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JÉSSIKA CRISTINA CHAGAS LESBON

Determinação de potenciais alvos terapêuticos epigenéticos em câncer de pulmão de células não-pequenas

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Mônica Mazalli

Profa. Dra. Mônica Roberta Mazalli
Coordenadora do CEPH

Av. Duque de Caxias Norte, 225 - 13635-900 – Pirassununga, SP
Fone: (19) 3565-6759 - E-mail: cepfzea@usp.br

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“I dedicate this work to all cancer patients, because for many it seems to be a war without victory and without end.”

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“Our greatest weakness lies in giving up. The most certain to succeed is always to try just one more time.”

Thomas Alva Edison

RESUMO

Lesbon, J. C. C. **Determinação de potenciais alvos terapêuticos epigenéticos em câncer de pulmão de células não-pequenas**. 2022. 98 p. Dissertação (Mestrado em Ciências) - Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, 2022.

Apesar dos avanços nas abordagens diagnósticas e terapêuticas, muitos pacientes com câncer de pulmão ainda evoluem para estágios avançados com lesões metastáticas e óbito. Assim, novas terapias visando mecanismos epigenéticos relacionados aos processos metastáticos são muito importantes para o controle de genes específicos do câncer. Neste estudo, selecionamos uma pequena biblioteca de inibidores epigenéticos em linhagens de células de câncer de pulmão de células não-pequenas (CPCNP) e avaliamos 38 potenciais alvos epigenéticos no CPCNP metastático. Os potenciais candidatos foram classificados por uma abordagem simplificada usando experimentos *in silico* e *in vitro* com base em bancos de dados publicamente disponíveis e avaliados por expressão do gene alvo qPCR em tempo real, ensaios de viabilidade celular e invasão, e análise transcriptômica. A taxa de sobrevida dos pacientes com adenocarcinoma pulmonar é inversamente correlacionada com a expressão gênica de oito potenciais alvos epigenéticos, e uma revisão sistemática da literatura confirmou que quatro deles já foram identificados como alvos para o tratamento do CPCNP. Usando doses não citotóxicas dos inibidores selecionados, KDM6A/B (*lysine demethylase 6A e 6B*) e PADI4 (*protein-arginine deiminase Type-4*) foi avaliado que ambos afetam a atividade de invasão e migração de linhagens de células de câncer de pulmão metastático. A análise transcriptômica das células tratadas com KDM6A/B e PADI4 mostrou expressão alterada de genes importantes relacionados ao processo metastático. Em conclusão, mostramos que KDM6B e PADI4 são alvos promissores para inibir a metástase de células tumorais de adenocarcinoma pulmonar.

Palavras-chave: Câncer de pulmão, CPCNP, alvos epigenéticos, metástase, KDM6B, PADI4

ABSTRACT

Lesbon, J. C. C. **Determination of potential epigenetic therapeutic targets in non-small cell lung cancer**. 2022. 98 p. Thesis (Master in Science) – Faculty of Animal Science and Food Engineering, University of São Paulo, Pirassununga, 2022.

Despite advances in diagnostic and therapeutic approaches for lung cancer, many patients still progress to advanced stages, with metastatic lesions and death. Thus, new therapies targeting metastasis by the specific regulation of cancer genes are needed. In this study, we screened a small library of epigenetic inhibitors in non-small-cell lung cancer (NSCLC) cell lines and evaluated 38 potential epigenetic targets for their role in metastatic NSCLC. The potential candidates were ranked by a streamlined approach using *in silico* and *in vitro* experiments based on publicly available databases, and evaluated by real-time qPCR target gene expression, cell viability and invasion assays, and transcriptomic analysis. The survival rate of patients with lung adenocarcinoma is inversely correlated with the gene expression of eight potential epigenetic targets, and a systematic review of the literature confirmed that four of them have already been identified as targets for the treatment of NSCLC. Using nontoxic doses of the remaining inhibitors, KDM6A/B (lysine demethylase 6A e 6B) and PADI4 (protein-arginine deiminase Type-4) were identified as potential targets affecting the invasion and migration of metastatic lung cancer cell lines. Transcriptomic analysis of KDM6A/B and PADI4 treated cells showed altered expression of important genes related to the metastatic process. In conclusion, we showed that KDM6B and PADI4 are promising targets for inhibiting the metastasis of lung adenocarcinoma cancer cells.

Keywords: Lung cancer**,** NSCLC, epigenetic targets, metastasis, KDM6B, PADI4.

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1. INTRODUÇÃO GERAL

O câncer de pulmão é caracterizado por neoplasias malignas que se originam no epitélio respiratório (ROSKOSKI, 2017), apresentando alta taxa de proliferação celular, heterogeneidade tumoral, elevado potencial de gerar metástases e vários distúrbios metabólicos associados (MA et al., 2017). Representa o segundo câncer mais diagnosticado no mundo, responsável por 1.796.144 mortes em 2020 segundo o GLOBOCAN (SUNG et al., 2021). As principais formas são o câncer de pulmão de pequenas células (CPPC), representando aproximadamente 10-15% e o câncer de pulmão de células não-pequenas (CPCNP), sendo o mais diagnosticado, representando 85% dos casos. O CPCNP é um grave problema de saúde pública em todo o mundo, devido ao seu fenótipo invasivo e metastático (RILEY et al., 2013). Histologicamente, o câncer de pulmão de células não-pequenas pode ainda ser classificado em adenocarcinoma, carcinoma de células escamosas e carcinoma de grandes células (BALGKOURANIDOU; LILOGLOU; LIANIDOU, 2013; MEHTA et al., 2015), sendo os subtipos mais prevalentes o adenocarcinoma, o carcinoma adenoescamoso e o carcinoma espinocelular representando 80% dos casos. A etiologia do câncer de pulmão inclui tabagismo, fator genético, idade, sexo, etnia, raça, dieta, obesidade, infecções, poluição do ar, exposição ocupacional e doenças respiratórias crônicas. (ANSARI; SHACKELFORD; EL-OSTA1, 2016a; DEVARAKONDA; MORGENSZTERN; GOVINDAN, 2015).

Entretanto, quase 50% dos casos de câncer de pulmão já são metastáticos ao diagnóstico, implicando em um prognóstico ruim e opções terapêuticas limitadas, resultando em sobrevida global de 5 anos em 10% e 1% em pacientes com estágio IVA e IVB, respectivamente (KHAJURIA; SHARMA, 2021). A intervenção cirúrgica, a radioterapia, quimioterapia e, tratamento específico em genes mutacionais e a imunoterapia são fundamentais para o tratamento do câncer de pulmão (CHENG et al., 2016). O tratamento é estabelecido conforme a classificação do estágio, de acordo com o sistema TNM (Classificação de tumores malignos) que é o mais utilizado pelos oncologistas, sendo recomendado pela AJCC (American Joint Committee on Cancer) e pela UICC (Union for International Cancer Control), e em janeiro de 2017 foi realizada uma revisão na classificação

dos estágios (I, II, IIIA, IIIB, IVA, IVB) e atualizado para a oitava edição lançada em janeiro de 2018 nos Estados Unidos (DETTERBECK, 2018). Utiliza três critérios para avaliar o estágio do câncer: o próprio tumor (T), os linfonodos regionais (N) e disseminação tumoral para outros órgãos (M) (KHAJURIA; SHARMA, 2021).

Apesar dos avanços nas abordagens diagnósticas e terapêuticas, muitos pacientes progridem para estágios avançados com lesões metastáticas. A invasão e migração das células tumorais são fatores importantes de prognósticos desfavoráveis, e resultam na grande maioria das vezes em falhas no tratamento instituído (D'ANTONIO et al., 2014). Deste modo, se faz de grande importância os estudos relacionados aos mecanismos moleculares que avaliam o fenótipo agressivo do câncer de pulmão. As baixas taxas de sobrevida podem ser atribuídas aos efeitos moderado da terapia à base de platina, ao desenvolvimento de quimiorresistência e falhas na detecção de genes mutacionais em cerca da metade de todos os casos de CPCNP, o que compromete a determinação da terapia alvo (ANSARI; SHACKELFORD; EL-OSTA1, 2016a; HILDEBRANDT; GU; WU, 2009; YUAN et al., 2019). Com isso, a busca por novos alvos terapêuticos são de extrema importancia (BAJBOUJ et al., 2021).

O câncer de pulmão desenvolve-se a partir de eventos genéticos e epigenéticos no epitélio respiratório (CHAMBERS; GROOM; MACDONALD, 2002). As aberrações genéticas somáticas, como mutações e alterações no número de cópias dos genes, desempenham um papel bem conhecido na oncogênese, porém, está se tornando cada vez mais evidente a importância das alterações epigenéticas no processo metastático (JAENISCH; BIRD, 2003).

As alterações epigenéticas são normalmente dinâmicas e reversíveis, afetando a estrutura da cromatina para a regulação da expressão gênica sem modificar as sequências do DNA (KOUZARIDES, 2007). Ao longo da última década, as alterações epigenéticas têm sido cada vez mais estudadas e utilizadas como marcadores para a detecção precoce do câncer. Os mecanismos epigenéticos abrangem modificações das histonas, reposicionamento de nucleossomos, metilação de DNA e alterações na expressão de RNAs não codificantes e microRNAs. O impacto das alterações epigenéticas no câncer é

refletido pela expressão gênica alterada, reativação de retro-elementos endógenos e instabilidade genômica (ROCHE; GEMMILL; DRABKIN, 2017).

O DNA pode ser covalentemente modificado por metilação, geralmente em resíduos de citosinas, nas ilhas CpGs, sem alterar a sequência de pares de bases. Além disso, as ilhas CpGs dos promotores dos genes de supressão tumoral são altamente metiladas levando à repressão transcricional, enquanto outros genes envolvidos em processos como reparo de DNA, apoptose, transição epitélio-mesenquimal (EMT), migração, invasão celular e metástase são desregulados pela metilação aberrante da citosina (MEHTA et al., 2015).

As modificações pós-traducionais das histonas são outro tipo importante de mecanismos epigenéticos que ocorrem em resíduos de aminoácidos específicos das proteínas histonas. Os tipos de modificações que podem ocorrer nas caudas de histonas incluem acetilação, metilação, fosforilação, ADP-ribosilação, desiminação, isomerização, ubiquitinação, parilação, citrulinação e sumoilação. Essas modificações têm sido associadas a mudanças dinâmicas nas estruturas da cromatina, com impacto, por exemplo, na transcrição, replicação e reparo do DNA (MAJCHRZAK-CELINSKA; WARYCH; SZOSZKIEWICZ, 2021). Eles também são alvos potenciais de medicamentos antitumorais. A metilação do DNA e as modificações de histonas são adicionadas por proteínas denominadas de "*writers*" e removidas por proteínas denominadas "*erasers*", e por sua vez, essas modificações são reconhecidas por proteínas chamadas "*readers*" que modificam a expressão de vias gênicas (ARROWSMITH et al., 2012).

Portanto, os reguladores epigenéticos são baseados em metiltransferases de DNA (DNMTs); histonas acetiltransferases (HATs) e histonas desacetilases (HDACs); bromodomínios e proteínas do domínio extra-terminal (BET); proteína arginina metiltransferase (PRMT); histonas lisina metiltransferases (HMTs); histonas lisina desmetilases (KDMs) (BAIS, 2019).

As DNAs metiltransferases (DNMTs) são uma família conservada de metilases de citosina. A atividade destas metiltransferases consiste na transferência de um grupo metila da S-adenosil-L-metionina para a posição C5 dos resíduos de citosina (JUBIERRE et al., 2018). As DNMTs estão envolvidas no *splicing* alternativo, duplicação gênica, silenciamento de genes, ativação

transcricional e regulação pós-transcricional. Os mecanismos regulatórios da proteína DNMT também incluem interações moleculares com outras proteínas (LYKO, 2018). A Azacitidina (AZA) e 5-aza-2'-desoxicitidina (decitabina) são dois inibidores de DNMTs clinicamente aprovados (DNMTi). Após um longo período de desenvolvimento foram aceitos como novos padrões de tratamento como linha de frente para pacientes (principalmente idosos) com síndrome mielodisplásica (SMD) e leucemia mieloide aguda (LMA), melhorando as taxas de remissão, sobrevida e qualidade de vida dos pacientes (SCHIFFMANN et al., 2016).

As atividades das histonas acetiltransferases (HATs) e histonas desacetilases (HDACs) são responsáveis no controle da acetilação das histonas. Quando as proteínas histonas estão acetiladas, resultam em uma conformação mais “aberta” da cromatina, pois ocorre a neutralização da carga positiva da lisina enfraquecendo assim, a interação eletrostática entre as histonas e o DNA que apresenta carga negativa, desta forma, colabora para a expressão de genes que estavam “silenciados” (BAIS, 2019). Os inibidores de HDAC (HDACi) exibem atividade antineoplásica em células tumorais por inibirem a proliferação e a angiogênese. Além disso, induzem a apoptose regulando genes pró e antiapoptóticos. Seis categorias estruturais diferentes de HDACi foram descritas e três delas foram aprovadas para o tratamento do câncer: Vorinostat, belinostat, chidamida e romidepsina para linfoma cutâneo e periférico de células T e panobinostat para mieloma múltiplo (SCHIFFMANN et al., 2016).

Os chamados bromodomínios e domínio extra-terminal (BET) são proteínas caracterizadas por serem leitoras de “marcas” epigenéticas, sendo representadas por BRD2 (*Bromodomain-containing protein 2*), BRD3 (*Bromodomain-containing protein 3*), BRD4 (*Bromodomain-containing protein 4*) e BRDT (*Bromodomain testis-specific protein*), com reconhecimento principalmente da lisina acetilada da histona 4. Um estudo recente mostrou que a inibição genética e farmacológica da BRD4 reduz a viabilidade de células que demonstraram resistência adquirida e intrínseca ao cetuximabe (LEONARD et al., 2018).

As proteínas arginina metiltransferases (PRMTs) desempenham um papel importante na regulação gênica devido à capacidade de transferirem grupos metil para resíduos de arginina em histonas ativadoras (histonas H4R3me2a,

H3R2me2s, H3R17me2a e H3R26me2a) ou repressivas (histonas H3R2me2a, H3R8me2a, H3R8me2s e H4R3me2s) (BAIS, 2019).

As histonas lisina metiltranferases (HMTs) são responsáveis em mono-, di- ou trimetilar resíduos de lisinas na proteína histona, portanto, são as "*writes*" das marcas de metilação nas histonas (BAIS, 2019). A metilação da lisina em posições específicas da histona (H3K9, H3K27, H3K37 e H4K20) mantém a cromatina "enovelada" às proteínas histonas promovendo o silenciamento de genes específicos. Entretanto, a metilação de H3K4, H3K36 e H3K39 está associada a regiões ativamente transcritas (BAIS, 2019). Alguns estudos demonstraram que as HMTs, como NSD1 (*Nuclear Receptor Binding SET Domain Protein 1*), EZH2 (*Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit*), KMT5A (*Lysine Methyltransferase 5*), SMYD2 (*SET And MYND Domain Containing 2*) e MLL *(Lysine Methyltransferase 2)*, promovem o crescimento do carcinoma de células escamosas de cabeça, ativando vias de sinalização oncogênicas co-dependentes e também estão envolvidas nos mecanismos de quimiorresistência (BRENNAN et al., 2017; BUI et al., 2018; HUANG et al., 2016; LEE et al., 2018a; LIAO et al., 2018; LUO et al., 2016; SUN et al., 2016). Portanto, as HMTs são amplamente estudadas como alvos terapêuticos (BAIS, 2019).

As histonas lisina desmetilases (KDMs) são aminas oxidases que catalisam a remoção de grupos metil dos resíduos de lisina das histonas, podendo ser de duas classes (desmetilases específicas de lisina 1 / 2 - LSD1 / 2 ou lisina desmetilase 1 A / 1B - KDM1A / KDM1B) e proteínas contendo o domínio JmJC (*domain-containing proteins*) dependentes de ferro e α-cetoglutarato (lisina desmetilase 2-6 / KDM 2–6) (BAIS, 2019). O KDM1A pode desmetilar a lisina em posições específicas das histonas com o objetivo de reprimir a expressão de um gene específico (por exemplo, H3K4) ou estimular a transcrição (por exemplo, H3K9) dependente do contexto na regulação transcricional (CAI et al., 2014). Recentemente, o KDM1B foi relacionado com a promoção do câncer de pulmão de pequenas células, regulando a expressão de *TFPI2 (Tissue Factor Pathway Inhibitor 2)* através da mediação da expressão de *DNMT3B (DNA Methyltransferase 3 Beta*) ou através da regulação da desmetilação de H3K4me1 na região promotora do gene *TFPI2* (CAO et al., 2018).

Portanto, o efeito da metilação das histonas na expressão do gene é muito mais complicado e dependente dos locais alvos. Três estados de metilação de lisina podem ser distinguidos - mono-, di- e trimetilação (me1, me2 e me3, respectivamente), nenhum dos quais altera a carga eletrônica da cadeia lateral de aminoácidos. Assim, não são os próprios grupos de metilação que influenciam a expressão gênica, mas as ações são exercidas pelas moléculas efetoras da cromatina ("*readers*") reconhecendo os resíduos metilados e fazendo com que o recrutamento de outras moléculas altere a cromatina e/ou a transcrição. Geralmente, as metilações H3K4, H3K36 e H3K79 estão associadas à ativação do gene, enquanto as metilações H3K9, H3K27 e H4K20 estão associadas à repressão do gene devido a estados de cromatina silenciados (BANNISTER; KOUZARIDES, 2011). Como as proteínas relacionadas à metilação de histonas (metiltransferases, desmetilases e proteínas de ligação à metil-lisina) são desreguladas no câncer e são estudadas como potenciais alvos para medicamentos. Os mais relevantes, em termos de terapia antitumoral, são os seguintes: H3K79 metiltransferase DOT1L (*DOT1 Like Histone Lysine Methyltransferase*), H3K4 visando leucemia de linhagem mista (MLL) e desmetilase 1 específica de lisina (LSD1), e H3K27 metiltransferase EZH2 (MAJCHRZAK-CELINSKA; WARYCH; SZOSZKIEWICZ, 2021).

Desta forma, terapias combinadas usando moduladores epigenéticos estão se tornando uma alternativa promissora para o tratamento do câncer, principalmente quando aplicadas em conjunto com outras modalidades como a imunoterapia (JUERGENS; RUDIN, 2013). Como os processos epigenéticos, a metilação do DNA e as modificações em proteínas histonas, muitas vezes estão associados entre si, uma atenção considerável está sendo dada aos testes de combinações de medicamentos com vários alvos epigenéticos, o que poderá aumentar a eficácia de cada um dos agentes isolados (JONES et al., 2019).

Atualmente também, o estudo de terapias epigenéticas que demonstram a redefinição do epigenoma em relação à expressão de genes codificadores de proteínas está sendo correlacionado com a imunoterapia, no interesse de ativar uma resposta imune adquirida, permitindo assim um maior reconhecimento do tumor pelo sistema imune do hospedeiro, além do silenciamento da expressão de oncogenes ou ativação da expressão de genes supressores (JONES et al., 2019).

Estudos têm demonstrado que as regulações epigenéticas estão relacionadas à manutenção de subpopulações de células tumorais com resistência à terapia citotóxica e maior malignidade (SHARMA et al., 2010). Também, diferente das mutações oncogênicas que se mantêm fixas no genoma do câncer, as alterações epigenéticas são potencialmente reversíveis, favorecendo o estudo de potenciais alvos epigenéticos terapêuticos e terapias promissoras para o câncer (JUERGENS et al., 2011). Outros trabalhos também evidenciaram que alterações na hipermetilação da citosina tem valor diagnóstico e prognóstico no câncer de pulmão e, em alguns casos, parece predizer as respostas ao tratamento (ANSARI; SHACKELFORD; EL-OSTA, 2016), como foi observado em um estudo que demonstrou que em amostras de 64 pacientes com câncer de pulmão, pacientes com quatro ou mais genes metilados referentes a um painel de 15 genes *APC (APC Regulator Of WNT Signaling Pathway*), *CHD13 (Cadherin 13*), *KLK13* (*Kallikrein Related Peptidase 13*), *DLEC1 (DLEC1 Cilia And Flagella Associated Protein*), *RASSF1A (Ras Association Domain Family Member 1*), *EFEMP1 (EGF Containing Fibulin Extracellular Matrix Protein 1*), *SFRP1 (Secreted Frizzled Related Protein 1*), *RAR (Retinoic Acid Receptor Alpha*), *p16INK4A (Cyclin Dependent Kinase Inhibitor 2*), *RUNX3 (RUNX Family Transcription Factor 3*), *HMLH1 (MutL Homolog 1*), *DAPK (Death Associated Protein Kinase 1*), *BRAC1 (BRCA1 DNA Repair Associated*), *p14ARF* (*Cyclin Dependent Kinase Inhibitor 2*) apresentaram uma sobrevida de 13,8 meses e pacientes com menos de quatro genes metilados apresentaram sobrevida de 17,8 meses (ZHANG et al., 2011). Outro estudo ressaltou que a desmetilação de quatro genes silenciados epigeneticamente associados ao câncer de pulmão *APC*, *RASSF1a*, *CDH13* e *p14ARF* foram associados à melhora da sobrevida livre e global da progressão tumoral (JUERGENS et al., 2011).

A utilização de moléculas moduladoras do epigenoma são capazes de inibir ou atenuar uma série de características relevantes para o desenvolvimento tumoral, como a capacidade metastática e de resistência a terapias convencionais. Além disso, moléculas reguladoras do epigenoma utilizadas em doses adequadas podem agir em alvos específicos, inibindo ou atenuando fenótipos tumorigênicos sem, necessariamente, apresentar citotoxidade as células de tecidos saudáveis (NERVI; DE MARINIS; CODACCI-PISANELLI, 2015). Já foram identificados mais de 400

domínios de alvos epigenéticos envolvidos na metilação do DNA, na modificação de histonas (e alguns não-histonas) ou na tradução dessas modificações em expressão gênica. Embora o número destas moléculas químicas epigenéticas seja pequeno em relação ao número de alvos epigenéticos, estas moléculas são muito utilizadas para descobrir e melhor compreender a complexa biologia dos mecanismos epigenéticos (MÜLLER, SUSANNE; BROWN, 2012). As moléculas epigenéticas são caracterizadas pelas seguintes propriedades: uma potência de menos de 100 nM em um ensaio bioquímico ou biofísico; seletividade de mais de 30 vezes em relação aos outros membros da mesma família; e atividade celular específica no alvo de menos de 1 μM (https://www.thesgc.org/chemical-probes). Portanto, são consideradas como moléculas inibidoras potentes, seletivas e permeáveis às células com afinidade de ligação em regiões proteicas específicas, sendo muito utilizadas na pesquisa básica e aplicada e são essenciais nos estudos iniciais para a descoberta de novos medicamentos, permitindo a validação de alvos pré-clínicos em laboratórios acadêmicos e industriais (https://www.thesgc.org/chemical-probes).

A combinação de terapia convencional ou tratamentos antitumorais inovadores com medicamentos epigenéticos podem oferecer uma alternativa à quimioterapia clássica e otimizar o efeito terapêutico. Compostos epigenéticos, como DNMTs e HDACs, podem aumentar a acessibilidade da cromatina para os medicamentos quimioterápicos através da descompactação da cromatina. Estudos pré-clínicos e clínicos mostram inúmeros benefícios na combinação de vários DNMTs e HDACs com diversos quimioterápicos, particularmente em neoplasias hematológicas, mas também em neoplasias sólidas. As vantagens de tais combinações, em comparação com a quimioterapia padrão, são as seguintes: os compostos epigenéticos podem ser usados para preparar as células tumorais para a quimioterapia por quimiossensibilização e imunopotenciação; podem ter efeitos sinérgicos com outras terapias antitumorais, ou podem ser usados para reverter a resistência adquirida à terapia (MAJCHRZAK-CELINSKA; WARYCH; SZOSZKIEWICZ, 2021).

Portanto, principalmente dois grupos de pacientes com câncer de pulmão podem obter favoráveis benefícios através de medicamentos de inibidores epigenéticos: pacientes que não estão aptos o suficiente para quimioterapia e pacientes com CPCNP de alto risco. O primeiro grupo, ou seja, pacientes não elegíveis para quimioterapia, ainda podem estar aptos o suficiente para a terapia epigenética, por não apresentar muitos efeitos colaterais devido a baixa citotoxicidade em comparação aos quimioterápicos convencionais. As terapias epigenéticas induzem apoptose e/ou diferenciação revertendo o silenciamento ou ativação aberrante de genes, afetando apenas as células tumorais. O segundo grupo compreende pacientes com CPCNP de alto risco, ou seja, aqueles com menor sobrevida livre que parecem estar propensos a alterações epigenéticas relevantes (SCHIFFMANN et al., 2016).

Entretanto, o grande desafio para esses compostos seria como traduzir a eficácia *in vitro* com as concentrações em escala nanomolar para o uso clínico, com concentrações baixas e eficientes. O efeito *off-target* desses compostos é outra questão problemática. Como a regulação epigenética é dinámica, reversível e interdependente, o mecanismo por trás ainda não é bem esclarecido, podendo ter ação em mais de um alvo específico e regular diversas proteínas, ou até mesmo, uma mesma proteína ter mais de uma função em tecidos e locais diferentes. Portanto, um perfil de segurança bem estabelecido dessas epi-drogas é necessário para facilitar sua aplicabilidade na terapia clínica (LU et al., 2020a).

Em conclusão, a terapia epigenética tem sido aplicada em diferentes tipos de câncer como tratamento principal ou em combinação com outras terapias, apresentando resultados muito convincentes, apesar dos desafíos que ainda devem ser enfrentados.

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2. MANUSCRIPT:

A SCREENING OF EPIGENETIC THERAPEUTIC TARGETS FOR NON-SMALL CELL LUNG CANCER REVEALS PADI4 AND KDM6B AS PROMISING CANDIDATES

2.1. ABSTRACT

Despite advances in diagnostic and therapeutic approaches for lung cancer, new therapies targeting metastasis by the specific regulation of cancer genes are needed. In this study, we screened a small library of epigenetic inhibitors in non-small-cell lung cancer (NSCLC) cell lines and evaluated 38 epigenetic targets for their potential role in metastatic NSCLC. The potential candidates were ranked by a streamlined approach using in silico and in vitro experiments based on publicly available databases and evaluated by real-time qPCR target gene expression, cell viability and invasion assays, and transcriptomic analysis. The survival rate of patients with lung adenocarcinoma is inversely correlated with the gene expression of eight epigenetic targets, and a systematic review of the literature confirmed that four of them have already been identified as targets for the treatment of NSCLC. Using nontoxic doses of the remaining inhibitors, KDM6B and PADI4 were identified as potential targets affecting the invasion and migration of metastatic lung cancer cell lines. Transcriptomic analysis of *KDM6B* and *PADI4* treated cells showed altered expression of important genes related to the metastatic process. In conclusion, we showed that KDM6B and PADI4 are promising targets for inhibiting the metastasis of lung adenocarcinoma cancer cells.

Keywords: Lung cancer, NSCLC, epigenetic targets, metastasis, KDM6B, PADI4.

2.2. INTRODUCTION

Lung cancer is the second most diagnosed cancer worldwide and was responsible for 1,796,144 deaths in 2020, according to GLOBOCAN (SUNG et al., 2021). Non-small cell lung cancer (NSCLC) represents 85% of the cases, of which 80% are adenocarcinomas (AdCs), adenosquamous carcinomas, or squamous-cell carcinomas (SqCCs) (ANSARI; SHACKELFORD; EL-OSTA1, 2016a; DEVARAKONDA; MORGENSZTERN; GOVINDAN, 2015). Unfortunately, almost 50% of lung cancer cases are metastatic resulting in a poor prognosis and limited therapeutic options, with a critical five-year overall survival (OS) of only 10% and 1% in patients with stage IVA and IVB respectively (ANSARI; SHACKELFORD; EL-OSTA1, 2016a; HARDTSTOCK et al., 2020; NIU et al., 2016). The low survival rates are mainly attributed to chemoresistance, low detection rate of mutations in target genes, compromised choice of targeted therapy, and late diagnosis of lung cancer patients (ANSARI; SHACKELFORD; EL-OSTA1, 2016a; HILDEBRANDT; GU; WU, 2009; YUAN et al., 2019). Therefore, the identification of novel therapeutic targets and their inhibitors is urgent (BAJBOUJ et al., 2021).

Proteins that modify the epigenetic code are promising targets for the development of new anti-metastasis and anti-invasion drugs for NSCLC (BAJBOUJ et al., 2021). Histone posttranslational modifications (PTMs) represent epigenetic modifications that are frequently altered in cancer and contribute to tumor migration, metastasis, and aberrant cellular growth (AUDIA; CAMPBELL, 2016). Many histone deacetylase (HDAC) inhibitors (HDIs), such as vorinostat and panobinostat, have shown promising results in preclinical and clinical investigations of NSCLC (AUDIA; CAMPBELL, 2016) and new molecules for epigenetic targets are being developed and explored for their use in the treatment of diverse cancers (YU et al., 2021). However, there remains a need to validate these targets in large-scale clinical trials (BAJBOUJ et al., 2021).

The Structural Genomics Consortium (SGC) is an international public–private partnership with the goal of supporting research for a better understanding of human disease biology and to enable the discovery of new medicines (https://www.thesgc.org, accessed on 25 January 2019). To this end, SGC develops and makes available highly specific inhibitors (chemical probes) to the scientific community (ACKLOO; BROWN; MÜLLER, 2017; BROWN; MÜLLER, 2015; MÜLLER et al., 2018). Most available epigenetic probes are inhibitors of bromodomains (BRDs) and protein methyltransferases (PMTs). These molecules have been shown to be effective in several tumor models by inhibiting or attenuating several characteristics relevant to tumor development, such as metastatic capacity and resistance to conventional treatment (WU et al., 2019). However, epigenetic inhibitors used at adequate doses can inhibit tumorigenic phenotypes without being overtly cytotoxic to cells from healthy tissues (NERVI; DE MARINIS; CODACCI-PISANELLI, 2015).

Here, we performed a streamlined set of in silico and in vitro experiments to rank and validate epigenetic targets that regulate the metastatic process in lung cancer cells, using SGC chemical probes as specific inhibitors.

2.3. MATERIAL AND METHODS

A streamlined set of in silico analyses coupled with in vitro analyses (Figure 1) was performed to evaluate and rank potential epigenetic targets based on epigenetic probes from the SGC (https://www.thesgc.org, accessed on 25 January 2019). The screening was based on a list of available epigenetic inhibitors from SGC, followed by an analysis of the association of the epigenetic target with NSCLC survival, subsequent selection by a systematic review of potential new cancer targets, in silico analysis of protein expression in NSCLC cell lines, and real-time qPCR expression to evaluate target expression in the cell lines.

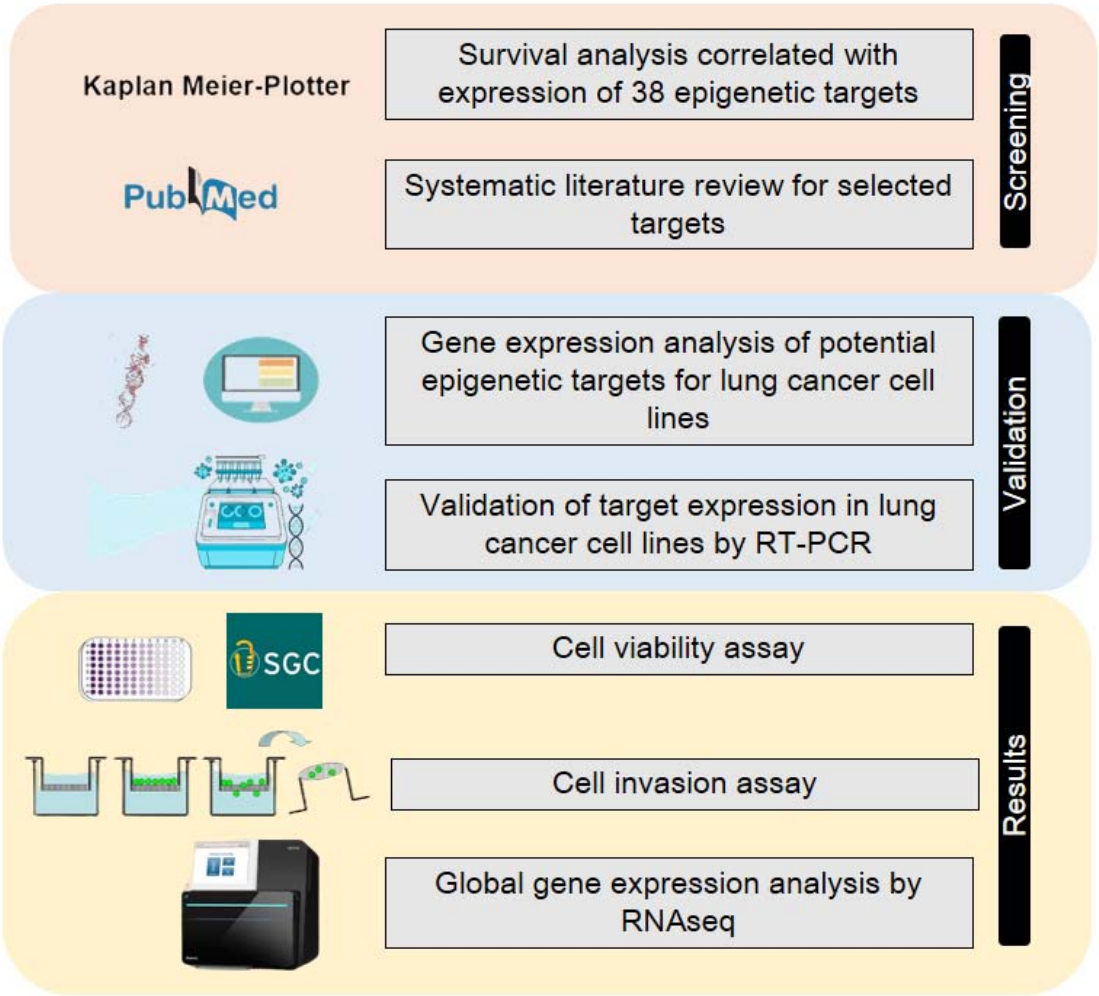


Figure 1**.** The streamlined approach using in silico and in vitro experiments. The initial screening was performed using publicly available data of lung cancer samples from TCGA using the KMplotter. Then, a systematic review of the significant candidates was performed to exclude the already described targets in the literature for lung cancer. The potential candidates were evaluated by gene expression in cancer cell lines, first, in silico and, then, experimentally in cancer cells. Lastly, experiments for cytotoxicity, an invasion assay and transcriptomic analyses were performed.

2.3.1. In silico analysis for determination of potential epigenetic targets in patientes with non-small cell lung câncer

The Kaplan-Meier Plotter software (GYORFFY et al., 2013) (http://kmplot.com/analysis, accessed on 4 February 2019) was powered with public

data from 14 repositories with information on gene expression and clinical samples, totaling 2438 cases of NSCLC. Thirty-eight epigenetic targets (Table S1) were individually analyzed for their association with the survival rates of 590 patients diagnosed with adenocarcinoma and 492 patients with squamous cell carcinoma (Table 1). The patient selection criteria considered the histological types, grouping them into adenocarcinoma and squamous cell carcinoma, patients in stages I, II, III, and IV of the disease, of both sexes, smokers and non-smokers, and if patients had started any type of treatment, such as surgery, chemotherapy, and radiotherapy. The follow-up time for each patient was evaluated from the time of diagnosis to the time of death. For all analyses, the results ($p < 0.05$) were considered, according to the log-rank test (chi-square), to compare whether there was a statistical difference between the curves of high and low gene expression and the use of the hazard ratio (HR) with a 95% confidence interval. A hazard ratio equal to one means no association between treatments, a rate greater than one suggests an increase in risk and below one suggests a decrease in risk.

For the selection of the cases, the following inclusion criteria were applied: use of cases that presented patient survival; quality control of array chips excluding chips with outliers (>95% of total arrays) from analysis for any of the following parameters: percentage of calls present, background, rawQ, bioB-/C-/D-spikes, *GAPDH* (Glyceraldehyde-3-Phosphate Dehydrogenase) and *ACTB* (Actin Beta) 3 ratio for 5. As recommended by the authors, the Jetset Best probe set was always used to analyze the expression of genes of interest, and a high and low expression group based on quartiles (25%, Q1 × Q4) was created for survival analysis. Analyses were performed independently for adenocarcinoma and squamous cell carcinoma cases. Statistical analysis was performed using univariate Cox regression, generating *p*-values and hazard ratios.

Table 1. Clinical data from 590 patients with adenocarcinoma evaluated for expression of epigenetic targets with the Kaplan-Meier Plotter.

Clinical Data	***n***	**Freq. (%)**
Histology		
Adenocarcinoma	590	100%
Stage		
1	277	(46.95%)
2	115	(19.49 %)
3	16	(2.71 %)
4	4	(0.68%)
Staging		
T1	123	(20.85%)
T2	103	(17.46%)
T3	4	(0.68%)
T4	0	0%
N0	184	(31.19%)
N1	42	(7.12%)
N2	3	(0.51%)
M0	229	(38.81%)
M1	1	(0.17%)
Gender		
Women	247	(41.86%)
Men	289	(48.98%)
Smoking history		
Exclude those never smoked	180	(30.51%)
Only those never smoked	92	(15.59%)
Surgery success		
Only surgical margins negative	127	(21.53%)
Chemotherapy		
Yes	14	(2.37%)
No	8	(1.36%)

2.3.2. Systematic literature review for selection of new epigenetics targets for NSCLC

An independent systematic literature review was performed for each epigenetic target in the PubMed database (https://www.ncbi.nlm.nih.gov/pubmed, accessed on 7 February 2019). For literature selection, a specific set of keywords was used, presenting the abbreviation of the name of the epigenetic target and the term “lung cancer,” as being mandatory in the titles in order to find studies that specifically

evaluated epigenetic targets and lung cancer. The analysis was performed on publications published from 2000 to 2019 (Appendix 1).

2.3.3. In silico analysis of epigenetic target expression

The CellExpress software (LEE et al., 2018b) (http://cellexpress.cgm.ntu.edu.tw, accessed on 11 March 2019) was used to perform gene expression analysis on more than 4000 tumor cell lines and clinical samples obtained from public datasets. For expression analysis, the databases of gene expression studies of cell lines NCI-60 Human Tumor Cell Lines Screen (GSE32474), Cancer Cell Line Encyclopedia-CCLE (GSE36133), and Sanger Cell Line Project (GSE68950) were used. Microarray data obtained on the same platform were normalized using a quartile normalization algorithm to remove systematic biases. Expression data from the GSE36133 study from the CCLE database were used, which presented a more complete list of cell lines of interest, using the selection of the Jetset Best probe and the expression of the four potential epigenetic targets in lung adenocarcinoma cell lines. The probes selected to assess gene expression levels in cells were from the Jetset Best probe set, being (41386_at) for *KDM6B* (212512_at) for *CARM1*, (201353_at) for *BAZ2A* and (220001_at) for *PADI4*, which were also used in the Kaplan-Meier survival analysis. The endogenous gene, *GAPDH,* was used to normalize the expression levels of the genes of interest. The results were generated by calculating the relative expression, which showed similar gene expression values between the analyzed cells. Evaluation of the expression of targets in healthy lung tissue was performed using the CellNavigator software (https://medicalgenomics.org/rna_seq_atlas, accessed on 15 March 2019) through microarray analysis coupled to the RNA-seq Atlas platform through the Human Genome Set U133 (HG-U133). Background correction, normalization and summarization was performed by applying the frma function from the fRMA package to AffyBatch with default options. The Z-Score transformation was calculated using the barcode function of the fRMA package to standardize gene values from the Microarray data. The barcode options were set for the corresponding platform and the output method was set to ‘z-score’. Then, the Z-Scores were averaged for each tissue and each pathological state (healthy, cancer), Z-Score > 5 suggests that the gene is

expressed in that tissue. Finally, the Z-Score was averaged for each tissue and state (healthy, cancer) and stored in the PostgreSQL database.

2.3.4. Cell culture

The lung cancer cell lines A549, H1568, and H2126 were donated by Dr. Lucy M. Anderson from the Laboratory of Comparative Carcinogenesis at the Frederick National Laboratory for Cancer Research, Frederick, Maryland, United States of America and maintained as previously described (FUKUMASU et al., 2014). Briefly, cell lines were maintained in 75 cm^2 flasks at 37 °C and 5% CO2 in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic (Pen-Strep). Cell passaging was performed when the cells were 85% confluent using TrypLE™ Express trypsin. Culture evolution was evaluated daily using optical microscopy (Axio Vert A1, Zeiss, Jena, Germany). All the reagents used for cell culture were purchased from Thermo Fisher Scientific (USA). All cell lines were authenticated at the Laboratory of Molecular Diagnosis of the Cancer Hospital of Barretos (Hospital de Amor HA) as previously reported (BOURNE, 2010) before the experiments and were free for Mycoplasma spp. by real-time PCR (Myco-Sniff-Valid™ Mycoplasma PCR Detection Kit).

2.3.5. RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from A549, H1568, and H2126 cell lines using TRIzol (Invitrogen; Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's protocol. cDNA was synthesized from total RNA (1000 ng) using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the following parameters: 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min. qPCR was performed using the SYBR Master Mix (Roche Diagnostics, Basel, Switzerland). Gene expression analyses were performed by real-time PCR using the StepOne System (Thermo Fisher Scientific). Specific primers were designed using Primer-BLAST (YE et al., 2012) and dimers and hairpins were verified using AutoDimer software (VALLONE; BUTLER, 2004). Primers were

also analyzed using in silico polymerase chain reaction (PCR) (https://genome.ucsc.edu/cgi-bin/hgPcr, accessed on 7 March 2019) to confirm specificity. The primer sequences are listed in (Table S2). PCR was carried out using Fast SYBR Green Master Mix in a final volume of 10 µL. The conditions for quantitative PCR were as follows: 95 °C for 20 s; 40 cycles at 95 °C for 3 s for denaturation and 60 °C for 30 s for anneal/extension; melt curve analysis was performed at 95 °C for 15 s and 60 °C for 60 s. The housekeeping gene used was *18 s* ribosomal RNA, and the analysis of relative gene expression data was performed according to the ΔΔCt method (LIVAK; SCHMITTGEN, 2001). The experiments were performed twice and in triplicate. All reagents were purchased from Thermo Fisher Scientific. The experiment was performed twice and in triplicate.

2.3.6. Epigenetic probes cell viability assay

The epigenetic probes (Cayman Chemical, Ann Arbor, Michigan, USA) were dissolved in dimethylsulfoxide (DMSO) to a concentration of 20 mM. A549 cells were seeded at 5000/well in 96-well plates (Corning, New York, USA) containing 100 µL of supplemented media, as described previously. After 24h, the medium was replaced with fresh culture medium containing different concentrations of epigenetic probes, ranging from 10 µM to 13.72 nM. Epigenetic probes were added in six replicates per concentration and the experiments were performed in triplicate. After 72h, 10 µL of 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT, 5 mg/mL) was added to each well and formazan crystals were produced over a 2h incubation period. One hundred microliters of DMSO were added to dissolve the crystals. The optical density at 540 nm was measured using Fluorstar Optima (BMG Labtech, Ortenberg, Germany). The concentration of the compound corresponding to the IC_{50} was calculated using a nonlinear regression test performed in GraphPad Prism (version 6.00 for Windows, GraphPad Software, USA).

2.3.7. Invasion assay

The cells were treated with DMSO (control) or 1000 nM of TP-064, GSK2801, GSK-J4, and GSK484 for 72h. Cells were cultured for 24 h in serum-free medium.

Transwell inserts were placed in 24 well plates and filled with 100 µL of ECM gel (Sigma Aldrich Saint Louis, MO, USA) in RPMI-40 medium (1:5). After, 2 × 10^4 A549 and 2.5 × 10^4 H1568 cells were resuspended in 100 µL serum-free medium and plated on inserts. The bottom well was filled with 600 µL of RPMI-40 medium supplemented with 20% fetal bovine serum (FBS), used as a chemoattractant, and after 48 h, a cotton swab was used to remove non-invasive cells from the top of the inserts. As a fixative, 5% glutaraldehyde was used for 10 min at room temperature and inserts were stained with 1% crystal violet in 2% ethanol for 20 min. The invasive cells were observed and photographed under an optical microscope in five random fields at 100× magnification using the ZEISS ZEN 2 Microscope Software (ZEISS, Germany). Finally, the invasive cells were counted using ImageJ software version 1.8.0_112 (BOURNE, 2010). The experiment was performed thrice in duplicate.

2.3.8. RNA-seq data generation

To assess the genes affected by treatment with GSK 484 (PADI4) and GSK-J4 (KDM6A/B), A549 cells were treated with 1000 nM of these inhibitors. Duplicates of each treatment and control group were prepared. A549 cells were treated with 1000 nM GSK-J4, GSK484, and DMSO (control) for 72h and then RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). RNA quality and quantity were assessed using automated capillary gel electrophoresis on a Bioanalyzer 2100 with RNA 6000 Nano Labchips, according to the manufacturer's instructions (Agilent Technologies, County Cork, Ireland). Only samples that presented an RNA integrity number (RIN) higher than 8.0 were considered for sequencing. RNA libraries were constructed using the TruSeq™ Stranded mRNA LT Sample Prep Protocol and sequenced on an Illumina HiSeq platform. 2500 equipment in HiSeq Flow Cell v4 using a HiSeq SBS Kit v4 (2 × 100 bp).

2.3.9. Alignment and differential expression.

Sequencing quality was evaluated using FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc, accessed on 3 March 2022), and no additional filtering was performed. Sequence alignment against the

human reference genome (GRHC38) was performed using STAR (DOBIN et al., 2013), according to standard parameters and including the annotation file (Ensembl release 89). Secondary alignments, duplicated reads, and reads failing vendor quality checks were removed using Samtools (LI et al., 2009). The alignment quality was confirmed using Qualimap (GARCÍA-ALCALDE et al., 2012). Gene expression was estimated by read counts using HTseq (ANDERS; PYL; HUBER, 2015) and normalized to counts per million reads (CPM). Only genes presenting at least one CPM in at least four samples were retained for differential expression (DE) analysis. DE was performed using the EdgeR package (ROBINSON; MCCARTHY; SMYTH, 2010) in the R environment based on a negative binomial distribution. The Benjamini-Hochberg procedure was used to control the false discovery rate (FDR), and transcripts with FDR ≤ 0.05, and log-fold change (LogFC) > 1; <−1 were considered differentially expressed (DE). Functional enrichment analysis of the DE genes was performed using STRING (SZKLARCZYK et al., 2019).

2.3.10. Statistical Analysis

The IC_{50} was calculated using a nonlinear regression test. Gene expression was analyzed by one-way ANOVA with Tukey's post-hoc test. One-way ANOVA followed by Student's t-test was used for invasion assays. For functional enrichment analyses, *p*-values were adjusted for multiple tests, and the Benjamin and Hochberg method was used to test multiple categories in a group of functional gene sets. Differences were considered statistically significant at $p < 0.05$.

2.4. RESULTS

2.4.1. Evaluation of epigenetic targets in non-small cell lung cancers using publicly available data

All 38 epigenetic targets were analyzed and ranked based on the significance of inverse association between survival of patients with NSCLC and gene expression.

According to the selection criteria, from 1082 patients, 590 and 492 patients were selected for adenocarcinoma and squamous cell carcinoma, respectively.

Of the 38 epigenetic targets, eight were inversely associated with low survival of lung adenocarcinoma patients (Hazard Ratio (HR) > 1, $p < 0.05$, Table 2), and none were associated with patients with squamous cell carcinoma ($n = 492$).

Table 2. The eight significant potential epigenetic targets selected by inverse association with survival rates of patients diagnosed with pulmonary adenocarcinoma. The survival medians of the low and high expression groups of the targets were also analyzed.

Epigenetic Targets	**Enzyme Class**	***p* Value**	**Hazard Ratio (HR)**	**Low Expression Survival (Median in Months)**	**High Expression Survival (Median in Months)**
PRMT1	Methyltransferase	5.8×10^{-9}	(HR = 3.22; IC = 95%; 2.12–4.88)	75	21
KDM6B	Demethylase	6.3×10^{-9}	(HR = 2.81; IC = 95%; 1.95–4.03)	150	34
CARM1	Methyltransferase	9.2×10^{-8}	(HR = 2.73; IC = 95%; 1.86–4.00)	48	18
BAZ2A	Bromodomain	7.8×10^{-6}	(HR = 2.23; IC = 95%; 1.56–3.20)	175	52
BRD4	Bromodomain	0.0025	(HR = 1.81; IC = 95%; 1.23–2.68)	117	69
EZH2	Methyltransferase	0.024	(HR = 1.50; IC = 95%; 1.05–2.13)	126	70
PADI4	Deiminase	0.025	(HR = 1.47; IC = 95%; 1.05–2.06)	107	80
BRD9	Bromodomain	0.033	(HR = 1. 45; IC = 95%; 1.03–2.05)	103	52

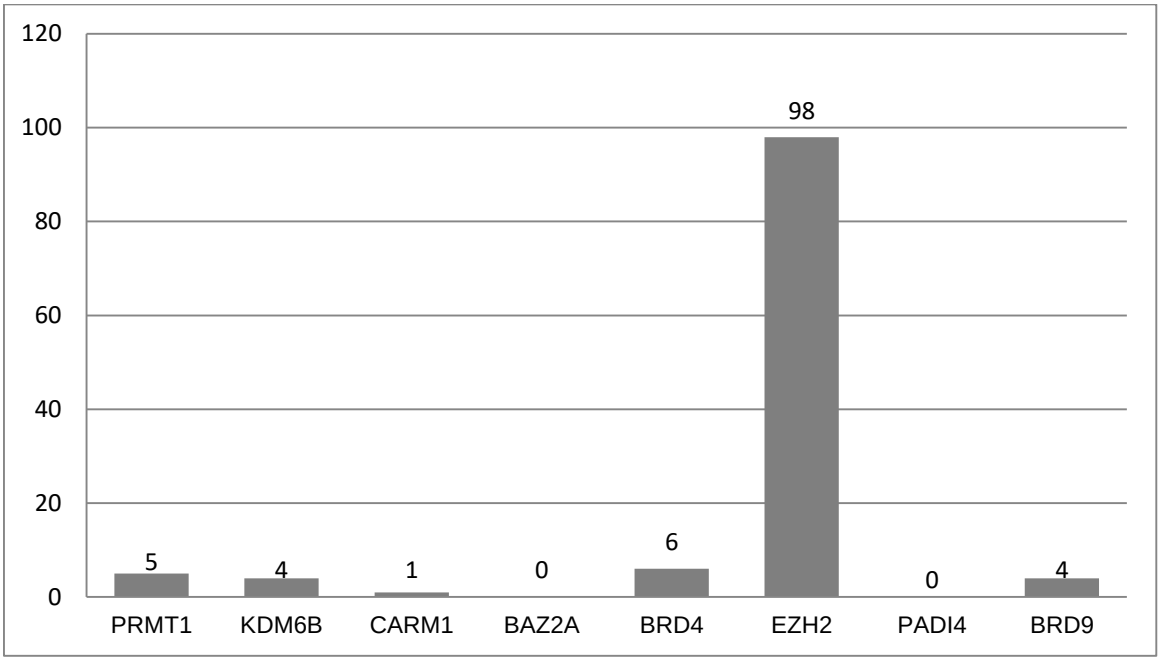
Source: Lesbon, J. C. C. (2022).

2.4.2. Systematic literature review analysis of potential epigenetic targets

The systematic review initially resulted in 98 publications related to the epigenetic target Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit (EZH2), 6 related to Bromodomain Containing 4 (BRD4), 5 related to Protein Arginine Methyltransferase 1 (PRMT1), 4 related to each Lysine Demethylase 6B (KDM6B) and Bromodomain Containing 9 (BRD9) targets; one related to Coactivator Associated Arginine Methyltransferase 1 (CARM1), and no work related to Bromodomain Adjacent To Zinc Finger Domain 2 (BAZ2A) or Peptidyl Arginine Deiminase 4 (PADI4) as targets in lung cancer (Figure 2). Specific targets, such as PRMT1, KDM6B, CARM1, BRD4, and EZH2, have been shown to be associated with malignant phenotypes of lung cancer, influencing cell proliferation and metastatic processes (regulation of epithelial–mesenchymal transition and cell invasion). Lysine Demethylase 6A (KDM6A) expression was not correlated with poor survival in lung cancer patients.

Thus, we selected the targets that presented the highest risk rate (HZ) and the lowest number of publications in the literature (<5), with the aim of studying potential new epigenetic targets for lung cancer. Therefore, the epigenetic targets selected for further analysis were KDM6B, CARM1, BAZ2A and PADI4

Figure 2. Systematic literature review of the 8 potential epigenetic targets presenting the number of publications in the PubMed database in the 2000-2019 range related to specific targets and lung cancer.

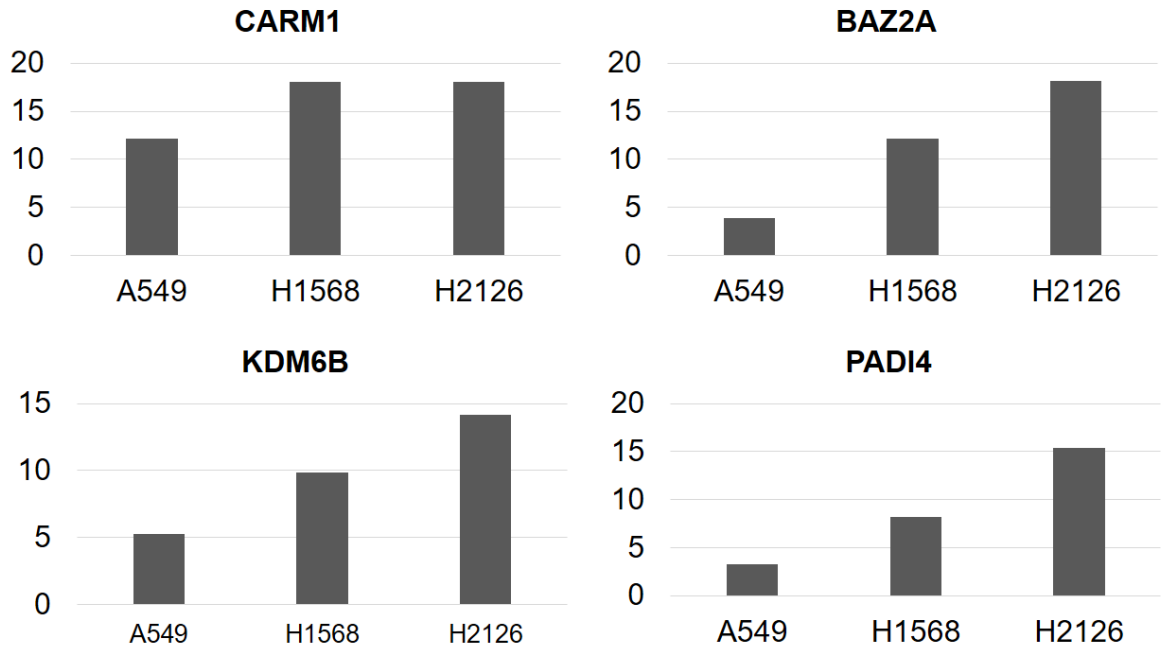


Source: Lesbon, J. C. C. (2022).

2.4.3. In silico analysis of expression of the 4 target genes in lung cancer cells

Through the analysis of gene expression *in silico*, three cell lines showed elevated expression of the chosen potential targets (A549, H2126 and H1568). The cell lines (H2126 and H1568) were collected from metastatic sites, pleural effusion, and lymph nodes, whereas A549 cells were collected from the primary tumors (Figure 3). However, the H2126 cell line does not have the potential for in vitro invasion [18]. The expression of the epigenetic targets in healthy lung tissue showed the following results: KDM6B (Z-score = 2.8), CARM1 (Z-score = −0.5), BAZ2A (Z-score = −1.0) and PADI4 (Z-score = 0.58), Z-score < 5, suggesting non-expression in healthy lung tissue.

Figure 3. In silico gene expression analysis of 4 epigenetic targets in pulmonary adenocarcinoma cell lines. Graphs are presented as the mean of normalized expression values.

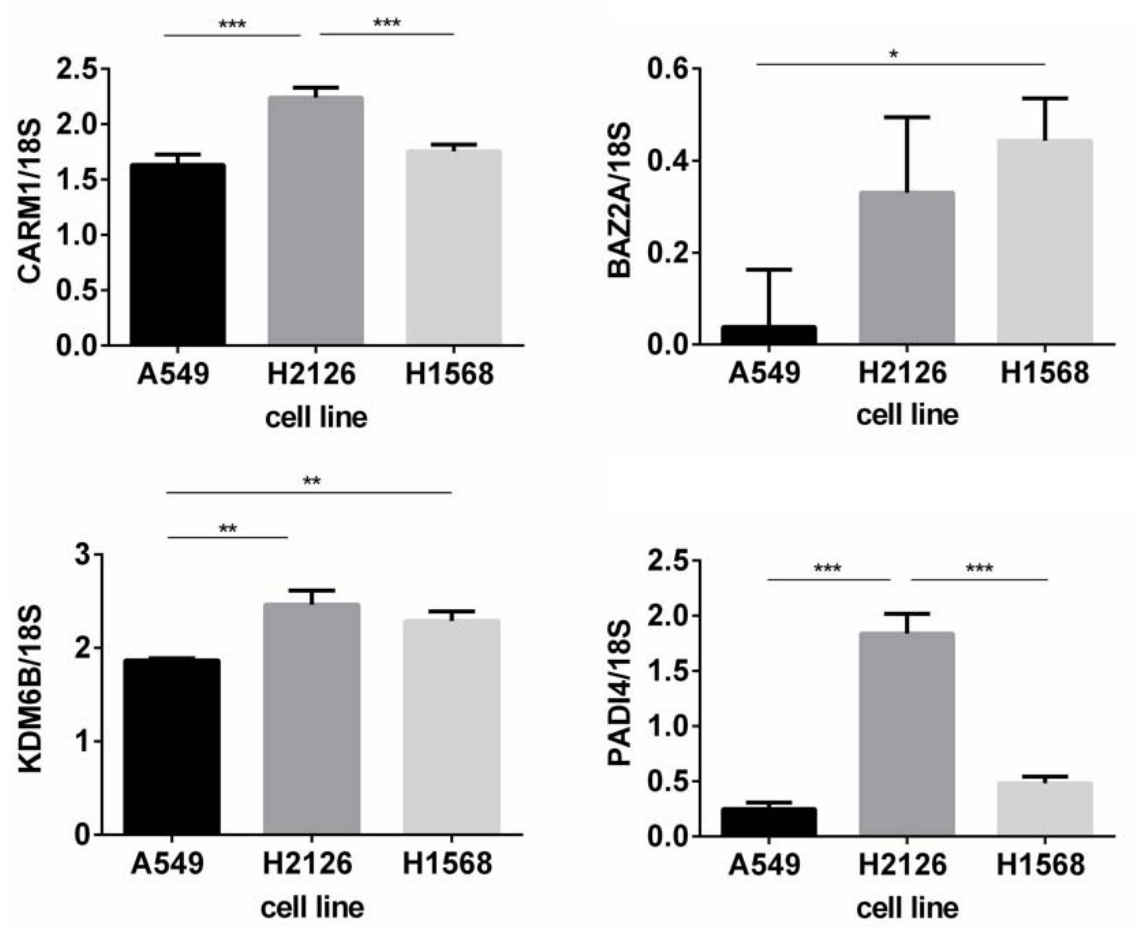


Source: Lesbon, J. C. C. (2022).

2.4.4. Epigenetic targets gene expression by real-time PCR

The expression of *CARM1*, *BAZ2A*, *KDM6B* and *PADI4* was evaluated in the cell lines H2126, H1568 and A549. All three cell lines, A549, H2126, and H1568, showed a higher level of target expression in general (Figure 4).

Figure 4. Epigenetic target gene expression (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ - One way ANOVA followed by multiple comparisons test from Tukey).

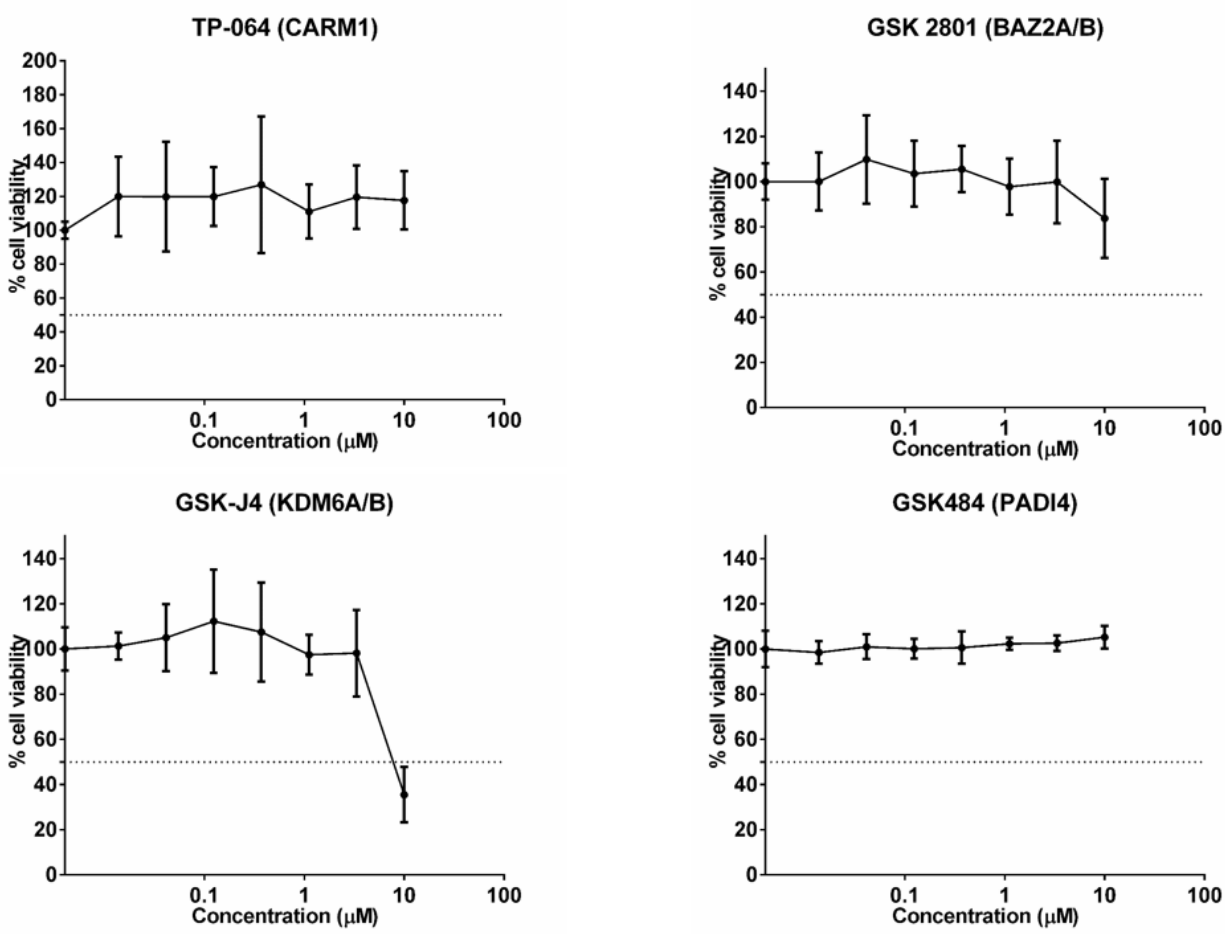


Source: Lesbon, J. C. C. (2022).

2.4.5. Cytotoxic potential (IC50) and selection of the maximum concentration without cytotoxic effect of epigenetic molecules

Cytotoxic potential of the four epigenetic inhibitors, TP-064 (CARM1), GSK2801 (BAZ2A/B), GSK-J4 (KDM6A/B), and GSK484 (PADI4) in A549 cells was assessed GSK-J4 (KDM6A/B) had an IC_{50} value of 8.21 µM, whereas the other probes were not cytotoxic, even at 10 µM. Therefore, the inhibitors showed low cytotoxic potential, even when using very high doses not recommended for use, demonstrating high safety. However, we selected a concentration of 1000nM for further experiments (Figure 5).

Figure 5. Cytotoxic potential of the 4 specific epigenetic molecules in the A549 cell line. The GSK-J4 (KDM6A/B) molecule had an IC_{50} value of 8.21 µM, the others had a value greater than 10 µm.



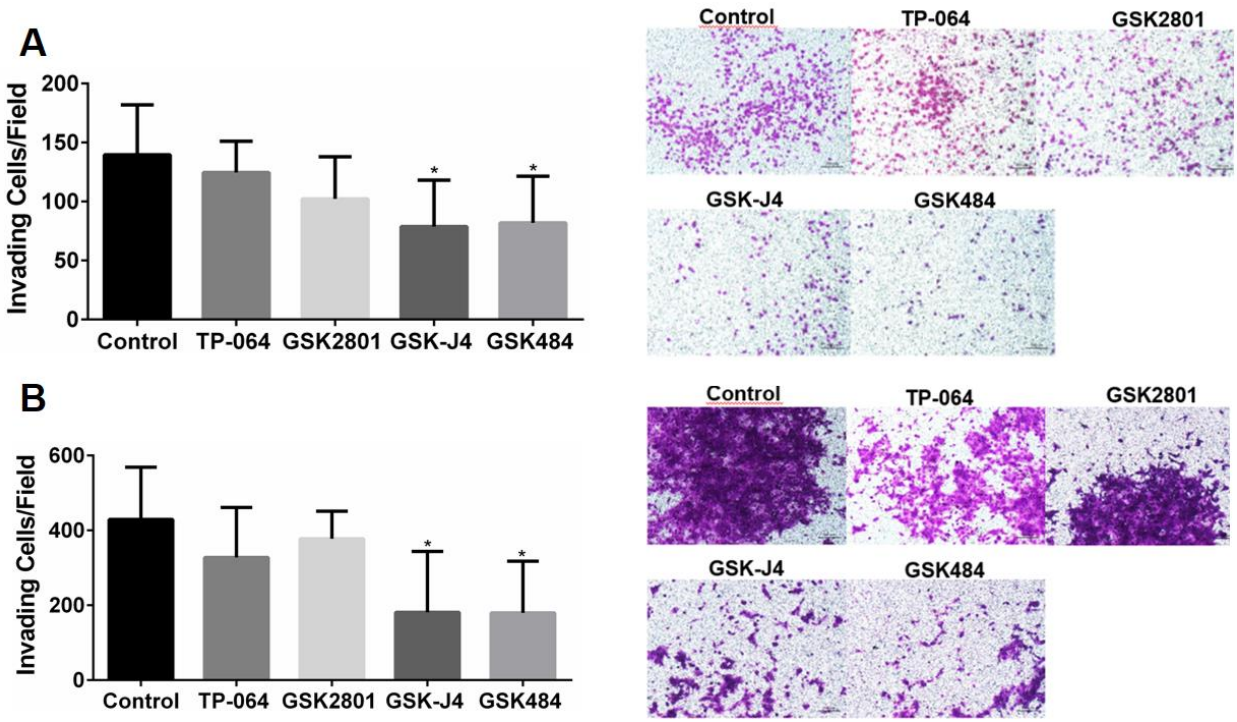
Source: Lesbon, J. C. C. (2022).

2.4.6. Epigenetic inhibitors effects on cancer cell migration and invasiveness

Inhibition of the epigenetic targets KDM6A/B and PADI4 reduced cell invasiveness compared to the control group ($p < 0.05$) in the A549 and H1568 cell lines (Figure 6).

Figure 6. (A) Analysis of cell invasiveness after treatment with epigenetic molecules in the A549 cell line. (B) Analysis of cell invasiveness after treatment with epigenetic

molecules in the H1568 cell line. Transwell invasion test evaluated after 48 h (* $p < 0.05$, One-way ANOVA followed by Student's t-tests).



Source: Lesbon, J. C. C. (2022).

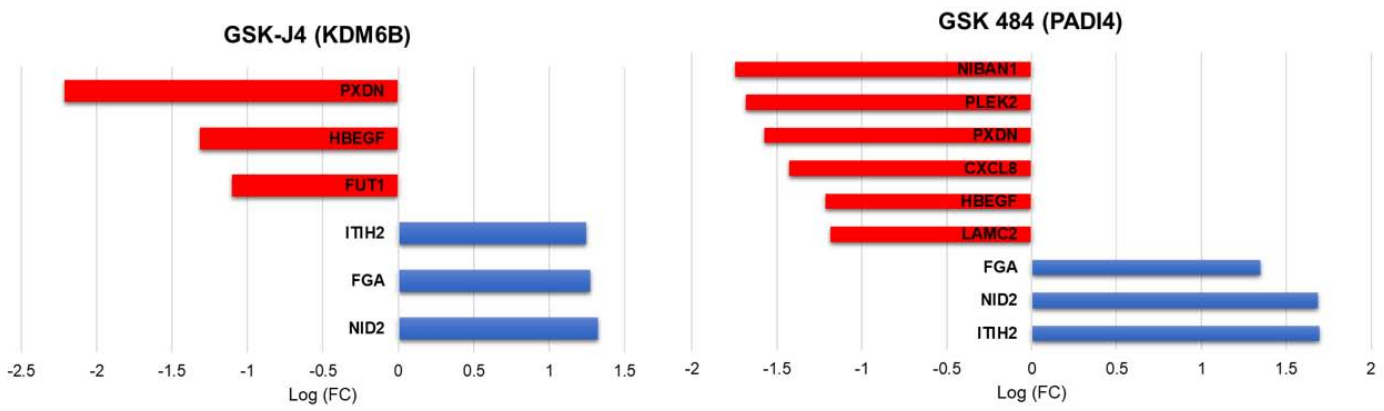
2.4.7. Global gene expression change by treatment with inhibitor probes for KDM6A/B and PADI4

Treatment with the PADI4 inhibitor GSK484 led to 152 differentially expressed genes, of which 62 genes were downregulated and 90 were upregulated in A549 cancer cells, whereas the KDM6A/B inhibitor, GSK-J4, altered the expression of 190 genes, of which 56 genes were downregulated and 134 were upregulated in A549 cancer cells (FDR < 0.05 and LogFC > 1; <−1) (Table S3). Functional enrichment analysis showed that treatment with PADI4 and KDM6A/B inhibitors was associated with processes linked to the collagen-containing extracellular matrix, extracellular matrix, extracellular space, cell periphery-related genes, and processes related to metastasis.

In cells treated with the PADI4 inhibitor, we found nine genes differentially regulated and six genes in cells treated with the KDM6A/B inhibitor, of which the

following five genes were common among the treatments: the genes for Fibrinogen Alpha Chain (*FGA)*, Nidogen 2 (*NID2)*, Inter-Alpha-Trypsin Inhibitor Heavy Chain 2 (*ITIH2)*, Peroxidasin (*PXDN)* and Heparin Binding EGF Like Growth Factor (*HBEGF)*. All of the five genes common among the treatments are related to adhesion proteins, cell ligands, and protein stabilizing proteins of the extracellular matrix, suggesting that these genes participate in the regulation of metastasis (Figure 7).

Figure 7. Representation of log-fold change value of EMT-related gene regulation after treatment with PADI4 and KDM6B inhibitors.



Source: Lesbon, J. C. C. (2022).

2.5. DISCUSSION

Metastasis, one of the biggest problems of solid epithelial cancers, begins with the migration of tumor cells from the confined primary tumor to adjacent tissue, where tumor cells cross the basement membrane and lamina propria to invade the underlying connective tissue. Unlike normal epithelial cells, which undergo apoptosis when they lose contact with their native extracellular matrix, tumor cells develop mechanisms to detach from the primary tumor associated with epithelial organization, closely followed by the expression of mesenchymal markers (PACHMAYR; TREESE; STEIN, 2017). These changes are the result of altered gene expression, which can be driven by epigenetic processes, thereby opening the possibility of affecting these changes by epigenetic regulation. Here, we performed a streamlined approach with in silico and in

vitro analyses starting from 38 epigenetic targets to select the most relevant for lung cancer cell treatment and showed that the inhibition of PADI4 and KDM6B proteins controls the metastatic process, inhibiting cancer cell migration and invasion by altering their transcriptomes.

Protein-arginine deiminase Type-4 (PADI4) is a calcium-dependent enzyme that is known for its role in converting arginine to citrulline residues. Its downstream signaling has been studied in the progression of a variety of human cancers, but there is a lack of studies showing the efficacy of PADI4 in lung cancer (LIU et al., 2019; ZHAI et al., 2017). Recently, Liu et al. (2019) demonstrated that PADI4 is overexpressed in lung cancer and contributes to cell growth and metastasis. Knockdown of PADI4 in A549 lung cancer cells resulted in a striking reduction in the EMT-associated Snail Family Transcriptional Repressor 1 (Snail1/mothers) against the decapentaplegic homolog ¾ transcriptional complex, which was consistent with alterations in migratory and invasive phenotypes of A549 lung cancer cells. On the other hand, the lysine demethylase 6B (KDM6B) is a histone demethylase that removes methyl groups from lysine and arginine residues on histone tails. It is a member of the Fe(II)- and α-ketoglutarate-dependent demethylases that activates gene expression by removing H3K27me3 marks on gene promoters (RAMADOSS; CHEN; WANG, 2012). KDM6B has been shown to be involved in tumor progression via the regulation of cell proliferation, migration, and senescence (XUN et al., 2021). High levels of KDM6B induce the expression of mesenchymal genes, such as *Snail* and *Slug* (Snail Family Transcriptional Repressor 2), which promote *TGF-β*-induced (Transforming Growth Factor Beta 1) EMT and tumor metastasis (SH et al., 2021). Knockdown of KDM6B inhibited EMT induced by *TGF- β*, inhibiting breast cancer cell invasion (RAMADOSS; CHEN; WANG, 2012). Another study provided evidence of pulmonary metastasis of osteosarcoma in an in vivo model in which osteosarcoma cells were injected into the medullary cavity of nude mice. Intraperitoneal administration of GSK-J4 at concentrations above 5 mg/kg significantly inhibited the pulmonary metastasis of osteosarcoma cells in vivo. These results strongly suggest the potential of KDM6B as a target for highly metastatic osteosarcoma (Y et al., 2021). Thus, KDM6B may present a target for cancer metastasis. One point to consider is that GSK-J4 could also inhibit lysine demethylase 5B (KDM5B) histone demethylase and not only KDM6A/B. KDM5B has been implicated in several cancers, including NSCLC, and was recently described

as a therapeutic target for cancer therapy (JOSE et al., 2020). However, GSK-J4 is more selectively potent for KDM6B than for KDM5B.

Interestingly, the treatment of cancer cells with non-cytotoxic doses of PADI4 and KDM6B inhibitors induced similar transcriptomic profiles, regulating genes related to cell adhesion and the extracellular matrix, which was associated with decreased capacity of cancer cells to invade and migrate in the in vitro model. For both inhibitors, upregulation of *FGA*, *NID2* and *ITIH2* genes, and downregulation of *PXDN* and *HBEGF*, was observed. Fibrinogen is an extracellular matrix protein composed of three polypeptide chains, fibrinogen alpha (FGA), beta (FGB), and gamma (FGG), and is involved in tumor angiogenesis and metastasis. *FGA* may play a suppressive role by inhibiting tumor growth and metastasis. FGA administration is considered a novel therapeutic approach to inhibit the growth and metastasis of lung adenocarcinoma (WANG et al., 2020). Nidogen-2 (NID2) is ubiquitously present in the basement membrane and maintains its integrity and stability of the basement membrane by connecting laminin and collagen IV networks in the extracellular matrix (ECM). The restoration of *NID2* expression in cancer cells was shown to have a negative regulatory role in Epidermal Growth Factor Receptor (*EGFR*) and integrin signaling pathways, suggesting that *NID2* elicits in vitro migration/invasion suppression and in vivo metastasis inhibition effects through negative modulation of these two oncogenic pathways (CHAI et al., 2016). The other gene upregulated by both inhibitors was *ITIH2*, the inter-alpha-trypsin inhibitor 2, belonging to a family of plasma protease inhibitors, contributing to the stability of the extracellular matrix by covalently binding to hyaluronan. Loss or downregulation of *ITIH2* expression was observed in 70%, 71%, and 70% of breast, lung, and kidney tumors, respectively. In addition, careful densitometric evaluation of hybridization signals revealed downregulation in 56% of gastric cancers, 61% of rectal carcinomas, and 50% of prostate cancers (HAMM et al., 2008).

Epigenetic inhibitors downregulated two genes in common: *PXDN* and *HB-EGF*. Peroxidasin (PXDN) is an extracellular matrix protein with peroxidase activity and has been reported to participate in epithelial mesenchymal transition processes, playing a promoting role in the proliferation, invasion, and migration of ovarian cancer cells through the regulation of *PI3K* (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase

Catalytic) pathway activation *Pl3k/Akt* (AKT Serine/Threonine Kinase), and is considered a potential target for therapy (ZHENG; LIANG, 2018). Heparin-bound epidermal growth factor-like growth factor (HB-EGF) is a member of the heparin-bound EGF family (Epidermal Growth Factor) and is more widely expressed in tumors than in normal tissues. HB-EGF can be produced in a membrane-anchored form (pro-HB-EGF) and further processed into a soluble form (s-HB-EGF), although a significant amount of pro-HB-EGF remains cleaved on the surface of the cell. In addition, wild-type s-HB-EGF or HB-EGF induced the expression and activity of the metalloproteases MMP-9 (Matrix Metallopeptidase 9) and MMP-3 (Matrix Metallopeptidase 3), leading to increased cell migration (ONGUSAHA et al., 2004).

PADI4 inhibitor treatment in cancer cells downregulated four genes related to metastatic cancer phenotypes: Laminin Subunit Gamma 2 (LAMC2), C-X-C Motif Chemokine Ligand 8 (CXCL8), Niban Apoptosis Regulator 1 (FAM129A), and Pleckstrin 2 (PLEK2). Laminin Subunit Gamma 2 (LAMC2) is a subunit of the heterotrimeric glycoprotein laminin-332 (LAM-332, formerly laminin-5) consisting of α3, β3, and γ2 chains. Although LAMC2 is an important structural component of the epithelial basement membrane (BM) in various normal tissues, there is emerging evidence of a pathological role for the LAMC2 monomer in cancer (MOON et al., 2015). *LAMC2* promotes migration and invasion via EMT, which is dependent on TGF-β1 and ZEB1 (Zinc Finger E-Box Binding Homeobox 1) integrin (MOON et al., 2015). CXCL8, also known as interleukin-8 (IL-8), is a prototypic chemokine belonging to the CXC family and is responsible for the recruitment and activation of neutrophils and granulocytes to the site of inflammation (XIONG et al., 2022). Recent studies have shown that CXCL8 is essential for tumor cells to acquire and maintain this aggressive phenotype. A member of the family with sequence similarity 129, member A (FAM129A), inhibited apoptosis and promoted migration and proliferation in human cancers. One study revealed that *FAM129A* promoted tumor invasion and proliferation by upregulating the expression of MMP2 (Matrix Metallopeptidase 2) and cyclin D1, which was due to increased FAK (Protein Tyrosine Kinase 2) phosphorylation at Tyr 397 and Tyr 576. Overexpression of *FAM129A* was associated with tumor progression and predicted low survival of NSCLC patients (ZHANG et al., 2019). Pleckstrin2 (PLEK2) is a 353 amino acid protein that is widely expressed in a variety of tissues and is highly expressed in NSCLC. *PLEK2* promotes NSCLC proliferation and metastasis

via a BRD4-dependent *PI3K/AKT* signaling pathway that functions as an epigenetic reader and binds to acetylated lysine residues (KAc) that regulate chromatin structure and gene expression (CAI et al., 2022).

Treatment of cancer cells with a KDM6B inhibitor downregulated *FUT1* (Fucosyltransferase 1) gene, which is also related to metastasis. Fucosylation is a posttranslational modification that links fucose residues with protein- or lipid-linked oligosaccharides. Certain genes in the fucosylation pathway are aberrantly expressed in several types of cancer, including non-small cell lung cancer, and this aberrant expression is associated with poor prognosis in cancer patients. Fucosylation pathway genes, including fucosyltransferase 1/2/3/6/8 (*FUT1*, *FUT2*, *FUT3*, *FUT6*, *FUT8*) and GDP-L-fucose synthase (*TSTA3*), were correlated with poor patient survival in these patients. In this study, the inhibition of *FUT*s by 2F-peracetyl-fucose (2F-PAF) suppressed transforming growth *factor β* (*TGFβ*)-mediated *Smad3* (SMAD Family Member 3) phosphorylation and nuclear translocation in NSCLC cells. Furthermore, transwell wound healing and migration assays demonstrated that 2F-PAF inhibited the TGFβ-induced migration and invasion of NSCLC cells (PARK et al., 2020).

Our work emphasized the inhibition of important epigenetic targets related to the process of migration and invasion of tumor cells that favor cancer metastasis. Thus, these inhibitors have great potential to add to antitumor therapy, and can be added to other drugs already in clinical use, such as chemotherapy, immunotherapy and targeted therapy, contributing to the increase of antitumor effects, overcoming resistance to drugs already used and activation of the host's immune response. Indeed, chemotherapy is still a traditional method in advanced cases, in which surgical excision is not possible, so the emergence of chemoresistance remains a major problem in cancer therapy. Thus, the combination of epigenetic drugs with other chemotherapeutics can not only promote a potent suppression of tumorigenesis, but also resensitize tumor cells to radiotherapy and chemotherapy (LU et al., 2020b). Immunotherapy has been used as a promising candidate for both first- and second-line treatment in metastatic NSCLC. However, about 50% of NSCLC expressed PD-L1. There is no consensus predictive biomarker and resistance to immunotherapy can occur (BODOR; BOUMBER; BORGHAEI, 2020; DE MARCHI et al., 2021, 2022). This fact limits the use of immunotherapy and overcoming immunotherapy resistance can

be challenging due to the complex and dynamic interplay between malignant cells and the defense system. In the case of resistance, the epigenetic inhibitors could act as reactivating tumor suppressor genes and repress cancer cell growth. Some studies have shown that epigenetic inhibitors, such as BET, LSD1 and EZH2 inhibitors, are already used in combination with anti-PD1 therapy activating the antitumor immune response by increasing the persistence of T cells in the tumor microenvironment (ANSARI; SHACKELFORD; EL-OSTA1, 2016b).

A study by Rohrbach and collaborators elucidates the relation between PAD4 activation and immune cells. PAD4 is expressed in granulocytes, which are essential for innate immunity and the formation of neutrophil extracellular traps (NETs). Anti-PAD4 therapies have been proposed for inflammatory and cancer conditions, but we need a better understanding regarding the role of neutrophils in cancer. The tumor microenvironment is composed of adaptive immune cells, which play important roles in tumor growth and metastasis (ROHRBACH et al., 2012). Shi et al., 2020, transplanted Padi4 wild-type and Padi4-knocknout breast cancer cells into inguinal mammary fat pad areas of immunodeficient mice, which lacked functional T cells, B cells and NK, and found that tumor derived PADI4 facilitated metastasis, at least partially independent of the adaptative immune cells. Those findings together suggested that PADI4 inhibition can negatively affect the immune cells; however, the effects on metastatic cancer cells remained (SHI et al., 2020).

Lysine demethylase 6b (KDM6B) is essential for the generation and proper functioning of CD8+ effector T cells during acute infection and tumor eradication, being indispensable for proper effector functions and tumor protection, and KDM6B inhibition exhibits a memory-defective T cell response. Therefore, KDM6B may act as an epigenetic modulator of CD8+ T cell fate determination by regulating effector-associated gene expression and chromatin accessibility (XU et al., 2021). As members of the KDM6 family have been therapeutic targets for several cancers, it is necessary to properly understand their intrinsic role in T cell function. More studies are necessary to better understand the interaction between epigenetic protein inhibition and immunotherapy.

2.7. CONCLUSION

In summary, a streamlined approach of in silico and in vitro experiments allowed us to select, from 38 different epigenetic targets, the two most promising candidates for NSCLC drug development: PADI4 (GSK 484) and KDM6B (GSK-J4). The inhibition of these epigenetic proteins regulates molecular pathways in NSCLC, affecting the ability of cancer cells to migrate and invade, thereby controlling the metastatic cascade. Treatment with the identified inhibitors regulates common genes linked to tumor metastasis.

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Supplementary Table 1. Inhibitory molecules acquired in collaboration with Structural Genomics Consortium (SGC).

Alvos epigenéticos	Classe enzimática	Sonda
BRD2	Bromodomains	JQ1
BRD3	Bromodomains	JQ1
BRD4	Bromodomains	JQ1
BRDT	Bromodomains	JQ1
BAZ2B	Bromodomains	GSK2801/BAZ2-ICR
BAZ2A	Bromodomains	GSK2801/BAZ2-ICR
BRPF1	Bromodomains	NI-57
BRD1	Bromodomains	NI-57
BRPF3	Bromodomains	NI-57
SMARCA2	Bromodomains	PFI-3
SMARCA4	Bromodomains	PFI-3
PBRM1	Bromodomains	PFI-3
SMYD2	Metiltransferases	BAY-598
KDM6B	Demetilases	GSK-J4
BPTF	Bromodomains	TP-238
CECR2	Bromodomais	NVS-CECR2-1/TP-238
PADI4	Deiminases	GSK484
DOT1L	Metiltransferases	SGC0946
WDR5	Metiltransferases	OICR-9429
SETD7	Metiltransferases	R-PFI-2
IDH1	IDH1 mutant inhibitor	GSK864
EZH2	Metiltransferases	GSK343
KDM1A	Demetilases	GSK-LSD1
L3MBTL3	Reader de lisinas metiladas	UNC1215
BRD9	Bromodomains	Bi-9564
BRD7	Bromodomains	Bi-9564
EZH1	Metiltransferases	UNC1215
KMT5B	Metiltransferases	A-196
KMT5C	Metiltransferases	A-196
EHMT2	Metiltransferases	A-366
CREBBP	Acetilases	SGC-CBP30
EP300	Acetilases	SGC-CBP30
PRMT1	Metiltransferases	MS023
PRMT3	Metiltransferases	MS023
CARM1	Metiltransferases	TP-064/MS023
PRMT5	Metiltransferases	MS023
PRMT6	Metiltransferases	MS023
PRMT8	Metiltransferases	MS023

Source: Lesbon, J. C. C. (2022)

Supplementary Table 2. Primer sequences to reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Gene	Sequence (5'-3')
CARM1 fw	CACACCGACTTCAAGGACAA
CARM1 rev	AAAAACGACAGGATCCCAGA
BAZ2A fw	AAGATGTGTGGCTACAATGG
BAZ2A rev	TCTGCACCCATCAGCTCCG
KDM6B fw	GCCTCTTCTCCACCAAGACC
KDM6B rev	GCCTGGTACTGTGCGTACTT
PADI4 fw	GGCAAAGTGAAGCCAACCAG
PADI4 rev	GTCACAGTTCACCAGCAGGA

Source: Lesbon, J. C. C. (2022).

Supplementary Table 3. Differential gene expression analysis for A549 cell line after the treatment with PADI4 and KDM6A/B epigenetic inhibitors. The results selected genes based on FDR < 0.05 and LogFC > 1; < -1.

PADI4 (downregulated genes)				
Gene	**logFC**	**logCPM**	**P value**	**FDR**
ABLIM3	-2,16598	3,896844	6,68E-33	2,18E-29
RFTN1	-2,07843	2,583859	1,05E-13	4,42E-11
NIBAN1	-1,74611	4,702511	1,19E-06	7,71E-05
PLEK2	-1,68459	1,889142	1,57E-08	2,16E-06
RYR1	-1,65518	1,805821	6,53E-08	7,12E-06
DHRS2	-1,62446	4,79535	6,82E-16	4,06E-13
UNC5B	-1,5814	2,046634	2,96E-08	3,52E-06
PXDN	-1,57246	1,364956	1,79E-06	0,000109
ATF3	-1,5494	4,000687	4,80E-20	4,46E-17
SERPINA3	-1,47609	1,680433	0,002389	0,02837
RAB39B	-1,44302	2,990452	1,65E-08	2,23E-06
CXCL8	-1,42821	2,971476	2,47E-08	3,06E-06
IL11	-1,41114	2,883052	8,46E-06	0,000386
SYT13	-1,40973	4,231187	1,99E-20	2,00E-17
DRD1	-1,40966	3,293555	2,86E-11	8,70E-09
BARX1	-1,39148	0,826938	0,000908	0,014122
LINC00941	-1,37827	2,190588	2,83E-06	0,000151

KIF1A	-1,35548	1,89791	2,09E-05	0,000803
SNORD83A	-1,34829	0,901237	0,000353	0,007312
SCO2	-1,30857	0,697471	0,002042	0,025266
LYPD1	-1,28858	1,07346	0,00179	0,023015
SNORD63B	-1,28674	0,890429	0,00082	0,013243
NLGN4Y	-1,27874	0,819972	0,00344	0,037143
NCMAP	-1,27447	1,246873	0,000127	0,003378
ZFP3	-1,26491	2,649588	5,98E-07	4,35E-05
NIPAL4	-1,25077	2,722688	1,46E-07	1,31E-05
SNORA64	-1,23225	1,610212	0,000136	0,003553
KCNMA1	-1,21997	4,878998	6,12E-21	7,28E-18
ZSCAN18	-1,21976	1,164495	0,000362	0,007412
HBEGF	-1,21365	1,838905	3,34E-05	0,001181
STC1	-1,19296	0,912192	0,001193	0,017404
KCNH1	-1,18789	3,380463	0,000165	0,004105
BCL2A1	-1,18785	0,883621	0,004105	0,042303
LAMC2	-1,18455	4,414846	1,67E-09	2,84E-07
ZNF542P	-1,18364	1,63012	0,002579	0,030007
EREG	-1,18328	6,424183	3,99E-26	8,71E-23
MIAT	-1,15437	2,792116	1,67E-06	0,000102
SNORA73B	-1,1463	1,702368	8,07E-05	0,002373
TAS2R5	-1,13598	0,88959	0,003545	0,038005
SLC16A6	-1,12895	1,024322	0,004371	0,044111
DGAT2	-1,11783	2,108499	0,000226	0,005254
LINC00471	-1,11283	1,409989	0,000427	0,008256
TRIB3	-1,11014	8,616827	2,38E-05	0,000889
CITED4	-1,10676	3,71473	4,62E-06	0,000229
NCKAP5	-1,09288	2,909523	2,99E-06	0,000158
TNFRSF12A	-1,09209	6,180822	6,35E-15	3,32E-12
FILIP1	-1,08797	2,663604	2,01E-05	0,000776
DMRTA1	-1,08438	1,604028	0,000895	0,014027
LRRC37A6P	-1,07934	1,242138	0,001906	0,024059
DGUOK-AS1	-1,06685	1,644268	0,000254	0,005752
ANKRD1	-1,06572	2,657378	2,29E-06	0,000131
NOS1	-1,05983	1,248634	0,004645	0,045741
ZNF239	-1,05911	2,827497	7,10E-07	5,00E-05
KIRREL3	-1,04891	3,707308	6,74E-10	1,24E-07
SLFNL1	-1,04426	2,31261	0,000421	0,008245
PPP1R15A	-1,03949	7,1728	3,39E-14	1,53E-11
LOC102725231	-1,03552	2,039582	0,000344	0,007163
NAV2	-1,03463	4,876648	3,60E-08	4,17E-06
PABPC1L	-1,01848	6,364791	4,55E-14	1,99E-11
XDH	-1,00992	1,135906	0,005201	0,049397
ZNF420	-1,00923	1,748509	0,000526	0,009627
AKNA	-1,0008	5,240594	2,85E-05	0,001034

PADI4 (upregulated genes)				
Gene	**logFC**	**logCPM**	**P value**	**FDR**
FGB	2,570617	6,585985	1,22E-99	1,60E-95
PPARGC1A	2,345716	1,71671	1,19E-10	2,64E-08
SLC16A7	2,111457	3,161757	8,55E-23	1,24E-19
DOCK8	1,884747	1,348758	2,38E-08	3,03E-06
MYOCD	1,845663	1,611589	9,80E-09	1,44E-06
F5	1,767262	3,026287	1,40E-16	8,73E-14
TMEM151A	1,737155	2,251023	6,47E-11	1,63E-08
NRXN3	1,702303	0,867355	4,99E-05	0,001634
ITIH2	1,694332	1,402564	9,44E-08	9,15E-06
NID2	1,688522	1,8109	1,82E-09	2,94E-07
TM4SF4	1,635597	5,034187	8,97E-21	9,79E-18
A1CF	1,633534	2,74931	3,81E-13	1,39E-10
CFH	1,577096	7,470666	1,74E-58	1,14E-54
MUC16	1,52822	5,408622	5,67E-26	1,06E-22
FAXDC2	1,525156	1,018501	4,87E-05	0,001613
CFHR1	1,511834	1,975166	8,11E-08	8,11E-06
KCNJ2	1,501049	1,357311	1,70E-05	0,000679
ADH6	1,494147	1,897476	8,87E-08	8,80E-06
SPTLC3	1,450007	1,662017	6,30E-07	4,55E-05
MROH2A	1,392993	1,010119	0,000121	0,003244
LINC01474	1,392282	0,659862	0,000595	0,010531
LRRC29	1,388649	0,966035	8,24E-05	0,002413
SOX2	1,383915	0,979558	0,000455	0,008675
CHL1	1,37935	0,729878	0,000382	0,007721
HOOK1	1,37373	2,231556	9,18E-07	6,22E-05
GOT1-DT	1,353048	1,383392	0,00044	0,008445
UGT1A7	1,352245	2,208636	6,87E-08	7,23E-06
FGA	1,346355	5,988379	4,64E-33	2,03E-29
TM4SF20	1,334241	6,238076	5,25E-28	1,37E-24
ENTPD2	1,330111	2,454085	2,68E-07	2,11E-05
CEACAM16-AS1	1,327011	0,533268	0,005012	0,048227
STAG3L1	1,321903	0,513889	0,003226	0,035388
TM4SF5	1,321381	0,829997	0,000782	0,012869
ASB4	1,320708	2,539827	3,16E-06	0,000166
KCNJ16	1,320141	1,168848	0,001778	0,022919
KCNQ1	1,313789	0,727692	0,000572	0,010216
DPEP1	1,312026	2,063608	1,30E-06	8,24E-05
TENM3	1,309977	4,936187	4,28E-25	7,00E-22
DDIT4L	1,303926	1,90864	6,49E-06	0,000303
CEACAM1	1,303799	3,445515	4,59E-11	1,28E-08
TNFRSF13C	1,281089	1,668056	0,000149	0,003785
NPY1R	1,27707	1,034076	0,000558	0,010053
LOC105377744	1,276757	0,772785	0,001068	0,016102
RNF224	1,274336	0,983359	0,000293	0,006386

NEB	1,263852	3,473212	2,50E-08	3,06E-06
UNC13D	1,260612	4,64725	1,33E-16	8,69E-14
LOC101926887	1,258073	1,008998	0,002482	0,029194
CDH16	1,254207	0,84291	0,001268	0,018054
COL1A1	1,248641	3,175263	1,60E-09	2,76E-07
ZNF554	1,229245	1,240794	0,000205	0,00489
BCAS1	1,221997	4,132543	1,79E-10	3,85E-08
IFIT1	1,178667	1,761142	0,000228	0,00529
ARHGEF16	1,174711	3,00665	2,23E-07	1,86E-05
NRIP3	1,170341	0,95106	0,001669	0,021906
ANK3	1,150479	2,475505	3,94E-05	0,001356
PHEX	1,148861	1,189953	0,000452	0,008644
SH2B2	1,148361	3,474898	1,70E-10	3,71E-08
UPK3B	1,146103	1,910386	7,17E-05	0,002173
FLRT2	1,145093	1,445011	0,000172	0,004253
KCTD13-DT	1,131037	1,473627	0,000131	0,003437
PCAT7	1,126435	1,122828	0,001979	0,024672
CORO2A	1,125714	4,747707	1,01E-18	8,28E-16
ARID5B	1,105971	3,272508	6,83E-08	7,23E-06
CIDEC	1,104077	1,379261	0,000362	0,007412
S1PR5	1,09904	1,482257	0,00126	0,018019
RORA	1,093546	0,839522	0,002436	0,028766
KCNQ1OT1	1,092971	1,895612	7,84E-05	0,002321
KLF9	1,089679	3,878726	6,37E-10	1,19E-07
VIL1	1,087472	1,070202	0,002196	0,026522
FBXO2	1,084228	2,155773	6,36E-05	0,001973
VASH2	1,082665	2,676476	2,68E-06	0,000146
GPR199P	1,079519	0,964713	0,003169	0,034947
TJP3	1,074671	3,837274	3,42E-11	9,94E-09
QPRT	1,068836	3,531338	5,90E-10	1,12E-07
KRT4	1,066978	4,705169	2,42E-13	9,60E-11
FGG	1,06431	5,238643	5,11E-20	4,46E-17
C1orf21	1,052845	1,703124	0,000165	0,004105
MYO1D	1,043369	2,764934	1,28E-06	8,16E-05
BCL2L15	1,042217	1,243708	0,003053	0,034062
LOC107985728	1,040762	4,791001	1,37E-12	4,61E-10
LOC105373424	1,03886	1,63829	0,003362	0,036572
FAM83A	1,037041	1,849497	0,000478	0,008969
ALDOC	1,033297	1,424643	0,003119	0,03461
SKIDA1	1,031406	1,393316	0,000675	0,011569
PDK4	1,028369	7,009559	2,62E-18	2,01E-15
DCDC1	1,023172	1,854705	0,000833	0,013374
NPNT	1,020227	3,10865	4,66E-07	3,45E-05
EIF2AK3-DT	1,018657	0,910279	0,004377	0,044134
LIN7A	1,011893	4,496597	3,58E-13	1,34E-10
ATP6V0D1-DT	1,004192	1,334714	0,002926	0,033018

KDM6A/B (downregulated genes)				
Gene	**logFC**	**logCPM**	**P value**	**FDR**
PRDM1	-1,00918	2,289796	3,07E-05	0,000457
ANKRD1	-1,01703	2,662901	2,39E-06	4,89E-05
KIF1A	-1,01714	1,979568	0,003475	0,022257
MYPN	-1,03131	0,811733	0,008964	0,045505
HK2	-1,03778	1,076437	0,004464	0,026911
ZNF583	-1,05113	1,023525	0,001577	0,012154
KCNMA1	-1,05279	4,928109	1,12E-29	6,05E-27
RFTN1	-1,06209	2,805277	1,24E-07	3,67E-06
KLC2-AS1	-1,0629	0,872893	0,003692	0,023349
ZSCAN18	-1,07563	1,184041	0,000509	0,004875
SNORA73B	-1,07615	1,707718	4,21E-05	0,000605
ZNF561-AS1	-1,07662	0,97798	0,001467	0,011502
MSS51	-1,07841	1,101731	0,001592	0,012255
ZFP3	-1,09129	2,68986	1,09E-07	3,27E-06
ZFP82	-1,09731	1,561737	0,000181	0,002067
MATN1-AS1	-1,10297	1,143075	0,001105	0,009121
LOC112268035	-1,10403	0,862505	0,00171	0,012948
FUT1	-1,10483	1,335725	0,000239	0,002597
CDCP1	-1,13683	3,919086	2,12E-15	2,15E-13
ZNF239	-1,13949	2,797196	4,06E-09	1,68E-07
LOC112268412	-1,14073	1,084645	0,000505	0,004847
SCO2	-1,15653	0,715384	0,002841	0,019163
LAMP3	-1,16051	0,247896	0,010156	0,049873
SLCO2B1	-1,16589	1,126291	0,001799	0,013444
ATF3	-1,18165	4,09472	8,54E-18	1,21E-15
SYT13	-1,18472	4,290846	1,23E-17	1,73E-15
THBD	-1,1887	2,697802	1,19E-06	2,65E-05
FBXW10	-1,18972	2,398985	1,32E-06	2,89E-05
ACY1	-1,19254	0,737035	0,001834	0,01364
FILIP1	-1,20499	2,622781	1,90E-06	3,97E-05
ABI3BP	-1,21655	0,05122	0,008286	0,043073
ZDHHC14	-1,2228	1,543091	0,00025	0,002696
MSX1	-1,22676	0,801199	0,00179	0,013418
SLIT1	-1,23374	0,550856	0,0019	0,014031
ADGRE1	-1,23723	0,4614	0,003585	0,022823
RAB39B	-1,24426	3,033895	2,07E-07	5,84E-06
CXCL8	-1,26235	3,005777	2,57E-10	1,33E-08
DHRS2	-1,28607	4,881452	6,15E-20	1,20E-17
EGLN2	-1,29528	1,498515	0,000137	0,001622
SLC2A1-DT	-1,31344	0,051177	0,007315	0,039311
HBEGF	-1,31694	1,798411	7,91E-07	1,84E-05
LINC00638	-1,3413	0,575132	0,002282	0,016197
EXOC3L2	-1,37088	0,736812	0,000228	0,002496
HNRNPR	-1,51659	0,119557	0,001237	0,010007

PLAC1	-1,59695	0,611281	8,53E-05	0,001099
SLC16A6	-1,67655	0,871747	3,47E-05	0,000509
NOS1	-1,68312	1,082928	2,83E-05	0,000427
ZNF420	-1,6859	1,566053	2,33E-08	8,23E-07
DMGDH	-1,78063	0,307399	0,000319	0,003305
TTC41P	-1,85169	0,032922	0,000185	0,002109
ABLIM3	-1,86819	3,948763	4,34E-33	3,26E-30
BARX1	-1,87199	0,703033	1,42E-05	0,000236
NLGN4Y	-1,97709	0,657243	6,57E-06	0,000121
SUSD5	-2,15096	0,433536	4,78E-06	9,08E-05
PXDN	-2,21284	1,228312	8,45E-10	3,99E-08
CIART	-2,7148	-0,37603	1,02E-05	0,000178

KDM6A/B (upregulated genes)				
Gene	**logFC**	**logCPM**	**P value**	**FDR**
LOC100507634	2,91893	-0,17269	8,84E-08	2,74E-06
TMEM132D-AS1	2,55070	1,53118	5,74E-14	4,70E-12
LOC105373180	2,49009	-0,49535	1,73E-05	0,00027748
FGB	2,42660	6,45541	2,99E-266	4,04E-262
TMED2-DT	2,38016	-0,30233	1,22E-05	0,00020591
HS3ST3B1	2,30133	0,15776	4,56E-07	1,15E-05
ADGRV1	2,20827	0,00157	1,06E-05	0,00018409
SLC16A7	2,18723	3,21911	1,47E-37	1,53E-34
F13B	2,15555	0,28386	1,99E-06	4,14E-05
BCHE	2,10230	0,23827	0,0007448	0,00671062
MYOCD	2,08913	1,81244	3,62E-14	3,06E-12
F5	2,02463	3,22218	3,36E-32	2,27E-29
CTTNBP2	2,00077	1,03086	1,23E-08	4,69E-07
BCO2	1,92912	0,25348	0,00013704	0,00162185
TEX19	1,88915	-0,13298	0,00033765	0,00347018
DOCK8	1,87354	1,35195	3,65E-09	1,53E-07
VIL1	1,86738	1,62090	3,33E-11	2,04E-09
LOC101927699	1,82739	0,31321	2,75E-05	0,00041889
LOC102724908	1,78606	0,65342	5,15E-06	9,70E-05
LOC107986087	1,77341	-0,21533	0,00049751	0,00478227
ACSL5	1,75182	0,07265	0,00011311	0,00137474
LOC105377744	1,68256	1,04906	6,21E-06	0,00011457
LOC105378539	1,66500	-0,37244	0,00234837	0,01658213
IDI2-AS1	1,62544	-0,15317	0,00154094	0,01195511
SMAD9	1,62518	0,71061	0,0002376	0,00258752

TM4SF4	1,61606	5,00907	1,92E-51	5,20E-48
KCNJ16	1,58668	1,34369	2,45E-06	4,99E-05
MROH2A	1,57543	1,13476	1,12E-06	2,49E-05
LOC105370941	1,56653	-0,19430	0,00197322	0,01446989
ADH6	1,56473	1,95099	2,22E-10	1,16E-08
FAM110B	1,56337	0,86866	9,71E-06	0,00017036
LOC105376506	1,53950	0,38498	0,00089339	0,00777975
CFH	1,51705	7,42085	1,21E-149	8,21E-146
CHL1	1,48821	0,81750	5,29E-05	0,00072601
LOC105377685	1,47954	0,25193	0,00069772	0,00634569
BMPR1B-DT	1,47433	0,43973	0,00046858	0,00455277
CFHR1	1,47310	1,95477	1,34E-08	5,03E-07
A1CF	1,47047	2,63674	3,22E-14	2,74E-12
DCDC1	1,44488	2,14591	2,05E-09	9,05E-08
PPARGC1A	1,43509	1,03928	1,18E-05	0,00020147
DNAJC3-DT	1,40363	0,08976	0,00222498	0,01591038
LGI4	1,39660	0,34184	0,00511923	0,02998355
CCNE2	1,39523	0,34174	0,00095873	0,00820598
ENG	1,38111	0,81702	0,000215	0,00238953
RTKN2	1,37257	1,79983	1,41E-07	4,12E-06
ABR	1,36219	-0,05483	0,0047209	0,02813807
LAD1	1,35047	0,68705	0,00021264	0,0023672
LOC105374868	1,34845	0,26725	0,00209957	0,01528041
LOC105371419	1,34113	0,15696	0,00436661	0,02645217
LOC102724378	1,33918	0,05435	0,00404719	0,02496662
LINC01389	1,33174	-0,13322	0,00529932	0,0307316
PRR15L	1,32871	0,74299	0,00107163	0,00895615
NID2	1,32283	1,56492	1,50E-06	3,25E-05
TENM3	1,32271	4,93870	1,27E-42	2,46E-39
CALML6	1,32026	-0,01767	0,00391348	0,02436238
COLCA1	1,31428	0,58137	0,00695685	0,03794263
MIR29B2CHG	1,30943	1,09971	9,75E-05	0,00122629
BCL2L15	1,30728	1,40779	4,53E-05	0,00064199
ZNF608	1,30548	1,88240	6,61E-06	0,00012121
C6orf201	1,30452	0,38352	0,00839665	0,04335283
SOX2	1,30061	0,91817	0,00030358	0,00317071
SPTLC3	1,28716	1,56399	5,09E-06	9,62E-05
ENTPD2	1,28382	2,42463	2,50E-08	8,67E-07

SEPTIN3	1,27438	0,31230	0,00266797	0,01817422
FGA	1,27204	5,92917	8,47E-55	2,86E-51
PAX7	1,27155	3,93623	3,68E-22	1,01E-19
NRXN3	1,26894	0,60433	0,00457801	0,02748636
SHH	1,26680	3,05432	8,53E-10	4,01E-08
UNC13D	1,26412	4,64299	9,43E-26	3,75E-23
NRTN	1,25603	0,76442	0,00080912	0,00717534
SEC24B-AS1	1,24466	0,72096	0,00151821	0,0118278
COL1A1	1,24446	3,17070	1,89E-09	8,41E-08
STAG3L1	1,24439	0,46580	0,00275711	0,01865908
ITIH2	1,24337	1,10832	0,0001004	0,00125644
MUC16	1,23856	5,19335	1,73E-29	8,99E-27
LOC105370092	1,23185	0,72001	0,00149433	0,01169415
ANXA8	1,23078	1,65457	2,98E-06	5,93E-05
INHA	1,22899	-0,07435	0,0078922	0,0417305
LOC102724030	1,22665	0,03674	0,00877312	0,04475979
SOX21-AS1	1,22348	0,53074	0,00140991	0,01114975
FLJ16779	1,21312	1,90104	6,52E-07	1,56E-05
UGT1A7	1,21281	2,12009	1,20E-07	3,57E-06
LOC100506990_1	1,19371	0,12306	0,00861269	0,04411504
TM4SF20	1,19082	6,13098	1,82E-50	4,11E-47
ENTPD8	1,18210	0,58041	0,00174691	0,0131823
LOC107985000	1,17830	0,46564	0,00259243	0,01783543
LOC105373449	1,17364	0,15660	0,00826702	0,04298916
LOC105376505	1,17040	0,75244	0,00656816	0,03644036
HES2	1,16912	1,02996	0,00276148	0,01866068
CCDC39	1,16847	1,46737	9,25E-05	0,00117375
SH2B2	1,16345	3,48421	2,90E-14	2,50E-12
KCTD13-DT	1,16325	1,50051	1,70E-05	0,00027402
HNMT	1,15876	0,64002	0,00505764	0,02973206
MYO1D	1,15479	2,84093	2,77E-09	1,18E-07
ASPDH	1,14225	0,55562	0,00236215	0,01662731
CIDEC	1,13750	1,40058	0,00061523	0,00572646
MIATNB	1,13275	1,18992	0,00177363	0,01331704
LOC105373195	1,13146	1,53314	0,00012194	0,00146621
LOC107985728	1,12502	4,84150	6,84E-22	1,85E-19
LOC105369308	1,12494	0,39725	0,0088978	0,04524218
FGG	1,12300	5,27165	8,02E-32	4,93E-29

SNORD87	1,12204	0,46593	0,00561186	0,03226046
KCNT2	1,11135	2,38230	2,35E-07	6,43E-06
SCX	1,11058	0,80582	0,0085579	0,04397722
EPHA5	1,11047	0,91729	0,0013245	0,01057333
BCAS1	1,10178	4,04358	3,10E-13	2,36E-11
UPK3B	1,09826	1,88529	2,92E-05	0,0004379
TMEM151A	1,09460	1,80913	1,66E-05	0,00026901
GPR199P	1,09131	0,98394	0,00129707	0,01039735
NEB	1,08543	3,34670	1,66E-09	7,42E-08
LOC100130027	1,08285	1,59439	4,66E-05	0,00065553
PRG4	1,08132	1,74381	0,00020193	0,00227761
ADGRG5	1,07911	0,32629	0,00913474	0,04613452
PDZD3	1,07822	0,47883	0,00632528	0,035442
RND1	1,07811	4,48559	5,20E-19	9,12E-17
BTNL9	1,07759	3,00116	8,81E-08	2,74E-06
LINC02747	1,07558	1,28383	0,00103141	0,00869589
LOC105377743	1,06443	1,07302	0,00102693	0,0086721
WHAMMP4	1,06112	0,79636	0,0059365	0,03386736
ASB4	1,05791	2,35983	1,43E-06	3,10E-05
PLXNC1	1,04132	0,92704	0,00249928	0,01730418
LOC102723961	1,04029	1,62333	0,00151199	0,01179827
GGT5	1,03605	2,40541	3,53E-06	6,90E-05
LOC107984421	1,03445	0,49158	0,00778925	0,04138041
ELMO1	1,03194	3,80243	2,52E-14	2,21E-12
GLIPR1L1	1,02675	0,55544	0,00697232	0,03801167
MYO1A	1,02611	2,62836	1,77E-06	3,71E-05
OVGP1	1,01537	1,90420	7,02E-05	0,00092966
ARID5B	1,01418	3,20809	4,14E-08	1,39E-06
CD82	1,01401	2,32026	2,46E-06	5,01E-05
CABCOCO1	1,01294	0,90785	0,00455071	0,02734673
AFF1-AS1	1,01114	0,96449	0,00246386	0,01715559
SYNDIG1	1,01054	1,19860	0,00253554	0,01751935
LOC107987266	1,00975	0,74164	0,00521019	0,03037141

Source: Lesbon, J. C. C. (2022)

Appendix 1. List of articles presented by the PubMed database through a systematic literature review.

PRMT1

1: Sundar IK, Rahman I. Gene expression profiling of epigenetic chromatin modification enzymes and histone marks by cigarette smoke: implications for COPD and lung cancer. Am J Physiol Lung Cell Mol Physiol. 2016 Dec 1;311(6):L1245-L1258. doi: 10.1152/ajplung.00253.2016. Epub 2016 Oct 28. PubMed PMID: 27793800; PubMed Central PMCID: PMC5206398.

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Cancer. J Biol Chem. 2015 May 22;290(21):13479-89. doi: 10.1074/jbc.M114.636050.

Epub 2015 Apr 6. PubMed PMID: 25847239; PubMed Central PMCID: PMC4505594.

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Guéant JL, Namour F. CARM1 and PRMT1 are dysregulated in lung cancer without

hierarchical features. Biochimie. 2014 Feb;97:210-8. doi:

10.1016/j.biochi.2013.10.021. Epub 2013 Nov 6. PubMed PMID: 24211191.

KDM6B

1: DiBardino DM, Rawson DW, Saqi A, Heymann JJ, Pagan CA, Bulman WA.

Next-generation sequencing of non-small cell lung cancer using a customized,

targeted sequencing panel: Emphasis on small biopsy and cytology. Cytojournal.

2017 Mar 20;14:7. doi: 10.4103/1742-6413.202602. eCollection 2017. PubMed PMID:

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Jiang L, Qin W. KDM6B Elicits Cell Apoptosis by Promoting Nuclear Translocation

of FOXO1 in Non-Small Cell Lung Cancer. Cell Physiol Biochem. 2015;37(1):201-13.

doi: 10.1159/000430345. Epub 2015 Aug 20. PubMed PMID: 26303949.

3: Sato T, Kaneda A, Tsuji S, Isagawa T, Yamamoto S, Fujita T, Yamanaka R, Tanaka

Y, Nukiwa T, Marquez VE, Ishikawa Y, Ichinose M, Aburatani H. PRC2 overexpression

and PRC2-target gene repression relating to poorer prognosis in small cell lung

cancer. Sci Rep. 2013;3:1911. doi: 10.1038/srep01911. PubMed PMID: 23714854;

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4: Tian C, Deng H, Tang X, Hu H, Liu X, Luo F. [Effect of Jumonji

domain-containing protein-3 on the proliferation and migration of lung cancer

cell line]. Sheng Wu Yi Xue Gong Cheng Xue Za Zhi. 2012 Jun;29(3):514-8. Chinese.

PubMed PMID: 22826950.

CARM1

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CARM1 and PRMT1 are dysregulated in lung cancer without hierarchical features.

Elakoum R(1), Gauchotte G(1), Oussalah A(1), Wissler MP(1), Clément-Duchêne C(1),

Vignaud JM(1), Guéant JL(2), Namour F(3).

BRD4

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Serrano M, Brooks HB, Campbell RM, Barrero MJ. NSD2 contributes to oncogenic

RAS-driven transcription in lung cancer cells through long-range epigenetic

activation. Sci Rep. 2016 Sep 8;6:32952. doi: 10.1038/srep32952. PubMed PMID:

27604143; PubMed Central PMCID: PMC5015087.

2: Liao YF, Wu YB, Long X, Zhu SQ, Jin C, Xu JJ, Ding JY. High level of BRD4

promotes non-small cell lung cancer progression. Oncotarget. 2016 Feb

23;7(8):9491-500. doi: 10.18632/oncotarget.7068. PubMed PMID: 26840017; PubMed

Central PMCID: PMC4891054.

3: Lenhart R, Kirov S, Desilva H, Cao J, Lei M, Johnston K, Peterson R, Schweizer L, Purandare A, Ross-Macdonald P, Fairchild C, Wong T, Wee S. Sensitivity of Small Cell Lung Cancer to BET Inhibition Is Mediated by Regulation of ASCL1 Gene Expression. Mol Cancer Ther. 2015 Oct;14(10):2167-74. doi: 10.1158/1535-7163.MCT-15-0037. Epub 2015 Aug 7. PubMed PMID: 26253517.

4: Wang X, Zhang Y, Nilsson CL, Berven FS, Andrén PE, Carlsohn E, Horvatovich P, Malm J, Fuentes M, Végvári Á, Welinder C, Fehniger TE, Rezeli M, Edula G, Hober S, Nishimura T, Marko-Varga G. Association of chromosome 19 to lung cancer genotypes and phenotypes. Cancer Metastasis Rev. 2015 Jun;34(2):217-26. doi: 10.1007/s10555-015-9556-2. Review. Erratum in: Cancer Metastasis Rev. 2015 Jun;34(2):227. PubMed PMID: 25982285.

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EZH2

1: Feng Q, He H, Gao T, Zhang Q, Liu Z, Tao X, Zhu Y, Zhang L, Yu L. Synthesis and biological evaluation of benzomorpholine derivatives as novel EZH2 inhibitors for anti-non-small cell lung cancer activity. Mol Divers. 2019 Jan 5. doi: 10.1007/s11030-018-9903-7. [Epub ahead of print] PubMed PMID: 30612258.

2: Fang S, Shen Y, Chen B, Wu Y, Jia L, Li Y, Zhu Y, Yan Y, Li M, Chen R, Guo L, Chen X, Chen Q. H3K27me3 induces multidrug resistance in small cell lung cancer by affecting HOXA1 DNA methylation via regulation of the lncRNA HOTAIR. Ann Transl Med. 2018 Nov;6(22):440. doi: 10.21037/atm.2018.10.21. PubMed PMID: 30596070; PubMed Central PMCID: PMC6281536.

3: Cheng H, Ge X, Zhuo S, Gao Y, Zhu B, Zhang J, Shang W, Xu D, Ge W, Shi L. β-Elemene Synergizes With Gefitinib to Inhibit Stem-Like Phenotypes and Progression of Lung Cancer via Down-Regulating EZH2. Front Pharmacol. 2018 Nov 30;9:1413. doi: 10.3389/fphar.2018.01413. eCollection 2018. PubMed PMID: 30555330; PubMed Central PMCID: PMC6284059.

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vulnerabilities. J Exp Med. 2018 Dec 3;215(12):3115-3135. doi:

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RNA PCAT6 functions as an oncogene by binding to EZH2 and suppressing LATS2 in

non-small-cell lung cancer. EBioMedicine. 2018 Nov;37:177-187. doi:

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N, Takamori S, Akamine T, Hirai F, Yamada Y, Hamamoto R, Oda Y, Maehara Y. Prevalence of Enhancer of Zeste Homolog 2 in Patients with Resected Small Cell Lung Cancer. Anticancer Res. 2018 Jun;38(6):3707-3711. doi: 10.21873/anticanres.12649. PubMed PMID: 29848731.

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