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UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA

Ana Laura Seneda

Identificação de novos microRNAs associados ao metabolismo maligno do câncer de pulmão

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de Botucatu, para obtenção do título de Doutora em Cirurgia e Medicina Translacional.

Orientadora: Profa. Associada Patricia Pintor dos Reis
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FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: MARIA CAROLINA A. CRUZ E SANTOS-CRB 8/10188

Seneda, Ana Laura.
Identificação de novos microRNAs associados ao metabolismo maligno do câncer de pulmão / Ana Laura Seneda. - Botucatu, 2023

Tese (doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Faculdade de Medicina de Botucatu
Orientador: Patricia Pintor dos Reis
Coorientador: Luis Alejandro Jose Mur
Capes: 40102050

1. Pulmões - Câncer. 2. Genes. 3. Tumores malignos. 4. MicroRNAs.

Palavras-chave: Câncer de pulmão; Genes alvos; Metabolismo maligno; Novos microRNAs.

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UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Botucatu

ATA DA DEFESA PÚBLICA DA TESE DE DOUTORADO DE ANA LAURA SENEDA, DISCENTE DO PROGRAMA DE PÓS-GRADUAÇÃO EM CIRURGIA E MEDICINA TRANSLACIONAL, DA FACULDADE DE MEDICINA.

Aos 11 dias do mês de agosto do ano de 2023, às 14:00 horas, por meio de Videoconferência, realizou-se a defesa de TESE DE DOUTORADO de ANA LAURA SENEDA, intitulada **Identificação de novos microRNAs associados ao metabolismo maligno de câncer de pulmão**. A Comissão Examinadora foi constituida pelos seguintes membros: Profa. Dra. PATRICIA PINTOR DOS REIS (Orientador(a) - Participação Presencial) do(a) Depto. de Cirurgia e Ortopedia / FM/Botucatu - Unesp, Prof. Dr. CRISTIANO DE PÁDUA SOUZA (Participação Virtual) do(a) Hospital de Câncer de Barretos - Fundação Pio XII, Prof. Dr. ROBSON FRANCISCO CARVALHO (Participação Presencial) do(a) Depto. de Biologia Estrutural e Funcional / IB/Botucatu - Unesp. Após a exposição pela doutoranda e arguição pelos membros da Comissão Examinadora que participaram do ato, de forma presencial e/ou virtual, a discente recebeu o conceito final: APROVADA. Nada mais havendo, foi lavrada a presente ata, que após lida e aprovada, foi assinada pelo(a) Presidente(a) da Comissão Examinadora.

Profa. Dra. PATRICIA PINTOR DOS REIS

Faculdade de Medicina - Câmpus de Botucatu -
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Dedicatória

A Deus por me permitir chegar até aqui e concluir este trabalho.

À minha família, meus pais, Mariza da Silva Seneda e Carlos Alberto Seneda, e minhas irmãs, Letícia Fernanda Seneda e Maria Júlia Seneda, por todo carinho e apoio incondicional, compreendendo minhas ausências, e sempre me incentivando a buscar por meus objetivos, e por não medirem esforços para que eu chegasse até aqui.
A vocês, minha imensa e eterna gratidão.

Dedico, ainda, a todos os pacientes e seus familiares que são afetados por esta doença. Espero fortemente que este trabalho possa contribuir significativamente para melhorar sua qualidade de vida, bem como contribuir para futuras diretrizes terapêuticas.

Agradecimentos

Agradeço imensamente à minha orientadora, Dra. Patrícia Pintor dos Reis, pelas orientações, pela paciência, pela atenção, pelo tempo disposto e incentivo.
Você foi fundamental para a conclusão deste trabalho. Admiro demais a pesquisadora que é, um grande exemplo a ser seguido, a qual me inspiro muito. Sou muito grata por nossa parceria ao longo desta jornada. Você me ensinou muito. Obrigada por tudo.

Ao meu co-orientador, Prof. Dr. Luis Alejandro Jose Mur, pelo tempo disposto, atenção, ensinamentos e incentivo.

Aos professores membros da banca, Dr. Cristiano Pádua de Souza e Dr. Robson Francisco Carvalho pelas valorosas contribuições.

A todos os amigos e amigas, que me acompanharam e torceram por mim nesta jornada, especialmente Carolina Fazio Campos, Iael Weissberg Minutentag e Tainara Francini Felix, pelo carinho, ajuda, suporte aos dias difíceis e mais

ainda por torná-los mais leves com palavras de apoio. Vocês são incríveis.

À equipe do Laboratório de Oncologia Molecular e Translacional, especialmente ao Dr. Márcio de Carvalho por toda ajuda técnica e à Dra. Sandra Aparecida Drigo Linde pelo apoio.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela bolsa concedida.

Aos colaboradores do projeto, Dr. Mateus Barros-Filho e Dr. Wan Lam.

À Faculdade de Medicina de Botucatu (FMB-UNESP) e à Unidade de Pesquisa Experimental (UNIPEX) pela estrutura disponibilizada e a todos os seus funcionários.

A todos os funcionários da Biblioteca e da Seção Técnica de Pós-Graduação, em especial à secretária do Programa de Cirurgia e Medicina Translacional, Márcia Fonseca Piagentini Cruz, por toda ajuda e apoio.

Ao NCBI dbGaP e ao TCGA por autorização a obtenção dos dados utilizados para a análise computacional.

A todos que, direta ou indiretamente, contribuíram para a realização desse trabalho.

"Em algum lugar, algo incrível está esperando para ser descoberto"

Carl Sagan

RESUMO

Introdução: O câncer de pulmão é a principal causa de morte por câncer no mundo e compreende dois principais subtipos histológicos: adenocarcinoma (LUAD) e carcinoma de células escamosas (LUSC). MicroRNAs (miRNAs) são pequenos RNAs não codificantes que regulam a expressão gênica e são conhecidos por modular processos celulares, incluindo o metabolismo, que está alterado nas células cancerígenas. De acordo com o miRBase, um banco de dados de sequências de miRNAs, atualmente são conhecidos 2.654 miRNAs humanos maduros. No entanto, estudos recentes sugerem a existência de novos miRNAs com um potencial papel no desenvolvimento e progressão da doença. **Objetivos:** Identificar novas sequências de miRNAs no câncer de pulmão. **Métodos:** Os dados de sequenciamento de miRNA publicamente disponíveis (miRNA-Seq) do LUAD e LUSC foram obtidos do *The Cancer Genome Atlas* (TCGA). O miRMaster foi utilizado para predição de novos miRNAs, com critério de filtragem de FC> 2 e p <0,001. Os genes alvo dos miRNAs foram investigados usando mirDB. Em seguida, a expressão dos genes alvo foi validada (*in silico*) usando conjuntos de dados de RNA-seq (LUAD e LUSC) obtidos do *Xena Browser*. Análise de correlação de Spearman entre miRNAs e genes alvo foi realizada. PathDB foi usado para identificar vias metabólicas incluindo genes alvo de miRNA. **Resultados:** 126 sequências de novos miRNAs previstos foram identificadas no LUAD e 225 no LUSC. Destas, 18 novas sequências foram correlacionadas negativamente (r> 50%; p <0,0001) com 47 genes alvo no LUAD, e 66 foram correlacionadas negativamente com 473 genes alvo no LUSC. Destes, 13 novos miRNAs foram comumente superexpressos em LUAD e LUSC. Em seguida, identificamos (*in silico*) o papel dos novos miRNAs no metabolismo do câncer. Encontramos duas vias principais: metabolismo de carboidratos (136 vias incluindo 30 genes alvo de miRNA) e metabolismo energético (64 vias e 26 genes alvo de miRNA). **Conclusões:** Um subconjunto de novos miRNAs está desregulado no câncer de pulmão versus tecidos pulmonares normais, com papéis potenciais no metabolismo maligno associado à

tumorigênese pulmonar. Os miRNAs identificados modulam a expressão de genes alvo com funções biológicas nas vias do metabolismo de carboidratos e energia.

Palavras-chaves: novos microRNAs, câncer de pulmão, genes alvos, metabolismo maligno.

ABSTRACT

Introduction: Lung cancer is the main cause of cancer death worldwide and comprises two main histological subtypes: lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC). MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression, and are known to modulate cellular processes, including metabolism, which is altered in cancer cells. According to miRBase, a database of miRNA sequences, 2,654 mature human miRNAs are currently known. However, recent studies suggested the existence of novel miRNAs with a potential role in disease development and progression. **Objectives:** To identify novel miRNA sequences in lung cancer. **Study design and methods:** Publicly available miRNA sequencing (miRNA-Seq) data from LUAD and LUSC were obtained from The Cancer Genome Atlas (TCGA). miRMaster was used for novel miRNA prediction, with a filtering criterion of FC>2 and $p<0.001$. miRNA target genes were investigated using mirDB. Next, target gene expression was validated (*in silico*) using LUAD and LUSC RNA-seq datasets obtained from Xena Browser. Spearman correlation analysis between miRNAs and target genes were performed. PathDB was used to identify metabolic pathways including miRNA target genes. **Results:** 126 sequences of predicted novel miRNAs were identified in LUAD, and 225 in LUSC. Of these, 18 novel sequences were negatively correlated (r>50%; $p<0.0001$) with 47 target genes in LUAD, and 66 were negatively correlated with 473 target genes in LUSC. Of these, 13 novel miRNAs were commonly overexpressed in LUAD and LUSC. Next, we identified (*in silico*) the role of novel miRNAs in cancer metabolism. We found two main pathways: carbohydrate metabolism (136 pathways including 30 miRNA target genes) and energy metabolism (64 pathways and 26 miRNA target genes). **Conclusions:** A subset of novel miRNAs are deregulated in lung cancer *vs*. normal lung tissues, with potential roles in malignant metabolism associated with lung tumorigenesis. The identified miRNAs modulate the expression of target genes with biological roles in pathways of carbohydrate and energy metabolism.

Key-words: novel microRNAs, lung cancer, target genes, malignant metabolism.

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1. INTRODUCTION

1.1 Lung Cancer: Epidemiology and Risk Factors

The World Health Organization (WHO) (2020) reported that lung cancer is the fourth most prevalent and the second most incident cancer, and the leading cause of cancer death worldwide (**Figures 1A, 1B**, **1C**) (1). In Brazil, according to the National Institute of Cancer (INCA, 2023), lung cancer is the third most incident cancer among men (7,5% of all new cases), and the fourth most incident (6%) in women. Regarding mortality, lung cancer is the major cause of death in men (13,6% of total deaths related to cancer) and the second (11,6%) in women (2). The high mortality index is mainly related to diagnosis of advanced stage disease (3).

Although lung cancer is more commonly observed in older patients, with a median age of diagnosis of 71 years, in the United States (4), the number of new cases is increasing in individuals from 45 years of age, showing that diagnosis of lung cancer is now becoming more common in younger individuals (3).

Smoking is still the main risk factor for lung cancer development. According to INCA (2023), cigarette smoking is associated with approximately 90% of deaths by lung cancer. Although, about 10% of patients diagnosed with lung cancer have never smoked (3), showing that there are other risk factors that play a role in disease development, such as air pollution, ionizing radiation, secondhand smoke, and occupational exposure to carcinogens such as asbestos, radon, and metals (mainly arsenic, chromium and nickel). A history of previous lung diseases, and human immunodeficiency virus (HIV) infection (4). Diseases that lead to pulmonary chronic tissue inflammation, damage and fibrosis, as well as gene expression changes, including pulmonary tuberculosis (5,6) and chronic obstructive pulmonary disease (COPD) (7), also elevate the risk of lung cancer development. These chronic diseases may be associated with lung cancer cases in younger individuals (8).

It is noteworthy that smoking is more strongly related to squamous cell carcinoma and small cell lung cancer subtypes (9).

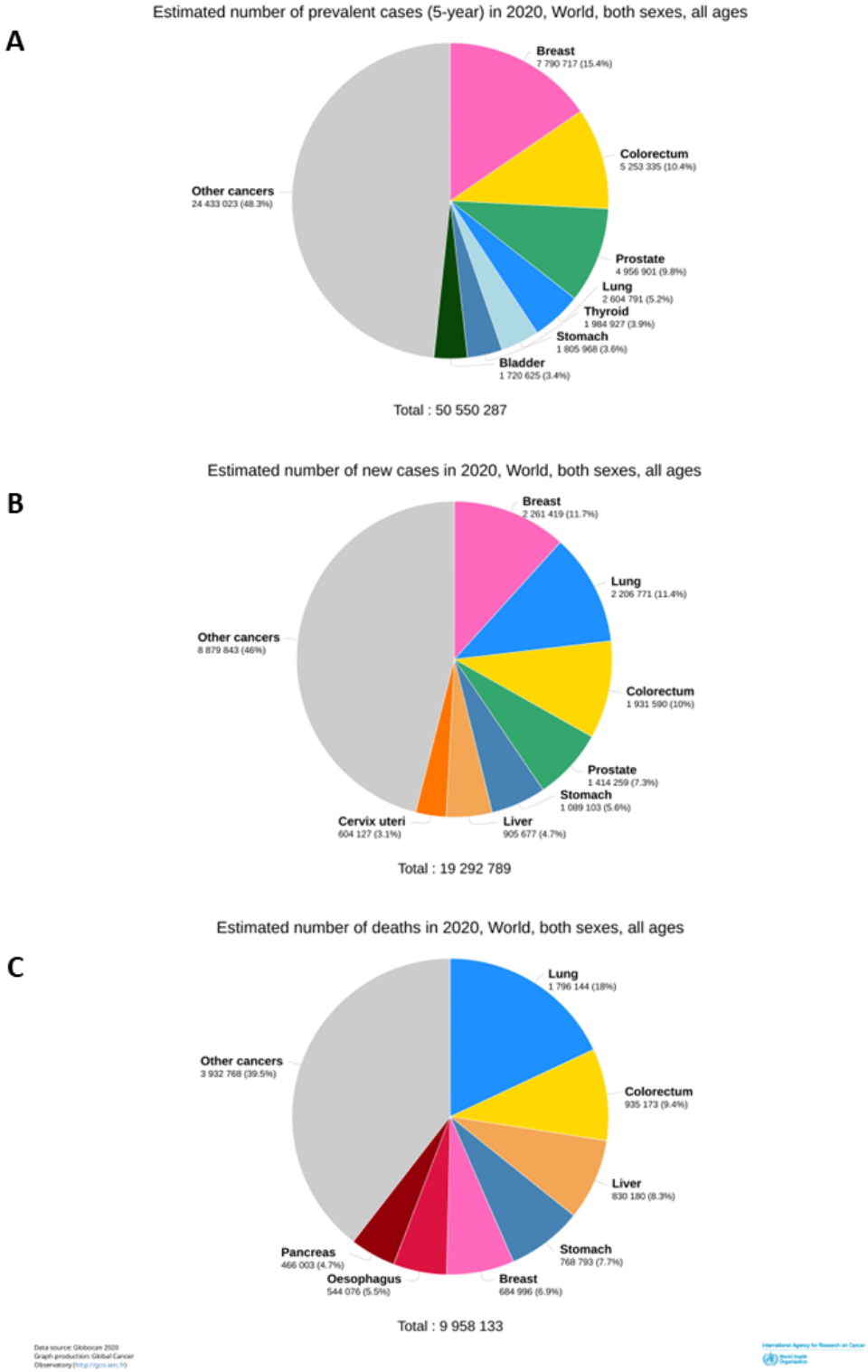

Figure 1. Prevalence, Incidence and Mortality by cancer. Lung cancer is shown in light blue. (**A**) Estimated number of cases for the most prevalent cancers; (**B**) Number of new cases (incidence) of cancer; (**C**) Estimated number of cancer-related deaths. Charts show worldwide data from both genders and for all ages for the year of 2020 (1). Accessed on: Dec., 7th 2022**.**

1.2 Histological Classification

According to the WHO, there are two main types of lung cancer: non-small cell lung cancer (NSCLC), which accounts for approximately 85% of cases, and small cell lung cancer (SCLC), which accounts for about 15% of cases. Among the NSCLC types, the most common are lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) (3,4).

1.2.1 Lung Adenocarcinoma

Lung Adenocarcinoma (LUAD) is the most common subtype of lung cancer, comprising 40% of NSCLC cases. In the lungs, adenocarcinoma arises from alveolar cells, which are located in the epithelium of the smaller airways, and tumor cells express the thyroid transcription factor-1 (TTF1) or napsin A (3,4) immunohistochemical markers. The presence of mucin granules (increased mucus production) in the cell cytoplasm (more than 5 cells in the field of view) also serves to classify lesions as adenocarcinoma (3).

The study by Noguchi *et al*. (10) described six subtypes of LUAD in 1995. This study became the basis for the most recent histological tumor classifications defined by The International Association for the Study of Lung Cancer (IASLC), The American Thoracic Society (ATS), The European Respiratory Society (ERS), and WHO (11).

Currently, it is accepted that LUAD starts and gradually develops from atypical adenomatous hyperplasia (AAH) to adenocarcinoma *in situ* (AIS), to minimally invasive adenocarcinoma (MIA) and, finally, to invasive cancer (12). AIS is defined as a lesion with a lepidic pattern and less than 3 cm in diameter. MIA measures over 3 cm in diameter (3,4); and invasive adenocarcinoma is classified according to its predominant histological pattern and morphology, with subtypes of adenocarcinoma being lepidic, acinar, papillary, micropapillary, and solid. About 90% of LUAD have mixed histology, and in this case are classified according to the predominant histological subtype (13,14). There are other variations to invasive LUAD that have received their own categories, namely: mucinous, mixed non-

mucinous and mucinous, colloid, fetal, and enteric invasive (14).

It is important to determine the histological pattern of disease, since it has implications in patient prognosis. Upon complete resection, non-invasive lesions such as adenocarcinoma *in situ* or minimally invasive adenocarcinoma, are not expected to recur; the same is observed for patients diagnosed with the lepidic pattern, which have better prognosis. The acinar and papillary subtypes have an intermediate risk of recurrence, while the micropapillary and solid subtypes have a higher risk of recurrence (14).

1.2.2. Lung Squamous Cell Carcinoma

Lung Squamous Cell Carcinoma (LUSC) corresponds to 25-30% of cases and presents a rapid progression and aggressive growth, explaining the diagnosis of advanced stage disease in most patients with squamous cell cancer of the lung (8). The presence of the immunohistochemical markers p40, p63, desmoglein-3 and cytokeratins 5 and 6, together with the absence of keratinization, are useful to confirm the histological subtype of squamous cell carcinoma, as they allow the tumor to be distinguished from adenocarcinomas that have similar morphology (3,4). LUSC can be distinguished in main four subtypes: keratinizing, non-keratinizing, basal cell carcinoma, and pre-invasive lesions (*in situ* squamous cell carcinoma) (3).

Squamous carcinoma of the lungs originates in the epithelial airway cells, which suffer morphological alterations usually associated with chronic inflammation, progressing from basal cell hyperplasia (BCH), squamous metaplasia (SM), grade I–III dysplasia, and carcinoma *in situ* (CIS) (3,4,8).

Basal Cell Hyperplasia occurs when there are three or more layers of basal cells and ciliated cells, with absence of goblet cells. The substitution of the cylindrical ciliated epithelium by the squamous epithelium is defined as the SM. Both subtypes, BCH and SM appeared commonly in smokers and in patients with COPD. Interestingly, these early, pre-neoplastic lesions may be reversible by cessation of exposure, as they result from physiological responses to damages

caused by air pollution, smoking, and inflammation. In fact, patients with pulmonary inflammatory diseases affecting the respiratory epithelium and/or exposed to high rates of air pollution frequently present SM (8).

Regarding dysplastic lesions, there are distinct grades of dysplasia: grade I (mild), which shows minimal abnormalities, slightly enlarged cells, vertically oriented nuclei, and rare mitoses; II (moderate), which presents more evident alterations, and mitoses limited to the lower third of epithelial layer; and III (severe), which exhibit cellular pleomorphism, basilar zone expanded with cellular crowding into the upper third, and mitoses confined to the lower two thirds of the airway epithelial layer (8).

1.3. Diagnosis, Staging, and Treatment

The differential diagnosis of lung cancer subtypes is defined by histopathological analysis, based on cell morphology and detection of immunohistochemical markers in tissue samples (biopsies). The clinical outcome of patients with NSCLC is strongly related to the stage of the disease at the time of diagnosis, evidencing the importance of an early diagnosis. Besides, the histological diagnosis is associated with several molecular alterations, evidencing the importance to differentiate the histological subtypes (3).

Mostly, the symptoms of lung cancer are nonspecific, the most common being cough, followed by hemoptysis, chest pain and dyspnea; other less common symptoms are laboratory alterations or paraneoplastic syndromes (4).

Reliable biomarkers for early disease detection have not been identified yet. However, several studies have demonstrated that miRNAs have a potential value as diagnostic biomarkers. The detection of miRNAs in serum was associated with image exams, such as tomography, and showed increased diagnostic performance (15,16).

Disease staging follows the TNM staging system (tumor, nodule, metastasis) and it is performed at the time of diagnosis, to guide treatment decisions (4).

Regarding therapeutic options for patients with lung cancer, surgery is an

effective treatment for early disease stages and depending on patient status, which determine eligibility to surgery. However, disease recurrence may be observed, with a 5-year overall survival varying from 83% to 36% for stage IA to stage IIIA disease, respectively. For patients with unresectable tumors, the standard treatment is the association of thoracic radiation and cytotoxic therapy (9). In patients with advanced disease stage, treatment is mainly based on combinations of platinum, such as cisplatin, with other cytotoxic agents (9).

In recent decades, treatment for lung cancer has evolved significantly, from the use of nonspecific cytotoxic therapies to more precise targeted treatments. Novel treatment strategies are directed towards histological and molecular subtypes of disease, often determined by a panel of mutations specific to LUAD, with therapies still under development for LUSC. The use of target therapies for lung cancer treatment started by the end of the 1990s, with the development of gefitinib, a tyrosine kinase inhibitor (TKI) targeting *EGFR* driver mutations. Another TKI targeting *EGFR* mutations, erlotinib, improved survival of patients with advanced NSCLC. Furthermore, the identification of new driver mutations led to development of new target therapies, targeting *ROS1* fusions, *BRAF* mutations, and *ALK* rearrangements (9).

Another class of therapies that are showing promising results in NSCLC are the immunotherapies, which consist of using immune checkpoints blockades, such as monoclonal antibodies targeting PD1/PD-L1 and CTLA-4. These treatments stimulate immune response against the tumor. Examples of FDA-approved immunotherapies for the NSCLC treatment are nivolumab and pembrolizumab, both anti-PD1. Immunotherapy has demonstrated an improvement in patient overall survival (9). However, there is still a need for the identification of biomarkers to improve disease treatment, since not all patients respond to targeted therapies and immunotherapies.

1.4 Molecular Classification

Similarly, to other solid malignancies, lung cancer is composed of cellular

subpopulations that may contain distinct molecular characteristics, defined as tumor heterogeneity (9). Such features influence molecular diagnosis and treatment decisions, as well as and the arising of drug resistance. Furthermore, a percentage of mutations in neoplastic cell subclones may be associated with disease recurrence in patients with localized lung adenocarcinoma (17). The understanding gained from previous scientific studies led to advancements in clinical practice for treating lung cancer. Previously reliant on broad cellular cytotoxic agents, therapeutic approaches have included personalized medicine tailored based on genetic alterations. In LUAD, molecular diagnostics have resulted in more effective treatments (9).

The epidermal growth factor receptor (*EGFR*) and the Kirsten rat sarcoma (*KRAS*) are among the most frequent gene mutations identified in LUAD. Interestingly, these alterations are generally found in clone-cells denominated "founders", since they play an important role in tumorigenesis and are useful for targeted therapeutics. Usually, such gene mutations are mutually exclusive, that is, if *EGFR* is mutated, *KRAS* is wild type and vice-versa; however, when they coexist, they may confer resistance to treatment with tyrosine kinase inhibitors. Other molecular changes such as *TP53* mutations, are more frequently observed in advanced-stage tumors, suggesting a role in tumor progression (9).

Regarding tumor suppressor genes, the most commonly mutated in LUAD are *TP53*, *KEAP1*, *STK11*, and *NF1*. It has been reported that *KEAP1* inactivation accompanied by *KRAS* mutations may contribute to sensitivity to glutaminase inhibition in preclinical models, showing its potential used for targeted treatment (9).

Commons alterations present in LUSC are *TP53* (>90% of cases) and *CDKN2A* inactivation. *EGFR* amplification has been also reported; however, *EGFR* mutations are not common in the squamous subtype (9).

Additionally, molecular profiles of lung cancer in smokers is different from that of non-smokers. It is common to find significantly higher frequency of mutations in patients with a history of smoking including *KRAS* and *TP53*

mutations. In non-smokers, it is more common to find changes in actionable driver genes, such as activating mutations in *EGFR* and translocations in *ROS1* and *ALK* (9). In fact, *EGFR* mutations were significantly associated with tumors arising in non-smokers while *KRAS* mutations were significantly associated in lung adenocarcinoma in smoker patients (12).

1.5 microRNAs (miRNAs)

The first miRNA, lin-4, was identified in 1993 by Ambros and colleagues in *Caenorhabditis elegans* (*C. elegans*) (18), and was described as a "small, non-protein-coding RNA, regulating the expression of a protein-coding gene" (18,19). The second, let-7, also discovered in *C. elegans*, was identified seven years later by Ruvkun and colleagues (20). To date, there are 2,654 mature miRNAs annotated in the human genome (21).

miRNAs are small (about 18-22 nucleotides in length), highly conserved, endogenous, non-coding RNAs that act post-transcriptionally to regulate gene expression. Their mechanism of action consists basically to prevent the target messenger RNA (mRNA) translation and stability, blocking protein synthesis. Thereby, miRNAs act in several cellular processes, controlling genes related to cell cycle regulation, proliferation and apoptosis, differentiation, migration, inflammation and immunity, stress response, and metabolism, among other processes. miRNAs are deregulated in several diseases, including cancer, playing an important role in tumorigenesis, including disease development and progression. Moreover, miRNAs have tissue-specific expression patterns (19,22,23).

miRNA biogenesis (canonical pathway) starts in the cell nucleus with the transcription of the miRNA gene, by an RNA polymerase II (Pol II), in a long hairpin structure, named primary miRNA (pri-miRNA). The pri-miRNA is then cleaved by a microprocessor protein complex in a precursor miRNA molecule (pre-miRNA), which contains approximately 70 nucleotides in length, and shapes a stem-loop structure, being capped at 5' and polyadenylated at 3'. This microprocessor complex comprises the DGCR8 (DiGeorge syndrome critical

region gene 8), a double-stranded RNA protein that binds to the RNase III endonuclease Drosha (19,22–24).

The pre-miRNA is then exported from the nucleus to the cytoplasm through Ran/GTP/exportin-5 (EXP-5). Following, the next cleavage by Dicer (a type of RNase III protein) generates a duplex (miRNA-3p/miRNA-5p) with approximately 18-22 nucleotides in length in each strand, and both may be functional. The mature miRNA strand is finally bound to the Argonaute (Ago) protein to form the RNA-induced silencing complex (RISC), which will bind to the 3' untranslated region (UTR) of a target mRNA, promoting its decaying and suppressing its translation (19,22,23).

The majority of miRNA genes are mapped in the human genome in intronic clusters in regions with protein-coding pre-mRNAs, some others may be transcribed as autonomous gene units, besides other miRNAs that can be encoded in long non-coding RNAs (lncRNA) (22).

The binding between miRNAs and target mRNAs is through base complementarity. Base pairing between miRNA and mRNA occurs in the seed region, which usually comprises the nucleotides 2 to 7. It has been demonstrated that only the miRNA seed sequence inside the AGO protein is accessible to bind to the target mRNA, being the seed region important to stabilize the pairing (25). Commonly, partial complementarity between miRNA and target mRNA leads to mRNA destabilization and translational repression (19,25).

1.6 miRNAs in lung cancer

All steps of miRNA biogenesis can be altered in cancer. Alterations include genomic changes such as amplifications, deletions, or translocations, point mutations, and changes in the expression of proteins and enzymes involved in miRNA biogenesis. Such alterations can lead to deregulated miRNA expression, and as a consequence miRNA-target gene expression in cancer (19,23,26).

miRNAs can have roles as oncogenes (oncomiRs) or as tumor suppressors (oncosuppressor miR), having as target genes, tumor suppressors or oncogenes,

respectively. An example of an oncomiR is miR-21, which is upregulated in the vast majority of tumors, with roles in cell proliferation and migration, and inhibition of apoptosis, helping in tumor maintenance, growth, and survival. On the other hand, a classical example of an oncosuppressor miRNA is let-7, which acts by targeting *RAS* or *MYC* genes, with roles in cancer development (19,23).

Several studies have demonstrated that miRNAs are deregulated in lung cancer cells, tissues, and body fluids. Examples of deregulated miRNAs in lung cancer include miR-205-3p, miR-205-5p, and miR-21-3p. The first two were found upregulated in NSCLC tissues and serum, with expression levels significantly higher in LUSC than LUAD; while all three miRNAs were upregulated in patient serum. Also, a significant correlation was found between relative expression levels of miR-21-3p and miR-205-3p in tissues and serum. In this same study, a potential use of miR-205-5p as a biomarker for NSCLC was suggested (27). Another study analyzed the role of miR-205-3p in cell lines, demonstrating its importance to promote cellular viability and apoptosis inhibition, by targeting the amyloid β precursor protein-binding family B member 2 (APBB2) (28).

Among miRNAs correlated with advanced disease stage in NSCLC, miR-10a-5p and miR-196a-5p were upregulated in tissues and serum, and both showed a positive correlation with lymph node metastasis (29).

Upregulation of miR-4652-5p was negatively correlated with expression of *RND1* (Rho family GTPase 1) gene in LUSC. This study suggested that RND1 under-expression, due to high miR-4652-5p levels, contributes to cell proliferation, migration, and invasion, also affecting cell adhesion, and promoting disease progression (30).

It is known that cancer development and progression occur due to a series of complex events, known as the hallmarks of cancer: (I) self-sufficiency in growth signals, (II) insensitivity to anti-growth signals, (III) tissue invasion, and metastasis, (IV) limitless replicative potential, (V) sustained angiogenesis, and (VI) evading apoptosis (31). In 2011, Hanahan & Weinberg reported additional properties of tumor cells, including more complexity to the cancer hallmarks. These were four

additional characteristics: (VII) avoiding immune destruction, (VII) tumor-promoting inflammation, (IX) genome instability and mutation, and (X) deregulating cellular energetics (32). In 2022, Hanahan reported other four important hallmarks of cancer: (XI) non-mutational epigenetic reprogramming, (XII) polymorphic microbiomes, (XIII) senescent cells, and (XIV) unlocking phenotypic plasticity (33). Of note, miRNAs were associated with each of the cancer hallmarks, highlighting the importance of these molecules in tumor development, tumor maintenance, and progression (34).

1.7 novel microRNAs

Several studies (quoted below) have indicated the existence of novel miRNAs in the human genome. In 2019, Alles *et al*. published a study suggesting a real estimate of the number of human miRNAs. They analyzed 28,866 small RNA sequencing data sets and, after northern blotting validation, they extrapolated 2,300 true human mature miRNAs, of which 1,115 were annotated on miRBase (35). Another large study using 13 different human cell lines analyzed 1,323 short RNA sequencing samples (RNA-seq) and identified 3,707 novel miRNA mature sequences. They also demonstrated that these novel sequences showed a specific tissue expression profile (36).

Furthermore, the presence of novel miRNAs has been demonstrated in several cancer types. In malignant B cells, 286 novel miRNAs were identified in a study performed with 31 samples of tumor and normal B cells (37). Novel miRNAs were also identified in breast, bladder, colon, and lung tumors (n=23 samples from each tumor type), and 12 novel miRNAs were validated (38). In addition, 146 novel miRNAs were reported in head and neck squamous cell carcinoma (HNSCC) (n=43 paired samples of tumor and normal) (39). In papillary thyroid carcinoma (PTC) (n=504 tumor samples; n=59 normal tissues), 234 novel miRNAs were identified (40). In gastric adenocarcinoma, 143 novel miRNA sequences were significantly deregulated (n=434 tumor samples; n=41 normal samples) (41). Finally, in breast cancer cell lines, 189 novel miRNA candidates were described (42). In normal liver

tissues, 103 novel miRNAs were identified, and suggested to have a potential role in hepatocellular carcinoma (n=47) (43).

The existence of novel miRNAs has also been explored in other diseases. In brain tissues of patients with Huntington's disease (n=28) and Parkinson's disease (n=29), 99 novel miRNAs were identified, and suggested to play a role in disease pathogenesis (44).

Some evidence showed that these novel miRNAs have a low expression pattern in tissues, which can explain why they were not identified up to now. Experiments with MCF-7 breast cancer cells lines demonstrated that the expression of these transcripts was reduced when Dicer was knocked-down, supporting that these sequences are participating of the canonical miRNA's biogenesis. Moreover, it was also possible to verify that these novel sequences have the same evolutive origin of the known miRNAs, however, the novel sequences presented an evolutive origin more recent than the known ones (45).

Mostly, known miRNAs are commonly located in intronic or intergenic regions, on the other hand, the novel ones were more present in exons and repeats regions. The target genes prediction also uncovered that the novel miRNAs are capable to bind their target mRNAs with higher affinity and with more strength than the known miRNAs (45).

All of the above studies support the hypothesis of the existence of novel miRNAs, but also highlight the need for validation and additional investigation about the function of novel miRNAs in disease, considering the different cellular contexts and different tissues. It is important to highlight that most studies did not include a large number of samples, neither showed validation of their results in clinical samples.

1.8 Cancer metabolism and microRNAs

Pavlova and Thompson proposed six cancer metabolic hallmarks: (I) deregulated uptake of glucose and amino acids, (II) use of opportunistic modes of nutrient acquisition, (III) use of glycolysis/TCA cycle intermediates for

biosynthesis and NADPH production, (IV) increased demand for nitrogen, (V) alterations in metabolite-driven gene regulation, and (VI) metabolic interactions with the microenvironment. The cancer cells can show some or all of these hallmarks (46).

Deregulating cellular energetics is a cancer hallmark, once the cancer cells uncontrollably proliferate with a need for energetic compensation to support the high rate of cell division. This energetic disbalance occurs mainly because the cancer cells, even in the presence of oxygen, metabolize glucose in the glycolysis pathway, without using mitochondria, even if this means a lower ATP production (32,46). This reprogramming was first observed by Otto Warburg and since then it has been known as the "Warburg effect" (47) or "aerobic glycolysis" (46).

Aerobic glycolysis produces a small amount of ATP (not good to support the high proliferation rate and energetic requirement by cancer cells); however, it generates other metabolic intermediates that will support the energetic demand, by alternating between aerobic glycolysis and oxidative phosphorylation (48).

It has been demonstrated that cancer cells upregulate the glucose transporters, mainly GLUT1, increasing the influx of glucose to the cytoplasm. The glucose supply is correlated with oncogenes, such as *RAS* and *MYC*, and mutated tumor suppressor genes. Such alterations are beneficial to cancer cells once they give them proliferation competence and ability to evade apoptosis. Also, within tumors there are hypoxic conditions, which contribute to increasing the dependence on glycolysis. The real reasons behind this glycolytic switch in cancer cells are poorly understood; however, some hypotheses were raised: first, with the increased glycolysis, there is the deviation of several glycolytic intermediates that act in many biosynthetic pathways, and this can contribute to synthesizing novel macromolecules and organelles for nascent cells. Second, in some normal conditions when it is necessary a fast cell division such in embryonic tissues, it is possible to observe the Warburg-like metabolism (32).

Glutamine metabolism has also been highlighted as a metabolic pathway associated with oncogenesis. Glutamine is important once it provides nitrogen for

biosynthesis of non-essential amino acids, nucleotides, and glucosamine-6-phosphate. Thus, cancer cells present a high consumption of glutamine, again, to support the high proliferation rates (46). Overall, metabolic alterations were reported in LUAD and LUSC, with differences according to the histological subtype. LUSC showed differential glutamine and glucose catabolism when compared to LUAD and normal tissues (49).

Additionally, lung cancers showed upregulation of glycine/serine/threonine and pyrimidine pathways and, consequently, a low level of threonine and histidine both utilized in those pathways. Lipid metabolism is also altered in patients with lung cancer. Choline is a precursor to membrane phospholipids largely used by proliferation cells, and it showed low levels in the serum of patients with lung carcinoma (50).(51)

Several miRNAs have been previously associated with metabolism reprogramming in lung cancer. Examples of altered miRNAs include miR-144 and miR-124, which play roles in glucose metabolism by targeting GLUT1 (51), HK2, p-Akt1/2 (52), and also enhancing the Warburg effect and ATP generation, respectively. Furthermore, miR-33b was associated with lipid metabolism, targeting LDHA, and enhancing aerobic glycolysis and tumor invasion (53). These and other miRNAs associated with lung cancer metabolism were reviewed (48) and are summarized in **Table 1.**

Table 1. microRNAs deregulated in lung cancer and associated with metabolic pathways*.

Down-regulated microRNAs				
microRNA	**Target Gene(s)**	**Metabolic Pathway**	**Function(s)**	**Reference**
miR-29a	*ENO1*, *PGAM1*, *PGK1*, *CMPK1*	Glucose metabolism	Facilitates glycolysis	(54)
miR-33b	*LDHA*	Lipid metabolism	Enhances aerobic glycolysis and tumor invasion	(53)
miR-124	*GLUT1*, *HK2*, *p-Akt1/2*	Glucose metabolism	Enhances Warburg effect and ATP generation	(52)
miR-126	*SLC7A5*	Amino acid metabolism	Facilitates amino acid exchange and activate mTOR	(55)
miR-133b	*PKM2*	Glucose metabolism	Enhances Warburg effect and radiation resistance	(56)
miR-143	*HK2*	Glucose metabolism	Enhances Warburg effect	(57)
miR-144-3p	*GLUT1*	Glucose metabolism	Enhances Warburg effect	(51)
miR-206	*HK2*	Glucose metabolism	Enhances Warburg effect	(58)
miR-451	*c-Myc*, *GSK3*	c-Myc	Enhanced metabolic dysregulation and migration	(59)
Up-regulated microRNAs				
microRNA	**Target Gene**	**Metabolic Pathway**	**Function(s)**	**Reference**
miR-21	*HBP1*, *CD36*, *SOCS1*, *SOCS6*, *PTEN*	Lipid metabolism and PI3K/Akt/PTEN	Promotes proliferation and increase intracellular lipid influx; PTEN inhibition	(60,61)
miR-31-5p	*FIH*	Hypoxia	Promotes hypoxia and enhances aerobic glycolysis	(62)
miR-34	*SIRT1*	Hypoxia	Facilitate mutant p53 expression via a feedback loop	(63,64)
miR-125b	*p-Akt*, *GSK3*	PI3K/Akt/PTEN	PI3K/Akt and GSK3/Wnt/-catenin activation	(65)
miR-155	*SOCS1*, *SOCS6*, *PTEN*	PI3K/Akt/PTEN	PTEN inhibition	(60)
miR-182	*PDK4*/*PDH* axis	Lipid metabolism	Enhances de novo lipogenesis and JNK activation	(66)
miR-199-5p		Hypoxia	Activates HIF-1	(67)
miR-210	*NDUFA4*, *SDHD*	Hypoxia	Mitochondria disfiguration and cristae formation	(68)

*Reviewed in (48).

8. CONCLUSIONS

We identified 13 potential novel miRNAs in lung cancer and normal lung tissues; novel sequences have similar molecular characteristics regarding structure and composition when compared to known miRNAs: GC content and length of sequences.

Novel miRNAs were able to differentiate tumors from non-malignant tissues. Additionally, we predicted the target genes which are regulated by these potential novel miRNAs and related them to several metabolic pathways, which have been reported in the literature as deregulated in lung cancer. Such findings indicate that novel miRNAs identified in our study may be functional and with relevance to adenocarcinoma and squamous cell subtypes of lung cancer. These findings are important to support the real existence of these novel miRNAs.

Our results provide new insights about miRNAs involved in lung cancer and malignant metabolism.

9. FUTURE DIRECTIONS

In order to expand this study, experimental validation will be performed to demonstrate novel miRNA sequences in lung cancer. Validation experiments will be carried out in clinical samples of LUAD, LUSC, and histologically normal adjacent lung tissues, by quantitative real-time PCR.

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