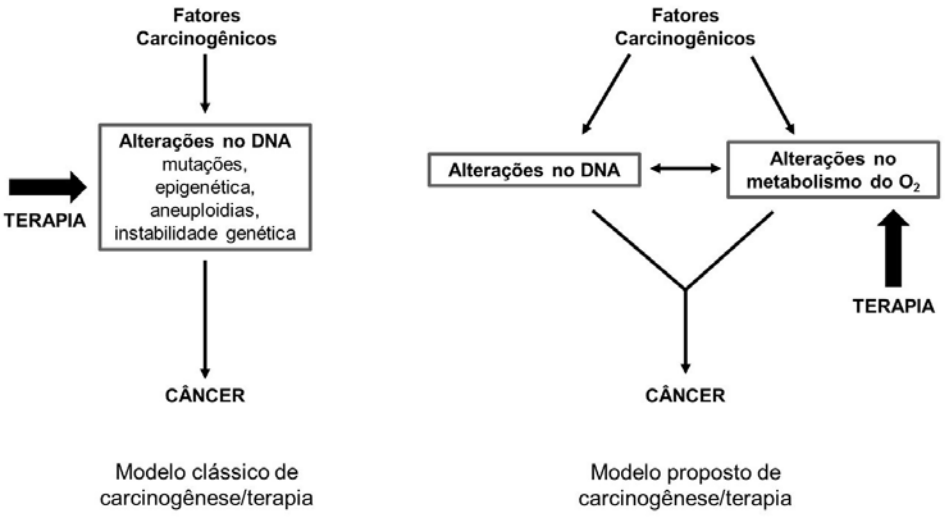


Figura 10 Proposta de atacar terapeuticamente o fenótipo agressivo (LÓPEZ-LÁZARO, 2010).

Um bom exemplo desta abordagem são os TKI'S (do inglês *tyrosine kinase inhibitors*) gefitinib e erlotinib. Inicialmente, a maioria dos casos de NSCLC tratados com TKI's não apresentavam boa resposta. Ao estudar os casos onde a droga era eficiente, descobriu-se que TKI's são eficazes especificamente contra carcinomas com hiperativação de EGFR (do inglês,

* Drogabilidade é a possibilidade de algo ser modulado farmacologicamente. Acredita-se que somente 10% do genoma humano seja drogável (http://www.nature.com/nrd/journal/v6/n3/full/nrd2275.html).

epidermal growth fator receptor), comum especialmente em adenocarcinomas e pacientes não fumantes (LYNCH et al., 2004). Assim, testes para alterações em EGFR tornou-se rotina no direcionamento terapêutico de câncer de pulmão (DEWEERDT, 2014). Ou seja, a identificação da mutação-chave veio posteriormente e direciona a terapia.

Portanto, levando em consideração o impacto da doença e a dificuldade em criar novas terapias baseadas em mutações, a caracterização de fenótipos agressivos contribuirá para a evolução da oncologia.

OBJETIVO & JUSTIFICATIVA

O câncer ainda é umas das maiores causas de mortalidade da humanidade, apesar de muito investimento e esforço. Por ser considerada uma doença genética, ou da expressão gênica (ver Introdução página 11), é natural que muitas abordagens terapêuticas tenham focado em mutações. Porém, o número de mutações existentes – aproximadamente 5 milhões (ALEXANDROV et al., 2013) – torna esta tarefa muitas vezes inglória. Para piorar, muitas vezes uma mutação relevante tem pouca utilidade terapêutica (EISENSTEIN, 2014).

Entretanto, muitas alterações convergem para um mesmo fenótipo (HANAHAN; WEINBERG, 2011). Alguns destes, já possuem terapias direcionadas eficazes, como a inibição dos promotores de proliferação EGFR por trastuzumab ou gefitinib. Assim, a alteração do prisma para o estudo de fenótipos agressivos é uma alternativa promissora para a evolução da oncologia (LÓPEZ-LÁZARO, 2010).

Portanto, o objetivo do presente trabalho foi investigar fenótipos agressivos em tumores sólidos, buscando caracterizar alterações e propor alvos e terapias.

Neste contexto, diferentes aspectos biológicos são promissores e cada capítulo deste trabalho focou em um fenótipos agressivo :

- **Capítulo I: Metabolismo Redox** (DE OLIVEIRA; AMOÊDO; RUMJANEK, 2012; JORGENSON; ZHONG; OBERLEY, 2013)
- **Capítulo II: Reprogramação Metabólica** (WARD; THOMPSON, 2012)
- **Capítulo III: Hipóxia e Epignética** (MCINTYRE; HARRIS, 2015; WARD et al., 2013)

Os objetivos específicos serão apresentados em cada capítulo.

PARTE II

CAPÍTULO I: METABOLISMO REDOX

Artigos publicados:

1 – Imbalance in redox status is associated with tumor aggressiveness and poor outcome in lung adenocarcinoma patients.

J Cancer Res Clin Oncol. 2014 Mar;140(3):461-70. doi: 10.1007/s00432-014-1586- 6. Epub 2014 Jan 22.

Periódico: Journal of Cancer Research and Clinical Oncology.

Fator de Impacto (JCR-2015): 3.009

Qualis: B1 (Ciências Biológicas, Medicina)

2 – Oxidative stress associates with aggressiveness in lung large-cell carcinoma.

Tumor Biol. 2015 Feb 1. [Epub ahead of print]

Periódico: Tumor Biology

Fator de Impacto (JCR-2015): 2.840

Qualis: B1 (Ciências Biológicas, Farmácia, Medicina)

OBJETIVO GERAL DO CAPÍTULO

Caracterizar o perfil redox e relacioná-lo com agressividade em 4 linhagens de NSCLC. Posteriormente, validar os achados em coortes clínicas e propor abordagens terapêuticas.

OBJETIVOS ESPECÍFICOS

- Avaliar em linhagens celulares:
  - Agressividade celular: invasividade e quimioresistência
  - Expressão da rede HAG (do inglês *Human Antioxidant Genes*)
  - Perfil redox: atividade de enzimas antioxidantes, lipoperoxidação, sulfidrilas reduzidas, potencial antioxidante total e produção de espécies reativas (ROS).
  - Resposta à modulação do ambiente redox
- Avaliar em coortes clínicas:
  - Expressão da rede HAG (do inglês *Human Antioxidant Genes*)
  - Níveis do estresse oxidativo 4-HNE e seu valor prognóstico
  - Valor prognóstico dos genes alterados nas linhagens

FENÓTIPO AGRESSIVO CARACTERIZADO

Sugerimos que NSCLC agressivos possuem um desbalanço redox favorecendo o estresse oxidativo intracelular.

PROPOSTA TERAPÊUTICA

A reversão do referido desbalanço pode afetar a progressão tumoral, por retirar-lhe um combustível que alimenta mutações, proliferação e migração, as espécies reativas (ROS).

Estes achados incentivaram uma dissertação de mestrado que demonstrou efeitos sinérgicos da combinação do antioxidante catalase com quimioterápicos (ver Anexo A).

Artigo 1

J Cancer Res Clin Oncol
DOI 10.1007/s00432-014-1586-6

ORIGINAL PAPER

Imbalance in redox status is associated with tumor aggressiveness and poor outcome in lung adenocarcinoma patients

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Received: 12 November 2013 / Accepted: 12 January 2014

Abstract
Purpose The expression levels of human antioxidant genes (HAGs) and oxidative markers were investigated in light of lung adenocarcinoma aggressiveness and patient outcome.
Methods We assayed in vitro the tumoral invasiveness and multidrug resistance in human lung adenocarcinoma (AdC) cell lines (EKVX and A549). Data were associated with several redox parameters and differential expression levels of HAG network. The clinicopathological significance of these findings was investigated using microarray analysis of tumor tissue and by immunohistochemistry in archival collection of biopsies.
Results An overall increased activity (expression) of selected HAG components in the most aggressive cell line (EKVX cells) was observed by bootstrap and gene set enrichment analysis (GSEA). In vitro validation of oxidative markers revealed that EKVX cells had high levels of oxidative stress markers. In AdC cohorts, GSEA of microarray datasets showed significantly high levels of HAG components in lung AdC samples in comparison with normal tissue, in advanced stage compared with early stage and in patients with poor outcome. Cox multivariate regression analysis in a cohort of early pathologic (p)-stage of AdC cases showed that patients with moderate levels of 4-hydroxynonenal, a specific and stable end product of lipid peroxidation, had a significantly less survival rate (hazard ratio of 8.87) ($P < 0.05$).
Conclusions High levels of oxidative markers are related to tumor aggressiveness and can predict poor outcome of early-stage lung adenocarcinoma patients.

Keywords Oxidative stress · Tumor progression · 4-Hydroxynonenal · Catalase · Lung cancer · Hydrogen peroxide

Electronic supplementary material The online version of this article (doi:10.1007/s00432-014-1586-6) contains supplementary material, which is available to authorized users.

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Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide, presenting in many countries a mortality rate that outranks prostate, colorectal and pancreatic cancer cases combined (Siegel et ai. 2013). Despite recent advances in therapy protocols (Coate et ai. 2009), 5-year survival rates of advanced stages remain as low as 2 % (Siegel et ai. 2013). Thus, it is essential to uncover any biological processes or molecular mechanisms associated with the initiation and progress of the aggressive malignant phenotype of lung cancer cells in order to identify potential targets for novel interventional strategies.

Lung tumor cells are able to grow in a highly oxidative environment (Melloni et ai. 1996; Brennan et ai. 2000; Ho et al. 2007; Esme et ai. 2008; Chan et ai. 2009), which is believed to contribute with tumor progression and metastasis (Sotgia et ai. 2011). Lung tumors are especially exposed to a pro-oxidative milieu due to the factors such as tobacco smoke, high atrnospheric oxygen pressure (Rahman et al. 2006) and the bulk amount of reactive species (RS) generated by pro-inftammatory cells in the pulmonary circulation (IIonen et ai. 2009). Moreover, clinicai data have shown that lung cancer patients have increased oxidative stress markers in peripheral blood (Esme et ai. 2008), erythrocytes (Kaynar et ai. 2005; Ho et ai. 2007), epithelium lining ftuid (Melloni et ai. 1996), breath condensate (Chan et ai. 2009) and tumor biopsies (Jaruga et ai. 1994; Coursin et ai. 1996; Blair et ai. 1997), and the inadequate ingest of antioxidants constitutes a risk factor for lung cancer development (Brennan et ai. 2000). Although pulmonary cells have these reductionl oxidation (redox) particularities, the inftuence of oxidative stress in lung cancer biology is poorly understood.

The present study focused on redox state and cancer cell growth in vitro, comparing a more aggressive human lung AdC cell line (EKVX) to a less aggressive one (A549). We found that the most aggressive AdC cell line presents high leveis of oxidative stress markers. Follow-up experiments indicate that this oxidative imbalance may support malignant features of AdC cells and has clinicai significance, since we found that human antioxidant gene (HAG) components and 4-hydroxynonenal (4-HNE) leveis have prognostic value in predicting lung AdC patient outcome.

Materiais and methods

Cell lines and chemicals

The human lung adenocarcinoma cell lines A549 and EKVX were obtained from NCI-Frederick cell line repository. Exponentially growing cells were maintained in RPMI 1640 medium (Invitrogen®) containing 10 % fetal bovine serum, ❙ J.g/mL of amphotericin B and 50 11-g!L of garamycin at 37 0C in a humidified atmosphere of 5 % of co2. Protein concentration was deterrnined by Lowry's method. Chernicals were obtained from Sigma® Chemical Co.

Cellular aggressiveness and redox parameters

The BioCoat Matrigel Invasion Chamber System (BD Bioscience®) was used to access the invasion index. Briefty, cells were seeded in the upper wells, while the chemoattractant (medium RPMI with 10 % of SFB) was added to the lower wells. After 22-h incubation, the trans-well movement of cells through the pore was determined. Cells that penetrated to the underside surfaces of the inserts were fixed and stained with HEMA 3 staining kit (Fisher Scientific®) and counted under the microscope. Cells were considered *migratory* when moved through uncoated pores and *invasive* when moved through Matrigel-coated pores. Data are expressed as the percentage of invasive/migratory and expressed as "invasion index."

Multidrug resistance was determined based on drug dose-response curves of Cisplatin, Carboplatin, Daunorubicin, Doxorubicin, 5-Fluorouracil, Hydroxyurea and Taxol (Sigma® Chemical Co.) using the sulforhodamine B (SRB) assay, following NCI-60 protocol (Vichai and Kirtikara 2006).

Superoxide dismutase (SOD) (E.C. 1.15.1.1) activity was measured by inhibition of superoxide-dependent epinephrine auto-oxidation at 480 nm (Misra and Fridovich 1972). Catalase (CAT) (E.C. 1.11.1.6) activity was measured by H_2O_2 consumption at 240 nm. Glutathione peroxidase (GPX) (E.C. 1.11.1.9) activity was measured by NADPH oxidation at 340 nm (Wendel 1981).

Nonenzymatic antioxidant potential was determined by total radical-trapping antioxidant potential (TRAP) assay (Dresch et ai. 2009). Elman's sulfhydryl group (-SH) levei was determined with 5-thio-2-nitrobenzoic acid at 412 nm (E412 nm = 27,200/M em) and expressed as nmol -SH/mg protein. Thiobarbituric acid reactive species (TBARS) assay was used as a lipoperoxidation index. TBARS were assayed at 532 nm and expressed as nmol MDA equivalents/mg protein. DCF-DA (2',7'-dichlorodihydroftuorescein diacetate) oxidation and Amplex Red® were used to determine intracellular generation of RS in a 96-well plate reader (Spectra Max GEMINI XPS, Molecular Devices®).

Proliferation and growth inhibition assays

Cells were treated with active or heat-inactivated Catalase, Trolox® or N-acetylcysteine (NAC). Cell growth inhibition was evaluated for 72 h with SRB assay. To investigate whether the growth inhibition by CAT was reversible, the assay was repeated removing CAT. The effect of bolus

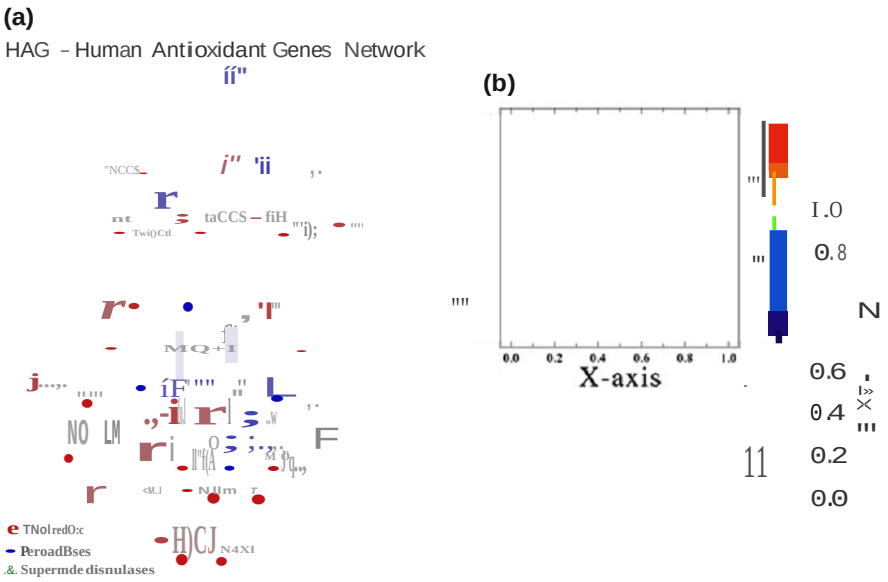

Fig. 1 Differential gene expression of HAG network in human lung AdC cell lines. a STRING representation of HAG network gene interactions. b Two-state landscape analysis of HAG expression between cell lines (GSE5846 dataset). Coordinates (X- and Y-axis) represent normalized values of the input network topology. *Color gradient* (Z-axis) represents the relative functional state mapped onto graph according to the data input from the adenocarcinomas EKVX-a versus A549-b, where $z = a/(a + b)$. The landscape was generated with ViaComplex® V1.0

amount of H_2O_2 addition or CAT inhibition with aminotriazole on cell growth was also evaluated.

Human antioxidant gene (HAG) network and microarray datasets

The HAG was designed to cluster functional gene network to facilitate high-throughput analysis of redox processes (Gelain et al. 2009). HAG is composed of 63 genes whose products are thiol-containing proteins or enzymes that react directly with RS and was subclassified into three functional groups: peroxidases, superoxide dismutases and thiol-containing redox proteins (Fig. 1a).

Microarray expression profiles were extracted from the gene expression omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/). For the human lung AdC cell lines, we used GSE5846 dataset. For cohort analysis, we used three microarray datasets: 21 surgically resected tumor tissue and adjacent normal tissue from primary NSCLC patients, with histological and staging details (GSE21933); 269 NSCLC specimens containing histological, staging, survival and recurrence details (GSE41271); and 174 NSCLC specimens containing histological, staging and survival details (GSE42127).

Differential gene expression and enrichment analysis

Differential gene expression was evaluated using ViaComplex® software (Castro et al. 2009, 2010). Gene set enrichment analysis (GSEA) was used to identify genes that contribute individually to global changes in expression levels in a given microarray dataset (Subramanian et al. 2005).

Retrospective cohort and immunohistochemistry

Formalin-fixed paraffin-embedded lung AdC tumors from patients diagnosed between 1998 and 2004 were obtained from the Pathology Service at the Santa Casa de Misericórdia de Porto Alegre (Porto Alegre, Brazil) (Sánchez et al. 2006). The pathological diagnoses were reviewed and classified by two independent pathologists, according to World Health Organization criteria. Inclusion criteria were lung adenocarcinomas as primary tumor and clinical follow-up data of at least 5 years available. Gender, age, height, weight, histology, pathological stage, smoking history and lung function information were collected. The research program was approved by the local Research Ethics Committee (#1852/08). The Helsinki Declaration of Human Rights was observed when performing these experiments, and written informed consent was provided.

Sections of 4 μm were deparaffinized and rehydrated, antigen retrieval was performed by pepsin (Zytovision®), endogenous peroxidase was blocked with 5 % H_2O_2 in methanol, and nonspecific blocking was done with 1 % bovine serum albumin (BSA). The slides were incubated overnight at 4 °C with rabbit polyclonal antibody against 4-hydroxynonenal (4-HNE) (Abcam ab46545) 1:1,200 in 1 % BSA and rinsed and incubated with HRP-labeled-polymer-conjugated kit (Invitrogen®). Sections were counterstained with hematoxylin. Negative control was obtained performing the same protocol without the primary antibody. The assessment of immunostaining intensity was performed semiquantitatively and in a blinded fashion (0 = no staining; 1 = weak staining; 2 = moderate staining; and 3 = intense staining) (Rahman et al. 2002).

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Table 1 In vitro evaluation of tumoral aggressiveness and redox parameters in human lung AdC celllines

	Human lung AdC cell !ines		p
	A549	EKVX	
Invasiveness (invasive/migratory cells)			
Invasion index	3.70 ± 1.2	19.22 ± 1.8***	0.0004
Multidrug resistance (GI_{50} value) (1.1.M)			
Cisplatin	3.04 ± 0.14	4.94 ± 0.13***	<0.0001
Carboplatin	81.9±9.1	152.8 ± 16.5**	0.0093
Daunorubicin	0.052 ±0.1	0.408 ± 0.06**	0.0012
Doxorubicin	0.07 ± 0.01	0.64 ± 0.04***	<0.0001
5-Fiuorouracil	5.18±1.1	355.7 ± 35***	<0.0001
Hydroxyurea	366.5 ±53	2425 ± 435**	0.0034
Taxo!	0.019 ± 0.1	0.203 ± 0.06*	0.0256
Antioxidant enzyme activities			
SOD (U/mg)	27.7 ± 1.3	33.2 ± 0.7*	0.0201
CAT (U/mg)	1.05 ± 0.10	0.62 ± 0.04**	0.0070
GPX (U/mg)	1.73 ± 0.21	0.89 ± 0.16*	0.0129
Nonenzymatic parameters TBARS			
(nmol eq.MDA/mg) Sulfhydryl	0.43 ± 0.09	1.05 ± 0.08**	0.0015
groups (1.1.mol –SH/mg) Total	250± 4.7	194 ± 11.4***	0.0010
antioxidant potential (TRAP)	0.73 ± 0.04	0.46 ± 0.07*	0.0234
Reactive species production			
DCF oxidation (RFU/min/10^6 cells)	28.2 ± 2.6	40.3 ± 1.8*	0.0196
Amplex Red® (RFU/min/10^6 cells)	19.9 ± 2.9	36.5 ± 2.6**	0.0056

Data represent mean ± SEM of at least four independent experiments ($n = 4$). Invasion index is defined as "number of invading cells/number of migrating cells" as described in "Materiais and methods." TRAP unit is expressed as "1-AUC" where high values correspond to higher antioxidant potential. Differences between both cells considered statistically significant when * ($P < 0.05$); ** ($P < 0.01$); *** ($P < 0.001$) (Student's t test)

SOD superoxide dismutase, *CAT* catalase, *GPX* glutathione peroxidase, *TBARS* thiobarbituric acid reactive species, *MDA* malondialdehyde

Statistical analysis

Data are expressed as mean ± SEM of at least three independent experiments carried out in triplicate, and Student's t test was used ($P < 0.05$) (GraphPad® Software 5.0). Multivariate Cox proportional hazards regression models were used to test the independent contribution of each variable on mortality, and the results were summarized by calculating hazard ratios (HR) and corresponding 95 % confidence intervals. Chi-squared test was used to assess the independence of the staining groups related to the cohort baseline characteristics. A chi-squared approximation for low-frequency groups was obtained using Monte Carlo simulated P values, based on 2,000 replicates in R (http://www.R-project.org/).

Results

Cellular aggressiveness, HAG activity and oxidative stress markers

In vitro analysis of basal invasion index and multidrug resistance was used to establish the aggressiveness between two human lung adenocarcinoma cell !ines (Table 1). Comparing cells, EKVX presents fivefold higher invasive potential ($P = 0.0004$) and a significant cross-resistance to ali seven drugs evaluated (range of 1.61- to 68.66-fold increase in drug GI_{50} values) and was established as the most aggressive cellline.

In addition, we assayed the cellular redox status of both cell !ines by analyzing HAG network activities. To do that, landscape maps of gene expression were built (Fig. 1a), and using the open-source software ViaComplex®, we observe that EKVX cell line has increased expression of HAG components ($P = 0.016$) (Fig. 1b). The specific genes that contribute to this difference, obtained by GSEA analysis, are summarized in Supplementary Table I. As an example, metallothioneins (1E/F/H/X and 2A), the mitochondrial SOD (SOD2) and components of the thioredoxin system (TXN2/TXNIP/TXNRD2) were found to be upregulated in the most aggressive phenotype. To further validate the differences obtained with microarray data, several redox parameters were accessed in vitro in our AdC cell panel (Table I). We found a significant imbalance in antioxidant enzyme (AOE) activities between cell !ines, more specifically an increased SOD activity with a concomitant decrease in CAT/GPX activities, suggesting H_2O_2 accumulation in EKVX cells. Consistent with this, EKVX cell line generates higher *steady-state* leveis of RS and H_2O_2 (Table I). In addition, regarding nonenzymatic parameters, basal lipoperoxidation (TBARS leveis) is twofold

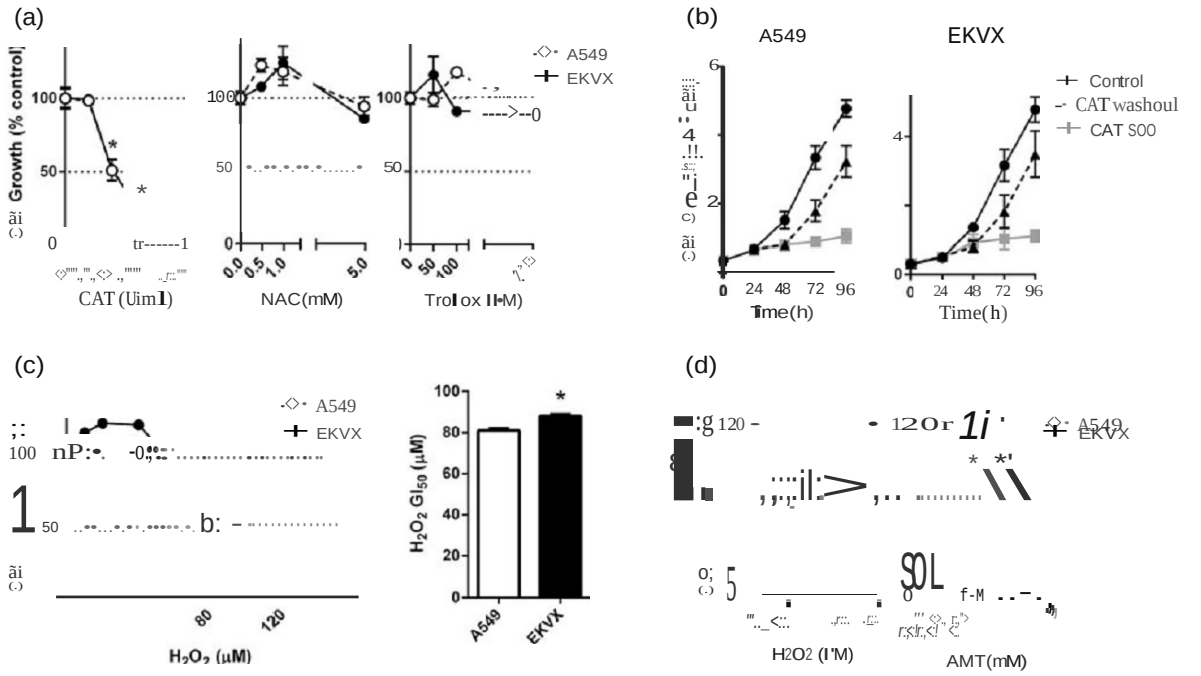


Fig. 2 Cell growth is dependent on H_2O_2 in human lung adenocarcinoma cell lines. a Exogenous addition of catalase (125-1,000 U/mL) for 72 h causes dose-dependent inhibition in cell proliferation of AdC cell lines. b CAT washout after 48 h of incubation allowed cells to return to its original proliferation rate. c Dose-response curve against H_2O_2 lung AdC cell lines. d Sublethal doses (<40 11-M) of H_2O_2 and aminotriazole stimulate cell growth on EKVX. Data represent mean ± SEM of at least three independent experiments ($n = 3$), performed in triplicate *differen t from respective control group ($P < 0.05$) (Student's t test)

higher and there is a significant decrease in total antioxidant capacity (TRAP) and reduced sulfhydryl leveis (-SH) in EKVX cells. Therefore, high leveis of intracellular oxidative markers might be associated with cellular aggressiveness in AdC cells, possibly due to the combination of imbalance in AOE activities and high RS generation.

Intracellular oxidative state modulates the proliferative rates of lung AdC cell lines

To deepen the question whether oxidative state influences cellular aggressiveness or is a by-product, we evaluated the effect of antioxidant treatments in the growth rates of human lung AdC cells. Exogenous addition of CAT specifically decreases H_2O_2 leveis since this oxidant diffuses through membranes (Policastro et ai. 2004) and caused a dose-dependent inhibition of both lung AdC cells' growth (Fig. 2a). CAT washout restored proliferation rate of cells (Fig. 2b), arguing that CAT addition caused a cytostatic (not cytotoxic) effect, since there was no decrease in cell viability (data not shown). This phenomenon seems to be specifically related to H_2O_2 scavenging and not to a general antioxidant effect, since Trolox® (synthetic analog of alpha-tocopherol) and N-acetyl-cysteine (NAC, a glutathione precursor) treatment did not inhibit cell proliferation (Fig. 2a). Although the proliferative rates of A549 and EKVX were equally inhibited by CAT treatment, a dose-response curve showed that the most aggressive AdC cell line presents a significantly higher resistance to H_2O_2 toxicity (increase in GI_{50} value) ($P < 0.05$) (Fig. 2c). Moreover, sublethal doses of H_2O_2 (<40 -tM) and aminotriazole (a specific catalase inhibitor) can consistently enhance the proliferative rates in EKVX cells (Fig. 2d). Collectively, these data suggest that an intracellular pro-oxidative state accompanies tumor progression and H_2O_2 plays a major role in cellular aggressiveness in lung AdC.

HAG is up-regulated in clinicallung adenocarcinoma samples

In order to access the clinicai value of the redox imbalance found in vitro, we analyzed differential gene expression leveis of HAG components using severa! microarray datasets derived from human cohorts of lung AdC samples (Table 2). Even though we found collectively a significant difference in HAG activity between groups, the contributions of each subgroup of HAG components (e.g., peroxidases, thiol-containing proteins and superoxide

Table 2 Gene set enrichment analysis of the HAG network in different human lung adenocarcinoma cohorts

GEOID	Cohort description	Experimental groups	Enrichment analysis (adjusted *P* value)			
			HAG	PER	Thiol	SOD
GSE21933	21 tumor tissues and adjacent normal tissue	Tumor versus normal tissue	0.335	0.022*	0.781	0.096
GSE41271	269 lung cancer specimens	Advanced versus early stages•	0.030*	0.538	0.016*	0.032*
GSE42127	174 lung cancer specimens with clinicopathological information	Dead versus aiive	0.014*	0.502	0.005**	0.772

Transcript profiles of human lung adenocarcinoma patients were obtained from GEO. Nominal *P* value of enrichment analysis obtained from GSEA ($P < 0.05$)

HAG human antioxidant gene network, *PER* peroxidase gene set, *Thiol* thiol-containing gene set, *SOD* superoxide dismutase gene set

• Patients were grouped as early (1-II) or advanced (III-JV) clinicai stages

dismutases) were specific to each comparison performed (Table 2). As an example, the peroxidase set of genes was enriched only in tumor samples as compared to healthy tissues ($P = 0.022$). In contrast, increases in thiol-containing gene set were found to be significantly up-regulated in advanced (lll-IV) stages as compared to early (1–II) stages ($P = 0.016$), and in patient with poor prognosis (death) as compared to good prognosis (alive) ($P = 0.005$). SOD gene set was found to be up-regulated only in advanced stage of disease. Ali in ali, our metadata analysis showed that HAG components are significantly altered in different aspects of l ung tumor cells and have a strong prognostic impact for AdC patients.

Imbalance in redox marker has prognostic value for lung adenocarcinoma patient

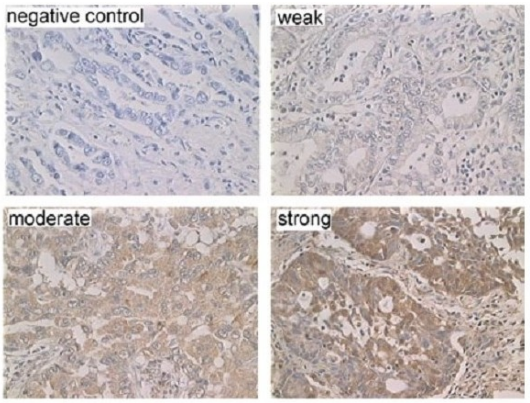
The immunostaining of 4-HNE (a specific end product of

early-stage lung AdC (Fig. 3a). Table 3 shows the complete description of the patient cohort baseline characteristics, which are well balanced among the staining groups. Ali baseline conditions are independent from the HNE leveis. The moderate immu nostaining of 4-HNE was the only parameter significantly associated with higher risk of poor outcome (HR 8.87; 95 % Cl 1.04-75.35; $P < 0.05$) (Fig. 3b).

Discussion

In many malignancies, footprints of oxidative damage, the markers of the imbalance between oxidants and antioxidants, have been detected and associated with severa! cancer-related processes, such as resistance to chemotherapy (Yoo et ai. 2008; Wang et ai. 2009), angiogenesis (Marikovsky et ai. 2002), cellular immortalization (Pani et ai. 2010) and cell death (Klamt and Shacter 2005; Circu and Aw 2010). Additionally, we recently described that patients

(a)



(b)

Hazard Ratio for patient's survival

Variables	Overall survival HR (95% CI)	P-value
Age (years)	1.08 (0.97-1.2)	0.140
Marker (4-HNE)		
weak	3.52 (0.54-23.08)	0.190
moderate	8.87 (1.04-75.35)	0.046
strong	1.00	
Smoking	1.26 (0.17 – 9.59)	0.821
BMI	0.95 (0.81 – 1.1)	0.423
Tumor staging		
IA	1.00	
IB	0.47 (0.06-3.85)	0.483
liA	5.56 (0.65-	0.118
47.72) IIB	2.10 (0.29-15.07)	0.461
FEV1	0.99(0.94-1.041)	0.755
FVC	0.98 (0.93 – 1.03)	0.326

• Cohort description in Table 3 (n = 34)

Fig. 3 4-Hydroxynonenal leveis in human lung adenocarcinoma samples. a Representative immunostaining of 4-HNE in AdC biopsies is shown. IHC images represent negative controls (absence of primary antibody) (grade 0), weak (grade 1), moderate (grade 2) and strong stain (grade 3). lmages are at x400 magnification. b Cox multivariate regression analysis was used to estimate HR for cohort clinicai covariates and 4-HNE leveis. C/ confidence interval, *BMI* body mass index, *FEVI* forced expiratory volume in I s, *FVC* forced vital capacity

Table 3 Cohort baseline characteristics according to 4-hydroxynonenal levels

Characteristic	Total	4-Hydroxynonenal staining			*P* value[a]
		Weak	Moderate	Strong	
Cohort (*n* = 34)	100 %	13 (38.2 %)	12 (35.3 %)	12 (35.3 %)	0.755
Age (years)	64.5 ± 9.51	63 ± 9.8	63.4 ± 10.5	68.3 ± 7.4	
Sex[b]					0.657
Men	20 (58.8 %)	9 (69.2 %)	6(50 %)	5 (55.5 %)	0.621
Women	14 (41.2 %)	4 (30.8 %)	6(50 %)	4 (44.5 %)	0.838
Tumor staging[b]					0.355
IA	8 (23.5 %)	3 (23.1 %)	2 (22.2 %)	3 (33.3 %)	1.000
m	13 (38.2 %)	6(46.1 %)	5(41.7 %)	2 (22.2 %)	0.477
liA	6 (17.7 %)	I (7.7 %)	4 (33.3 %)	I (11.1 %)	0.395
118	7 (20.6 %)	3 (23.1 %)	I (8.3 %)	3 (33.3 %)	0.720
Smoking[b]					0.060
Smoker	23 (67.7 %)	lO (76.9 %)	5 (41.7 %)	8 (88.9 %)	0.467
Nonsmoker	l i (32.3 %)	3 (23.1 %)	7 (58.3 %)	1 (11.1 %)	0.478

[a] Chi-squared test for given probabilities with Monte Carlo simulated *P* values (based on 2,000 replicates)

[b] 20-tabulated according to the respective groups

with p53 mutations, the most common mutational status of human cancer and the underlying defect of Li-Fraumeni syndrome (LFS), present high leveis of oxidative markers (Macedo et ai. 2012). Nonetheless, it is surprising that no antioxidant drug or intervention has been successfully translated to the oncologic clinicai setting (Sotgia et ai. 2011). So, the specific role played by oxidative stress in human lung adenocarcinoma biology is currently unknown and under investigation. In the present study, we took the advantage of an in vitro cell system to establish an association between tumoral aggressiveness and cellular redox imbala nce. Despite intrinsic limitations of in vitro studies (e.g., lack of intercellular interactions), cell !ines are reliable experimental models for cancer research since they retain relevant properties of primary tumors (Wistuba et ai. 1999). Moreover, we used a bioinformatics tool, the HAG network, to explore the expression leveis of major antioxidant system in cell systems and in clinicai samples. Finally, we demonstrated that quantification of the oxidative marker 4-hydroxynonenal (an end product of lipoperoxidation) has prognostic value for lung AdC patient outcome.

The redox state in the lung is controlled by complex and cell-specific antioxidant mechanisms. In addition to classical antioxidant enzymes (AOEs) (e.g., SOD, CAT, GPX), human lung tissue expresses severa! thiol-containing proteins and small molecules, including thioredoxins (TRXI and TRX2), metallothioneins (MTs 1-4), glutathione (GSH) and peroxidases such as thioredoxin reductases (TRXR I and TRXR2) and peroxiredoxins (also called thioredoxin peroxidases) (PRXs I-VI), which ali contain the amino acid cysteine in their active centers (Blair et ai. 1997; Kinnula et ai. 2004; Ho et ai. 2007). These molecules collectively participate not only in reactions to break down or scavenge H_2O_2, but also in the regulation of signal transduction pathways. Although dysregulation in these redox processes can be hypothesized to have fundamental role in carcinogenesis, tumor progression and drug resistance, very Iittle is known about their in vivo properties, especially with respect to alteration in their expression and functions in human lung AdC (Lehtonen et ai. 2004). As an example, the multifunctional protein thioredoxin (TRX) is responsible for catalyzing protein disulfide reductions. In tumors, TRX increases cell proliferation and resistance of various cells to oxidants and drugs. Moreover, metallothioneins (MTs) are proteins involved in metal binding and free radical scavenging activities, being associated with drug resistance and associated with lung cancer progression and poor patient outcome (Cherian et ai. 2003). Corroborating with these, in our study we found both protein families to be enriched in the most aggressive AdC cell line, which could be related to the multidrug resistance presented by EKVX cells, and involved clinically in tumor progression and poor patient outcome. Our data also pointed to the involvement of peroxidase gene set in the initiation of carcinogenesis (tumor vs. normal tissue). The previous study suggests that, in general, human lung AdC may contain increased leveis of PRXs, specifically in PRXI, II, IV and VI (Lehtonen et ai. 2004). Moreover, in the same study, PRX Ii expression was shown to be associated with advanced tumor stage (IIB-IV) in lung AdC, corroborating with our data that showed enrichment in PRXII expression in the most aggressive cellline.

To overcome the oxidizing microenvironment of lung tissue, it was thought that malignant cells have overexpression of antioxidant defenses. However, our data and from others (Laurent et ai. 2005; Myung et ai. 2010) show that high leveis of oxidative markers are present in cancer cells and are associated with tumoral aggressiveness (Chaiswing

et al. 2007; Jorgenson et al. 2013). Chemically, oxidative stress is associated with increased production of oxidizing species or a significant decrease in the effectiveness of antioxidant defenses and repair systems. Moreover, some RS act as cellular messengers (e.g., H_2O_2). Thus, oxidative stress can cause disruptions in normal mechanisms of cellular signaling. In our study, the stimulatory effect of RS in tumor growth seems to be specifically related to H_2O_2, because we could not find any effects of the supplementation of NAC and Trolox® on the proliferation rates of AdC cells. In this context, it was already suggested that tumors could not properly detoxify H_2O_2 (Coursin et al. 1996). This specific physiological oxidant can stimulate cell proliferation (Burhans and Heintz 2009), migration and invasion (Polytarchou et al. 2005; Connor et al. 2007) and is involved with the resistance against chemotherapy (Yoo et al. 2008; Wang et al. 2009). We showed that both lung AdC celllines are highly dependent on H_2O_2 to proliferate, since we could not find any differences between the two cell lines in relation to the growth inhibition in response to CAT treatment and the stimulatory effect of the CAT inhibitor AMT. Moreover, the most aggressive cell line (EKVX) has increased leveis of oxidative markers, generates higher *steady-state* leveis of H_2O_2 and, more importantly, presents an additional stimulatory effect in the proliferation rate in response to sublethal doses of H_2O_2. Ali in ali, collectively our data support that an imbalance in redox status is important for the pathological homeostasis of lung AdC and is associated with tumor progression. So, in fully developed cancer cells, the generation of high rates of reactive oxygen species may act as a driving force to induce oxidative damage to lipids, mutations in DNA bases and posttranslational modifications in proteins, contributing so to the genetic instability and metastatic potential of tumor cells (Cairns et al. 2011).

Despite the positive correlation between tumor aggressiveness and oxidative stress demonstrated here in vitro, we found a *bell-shape* curve effect of the oxidative stress marker 4-HNE in predicting the outcome of early-stage lung AdC patients. Only moderate leveis of 4-HNE were significantly associated with poor patient outcome. The biological effects of oxidative stress depend upon the size of these changes, with a cell system being able to overcome small perturbations by inducing the expression of AOE and regain its original state, a process known as cellular adaptation. However, more severe oxidative stress or chronic exposure to oxidants can cause extensive cellular damage, leading to cell death by apoptosis or necrosis (Kiamt et al. 2009) (Englert and Shacter 2002). Possibly, strong 4-HNE leveis in tumors reftect an extremely increased ROS levei that reaches toxic effect in cellular functions. On the other hand, moderate leveis of 4-HNE possibly reftect the stimulatory levei where high amount of RS fuels malignant features specifically in tumor cells (Lisanti et ai. 2011). Even though, to our knowledge, this is the first demonstration of the prognostic role of 4-HNE leveis in lung AdC, unfortunately, the determination of RS leveis and oxidative markers does not provide mechanistic insight conceming cancer development and progression.

A recent meta-analysis of randomized controlled trials that evaluated the efficacy of antioxidant supplementation in cancer indicated that there is no clinicai evidence to support preventive effect of antioxidant supplementation (Myung et ai. 2010). Moreover, the relationship of redox imbalances with different aspects of cancer biology can be systematically studied with the use of high-throughput experimental tools, such as redox proteome (Kiamt et ai. 2009) or differential gene expression leveis of the HAG network with microarray data (Gelain et ai. 2009). Along with other studies, we support the idea that compou nds with H_2O_2 rscavenging capacity might be a good approach for cancer management. As already shown in the literature, catalase overexpression reverted malignant features in different cell lines (Policastro et ai. 2004) and prevented tumor growth and metastasis in mouse lung (Nishikawa et ai. 2009). Moreover, mitochondrial-targeted catalase suppresses invasive breast cancer in mice (Goh et al. 2011), and the role of catalase has alread y been established for in vivo models (Nishikawa et ai. 2009) and should be further considered for human clinicai trials in lung AdC patients.

In summary, we demonstrated an association between redox imbalance and tumor aggressiveness in human lung adenocarcinoma samples. To our knowledge, this is the first study suggesting 4-HNE as a possible prognostic marker. Thereby, it seems plausible that imbalance in redox metabolism is pivotal to tumor malignancy, and besides consistent evidence of increased oxidative stress exists for lung cancer patients, future studies should focus on the specific mechanism of redox imbalances that mediates different aspects of tumor aggressiveness for the improvement of cancer therapy.

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Conflict of interest The authors declare none.

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RESEARCH ARTICLE

Oxidative stress associates with aggressiveness in lung large-cell carcinoma

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Abstract Oxidative stress is involved in many cancer-related processes; however, current therapeutics are unable to benefit from this approach. The lungs have a very exquisite redox environment that may contribute to the frequent and deadly nature of lung cancer. Very few studies specifically address lung large-cell carcinoma (LCC), even though this is one of the major subtypes. Using bioinformatic (in silico) tools, we demonstrated that a more aggressive lung LCC cell line (HOP-92) has an overall increase activity of the human antioxidant gene (HAG) network (P=0.0046) when compared to the less aggressive cell line H-460. Gene set enrichment analysis (GSEA) showed that the expression of metallothioneins (MT), glutathione peroxidase I (GPx-1), and catalase (CAT) are responsible for this difference in gene signature. This was validated in vitro where HOP-92 showed a pro-oxidative imbalance, presentin higher antioxidant enzymes (superoxide dismutase (SOD), CAT, and GPx) activities, lower reduced sulfhydryl groups and antioxidant potential, and higher lipoperoxidation and reactive species production. Also, HAG network is upregulated in lung LCC patients with worst outcome. Finally, the prognostic value of genes enriched in the most aggressive cell line was assessed in this cohort. Isoforms of metallothioneins are associated with bad prognosis, while the thioredoxin-interacting protein (TXNIP) is associated with good prognosis. Thus, redox metabolism can be an important aspect in lung LCC aggressiveness and a possible therapeutic target.

Keywords Tumor progression · Catalase · Lung cancer · Hydrogen peroxide · H661

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Introduction

Oxidative stress is involved in many processes during carcinogenesis; however, it is not yet possible to benefit patients with this approach [1, 2]. Free radicais are believed to initiate tumorigenesis, causing DNA mutations, and promote cancer by regulating cell survival, proliferation, apoptosis [3, 4], angiogenesis [5], migration, and metastasis [6, 7]. Although it was thought that antioxidant treatrnent could impair cancer cell homeostasis [8], severa! studies showed that this approach can enhance tumor progression [2, 9]. Since cancer cells survives in an already highly stressed environment, pro-oxidative treatments could push cancer cells over the edge via selective toxicity [1]. Thus, the way cancer responds to oxidative stress is still in debate.

The lungs have a unique redox balance, since it is exposed to a variety ofboth endogenous and exogenous oxidants that can contribute to tumor promotion [10]. These factors include environmental pollutants, tobacco smoke, high oxygen pressure [11], and reactive species (RS) from pro-inflammatory cells in the pulmonary circulation [12]. Moreover, lung cancer patients were found to have elevated oxidative stress markers in peripheral blood [13], erythrocytes [14, 15], epithe1ium lining fluid [16], breath condensate [17], and tumor biopsies [18–20], and the inadequate ingest of antiox.idants may constitute a risk factor for lung cancer [21). Despite this, the association between a redox imbalance and lung LCC was never investigated.

Lung cancer is the most prevalent and deadly malignancy worldwide [22] and often classified into small-cell and non-small-cell lung cancer (NSCLC). Of the latter, large-cell

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carcinoma (LCC) is one of its most common subtypes (5-10% of ali Jung cancer cases). Despite its importance, there are few studies focusing on lung LCC and its classification by the WHO is vague: undifferentiated NSCLC that lacks the cytological and architectural features of small-cell lung cancer and glandular or squamous differentiation [23]. Besides, half diagnosed lung LCC have been shown to belong to another category when molecular markers were used [24, 25].Therefore, studies focusing specifically on lung LCC are needed [26].

In light of the above, this study aimed to establish a relationship bctwccn oxidativc strcss and lung LCC aggrcssivcness. To achieve this, we characterized the aggressiveness of two human lung LCC cell !ines and found that HOP-92 is more aggressive than H-460. Then, we investigated the redox profile of the cell !ines with bioinformatic and in vitro tools. These demonstrated that the most aggressive cell line has a pro-oxidative imbalanced profile and that changes in the redox environment can modulate the behavior of the cells. Also, human antioxidant gene (HAG) network is upregulated in lung LCC patients with worst outcome and genes enriched in the most aggressive cell line have prognostic value in this cohort. Thus, redox metabolism can be an important aspect in lung LCC aggressiveness and a possible therapeutic target.

Materiais and methods

Cell !ines and chemicals

The human lung LCC cell !ines H-460 and HOP-92 were obtained from the NCI-Frederick cell line repository. Exponentially growing cells were cultivated in RPMI 1640 medium (Invitrogen®) containing 10 % fetal bovine serum (FBS), amphotericin B (1 -tg/mL), and garamycin (50 l-l-g/L) at 37 °C in a humidified atrnosphere of 5 % of C02.

Cellular aggressiveness

The invasion index was measured with the BioCoat Matrigel lnvasion Chamber System (BD Bioscience®). Briefly, cells were seeded in the upper wells, while the chemoattractant (RPMI medium with I0 % of FBS) was added to the lower wells. After 22-h incubation, the movement of cells through the pore was determined. Cells that penetrated to the underside surfaces of the inserts were fixed and stained with HeMa3 staining kit (Fisher Scientific®) and counted under the microscope. Cells were considered migratory when moved through uncoated pores and invasive when moved through Matrigel-coated pores. Data are expressed as the percentage of invasive/migratory and expressed as "invasion index."

Multidrug resistance was determined based on drug dose-response curves of cisplatin, carboplatin, daunorubicin, doxorubicin, 5-fluorouracil, hydroxyurea, and taxo!(Sigma® Chemical Co.) using the sulforhodamine B (SRB) assay, following NCI-60 protocol [27].

HAG network and microarray datasets

The HAG network was designed to cluster functional gene network to facilitate high-throughput analysis of redox processes [28]. HAG is composed of 63 genes whose products are thiol-containing proteins or enzymes that react directly with rcactivc spccics and is subclassificd into thrcc functional groups: perox idases, superoxide dismutases, and th iol-containing redox proteins (Fig. 1a).

Microarray expression profile was extracted from the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/). For the comparison with the human lung LCC cell !ines H-460 and HOP-92, GSE5846 dataset was used. For the validation including H661, GSE4824 and GSE14925 datasets were used. For cohort analysis, we used a microarray dataset with 24 lung LCC specimens containing survival details (GSE37745).

Differential gene expression and enrichment analysis

Differential gene expression was evaluated using ViaComplex® software [29]. To determine the significantly altered groups of functionally associated genes (GFAGs), ViaComplex uses resampling analysis with replacement (bootstrapping) in order to estimate the sampling distribution ofboth relative diversity and relative activity in the microarray dataset. Given that this analysis considers genes in the context of functional groups, the statistical design is constructed to compare groups of genes. The raw P values from the bootstrap analysis are controlled for multiple comparisons by false discovery rate (FDR) analysis. This procedure is used to identifY GFAGs exhibiting significant differential expression with a FDR no greater than 5 % (i.e., a 5 % FDR indicates that among ali GFAGs identified as being differentially expressed, 5 % of them are truly not significant) [30].

Gene set enrichment analysis (GSEA) was used to identifY genes that contribute individually to global changes in expression leveis in a given microarray dataset. GSEA considers experiments with genome-wide expression profiles from samples belonging to two classes (i.e., more aggressive and less aggressive cancer cells). Genes are ranked based on the correlation between their expression and the class distinction by using a suitable metric. Given a prior defined set of genes (i.e., HAG network), the goal ofGSEA is to determine whether the members of these set of genes are randomly distributed or primarily fou nd at the top or bottom of the ranking [31].

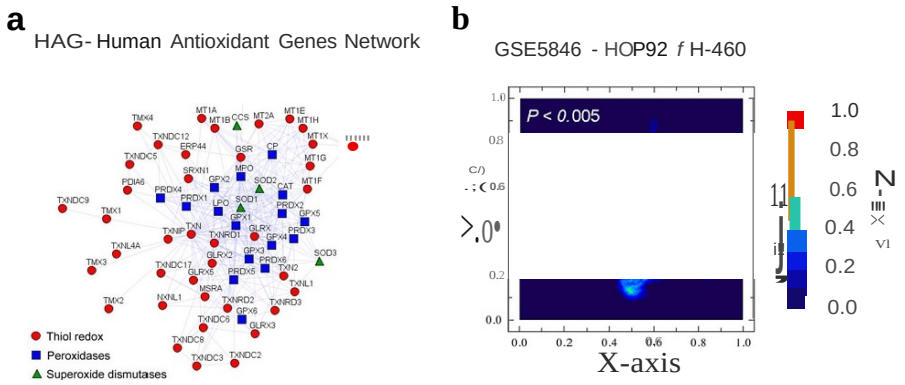

Fig.1 Expression ofhuman antioxidant gene (HAG) networl< in human lung large-cell carcinoma (LCC) cell !ines. a STRING representation of HAG network gene interactions. b Landscape analysis demonstrating elevated expression of HAG network in HOP-92 compared to H-460 (GSE5846 dataset), generated with ViaComplex® Vl.O. Color gradient (Z-axis) represents the relative functional state mapped onto graph according to the data input from the lung LCC HOP-92-a versus H-460-b, where *z=a f (a+b)*. *P* value refers to bootstrap analysis comparing cell !ines

Redox parameters

Superoxide dismutase (SOD) (E.C. 1.15.1.1) activity was measured by inhibition of superoxide-dependent epinephrine auto-oxidation at 480 nm [32). Catalase (CAT) (E.C. 1.11.1.6) activity was measured by H_2O_2 consumption at 240 nm. Glutathione peroxidase (GPX) (E.C. 1.11.1.9) activity was measured by NADPH oxidation at 340 nm [33).

Non-enzymatic antioxidant potential was determined by total radical-trapping antioxidant potential (TRAP) assay [34). Sulfhydryl group (–SH) levei was determined with 5-thio-2-nitrobenzoic acid at 412 nm (E412 nm=27,200/M/em) and expressed as nanomoles of -SH per milligram of protein. Thiobarbituric acid reactive species (TBARS) assay was used as a lipoperoxidation index. TBARS were assayed at 532 nm and expressed as nanomoles ofMDA equivalents per milligram of protein. 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) oxidation was used to determine intracellular generation ofRS in a 96-well plate reader (Spectra Max GEMINI XPS, Molecular Devices®).

Proliferation and growth inhibition assays

Cells were treated with aclive or heat-inactivated CAT, and cell growth inhibition was evaluated for 72 h with SRB assay. To investigate ifthe growth inhibition by CATwas reversible, the assay was repeated removing CAT. The effect of bolus amount of H_2O_2 addition on cell growth was also evaluated.

Statistical analysis

Cellline data are expressed as means±SEM of at least three independent experiments carried out in triplicate, and Student's *I* test was used (?<0.05) (GraphPad® Software 5.0). Expression analysis were evaluated as mentioned above. Survival graphs were made in GraphPad® Software 5.0, and log-rank (Mantel-Cox) test and hazard ratio (Mantel-Haenszel) were obtained.

Results

Cellular aggressiveness

Comparing in vitro invasion index and multidrug resistance between the two human lung large LCC cell tines, HOP-92 was established as the most aggressive (Table **1**). HOP-92 cells had 6-fold higher invasive index and a signficantly higher resistance to ali seven drugs evaluated (2.79-40.11-fold increase in drug GI_{50} values).

HAG expression

The most aggressive cell line (HOP-92) has higher expression of antioxidant genes, as demonstrated in the landscape map (?=0.0046) (Fig. 1b). To confirm this fmding, we looked for another lung LCC cell tine with freely available expression data and direct aggressiveness comparison between two or more celllines as part ofone published study. Then, we compared HAG expression in H-460 and H661 lung LCC cell lines. Among the cell lines used in this study, H661 is the less aggressive one, since H-460 has higher migratory behavior, higherexpression ofthe pro-angiogenic protein EphA4, and is more resistant to radiotherapy and the antineoplastic agent AZDII52-HQPA [35, 36). Once more, the more aggressive cellline has a higher expression of HAG (Fig. SI).

The genes that specifically contribute to enrichment in HOP-92 compared to H-460 are summarized in Table 2 and

Table 1 Aggressiveness of human lung large-cell carcinoma cell lines

	Large-cell carcinoma cell !ines		
	H-460	HOP-92	Fold increase
lnvasiveness (invasive/migratory cells)			
Invasion Index	2.15±0.3	12.48±2.6**	5.80
Multidrug resistance (GI_{50} value) (J.LM)			
Cisplatin	0.90±0.11	2.51±0.5*	2.79
Carl>oplatin	23.9±4.9	754±18.3*	3.15
Daunorubicin	0.027±0.007	0.109±0.03*	4.04
Doxorubicin	0.029±0.01	0.123±0.03*	4.24
5-Fluorouracil	4.41±1.35	1769±65.4*	40.11
Hydroxyurea	315.9±11.1	713.4±98**	2.26
Taxo!	0.026±0.021	0.095±0.02*	3.65

Data are presented as means±SEM of at least four independent experiments (n=4). lnvasion index is "invading cells/migrating cells." Bold values indicate higher values in each comparison

*$P<0.05$; **P<O.OI (Student's *t* test)

include metallothioneins, peroxidases, and components ofthe thioredoxin system (obtained with GSEA).

Oxidative stress in lung LCC cell lines

To validate in silico results, severa!redox parameters were evaluated in vitro in the lung LCC cell lines (Fig. 2). We found a significant upregulation in ali antioxidant enzyme (AOE) activities in the most aggressive cellline. HOP-92 has higher activities of SOD, CAT, and GPx (1.83, 4.76, and 2.1-fold increase, respectively) (Fig. 2a). This suggests an adaptation to higher leveis of RS. Consistently with this, HOP-92 was found to have higher DCF oxidation rate indicating elevated production of RS (Fig. 2b). On the other hand, basal lipoperoxidation (TBARS leveis) was found to be 2.26-fold higher and the total antioxidant capacity (TRAP) and the leveis of reduced sulfhydryl groups (–SH) in HOP-92 cells (Fig. 2c) were found to be decreased. Collectively, this indicates that despite the enzyrnatic adaptation, the most aggressive phenotype has higher leveis of intracellular oxidative stress.

Also, CAT treatrnent caused a dose-dependent inhibition of cell's growth and CAT washout restored proliferation. Finally, the treatrnent with sublethal dose of H_2O_2 increased growth of HOP-92, but not H-460 (Fig. S2). Therefore, the redox environment can modulate the behavior of cancer cells.

Table 2 Genes from human antioxidant gene (HAG) significantly enriched in most aggressive lung large-cell carcinoma cell line (HOP92)

Gene symbol	Gene name
CAT	Catalase
GPXI	Glutathione peroxidase I
MTIE	Metallothionein IE
MTIF	Metallothionein IF
MTIG	Metallothionein IG
MTIH	Metallothionein IH
MTIM	Metallothionein IM
MTIX	Metallothionein IX
MT2A	Metallothionein 2A
SEPPI	Selenoprotein P, plasma, I
TXNIP	Thioredoxin interacting protein

Data generated with gene score enrichment analysis (GSEA) comparing HOP92 versus H-460 transcripts obtained from GSE5846 dataset (Gene Expression Omnibus) *($P<0.05$)*

Oxidative stress in clinicai lung LCC patients

Ultimately, the value ofthe pro-oxidative imbalance was tested in a patient cohort. In accordance with the previous findings, HAG was found upregulated in patients with worst outcome (Fig. 3a).

Also, the prognostic value ofthe genes enriched in the most aggressive cell tines was assessed. Different isoforms of metallothioneins (MTlF, MTlG, MTlM, and MTlX) were found to be associated with bad prognosis, while TXNIP was associated with good prognosis (Fig. 3b).

Discussion

Despite evidence of oxidative stress involvement in severa! cancer-related processes, such as resistance to chemotherapy [37, 38], angiogenesis [5], cellular immortalization [7], and cell death [39, 40], it is disappointing that to date no

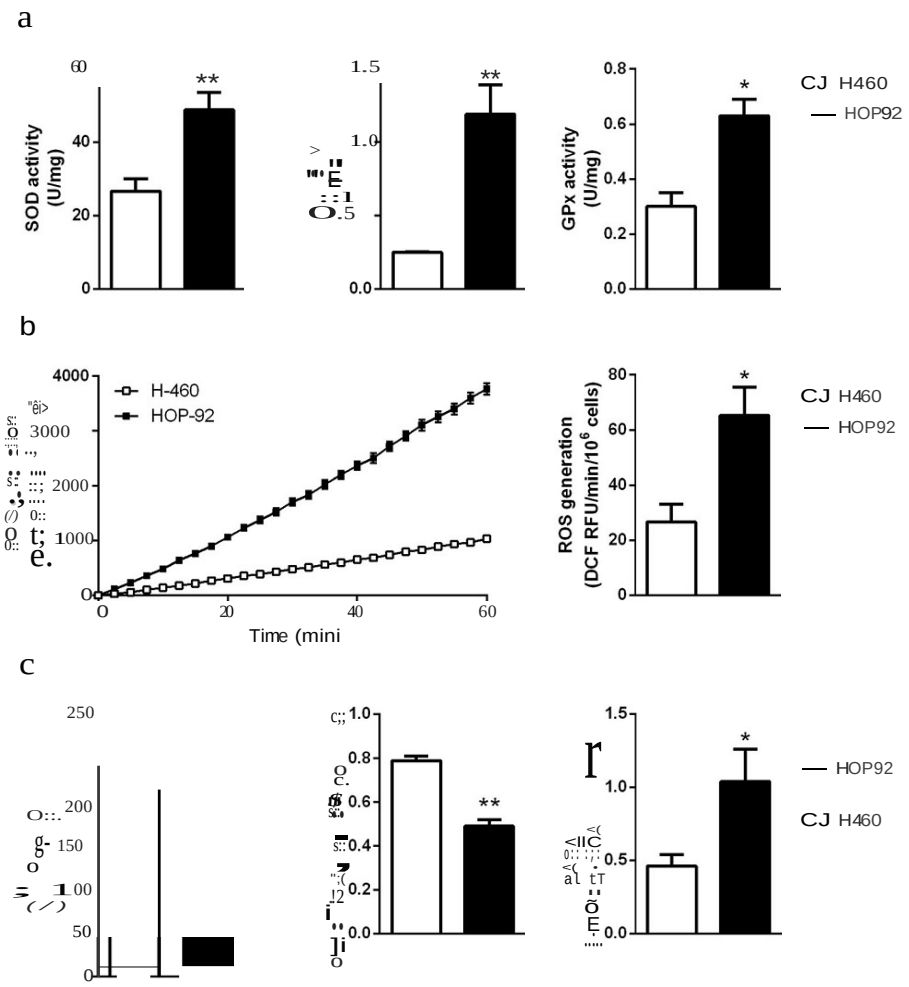

Fig.2 Redox characterization of Jung Jarge-cell carci noma cell !ines. Activity of antioxidant enzymes SOD, CAT, and GPx (a), ROS generation (b), and non-enzymatic parameters (c). Data are means±SEM of at least three independent experiments *(n=3)*, performed in triplicate. *P<0.05 or **P<O.Ol (Student's *t* test between cell lines)

antioxidant approach has been successfully translated to the oncologic clinicai setting [8]. Additionally, it has been shown that lung cancer patients have elevated oxidative stress markers in peripheral blood [13], erythrocytes [14, 15], epithelium lining tluid 116J, breath condensate 117J, and in tumor biopsies [18–20], and the inadequate ingestion of antioxidants constitutes a risk factor for lung cancer development [21]. Here, we demonstrated that the most aggressive cellline has a pro-oxidative imbalance and that oxidative stress can modulate tumoral cell's behavior. Also, this imbalance was confmned in a lung LCC cohort and genes enriched in the most aggressive cellline were shown to have prognostic value. The results will be further explored below.

Redox biology is intricately overlapped with severa(metabolic pathways, so we felt this was best evaluated using a systems biology approach [41]. We demonstrated an association between tumoral aggressiveness and higher expression of antioxidant genes using an in vitro cell system and in silico tools. This was corroborated by in vitro redox characterization.

Severa! isoforms of MT were found to be upregulated in the most aggressive cell line and were associated with bad prognosis. We found it to be upregulated also in a more aggressive lung adenocarcinoma cell line [3]. This is in accordance with data showing that MT are associated with drug resistance, lung cancer progression, and poor patient outcome l42J. Theretore, metallothioneins seems to be a very good candidate for lung cancer studies focusing on oxidative stress.

The thioredoxins (TXN) are antiox ida nts usu ally overexpressed and correlated with bad prognosis in severa! malignancies including lung cancer [43, 44]. On the other hand, the TXN inhibitor thioredoxin-interacting protein (TXNlP) has a negative correlation with TXN, being found underexpressed in tumors and correlating with good prognosis. Moreover, TXNlP is considered a tumor suppressor involved in reduced tumor growth, metastasis, and angiogenesis in different types of both solid tumors (as breast, gastric, thyroid, bladder, and liver) and leukemia [45–50]. However, we found TXNlP to be overexpressed in the most aggressive cell line. Despite the clear contrast with the abovementioned, this is not the first time we report this. TXNlP was found to be overexpressed in more aggressive lung adenocarcinoma cell line and patients [3]. Also, TXNIP overexpression results in

a

HAG expression in lung LCC cohort

GEO lD	Cohort	Experimental groups	Viacomplex Bootstrap	GSEA (nominal p-value)
GSE37745	24 lung LCC cancer specimens	Dead *vs.* Alive	0.042	0.025

b

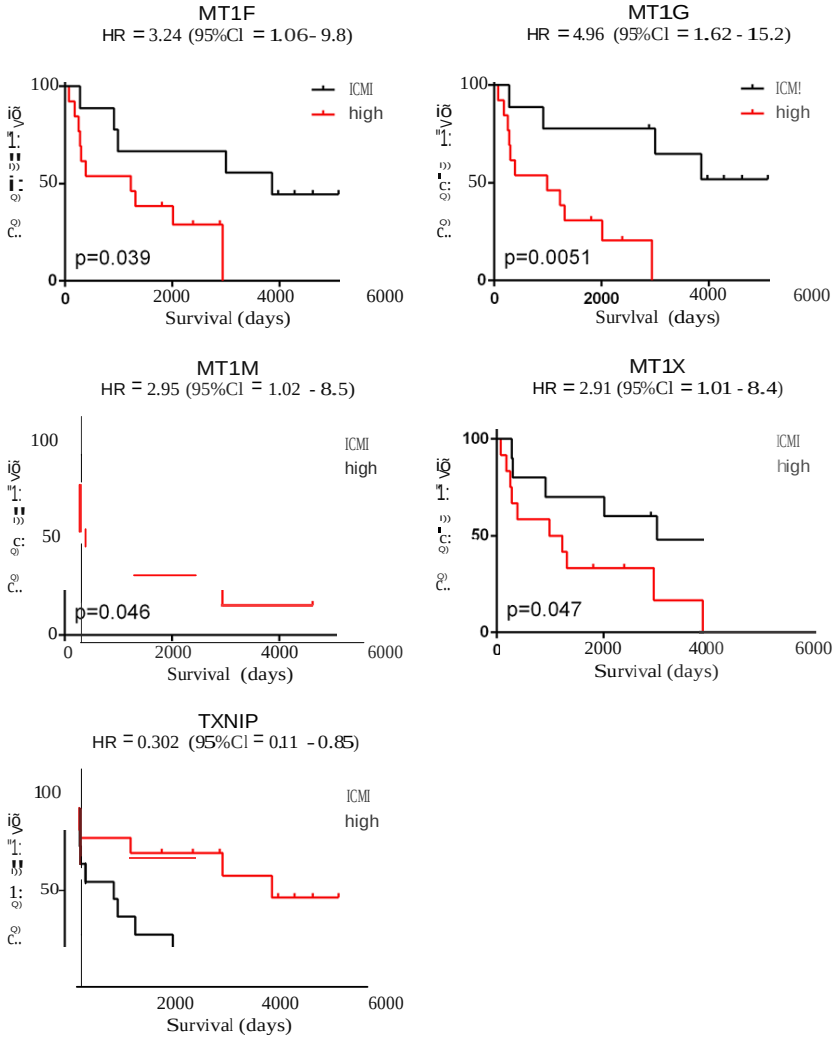

Fig. 3 Pro-oxidative imbalance in lung LCC cohort. Patients with worsl oulcome have a higher expression ofthe human anlioxidanl gene (HAG) network (a). Five genes lhat are enriched in lhe mosl aggressive lung LCC cell !ines have prognostic value; metallothioneins (MTIF, MTIG, MTIM, and MTIX) are associated with bad prognosis while TXNIP is associaled wilh good prognosis (b). Transcripl profiles ofhuman lung LCC palients were oblained from Gene Expression Omnibus (GEO). The 24 lung LCC patients were divided according to the value of expression of each gene in two groups: the lop or below the median of lhe entire cohort (GSE37745). Survival graphs were made in GraphPad® Software 5.0, and log-rank (Mantel-Cox) test and hazard ralio (Manlel-Haenszel) were obtained. *P<0.05*. *HR* hazard ralio, *C/* confidence inlerval

increased leveis of reactive species [45], corroborating our main hypothesis. In the meantime, TXNIP associates with good prognosis in the lung LCC cohort tested. Thus, more studies are necessary to fully comprehend how this gene, and the TXN pathway as a whole, can affect lung LCC aggressiveness.

Catalase and GPx were found to have higher expression and activity in the most aggressive cell line and could be an interesting target for future studies, particularly because both can detoxify H_2O_2. The higher activity and expression of antioxidant enzymes coupled with higher production of ROS suggest that most aggressive lung LCC are adapted to deal with more oxidizing environments. Since malignant turnors are resistant to cell death, they can benefit from ROS stimuli for proliferation and cell growth [51]. In accordance with this, patients with worst outcome presented higher expression of

HAG and the most aggressive cell line was the only one that enhanced its growth rate when treated with H_2O_2. Taken together, this indicates that aggressive lung LCC can have a pro-oxidative imbalance, which could have therapeutic implications.

Nevertheless, recent studies demonstrated that the antioxidants N-acetylcysteine (NAC) and vitamin E increases tumor progression and worsen outcome in in vivo model of lung cancer [2]. On the other hand, catalase overexpression [52] and H_2O_2 scavenging [53] were shown to revert malignant features in different celllines; mitochondrial-targeted catalase suppresses invasive breast cancer in mice [54] and prevented tumor growth and metastasis in mouse lung studies [55]. Another important detail is the quality of the antioxidant: catalase and other H_2O_2 scavengers have a specific target, while NAC and vitamin E do not. Our data supports the hypothesis that oxidants, especiaUy H_2O_2, fuel the behavior of tumor cells and thus specific antioxidants could impair malignant cell homeostasis by ROS starvation.

To the knowledge of the authors, this is the frrst study demonstrating an association between redox imbalance and tumor aggressiveness in human lung LCC. FinaUy, besides consistent evidence of elevated oxidative stress occurring in lung cancer patients, future studies should focus on the specific redox mechanism that mediates tumor aggressiveness for the improvement of lung LCC therapy.

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Conflicts of interest None

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Figure S1

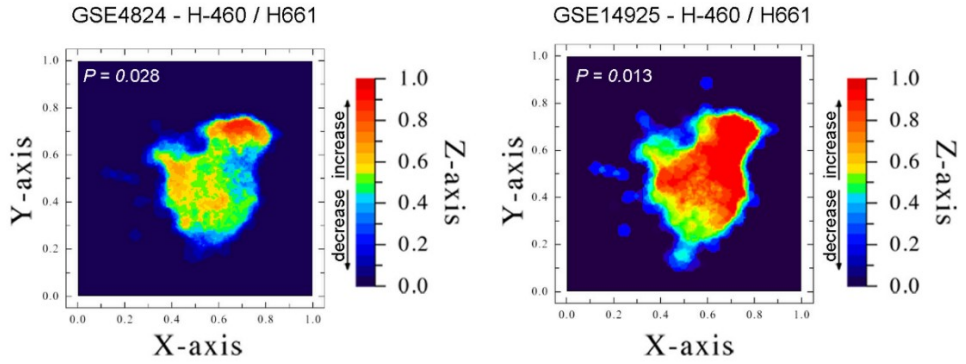

Figure S1: Landscape analysis comparing the expression of Human Antioxidant Gene (HAG) network in human lung large cell carcinoma (LCC) cell lines H-460 and H661, using two datasets, GSE4824 (a) and GSE14925 (b), generated with ViaComplex® V1.0. Color gradient (Z-axis) represents the relative functional state mapped onto graph according to the data input from the lung LCC H-460-a vs. H-661-b, where z=a/(a+b). P value refers to Bootstrap analysis comparing cell lines.

Figure S2

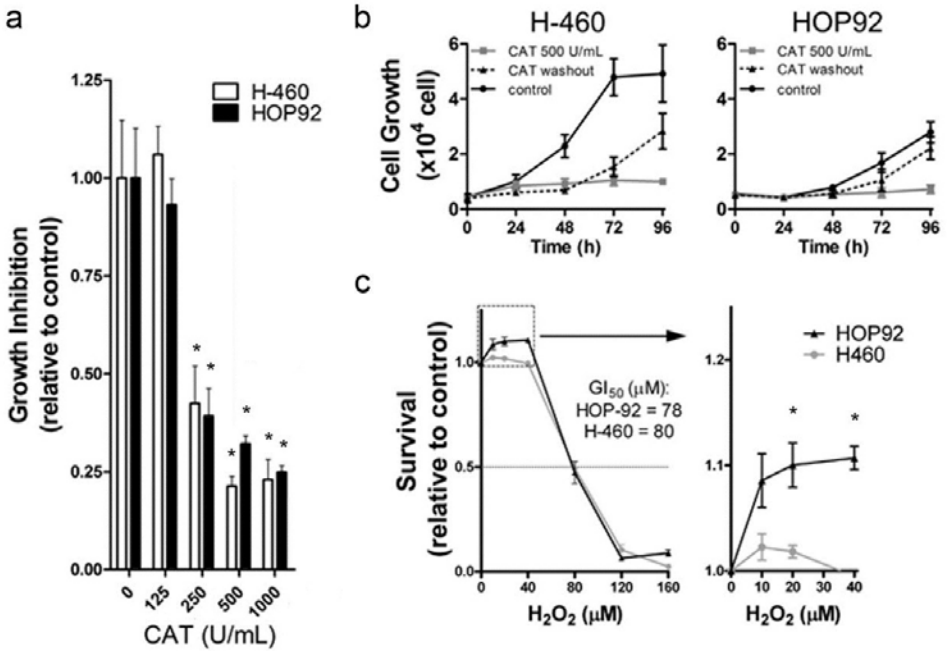

Figure S2: Redox state influences growth of lung large cell carcinoma cell lines. Treatment with exogenous catalase (125–1000 U/mL) for 72 h cause dose-dependent inhibition in cell proliferation of lung LCC cell lines (a). CAT washout after 48 h of incubation allowed cells to return to its original proliferation rate (b). Dose response curve against H2O2 in lung LCC cell lines. Sub-lethal doses (<40 μM) of H2O2 stimulate cell growth of HOP-92 (c). Data is mean ± S.E.M. of at least three independent experiments (n = 3), performed in triplicate. * $P < 0.05$ (Student's t-test compared to untreated group).

CAPÍTULO II: REPROGRAMAÇÃO METABÓLICA

Dados prontos para elaboração de manuscrito a ser submetido para periódico Qualis A2 ou B1.

OBJETIVO GERAL DO CAPÍTULO

Caracterizar o perfil bioenergético de 2 linhagens de adenocarcinoma de pulmão relacionando-o com a agressividade, caracterizado anteriormente (ver Capítulo I). Posteriormente, buscou-se validar os achados em coortes clínicas. Por fim, foram propostos novos alvos terapêuticos com foco na reprogramação metabólica.

OBJETIVOS ESPECÍFICOS

- Avaliar em linhagens celulares:
  - Respirometria de alta resolução
  - Atividade de enzimas relacionadas ao metabolismo bioenergético
  - Expressão gênica de isoformas comum à reprogramação metabólica tumoral
  - Sensibilidade a inibidores metabólicos
- Avaliar em coortes clínicas:
  - Valor prognóstico dos genes alterados nas linhagens

FENÓTIPO AGRESSIVO CARACTERIZADO

Sugerimos que adenocarcinomas pulmonares agressivos apresentam um fenótipo glicolítico com dependência de transportadores para glicose (GLUT's) e lactato/piruvato (MCT's).

PROPOSTA TERAPÊUTICA

Como as isoformas expressas em tumores são diferentes daquelas de tecidos sadios, o desenvolvimento de inibidores específicos pode ter um impacto imediato na terapia de adenocarcinoma de pulmão, que atualmente não tem uma terapia específica.

Introdução específica

O câncer pode ser visto como uma doença metabólica, sendo os outros *hallmarks* fenômenos posteriores (SEYFRIED et al., 2014). A atenção ao metabolismo energético tumoral foi despertada especialmente após a descoberta de ligações entre rotas metabólicas e oncogênicas (KROEMER; POUYSSEGUR, 2008)(Fig.11). Alterações metabólicas beneficiam as células tumorais e, portanto, podem ser potenciais alvos terapêuticos. Porém, a compreensão deste fenótipo na sua totalidade tem se mostrado um desafio incrivelmente complexo (SCHULZE; HARRIS, 2012; TENNANT; DURÁN; GOTTLIEB, 2010).

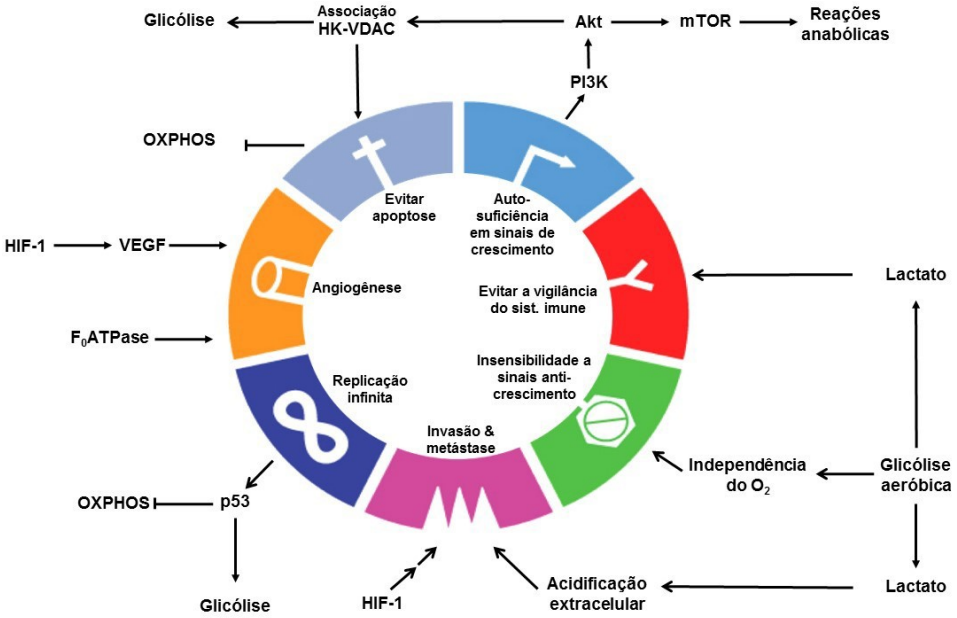

Figura 11 Associações entre os *hallmarks* do câncer e diferentes aspectos do metabolismo bioenergético.(KROEMER; POUYSSEGUR, 2008).

O Efeito Warburg

Há quase 1 século, Otto Warburg* observou que tecidos tumorais metabolizam dez vezes mais glicose a lactato que um tecido sadio mesmo na presença de oxigênio, e sugeriu que

* Vencedor do Prêmio Nobel de 1931 em Fisiologia e Medicina por sua descoberta da enzima respiratória citocromo c oxidase (KOPPENOL; BOUNDS; DANG, 2011).

aqueles realizam glicólise aeróbica (KOPPENOL; BOUNDS; DANG, 2011; WARBURG; WIND; NEGELEIN, 1927). Este fenômeno ficou conhecido como "efeito Warburg" (Fig.12). Estes achados levaram-no a postular que células malignas utilizam preferencialmente a via glicolítica e a fermentação lática para obtenção de energia. À época, esta afirmação gerou um paradoxo devido ao efeito Pasteur, segundo o qual o oxigênio inibe a fermentação . Porém, o pesquisador alemão acreditava que a origem do câncer se dava por um defeito mitocondrial que incapacitava a célula de realizar respiração oxidativa (WARBURG, 1956). Enquanto na realidade, muitos tumores exibem o efeito Warburg, mas retêm a capacidade de respirar (BIRSOY et al., 2014; DIERS et al., 2012; KOPPENOL; BOUNDS; DANG, 2011; WARD; THOMPSON, 2012). Assim, o efeito Warburg demorou a ser aceito, pois além de se opor ao efeito Pasteur, a via glicolítica é menos eficiente do ponto de vista energético, portanto como poderia ser a via preferida em uma célula com metabolismo acelerado como as células tumorais?

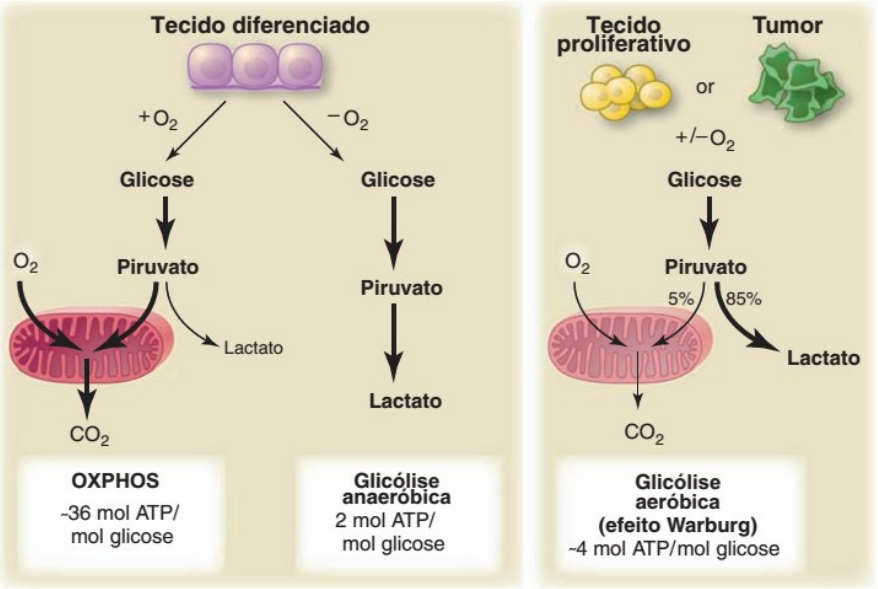

Figura 12 Destinos da glicose em tecidos diferenciados/sadios e proliferativos ou tumorais. OXPHOS – fosforilação oxidativa *(VANDER HEIDEN; CANTLEY; THOMPSON, 2009)*.

Entretanto, após algumas décadas de esquecimento, o efeito Warburg foi resgatado nos anos 90 para fins diagnósticos (GATENBY; GILLIES, 2004). Foi observado que muitos tumores apresentam uma maior captação de um 18F-fluorodeoxiglicose (FDG), análogo da glicose e,

portanto, podem ser mais facilmente diagnosticado com tomografia por emissão de pósitrons (PET scan, do inglês *positron emission tomography*) (HAGEN et al., 2004; ROHREN; TURKINGTON; COLEMAN, 2004). Além disso, é possível acompanhar remissão do tumor ao longo de um tratamento (VANDER HEIDEN; CANTLEY; THOMPSON, 2009)(Fig.13).

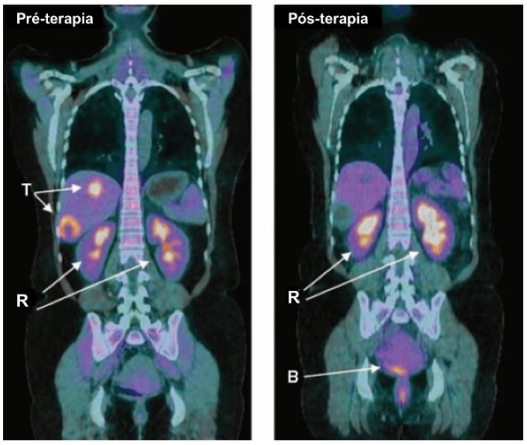

Figura 13 Captação de glicose por tumores (T), visualizada por PET com FDG em resposta à terapia. O tumor (T) é facilmente identificado antes da terapia (esquerda) e não observado após terapia (direita). O excesso de FDG é excretado pela urina e, portanto, é possível visualizar também os rins (R) e a bexiga (B) (VANDER HEIDEN; CANTLEY; THOMPSON, 2009).

Além disso, hoje podemos explicar este aparente paradoxo de diversas formas. A primeira explicação é a adaptação imposta pelo ambiente, como hipóxia e privação de nutrientes**,** oriundo do crescimento exacerbado do tumor em relação à vascularização existente (GATENBY; GILLIES, 2004). A privação de glicose em células de câncer de cólon exerce uma pressão seletiva sobre KRAS, gene que pode conferir tolerância à baixos níveis de glicose (YUN et al., 2009). Da mesma forma, células em cultura podem compensar a perda de glicose ou glutamina utilizando um ou outro nutriente para suprir a falta de metabólitos (CHENG et al., 2011; YANG et al., 2009). Neste contexto, células adaptadas ao metabolismo glicolítico possue m uma vantagem seletiva. Desta forma, o metabolismo glicolítico pode ser mantido mesmo após o término da pressão seletiva. Até mesmo terapias anti-angiogênicas podem induzir hipóxia, selecionando células com metabolismo glicolítico (CURTARELLO et al., 2015). Outra variável muito importante a uma célula proliferativa é a necessidade de biossíntese (Fig.14). Portanto,

uma rota extremamente catabólica como a fosforilação oxidativa não parece interessante neste contexto (DEBERARDINIS et al., 2008; VANDER HEIDEN; CANTLEY; THOMPSON, 2009).

Reprogramação Metabólica Tumoral

Porém, o efeito Warburg é apenas uma parte do todo, pois sabemos hoje que células neoplásicas possuem alterações em diversas rotas, assim o fenômeno é denominado reprogramação metabólica tumoral (BOULAHBEL; TENNANT, 2009; CHEN; RUSSO, 2012; HERLING et al., 2011). Atualmente, a reprogramação metabólica em tumores é considerada um dos novos *hallmarks* do câncer (HANAHAN; WEINBERG, 2011; WARD; THOMPSON, 2012) (Fig.8). Interessantemente, este fenômeno não acrescenta novas reguladores, mas sim é regulado por oncogenes e supressores tumorais clássicos – como myc e p53 (JONES; THOMPSON, 2009; SOGA, 2013) (Fig.14).

O metabolismo da glicose, e também da glutamina, são reprogramados por mutações em MYC, TP53, oncogenes relacionados a Ras, LKB1-AMP quinase (AMPK) e PI3 quinase (PI3K), ente outros (BOROUGHS; DEBERARDINIS, 2015). As proteínas oncogênicas Ras, AKT e Myc podem regular o fluxo glicolítico, uma vez que controlam a expressão de genes responsáveis pela captação e metabolização da glicose, como transportadores de glicose (GLUT's), hexoquinase (HK), fosfofrutoquinase (PFK) e lactato desidrogenase (LDH) (BOROUGHS; DEBERARDINIS, 2015; KROEMER; POUYSSEGUR, 2008; LEVINE; PUZIO-KUTER, 2010). A perda de supressor tumoral PTEN promove captação de glicose via rota da PI3K/Akt/mTOR enquanto a perda do supressor tumoral Von-Hippel-Lindau (VHL) promove um fenótipo metabólico similar via estabilização do fator de transcrição HIF-1α (do inglês, *hypoxia inducible fator*)(FAUBERT et al., 2014). Por fim, o supressor tumoral p53 também tem um papel destacado na regulação da glicólise, respiração mitocondrial e via das pentose fosfato (PPP – do inglês *pentose phosphate pathway*) (JIANG et al., 2011; MADDOCKS; VOUSDEN, 2011; VOUSDEN; RYAN, 2009), bem como

na modulação de espécies reativas do oxigênio (ROS – do inglês *reactive oxygen species*) (SABLINA et al., 2005).

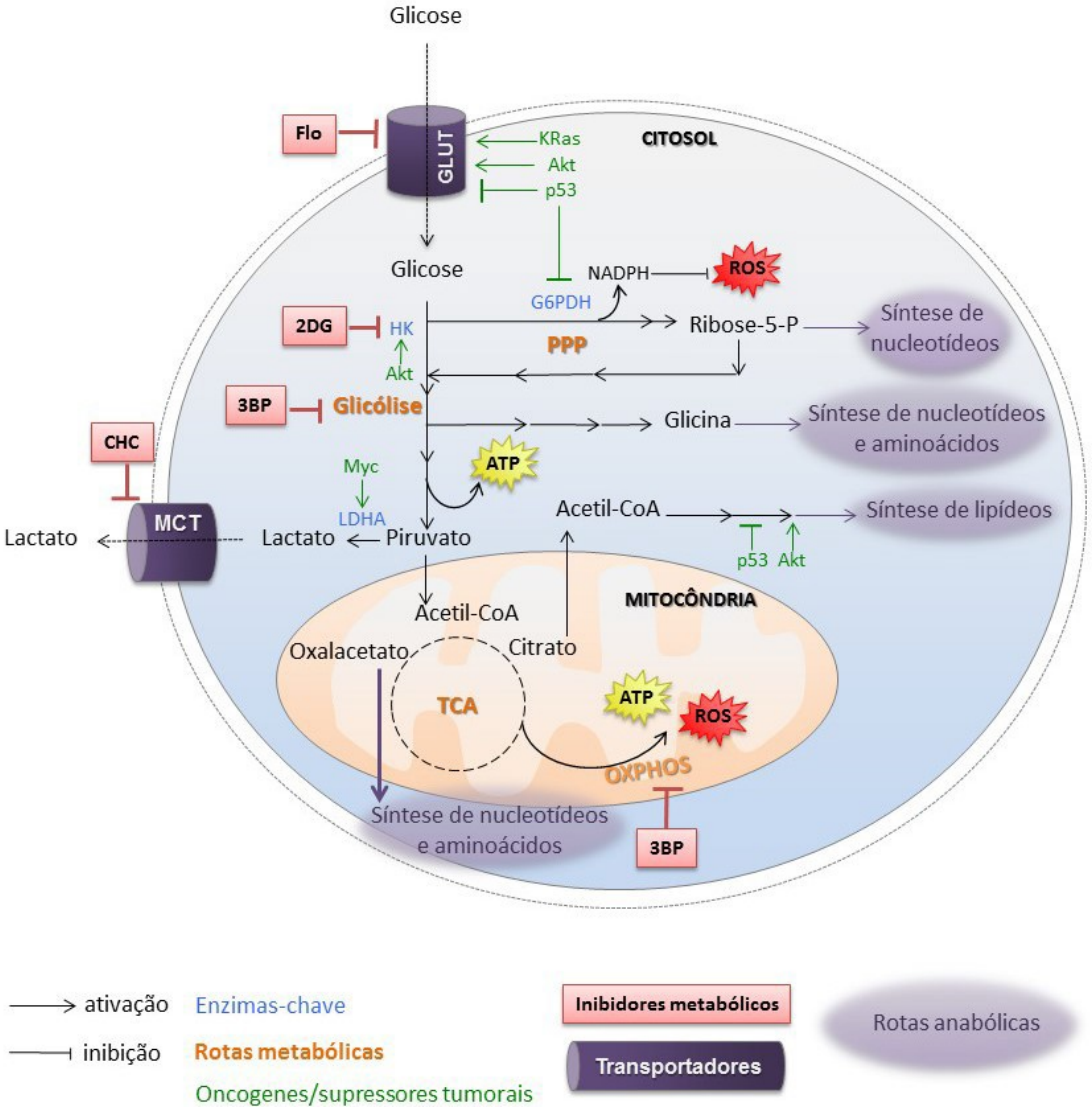

Figura 14 Rotas alteradas na reprogramação metabólica tumoral. 2DG – 2-desoxiglicose; 3BP – 3-bromopiruvato CHC –ácido α-hidroxicinâmico; Flo – floretina; G6PDH – glicose-6-fosfato desidrogenase; GLUT – transportadores de glicose; HK – hexoquinase; LDHA – lactato desidrogenase A; MCT – transportador de monocarboxilato; PPP – via das pentoses-fosfato; ROS – espécies reativas do oxigênio; TCA – ciclo do ácido tricarboxílico. (Figura gentilmente elaborada por Fernanda Stapenhorst França).

Muitas rotas metabólicas estão interligadas entre si e com o equilíbrio redox (HERLING et al., 2011; JIANG et al., 2011; MENENDEZ; LUPU, 2007). A PPP sustenta reações anabólicas e o equilíbrio redox através da produção de NADPH. A síntese de lipídios precisa de NADPH e acetil-CoA, resultando em aumento do consumo de glicose e consumindo piruvato, produto final da

glicólise. Este processo ocorre numa série de passos catalisados pela enzima ácido graxo sintase (FASN – do inglês *fatty acid synthase*), que está superexpressa em muitos tumores (MENENDEZ; LUPU, 2007). Além disso, os ácidos graxos são capazes de modular a produção de ROS. Sabe-se que alterações no metabolismo da glicose podem modular a produção de ROS, causar citotoxicicade via estresse oxidativo e alterar o potencial antioxidante intracelular (SCHÖNFELD; WOJTCZAK, 2008). Por fim, as próprias espécies reativas podem mediar rotas oncogênicas, alterar a população celular peritumoral e participar ativamente na reprogramação metabólica em células (COSTA; SCHOLER-DAHIREL; MECHTA-GRIGORIOU, 2014). A SOD2 pode alimentar o fenótipo glicolítico pela produção de H_2O_2 e consequente ativação da AMPK (HART et al., 2015).

Além disso, dentro de um mesmo tumor podem ocorrer células com diferentes fenótipos bioenergéticos que estabelecem entre si uma relação de simbiose. Nesta, as células hipóxicas são glicolíticas e liberam lactato que será oxidado pelas células oxigenadas (SEMENZA, 2005). Ainda, o fenótipo metabólico exibido por tumores depende tanto do genótipo quanto do tecido de origem (YUNEVA et al., 2012). Assim, a compreensão da reprogramação metabólica específica em cada tipo de câncer tem profundas implicações terapêuticas.

Terapia Anti-metabólica

Ainda hoje, o câncer é definido como uma doença genética e sua terapia foca na reversão de mutações. Porém, além do número de mutações ser enorme (ALEXANDROV et al., 2013), o ataque inespecífico a células em proliferação frequentemente leva a efeitos colaterais indesejados e se mostrou insuficiente até o momento (LÓPEZ-LÁZARO, 2010). Por outro lado, a reprogramação metabólica se mostra fundamental para a tumorigênese e progressão tumoral (OBRE; ROSSIGNOL, 2014; TENNANT; DURÁN; GOTTLIEB, 2010) e, apesar de sua complexidade, converge para três necessidades básicas de células proliferativas: ATP, biossíntese e equilíbrio

redox (CAIRNS; HARRIS; MAK, 2011) (Fig.14). Assim, a identificação de inibidores metabólicos capazes de afetar uma delas pode ser eficiente na terapia anti-tumoral.

Ensaios in vitro com compostos capazes de reverter o metabolismo glicolítico se mostraram eficientes na inibição da migração sem afetar a proliferação, indicando especificidade a tumores avançados (YIZHAK et al., 2014). Atualmente, diversos ensaios clínicos encontram-se em andamento com abordagens anti-metabólicas (OBRE; ROSSIGNOL, 2014).

As abordagens terapêuticas visando o metabolismo tumoral podem ser baseadas na disponibilidade de nutrientes ao tumor ou inibição farmacológica, direta ou indireta.

A primeira estratégia se baseia em uma dieta cetogênica, por matar células dependentes da via glicolítica por inanição (SEYFRIED et al., 2014). Existem resultados promissores em modelos animais e pacientes (KLEMENT, 2014; NEBELING et al., 1995; OTTO et al., 2008; SEYFRIED et al., 2015; ZHOU et al., 2007). Por outro lado, alguns estudos não observaram benefício algum (CHU-SHORE; THIELE, 2010), sugerindo até mesmo cautela devido ao risco de má nutrição (HUEBNER et al., 2014). Thomas Seyfried é um defensor da dieta cetogênica até mesmo como monoterapia (SEYFRIED et al., 2014), mas a maioria dos estudos sugere que qualquer efeito advindo desta abordagem, se comprovado, deverá ser usado como adjuvante a terapias convencionais (ALLEN et al., 2014; TENNANT; DURÁN; GOTTLIEB, 2010).

Os alvos indiretos são os reguladores *upstream* de rotas metabólicas, incluindo HIF, MYC, PI3K, Akt, mTOR e AMPK (Fig.14). A inibição destes reverteria adaptações necessárias à homeostase tumoral. Neste contexto, o eixo AMPK/mTOR aparenta ser o alvo mais próximo da clínica, pois há menor incidência de câncer em paciente tratados com metformina, um ativador de AMPK e inibidor de mTOR receitada para diabetes tipo 2 (DOWLING et al., 2007; LIBBY et al., 2009). Ensaios pré-clínicos demonstraram uma série de efeitos antitumorais desta droga em modelos *in vitro* e *in vivo*: inibição de proliferação, crescimento tumoral e angiogênese, indução

de apoptose e ainda sinergia com paclitaxel (LIU et al., 2009; ORECCHIONI et al., 2014; ROCHA et al., 2011). Entretanto, por estas moléculas influenciarem múltiplos alvos, usar sua inibição especificamente para atacar o metboalismo energético não se mostrou simples até o momento (DANG, 2012; ENGELMAN, 2009; JONES; HARRIS, 2012).

Por fim, temos os alvos diretos, ou seja enzimas de rotas metabólicas diretamente envolvidas com geração de ATP ou biossíntese. Atacar a biossíntese de moléculas necessárias à proliferação não é uma ideia nova e os primeiros quimioterápicos denominados antimetabólicos inibem a síntese de nucleotídeos, como o 5-fluorouracil (5-FU) (EWALD; SAMPATH; PLUNKETT, 2008). Entretanto, sua eficácia é limitada pela pouca especificidade tumoral (TENNANT; DURÁN; GOTTLIEB, 2010). Assim, o metabolismo da glicose e surge como um alvo muito promissor, por alimentar diversas rotas fundamentais às células malignas.

Muitos tumores apresentam expressão aumentada das proteínas responsáveis pelo primeiro passo no metabolismo da glicose, os transportadores (GLUT, do inglês *glucose transporter*). Alguns GLUT's respondem à demanda metabólica do momento – GLUT2 e GLUT4 – enquanto outros mantém o fluxo de glicose contínuo - GLUT1 e GLUT3 – e são fundamentais a célula como eritrócitos, desprovidas de mitocôndrias e, portanto, dependentes da glicólise para obtenção de ATP. A elevada expressão de GLUT1 está associada à progressão tumoral e pior prognóstico (STARSKA et al., 2015; SZABLEWSKI, 2013). A Floretina, um fenol natural de origem vegetal, inibe a captação de glicose via GLUT1 (Fig.14) e é capaz de inibir crescimento tumoral em modelo animal (NELSON; FALK, 1993) e sensibilizar células quimioresistentes à apoptose (CAO et al., 2007). Entretanto, nenhum ensaio clínico com essa abordagem foi iniciado.

Após ser captada, a glicose é metabolizada pela enzima hexoquinase (HK, do inglês *hexokinase*) e pode seguir diversas rotas intracelulares. A glicose-6-fosfato (G6P), produto da HK, pode gerar ATP e piruvato ao seguir pela via glicolítica ou NADPH, lipídeos, nucleotídeos e até mesmo manter o equilíbrio redox se for desviado para a PPP (Fig.14). Apesar da PPP estar

envolvida em várias rotas importantes, nenhum inibidor desta rota chegou a ensaios clínicos. Assim, a glicólise e a fermentação lática tem sido o principal foco na busca de terapias tumorais anti-metabólicas (TENNANT; DURÁN; GOTTLIEB, 2010).

O inibidor da HK mais utilizado é a 2-desoxiglicose (2-DG), molécula análoga à glicose (ZHANG et al., 2014) (Fig.14). Apesar de promissora incialmente (ELY, 1954), a 2-DG tem pouco efeito como agente monoterápico. Inclusive, já foi demonstrado que 2-DG pode ativar rotas pró-sobrevivência via AKT/PI3K (ZHONG et al., 2009). Porém, ela é capaz de sensibilizar tumores a quimio e radioterapia e deve ser pensado como um agente adjuvante. Já existem estudos clínicos em andamento com esta abordagem em diversos tipos de câncer, incluindo pulmão e mama (TENNANT; DURÁN; GOTTLIEB, 2010; ZHANG et al., 2014).

A enzima final da glicólise é a PK (do inglês *pyruvate kinase*), e catalisa um passo importante na produção de ATP e na regulação de toda a via. O composto TLN-232 inibe a PK e está em fase de ensaio clínico. A isoforma PKM2 é mais comum em células tumorais (MAZUREK et al., 2005) e sabe-se que a isoforma comum a tecidos não-proliferativos (PKM1) é incompatível com crescimento tumoral (CHRISTOFK et al., 2008). Destacando assim o potencial terapêutico de um inibidor específico para a isoforma tumoral.

O 3-bromo-piruvato (3-BP) é um inibidor metabólico que apresenta efeito antitumoral notável em diversos modelos de câncer (CARDACI; DESIDERI; CIRIOLO, 2012; GANAPATHY-KANNIAPPAN; KUNJITHAPATHAM; GESCHWIND, 2013; GANAPATHY-KANNIAPPAN et al., 2010; SHOSHAN, 2012). A HK-II é a isoforma associada a reprogramação metabólica tumoral (PATRA et al., 2013) e inicialmente o 3-BP foi descrito como seu inibidor, porém as evidências não dão suporte a esta afirmação (GALINA, 2014). O 3-BP é um potente depletor de ATP inibindo não somente a glicólise, mas também o metabolismo mitocondrial (SHOSHAN, 2012) (Fig.14). Evidências apontam que seu principal alvo é a enzima GAPDH e que seu mecanismo de ação envolva estresse oxidativo, estresse de retículo, inibição de síntese protéica (CARDACI;

DESIDERI; CIRIOLO, 2012; GANAPATHY-KANNIAPPAN et al., 2010). A analogia do 3-BP com o piruvato não explica sua letalidade – que se deve ao efeito alquilante. Porém, esta analogia pode explicar sua especificidade, uma vez que ambas moléculas são captadas por MCTs (do inglês *monocarboxylate transporter*), transportadores comumente hiper-expressos em células tumorais (GANAPATHY-KANNIAPPAN; KUNJITHAPATHAM; GESCHWIND, 2013).

O produto final da glicólise é o piruvato, que é reduzido a lactato em células glicolíticas pelo processo de fermentação lática pela enzima LDH (do inglês *lactate dehydrogenase*) (Fig.14). Outra enzima importante no favorecimento da fermentação lática é a PDK1 (do inglês *pyruvate dehydrogenase kinase 1*), por impedir a metabolização do piruvato a acetil-CoA e consequente oxidação mitocondrial. A fermentação lática é muito importante para tumores, pois a inibição destas duas enzimas leva a redução do crescimento tumoral em modelos animais (BONNET et al., 2007; FANTIN; ST-PIERRE; LEDER, 2006; XIE et al., 2014). Existem ensaios clínicos para diferentes tipos de câncer com o inibidor da PDK1 Dicloroacetato (https://clinicaltrials.gov/ct2/results?term=Dichloroacetate&no_unk=Y).

Os MCT's (do inglês *monocarboxylate transporter*) são responsáveis pelo fluxo de lactato/piruvato comumente expressos acima do normal em tumores e, portanto, constituem outro alvo terapêutico potencial (Fig.14). Estes transportadores permitem à célula preservar seu pH intracelular (TENNANT; DURÁN; GOTTLIEB, 2010). Os mais importantes para as células tumorais são o MCT1 – fundamental na captação de lactato em células tumorais oxidativas (SONVEAUX et al., 2008) – e o MCT4 – associado a tumores glicolíticos pois otimiza a liberação e produção de lactato devido à sua baixa afinidade por piruvato (PARKS; CHICHE; POUYSSÉGUR, 2013). O silenciamento de MCT1 e MCT4 reduz o fluxo glicolítico e o crescimento tumoral *in vivo* (LE FLOCH et al., 2011). O MCT4 está associado ao comportamento metastático pela manutenção das taxas glicolíticas necessárias para alimentar este processo (GALLAGHER; CASTORINO; PHILP, 2009). Entretanto, não há nenhum inibidor descrito para inibir MCT4. O CHC

(do inglês *α-cyano-4-hydroxycinnamic acid*) é um inibidor não-específico de MCT1 com efeitos antitumorais *in vivo* promissores. O MCT1 em células tumorais capta o lactato secretado por células hipóxicas e a sua inibição por CHC induz comportamento glicolítico, reduz o crescimento tumoral e sensibiliza as células remanescente à radioterapia (SONVEAUX et al., 2008). Ainda, o CHC pode induzir necrose tumoral e reduzir invasão (COLEN et al., 2011).

Apesar dos efeitos promissores oriundos da inibição de MCT1, esta abordagem é controversa pois o lactato é importante a órgãos como músculo esquelético e cérebro. Assim, entende-se que esta inibição deveria ter efeitos muito rápidos, de modo a não afetar os tecidos sadios, ou então ser utilizada como adjuvante a outras terapias, a fim de reduzir as doses (TENNANT; DURÁN; GOTTLIEB, 2010). Já foi demonstrado que o co-tratamento de CHC com inibidores mitocondriais leva a célula a uma "catástrofe metabólica" (MARCHIQ et al., 2015). Outra combinação terapêutica promissora seria com inibidores de membros da família anidrase carbônica (CA), importante reguladores de pH em tumores (CHICHE et al., 2009).

Assim, justifica-se a euforia dos pesquisadores frente à oportunidade terapêutica apresentada pela reprogramação metabólica. Entretanto, o ramo ainda carece de drogas, ou outras abordagens, que se mostrem eficazes em pacientes. Por fim, a correta caracterização da reprogramação metabólica presente em cada tipo de câncer é fundamental para que futuras abordagens terapêuticas sejam bem sucedidas.

Carcinoma Pulmonar e Reprogramação Metabólica

O pulmão é um órgão único na sua relação com o oxigênio, o que tem implicações diretas no metabolismo das suas células e consequentemente em qualquer tumor que venha a se originar neste sítio. Assim, em um momento onde os olhos da oncologia estão voltados para o metabolismo, é fundamental que o câncer mais letal seja estudado neste contexto.

O acúmulo de ^{18}F-FDG, um análogo radioativo da glicose, observado por *PET scan* é uma ferramenta diagnóstica aceita para vários tipos de câncer. Em carcinomas pulmonares, o acúmulo é maior em tumores pouco diferenciados, considerados mais agressivos (KAIRA et al., 2011). Porém, não há um consenso sobre reprogramação metabólica em câncer de pulmão, uma vez que outros trabalhos indicam a OXPHOS como rota preferencial para obtenção de energia em câncer de pulmão (MORENO-SÁNCHEZ et al., 2007).

Já foi demonstrado que linhagens de NSCLC são dependentes da glicólise, pois são incapazes de compensar a síntese de ATP quando esta via é inibida. Esta reprogramação metabólica relaciona-se com crescimento tumoral e resistência a apoptose, conferindo, portanto, um fenótipo mais agressivo (WU et al., 2007). A perda do supressor tumoral LKB1 associa-se com comportamento glicolítico e tumorigênese (FAUBERT et al., 2014) e a inibição da enzima-chave à fermentação lática LDH-A (do inglês *Lactate Dehydrogenase-A*) reduz a tumorigênese e a progressão tumoral em modelos animais de NSCLC (XIE et al., 2014). Muitos casos de NSCLC apesentam expressão de HIF (GIATROMANOLAKI et al., 2001), mantendo ativa a via glicolítica e o controle da acidez, quando estes deveriam se restringir a momentos de hipóxia (BERTOUT; PATEL; SIMON, 2008). Em NSCLC glicolíticos, a enzima Piruvato Carboxilase se mostrou essencial à tumorigênese por alimentar o Ciclo de Krebs para fins anapleróticos, evidenciando a importância da biossíntese (SELLERS et al., 2015).

K-RAS mutada é comum em NSCLC, especialmente em AdC (RIELY; MARKS; PAO, 2009), e associa-se com desbalanço redox, quimioresistência, pior prognóstico (MENG et al., 2013; SHAW et al., 2011) e promove comportamento glicolítico (VIZAN et al., 2005). Mutações em K-RAS e EGFR são mutuamente excludentes (KARACHALIOU et al., 2013). Porém, pacientes com EGFR mutado respondem a TKI's (do inglês *tyrosine kinase inhibitors*). Portanto, qualquer abordagem terapêutica para NSCLC glicolíticos, terá impacto clínico imediato.

A elevada expressão de GLUT1 e MCT4 em NSCLC está relacionada a tumores pouco diferenciados, linfonodos comprometidos e menor sobrevida (MEIJER et al., 2012). O metabolismo é diferente mesmo entre os dois subtipos mais comuns de NSCLC: SQC e AdC. SQC possui maior expressão de GLUT1 e a sua expressão de GLUT1 e MCT4 aumenta com a distância dos vasos. Por outro lado, as amostras de AdC apresentam expressão de MCT4 independente da distância da vasculatura. Assim, acredita-se que AdC é o subtipo mais glicolítico de NSCLC e possa se beneficiar de terapias metabólicas (MEIJER et al., 2012).

Objetivo

Esta etapa do trabalho buscou caracterizar o perfil bioenergético de duas linhagens de adenocarcinoma pulmonar com diferentes níveis de agressividade: EKVX mais agressiva quando comparada à A549 (ver Capítulo I). A partir desta, será investigado o valor prognóstico destes achados em coortes clínicas para então sugerir-se novos alvos terapêuticos.

Metodologia

Cultura de células

Foram utilizadas as linhagens humanas de NSCLC: A549 e EKVX (NCI-Frederick Cancer DCTD tumor/cell line repository). As células foram cultivadas em meio RPMI-1640 com 2mM L-glutamina suplementado com 10% de SFB inativado e antibiótico (estreptomicina e penicilina). As células foram mantidas em fase exponencial de crescimento dentro de estufa com atmosfera úmida de 5% de CO_2 à 37°C.

Ensaio de inibição de crescimento

Foram realizados ensaios de inibição de crescimento celular pela técnica de sulforodamina B (SRB) (VICHAI; KIRTIKARA, 2006). Para tal as células foram plaqueadas em placas de 96 poços apropriadas para cultivo celular e, após 24h para permitir adesão, as células

foram tratadas com diferentes concentrações dos seguintes inibidores metabólicos: 2-desoxiglicose, 3-bromopiruvato, Floretina e CHC.

Após o tempo de incubação adequado as células foram fixadas com TCA 10% por 1 hora a 4°C e então são deixadas secar a temperatura ambiente. Posteriormente, o SRB foi adicionado (2% p/v em ácido acético 1% v/v). Após 15 minutos em temperatura ambiente o excesso do corante foi removido e o conteúdo no fundo do poço ressuspendido em Tris-HCl e agitado por 20 min em agitador automático. Por fim, foi feita uma leitura de ponto final a 490nm em espectrofotômetro de placa. Utilizando o grupo controle como referência, foi calculado o percentual de inibição de crescimento de cada dose e depois a partir de uma curva de regressão linear foi obtido o valor de IC_{50} (dose que inibe o crescimento em 50%).

Respirometria

O consumo basal de oxigênio foi medido utilizando respirometria de alta resolução (Oroboros Oxygraph-O2K). Para isto, as células foram cultivadas normalmente até o momento do experimento e então foram tripsinizadas e ressuspendidas em meio de cultura sem SFB.

O consumo de rotina, respiração independente de oligomicina (*proton leak*) e respiração máxima (estimulada por FCCP) foram avaliados. O software DatLab software (Oroboros Instruments, Innsbruck, Austria) foi utilizado para aquisição dos dados e análise.

Expressão gênica (RT-PCR)

O RNA foi isolado das células utilizando TRIzol (Invitrogen), seguindo instruções do fabricante. O RNA foi quantificado espectofometricamente e tratado (1 µg de RNA) com 1 U de DNAse livre de RNAse por 30 minutos a 37°C. As reações são paradas pela adição de 1 µL de 20 mM EDTA e aquecendo por 10 minutos a 65°C. A síntese de cDNA foi feita com RNA tratado com DNAse utilizando o kit "High Capacity cDNA Reverse Transcription" (Applied Biosystems),

seguindo instruções do fabricante. Para o experimento, foi utilizado o equipamento 7500 Real Time PCR (Applied Biosystems) e o kit power SYBR-GREEN PCR master MIX (Applied Biosystems).

A análise de expressão foi realizada utilizando pares de *primers* desenhados utilizando o GenBank (http://www.ncbi.nlm.nih. gov/genbank/), cujas sequências podem ser encontradas abaixo (Tab.2). O método de comparação de Ct foi utilizado para quantificar a expressão gênica relativa à actina, utilizado como controle endógeno.

Tabela 2 Sequência de *primers* utilizados para RT-PCR

Gene-alvo	Sequência
Actina	Forward 5'- TTCCTTCCTGGGCATGGAGTC -3' Reverse 5'- AGACAGCACTGTGTTGGCGT A -3'
GLUT 1	Forward 5'- AATGCTGATGATGAACCTGCT -3' Reverse 5'- CAGTACACACCGATGATGAAG -3'
GLUT 3	Forward 5'- CTTTCTCATCCCACGCACTC -3' Reverse 5'- CACTCGGTCTCTCCTAAGCA -3'
HK I	Forward 5'- GATCATCGGCACTGGCACCAA -3' Reverse 5'- CCAAAGGCTCCCCATTCTGTA -3'
HK II	Forward 5'- ATGAGGGGCGGATGT GTATCA -3' Reverse 5'- GGTTCAGTGAGCCCATGTCAA -3'
LDH A	Forward 5'- GCAGATTTGGCAGAGAGT ATAATG -3' Reverse 5'- GACATCATCCTTTATTCCGTAAAGA -3'
LDH B	Forward 5'- GATGGATTTTGGGGGAACAT -3' Reverse 5'- AACACCTGCCACATTCACAC -3'
MCT 1	Forward 5'-TGGGTACTGGAACAAGCAAA -3' Reverse 5'- GCAGGTCAAATCCAAATATC G -3'
MCT 4	Forward 5'- GAGTTTGGGATCGGCTACAG -3' Reverse 5'- CGGTTCACGCACACACTG -3'

Atividade enzimática

O lisado das células foi preparado expondo as culturas a nitrogênio líquido em tampão de lise (Tris 10mM pH 8, DTT 1mM) com 5 µl/mL inibidor de proteases (Sigma® P8340). As reações foram iniciadas adicionando-se 10-100 µg de proteína ao meio de reação adequado (Tab.3), de acordo com a linearidade de cada ensaio, a 37°C em placa de 96 poços. A absorbância foi medida a 340nm. A atividade específica da enzima foi definida como quantidade de substrato formado por miligrama de proteína por minuto.

Tabela 3 Meios de reação utilizados para ensaios de atividade enzimática

	ENZIMAS				
Reagentes	HK	PYK	LDH	G6PDH	Unidade
Tris-HCl pH7.4	20	50	50	20	**mM**
Triton X-100	0.05	0.05	0.05	0.05	**%**
$MgCl_2$	10	5		10	**mM**
β-NAD^+	1				**mM**
ATP	1				**mM**
Glicose	5				**mM**
G6PDH	2				**U/mL**
KCl		50			**mM**
ADP		2.5			**mM**
PEP		5			**mM**
LDH		0.5			**U/mL**
β-NADH		0.2	0.2		**mM**
EDTA			1		**mM**
Piruvato			1		**mM**
β-$NADP^+$				1	**mM**
G6P				5	**mM**

HK – hexocinase; PYK – piruvato cinase; LDH – lactato desidrogenase; G6PDH – glicose-6-fosfato desidrogenase; PEP – fosfoenolpiruvato

Análise de risco associado à expressão de transportadores

A taxa de risco (*hazard ratio*) prevê a probabilidade de um evento, em uma população no tempo "t", associado à variável investigada.

Os valores de expressão gênicas de pacientes foram obtido junto à plataforma Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.ni h.gov/geo/). Foram utilizados os seguintes banco de dados: GSE37745 (106 casos); GSE41271 (177 casos); GSE42127 (132 casos). Antes de serem utilizados, os valores foram normalizados (z-score). Todos receptores foram testados em todas coortes utilizando o pacote estatístico R (http://www.r-project.org/) e aqueles que apresentaram diferença estatística foram levados adiante.

Análise estatística

Os resultados estão expressos como media ± desvio padrão de pelo menos 3 experimentos independentes em triplicata. Os dados foram analisados por teste t de student ou

ANOVA de uma via, seguidos de teste Neuman-Keuls. Diferenças foram considerada s estatisticamente significativas quando $p < 0,05$ (GraphPad Prism® v6.01).

Resultados

Respirometria demonstra que A549 é mais oxidativa que EKVX

A linhagem A549 apresenta perfil de consumo de O_2 mais oxidativo quando comparado com a linhagem EKVX (Fig.15). O consumo de rotina já evidencia que A549 utiliza mais oxigênio em estado basal. O segundo parâmetro aumentado é o consumo acoplado à síntese de ATP oxidativa, obtido a partir da adição do inibidor da cadeia transportadora de elétrons oligomicina . Neste, a linhagem A549 reduz mais seu consumo de O_2. Isto indica que A549 apresenta maior consumo de O_2 destinado para a síntese de ATP pela OXPHOS. Ainda, a respiração máxima é maior na A549 e, consequentemente a capacidade de reserva. Este parâmetro é obtido a partir do uso de um desacoplador mitocondrial (FCCP). A reserva indica quantas vezes acima do basal o consumo pode ser elevado. Novamente, eles indicam que a A549 é mais oxidativa.

Por fim, o consumo não associado à OXPHOS está aumentado na EKVX, indicando que além desta linhagem ter um consumo menor, ela usa mais do seu consumo em reações não relacionadas ao metabolismo oxidativo.

A atividade enzimática e a expressão gênica confirmam que EKVX é mais glicolítica

Foi investigado a atividade enzimática e a expressão gênica de proteínas-chave no metabolismo energético para melhor caracterizar as linhagens. A linhagem EKVX apresenta maior atividade de HK, enquanto a A549 apresenta maior atividade da G6PDH, PK, LDH (Fig. 16). Tanto HK como LDH apresentam maior atividade em tumores glicolíticos e, paradoxalmente , cada uma destas enzimas apresentou maior atividade em uma linhagem. Ainda, A549 apresenta maior atividade de G6PDH, indicando uma maior utilização da via das pentoses.

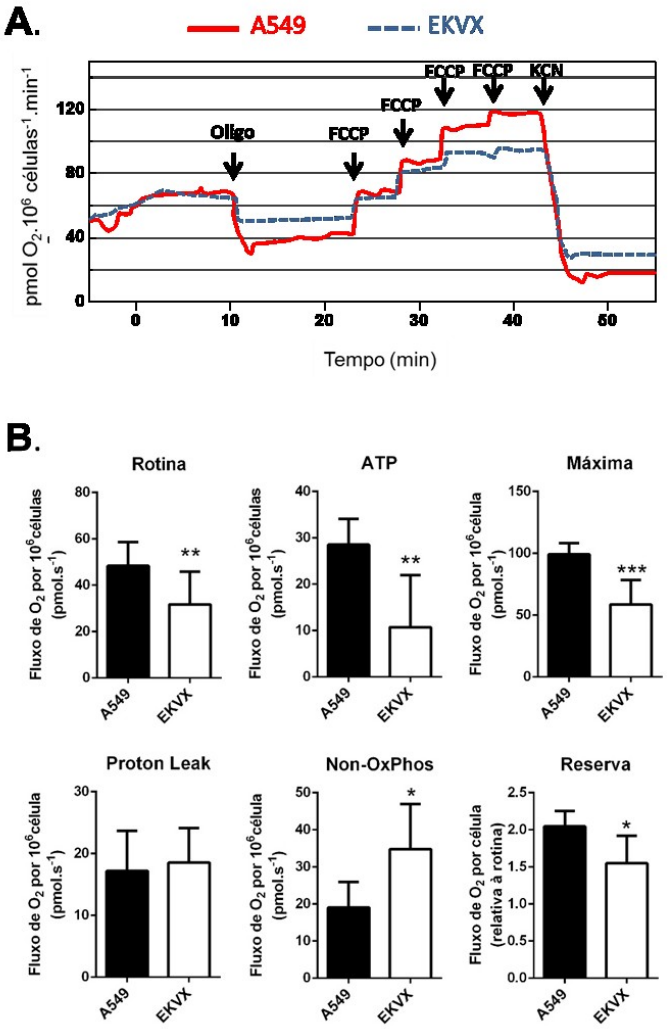

Figura 15 Respirometria de alta resolução das linhagens de AdC de pulmão. (A) Análise representativa do consumo de oxigênio das linhagens A549 e EKVX.(B) Parâmetros respiratórios: Rotina – consumo basal; ATP – consumo acoplado à síntese de ATP medido com adição de oligomicina; Máxima – consumo máximo após uso do desacoplador FCCP; Proton leak – consumo de oxigênio após adição de oligomicina, portanto, não acoplada à síntese de ATP; Non-OxPhos – consumo após adição de KCN, ou seja, independente do consumo via cadeia transportadora de elétrons; Reserva – aumento relativo do consumo após desacoplamento. Média ± desvio padrão; N = 9, teste t não-pareado. * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$.

A EKVX apresenta maior expressão de diversos genes associados ao perfil glicolítico (Fig.17). Especialmente HK 2, isoforma mitocondrial associada à reprogramação metabólica em tumores glicolíticos (GALINA, 2014; PATRA et al., 2013). A expressão de transportadores de glicose não-responsivos à insulina (GLUT 1 e 3), normalmente aumentada em tecidos glicolíticos, é maior na linhagem EKVX. A isoforma LDH-A também está aumentada na EKVX e é associada à fermentação lática. Por fim, o transportador induzível por hipóxia MCT4, normalmente expresso

em tumores glicolíticos (PARKS; CHICHE; POUYSSÉGUR, 2013), também está aumentado em EKVX. Por outro lado, A549 tem maior expressão do MCT1, associado ao metabolismo oxidativo (PINHEIRO et al., 2012; SONVEAUX et al., 2008).

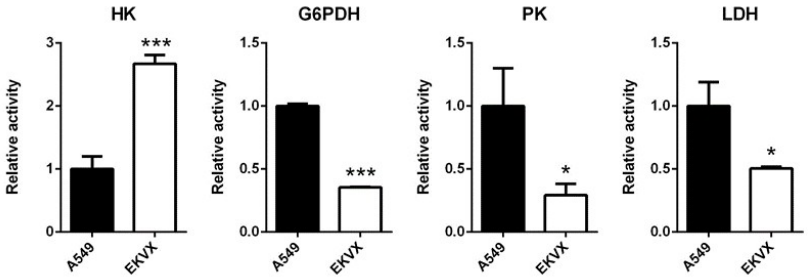

Figura 16 Atividades enzimáticas das linhagens de adenocarcinoma pulmonar. HK – hexoquinase; G6PDH – glicose-6-fosfato desidrogenase; PK – piruvato quinase; LDH – lactato desidrogenase. Os valores representam média ± desvio padrão; N = 3, teste t não-pareado. * p < 0,05; *** p < 0,001.

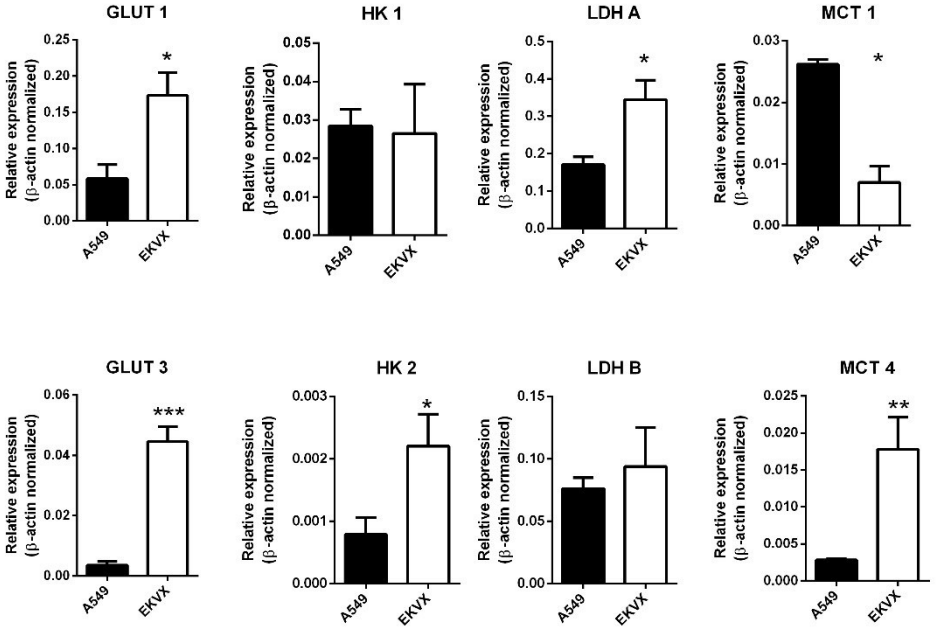

Figura 17 Expressão gênica das linhagens de adenocarcinoma pulmonar. GLUT – transportador de glicose; HK – hexoquinase; LDH – lactato desidrogenase; MCT – transportador de monocarboxilato. Os valores representam média ± desvio padrão; N = 3, teste t não-pareado. * p < 0,05; ** p < 0.01; *** p < 0,001.

Sensibilidade a anti-metabólicos aponta transportadores como um possível alvo

A linhagem glicolítica EKVX foi mais sensível (menor IC_{50}) ao 3-BP, FLO e CHC, enquanto a A549 foi mais sensível a 2-DG e à oligomicina (Fig.18). A maior sensibilidade à oligomicina

indica uma maior dependência da OXPHOS. Baseado nos alvos conhecidos de cada composto, a linhagem mais glicolítica EKVX é mais sensível à inibição de transportadores, GLUT's e MCT's.

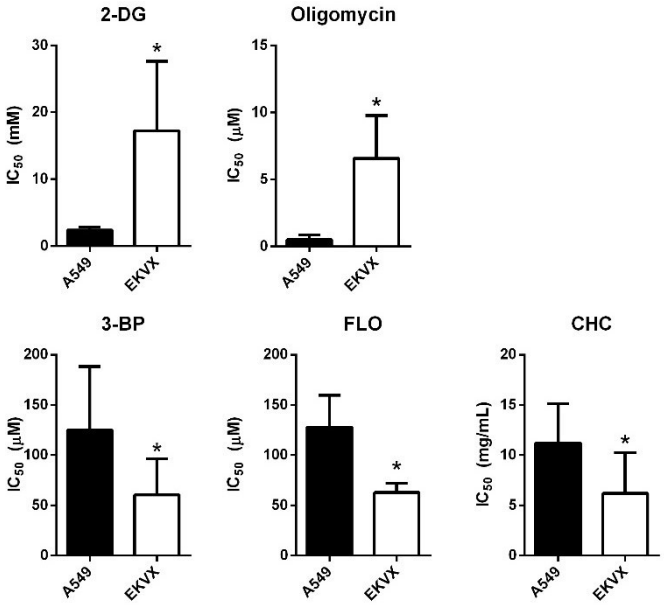

Figura 18 Sensibilidade a moléculas anti-metabólicas das linhagens de adenocarcinoma de pulmão. As linhagens foram tratadas com diferentes doses de cada composto e a partir da curva de crescimento celular foi inferido a dose que inibe o crescimento em 50% (IC_{50}). 2-DG – 2-deosoxiglicose; 3-BP – 3-bromopiruvato; FLO – floretina; CHC – ácido α-hidroxicinâmico. Os valores representam média ± desvio padrão; N = 3, teste t não-pareado. * $p < 0,05$.

Expressão de transportadores se correlaciona com pior prognóstico

Sabendo-se que a linhagem mais agressiva é também mais glicolítica e mais sensível a inibidores de transportadores, naturalmente foi questionado qual seria o valor clínico desta informação. Assim, foi obtido dados de expressão de 3 coortes de adenocarcinoma de pulmão no qual foi investigado o valor prognóstico dos GLUT e MCT.

Os transportadores GLUT1, GLUT2, MCT1, MCT3 e MCT10 se correlacionaram com pior prognóstico em coortes de adenocarcinoma pulmonar (Fig.19). O transportador MCT3 foi o mais consistente, estando relacionado a pior prognóstico em duas coortes.

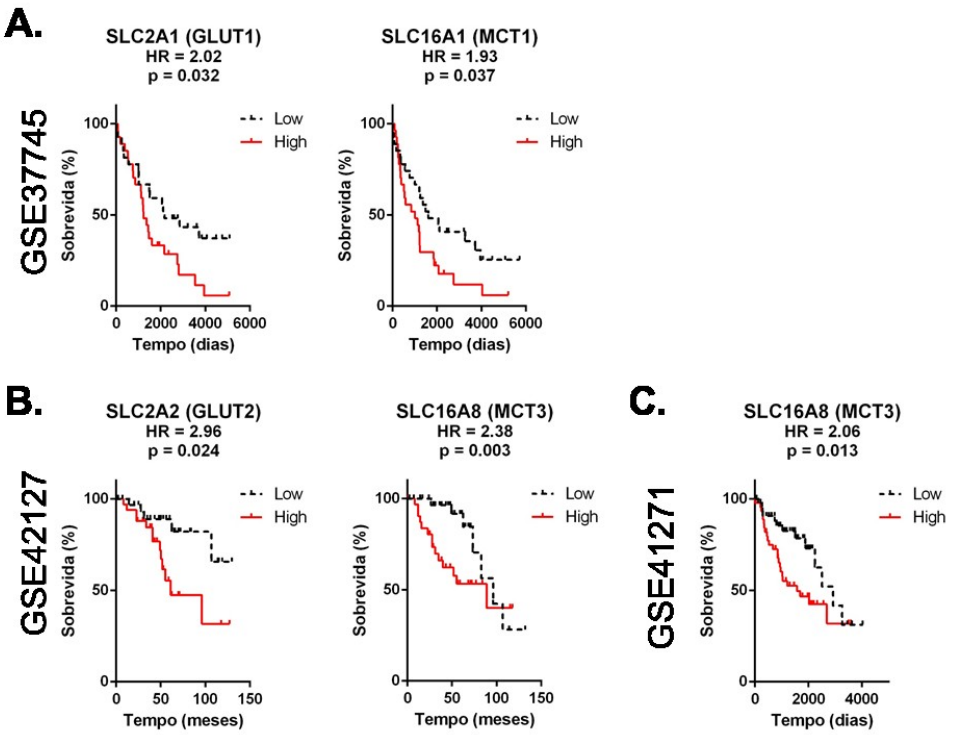

Figura 19 Valor prognóstico de genes de transportadores de glicose (GLUT) e monocarboxilato (MCT) em adenocarcinoma de pulmão. (A) A expressão do gene do transportador GLUT1 e MCT1 se correlaciona com pior prognóstico na coorte GSE37745, composta por 106 casos de adenocarcinoma . (B) A expressão do gene dos transportadores GLUT2 e MCT3 se correlaciona com pior prognóstico na coorte GSE42127, composta por 132 casos de adenocarcinoma. (C) A expressão do gene do transportador MCT3 se correlaciona com pior prognóstico na coorte GSE41271, composta por 177 casos de adenocarcinoma. Os dados de expressão foram obtidos no banco de dados GEO (http://www.ncbi.nlm.nih.gov/geo/). Os gráficos e análises estatísticas foram feitos no GraphPad Prism® v6.01. HR – *hazard ratio*.

Discussão

O presente estudou reprogramação metabólica em AdC pulmonar e demonstrou que a linhagem menos agressiva A549 é mais oxidativa, enquanto a linhagem mais agressiva EKVX é mais glicolítica por diferentes ferramentas: respirometria de alta resolução, atividade enzimática, expressão gênica, sensibilidade a compostos anti-metabólicos e dados de microarranjo. Por fim, foi demonstrado o valor prognóstico de diferentes transportadores em coortes de adenocarcinoma pulmonar. Baseados nestes resultados, sugerimos que a reprogramação metabólica é um fenômeno relevante em câncer de pulmão e sugerimos os transportadores (GLUT's e MCT's) como um promissor alvo terapêutico. Estes resultados serão discutidos em maior profundidade a seguir.

Primeiramente, nossos dados indicam uma associação entre agressividade e metabolismo glicolítico, validando o estudo sobre reprogramação metabólica nestas linhagens de AdC pulmonar. Previamente, nosso grupo caracterizou a linhagem EKVX como mais agressiva comparada à A549 (CASTRO et al., 2010a; LISBÔA DA MOTTA et al., 2014). A linhagem A549 demonstrou maior consumo de O_2 em uma série de parâmetros associados com comportamento oxidativo e ainda foi mais sensível ao tratamento com oligomicina. As atividades enzimáticas não mostram claramente uma linhagem mais glicolítica pois tanto HK como LDH apresentam atividade aumentada em tumores glicolíticos e cada linhagem apresentou atividade aumentada em uma delas. Entretanto, a expressão de diversos genes associados ao metabolismo glicolítico é maior na EKVX. Coletivamente, estes resultados indicam que, nesta comparação, a EKVX é a linhagem de AdC pulmonar mais glicolítica. Este achado tem impacto imediato para estudos *in vitro* de reprogramação metabólica em câncer de pulmão.

A expressão de GLUT1, GLUT3 e MCT4 é maior na linhagem mais agressiva EKVX e esta linhagem se mostrou mais sensível aos inibidores de transportadores (FLO e CHC) e expressão de GLUT1 se relaciona com pior prognóstico em coorte clínica de AdC pulmonar. Sabe-se que GLUT1 e GLUT3 tem expressão aumentada em diversos tumores, são marcadores de hipóxia e de metabolismo glicolítico e correlacionam-se com pior prognóstico (GATENBY et al., 2007). Ainda, em AdC pulmonar, alta expressão de GLUT1 se correlaciona com agressividade (MEIJER et al., 2012), corroborando os resultados apresentados aqui. Por fim, anticorpos contra GLUT1 são capazes de inibir proliferação em NSCLC e também de aumentar a sensibilidade a quimioterápicos como daunorubicina, cisplatina, paclitaxel e gefitinib (CAO et al., 2007; SZABLEWSKI, 2013). Portanto, nossos achados suportam o transportador GLUT1 como um possível alvo terapêutico em adenocarcinoma de pulmão.

O MCT4 também associa-se ao comportamento glicolítico. Este é induzido por hipóxia e, devido à sua baixa afinidade por piruvato, estimula a produção de lactato (PARKS; CHICHE;

POUYSSÉGUR, 2013). Em adenocarcinomas de pulmão, a expressão elevado de MCT4 e GLUT1 associa-se com menor sobrevida, indicando maior agressividade (MEIJER et al., 2012). Ainda, a expressão de MCT4 pode estar associada à metástase e invasão (COLEN et al., 2011; GALLAGHER; CASTORINO; PHILP, 2009), a maior causa de morte em casos de câncer avançados. E sabemos que a linhagem EKVX apresenta a maior capacidade invasiva do painel de linhagens de câncer de pulmão do NCI-60 (CASTRO et al., 2010a; LISBÔA DA MOTTA et al., 2014). Assim, estratégias inibindo estes transportadores podem atacar seletivamente AdC pulmonares mais glicolíticos e agressivos. No entanto, não há até o momento opções terapêuticas para inibir especificamente este transportador.

Os resultados relativos à expressão de MCT1 são paradoxais. Por um lado, a sua expressão é maior na linhagem menos agressiva e menos glicolítica, a A549, corroborando achados da literatura que descrevem a do MCT1 à células oxidativas do tumor (SONVEAUX et al., 2008). Por outro, sua maior expressão relaciona-se com pior prognóstico em coorte de pacientes de adenocarcinoma. Recentemente, a expressão de MCT1 no estroma tumoral foi apontado como um fator prognóstico independente para câncer de pulmão (EILERTSEN et al., 2014). Assim, remete-se à ideia de simbiose tumoral, apesar das células maligna estar mais associada com MCT4, o tumor pode apresentar aumento de MCT1 e, este, ter valor prognóstico.

Paradoxalmente, a linhagem EKVX foi mais resistente à 2-DG, o que é inesperado por ela ser mais glicolítica. Entretanto, a 2-DG é um inibidor competitivo da HK e, como a EKVX tem maior atividade desta enzima em condições basais, mais inibidor é necessário. Ainda, apesar da 2-DG ser capaz de inibir um passo importante do metabolismo da glicose, no contexto geral este composto é capaz de causar outros efeitos e até mesmo ativar vias pró-sobrevivência (ZHONG et al., 2009). A EKVX também se mostrou mais sensível ao 3-BP, um composto descrito como um depletor de ATP generalizado (GALINA, 2014), indicando uma célula mais metabolicamente ativa. Devido a seus efeitos inespecíficos, é difícil interpretar este resultado em termos de

mecanismo. Entretanto, sabe-se que o 3-BP apresenta efeitos notáveis em tumores glicolíticos com pouco ou nenhum efeito colateral (GESCHWIND et al., 2002; JAE et al., 2009; KO et al., 2004). O 3-BP não somente impede crescimento, mas também é capaz de erradicar tumores, portanto sendo um antimetabólico importante neste tipo de estudo (SHOSHAN, 2012).

A expressão de GLUT2 ter apresentado valor prognóstico é inesperada, pois não é a isoforma de GLUT mais associada com reprogramação metabólica tumoral. Este transportador possui baixa afinidade (alto Km) por glicose, sendo regulado pela concentração de glicose, sendo particularmente importante no intestino pós-prandial, fígado, ilhotas pancreáticas e retina. No entanto, a expressão de GLUT2 em tumores já foi reportada em câncer de estômago, mama, cólon, fígado e lesões pré-tumorais de pâncreas (SZABLEWSKI, 2013). Por outro lado, a expressão deste transportador pode ser reprimida por oncogenes e está associada com menor potencial invasivo (MACHEDA; ROGERS; BEST, 2005). É a primeira vez que a expressão de GLUT2 é reportada como fator prognóstico para câncer de pulmão.

O MCT3 é codificado pelo gene SLC16A8 que apresentou valor prognóstico para pacientes de adenocarcinoma de pulmão, porém poucos estudos investigaram este transportador. Sabe-se que ele exporta lactato, é expresso na retina e plexo coróide e possui a menor afinidade por monocarboxilato dentre os 4 MCT's comprovadamente funcionais em humanos, MCT 1 – 4 (HALESTRAP, 2013). A expressão de MCT3 em câncer de pulmão já foi reportada e associada com melhor prognóstico (EILERTSEN et al., 2014), em contraste com os resultados apresentados aqui. Este mesmo estudo descreve a expressão do MCT3 como citosólica, o que dificulta a compreensão do seu papel na reprogramação metabólica tumoral.

Assim, este estudo corrobora a importância da reprogramação metabólica em NSCLC e sugeriu alvos terapêuticos. Ao conhecimento dos autores, é a primeira vez que a linhagem EKVX é caracterizada sob esta ótica, o que contribuirá para investigações *in vitro*. Por fim, o MCT3 surge com um alvo pouco estudado em NSCLC que pode contribuir para pesquisa e terapia.

CAPÍTULO III: HIPÓXIA E EPIGENÉTICA

Manuscrito elaborado para ser submetido

1 –The Bet Inhibitor JQ1 Impairs Tumour Response To Hypoxia Downregulating CA9 and Angiogenesis In Triple Negative Breast Cancer

Periódico: Cancer Discovery

Fator de Impacto (JCR-2015): 15.929

Qualis: C (Ciências Biológicas)

OBJETIVO GERAL DO CAPÍTULO

Investigar o potencial do modulador epigenético JQ1 para inibir a expressão de CA9 e matar células resistentes à hipóxia em câncer de mama triplo-negativo.

OBJETIVOS ESPECÍFICOS

- Avaliar o efeito de JQ1 em linhagens de TNBC, quanto aos seguintes parâmetros:
  - Transcriptoma completo por microarranjo
  - Inibição de crescimento de culturas 2D e 3D
  - Expressão de CA9 e outros genes regulados por hipóxia
  - Ligação de HIF ao promotor de CA9 (*ChIP assay*)
  - Atividade de HIF (*luciferase gene repórter*)
- Avaliar o efeito de JQ1 em modelo animal de TNBC, quanto aos seguintes parâmetros:
  - Taxa de crescimento do tumor
  - Expressão de CA9 e outros genes regulados por hipóxia
  - Quantificar presença de vasos sanguíneos

FENÓTIPO AGRESSIVO ESTUDADO

A resistência à hipóxia associa-se com pior prognóstico em TNBC e contribui para progressão tumoral, quimio/radioresistência e metástase. Esta agressividade é mediada por vias normalmente utilizadas somente em momentos de estresse – regulação do pH, glicólise e angiogênese – que acabam ficando ativas continuamente, conferindo vantagem seletiva às células malignas (KEITH; JOHNSON; SIMON, 2012; MILANI; HARRIS, 2008; WILSON; HAY, 2011).

PROPOSTA TERAPÊUTICA

JQ1 é um potente inibidor de CA9, e outros genes induzidos por hipóxia, sendo capaz impedir a ligação de HIF ao seu promotor. Além disso JQ1 foi eficaz na redução do crescimento tumoral e angiogênese em modelo animal de TNBC.

The BET Inhibitor JQ1 Impairs Tumour Response to Hypoxia Downregulating CA9 and Angiogenesis In Triple Negative Breast Cancer

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Key words: bromodomain, I-BET151, I-BET762, VEGF-A, glycolysis, pH, MDA-MB-231, HCC1806

Running title: JQ1 impairs tumour hypoxia response in breast cancer

Introduction

Epigenetic alterations have recently emerged as one of the most promising targets in cancer therapy [1]. Bromodomain and extra-terminal (BET) proteins comprise a family that recognizes acetylated histone residues and then recruit transcriptional complex. BET inhibitors have performed extremely well in preclinical settings and clinical trials are on the way to access its therapeutic value [2,3]. Breast cancer is the leading cause of cancer deaths in women and 10-20% of these are triple-negative breast cancer (TNBC). This is the most lethal breast cancer subtype and owe its name to the lack of known specific markers, which means there is no targeted therapy currently available for TNBC [4].

JQ1 is a selective BET inhibitor (BETi) [5] that showed antitumoural effects in both solid and non-solid malignancies, including breast [6,7], lung [8], skin [9], prostate [10] and ovarian cancer [11], medulloblastoma [12,13], neuroblastoma [14,15], glioblastoma [16], osteosarcoma [17] and finally myeloma [18], leukemias [19–22] and lymphomas [21–24]. JQ1 demonstrated inhibit tumour growth and increase survival in animal models [1,12,14]. JQ1 also can induce cell cycle arrest [8,9,12,17,18,21], differentiation [1,5], senescence [17,18], autophagy [16] and reduce clonogenicity [1]. One non-consensual effect of JQ1 is apoptosis, it was already reported that JQ1 reduced apoptosis [12], while other studies found JQ1 to stimulate apoptosis [8,10,14,21] and some did not find any effect [9,17].

Although most of JQ1 effects is attributed to its capacity to downregulate MYC [6,9–12,14–16,18], it is unlikely that this is the only mechanism JQ1 impairs cancer [1]. MYC is a master regulator of cell proliferation and metabolism usually overexpressed in malignancies, including breast cancer [25,26]. MYC expression was suggested as predictor of JQ1 efficacy [9,11,12,18]. However, some studies do not corroborate this [8], since not always MYC downregulation is sufficient to inhibit cell growth [21] and it is possible to observe JQ1 effects without alteration of MYC expression [17]. In fact, BET protein can associate with different transcription factors [10,13,27] and a variety of genes were already found to be regulated by BET inhibitors, such as: p21[9,17,21], BCL-xl[10] and BCL2[16], AKT[16], FOSL1[8], cyclin D1, p27 and p57[9], RUNX2 [17] and IKK [24]. Once tumours overexpress different sets of genes to establish malignancy and JQ1 displaces BET proteins from chromatin [5], one can presume JQ1 acts downregulating pathways overexpressed in cancer.

Hypoxia occurs in most solid tumours due to outgrowth of vasculature and drives upregulation of glycolysis, angiogenesis and pH regulation genes, among other pathways. Most

of these are regulated by the hypoxia inducible factor (HIF) [28]. Physiologically, HIF adapt the cell to survival in a hostile environment, but under uncontrolled circumstances hypoxia may support tumour progression [29,30]. In fact, HIF-1α and HIF2 α expression associates with poor prognosis in breast cancer [28,30] as well as some HIF-induced genes like, VEFG[31], GLUT1 [32] and CAIX [32–34]. Eventually, hypoxia lead to chemo and radioresistance, increased risk of invasion and metastasis. Therefore, impairing hypoxic response in tumours is desirable, but since it is difficult to target transcription factors like HIF [28], targeting downstream HIF-targets seems to be more feasible and equally promising [35].

In this regard, the transmembrane protein carbonic anhydrase 9 (CA9) is a good candidate. CA9 regulates intra / extracellular pH gradient and is induced by HIF1α to protect the cells from acidic conditions. It is considered an endogenous marker of hypoxia and is overexpressed in many malignancies and associating with poor prognosis [32–34]. In breast cancer, CA9 significantly associated with the triple negative phenotype and is inversely correlated with the BRCA1 expression [34]. CA9 promotes tumour growth and invasion and associates with tumor size, grade, chemoresistance and metastasis [33,36,37]. CA9 inhibition reduces primary tumour growth and metastasis resulting in prolonged survival and minimal recurrence in animal model [33,38]. Also, CA9 inhibition sensitizes tumours to radiotherapy and anti-angiogenic treatment [36,38]. Finally, an antibody against CA9 administered in combination with interferon-α (IFNα) increased survival in patients with metastatic cancer [38]. This has clear impact in metastatic breast cancer management.

Therefore, this work aimed to investigate the effect of the BETi JQ1 in hypoxic tumour response, especially CA9 expression, in TNBC. Here, we demonstrate that JQ1 impairs tumor growth, reduces CA9 and other hypoxia-induced genes and can inhibit angiogenesis. Thus, we believe BET inhibition is a promising approach to TNBC therapeutic management.

Results

JQ1 affect response to hypoxia and downregulate CA9 in breast cancer cell lines

In order to investigate possible effects of JQ1 on hypoxic response of tumor cells, we first did a whole transcriptome analysis (Fig.1). Initially we observe that JQ1-treated cells present large differences in gene expression, 2,338 differentially expressed genes (DEG) in MDA-MB-231 and 2,993 in MCF-7. Hypoxia status has a lesser effect, 119 DEG in MDA-MB-231 and 1,286 in MCF-7. A large fraction of the DEG in hypoxia have their expression modulated by JQ1 in hypoxia, 44% in MDA-MB-231 and 29% in MCF-7 (Fig 1A and S1A). Interestingly, within these genes, approximately half of them are downregulated by JQ1 compared to untreated cell, while the other half is further upregulated.

In order to investigate whether JQ1 affects tumor cells response to hypoxia, we first defined which pathways were hypoxia–regulated and then investigated the expression of these pathways in JQ1-treated cells. Then, it was created a Hypoxia Network (HyN) (Fig.1B) containing the previously published Hypoxia Signature[29] and the complete list of genes (obtained from KEGG, Table S1) for each pathway found to be hypoxia-regulated. Hypoxic condition up-regulated most of the HyN clusters in both cell lines (Fig.1C and S1B), as expected. Gene Set Analysis (GSEA) informs that hypoxia upregulates angiogenesis, glycolysis, OXPHOS and PPP set of genes (Table S2) in MDA-MB-231. Interestingly, the Hypoxia Signature set of genes was upregulated, validating its use in cell lines. JQ1 treatment prevented hypoxic up-regulation of Hypoxia Signature, angiogenesis, OXPHOS and PPP gene data sets, but did not alter glycolysis neither MYC. Additionally, it downregulated cell cycle and TCA set of genes (Fig.1B and Table S2). MCF-7 results confirm most of these findings (Fig. S1B and Table S2).

CA9 was the most prominently gene downregulated by JQ1 in hypoxia (Fig.1A). Within the JQ1-DEG genes in hypoxia consistently affected in both tested cell lines 2 of them are associated with poor prognosis in TNBC: CA9 and LOX (Fig.1D). Therefore, JQ1 can affect response to hypoxia in breast cancer and modulate genes with clinical implication.

JQ1 reduce growth in 2D and 3D models of TNBC

Next, it was investigated if JQ1 affects TNBC cell lines growth. JQ1 dose-dependently reduced cell growth in monolayer (2D) cultures in all the 4 TNBC cell lines tested, both in hypoxia and normoxia (Fig.2A and S.2A). The non-active enantiomer (-)-JQ1 [5] was also tested and showed no effect on cell growth. Thus, the observed effect can be attributed to BET-bromodomain inhibition by JQ1 (Fig.2A and S2A). Interestingly, JQ1 effect on cell growth was not altered by hypoxia (Fig.2A and S2A).

On the contrary of other findings, MYC status was no predictor of JQ1 sensitivity (Fig. 2B). In fact, MDA-MB-231, which have no MYC alteration as informed by the CCLE[39–41] and corroborated by c-Myc immunoblot (Fig.2B and S2B), seems to be more sensitive to JQ1-induced growth inhibition than the MYC amplified cell line HCC1806. Although no statistic test was applied for this as it is not the focus of the present study. Another tested TNBC cell line tested Cal51 is also informed as non-amplified MYC.

JQ1 also inhibited tumor growth in spheroid (3D) model, which is "widely accepted as more physiologically relevant than conventional 2D cell culture methods and are believed to improve the prediction of drug candidates"[42] (Fig.2C, 2D and S2). The effect was consistent among the 3 cell lines tested. The cell line SUM159 was part of 2D panel but not the 3D because it was not able to grow spheroids. Once again, the non-active enantiomer (-)-JQ1 did not cause any observable effect (Fig.2C, 2D and S2), indicating the observed effect is due to BET inhibition.

JQ1 reduces CA9, Ki67 and cell number in spheroids of TNBC cell lines

Immunohistochemistry show that JQ1 extinguished any CA9 immunocontent in spheroids of both tested cell lines (Fig.3 and S3). Also, JQ1 reduced Ki67 staining, indicating anti-proliferative effect (Fig3 and S3). Finally, JQ1-treated MDA-MB-231 spheroids have less cell/spheroid, corroborating previous findings.

JQ1 reduces CA9 and other hypoxia up-regulated genes

To validate the expression findings and further investigate the JQ1-induced, quantitative PCR (qPCR) was performed. Results show that CA9 expression is consistently inhibited by JQ1,

without alteration of HIF expression in both mRNA level and protein immunocontent in all cell lines tested (Fig.4 and S4A).

A panel of 16 genes were selected including not only CA9, HIF-1α and HIf-2α, but also genes differentially expressed in the array analysis and other genes related to HIF or BET proteins. This panel is almost entirely upregulated by hypoxia and a set of genes has its expression prevented by JQ1 (Fig.4). Within these, CA9 is the most prominent and the others are: CXCR7, TMEM45A, LOX and VEGF A. Interestingly the profile for JQ1 effect in normoxia is very similar (Fig.S3). Other HIF-regulated genes like LDH-A or BNIP3 were not affected by JQ1 (Fig.3A), indicating that not all HIF-regulated genes are associated with BET proteins function. MYC is also downregulated either in normoxia or hypoxia, but only in MYC-amplified cell lines HCC1806 and MCF-7 (Fig.2B, 3A, S3A).

Other BET inhibitors (BETi) were tested (I-BET151 and I-BET762) and validate the previous findings (Fig.S4B, S4C). CA9 is consistently inhibited, either in normoxia or hypoxia, with the use of both BETi in MDA-MB-231 (Fig.S3C) and HCC1806 (Fig.S3B). Once again no difference was observed in HIF-1α, but there is a significantly up-regulation of HIF-2α. Regarding the rest of the panel, a similar pattern was observed: BETi reduced VEGF-A, LOX, CXCR7, TMEM45A and up-regulated PFKFB3. MYC was downregulated only in the MYC-amplified cell line HCC1806.

Although LOX and CXCR7 genes were consistently downregulated by JQ1, their immunocontent were not (data not shown). Maybe they rely on another level of regulation not investigated here or the incubation time should be longer than 24h to observe any difference. On the other hand, this strengthens CA9 as a JQ1 target.

JQ1 reduce binding of HIF-1β to CA9 promoter, but not activity in TNBC cell lines

CA9 is known to be regulated by HIF-1α, which dimerizes with HIF-1β prior to transcription induction. So, to further investigate how JQ1 prevents hypoxia-induced CA9 upregulation, chromatin immunoprecipitation (ChIP) assay was performed. As expected, both tested cell lines presented higher "%input" in hypoxia (Fig. 5A), indicating there was more binding between CA9 promoter and HIF-1β. When the cells were treated with JQ1, the values are similar to untreated normoxic cells and lower comparing to untreated hypoxia (Fig.5A). This indicates

that in the presence of JQ1 HIF is not able to bind, and thus express, CA9 and other genes found downregulated by BETi in hypoxia (Fig.1A, S1A, 3A, 3B, S3A and S3B, S3C).

To deepen the question, we used a stable MDA-MB-231 with HRE-luciferase reporter. Using this system, we observe that JQ1 induces higher chemoluminescece, 10-fold in normoxia and 4-fold in hypoxia (Fig.5B), indicating higher HIF transcription activity. Despite higher HIF activity, JQ1 still down-regulates CA9 expression in these cells (Fig.S5A), but not HIF. Other genes of selected panel follows the same pattern as wild type MDA-MB-231 (Fig.4 and S4). Thus, indicating the CA9 downregulation by JQ1 is unrelated to HIF activity.

Next, it was used transient silencing to investigate which BET protein is responsible for the observed effects. When BRD2, BRD3 and BRD4 is knockdown, CA9 expression is reduced in hypoxia without changes in HIF-1α (Fig.5C). JQ1 is known to block all BET proteins and this data suggest these 3 can regulate CA9 expression. On the other hand, only BRD4 knockdown reduced VEGF-A expression (Fig.5C).

Thus, it is likely that the presence of BET proteins is necessary for HIF binding to CA9, what explains why it is downregulated in JQ1 treated cells. On the other hand, it is not necessary for the ability of HIF to bind to all HRE-containing genes neither for HIF transcription activity. Thus, we suggest some HIF-target genes are BET-dependent, as CA9, while others don't, like LDHA.

JQ1 reduces tumor growth, CA9 and VEGF-A expression and the blood vessel marker CD31 immunostaining in TNBC xenograft model

Next, we investigated *in vivo* value of JQ1. In TNBC xenograft model, JQ1 reduced tumor growth (Fig.6A) and CA9 expression, but not HIF (Fig.6B). Other genes downregulated in cell lines were also downregulated by JQ1 *in vivo*: VEGF A, CXCR7 and MYC (Fig.6B). Interestingly, some HIF-regulated genes that were unaltered in HCC1806 cells were found upregulated in HCC1806 xenografts: LDH-A, BNIP3, PFKFB4 and TMEM45A.

Finally, it was investigated the role of JQ1 in angiogenesis due to the consistent downregulation of VEGF-A found in cell lines (Fig.4A and S4A, S4B ,S4C and S5A) and xenograft (Fig.6B) model along with the reduction of angiogenesis pathway expression (Fig.1C). For this, it was measured CD31 immunostaining. Tumors from JQ1-treated xenografts have less CD31 immunostaining, indicating and anti-angiogenic effect (Fig.6C).

Discussion

Hypoxia occurs in most solid tumors and triggers a response that supports tumor progression through chemoresistance, radioresistance, metabolic reprograming and associates with recurrence [28–30]. Here we demonstrate an epigenetic approach that modulate the Tumour Response to Hypoxia (TRH) and reduce tumour growth in TNBC models. The membrane protein CA9 is greatly induced by hypoxia, through HIF-1α, associates with poor prognosis and enhance tumour growth [32,33,36]. CA9 was downregulated by the BET inhibitor JQ1 in all tested models, with no alteration of HIF expression. Additionally, other important hypoxia-regulated genes like VEGF-A followed a similar pattern, but not all. It was also demonstrated that JQ1 impairs the ability of HIF to bind to CA9 promoter, but not its transcriptional capacity. Thus, we suggest some HIF-targets are BET dependents. Finally, JQ1 was found to downregulate angiogenesis *in vivo*, a basic hallmark of cancer associated with hypoxia. These findings will be further explored below.

There is large evidence for the importance of CA9 in tumor adaptation to hypoxia [36]. CA9 expression is considered an endogenous marker of hypoxia and is associated with poor prognosis in breast cancer [32]. Among breast cancers, it is particularly associated with the basal subtype[37] and can be associated with BRCA mutation [34], which is more common in TNBC. To date, there is no CA9 downregulating molecule for use in patients. Thus, JQ1-induced downregulation of CA9 can be exploited for benefit of cancer therapy.

Although BETi reduce the transcriptions of BET targets, they do not alter acetylation levels [43]. Thus, it seems likely that JQ1 acts by blocking BET proteins ability to bind to chromatin. In fact, we observed a reduction in the binding of HIF to CA9 promoter region. JQ1 was already found to impair the recruitment of a transcription factor to its target gene loci by disruption of the physical interaction between the BET protein BRD4 and the N-terminal domain of the androgen receptor (AR)[10]. On the other hand, HIF activity can be elevated by JQ1 treatment. Then, JQ1 probably do not interact physically with HIF to inhibit CA9 expression, but rather makes some HIF binding sites inaccessible due to the lack of BET protein to the transcriptional complex. This, way, we propose some HIF targets are BET dependents.

Angiogenesis happens in response to hypoxia and support tumour progression [44,45] and JQ1 was able to downregulate it. Many studies with JQ1, and other BETi, showed an anti-tumoral effect in models of solid tumors [7,8,10,14,17], however none of them considered a possible anti-

angiogenic effect. On one side, it makes BETi an even more attractive approach as it has an additional anti-tumor effect. On the other hand, it hinders the understanding of JQ1 effects, as anti-angiogenic therapy increases hypoxia and can select glycolytic phenotype [44,46].

In fact, we observed that JQ1 treatment increases the expression of LDHA and PFKFB4 in xenografts. Since this was not observed in cell culture, we believe this is not a direct effect of JQ1, but a response of the tumor to the effect of JQ1. It was already described that JQ1 downregulate LDHA[47]. However, we found that JQ1 reduced expression of OXPHOS, PPP, TCA gene data sets but not GLY. If this is the case, we might expect that a co-treatment with anti-glycolytic drugs could lead to a synergistic effect.

We demonstrated that JQ1 reduced tumour cell growth in several models, in accordance with the literature. Although we investigated JQ1 effect in hypoxia, we did not observe any differential effect in cell growth between cells in normoxia and hypoxia. The BET protein BRD4 is required for the transition from mitosis to G1 [48] and JQ1 is known to induce G1 cell cycle arrest [9,17]. However, hypoxia already induces cell cycle arrest. Thus, it might be the case that in hypoxic slowing proliferation cells, JQ1 does not further increases the growth inhibition. On the other hand, the main finding is that genes induced by hypoxia can be downregulated by JQ1. Meaning that the genes needed to maintain survival in hypoxia have their expression prevented by JQ1.

Most of the described effects of BETi are attributed to downregulation of MYC [6,9–12,14–16,18], however it is not the only target [8–10,16,17,24,49]. MYC expression was believed to predict JQ1 sensitivity [11], however our results are not in accordance with this. The MYC-amplified cell lines (MCF-7 and HCC1806) had their MYC expression downregulated by JQ1. However, the effects observed here are MYC-independent as MDA-MB-231 has no amplification of MYC, had no change in MYC expression and showed the described effects. Therefore, we do not exclude MYC as an important target for BETi. However, we believe other effects can be as important.

In conclusion, we showed for the first time that JQ1 can impair tumor response to hypoxia, inhibit CA9 expression and angiogenesis. Also, for the knowledge of the authors this is the first study investigating the interaction between HIF and BET proteins. Thus, we believe our findings have clear impact on the understanding of tumor response to hypoxia and opens a new possibility for epigenetic therapy in TNBC.

Methods

Cell culture

Cells were cultivated in DMEM with 10% (v/v) FBS, penicillin (100 U/ml), streptomycin (100mg/ml) (Life Technologies) in humidified incubator at 5% CO_2 and 37°C. Hypoxic incubations (0.1% O_2, 5% CO_2, 37ºC) were performed in INVIVO$_2$400 workstation (Baker Ruskinn, USA).

Cells treated with JQ1 were seeded for 72h using the concentrations indicated in the figures. Treatments with I-BET's followed the same protocol. For monolayer (2D) cell growth evaluation, cells were seeded (1.5 x 10^4.well^{-1}) in 96 well plate, treated with JQ1, as described above. Cells were kept in appropriate culture condition (normoxic or hypoxic) for 72h and cell number was indirectly measured following the Sulforhodamine B (SRB) assay [50] or the CyQUANT® kit (Invitrogen™ | Molecular Probes®, USA) following manufacturer's instructions.

Spheroid culture

Spheroids were generated seeding 5 x 10^3.0.1mL^{-1}.well^{-1} in 96-well round bottom plate (Costar® 7007, Corning, USA) in complete media supplemented with 1:20 v/v cold Matrigel® (BD Bioscience). Aggregation was induced by centrifugation (2000 rpm / 10 min). Cells were incubated under normal cell culture conditions for 48h prior to any manipulation for spheroids formation. JQ1 treatment started on day 2 and was daily renewed. Pictures were taken 3-days/week with an inverted microscope (EVOS® xl Core, AMG, USA). Neubauer chamber picture was used as reference for diameter measurement and spheroid volume estimation.

For immunohistochemistry, spheroids were fixed overnight with formalin 10%. After, they were embedded in agarose and wax and cut in microtome.

Gene expression array analysis and bioinformatics

To evaluate the effect of JQ1 in gene expression, Illumina whole genome gene expression profiling carried out transcriptome analysis was performed. Cell were incubated for 24h in normoxia or hypoxia (0.1% O_2) with or without 200nM JQ1. Triplicates were used for each experimental condition. Cells were lysed and RNA was extracted using TRIzol® (Life technologies) following manufacturer's instructions. RNA quality and quantity were confirmed with

the NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific). Complementary DNA (cDNA) was produced using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™).

Next, biotin labelled aRNA was hybridized according to Illumina whole-genome gene expression direct hybridization assay from Illumina #11286340 to high-density Illumina Human oligonucleotide arrays Human HT-12_V4_0_R1_15002873_B; designed to detect 47,231 transcripts. Of those 33,223 probes were analysed after the results were filtered to remove probes not detected in any sample. The Fluorescence emissions were quantitatively detected using iScanner and data were extracted using BeadStudio v2011.1 Software (Illumina Inc) which were imported to GeneSpring GX 12.1 (Agilent Technologies, Inc., Santa Clara, CA) ; normalized with Shift to 75 percentile and baseline transformed to median of all samples to identify significantly differentially expressed genes (DEG) with FDR (Benjamini-Hochberg) corrected P-value cut off at <0.01.

Functional and pathway analysis carried out on DEG to identify statistically over-represented ontologies in the list using Database for Annotation, Visualization and Integrated Discovery (http://david.abcc.ncifcrf.gov/). Differentially expressed genes (DEG) fulfilled the following criteria: fold change (FC) $\log_2 \geq 1$ and $p < 0.05$.

The prognostic value of the consistently differentially expressed genes (DEG) between the two cells lines were based upon data generated by The Cancer Genome Atlas pilot project established by the NCI and NHGRI. Information about TCGA and the investigators and institutions who constitute the TCGA research network can be found at http://cancergenome.nih.gov/.

Genecodis (http://genecodis.cnb.csic.es/) was used to investigate pathways that were possibly altered in our experimental conditions. Here, hypergeometric test was used and false discovery rate threshold was set to 0.05. Then the list of genes comprising those pathways were obtained from KEGG (http://www.genome. jp/kegg/), GO or from the literature [29]. Ensembl IDs were used as reference for gene names. Finally, the Hypoxic Response (HyR) network was designed in STRING (http://string-db.org/) containing all the selected pathways (Fig. 1B).

Gene Set Enrichment Analysis (GSEA) was used to evaluate pathway enrichment and to identify genes that contribute individually to global changes in each experimental condition [51]. Then, ViaComplex® [52] was used to generate representative landscape images of these results (Fig. 1C and S1B).

Real-time PCR (qPCR)

RNA extraction and quantification and cDNA synthesis were carried out as mentioned above. Real-time PCR (qPCR) reactions were performed with SensiMix™ SYBR® Hi-ROX Kit (Bioline, UK) following manufacturer's instructions in triplicate using the 7900HT Fast Real-Time PCR System (Applied Biosystems). Conditions of the PCR reaction were 2 min at 50°C, 10 min at 95 °C and then 40 cycles, each containing of 15 s at 95 °C, and 1 min at 60 °C. β-actin was used as a reference gene.

Immunoblotting

Cells were taken out of the incubator on ice, washed with cold PBS and lysed in RIPA buffer (Sigma). After protein quantification, samples were normalized in 2x sample buffer (125mmol/L Tris [pH 6.8], 4% sodium dodecyl sulfate, 0.01% bromophenol blue, 10% b-mercaptoethanol, 10% glycerol). Lysates were separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore), and probed with primary. Then, appropriate secondary horseradish peroxidase (HRP)-linked antibodies were used (Dako).

Horseradish peroxidase – conjugated anti- rabbit or anti-mouse antibodies (DAKO Inc.) were used with ECL system (Amersham Biosciences) to visualize immunoreactivea bands. Proteins were detected using antibodies according to manufacturer's instructions.

Primary antibodies were used at 1:1,000. The following antibodies were used: mouse anti-HIF-1α, (BD Transduction Laboratories, USA); anti-HIF-2α (Sigma); mouse anti-CAIX (Gift form J. Pastrorek, Institute of Virology, Slovak Republic); anti-c-Myc (Sigma); mouse anti-β-actin (Sigma). Bands were acquired with and quantified with image analysis in ImageJ.

Xenograft studies

Mice were housed at Cancer Research UK Laboratories (Clare Hall)/BMS, University of Oxford, UK, and procedures were carried out under a Home Office licence. HCC1806 cells were cultivated as previously described until the day of the inoculation.

On that day, the cells were trypsinized and washed twice in serum-free medium. Six to seven-week old female CD1 Nude mice were injected subcutaneously in the lower flank with

$2.5x10^6$ cells suspended in 50µl of serum-free medium and 50µL Matrigel® (BD Bioscience). Mice received isoflurane for anaesthesia / analgesia purpose.

Tumor growth was monitored 3 times per week measuring the length (L), width (W), and height (H) of each tumor with calipers. Volumes were calculated from the formula $1/6 \times \pi \times L \times W \times H$. When xenograft size reached 150 mm^3, animals received JQ1 or vehicle (10% DMSO, 10% hydroxypropyl beta cyclodextrin) IP at 50mg/kg daily. xenografts treatment – JQ1 dilution Animals were checked regularly for any side-effect.

When tumors reached 1.44 cm3 the mice were sacrificed by cervical dislocation. Ninety minutes before sacrifice, mice were injected intravenously with 2 mg of pimonidazole (Hypoxyprobe-1; Chemicon International, USA) as described previously [36].

Tumour have been quickly removed. Half of the tumour was frozen in liquid N_2, the other half was formalin-fixed overnight. Next day the formalin-fixed tumours were transferred into 70% ethanol. Blood plasma was collected for analysis and cardiac puncture was used to take blood.

Immunohistochemistry (IHC)

Immunohistochemistry was carried out as previously described [36]. Briefly, after being embedded in wax, cut in microtome, slides were heat (60ºC) dried for 10 min and dewaxed by sequential washes (5 min each): 2x histoclear, 2x 100% ethanol, 1x 50% ethanol and 1x water. Then the adequate antigen retrieval was applied for 2 min at 125ºC. Slides were then washed with PBS, air room temperature dried and a protein block solution was applied. Then, the primary antibodies were used at room temperature for 1 hour or overnight at 4ºC. After, the slides were washed with PBS and incubated with the anti-rabbit/anti-mouse secondary antibody (Dako) for 30 minutes at room temperature and washed in PBS. 3,3′-Diaminobenzidine (DAB; Dako) was applied to the sections for 7 minutes. The slides were counterstained by immersing in hematoxylin solution (Novocastra blue top, Sigma-Aldrich) for 20 seconds and mounted with Aquamount (VWR). Secondary-only control staining was done routinely, these were negative. One section from each xenograft was analyzed for each stain. Slides were analyzed quantitatively by image analysis in imageJ using color deconvolution by two different researchers, as described previously[36]. Where scores differed, sections were reviewed and a consensus result was decided.

Gene silencing by RNA Interference

Transfections of siRNA duplexes targeting BET proteins (BRD2, BRD3, BRD4 and BRDT) or a scramble control (ON-TARGETplus SMARTpool) at a final concentration of 20 nM, were performed in Optimem (Invitrogen), using Oligofectamine® (Invitrogen). The sequences of siRNA used to target CAIX were 118898, 9567 and 9473 (Invitrogen). Reduction in CAIX protein expression, measured by Western blot analysis, was used to indicate gene silencing.

Chromatin immunoprecipitation assay (ChIP)

ChIP assay was performed to investigate the capacity of JQ1 to impair HIF binding to Hypoxia Responsive Element (HRE) of CA9 gene. Immunoprecipitation assays for HIF-1β was performed using the EZ-ChIP™ Chromatin Immunoprecipitation Kit (#17-371, Millipore), according to manufacture's instructions. Cells were seeded in 100mm culture dishes (#430167, Corning®) at 70% confluence maximum to avoid HIF activation in normoxic cultures, and treated with 200nM (+)-JQ1 for 24h. Then, cells were cross-linked with 1% formaldehyde, lysed and sonicated in 30s pulses for a total of 6 min (Diagenode Bioruptor® Ultrasonicator). Chromatin was immunoprecipitated using rabbit polyclonal antisera to HIF-1β (NB-100-110, Novus Biologicals, USA). DNA isolated from ChIP was quantified by qPCR using the CA9 HRE primer.

HRE Reporter Assay

Cells were transfected with 2 µg/mL HIF-1α reporter plasmid or pGL3 promoter control plasmid, and 0.02 µg/mL phRL-cytomegalovirus Renilla luciferase plasmid using Fugene 6 eukaryote transfection reagent kit (Roche, Welwyn Garden City, United Kingdom). After the appropriate experimental conditions, cells were lysed and luciferase activity was analyzed with a Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer' s instructions. Luciferase chemoluminescence was using FLUOstar Optima (BMG Labtech).

Statistical analysis

Statistical analysis and graphs were performed using GraphPad Prism® v6.0 software (GraphPad). Error bars represent mean ± Standard Deviation (SD). Different statistical tests were used according to the appropriate situation and are described in the figure legend.

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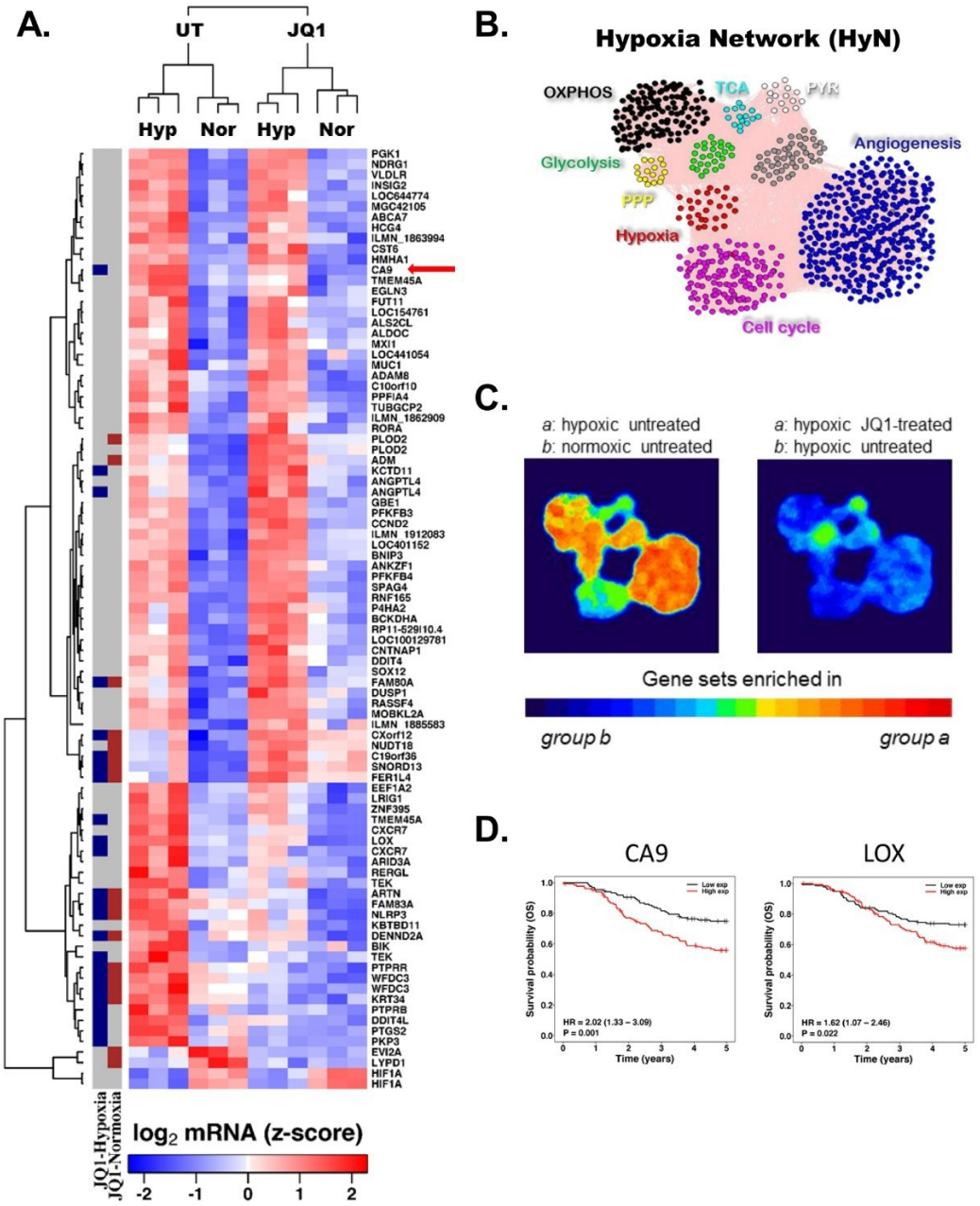

Figure 1. JQ1 downregulates expression of several hypoxia-regulated genes, especially CA9. (A) List of differentially expressed genes (DEG) under hypoxia for MDA-MB-231 cells obtained from microarray. Columns at the left denote DEG under JQ1 treatment, either in normoxia (red blocks) or hypoxia (blue dots). CA9 is the most prominently downregulated gene in hypoxia (red arrow). (B) Hypoxia Network (HyN) created including pathways regulated by hypoxia. (C) Most components of HyN are upregulated by hypoxia and downregulated by JQ1 treatment in MDA-MB-231 cells. (D) Kaplan-Meier curves, demonstrating prognostic value of two genes consistently inhibited by JQ1 in both cell lines tested (MDA-MB-231 and MCF-7) for triple-negative breast cancer patients. OXPHOS: oxidative phosphorylation; TCA: tricarboxylic acid cycle; PYR: pyruvate metabolism; PPP: pentose-phosphate pathway; DEG: differentially expressed genes.

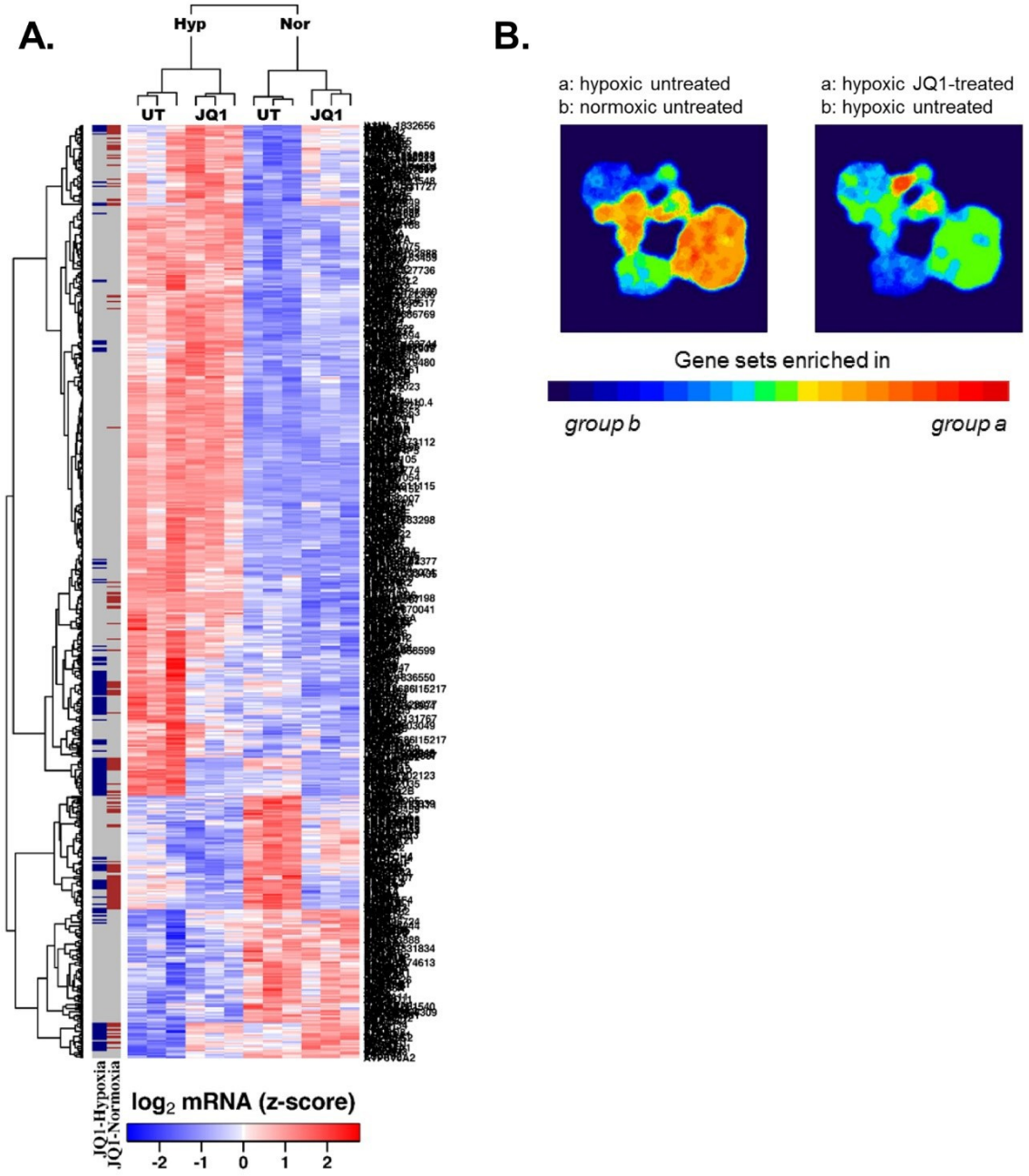

Figure S1. JQ1 downregulates expression of several hypoxia-regulated genes, especially CA9. (A) List of differentially expressed genes (DEG) under hypoxia for MCF-7 cells obtained from microarray. Columns at the right denote DEG under JQ1 treatment, either in normoxia (red blocks) or hypoxia (blue dots). (B) Many components of HyN are upregulated by hypoxia and downregulated by JQ1 treatment in MCF-7 cells.

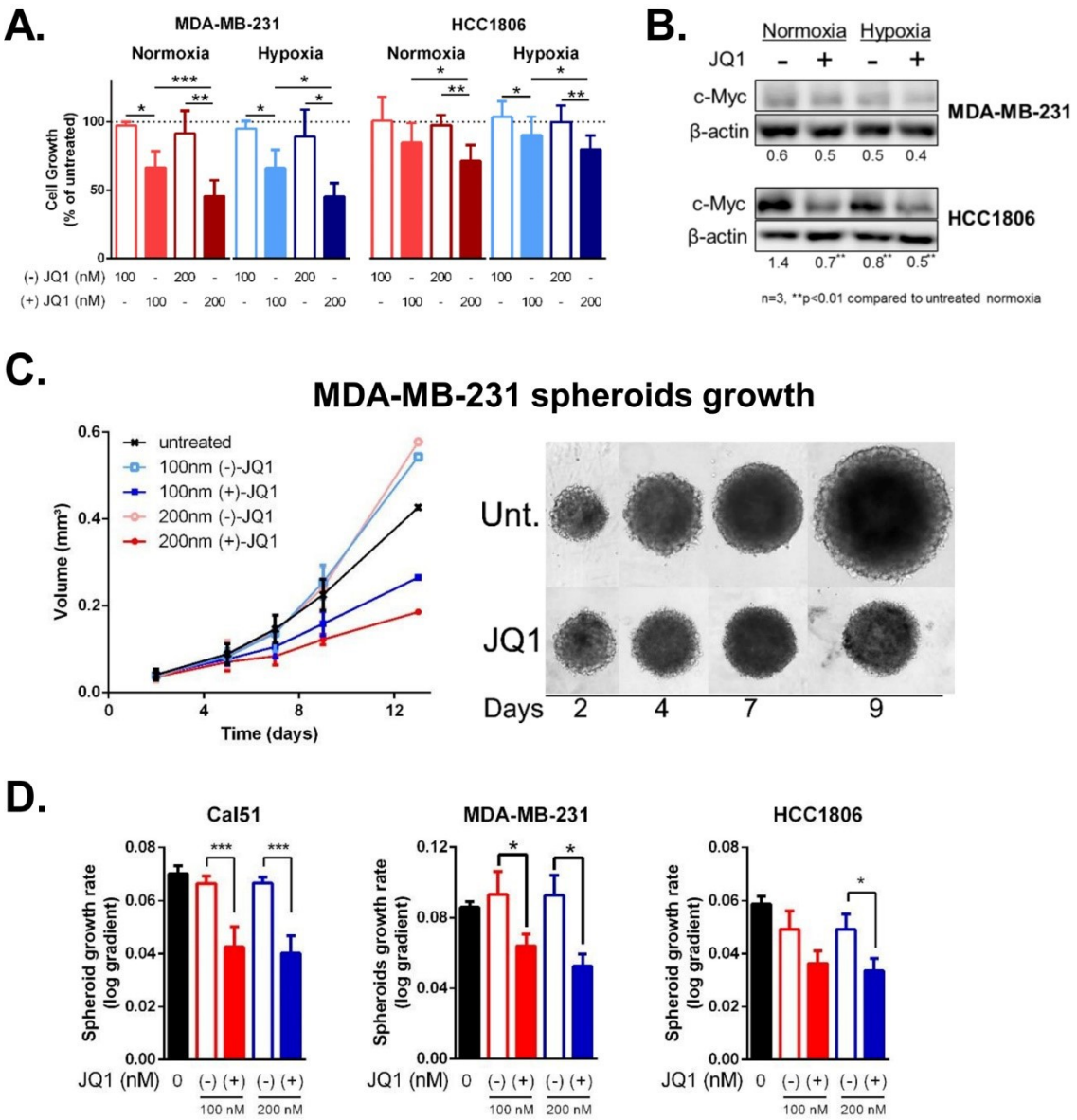

Figure 2. JQ1 reduces TNBC monolayer and spheroid growth, regardless of MYC. (A) (+)-JQ1 dose-dependently reduces monolayer (2D) cell growth of TNBC cell lines MDA-MB-231 and HCC1806 in normoxic and hypoxic conditions after 72h incubation, while (-)-JQ1 does not. JQ1 reduced c-Myc immunocontent only in the MYC amplified cell line HCC1806, while the non-mutated cell line MDA-MC-231 showed no difference in c-Myc (B). (C) Representative spheroid growth curve and pictures of MDA-MB-231 spheroids following JQ1 treatment. (+)-JQ1 reduces spheroid growth in MDA-MB-231 and other TNBC cell lines (D), while (-)-JQ1 does not. One-way ANOVA, n = 3, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

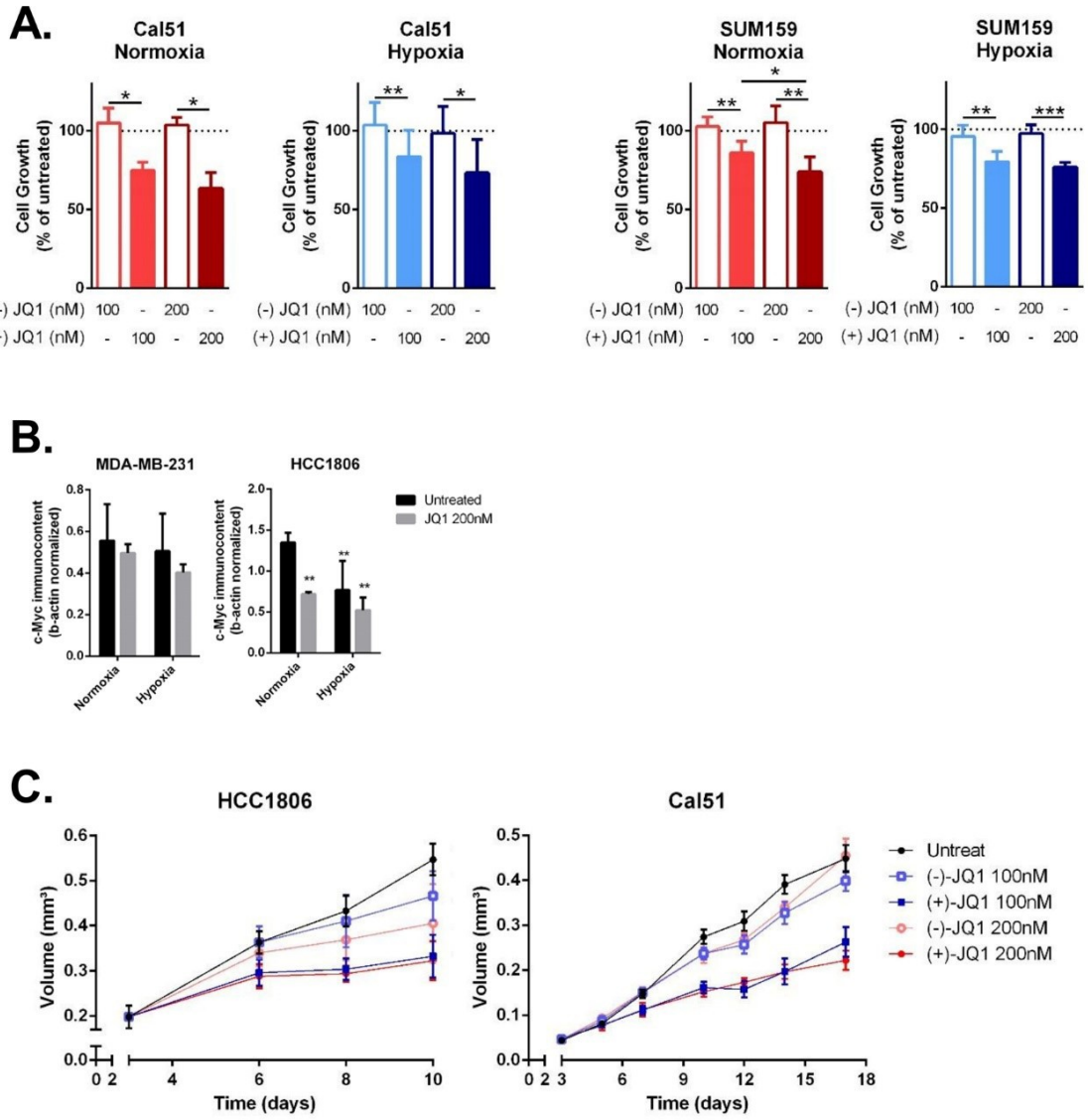

Figure S2. JQ1 reduces TNBC monolayer and spheroid growth. (A) (+)-JQ1 reduces monolayer (2D) cell growth of TNBC cell lines Cal51 and SUM159 in normoxic and hypoxic conditions after 72h incubation, while (-)-JQ1 does not. (B) Densitometry of c-Myc immunocontent in MDA-MB-231 and HCC1806 cell lines with and without (+)-JQ1 treatment. (C) Spheroid growth curves for HCC1806 and Cal51 cell lines following JQ1 treatment, (+)-JQ1 reduces spheroid growth in the cell lines, while (-)-JQ1 does not. One-way ANOVA, n = 3, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

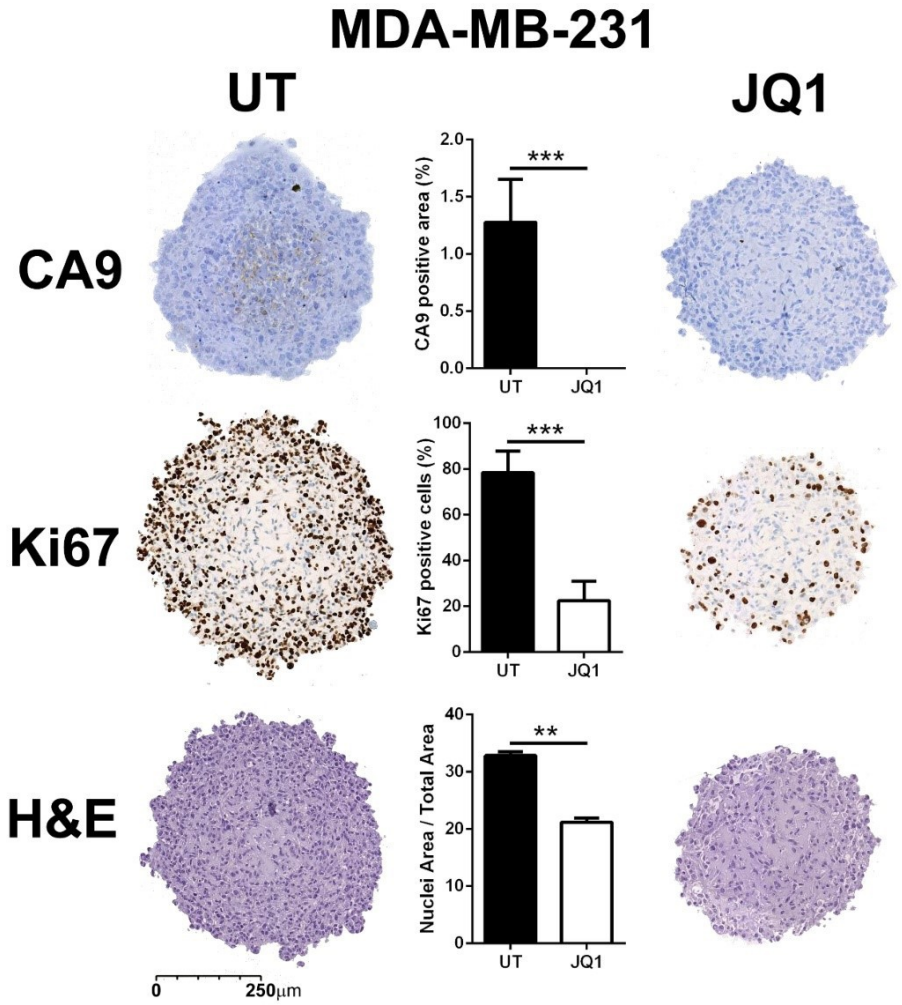

Figure 3. JQ1 reduces CA9 expression, Ki67 and number of cells in MDA-MB-231 spheroids. CA9 expression is visible in untreated (UT) spheroids, but undetectable in JQ1-treated spheroids. The proliferative marker Ki67 was also reduced in spheroids treated with JQ1. The treatment with JQ1 also reduced the ratio "nuclei area/total area" of the spheroid. Spheroids were formed and fixed for immunohistochemistry as described in Methods section. Student t-test, n = 3, ** $p < 0.01$, *** $p < 0.001$.

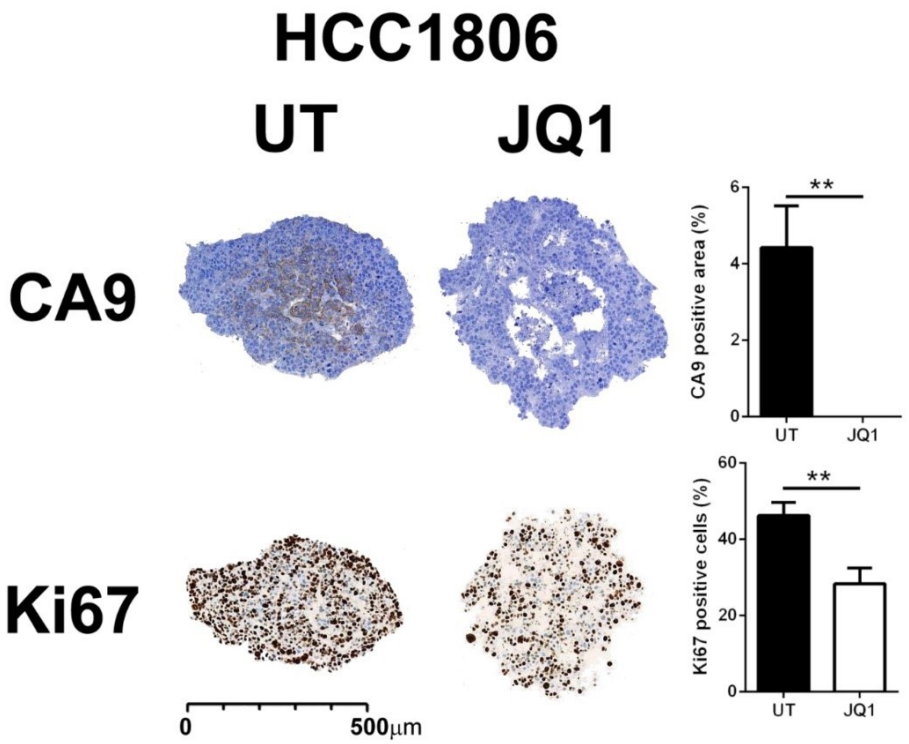

Figure S3. JQ1 reduces CA9 and Ki67 expression in HCC1806 spheroids. CA9 expression is visible in untreated (UT) spheroids, but undetectable in spheroids treated with JQ1. The proliferative marker Ki67 was also reduced in spheroids treated with JQ1. Spheroids were formed and fixed for immunohistochemistry as described in Methods section. Student t-test, n = 3, ** $p < 0.01$.

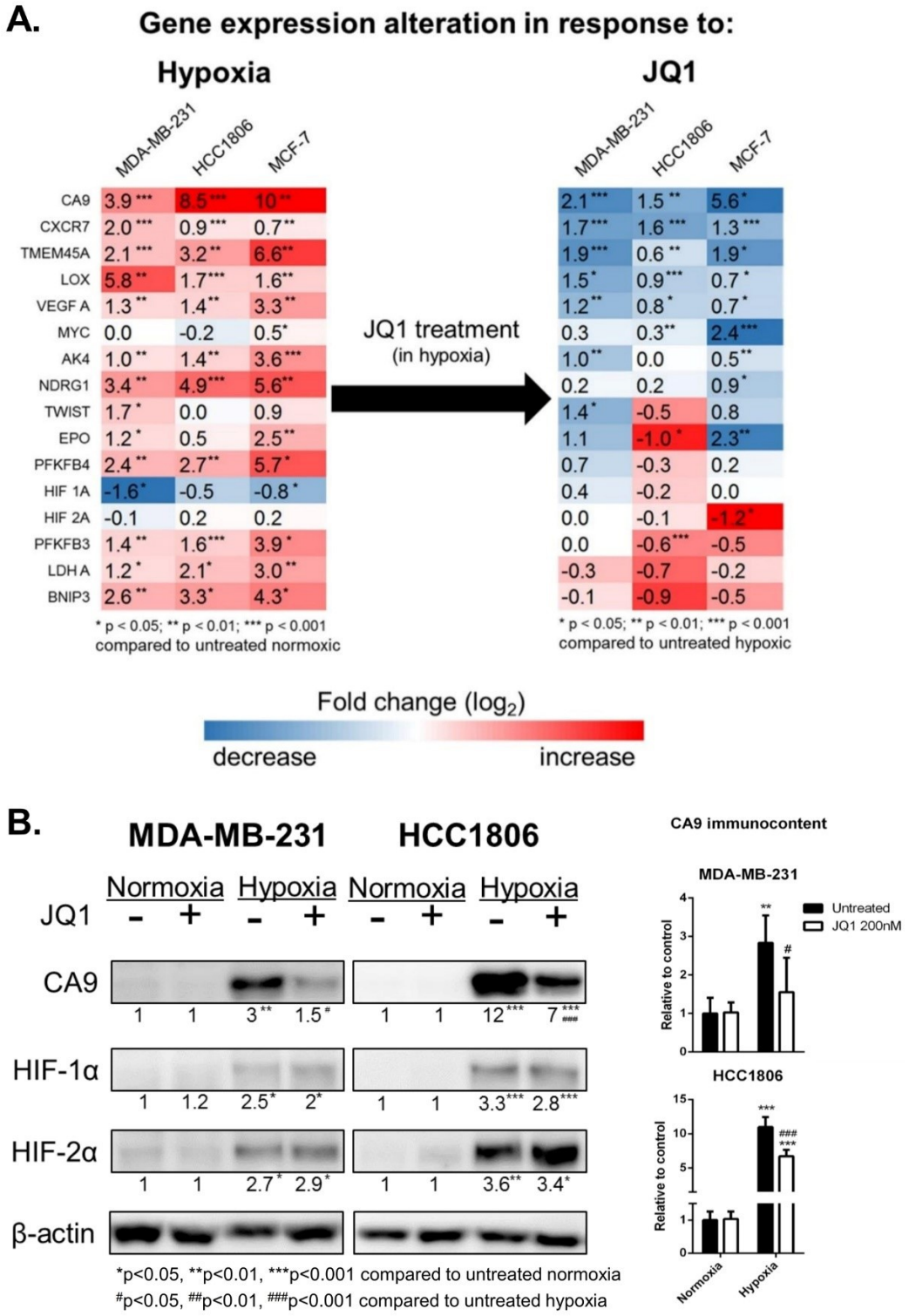

Figure 4. JQ1 reduces CA9 expression in TNBC cell lines. (A) Hypoxia up-regulates several genes and JQ1 downregulates a group of them, being CA9 is the most prominent across the 3 cell lines. HIF expression is not altered by JQ1. (B) CA9 is consistently downregulated by JQ1 in hypoxia, without any effect on HIF. Cells were treated with JQ1 for 24h prior to RNA or protein extraction, then gene expression was assessed by RT-qPCR and protein immunocontent was assessed by Western Blot. Two-way ANOVA, n = 3, * p < 0.05, ** p < 0.01, *** p < 0.001.

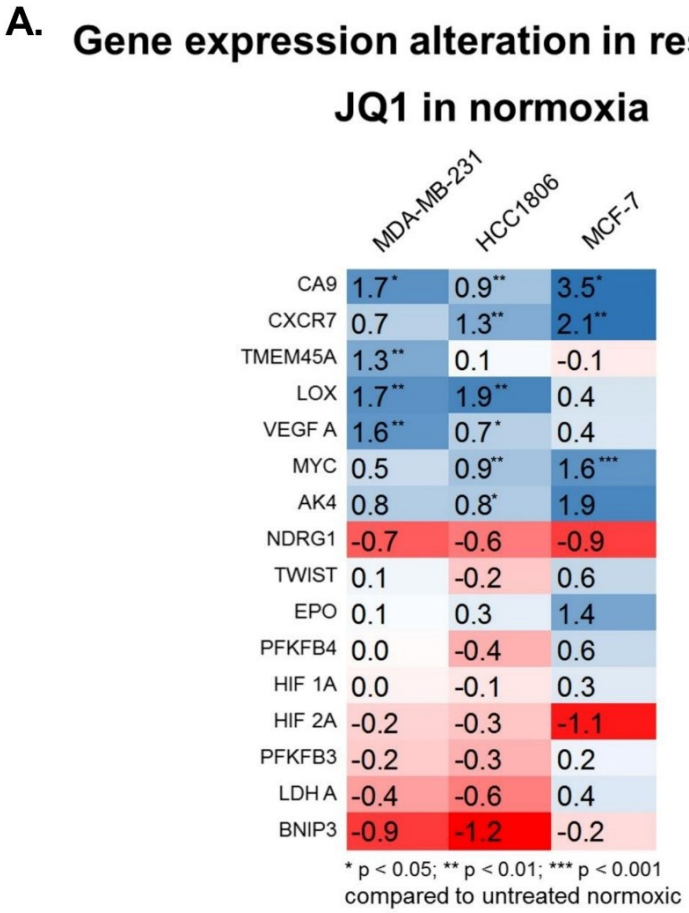

Figure S4. JQ1 reduces CA9 expression in TNBC cell lines. (A) Gene expression alterations due to JQ1 treatment in normoxia. CA9 is consistently downregulated by JQ1, without any effect on HIF. Cells were treated with JQ1 for 24h prior to RNA or protein extraction, then gene expression was assess ed by RT-qPCR. Two-way ANOVA, n = 3, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

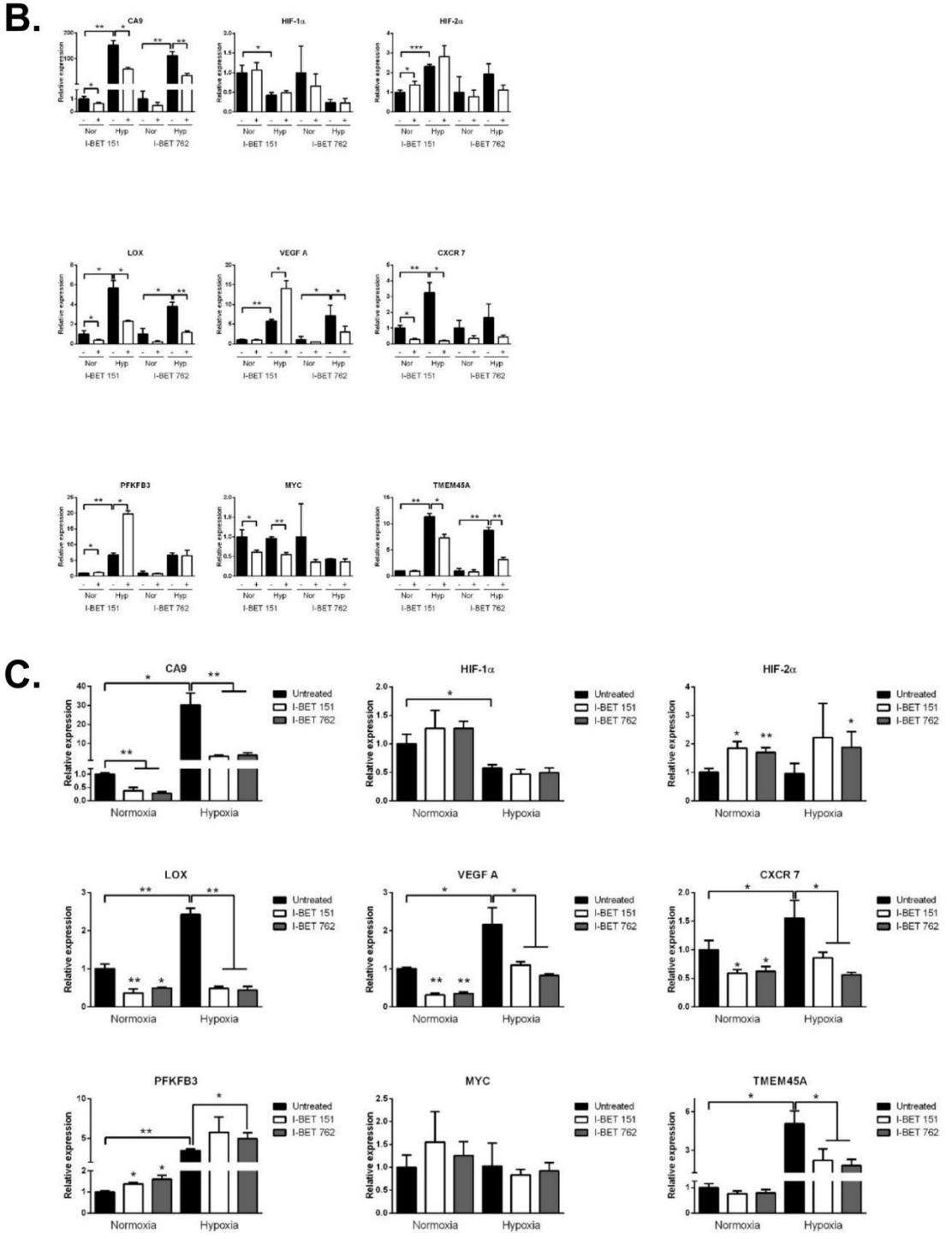

Figure S4. I-BET-151 and I-BET-762 reduces CA9 expression in TNBC cell lines. Other BET inhibitors induce similar gene expression modulation in TNBC cell lines MDA-MB-231(A) and HCC1806 (B). Cells were treated with I-BET-151 or I-BET-762 for 24h prior to RNA extraction, then gene expression was assessed by RT-qPCR. Two-way ANOVA, n = 3, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

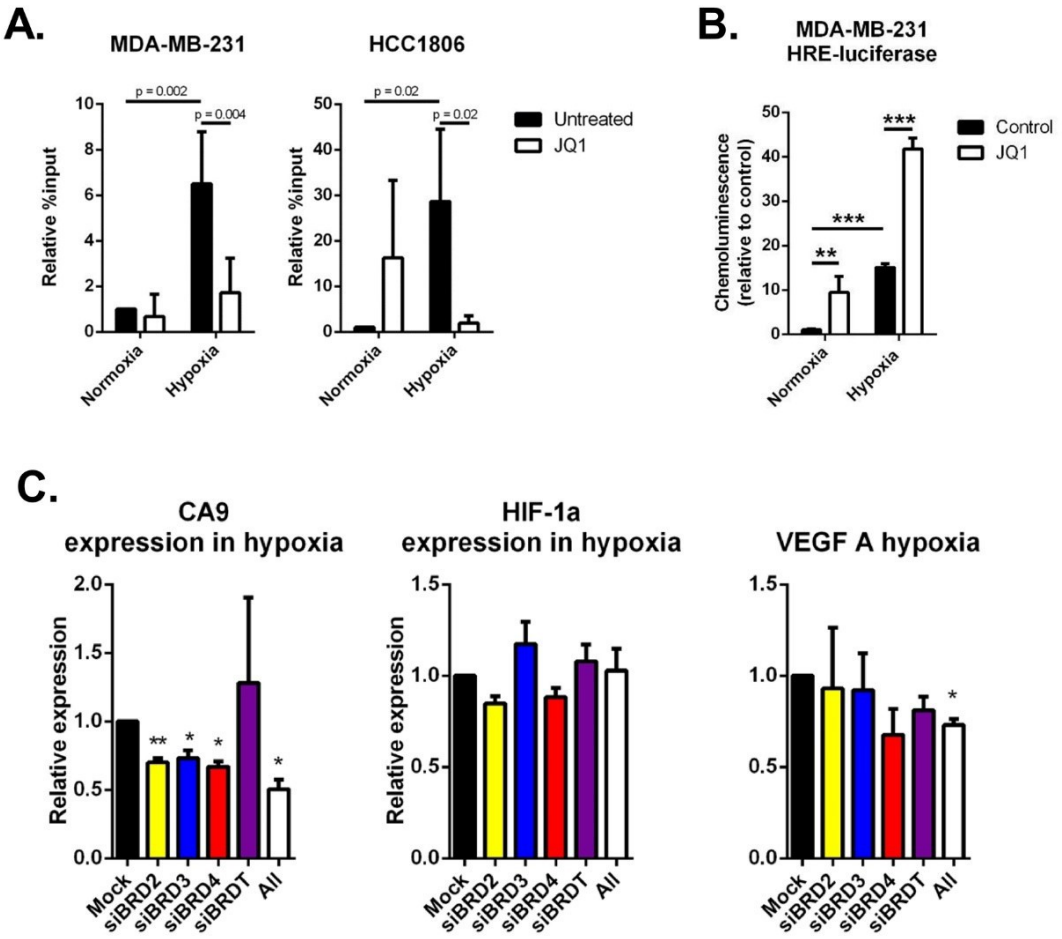

Figure 5. Mechanism of CA9 reduction by JQ1. JQ1 reduces HIF-1β binding to CA9 promoter (A), but does not affect HIF activity (B). (A) ChIP assay for CA9 promoter region with HIF-1β immunoprecipitation in 2 TNBC cell lines. Two-way ANOVA, n=3. (B) MDA-MB-231 HRE-luciferase cells were made and tested for HIF Capacity of transcribing luciferase gene with and artificial HRE in the presence of JQ1. Two-way ANOVA, n=3. (C) Transient siRNA for BRD2, 3 and 4 reduce CA9 expression in hypoxia, but not HIF-1α. One-way ANOVA, n=3, * $p < 0.05$; ** $p < 0.01$.

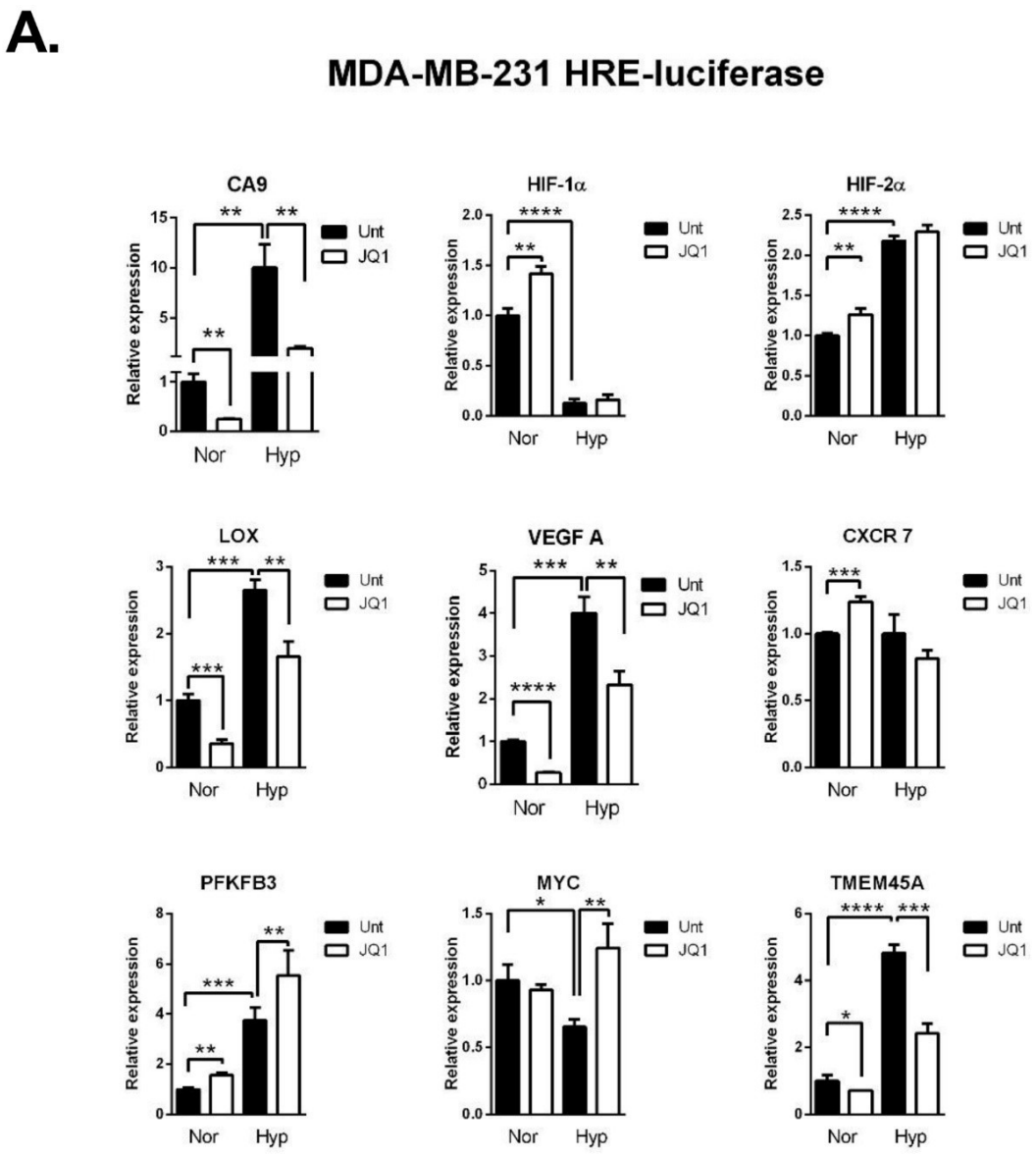

Figure S5. JQ1 reduces CA9 and VEGF-A expression in MDA-MB-231 HRE-luciferase cells. (A) RT-qPCR of selected genes in MDA-MB-231 HRE-luciferase. Two-way ANOVA, n=3.

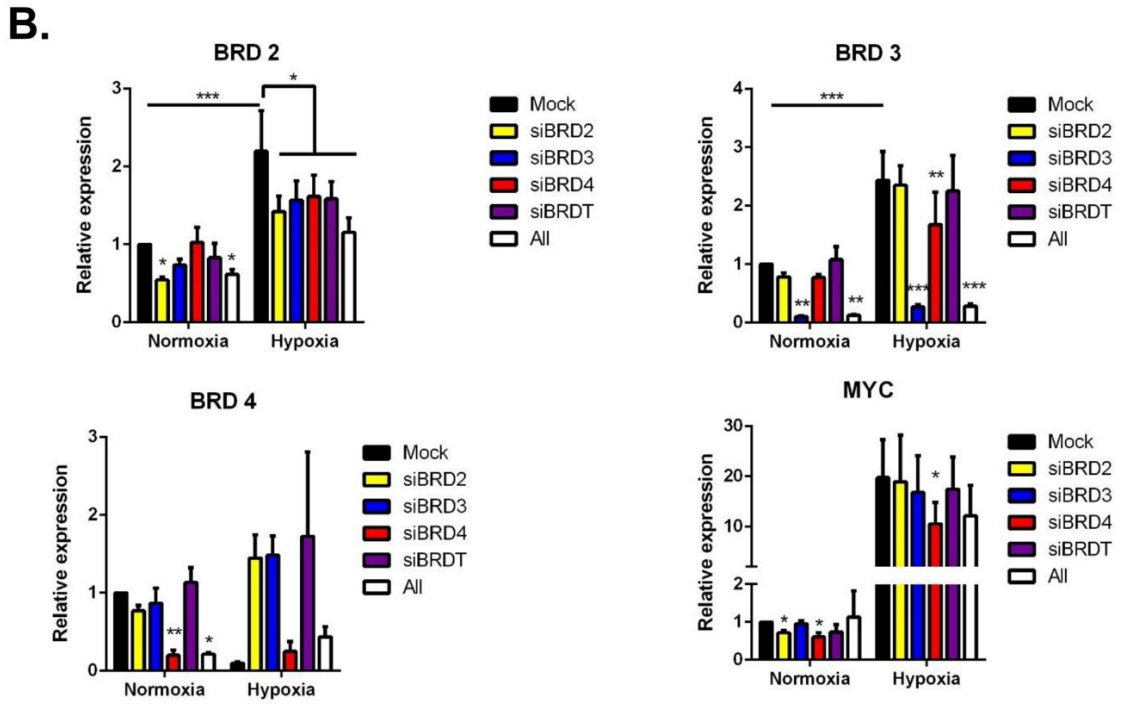

Figure S5. Silencing of BRD proteins in hypoxia and normoxia and MYC expression after silencing. Two-way ANOVA, n=3, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

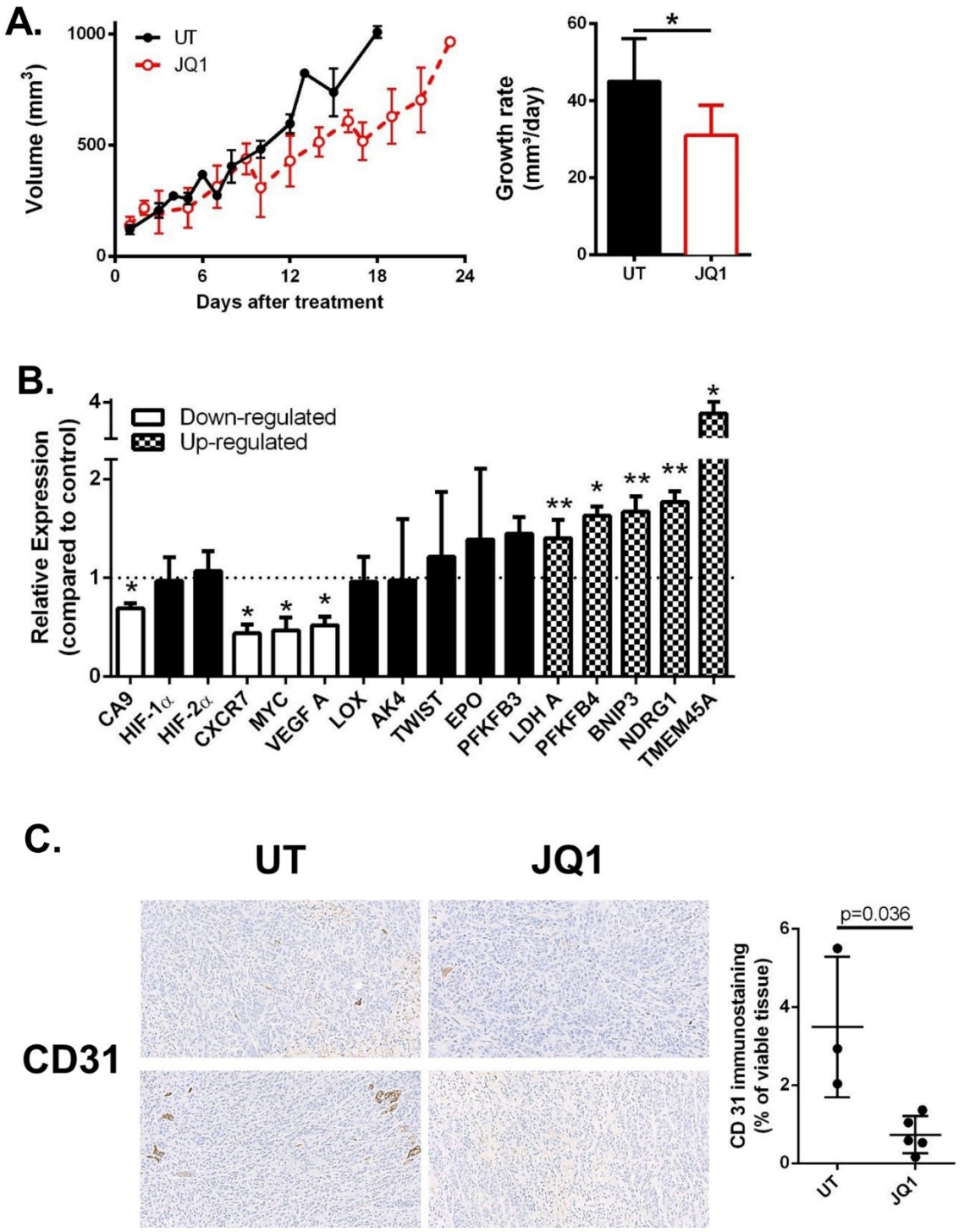

Figure 6. JQ1 reduces tumor growth, CA9 and VEGF-A expression and the blood vessel marker CD31 xenograft model of TNBC. (A) Growth curve and rate of xenografts treated with JQ1 or untreated (UT). Student t-test, n = 3 – 5, * $p < 0.05$. (B) Expression of selected panel of genes in xenografts treated with JQ1. Xenografts were grown using HCC1806 cells in 6-7 week old female CD1 nude mice. Student t-test, n = 3, * $p < 0.05$, ** $p < 0.01$. (C) Representative CD31 immunostaining in xenografts. Non-parametric Mann-Whitney test, n = 3 – 5

PARTE III

DISCUSSÃO & CONCLUSÃO

O câncer é responsável por aproximadamente 15% das mortes no Brasil e no mundo (INCA, 2014; OMS, 2015). Os casos de sucesso estão restritos quase que exclusivamente a aqueles diagnosticados em estágios iniciais o que se torna raro devido à natureza assintomática da doença. O câncer é um processo de evolução Darwiniana pelo qual células adquirem a capacidade de proliferar independente mente do equilíbrio do organismo. Neste, elas acumulam diferentes alterações que são selecionadas, resultando em um tumor avançado heterogêneo (BURRELL et al., 2013). Deste modo, muitas vezes a terapia elimina somente uma parcela das células malignas, mas acaba selecionando as resistentes (CURTARELLO et al., 2015; MCINTYRE; HARRIS, 2015) (Fig.9). No entanto, apesar das alterações genéticas serem praticamente incalculáveis (ALEXANDROV et al., 2013), o comportamento tumoral muitas vezes converge para poucos fenótipos agressivos (LÓPEZ-LÁZARO, 2010).

No presente trabalho foram investigados diferentes fenótipos associados à agressividade tumoral e, com base nestes, foram propostos alvos e abordagens terapêuticas.

METABOLISMO REDOX

O desbalanço na homeostase redox pode estar associado a diversas patologias, como o câncer (COSTA; SCHOLER-DAHIREL; MECHTA-GRIGORIOU, 2014). O desbalanço pró-oxidativo é um fenótipo agressivo (ver Capítulo I), de acordo com os dados aqui apresentados e estudos em colaboração com nosso grupo de pesquisa (DA MOTTA et al., 2015; LISBÔA DA MOTTA et al., 2014; MACEDO et al., 2012). No entanto, este não é um fenômeno linear, ou seja, apesar do tumor se beneficiar de maiores níveis de estresse oxidativo, acima de um limiar o excesso é tóxico mesmo para as células tumorais (Fig.20).

Apesar da maioria dos estudos apontarem nesta direção (CHAISWING et al., 2007; HEMPEL et al., 2009, 2013; PANI; GALEOTTI; CHIARUGI, 2010; POLICASTRO et al., 2004), há quem

correlacione agressividade com desbalanço redutor (JORGENSON; ZHONG; OBERLEY, 2013). Aparentemente, diferentes tipos de tumores podem apresentar alterações redox opostas. Esta contradição não diminui o valor dos nossos achados, mas chama a atenção para a importância de se considerar o tipo de câncer estudado.

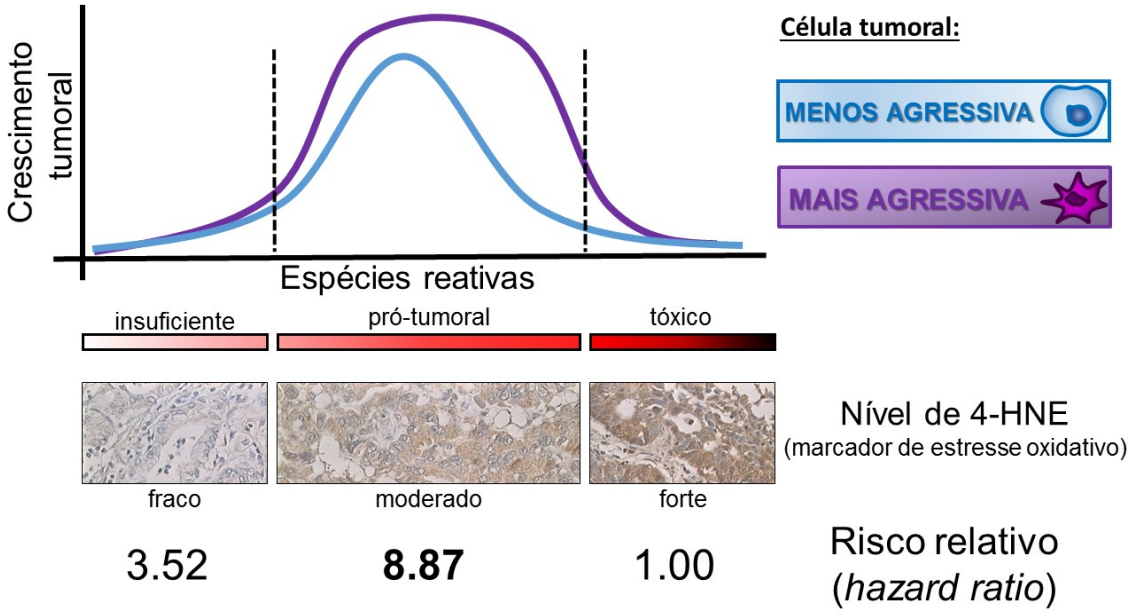

Figura 20 Fenótipo agressivo proposto para metabolismo redox (ver Capítulo I)(DA MOTTA et al., 2015; LISBÔA DA MOTTA et al., 2014).

De qualquer modo, é sabido que ROS regula comportamentos como proliferação (BURHANS; HEINTZ, 2009; IBAÑEZ et al., 2011) e migração (CONNOR et al., 2007; POLYTARCHOU; HATZIAPOSTOLOU; PAPADIMITRIOU, 2005). Assim, as espécies reativas em abundância nas células tumorais podem servir de combustível a comportamentos malignos e sua eliminação representaria um prejuízo à célula tumoral (LISANTI et al., 2011).

Apesar de muitos estudos caracterizarem o desbalanço pró-oxidativo como fenótipo agressivo, existem opiniões controversas quanto a uma proposta terapêutica que possa se beneficiar deste fenômeno (SAEIDNIA; ABDOLLAHI, 2013; YANG et al., 2014). Alguns estudos defendem que o aumento do estresse oxidativo levaria às células tumorais além do seu limite (GLASAUER; CHANDEL, 2014), mas é possível que tal abordagem cause efeitos colaterais

indesejado em tecidos sadios (HALLIWELL, 2007). Por outro lado, a terapia com antioxidantes poderia limitar o "combustível" das células tumorais diminuindo sua agressividade (SOTGIA; MARTINEZ-OUTSCHOORN; LISANTI, 2011). Estudos recentes concluem que as evidências não apoiam efeito preventivo de antioxidantes (GOODMAN et al., 2011; MYUNG et al., 2010). No entanto, do ponto de vista terapêutico já foi demonstrado que antioxidantes podem reduzir a agressividade de células tumorais (POLICASTRO et al., 2004) e impedir metástase em modelo animal (GOH et al., 2011).

A partir destes achados, foi desenvolvida uma tese de mestrado que investigou o potencial uso da enzima antioxidante catalase (CAT) em combinação com quimioterápicos (ver Anexo A). Este estudo encontrou que a CAT age sinergicamente com cisplatina, 5-FU e hidroxiuréia e antagonisticamente com paclitaxel em linhagem celular de NSCLC (Fig.21). Portanto, esta parte do trabalho conclui que abordagens antioxidantes podem ter impacto positivo na terapia de câncer de pulmão.

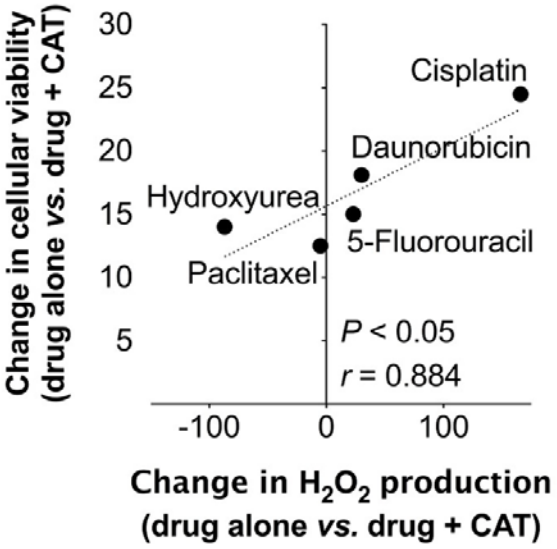

Figura 21 Efeito do uso combinado de CAT e quimioterápicos na viabilidade da linhagem A549 (DE OLIVEIRA, V.A., 2015. dados não publicados, ver Anexo A).

REPROGRAMAÇÃO METABÓLICA

A oncologia hoje aceita a ocorrência e relevância da reprogramação metabólica em tumores (BOROUGHS; DEBERARDINIS, 2015; WARD; THOMPSON, 2012) mas ainda se esforça para encontrar uma forma de utilizar este conhecimento terapeuticamente (KISHTON ; RATHMELL, 2015; SCHULZE; HARRIS, 2012; TENNANT; DURÁN; GOTTLIEB, 2010). As alterações metabólicas podem ser selecionadas durante a progressão tumoral (GILLIES; GATENBY, 2015; VERDUZCO et al., 2015) ou pior ainda, por terapias em uso na clínica (CURTARELLO et al., 2015; MCINTYRE; HARRIS, 2015). Sabe-se que diferentes alterações metabólicas têm profundo impacto no crescimento do tumor, na sua capacidade invasiva e também na resposta à terapia (ROBERTSON-TESSI et al., 2015). Portanto, definitivamente a reprogramação metabólica pode acrescentar à terapia oncológica.

As linhagens celulares utilizadas neste estudo mostraram-se um bom modelo para investigar reprogramação metabólica, uma vez que o metabolismo mais glicolítico está associado à maior agressividade – conforme caracterizado previamente no capítulo I (CASTRO et al., 2010b; LISBÔA DA MOTTA et al., 2014). Neste contexto, os transportadores de membrana surgiram como importante protagonista deste processo, fosse nas linhagens ou mesmo em coorte de pacientes. A maior captação de glicose é fundamental para a manutenção das necessidades metabólicas da célula tumoral (CAIRNS; HARRIS; MAK, 2011). Adicionalmente, a acidificação do ambiente extracelular é importante para agressividade, promovendo invasão e metástase de modo que está associado a pior prognóstico (GILLIES; GATENBY, 2015). Autores proeminentes na área chegam a defender que o principal benefício do tumor ao desenvolver o fenótipo glicolítico é o aumento da produção de lactato e consequente acidificação do meio (GILLIES; ROBEY; GATENBY, 2008). Portanto, a inibição de transportadores de glicose e lactato pode ter uma implicação clínica imediata.

Neste contexto, é importante observar que as isoformas destes transportadores possuem afinidades diferentes e são importantes em diferentes órgãos e momentos metabólicos tanto em tecidos sadios como no câncer (PINHEIRO et al., 2012; SZABLEWSKI, 2013). Justamente aí reside a maior limitação atual, os inibidores não são suficientemente seletivos quanto às isoformas. Os resultados aqui apresentados sugerem um importante papel de isoformas pouco estudadas, o GLUT2 e o MCT3. Portanto, novos estudos com enfoque nestas isoformas podem contribuir para o desenvolvimento de novas terapias.

A exemplo do Capítulo I, estes achados motivaram a elaboração de uma tese de mestrado investigando o papel de possíveis alvos terapêuticos, no contexto da reprogramação metabólica. Este trabalho está em andamento com previsão de conclusão para abril de 2016.

HIPÓXIA & EPIGENÉTICA

A resistência à hipóxia é um fenótipo agressivo bem caracterizado que induz metástase e se correlaciona com pior prognóstico (GATENBY et al., 2007; LIU; SEMENZA; ZHANG, 2015; WILSON; HAY, 2011). Dentre os comportamentos induzidos neste contexto, o controle do pH e a angiogênese são cruciais para a sobrevivência da células maligna (CHICHE et al., 2009; FOX; GENERALI; HARRIS, 2007; HICKEY; SIMON, 2006; SWIETACH et al., 2009). A proteína de membrana anidrase carbônica 9 (CA9) é fundamental no equilíbrio da acidez tumoral, sua expressão está associada a pior prognóstico e sua inibição reduz crescimento tumoral (MCINTYRE et al., 2012; TAN et al., 2009). Portanto, é grande a busca por terapias capazes de inibir CA9 ou de atacar de algum outro modo as células resistentes à hipóxia (MILANI; HARRIS, 2008; PETTERSEN et al., 2014; WARD et al., 2013; WILSON; HAY, 2011).

Em paralelo, a epigenética tumoral tem recebido muita atenção recentemente, em especial a família BET (DI COSTANZO et al., 2014; ESTELLER, 2008; FILIPPAKOPOULOS et al., 2010). Estas proteínas são importantes componentes da maquinaria de transcrição cuja inibição

apresentou resultados antitumorais promissores que levaram estes inibidores a ensaios clínicos em malignidades hematológicas (DA COSTA et al., 2013; DAWSON et al., 2011; DELMORE et al., 2011).

Aqui, demonstramos que o inibidor de BET (BETi) JQ1 reduz consistentemente o crescimento tumoral e a expressão de CA9 em modelos *in vitro* e *in vivo*. Além disso, esta molécula inibiu angiogênese e outras rotas reguladas por hipóxia. Sabe-se que terapias anti-angiogênicas podem selecionar fenótipo glicolítico (CURTARELLO et al., 2015; MCINTYRE; HARRIS, 2015), e dados experimentais apresentados aqui suportam que seja o caso também para JQ1. Portanto, sugere-se que novos estudos testem um possível co-tratamento de BETi com antiglicolíticos. Deste modo, sugere-se aqui que JQ1 é um candidato promissor na busca terapêutica por um agente capaz de eliminar células resistentes a hipóxia.

"Não é o mais forte nem o mais inteligente que sobrevive, mas o aquele que melhor se adapta às mudanças. " Este dito popular, equivocadamente atribuída ao pai da biologia evolutiva Charles R. Darwin, pode ser aplicada a diversos fenômenos da vida, inclusive à progressão de tumores agressivos. Não é a toa que a *Cancer Research UK* (CRUK), principal instituição de caridade financiadora da pesquisa em câncer da Inglaterra, já colocou lado a lado uma representação de progressão tumoral e a clássica ilustração do famoso naturalista inglês representando sua ideia de evolução (Fig. 22). Assim como a CRUK, acredito que se quisermos acelerar nosso sucesso rumo à cura da doença mais cruel e injusta que a humanidade já teve conhecimento, devemos voltar aos pensamentos de Darwin.

Cada vez mais a progressão tumoral é descrita como um processo de evolução Darwiniana, e a ocorrência de fenótipos agressivos como o produto final de um processo dinâmico. Para fortalecer a analogia podemos citar fatores centrais à teoria de Darwin que estão

presentes na biologia tumoral, como variabilidade hereditária definindo sobrevivência, reprodução e pressão seletiva – imposta por fatores intracelulares (instabilidade genética e energética) ou intratumorais (hipóxia, acidose, estresse oxidativo e sistema imune) ou ainda por fatores externos (radio e quimioterapia) – deriva e adaptação (DE BRUIN et al., 2014; GILLIES; VERDUZCO; GATENBY, 2012; PEPPER et al., 2009). Outra demonstração da aplicação de ideias evolutivas à oncologia são cladogramas e filogenia para compreender a progressão tumoral (NAXEROVA; JAIN, 2015). Temos inclusive fenômenos que retomam as ideias de Lamarck, quando pensamos na epigenética como um regulador do fenótipo que pode ser adquirido e é hereditário, mas não altera o código genético (DAWSON; KOUZARIDES, 2012; ESTELLER, 2008).

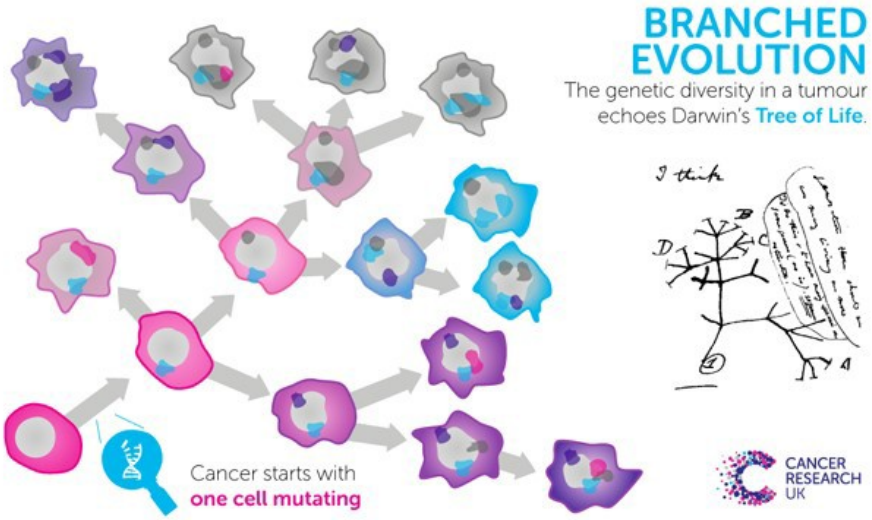

Figura 22 Analogia ente evolução e progressão tumoral em material de divulgação da Cancer Research UK (http://scienceblog.cancerresearchuk.org/2014/10/09/lung-cancer-evolution-a-journey-through-space-and-time/).

Evolutivamente falando, a célula agressiva é o ápice da seleção ao nível de célula e compreendendo o câncer como um processo dinâmico e adaptável, facilmente compreendemos porque é tão difícil curá-lo. Primeiro a sua heterogeneidade, prevê que jamais haverá uma droga capaz de lidar com todos tipos de câncer (GERLINGER et al., 2012). Segundo, muitas terapias impõe uma forte pressão seletiva que pode resultar em um novo tumor mais resistente (BURRELL; SWANTON, 2014; FISHER; PUSZTAI; SWANTON, 2013; MCGRANAHAN; SWANTON, 2015). Então, como tratar uma doença com tamanha variabilidade genética, heterogeneidade e

capacidade de adaptação? Se a biologia evolutiva nos ajuda a entender a ocorrência do câncer e a ineficácia das terapias atuais, ela certamente também nos aponta perspectivas.

Darwin teorizou toda a sua evolução por seleção natural sem ter nenhum conhecimento de genética. Mas não precisava, afinal de contas a natureza seleciona o fenótipo. A variabilidade genética tumoral é virtualmente infinita, mas apesar de conhecermos exemplos de genes que determinam uma característica, na maioria dos casos o fenótipo pode emergir a partir de diversos genótipos. Temos aqui nossa lição evolutiva para entender porque o foco terapêutico deve ser o fenótipo agressivo, e não a mutação subjacente.

Apesar da ocorrência de resistência ser inevitável, a proliferação da população resistente é controlável. Podemos antecipar a resistência, uma vez que o tumor só será selecionado ao ser tratado. Assim, o estudo de fenótipos agressivos pode apontar fraquezas e opções de fuga do câncer. Foi segundo esta filosofia que este trabalho foi desenvolvido, e é com muito orgulho que acredito ter contribuído no sentido de um melhor cenário clínico.

Biólogos evolutivos acreditam e confirmam a frase do russo Theodosius Dobzhansky que escreveu: "Nada em biologia faz sentido senão à luz da evolução" *.

Como um biólogo evolutivo por vocação, afirmo que a biologia tumoral não é exceção!

* frase de Theodosius Dobzhansky, biólogo evolutivo e membro da Igreja Católica Ortodoxa Russa. Foi primeiramente utilizada em 1964 no artigo intitulado "*Biology, Molecular and Organismic*" publicado no periódico *American Zoologist* para afirmar a importância do estudo da biologia a nível do organismo em resposta ao desafio colocado pela subida de popularidade da biologia molecular. Posteriormente, o russo reutilizou a expressão para criticar o criacionismo anti-evolução e defender o evolucionismo teísta, e foi quando a frase se popularizou e ficou famosa. Este ensaio foi publicado pela primeira vez na revista American Biology Teacher, volume 35, páginas 125-129.

PRODUÇÃO CIENTÍFICA E LEGADO ACADÊMICO

Além da contribuição científica, materializado na forma de 2 artigos e 1 manuscrito pronto, a realização deste trabalho originou outros produtos que merecem destaque: captação de técnicas, dois trabalhos de conclusão de curso (TCC) e posteriores projetos de mestrado, formação de recursos humanos e captação de colaboradores.

Primeiramente, o legado acadêmico que fica para o laboratório na forma de técnicas experimentais. Diversas técnicas eram inéditas no laboratório e tiveram de ser captadas, aprendidas, adaptadas e estabelecidas para o desenvolvimento deste trabalho. As mais relevantes sendo: medidas de perfil redox em células, respirometria de alta resolução, avaliação do perfil metabólico em linhagens celulares, cultivo celular em câmara de hipóxia, análise de expressão gênica, silenciamento gênico, imunoprecipitação de cromatina (ChIP), quantificação de luciferase como gene repórter, cultivo e imunohistoquímica de esferóides e análise quantitativa de imunohistoquímica.

Quanto à formação de recursos humanos, este trabalho gerou perguntas que originaram 2 TCC. As 2 alunas foram co-orientadas pelo autor deste trabalho e seguiram a linha de pesquisa em seus mestrados, produzindo, até o momento, mais um manuscrito (Anexo A).

Por fim, este trabalho tem característica multicêntrica incluindo diversos laboratórios em Porto Alegre, no Brasil e no exterior. Foram iniciadas e estabelecidas colaborações em: Universidade Federal do Rio de Janeiro (UFRJ), na pessoa do Prof. Dr. Antônio Galina Filho, o Tonhão, e também Prof. Dr. Franklin Rumjaneck e a Dra. Nívea Amoedo Dias; Hospital de Clínicas de Porto Alegre (HCPA), na pessoa da Prof. Dr. Patrícia Ashton-Prolla e do Me. Gabriel Macedo; Instituto de Cardiologia (IC/FUC), na figura da Dra. Melissa Markoski; University of Oxford, na pessoa do Prof. Adrian L. Harris FMedSci FRCP, e também Alan McIntyre, Professor na The University of Nottingham.

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ÍNDICE REMISSIVO

LISTA DE FIGURAS

ANEXO A

Research Article

In Vitro Evaluation of Antitumoral Efficacy of Catalase in Combination with Traditional Chemotherapeutic Drugs Against Human Lung Adenocarcinoma Cells

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Abstract

Lung cancer is the most lethal cancer-related disease worldwide. Since survival rates remain poor, there is an urgent need for more effective therapies that could increase the overall survival of lung cancer patients. Lung tumors exhibit increased levels of oxidative markers with altered levels of antioxidant defenses and previous studies demonstrated that the overexpression of the antioxidant enzyme catalase (CAT) could control tumor proliferation and aggressiveness. Herein we evaluated the effects of CAT treatment on the sensitivity of A549 human lung adenocarcinoma cells towards various anticancer treatments, aiming to establish the best drug combination for further therapeutic management of this disease. Exponentially growing A549 cells were treated with CAT alone or in combination with chemotherapeutic drugs (cisplatin, 5- fluorouracil, paclitaxel, daunorubicin and hydroxyurea). CalcuSyn® software was used to assess CAT/drug interactions (synergism or antagonism). Growth inhibition, NFκB activation status and redox parameters were also evaluated in CAT-treated A549 cells. CAT treatment caused a cytostatic effect, decreased NFκB activation, and modulated the redox parameters evaluated. Moreover, with exception of paclitaxel, CAT treatment exhibited a synergistic effect among the anticancer drugs tested, which is significantly correlated with an increased H_2O_2 production. These data suggest that combining CAT (or CAT analogs) with traditional chemotherapeutic drugs, especially cisplatin, is a promising therapeutic strategy for the treatment of lung cancer.

Key words: lung cancer, A549 cells, catalase, anticancer drug combination, chemotherapy, drug synergism.

Introduction

Lung cancer remains the most common and lethal cancer-related pathology with nearly 1.59 million deaths annually worldwide. [1] There are two main types of lung cancer with epithelial origin: small cell (SCLC) and non-small cell lung cancer (NSCLC). [2] The latter comprises more than 80% of all cases of lung cancer, and of these, roughly 50% are lung adenocarcinomas (AdC). [3] Despite progress in molecular research, the therapeutic armamentarium remains poor [4] and unable to improve NSCLC patients outcome, which have a 5 year survival rates of only 2% for advanced stages. [1]

As the initial stages of lung cancer are asymptomatic, the disease is typically diagnosed in advanced stages. For these patients, chemotherapy forms the foundation of their treatment, which is usually palliative. Currently, the gold standard treatment for lung AdC is based on platinum agents such as cisplatin, carboplatin or oxaliplatin and usually given in combination with other agents such as paclitaxel and etoposide. Unfortunately, despite these therapies, the disease is rarely curable and prognosis is poor. [5] Several factors contribute to the high mortality rate (which outranks prostate, colorectal and pancreatic cancer cases combined) and one of the most common includes resistance to cytotoxic drugs. [1] Anticancer drug resistance may be innate or acquired and may apply to a single agent or to a class of agents with similar antineoplasic mechanisms of action. [6-8] Therefore, there is an urgent need for more effective therapies, drugs, or treatments that could help increase the overall survival of lung cancer patients. With an estimated average cost between US$800 million and US$1.8 billion, and an accompanying timeline of 15 years, the development of novel drugs are an expensive and time consuming endeavor. [9] Thus, the practice of identifying additional therapeutic indications for existing compounds has become a needed and appropriated approach in many diseases, including cancer. [5] Indeed, compounds or drugs that interfere with any aberrant pathways or physiopathological processes known to be altered in lung cancer should be rationally explored in order to identify potential adjuvant strategies for novel

interventional regimens.

Lung cancer cells are consistently exposed to a particular pro- inflammatory and pro-oxidant environment, derived from the high environmental oxygen pressure and exogenous oxidants, such as cigarette smoke and air pollutants. [10] In this context, an imbalance in antioxidant mechanisms plays a significant role in the pathogenesis of lung cancer. Numerous reports showed that reactive oxygen species (ROS), manly hydrogen peroxide (H_2O_2), act as endogenous drivers of the oncogenic phenotype, by the modulation of cellular proliferation, migration, survival and multidrug resistance. [11,12] Most of these processes are regulated by redox-responsive transcriptional factors such as Nuclear Factor Kappa B (NFkB), Nuclear Factor (erythroid-derived 2)-like 2 (Nrf2) and activator protein-1 (AP-1). [11,13] Moreover, previous studies of our group demonstrated that lung AdC aggressiveness is associated with elevated intracellular oxidative stress, where H_2O_2 plays a crucial role, since the exogenous treatment with the antioxidant enzyme catalase (CAT) (E.C. 1.11.1.6), but not T rolox® (synthetic analog of alpha-tocopherol) or N-acetyl- cysteine (NAC, a glutathione precursor), attenuated tumor aggressiveness. [14] CAT overexpression reverts malignant features in different cell lines [15] and prevented tumor growth and metastasis in mouse. [16] Moreover, mitochondrial-targeted CAT suppresses invasive breast cancer in mice [16] and in others *in vivo* models. [15] Thus, compounds that are able to specifically scavenging H_2O_2 (such as catalase/peroxidase mimetics) could provide a future venue to the development of new therapeutics interventions.

This way, the present study aimed to establish the role of the combination of CAT treatment in the efficacy of different anticancer drugs. For this purpose, the human AdC cell line A549 was treated with exogenous CAT and the proliferative behavior, intracellular redox status and p65 nuclear and cytosolic immunocontent were evaluated. Also, CAT-treated cells were exposed to several chemotherapeutic drugs with different mechanisms of action: alkylating agents (Cisplatin); antimetabolites (5-Fluorouracil and

Hydroxyurea); microtubule stabilizing (Paclitaxel) and topoisomerase inhibitor (Daunorubicin), aiming to assess the potential synergistic, additive or antagonistic effect of CAT on these drugs.

Results

Growth inhibition, NFkB inactivation and redox modulation in CAT-treated A549 cells

Exogenous addition of CAT in exponentially growing A549 human lung AdC cells causes a dose dependent inhibition on cellular proliferation (Fig. 1a), [14] with a maximal dose effect of 1000 U/mL. This cellular growth inhibition was significantly observed after 24 h of treatment and maintained over 4 days (Fig.1b). Growth inhibition was related to a cytostatic (not cytotoxic) effect since CAT washout readily restored cellular proliferative rate similar to untreated cells (Fig.1b). We also observed an increased G0/G1 cell cycle phase without substantial increase in sub-G0/G1 population, nor trypan blue positive cells (data not shown). Based on the above-mentioned data, we used 1000 U/mL of CAT for 48 and 96 h of treatment in the following experiments. All inhibitory effects were dependent of CAT activity since the treatment with heat-inactivated CAT was ineffective to modulate the growth of A549 cells. The activation status of NFkB (a redox-responsive transcriptional factor that modulates tumoral proliferation, migration, survival and multidrug resistance) in CAT-treated A549 cells was determined by the evaluation of p65 subcellular distribution (Fig.1 c). We found that NFkB was constitutively activated in A549 cells (p65 was mainly present in the nuclear fraction). CAT-treatment caused a 2.9-fold increase in the cytosolic immunocontent of p65, suggesting that CAT treatment leads to a significantly inactivation of NFkB in A549 cells (Fig. 1c). CAT-treated A549 cells presented lower intracellular antioxidant potential and reduced thiol levels when compared to untreated cells (Fig. 1d). Notably, the decrease in antioxidant potential was time dependent for both groups. CAT washout was able to restore both reduced thiol and antioxidant potential levels. Interestingly, high GSH levels (Fig.1 d) and the

intracellular generation of H_2O_2 (Fig. 1e) were found significantly in CAT-treated cells as compared to untreated cells. These data suggest that A549 cells treated with CAT presented an imbalance in antioxidant defenses and that could be responsible for the NFkB inactivation and growth inhibition.

CAT-treatment in combination with anticancer drugs

Aiming to establish the best chemotherapeutic regimens that could benefit from the cytostatic and modulatory effects on redox parameters induced by catalase, we first obtained the cytotoxic curves (and GI_{50} values) of the anticancer drugs cisplatin (Cis), 5-fluorouracil (5-FU), paclitaxel (PT), daunorubicin (Dauno) and hydroxyurea (HU) in exponentially growing A549 human lung AdC cell line (Supplementary Figure 1). When co-administrated, CAT (1000 U/mL) significantly potentiated the cytotoxicity caused by all anticancer drug tested, with the exception of paclitaxel (Fig. 2). Table 1 presents the synergism/antagonism evaluation of the anticancer drugs in association with CAT, based on *Combination Index* (CI) values. Cisplatin, 5- fluorouracil and hydroxyurea exhibited a significant synergism with catalase, while the combination of daunorubicin with CAT showed only a slight synergism. In contrast, CAT treatment caused antagonistic effect when combined paclitaxel. We also evaluated the long-lasting effect of catalase treatment in the effectiveness of anticancer drugs. Even after 24 h of CAT washout, the increased cytotoxic effects presented by the combination of anticancer drug treatment with CAT was still significant in cisplatin-, 5- fluorouracil- and hydroxyurea-treated cells.

Association of H_2O_2 generation and cell death by the combination of CAT and anticancer drugs

Several studies in a variety of cell types have suggested that cancer chemotherapy drugs induce tumor cell death in part by increasing the formation of reactive oxygen species (ROS). In that regard, considering that we found synergistic,

slight synergistic and even antagonistic effect of the combination of CAT with anticancer drugs, we decided to assess the adjuvant effect of CAT treatment in the generation of H_2O_2 by the chemotherapeutic drugs evaluated in this study. Possible generation of intracellular H_2O_2 by the chemotherapy drugs was assessed by measuring the extent of oxidation of 10-acetyl-3,7- dihydroxyphenoxazine (Amplex Red® reagent) which is a substrate for horseradish peroxidase (HRP) that enables selective detection of H_2O_2. [23,24] Among the anticancer drugs tested, paclitaxel and hydroxyurea showed basal increase in H_2O_2 production when compared with vehicle alone (Fig. 3a). However, when chemotherapeutics drugs were co-treated with CAT, only the combination of paclitaxel plus CAT showed no difference in the rate of H_2O_2 production (Fig. 3b). Hydroxyurea plus CAT, as expected, had a decreased in H_2O_2 production when compared with the drug alone. In contrast, the drug cisplatin, although alone had no effect in H_2O_2 production, when treated with CAT presented almost a 3-fold increase in the rate of H_2O_2 generation. Again, the increase in H_2O_2 generation by the combination of CAT with the alkylating agent cisplatin was a long-lasting effect because it was sustained even 48 h after the remove of CAT (CAT wash-out group) (Fig. 3b). The increase in H_2O_2 production rate by the combination of CAT with the chemotherapeutic drugs were found to be significantly correlated with the potentiation of tumoral cell death ($P < 0.05$) ($r = 0.884$) (Fig. 4). These results support the notion that the effectiveness of the anticancer drug combination with CAT in eliminating tumoral cells could be explained, at least in part, by the increase in intracellular H_2O_2 generation.

Discussion

Among malignancies, lung cancer ranks as one of the most common and lethal. [1] Although chemotherapy presents efficacy for some patients with lung cancer, effective therapeutic options for advanced stages of the disease remain limited and cure rates are low. [27] Even though the treatment of NSCLC has been

revolutionized by the development of targeted agents *(e.g.,* the FDA- approved drugs *erlotinib* and *gefitinib* for patients harboring specific EGFR mutations), the decision in NSCLC management is still mainly based on the anatomic extent of the disease. [5] Among the factors associated with high mortality rate, multidrug resistance is one of the most common. [6-8] In this context, the search for new adjuvant therapies or two-drugs combinations using already approved molecules are important to overcome drug resistance and to improve the prognosis of patient with lung cancer.

Since lung tumors develop in an atypical pro-inflammatory and pro- oxidative microenvironment, [10] reactive species (RS) are believed to be especially important in this tumoral pathogenesis and chemotherapy effectiveness. As previously reported, lung AdC cells have an imbalance in redox parameters compared to normal cells, presenting a high *steady-state* generation of reactive species that follows tumor progression and, presumptively, must somehow confer a selective advantage. [14] Numerous reports suggest that intracellular oxidants are generated in response to cancer chemotherapeutic drugs, and that these are an essential component of the cell death process. Here we investigated to which extent the toxicity of several common anticancer agents to lung cancer cells is influenced by the modulation of the cellular antioxidant parameters with the administration of exogenous CAT, aiming to establish the best drug combination for further therapeutic management of this disease.

In A549 human lung adenocarcinoma cells, the exogenous addition of CAT causes an inhibition of cellular proliferation. Indeed, it is well known that basal H_2O_2 levels greatly participate in cancer cell proliferation, notably through the activation of mitogenic signaling pathways such as the ERK/MAPK pathway. [12,28-30] Surprisingly, our results demonstrated that CAT treatment significantly decrease endogenous thiol levels and the non-enzymatic antioxidant capacity.

In contrast, intracellular GSH and H_2O_2 levels were increased in CAT-treated cells. This can be explained by the fact that H_2O_2 is able to diffuse through membranes, [28] causing

a cellular efflux of H_2O_2. Assuming this reduction in intracellular H_2O_2 concentration, CAT-treated A549 cells may decrease the synthesis of endogenous antioxidants defenses (thus justifying the reduction of thiol and total reactive antioxidant potential - TRAP - levels) and will present an increase in GSH/GSSG levels as glutathione (GSH) can be oxidized by H_2O_2 to form glutathione disulfide (GSSG). Moreover, we found high rates in H_2O_2 production CAT-treated cells. Although puzzling at first inspection, these data could reflect an attempt of tumor cells to overcome the cytostatic effect of CAT-treatment, by generating a higher rate of H_2O_2 production.

Reactive species (RS) are predominantly known for causing cell damage. However, cumulative information has showed that increase in basal levels of RS is associated with tumor progression in different cancer cells, [30,31] since RS play a major physiological role in the control of redox-sensitive transcriptional factors such as the Nuclear Factor Kappa B (NFkB). NFkB is a transcription factor that modulates gene expression in response to growth factors, pro- inflammatory agents such as interleukin-1 (IL-1), tumor-necrosis factor (TNF), oxidants and to anticancer drugs. [32-35] The most common isoform of NFkB is the p65/p50 heterodimer that exist in the cytoplasm in an inactive state bound to inhibitory protein IkB and, when stimulated, translocate to the nucleus, where activates transcription of target genes. [36] Substantial studies indicate that NFkB regulates oncogenesis, tumor progression and resistance to chemotherapy by enhancing the expression of anti-apoptotic and antioxidant genes including Bcl- xL, Superoxide Dismutase 2 (SOD2), and Glutathione S-Transferase (GST). [33,35,37-39]

Furthermore, our study and numerous reports describe that NFkB is constitutively activated in lung cancer cells, being associated with tumoral resistance to different classes of anticancer drugs, such as cisplatin, daunorubicin, paclitaxel and 5-fluorouracil. [33,40] In our study, catalase treatment caused an increase in the cytosolic p65 immunocontent, indicating an inactivation of this transcriptions factor. However, NFkB

inactivation does not seem to be the only mechanism related to the selected sensitivity of A549 cells against the anticancer drug tested. Our *Combination Index* approach, a manner to evaluate the synergism, antagonism or additive effects of two-drug combination showed that paclitaxel, a drug that the cellular resistance has been described to be affected by NFkB activation, presented an antagonistic effect when associated with CAT.

Additionally, H_2O_2 may influence cellular response to antineoplasic drug such as cisplatin and other alkylating agents, which are the gold standard treatment for lung AdC. Even though, in AdC cell lines, H_2O_2 can up-regulate APE-1 (a key enzyme in DNA repair) expression, [41] which may confer cisplatin resistance, [42] we found a significant correlation between the increase in drug sensitivity with the high rates of H_2O_2 production, specially for the combination of cisplatin with CAT. In this way, catalase co-treatment may enhance cisplatin effectiveness. [43] Actually, the combination of cationized catalase with cisplatin was shown to decreases nephrotoxicity, one of the most serious side effects of cisplatin. Thus, catalase combination could postpone early termination of the treatment or avoid the significant limitation in the dose usage imposed by nephrotoxicity, while, and most important, improving antitumor activity. [44]

Therefore, compounds that are able to scavenge H_2O_2, such as catalase or catalase mimetic, could provide a future venue for adjuvant therapeutics interventions, enabling therapeutic selectivity and overcoming drug resistance.

In this context, the Ruthenium oxide (RuO_2) nanoparticulate peroxidase/catalase mimetic shows better specific activities than many natural catalases and peroxidases. [45] PEG-liposomes encapsulating EUK-134 (a strong SOD/CAT mimetic) coated with antibodies against to platelet-endothelial cell adhesion molecule (PECAM-1) target to the vascular endothelium showed *in vitro* and *in vivo* efficacy in alleviating acute pulmonary inflammation. [46] Since the design elements of this drug delivery system are already in

clinical use, and there are promising vascular lung tumoral markers being described, [47] it is an attractive strategy for translational interventions using targeted-derived catalase mimetic in combination with cisplatin to enhance anticancer drug toxicity.

Conclusions

Taken together, the data presented here suggest that adjuvant CAT treatment can act synergistically with Cisplatin, 5-Fluorouracil and Hydroxyurea and antagonistically with Paclitaxel chemotherapeutics drug. This effect is probably related to amplification in H_2O_2 generation by the combination of CAT with anticancer drugs. Our data pointed into a potential beneficial effect of CAT adjuvant therapy providing a new therapeutic strategy for the management of lung adenocarcinoma.

Material and methods

Reagents and equipment

Materials used in cell culture were acquired from Gibco®/Invitrogen (Sao Paulo, SP, Brazil). Chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA) except when indicated. Spectrophotometric measurements were assayed in a 96-well microplate reader (Spectra Max GEMINI XPS, Molecular Devices, USA).

Cell line and cell culture conditions

Exponential growing A549 human lung AdC cell line, obtained from NCI-Frederick Cancer DCTD cell line repository, was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Cripion Biotech, SP, Brazil) 2 mM L-glutamine, 10000 U/mL penicillin G, 10000 ^g/mL streptomycin sulfate and 25 ^g/mL amphotericin B at 37°C in a humidified atmosphere of 5% of CO_2.

Dose response curve of CAT and growth inhibition assays

A549 cells (2 x 10^4) were seeded in a 12 well plate and, after adherence, cells were incubated with 250 - 1000 U/mL active or heat-inactivated (not shown) CAT. Cell number was evaluated each 24 h treatment using Neubauer chamber cell counting and Sulforhodamine B (SRB) assay. [17] Data is expressed as "cell number" based on a standard curve (cell number X SRB_{abs}). To revert CAT effects in A549 cells, the enzyme was washed out after 48 h of treatment.

NFkB activity

NFkB activity was determined by western blot immune quantification of p65 levels in cytosolic and nuclear fractions of control and treated cells. Briefly, cells were scraped, harvested and collected by centrifugation (4000 rpm/4 min). Cells were then lysed by resuspension in hypotonic buffer (10mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM KCl, 1 mM phenylmethylsulfonyl fluoride, proteases inhibitor cocktail (Roche®), 1 mM sodium orthovanadate, 5 mM sodium fluoride) and incubated on ice for 15 min. After, 10% IGEPAL® was added and cells were disrupted by vortexing (every 15 s for 5 min). The resulting suspension was centrifuged at (14000 rpm/30 seg), and the supernatant (cytosolic extract) was separated and stored at -80°C. The nuclear pellet was washed with cytosolic extraction buffer and then resuspended in high salt buffer consisting 20 mM HEPES (pH 7.9), 400 mM NaCl, 1.5 mM $MgCl_2$, 0.25 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 mM sodium orthovanadate, proteases inhibitor cocktail (Roche®), and 25% glycerol and incubated for 40 min in ice and vortexed for 15 s every 5 min to releasing soluble proteins from the nuclei. After, the nuclear fraction was centrifuged (12000g/10 min) and supernatant containing soluble nuclear proteins was stored at -80°C until experiments. The protein concentration was determined by Bradford assay. [18]

Cytosolic and nuclear proteins extracts (25 ^g) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred

onto a PVDF membrane. Thereafter, nonspecific binding was blocked with 5% of BSA in TTBS for 1h at room temperature. Membranes were then incubated overnight at 4°C with primary antibodies rabbit anti-p65 (1:500) (Abcam®), rabbit anti-lamin B1 (1:1000) (Abcam®) as a nuclear marker and rabbit anti-p-actin (1:5000) (Cell Signaling®) as cytosolic marker. After washing in TTBS, membranes were incubated with peroxidase-conjugated secondary antibodies (1:2000) (Dako®, Glostrup, DK) for 2 h at room temperature. Bands were visualized with Super Signal West Pico Chemiluminescence Substrate (PIERCE®, Rockford, IL, USA) and quantified using Image-J 1.36b software (National Institutes of Health).

Samples preparation for redox analysis

For the total reactive antioxidant potential (TRAP) assay and evaluation of Elman's sulfhydryl group, cells (2 x 10^4 cells/well) were cultivated in 12 well plates, treated with CAT (1000 U/mL) for 48 and 96 h, washed with PBS and frozen-and-thawed in 10 mM PBS three times prior to harvesting and then centrifuged (400g/6 min). Protein concentration was determined by Lowry's [19] for data normalization.

For the evaluation of intracellular reduced glutathione levels (GSH) cells were seeded into flasks of 75 cm^3 (4 x 10^5) and treated with CAT (1000 U/mL) for 48 and 96 h. After that, washed with PBS, removed with a cell scraper and centrifuged at 1000g for 5 min at 4°C. The supernatant was discarded, pellet resuspended in lysis buffer (HEPES 20 mM/CHAPS 1%), incubated on ice for 15 min (cells were disrupted by vortexing every 5 min) and centrifuged at 1000g for 10 min at 4°C. The supernatant was collected, protein concentration determined by Bradford assay [18] for data normalization.

To access the hydrogen peroxide (H_2O_2) production, cells were seeded in 96-wells plate at density of 2 x 10^3 cells/well and incubated with CAT (1000 U/mL) or chemotherapeutic drug (drug GI_{50} value) or in combination. Also, the CAT-washout was performed at the time 48 h and to these cells added chemotherapy (GI_{50}) and incubated

for another 48 h.

Redox analysis

Total radical-trapping antioxidant potential

The non-enzymatic antioxidant capacity of cells was evaluated by total radical-trapping antioxidant potential (TRAP) assay. This test is based on oxidized luminol-chemoluminescence measurement induced by AAPH (2,2'- Azobis 2-amidinopropane) decomposition in glycine buffer (pH 8.6). [20] After system stabilization (buffer plus luminol and AAPH), sample was added (20 ^g of protein) and the chemoluminescence decreases proportionally to the amount of non-enzymatic antioxidants monitored in a Wallace 1450 MicroBetaTriLux Liquid Scintillation Counter & Luminometer (Perkin Elmer). A time per chemoluminescence curve was obtained and the relative "area under the curve" (AUC) in the recovery phase used to analysis, as previously established. [20]

Reduced thiol levels (-SH)

To measure the levels of reduced sulfhydryl groups (-SH), samples (35 ^g) were diluted in PBS 10 mM and buffer (10 mM boric acid, 0.2 mM EDTA pH 8.5). DTNB 10 mM (5, 5'-dithionitrobis 2-nitrobenzoic acid) was added and -SH
levels were determined by reacting samples with 5-thio-2-nitrobenzoic acid (Nbs) at 412 nm ($e_{412\ nm}$ = 27200 M^{-1} cm^{-1}). Results are expressed as nmol - SH/mg protein. [21]

GSH levels

To determine the intracellular reduced glutathione levels (GSH), after proteins precipitation with picric acid, samples were neutralized com Kpi 0,1 M (pH 7,0) and mixed with 5 U/mL Glutathione Reductase (GR) (E.C. 1.8.1.7) and Mix solution (KPi, NADPH 100 mM, DTNB 5 mM). Once GSH is oxidized by DTNB to form an yellow derivative 5'-

thio-2-nitrobenzoic acid (TNB) and glutathione disulfide (GSSG,) the formation rate of TNB is monitored spectrophotometrically at 412 nm and is proportional to the sum of GSSG and GSH present whereas GSSG formed can be recycled to GSH by GR in the presence of NADPH. Results are expressed as nmol GSH/^g protein. [22]

Measurement of H_2O_2 production

Amplex Red® fluorescence dye was used to determine intracellular *steady-state* generation of H_2O_2. After treatment, the medium was removed, cells were washed with PBS and the determination of H_2O_2 produced by treated cells was performed using 10-Acetyl-3,7-dihydroxyphenoxazine (Amplex Red® reagent) which is a substrate for horseradish peroxidase (HRP) that enables selective detection of H_2O_2. [23,24] In the presence of peroxidase, this reagent reacts with H_2O_2 to produce resorufin. Fluorescence due to resorufin formation from 50 ^M of Amplex Red® in the presence of 0,02 U/mL HRP was measured in a plate reader (Spectra Max GEMINI XPS, Molecular Devices, USA) with

excitation at 530 nm and emission at 590 nm at 37°C. Results are expressed as $RFU.min^{-1}$ /10^5 cells.

CAT combination with anticancer drugs

To evaluate the effect of CAT treatment in combination with anticancer drugs in A549 growth inhibition, cells (2 x 10^3) were seeded in a 96 well plate and treated with different concentrations of Cisplatin, 5-Fluorouracil, Paclitaxel, Hydroxyurea and Daunorubicin for 48 h to determine their GI_{50} values. After that, cells were co-treated with CAT (1000 U/mL) or chemotherapy (GI_{50}) or with a combination of both for 48h. The growth inhibition was accessed by SRB assay.

To evaluate the effect of drug interactions, we determined the Combination Index (CI) of CAT treatment with each drug tested. The CI, a measure of synergism and

antagonism, is calculated based on the method described by Chou and Talalay along with the release of the computer software CalcuSyn (Biosoft, Ferguson, MO, USA), designed to assess drug interactions. This method takes into account both the potency of each drug or combination of drugs and the shape of the dose-effect curve. Drugs CI was obtained and ranks as synergism, additive or antagonism as CI values of CI < 0.9, CI = 0.9-1.1 and CI > 1.1, respectively. [25,26]

Statistical analysis

Data are expressed as means ± S.E.M. of at least 3 independent experiments in triplicate (n = 3) with significance level considered $P < 0.05$. For statistical analysis, data were analyzed by one or two-way analysis of variance (ANOVA) followed by Tukey test with GraphPad Software Inc., San Diego, CA, USA version 5.0.

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Figure Legends

Figure 1. CAT treatment causes reversible growth inhibition, NFkB inactivation and modulation of redox parameters in A549 human lung adenocarcinoma cell line. (a) Exogenous CAT addition (125 -1000 U/mL) for 72 h causes a dose dependent growth inhibition. (b) Growth inhibition is reverted by CAT wash-out. Data are presented as mean ± S.E.M. of triplicates in three independent experiments (n = 3) (*P < 0.05) (One-way analysis of variance) (ANOVA). (c) Representative western blots (with densitometric values) of cytosolic and nuclear fraction of p65 and lamin B1 (loading control) immunocontent of A549 cells treated with CAT (1000 U/mL) for 24. (d) Total antioxidant potential, thiol and intracellular glutathione (GSH) levels in CAT-treated cells. Data are presented as mean ± S.E.M. of triplicates in three independent experiments (n = 3). Significant differences are expressed by letters, where equal letters represent no significant differences and different letters represent significant differences (P < 0.05) (two-way ANOVA and Tukey post-test among groups). (e) Intracellular generation of H2O2 in CAT-treated cells for 48h and 96h. Data are presented as mean ± S.E.M. of triplicates in three independent experiments (n=3). Significant differences are expressed by letters, where equal letters represent no significant differences and different letters represent significant differences (P < 0.05) (two-way ANOVA and Tukey post-test).

Figure 2. Co-treatment with CAT and different antineoplastic drugs in A549 human AdC cell line. A549 cells were treated with CAT (1000 U/mL) or chemotherapy (GI50) or with a combination of both. In the washout group, CAT was removed at time 48 h, chemotherapy agent (GI50 drug value) was added and incubated for another 48 h. Data are presented as mean ± S.E.M. *P < 0.05 using two-way ANOVA and Tukey post-test.

Figure 3. H2O2 generation by different anticancer drugs and the effect of adjuvant CAT-treatment in A549 human AdC cell line. Amplex Red® assay was performed to access the intracelular H2O2 production. (a) Cells were treated with GI50 of chemotherapeutics for 48h. (b) A549 cells were treated with CAT (1000 U/mL) or chemotherapy (GI50) or with a combination of both. In the washout group, CAT was removed at time 48 h, chemotherapy agent (GI50 drug value) was added and incubated for another 48 h. Data are presented as mean ± S.E.M. *P < 0.05 using two-way ANOVA and Tukey post-test.

Figure 4. Pearson correlation between drug combination toxicity potential and change in H2O2 production rate. Y axis shows the difference between treatment viability of drug + CAT combination and drug alone (% of viability from control). X axis show the difference between H2O2 production rate of drug + CAT combination and drug alone ($RFU.min^{-1}.10^5$ cells).

Table 1: **Quantitative analysis for assessing anticancer drug synergism in combination with Catalase using dose-response curves in A549 human lung adenocarcinoma cell lines.**

Drug tested	Drug Combination	CI value	Interpretation	Mechanism of action
Cisplatin	CAT (1000 U/mL) + Cis (13.3 |JM)	0.563	Synergism	Alkylating agent
5-Fluorouracil	CAT (1000 U/mL) + 5-FU (3.2 JM)	0.483	Synergism	Antimetabolic agent
Paclitaxel	CAT (1000 U/mL) + PT (7 nM)	1.475	Antagonism	Microtubule stabilizer
Daunorubicin	CAT (1000 U/mL) + Dauno (0.16 JM)	0.852	Slight Synergism	Topoisomerase inhibitor
Hydroxyurea	CAT (1000 U/mL) + HU (0.68 mM)	0.402	Synergism	Antimetabolic agent

Combination index values (CI) of cisplatin (Cis), 5-fluorouracil (5-FU), paclitaxel (PT), danunorubicin (Dauno), and hydroxyurea (HU) treatments with catalase (CAT). CI values were calculated from cellular proliferation and viability assays (presented in Figure 1a and Supplementary Figure 1). The data represent means of at least three independent experiments carried out in triplicates (n = 3).

Figure 1

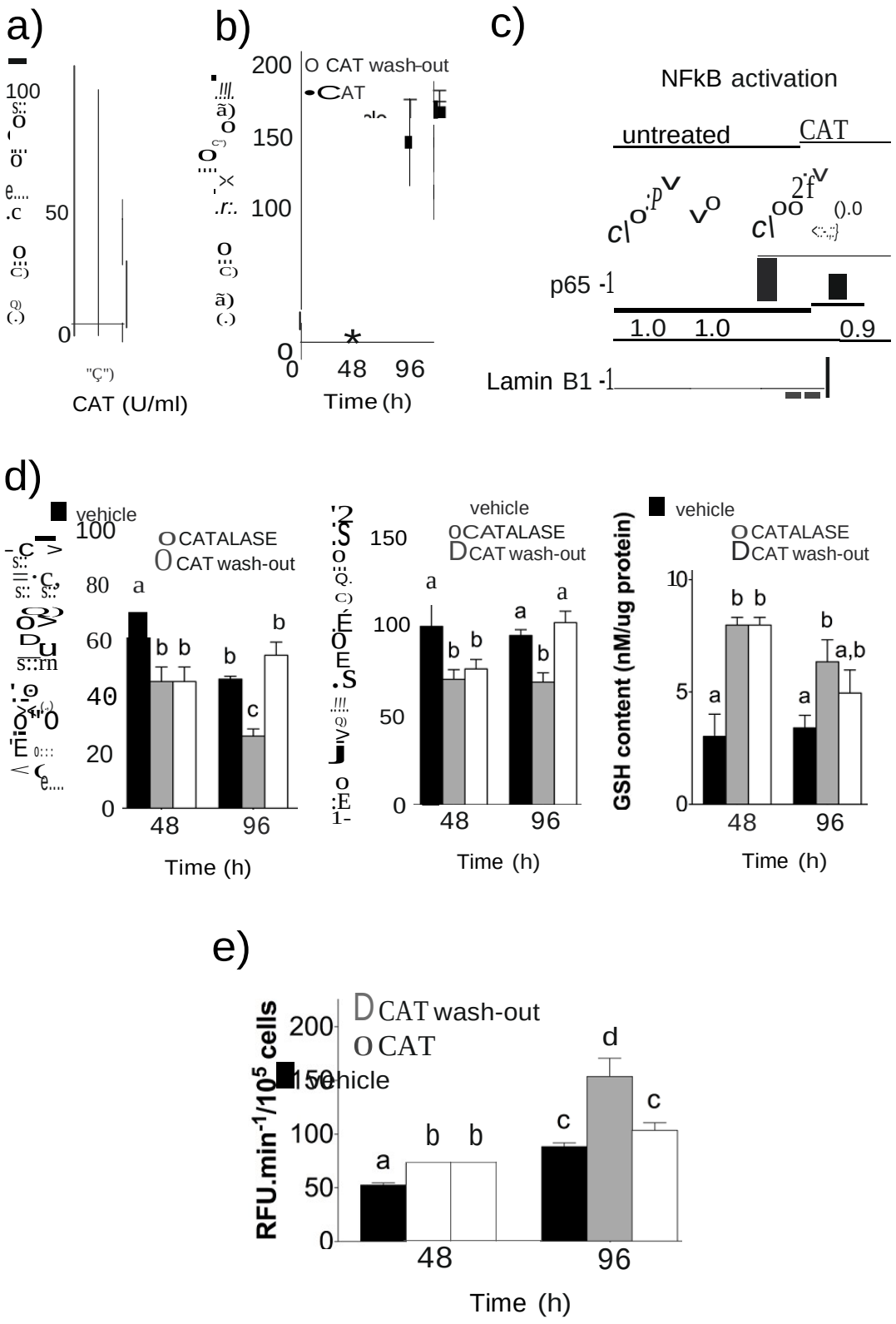

a)
CAT (U/ml)
b)
O CAT wash-out
CAT
Time (h)
c)
NFkB activation
untreated
CAT
p65
Lamin B1
1.0 1.0 0.9
d)
vehicle
CATALASE
CAT wash-out
Time (h)
GSH content (nM/ug protein)
e)
CAT wash-out
CAT
vehicle
RFU.min-1/10^5 cells
Time (h)

Figure 2

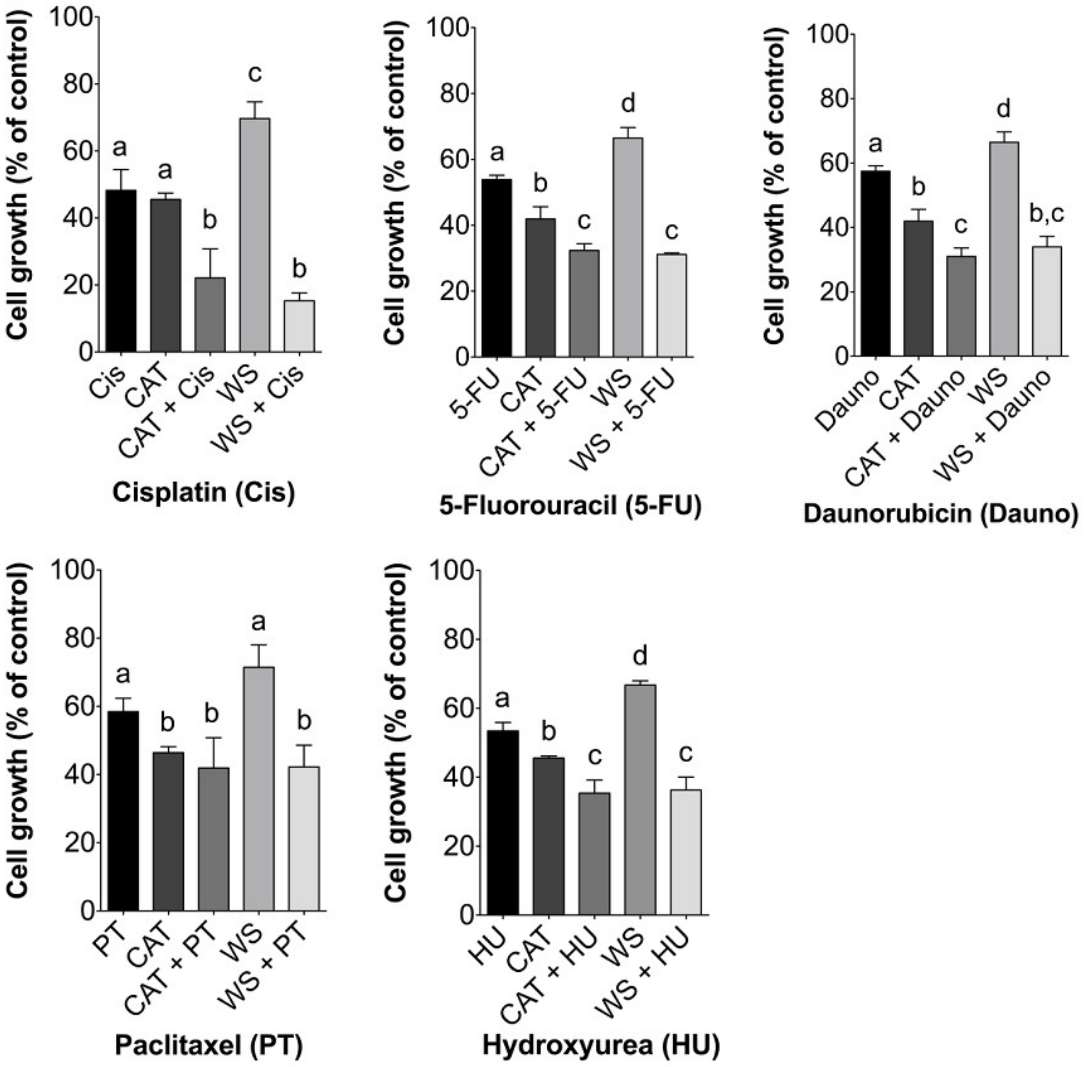
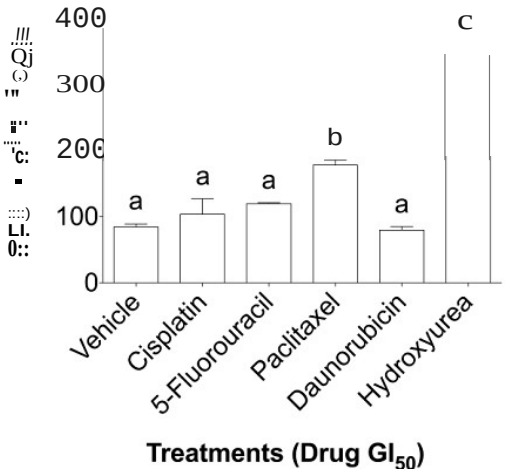

Cell growth (% of control)
100
80
60
40
20
0
a
a
b
c
b
Cis
CAT
CAT + Cis
WS
WS + Cis
Cisplatin (Cis)
a
b
c
d
c
5-FU
CAT
CAT + 5-FU
WS
WS + 5-FU
5-Fluorouracil (5-FU)
a
b
c
d
b,c
Dauno
CAT
CAT + Dauno
WS
WS + Dauno
Daunorubicin (Dauno)
a
b
b
a
b
PT
CAT
CAT + PT
WS
WS + PT
Paclitaxel (PT)
a
b
c
d
c
HU
CAT
CAT + HU
WS
WS + HU
Hydroxyurea (HU)

Figure 3

a)


400
300
200
100
0
a
a
a
b
a
c
Vehicle
Cisplatin
5-Fluorouracil
Paclitaxel
Daunorubicin
Hydroxyurea
Treatments (Drug GI50)

b)

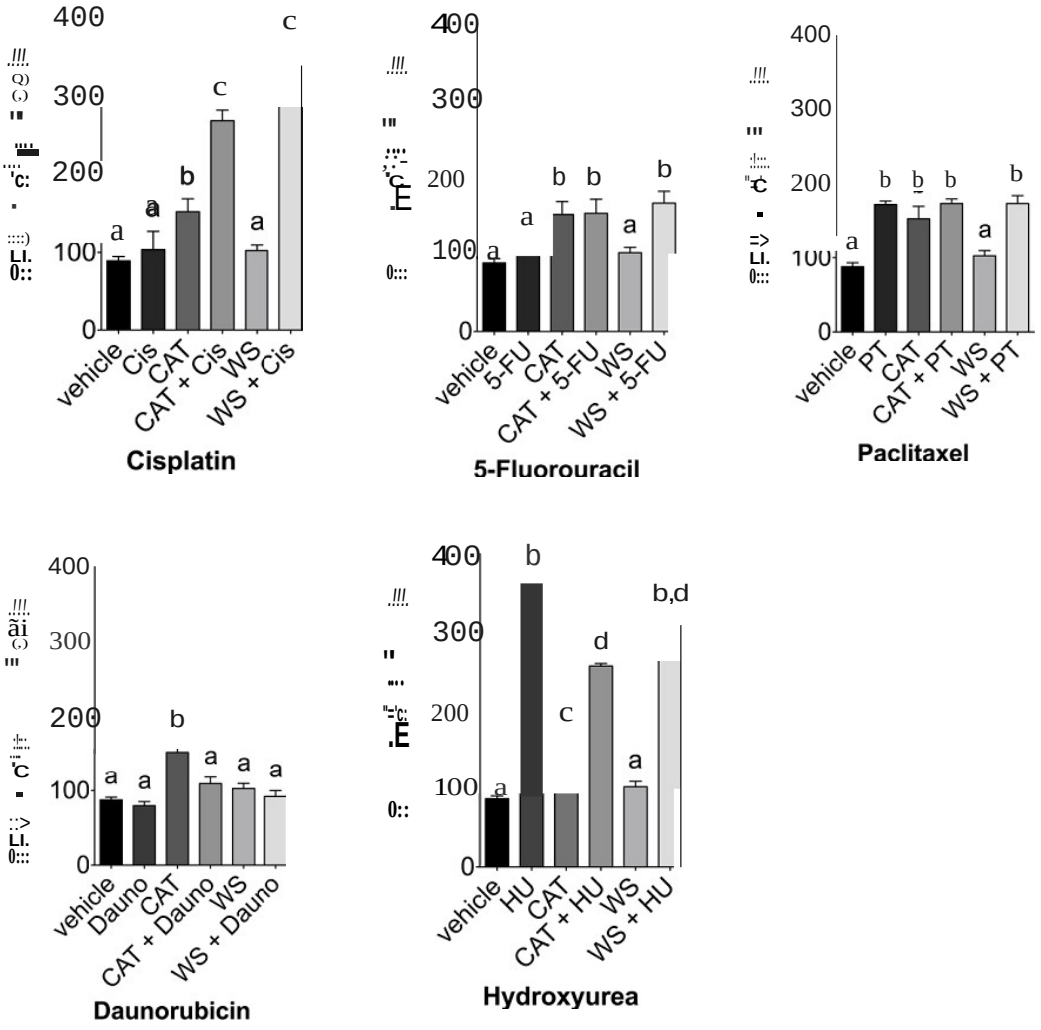

Cisplatin
vehicle
Cis
CAT
CAT + Cis
WS
WS + Cis
5-Fluorouracil
5-FU
CAT + 5-FU
WS + 5-FU
Paclitaxel
PT
CAT + PT
WS + PT
Daunorubicin
Dauno
CAT + Dauno
WS + Dauno
Hydroxyurea
HU
CAT + HU

Figure 4

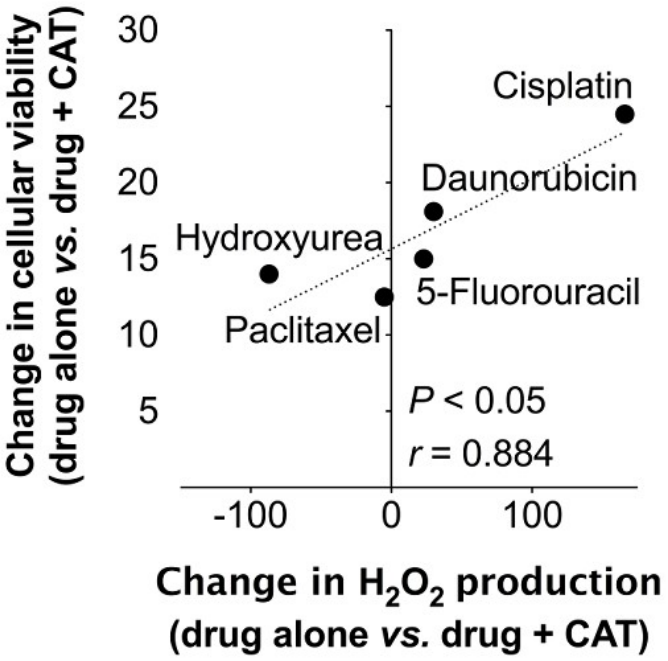
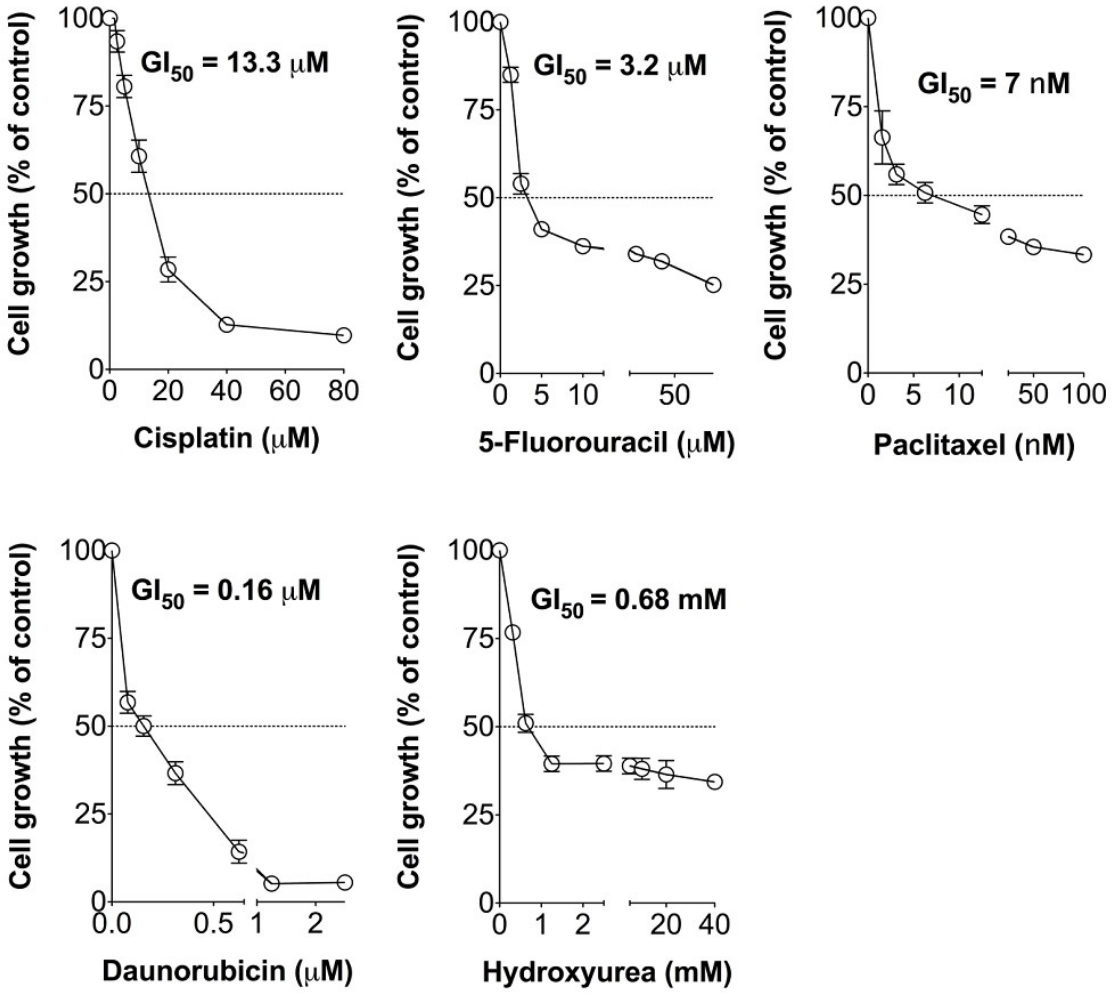

Change in cellular viability
(drug alone vs. drug + CAT)
30
25
20
15
10
5
Cisplatin
Daunorubicin
Hydroxyurea
5-Fluorouracil
Paclitaxel
P < 0.05
r = 0.884
-100
0
100
Change in H2O2 production
(drug alone vs. drug + CAT)

Figure S1



Supplementary Fig. 1. Cytotoxicity curves of the antineoplastic drugs in A549 human lung AdC cell line. Dose response curve of anticancer drugs were performed for 48 h of treatment using SRB assay. Dashed line represents GI50 value of drug. Data are presented as mean ± S.E.M. of five independent experiments carried out in quintuplicate (n = 5).