UNIVERSIDADE ESTADUAL PAULISTA
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Ana Laura Seneda

Perfil de Expressão de microRNAs e Análise Computacional de Vias Moleculares Moduladas por microRNAs em Tumor Carcinoide de Pulmão

Dissertação apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Botucatu, para obtenção do título de Mestra em Bases Gerais da Cirurgia.

Orientadora: Profa. Dra. Patricia Pintor dos Reis

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Palavras-chave: Invasão; MicroRNAs; Sistema imune; Tumor carcinoide de pulmão; Vias neuronais.

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“A tarefa não é tanto ver aquilo que ninguém viu, mas pensar o que ninguém ainda pensou sobre aquilo que todo mundo vê.”

Arthur Schopenhauer

“Devemos acreditar que temos vocação para alguma coisa, e que essa coisa deve, a qualquer custo, ser alcançada.”

Marie Curie

Resumo

Introdução: O tumor carcinoide do pulmão pertence ao tipo neuroendócrino das neoplasias pulmonares. Devido à sua baixa incidência (~2%), pouco se conhece sobre suas alterações moleculares. Os microRNAs (miRNAs) têm importante papel na regulação gênica e têm sido associados ao câncer como biomarcadores diagnósticos, prognósticos e preditivos. **Objetivos:** Determinar o perfil global de expressão de miRNAs em tumores carcinoides do pulmão e identificar (*in silico*) vias moleculares envolvendo os miRNAs desregulados e genes-alvo preditos. **Material e Métodos:** Dois fragmentos de um tumor carcinoide típico e sua metástase correspondente foram obtidos, o RNA extraído de cada amostra e analisado na plataforma *TaqMan Low Density Array* (TLDA), a qual contém sondas para 384 miRNAs. Os dados foram analisados no *Expression Suite software*. Adicionalmente, 7 tumores (5 carcinoides típicos e 2 atípicos) foram utilizados para análise de expressão de 2,578 miRNAs na plataforma GeneChip™ miRNA 4.0 e os dados analisados utilizando o *Transcriptome Analysis Console software*. A análise estatística dos dados foi realizada para identificação dos miRNAs significativamente ($p<0,05$) alterados. Métodos de análise *in silico* incluíram a identificação de mRNAs-alvo dos miRNAs e vias moleculares de tumorigênese. **Resultados e Discussão:** No tumor carcinoide típico e metástase (TLDA), 15 miRNAs estavam com expressão comumente diminuída, os quais regulam genes associados a vias de resposta imune adaptativa. Adicionalmente, a comparação dos carcinoides típicos ou atípicos *vs*. normal (GeneChip arrays) resultou na identificação de 9 miRNAs desregulados em tumores típicos e 21 miRNAs em atípicos ($p<0.01$ and $FDR<0.05$). Em tumores típicos, os miRNAs modulam genes-alvo envolvidos na regulação do receptor *FCℰRI*, *PDGF* e *NGF* via *TRKA*. Nos tumores atípicos, os miRNAs alterados regulam genes associados à resposta imune inata e adaptativa e mecanismos de desenvolvimento e diferenciação neuronal. **Conclusões:** (1) a expressão diminuída de um conjunto específico de 15 miRNAs deve modular mecanismos de resposta imune e invasão associados ao desenvolvimento e progressão de um caso raro de carcinoide típico metastático; (2) os carcinoides típicos e atípicos apresentam diferentes perfis de expressão de miRNAs e vias moleculares de tumorigênese. Esses dados contribuem para o melhor entendimento de alterações em miRNAs e vias moleculares associadas aos tumores carcinoides de pulmão.

Palavras-chave: tumor carcinoide de pulmão, microRNAs, sistema imune, invasão, vias neuronais.

Abstract

Introduction: Lung carcinoid tumors are a type of neuroendocrine lung neoplasia. Due to its low incidence (~2%), little is known about the molecular alterations associated with these tumors. microRNAs (miRNAs) have an important role in gene regulation and have been associated with cancer as diagnostic, prognostic and predictive biomarkers. **Objectives:** To determine the global expression miRNA profiles of lung carcinoid tumors and to identify (*in silico*) molecular pathways including deregulated miRNAs and predicted target-genes. **Material and Methods:** Two fragments of a typical carcinoid tumor and its corresponding metastasis were obtained, the RNA extracted and analyzed using the TaqMan Low Density Array (TLDA) platform containing 384 miRNAs. Data were analyzed using *Expression Suite software*. Additionally, 7 tumors (5 typical and 2 atypical carcinoids) were used for expression analysis of 2,578 miRNAs in the GeneChip™ miRNA 4.0 platform and data were analyzed using the Transcriptome Analysis Console software. Statistical analysis was performed to identify the significantly ($p<0,05$) deregulated miRNAs. *In silico* analyses methods included the identification of miRNA target genes (and enriched pathways. **Results and Discussion:** In the typical carcinoid tumor and metastasis (TLDA data), 15 miRNAs were commonly down-regulated and these modulate genes associated with the adaptive immune system pathway. Additionally, the comparison of typical carcinoids or atypical *vs.* normal (GeneChip arrays) showed 9 deregulated miRNAs in typical tumors and 21 miRNAs in atypical ($p<0.01$ and $FDR<0.05$).In typical tumors, identified miRNAs modulate the expression of target genes involved in the regulation of receptor *FCƐRI*, *PDGF* e *NGF* via *TRKA*. In the atypical tumors, altered miRNAs regulate genes with roles in adaptive and immune response and neuronal development and differentiation. **Conclusions:** (1) the 15 down-regulated miRNA subset may modulate mechanisms of immune response and invasion, associated with the development and progression in a rare case of metastatic carcinoid typical tumor; (2) typical and atypical lung carcinoids have different miRNA expression profiles and tumorigenesis pathways. These data contribute to our better understanding of miRNA alterations and pathways associated to lung carcinoid tumors.

Key-words: lung carcinoid tumor, microRNAs, immune system, invasion pathways.

Lista de abreviaturas e siglas

3'UTR – Região 3' não-traduzida; 3' untranslated region

AC – Carcinoide Atípico; Atypical Carcinoid

AJCC – American Joint Committee on Cancer

AKT – AKT serine/threonine kinase

ALK – ALK receptor tyrosine kinase

BAX – BCL2 associated X, apoptosis regulator

BCL2 – BCL2, apoptosis regulator

BTLA – B and T lymphocyte associated

CD200 – CD200 molecule

CD200R – CD200 receptor

CD28 – CD28 molecule

CDC42 – Cell Division Cycle 42

CDK – Cyclin Dependent Kinase (2 and 6)

CDKN1B – Cyclin Dependent Kinase Inhibitor 1B

CTLA-4 – Cytotoxic T-lymphocyte Associated Protein 4

E2F1 – E2F Transcription Factor 1

EGFR – Epidermal Growth Factor Receptor

ERK – MAPK1 Mitogen-activated Protein Kinase 1

FC – Fold Change

FCƐRI – Fc epsilon fragment of IgE receptor I

FDR – False Discovery Rate

FFPE – Tecido fixado, embebido em formalina; Formalin-fixed, paraffin-embedded

GEO – Gene Expression Omnibus

H&E – Hematoxilina e Eosina; Hematoxilin and Eosin

HMGA2 – High Mobility Group AT-hook 2

IASLC – Associação Internacional para o Estudo do Câncer de Pulmão; International Association for the Study of Lung Cancer

IgE – Immunoglobulin E

IKBKB – Inhibitor of Nuclear Factor kappa B Kinase Subunit Beta

MAPK – Mitogen-activated Protein Kinase 1

MHC II – Major Histocompatibility Complex, class II

MIEN1 – Migration and Invasion Enhancer 1

mirDIP – microRNA Data Integration Portal

miRNA – microRNA

MMP9 – Matrix Metallopeptidase 9

mRNA – RNA mensageiro; Messenger RNA

NF-κB – Nuclear Factor kappa B

NGF – Nerve Growth Factor

NSCLC – Câncer de pulmão de células não pequenas; Non-small cell lung cancer

p27 – p27 protein

PD-1 – Programmed Cell Death 1

PDGF – Platelet Derived Growth Factor (A, B, C and D)

PDGFR – Platelet Derived Growth Factor Receptor

PI3K – Phosphatidylinositol-4,5-bisphosphate 3-kinase

PTEN – Phosphatase and Tensin Homolog

QC – Controle de Qualidade; Quality Control

RAP2B – RAP2B, Member of RAS Oncogene Family

RAS – RAS Proto-oncogene, GTPase

RNA – Ácido Ribonucleico; Ribonucleic Acid

SCGN – Secretagogin, EF-hand Calcium Binding Protein

SEER – Surveillance, Epidemiology and End Results Program

SOCS1 – Suppressor of Cytokine Signaling 1

STAT3 – Signal Transducer and Activator of Transcription 3

TC – Carcinoide Típico; Typical Carcinoid

TCGA – The Cancer Genome Atlas

TLDA – TaqMan Low Density Array

TNF – Tumor Necrosis Factor

TNM – Tumor, Nódulo, Metástase; Tumor, Nodule, Metastasis

TRAF6 – TNF Receptor Associated Factor 6

TRK – Neurotrophic Receptor Tyrosine Kinase (A, B and C)

VEGFA – Vascular Endothelial Growth Factor A

Lista de símbolos

~ aproximadamente

< menor que

> maior que

≥ maior ou igual que

™ – Marca Registrada; Trade Mark

ε – epsilon

κ – kappa

Sumário

1 Introdução

De acordo com dados do GLOBOCAN (2018), o câncer de pulmão é o mais incidente e o maior em número de mortes em todo o mundo. O câncer de pulmão de células não pequenas representa a maioria dos casos (~85%), enquanto que o de células pequenas representa ~15%. O câncer de pulmão é mais comumente diagnosticado em pacientes com idade igual ou maior a 65 anos, sendo que o consumo excessivo de tabaco é um dos principais fatores de risco (1).

Os tumores neuroendócrinos compreendem um grupo heterogêneo de neoplasias que se originam de células neuroendócrinas, acometendo mais comumente os pulmões, intestino liso e reto (2). Os tumores neuroendócrinos pulmonares que ocorrem como células individuais ou pequenos clusters, também denominados de corpos neuroepiteliais (3), compreendem aproximadamente 25% das neoplasias pulmonares primárias (4).

Os tumores neuroendócrinos pulmonares compreendem quatro subtipos: (a) carcinoide típico bem diferenciado de baixo grau (2% das neoplasias pulmonares); (b) carcinoide atípico bem diferenciado de grau intermediário (<1%), (c) carcinoma de grandes células pobremente diferenciado de alto grau (3%) e (d) carcinoma de células pequenas pobremente diferenciado de alto grau (20%) (4,5).

Os subtipos carcinoide típico e atípico de tumores neuroendócrinos do pulmão são o foco da nossa pesquisa.

Ao contrário dos outros tipos de câncer de pulmão, os tumores neuroendócrinos são mais comuns em pacientes jovens e não estão na sua maioria associados ao tabagismo (5). Mesmo sendo considerados tumores menos frequentes, sua incidência vem aumentando nos últimos anos (6). Entretanto, ainda há um baixo reconhecimento desses tumores pelos especialistas os quais estão diretamente envolvidos no diagnóstico e tratamento dos pacientes. Este é um fator importante a ser considerado, visto que o diagnóstico corretamente aplicado e realizado precocemente está associado à implementação de estratégias de tratamento adequadas e tem impacto prognóstico (7).

Aproximadamente 2/3 dos tumores carcinoides se desenvolvem nos brônquios principais. Dessa forma, os sintomas clínicos mais comuns associados com o desenvolvimento desses tumores incluem dor torácica, dispnéia, tosse, a qual pode ou não ser acompanhada de sangue e pneumonia obstrutiva (8).

O diagnóstico é feito com base nas características histopatológicas dos tumores, contagem do número mitoses, presença ou ausência de necrose e positividade para

marcadores de imunohistoquímica específicos para células neuroendócrinas, como sinaptofisina e cromogranina A (9,10).

Quanto às características histopatológicas desses tumores, os carcinoides típicos são de baixo grau e os atípicos são de grau intermediário. Embora esses tumores possam ser diagnosticados em análises citológicas a partir de lavado brônquico ou por meio de biópsia, é difícil distinguir histologicamente os tumores típicos dos atípicos. O diagnóstico preciso geralmente requer análise histológica de um fragmento tumoral retirado por meio de biópsia ou cirurgia. A morfologia dos tumores carcinoides típicos e atípicos é similar com uma população uniforme de células tumorais organizadas em nichos organoides com uma quantidade moderada de citoplasma com tonalidade eosinofílica. Existe uma variedade de padrões histológicos predominantes desses tumores, incluindo células fusiformes, padrão oncocítico, glandular, folicular, células claras e melanocítico. Os carcinoides típicos apresentam menos de 2 mitoses por 2 mm² e ausência de necrose, enquanto os atípicos mostram um número aumentado de mitoses (2-10 mitoses por 2 mm²) e necrose, sendo que a presença de um grande número de figuras mitóticas é a característica mais importante para distinção dos subtipos típico e atípico (4,9,10).

Entretanto, a classificação e diagnóstico dos tumores carcinoides típicos e atípicos ainda são complexas, devido a fatores etiológicos não bem estabelecidos e sintomas inespecíficos da doença (7).

Os carcinoides atípicos têm maior probabilidade de recorrência ou aparecimento de metástases quando comparados com os tumores típicos, nos quais os pacientes têm melhor prognóstico (11,12). Dos tumores carcinoides, 10-30% são atípicos (8).

O estadiamento dos tumores carcinoides pulmonares primários é realizado de acordo com a classificação TNM (Tumor, Nódulo, Metástase). A Associação Internacional para o Estudo do Câncer de Pulmão (IASLC) aprovou essa classificação em 2009 quando foi determinado que o sistema de estadiamento TNM era útil para predizer o prognóstico de pacientes com tumores carcinoides (13). A aplicação desse sistema de estadiamento a casos registrados no Instituto Nacional do Câncer (*Surveillance, Epidemiology and End Results Program* – banco de dados SEER) determinou que a sobrevida de 5 anos dos pacientes com doença de estadiamento I é de 93%, estadiamento II de 74%, estadiamento III de 67-75% e estadiamento IV de 57% (13). Vários estudos indicam que a maioria dos pacientes com carcinoides típicos apresentam estadiamento I de doença (até 90% dos casos) enquanto os pacientes com carcinoides atípicos apresentam doença em estadiamento avançado (8).

A sobrevida é significativamente melhor para os pacientes com tumores típicos comparado com atípicos. Para pacientes com carcinoides típicos, a sobrevida de 5 e 10 anos é estimada em 87% e 86%, respectivamente e para aqueles com carcinoides atípicos, de 56% e 35%, respectivamente (14). Os fatores preditivos de sobrevida incluem o estadiamento, tamanho do tumor, presença de altos índices mitóticos (no subtipo atípico) e idade superior a 60 anos (11,13).

A cirurgia é a modalidade de tratamento primário e até o momento constitui a única opção curativa para pacientes com tumores carcinoides típicos e atípicos. Devido à localização central dos tumores, na maioria dos casos, a pneumonectomia ou bilobectomia é frequente, entretanto a maioria dos pacientes é submetida à lobectomia (15). Há um consenso na literatura que recomenda a dissecção completa de linfonodos no mediastino e, quando possível, a ressecção de linfonodos metastáticos (12), sendo associada à diminuição de recorrência local e melhoria da sobrevida (16). Entretanto, alguns autores consideram que há falta de consenso entre os protocolos de tratamento e manejo adequados de pacientes com tumores pulmonares neuroendócrinos, principalmente no contexto de doença não ressecável cirurgicamente e ou metastática, devido à falta de evidência clínica (7).

A terapia adjuvante para o tratamento dos tumores carcinoides ainda é questionável, principalmente devido à falta de dados. Outro fator importante é que, normalmente, eles apresentam resistência tanto à quimio- quanto à radioterapia. Ainda assim, a terapia adjuvante é indicada em casos de ressecção incompleta dos tumores ou quando há envolvimento de linfonodos do mediastino e das margens teciduais (17).

Alguns estudos têm sido realizados com o objetivo de encontrar novas terapias. Yao *et al* mostraram que a terapia com everolimus foi capaz de reduzir o risco de progressão da doença (18). Outro estudo, utilizando linhagens celulares de tumores carcinoides, mostrou que a combinação de octreotide, cabergolina e inibidores de mTOR foi capaz de reduzir a viabilidade celular e a fosforilação de Akt e ERK (19).

A maioria dos estudos sobre as bases moleculares do câncer de pulmão tem sido realizada em carcinomas de células não pequenas e levaram à identificação principalmente de mutações em genes condutores da tumorigênese (20). Muitos dos genes identificados levaram ao desenvolvimento de terapias mais precisas para os pacientes diagnosticados com o subtipo adenocarcinoma pulmonar, tais como os inibidores tirosina-quinase para mutações no receptor do fator de crescimento epidérmico (*EGFR*) (21).

Embora as características histopatológicas dos tumores carcinoides estejam bem descritas, a literatura é escassa em relação às alterações moleculares (8,22–30). Alguns estudos ainda mostram que essas alterações não são as mesmas encontradas em outros tipos de câncer de pulmão (26,28,31). Da mesma forma, os dados sobre microRNAs (miRNAs) em tumores carcinoides também são escassos (32–38).

A literatura evidencia que é necessária a realização de estudos sobre os mecanismos moleculares associados aos tumores carcinoides. Tais estudos devem levar à elucidação das vias moleculares implicadas no desenvolvimento e progressão neoplásica. Adicionalmente, podem contribuir para a identificação de biomarcadores para melhorar o valor diagnóstico, prognóstico ou tratamento dos pacientes.

Os miRNAs têm sido amplamente associados com processos de desenvolvimento e progressão de inúmeros tipos de câncer. Os miRNAs são RNAs pequenos, de ~18 a 22 nucleotídeos de comprimento, não codificam proteínas e são potentes reguladores da expressão gênica. O mecanismo de ação dos miRNAs é principalmente pela ligação à extremidades 3’ não traduzida (3’ UTR) do RNA mensageiro (mRNA), geralmente levando à inibição da tradução ou à degradação do mRNA (39).

Os miRNAs estão envolvidos em muitos processos biológicos importantes, tais como o desenvolvimento embrionário, diferenciação, apoptose e proliferação celular (40–42) e em mecanismos de oncogênese (43–45). Os miRNAs têm sido indicados como candidatos ideais a biomarcadores diagnósticos, prognósticos e como alvos terapêuticos potenciais no câncer (46–51).

2 Justificativa

O presente estudo faz-se necessário devido à escassez de dados sobre alterações genéticas e epigenéticas associadas ao desenvolvimento e progressão de tumores carcinoides do pulmão. Considerando que a identificação de vias moleculares condutoras da tumorigênese é de fundamental importância para determinar terapêuticas mais precisas para pacientes com câncer, a realização dessa pesquisa é plenamente justificada.

3 Objetivos

Objetivo 1. Determinar o perfil global de expressão de miRNAsem tumores carcinoides do pulmão;

Objetivo 2. Identificar (*in silico*) os mRNAs-alvo regulados pelos miRNAs e vias moleculares potencialmente condutoras da tumorigênese em tumores carcinoides do pulmão.

O presente trabalho de Dissertação de Mestrado está dividido em dois Capítulos, os quais compreendem os artigos científicos:

Capítulo 1: microRNA downregulation is associated with pathways of adaptive immune response and invasion in a rare metastatic typical lung carcinoid.

Capítulo 2: microRNA expression profiles and prognostic value in typical and atypical lung carcinoid tumors

Referências Bibliográficas

1. Testa U, Castelli G, Pelosi E. Lung cancers: Molecular characterization, clonal heterogeneity and evolution, and cancer stem cells. Cancers (Basel). 2018;10(8):1–81.
2. Yao JC, Hassan M, Phan A, Dagohoy C, Leary C, Mares JE, et al. One Hundred Years After " Carcinoid ": Epidemiology of and Prognostic Factors for Neuroendocrine Tumors in 35 , 825 Cases in the United States. J Clin Oncol. 2008;26(18):3063–72.
3. Filosso, Pier Luigi Asamura H, Brunelli A, Filosso PL, Garcia-Yuste M, Lim E, Papagiannopoulos K, Sarkaria I TP. Knowledge of Pulmonary N e u ro e n d o c r i n e Tu m o r s : W h e re A re We N o w ? Thorac Surg Clin. 2014;24(3):ix–xii.
4. Rekhtman N. Neuroendocrine Tumors of the Lung: An update. Arch Pathol Lab Med. 2010;134(11):1628–38.
5. Travis WD, Brambilla E, Nicholson AG, Yatabe Y, Austin JHM, Beasley MB, et al. The 2015 World Health Organization Classification of Lung Tumors: Impact of Genetic, Clinical and Radiologic Advances Since the 2004 Classification. J Thorac Oncol [Internet]. 2015;10(9):1243–60. Available from: http://dx.doi.org/10.1097/JTO.0000000000000630
6. Sackstein PE, Neil DSO, Neugut AI, Chabot J, Fojo T, Irving H, et al. Epidemiologic trends in neuroendocrine tumors : An examination of incidence rates and survival of specific patient subgroups over the past 20 years. Semin Oncol [Internet]. 2018;45:249–58. Available from: https://doi.org/10.1053/j.seminoncol.2018.07.001
7. Hendifar AE, Marchevsky AM, Tuli R. Neuroendocrine Tumors of the Lung: Current Challenges and Advances in the Diagnosis and Management of Well-Differentiated Disease. J Thorac Oncol [Internet]. 2017;12(3):425–36. Available from: http://dx.doi.org/10.1016/j.jtho.2016.11.2222
8. Fink G, Krelbaum T, Yellin A, Bendayan D, Saute M, Glazer M, et al. Pulmonary carcinoid: Presentation, diagnosis, and outcome in 142 cases in Israel and review of 640 cases from the literature. Chest. 2001;119(6):1647–51.
9. Pelosi G, Sonzogni A, Harari S, Albini A, Bresaola E, Marchiò C, et al. Classification of pulmonary neuroendocrine tumors: New insights. Transl Lung Cancer Res [Internet]. 2017;6(5):513–29. Available from: https://www.ncbi.nlm.nih.gov/pubmed/?term=10.21037%2Ftlcr.2017.09.04
10. Righi L, Gatti G, Volante M, Papotti M. Lung neuroendocrine tumors: Pathological characteristics. J Thorac Dis. 2017;9(Suppl 15):S1442–7.
11. Asamura H, Kameya T, Matsuno Y, Noguchi M, Tada H, Ishikawa Y, et al. Neuroendocrine Neoplasms of the Lung: A Prognostic Spectrum. J Clin Oncol [Internet]. 2006;24(1):70–6. Available from: https://www.ncbi.nlm.nih.gov/pubmed/16382115
12. Thomas CF, Tazelaar HD, Jett JR. Typical and atypical pulmonary carcinoids: Outcome in patients presenting with regional lymph node involvement. Chest [Internet]. 2001;119(4):1143–50. Available from: http://journal.publications.chestnet.org/article.aspx?doi=10.1378/chest.119.4.1143
13. Travis WD, Giroux DJ, Chansky K, Crowley J, Asamura H, Brambilla E, et al. The IASLC lung cancer staging project: Proposals for the inclusion of broncho-pulmonary carcinoid tumors in the forthcoming (seventh) edition of the TNM classification for lung cancer. J Thorac Oncol [Internet]. 2008;3(11):1213–23. Available from: http://dx.doi.org/10.1097/JTO.0b013e31818b06e3
14. Tumors C, Granberg DAN, Wilander E, Kjell O, Skogseid B. Prognostic Markers in Patients with Typical Bronchial. 2000;85(9):0–5.
15. Pusceddu S, Lo Russo G, Macerelli M, Proto C, Vitali M, Signorelli D, et al. Diagnosis and management of typical and atypical lung carcinoids. Crit Rev Oncol Hematol

[Internet]. 2016;100:167–76. Available from: http://dx.doi.org/10.1016/j.critrevonc.2016.02.009
16. Filosso PL, Guerrera F, Thomas P, Brunelli A, Lim E, Garcia-yuste M, et al. Management of bronchial carcinoids : international practice survey among the European Society of Thoracic Surgeons. Futur Oncol. 2016;12(17):1985–99.
17. Marquez-Medina D, Popat S. Systemic therapy for pulmonary carcinoids. Lung Cancer [Internet]. 2015;90(2):139–47. Available from: http://dx.doi.org/10.1016/j.lungcan.2015.08.018
18. Yao JC, Fazio N, Singh S, Buzzoni R, Carnaghi C, Wolin E, et al. Everolimus for the treatment of advanced, non-functional neuroendocrine tumours of the lung or gastrointestinal tract (RADIANT-4): A randomised, placebo-controlled, phase 3 study. Lancet. 2016;387(10022):968–77.
19. Pivonello C, Rousaki P, Negri M, Sarnataro M, Napolitano M, Zito F, et al. Effects of the single and combined treatment with dopamine agonist , somatostatin analog and mTOR inhibitors in a human lung carcinoid cell line : an in vitro study. Endocrine. :0–1.
20. Kris MG, Johnson BE, Berry LD, Kwiatkowski DJ, Iafrate AJ, Wistuba II, et al. Using multiplexed assays of oncogenic drivers in lung cancers to select targeted drugs. JAMA. 2014;311(19):1998–2006.
21. Travis WD, Brambilla E, Riely GJ. New pathologic classification of lung cancer: Relevance for clinical practice and clinical trials. J Clin Oncol. 2013;31(8):992–1001.
22. Asiedu MK, Thomas CF, Dong J, Schulte SC, Khadka P, Sun Z, et al. Pathways impacted by genomic alterations in pulmonary carcinoid tumors. Clin Cancer Res. 2018;24(7):1691–704.
23. Simbolo M, Mafficini A, Sikora KO, Fassan M, Barbi S, Corbo V, et al. Lung neuroendocrine tumours: deep sequencing of the four World Health Organization histotypes reveals chromatin-remodelling genes as major players and a prognostic role for TERT, RB1, MEN1 and KMT2D. J Pathol. 2017;241(4):488–500.
24. Lou G, Yu X, Song Z. Molecular Profiling and Survival of Completely Resected Primary Pulmonary Neuroendocrine Carcinoma. Clin Lung Cancer [Internet]. 2017;18(3):e197–201. Available from: http://dx.doi.org/10.1016/j.cllc.2016.11.014
25. Voortman J, Lee JH, Killian JK, Suuriniemi M, Wang Y, Lucchi M, et al. Array comparative genomic hybridization-based characterization of genetic alterations in pulmonary neuroendocrine tumors. Proc Natl Acad Sci U S A [Internet]. 2010;107(29):13040–5. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2919980/pdf/pnas.201008132.pdf
26. Armengol G, Sarhadi VK, Rönty M, Tikkanen M, Knuuttila A, Knuutila S. Driver Gene Mutations of Non-Small-Cell Lung Cancer are Rare in Primary Carcinoids of the Lung: NGS Study by Ion Torrent. Lung [Internet]. 2015;193(2):303–8. Available from: https://www.ncbi.nlm.nih.gov/pubmed/?term=10.1007%2Fs00408-015-9690-1
27. Gómez JMN, Bernal JFV, Arranz PG, Fernández SL, Roman JJG. Alterations in the expression of p53, KLF4, and p21 in neuroendocrine lung tumors. Arch Pathol Lab Med. 2014;138(7):936–42.
28. Fernandez-cuesta L, Peifer M, Lu X, Sun R, Ozretić L, Seidal D, et al. Frequent mutations in chromatin-remodeling genes in pulmonary carcinoids. Nat Commun [Internet]. 2014;3518(5):1–17. Available from: https://www.ncbi.nlm.nih.gov/pubmed/?term=10.1038%2Fncomms4518
29. Asiedu MK, Thomas CF, Tomaszek SC, Peikert T, Sanyal B, Sutor SL, et al. Generation and sequencing of pulmonary carcinoid tumor cell lines. J Thorac Oncol [Internet]. 2014;9(12):1763–71. Available from:

http://dx.doi.org/10.1097/JTO.0000000000000339
30. Swarts DRA, Claessen SMH, Jonkers YMH, Van Suylen RJ, Dingemans AMC, De Herder WW, et al. Deletions of 11q22.3-q25 are associated with atypical lung carcinoids and poor clinical outcome. Am J Pathol [Internet]. 2011;179(3):1129–37. Available from: http://dx.doi.org/10.1016/j.ajpath.2011.05.028
31. Rossi G, Bertero L, Marchiò C, Papotti M. Molecular alterations of neuroendocrine tumours of the lung. Histopathology [Internet]. 2018;72(1):142–52. Available from: http://doi.wiley.com/10.1111/his.13394
32. Di Fazio P, Maass M, Roth S, Meyer C, Grups J, Rexin P, et al. Expression of hsa-let-7b-5p, hsa-let-7f-5p, and hsa-miR-222-3p and their putative targets HMGA2 and CDKN1B in typical and atypical carcinoid tumors of the lung. Tumor Biol. 2017;39(10):1–8.
33. Yoshimoto T, Motoi N, Yamamoto N, Nagano H, Ushijima M, Matsuura M, et al. Pulmonary Carcinoids and Low-Grade Gastrointestinal Neuroendocrine Tumors Show Common MicroRNA Expression Profiles, Different from Adenocarcinomas and Small Cell Carcinomas. Neuroendocrinology. 2017;106(1):47–57.
34. Demes M, Aszyk C, Bartsch H, Schirren J, Fisseler-Eckhoff A. Differential miRNA-Expression as an adjunctive diagnostic tool in neuroendocrine tumors of the lung. Cancers (Basel). 2016;8(4):1–9.
35. Rapa I, Votta A, Felice B, Righi L, Giorcelli J, Scarpa A, et al. Identification of MicroRNAs Differentially Expressed in Lung Carcinoid Subtypes and Progression. Neuroendocrinology [Internet]. 2015;101(3):246–55. Available from: https://www.ncbi.nlm.nih.gov/pubmed/?term=25791280
36. Deng B, Molina J, Aubry MC, Sun Z, Wang L, Eckloff BW, et al. Clinical biomarkers of pulmonary carcinoid tumors in never smokers via profiling miRNA and target mRNA. Cell Biosci [Internet]. 2014;4(35):1–10. Available from: https://www.ncbi.nlm.nih.gov/pubmed/?term=25105010
37. Mairinger FD, Ting S, Werner R, Walter RFH, Hager T, Vollbrecht C, et al. Different micro-RNA expression profiles distinguish subtypes of neuroendocrine tumors of the lung: results of a profiling study. Mod Pathol an Off J United States Can Acad Pathol Inc [Internet]. 2014;27(12):1632–40. Available from: http://dx.doi.org/10.1038/modpathol.2014.74
38. Lee HW, Lee EH, Ha SY, Lee CH, Chang HK, Chang S, et al. Altered expression of microRNA miR-21, miR-155, and let-7a and their roles in pulmonary neuroendocrine tumors. Pathol Int. 2012;62(9):583–91.
39. Di Leva G, Calin GA, Croce CM. MicroRNAs: Fundamental facts and involvement in human diseases. Birth Defects Res C Embryo Today [Internet]. 2006;78(2):180–9. Available from: https://www.ncbi.nlm.nih.gov/pubmed/?term=16847883
40. Cummins JM, Velculescu VE. Implications of micro-RNA profiling for cancer diagnosis. Oncogene [Internet]. 2006;25(46):6220–7. Available from: http://www.nature.com/doifinder/10.1038/sj.onc.1209914
41. Harfe BD. MicroRNAs in vertebrate development. Curr Opin Genet Dev. 2005;15(4):410–5.
42. Bartel DP, Lee R, Feinbaum R. MicroRNAs : Genomics , Biogenesis , Mechanism , and Function Genomics : The miRNA Genes. 2004;116:281–97.
43. Reis PP, Tomenson M, Cervigne NK, Machado J, Jurisica I, Pintilie M, et al. Programmed cell death 4 loss increases tumor cell invasion and is regulated by miR-21 in oral squamous cell carcinoma. Mol Cancer. 2010;9:238.
44. Frankel LB, Christoffersen NR, Jacobsen A, Lindow M, Krogh A, Lund AH. Programmed cell death 4 (PDCD4) is an important functional target of the microRNA

miR-21 in breast cancer cells. J Biol Chem. 2008;283(2):1026–33.
45. Rinaldi A, Poretti G, Kwee I, Zucca E, Catapano C, Tibiletti MG, et al. Concomitant MYC and microRNA cluster miR-17-92 (C13orf25) amplification in human mantle cell lymphoma [2]. Leuk Lymphoma. 2007;48(2):410–2.
46. Iorio M V., Croce CM. MicroRNAs in cancer: Small molecules with a huge impact. J Clin Oncol. 2009;27(34):5848–56.
47. Yan L, Huang X, Shao Q, Huang M, Deng L. MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. Cold Spring Harb Lab Press. 2008;2348–60.
48. Yu SL, Chen HY, Chang GC, Chen CY, Chen HW, Singh S, et al. MicroRNA Signature Predicts Survival and Relapse in Lung Cancer. Cancer Cell. 2008;13(1):48–57.
49. Calin G, Ferracin M, Cimmino A. A microRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N Engl J Med. 2005;353:1793–801.
50. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. Nature [Internet]. 2005;435(7043):834–8. Available from: http://www.nature.com/doifinder/10.1038/nature03702
51. Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, et al. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res. 2004;64(11):3753–6.

Capítulo 1

microRNA downregulation is associated with pathways of adaptive immune response and invasion in a rare metastatic typical lung carcinoid.

microRNA Downregulation is Associated with Pathways of Adaptive Immune Response and Invasion in Metastatic Typical Lung Carcinoid Tumors

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Short Title: microRNAs in lung carcinoid tumors.

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Abstract

Background: Typical lung carcinoids are uncommon neuroendocrine tumors and not often metastatic. Molecular features of lung carcinoid tumors have been poorly defined, including the role of microRNAs (miRNAs), which are key players in gene expression regulation related to tumorigenesis.

Objectives: To identify commonly deregulated miRNAs in paired samples of tumor and metastasis and pathways including deregulated miRNAs and target-genes.

Method: Global miRNA expression profiles were assessed in typical carcinoid tumors, the corresponding lymph node metastasis and histologically normal lung tissues, using TaqMan Low Density Arrays. Computational analyses were performed to determine validated miRNA target genes and tumorigenesis pathways.

Results: We identified 28 deregulated miRNAs (21 down- and 7 up-regulated) in tumors and 40 in the lymph node metastasis (38 down- and 2 up-regulated) ($FC \geq 2$ and $p < 0.05$) compared to normal lung tissues. 16 miRNAs were commonly deregulated in tumor and metastasis; the majority (15 miRNAs) being significantly down-regulated and with decreasing levels in metastasis compared to the primary tumor. Deregulated levels of six miRNAs were validated in two external datasets (N=48 typical and 3 atypical carcinoids compared with 35 normal lung tissues). Low levels of miR-146b-5p were significantly associated with poor patient survival ($p < 0.02$) and HR=1.9 (1.14-3.16) in an independent external adenocarcinoma dataset. Down-regulated miRNAs have a tumor suppressive role in oncogenesis and are involved in biological pathways significantly enriched for adaptive immune response, invasion and metastasis.

Conclusions: A specific subset of miRNAs is widely down-regulated and modulates pathways of adaptive immune system response and invasion in typical lung carcinoid tumors.

Keywords: lung carcinoid tumor, microRNA, adaptive immune system, metastasis.

Introduction

Typical carcinoid tumors of the lung are low-grade lung neuroendocrine lesions that account for ~2% of all lung neoplasms. These tumors are most common in younger patients and are usually not related to smoking. They are also infrequently associated with metastasis at diagnosis and the 5-year overall patient survival is >80%. Diagnosis of typical carcinoids is based on histological examination and mitotic counting lower than 2 mitoses per 2 mm^2, without necrosis. Disease staging follows TNM categorization, and surgery remains as the standard treatment due to the high failure rates of chemo- or radiotherapy [1].

Although the histopathological features of lung carcinoid tumors are known, the molecular alterations associated with disease development and progression need to be elucidated. Armengol *et al.* [2] and Fernandez-Cuesta *et al.* [3] demonstrated that genetic mutations are uncommon in lung neuroendocrine tumors compared to other subtypes, such as adenocarcinoma or lung squamous cell cancers [4]. The few studies on microRNA (miRNA) changes in lung carcinoid tumors showed a wide range of differentially expressed miRNAs in tumor *vs*. normal lung tissue [5,6], among the different neuroendocrine tumor subtypes [7], and in typical *vs*. atypical tumors, and among atypical tumors with and without lymph node metastasis [8]. To the best of our knowledge, no other studies have reported miRNA changes in rare tumor subtypes such as typical carcinoids with lymph node metastasis.

Our hypothesis is that commonly deregulated miRNAs in primary tumor and lymph node metastasis are drivers of tumor progression. Our aim was to identify pathways including miRNAs and target genes as potential molecular drivers of metastasis in typical carcinoids.

Material and Methods

Patient and samples

Two fragments of the same typical carcinoid tumor and a lymph node metastasis were obtained from surgical resection in a female patient, 38-years-old at diagnosis, non-smoker and with no family history of cancer. This analysis was important to confirm that distinct parts of the same tumor were homogenous regarding their global miRNA expression levels. Disease diagnosis confirmed a locally invasive typical carcinoid tumor; T2aN1M0 (pathological stage IIB), according to the American Joint Committee on Cancer staging system (AJCC, 8^{th} edition). Formalin-fixed, paraffin-embedded (FFPE) tissues were obtained from the Pathology Department at Botucatu Clinical Hospital, FMB, UNESP, São Paulo, Brazil. Two FFPE tissue blocks were obtained from two different areas of the tumor, and one FFPE sample corresponding to the lymph node metastasis. FFPE samples were cut (10 sections of 10 µm each) for needle microdissection using the stereo microscope Leica EZ4 (Leica Microsystems, Wetzlar, Germany) before RNA extraction, in order to isolate the target cell populations (tumor or normal). An expert lung pathologist marked the tumor or normal areas on H&E-stained section. In addition, an RNA pool of 9 histologically normal lung tissues was used as a reference to calculate relative miRNA expression. Therefore, we have generated miRNA profiles using the following samples: tumor (N=2 fragments) and lymph node metastasis (N=1) from the same patient, and histologically normal lung tissues (N=9) as reference controls.

RNA extraction

RNA was extracted using the RecoverAll Total Nucleic Acid Isolation Kit for FFPE tissues (Ambion/Thermo Fisher), according to the manufacturer's instructions.

miRNA expression analysis

TaqMan Array Human microRNA card A v.3.0 (Life Technologies/Thermo Fisher Scientific) was used to identify commonly deregulated miRNAs in tumor and metastasis based on a panel of 384 miRNAs including controls as previously reported [9].

Computational analyses

miRNA target genes were predicted using *microRNA Data Integration Portal* (mirDIP) (http://ophid.utoronto.ca/mirDIP/) [10] and miRNA-target gene interactions were validated by miRTarBase. In addition, ToppGene Suite (https://toppgene.cchmc.org/) [11] was used to identify statistically enriched pathways, and miRBase (http://www.mirbase.org/) [12] and UCC Genomic database (https://genome.ucsc.edu/index.html) [13] were used to determine the genomic location of all 16 miRNAs.

Validation in external datasets

Publicly available miRNA expression data were retrieved from Gene Expression Omnibus (GEO) DataSets (https://www.ncbi.nlm.nih.gov/gds). Data were obtained from studies that followed the inclusion criteria of having original raw data available of global miRNA expression, in primary human lung carcinoids, of both typical and atypical histologies, and that included histologically normal lung tissues for comparison. Two studies [5,6] were considered eligible and used for data retrieval and analyzes. miRNA expression data were analyzed using the bioinformatics tool GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/) with the default parameters. miRNA expression was determined by comparing typical (N=48) and atypical (N=3) *vs*. corresponding normal samples (N=35) in these two external datasets (**Table 1**). Since one of these public datasets [5] contained two patients with typical carcinoids and lymph node metastasis, we refined our validation analysis by comparing only these cases against our data. Prognostic value of miRNA expression was assessed using Survmicro [14] in an independent adenocarcinoma dataset (esophageal cancer) [15].

Results

Tissue samples exhibited histological features and biomarkers characteristic of a typical carcinoid tumor

Histological analysis of surgically resected tumor and lymph nodes showed tumor cells having nuclei with small to intermediate size and heterogeneous chromatin. Tumor stroma was vascularized. There was 1 mitotic cell in 10 large magnification fields. No angiolymphatic or perineural invasion or necrosis was present. The Ki-67 proliferation index was 1%. Histopathological features of this typical carcinoid tumor are shown in **Figure 1A**; neuroendocrine differentiation is shown by immunopositivity of chromogranin and

synaptophysin [1] (**Figure 1B and 1C**). One of three resected lymph nodes showed cytomorphological aspects identical to the tumor. Immunohistochemical biomarker analysis of this lymph node sample confirmed epithelial histogenesis of tumor cells, showing immunopositivity to cytokeratins (AE1/AE3) (**Figure 1D**). Immunopositivity of chromogranin and synaptophysin confirmed the diagnosis of a typical lung carcinoid tumor with metastasis in 1/3 lymph nodes.

A specific subset of miRNAs is commonly deregulated in paired tumor and lymph node metastasis.

28 miRNAs were significantly deregulated (21 down- and 7 up-regulated) (FC≥2 and $p<0.05$) in the two fragments of the tumor and 40 (38 down- and 2 up-regulated) in the metastatic sample. Global miRNA expression profiles were highly correlated in the two tumor areas ($r=0.97$, Pearson correlation) demonstrating that the two tumor fragments were homogeneous regarding miRNA expression levels.

We were able to identify 16 commonly deregulated miRNAs (FC≥2 and $p<0.05$), most (15 miRNAs) being down- and 1 up-regulated. Of the 15 down-regulated miRNAs, 12 exhibited a notable further decreased expression in the metastasis compared to the tumor (**Table 2**).

Genomic mapping analysis showed that 5/16 miRNAs were mapped closely in the genome, with two miRNAs on chromosome 9q22.32-q34.3, and three on chromosome 14q32.2-q32.31 (**Figure 2**). These regions have been associated with genomic changes in cancer cells, and may be a potential mechanism of miRNA deregulation [16,17].

miRNA target genes control adaptive immune response, invasion and metastasis

mirDIP prediction analysis showed a total of 2,527 interactions encompassing all 16 miRNAs, with 1,698 targets being validated on miRTaRBase (**Supplementary Table 1 – Accessible at** https://www.dropbox.com/s/2yvqu49eueqzi6c/Supplementary%20table%201.xlsx?dl=0).
Genes were further mapped on 106 significantly ($p<0.05$) enriched pathways. Among pathways identified, 114 genes were involved in adaptive immune response and a large number of genes associated with pathways related to invasion and metastasis (**Supplementary Table 2 – Accessible at** https://www.dropbox.com/s/y34waonf3j9erut/Supplementary%20table%202.xlsx?dl=0**)**.
Interaction networks identified were significantly ($p<0.05$) enriched by deregulated miRNAs and genes that play roles in adaptive immune response (**Figure 3**). miRNA-modulated pathways of immune response, cellular proliferation and invasion are associated with tumor progression and lymph node metastasis formation, as illustrated in **Figure 4**.

Deregulated expression of tumor suppressive and oncomiRs were validated in external datasets

The 16 commonly deregulated miRNAs identified in tumor and lymph node metastasis (**Figure 5**) were validated against published GEO datasets, from which the original raw data were retrieved and re-analyzed, as outlined above. When we compared our miRNA data with the data generated by Yoshimoto *et al.*[5] and Deng *et al.*[6] in typical or atypical lung carcinoids *vs*. normal tissues, as well as in samples of two typical carcinoids with lymph node metastasis, six miRNAs were validated: five were down-regulated: hsa-let-7b-5p, hsa-miR-126-3p, hsa-miR-146b-5p, and hsa-miR-320a-3p, hsa-miR-494-3p and one was up-regulated:

hsa-miR-411-5p (p<0.05, **Table 3**). Validation of this six miRNA subset in these 3 cases of typical carcinoids (1 patient from our study and 2 patients from Yoshimoto´s study) indicates a likely involvement of these miRNAs as disease drivers in lung carcinoid tumorigenesis.

Discussion/Conclusion

Lung typical carcinoid tumors are not commonly observed and the presence of lymph node metastasis is reported in about 9% of the cases [18]. The case presented in our study matches the classification criteria for the typical carcinoid tumors, with less than 2 mitoses per 2 mm^2 and the absence of necrosis [1].

Considering that lung carcinoids do not have a high frequency of mutations [3], their underlying mechanisms of tumor development and progression may include deregulation of miRNAs, which are potent gene expression modulators. We were able to identify potential drivers of carcinogenesis and metastasis, with a particular focus on miRNAs in the analysis of matched tumor and metastasis from a same patient compared with histologically normal lung tissues.

We identified that a subset of miRNAs is mainly under-expressed in tumor and lymph node metastasis with marked further decrease in expression levels in metastasis compared to the primary tumor. Such miRNAs may have a tumor suppressive role associated with disease progression.

Our main findings agree with current evidence showing that metastatic progression accumulates further changes compared to the primary tumor (28 miRNAs deregulated in tumor and 40 miRNAs deregulated in metastasis, being 16 in common and with decreased levels in paired lymph node metastasis). Our data support existing evidence on metastatic dissemination upon immune system modulation, by identifying candidate miRNAs that play important regulatory roles in adaptive immune system [19].

The 16 commonly deregulated miRNAs in tumor and regional metastasis directly target carcinogenesis-associated genes, which was consistent with their tumor suppressive effect. 5/16 identified miRNAs (hsa-let-7e-5p, hsa-miR-186-5p, hsa-miR-24-3p, hsa-miR-29a-3p and hsa-miR-411-5p) have been previously reported in a comparison between typical *vs*. atypical lung carcinoid tumors [8], but their precise targets could not be unequivocally defined in that study since the specific miRNA strand was not reported.

Among the identified miRNAs, six were validated in external datasets: hsa-let-7b-5p, hsa-miR-126-3p, hsa-miR-146b-5p, hsa-miR-320a-3p, hsa-miR-494-3p and hsa-miR-411-5p; except the latter, all validated miRNAs were under-expressed in tumors compared to normal lung tissues.

The hsa-let-7 family of miRNAs has been characterized to have tumor suppressive effects by targeting *CDKN1B* ($p27^{kip1}$) whose over-expression is a feature of higher proliferation rates in some lung carcinoid tumors [20]. hsa-let-7e-5p, an important regulator of *RAS* family, has been linked to chemotherapeutic resistance, low E-cadherin expression and tumor aggressiveness in *ALK* positive lung adenocarcinoma [21]. miR-342-3p also has a prominent role in these tumors by targeting *RAP2B*, which is part of the RAS family [22]. The PI3K/Akt pathway is affected by miR-126 targeting of *VEGFA* and reduced expression of miR-126 is

linked to chemotherapy resistance in non-small cell lung cancer (NSCLC) and increased angiogenesis [23]. In NSCLC, lower miR-146-5p expression was associated with decreased survival and negatively correlated with *TRAF6* (TNF receptor-associated factor-6). In addition, miR-146-5p over-expression in NSCLC cells triggered cell cycle arrest at the G1 phase, reduced cell proliferation and decreased migration and invasion capabilities [24]. Interestingly, we applied SurvMicro analysis [14] to verify whether miRNAs were associated with survival in publicly available datasets of squamous cell carcinoma and adenocarcinoma; this analysis verified that low miR-146b-5p levels were associated with poorer survival of esophageal carcinoma patients (Supplementary Figure 1, analysis results based on dataset GSE 13937) [15]. Cell cycle regulation has been linked to miR-186 acting on cyclin D1, CDK2 and CDK6 [25] and miR-29a through CDC42 [26]. Two miRNAs belonging to the same family; miR-26a-5p and miR-26b-5p, had low levels in tumor and paired metastasis; miR-26a targets *HMGA2*, which has been linked to cisplatin chemoresistance in NSCLC and also E2F1 with consequent effects on *AKT* and *BCL2*[27]. The alternative isoform of this miRNA, miR-26b, targets *MIEN1*; which affected levels of *NF-kB*, *MMP9* and *VEGF* inNSCLC [28]. *BCL2* is also affected by miR-16-5p to influence the expression of BAX, p27 and procaspase 3 [29]. miR-494 influences *BCL2* to affect cycle arrest and apoptosis but also *SCGN* (Secretagogin, EF-Hand Calcium Binding Protein) to confer chemotherapy resistance [30].

Our bioinformatic analyses focusing on miRNA targets showed *N-RAS*, *RasGRP1*, *K-RAS*, *CDC42*, *CDKN1B*, *AKT3* and *TRAF6*, indicating a clear role in tumorigenesis. Additionally, we identified miRNA regulated genes that play a role in immune functions consistent with a modulator activity on the tumor immune response. Among pathways identified, the highest number of miRNA target genes was shown to play a role in disease processes driven by immunosuppression, angiogenesis and invasion [31].

Our study showed an enrichment of 8 miRNA target genes with an important role in adaptive immune system regulation: *CD200*, *BTLA*, *SOCS1*, *CD28*, *PTEN*, *AKT3*, *TRAF6* and *IKBKB*. Reduced PTEN expression can increase the production of immunosuppressive cytokines and reduced T cell recruitment to the tumor microenvironment to lessen cell death mediated by T cells [32]. TRAF6 mediates TNF receptor superfamily and Interleukin 1 receptor signaling, playing a central role in NF-kB activation [33]. BTLA (B- and T-lymphocyte attenuator) is a T cell co-inhibitory molecule that functions similarly to PD-1 and CTLA-4 in suppressing T cell activation [34]. CD28 is essential for T cell lymphocyte differentiation, proliferation, survival and cytokine production [35]. The potential role of CD200 is of particular interest in neuroendocrine tumors. *CD200* encodes a cell surface suppressor of the immune system through binding to CD200R. Tumor cells expressing CD200 may support growth [36] and metastatic progression [37] and especially in neuroendocrine tumor samples, so that it has been suggested as a diagnostic biomarker in this tumor type [38]. To our knowledge, ours is the first evidence of miRNA down-regulation having CD200 as one of the main direct targets in lung carcinoid tumor and paired metastasis.

In conclusion, we were able to identify and validate deregulated expression levels of 6 miRNAs in typical lung carcinoids with lymph node metastasis. These miRNAs act as transcriptional regulators in pathways enriched for genes that control immune system response and invasion. Validation of this miRNA subset in independent datasets suggests that these 6 miRNAs may be drivers of metastatic spread in typical lung carcinoids. Our data

contribute to the identification of miRNAs as biomarkers of typical carcinoids presenting with lymph node metastasis.

Statements

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Statement of Ethics

This study was approved by the Research Ethics Board of the Faculty of Medicine (FMB), São Paulo State University (UNESP), under the REB # 1.908.978.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Ana L Seneda: study design, data generation and interpretation, manuscript writing. **Rainer M L Lapa:** bioinformatic data analysis, data interpretation, manuscript writing. **Tainara F Felix:** data generation, manuscript writing. **Rogério A de Oliveira:** statistical review, data analysis, manuscript writing. **Cristiano C Oliveira:** histopathological analysis, manuscript writing. **Érica N Hasimoto:** collection of samples and clinical data, manuscript writing. **Daniele C Cataneo:** collection of samples and clinical data, manuscript writing. **Antonio J M Cataneo:** collection of samples and clinical data, manuscript writing. **Julio De Faveri:** histopathological analysis, manuscript writing. **Sandra A Drigo:** study design, data interpretation, manuscript writing. **Luis A J Mur:** study design, data interpretation, manuscript writing. **Patricia P Reis:** study design, data interpretation, study supervision, manuscript writing.

References

1 Pelosi G, Sonzogni A, Harari S, Albini A, Bresaola E, Marchiò C, et al.: Classification of pulmonary neuroendocrine tumors: New insights. Transl Lung Cancer Res 2017;6:513–529.

2 Armengol G, Sarhadi VK, Rönty M, Tikkanen M, Knuuttila A, Knuutila S: Driver Gene Mutations of Non-Small-Cell Lung Cancer are Rare in Primary Carcinoids of the Lung: NGS Study by Ion Torrent. Lung 2015;193:303–8.

3 Fernandez-cuesta L, Peifer M, Lu X, Sun R, Ozretić L, Seidal D, et al.: Frequent mutations in chromatin-remodeling genes in pulmonary carcinoids. Nat Commun 2014;3518:1–17.

4 Rossi G, Bertero L, Marchiò C, Papotti M: Molecular alterations of neuroendocrine tumours of the lung. Histopathology 2018;72:142–152.

5 Yoshimoto T, Motoi N, Yamamoto N, Nagano H, Ushijima M, Matsuura M, et al.: Pulmonary Carcinoids and Low-Grade Gastrointestinal Neuroendocrine Tumors Show Common MicroRNA Expression Profiles, Different from Adenocarcinomas and Small Cell Carcinomas. Neuroendocrinology 2017;106:47–57.

6 Deng B, Molina J, Aubry MC, Sun Z, Wang L, Eckloff BW, et al.: Clinical biomarkers of pulmonary carcinoid tumors in never smokers via profiling miRNA and target mRNA. Cell Biosci 2014;4:1–10.

7 Mairinger FD, Ting S, Werner R, Walter RFH, Hager T, Vollbrecht C, et al.: Different micro-RNA expression profiles distinguish subtypes of neuroendocrine tumors of the lung: results of a profiling study. Mod Pathol an Off J United States Can Acad Pathol Inc 2014;27:1632–40.

8 Rapa I, Votta A, Felice B, Righi L, Giorcelli J, Scarpa A, et al.: Identification of MicroRNAs Differentially Expressed in Lung Carcinoid Subtypes and Progression. Neuroendocrinology 2015;101:246–55.

9 Cervigne NK, Reis PP, Machado J, Sadikovic B, Bradley G, Galloni NN, et al.: Identification of a microRNA signature associated with progression of leukoplakia to oral carcinoma. Hum Mol Genet 2009;18:4818–29.

10 Tokar T, Pastrello C, Rossos AEM, Abovsky M, Hauschild AC, Tsay M, et al.: MirDIP 4.1 - Integrative database of human microRNA target predictions. Nucleic Acids Res 2018;46:D360-70.

11 Chen J, Bardes EE, Aronow BJ, Jegga AG: ToppGene Suite for gene list enrichment analysis and candidate gene prioritization. Nucleic Acids Res 2009;37:305–11.

12 Kozomara A, Griffiths-Jones S: MiRBase: Annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res 2014;42:68–73.

13 Casper J, Zweig AS, Villarreal C, Tyner C, Speir ML, Rosenbloom KR, et al.: The UCSC Genome Browser database: 2018 update. Nucleic Acids Res 2018;46:D762-9.

14 Aguirre-Gamboa R, Trevino V: SurvMicro: Assessment of miRNA-based prognostic signatures for cancer clinical outcomes by multivariate survival analysis. Bioinformatics 2014;30:1630–1632.

15 Mathé EA, Nguyen GH, Bowman ED, Zhao Y, Schetter AJ, Braun R, et al.: MicroRNA expression in squamous cell carcinoma and adenocarcinoma of the esophagus: associations with survival. Clin Cancer Res 2009;15:6192–6200.

16 Choi Y, Choi JS, Zheng LT, Lim YJ, Yoon HK, Kim YH, et al.: Comparative genomic hybridization array analysis and real time PCR reveals genomic alterations in squamous cell carcinomas of the lung. Lung Cancer 2007;55:43–51.

17 Girard L, Zochbauer-Muller S, Virmani AK, Gazdar AF, Minna JD: Genome-wide Allelotyping of Lung Cancer Identifies New Regions of Allelic Loss , Differences between Small Cell Lung Cancer and Non-Small Cell Lung Cancer, and Loci Clustering. Cancer Res 2000;60:4894–4906.

18 Wolin EM: Advances in the Diagnosis and Management of Well-Differentiated and Intermediate-Differentiated Neuroendocrine Tumors of the Lung. Chest 2017;151:1141–1146.

19 Lambert AW, Pattabiraman DR, Weinberg RA: Emerging Biological Principles of Metastasis. Cell 2017;168:670–91.

20 Di Fazio P, Maass M, Roth S, Meyer C, Grups J, Rexin P, et al.: Expression of hsa-let-7b-5p, hsa-let-7f-5p, and hsa-miR-222-3p and their putative targets HMGA2 and CDKN1B in typical and atypical carcinoid tumors of the lung. Tumor Biol 2017;39:1–8.

21 Kim H, Yang JM, Jin Y, Jheon S, Kim K, Taek Lee C, et al.: MicroRNA expression profiles and clinicopathological implications in lung adenocarcinoma according to EGFR, KRAS, and ALK status. Oncotarget 2017;8:8484–98.

22 Xie X, Liu H, Wang M, Ding F, Xiao H, Hu F, et al.: miR-342-3p targets RAP2B to suppress proliferation and invasion of non-small cell lung cancer cells. Tumor Biol 2015;36:5031–5038.

23 Zhu X, Li H, Long L, Hui L, Chen H, Wang X, et al.: miR-126 enhances the sensitivity of non-small cell lung cancer cells to anticancer agents by targeting vascular endothelial growth factor A. Acta Biochim Biophys Sin 2012;44:519–26.

24 Li Y, Zhang H, Dong Y, Fan Y, Li Y, Zhao C, et al.: MiR-146b-5p functions as a suppressor miRNA and prognosis predictor in non-small cell lung cancer. J Cancer 2017;8:1704–16.

25 Cai J, Wu J, Zhang H, Fang L, Huang Y, Yang Y, et al.: MiR-186 downregulation correlates with poor survival in lung adenocarcinoma, where it interferes with cell-cycle regulation. Cancer Res 2013;73:756–66.

26 Li Y, Wang Z, Li Y, Jing R: MicroRNA-29a functions as a potential tumor suppressor through directly targeting CDC42 in non-small cell lung cancer. Oncol Lett 2017;13:3896–904.

27 Yang Y, Zhang P, Zhao Y, Yang J, Jiang G, Fan J: Decreased MicroRNA-26a expression causes cisplatin resistance in human non-small cell lung cancer. Cancer Biol Ther 2016;17:515–25.

28 Li D, Wei Y, Wang D, Gao H, Liu K: MicroRNA-26b suppresses the metastasis of non-small cell lung cancer by targeting MIEN1 via NF-κB/MMP-9/VEGF pathways. Biochem Biophys Res Commun 2016;472:465–70.

29 Wang W, Chen J, Dai J, Zhang B, Wang F, Sun Y: MicroRNA-16-1 Inhibits Tumor Cell Proliferation and Induces Apoptosis in A549 Non-Small Cell Lung Carcinoma Cells. Oncol Res 2016;24:345–351.

30 Bai Y, Sun Y, Peng J, Liao H, Gao H, Guo Y, et al.: Overexpression of secretagogin

inhibits cell apoptosis and induces chemoresistance in small cell lung cancer under the regulation of miR-494. Oncotarget 2014;5:7760–7775.

31 Davidson-Moncada J, Papavasiliou FN, Tam W: MiRNAs of the Immune System: Roles in Inflammation and Cancer. Ann N Y Acad Sci 2011;1183:183–94.

32 Peng W, Chen JQ, Liu C, Malu S, Creasy C, Michael T, et al.: Loss of PTEN promotes resistance to T cell-mediated immunotherapy. Cancer Discov 2016;6:202–16.

33 Starczynowski DT, Lockwood WW, Deléhouzée S, Chari R, Wegrzyn J, Fuller M, et al.: TRAF6 is an amplified oncogene bridging the RAS and NF- κ B pathways in human lung cancer. J Clin Invest 2011;121:4095–105.

34 Watanabe N, Gavrieli M, Sedy JR, Yang J, Fallarino F, Loftin SK, et al.: BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. Nat Immunol 2003;4:670–9.

35 Miller J, Baker C, Cook K, Graf B, Sanchez-Lockhart M, Sharp K, et al.: Two pathways of costimulation through CD28. Immunol Res 2009;45:159–72.

36 Zhang S-S, Huang Z-W, Li L-X, Fu J-J, Xiao B: Identification of CD200+ colorectal cancer stem cells and their gene expression profile. Oncol Rep 2016;36:2252–60.

37 Stumpfova M, Ratner D, Desciak EB, Eliezri YD, Owens DM: The immunosuppressive surface ligand CD200 augments the metastatic capacity of squamous cell carcinoma. Cancer Res 2010;70:2962–72.

38 Love JE, Thompson K, Kilgore MR, Westerhoff M, Murphy CE, Papanicolau-Sengos A, et al.: CD200 Expression in Neuroendocrine Neoplasms. Am J Clin Pathol 2017;148:236–242.

Figures

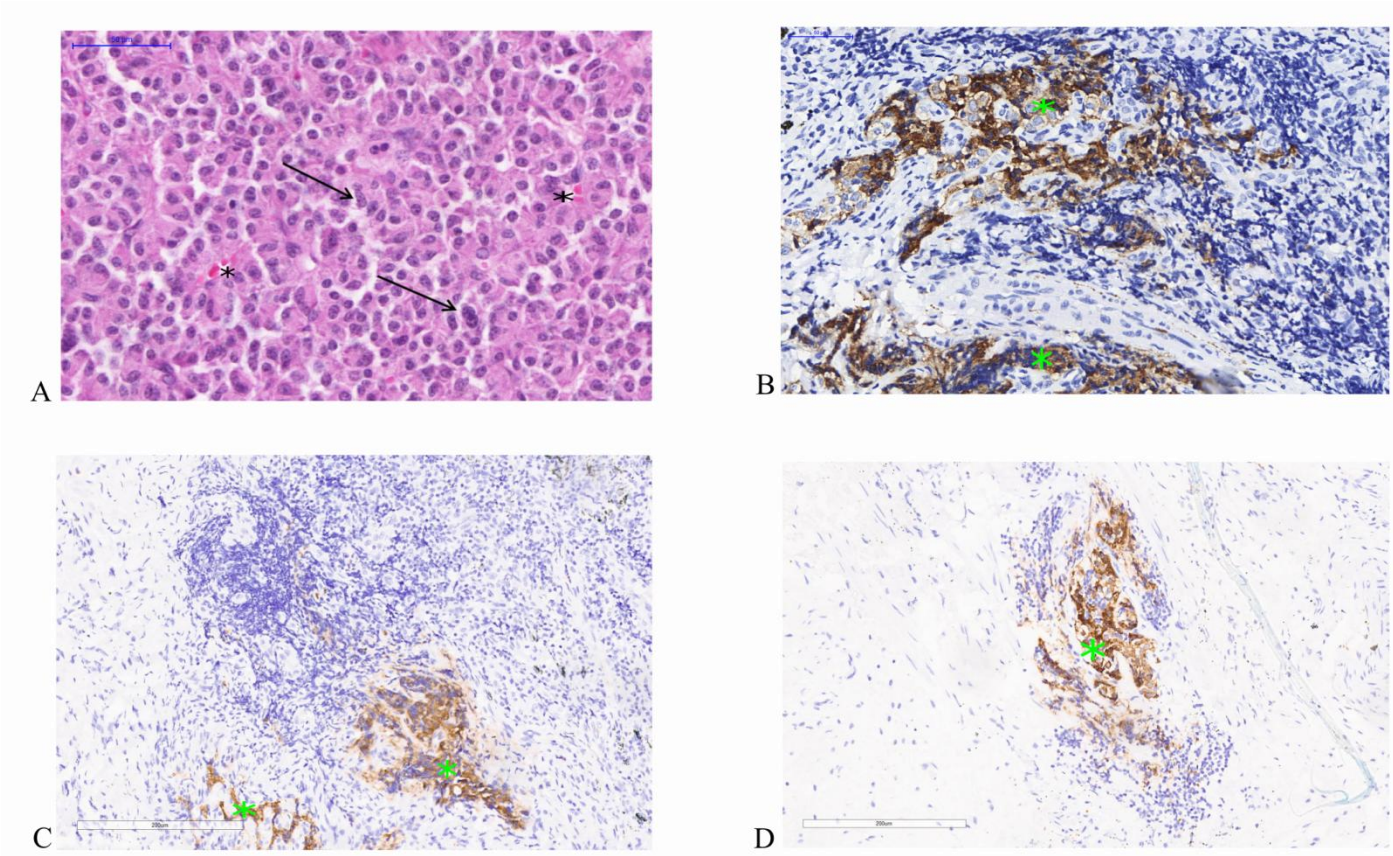


Figure 1. (A) Hematoxylin and Eosin (H&E) stained section of the low grade, typical carcinoid tumor of the lung. Chromatin in cell nuclei show evident "salt and pepper" patterning as indicated by black arrows. Tumor shows characteristic organoid tumor growth with vascularization (indicated by black *) and absence of necrosis and lack of mitotic activity, 400X magnification. **(B)** Immunohistochemical stained section showing positivity for synaptophysin, a specific biomarker expressed in the typical subtype of carcinoid tumors, 400X magnification. **(C)** Immunohistochemical stained section showing positivity for chromogranin A, 200X magnification. **(D)** Immunohistochemical stained section showing positivity for cytokeratins (AE1/AE3), 200X magnification. Positive immunostaining areas are indicated by the green stars *.

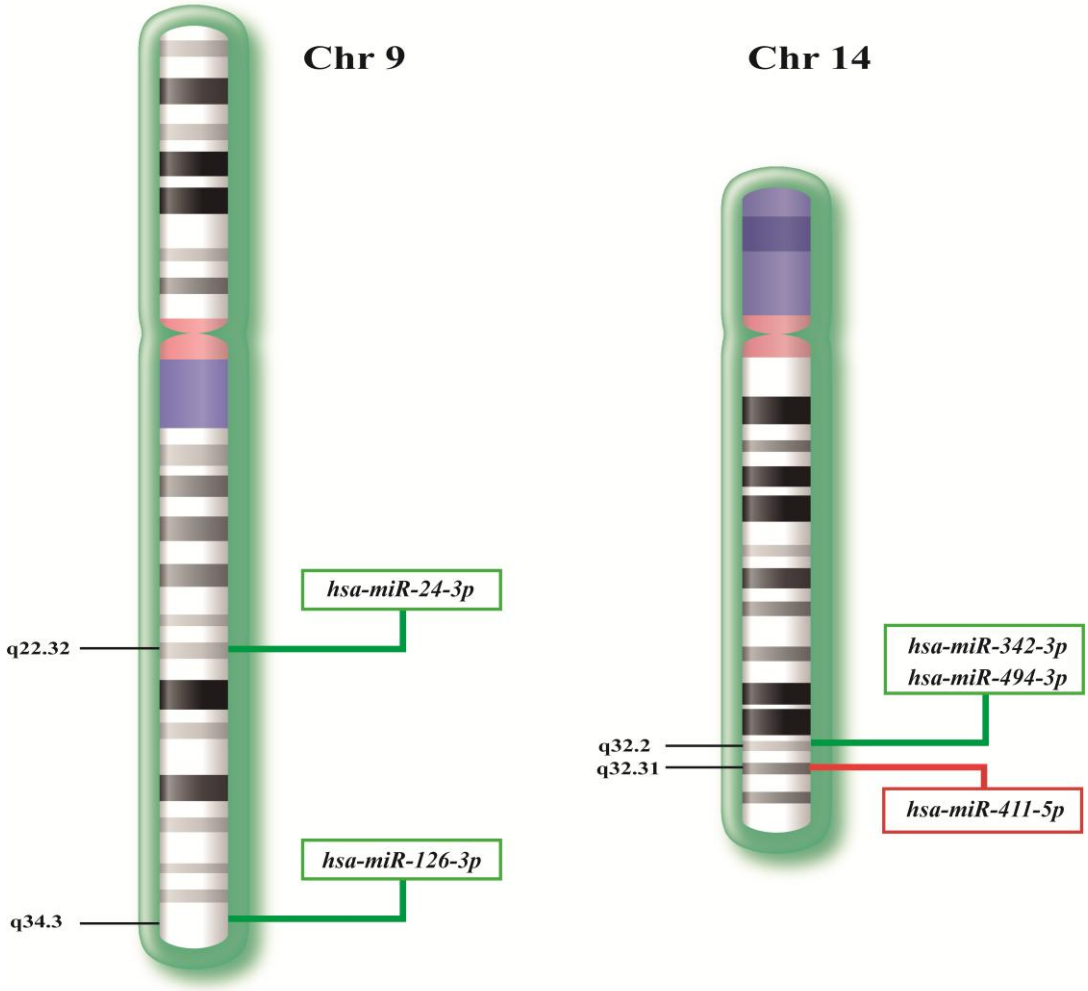


Figure 2. Genomic location of 5 miRNAs mapped closely in the human genome. Down-regulated miRNAs are shown as green boxes and up-regulated as the red box.

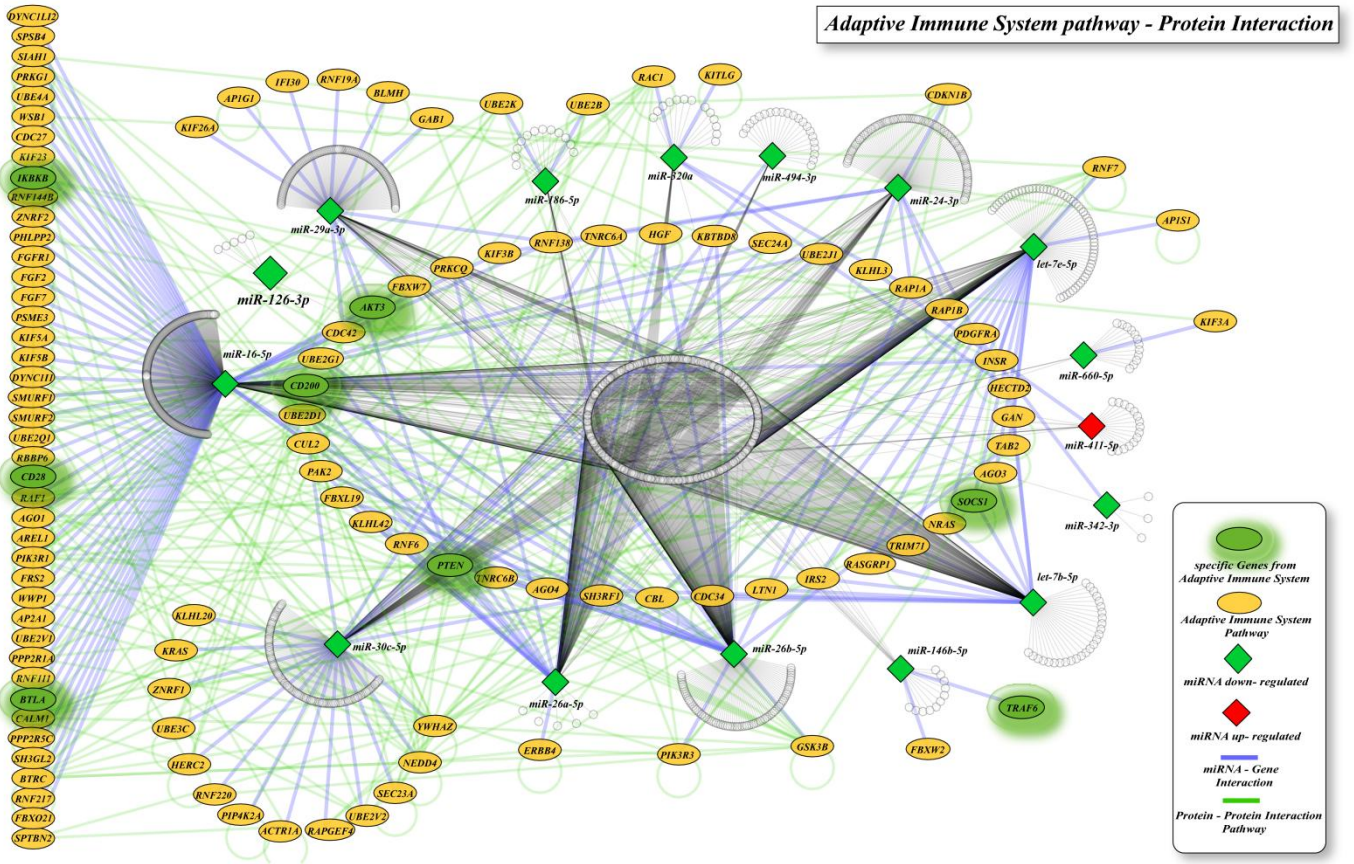


Figure 3. miRNA-mRNA network enrichment of target genes in adaptive immune response pathways.

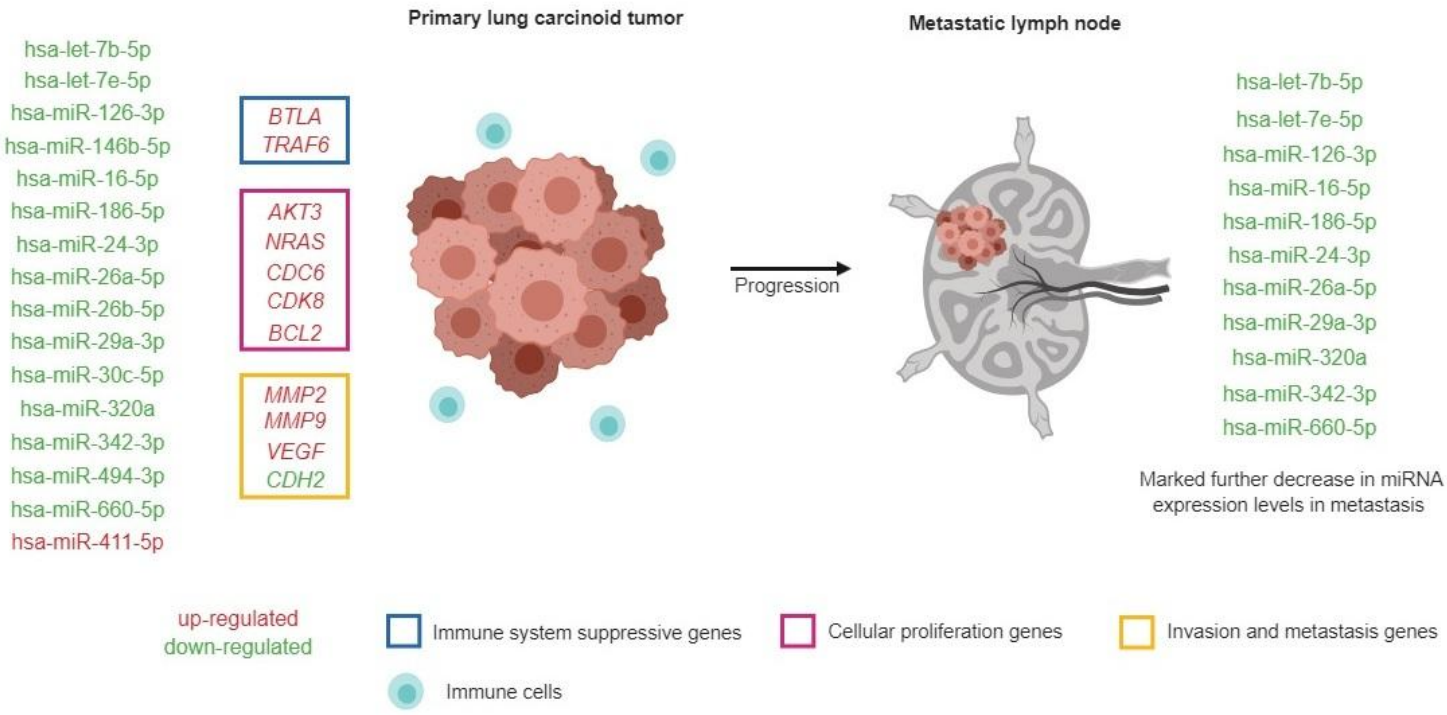


Figure 4. The 16 commonly altered miRNAs in primary tumor and lymph node metastasis regulate the expression of genes involved in immune response, cellular proliferation, invasion and metastasis. A subset of 12 miRNAs was further down-regulated in metastasis, suggesting a potential role for deregulated miRNA-mRNA networks in disease progression. This figure was made using BioRender software.

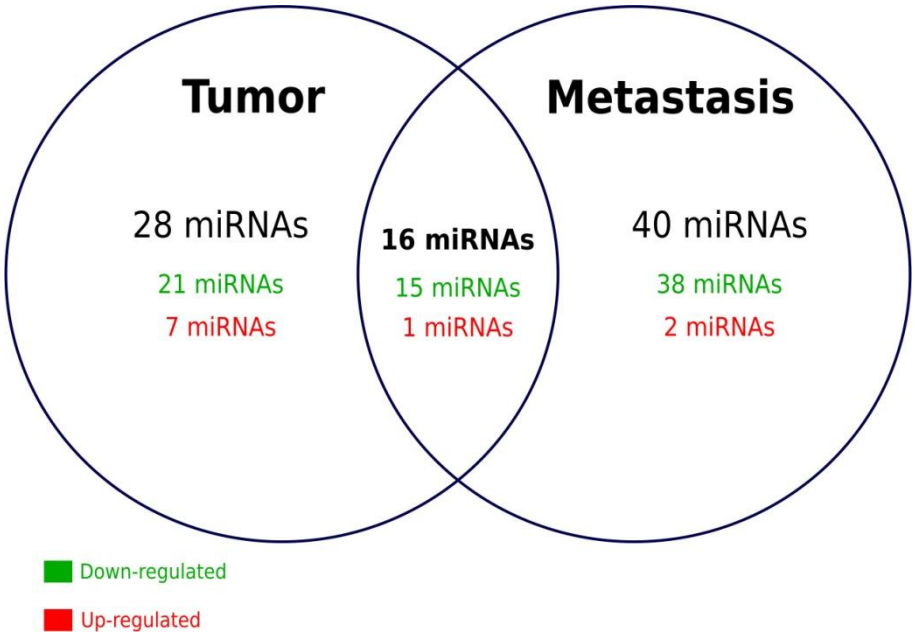
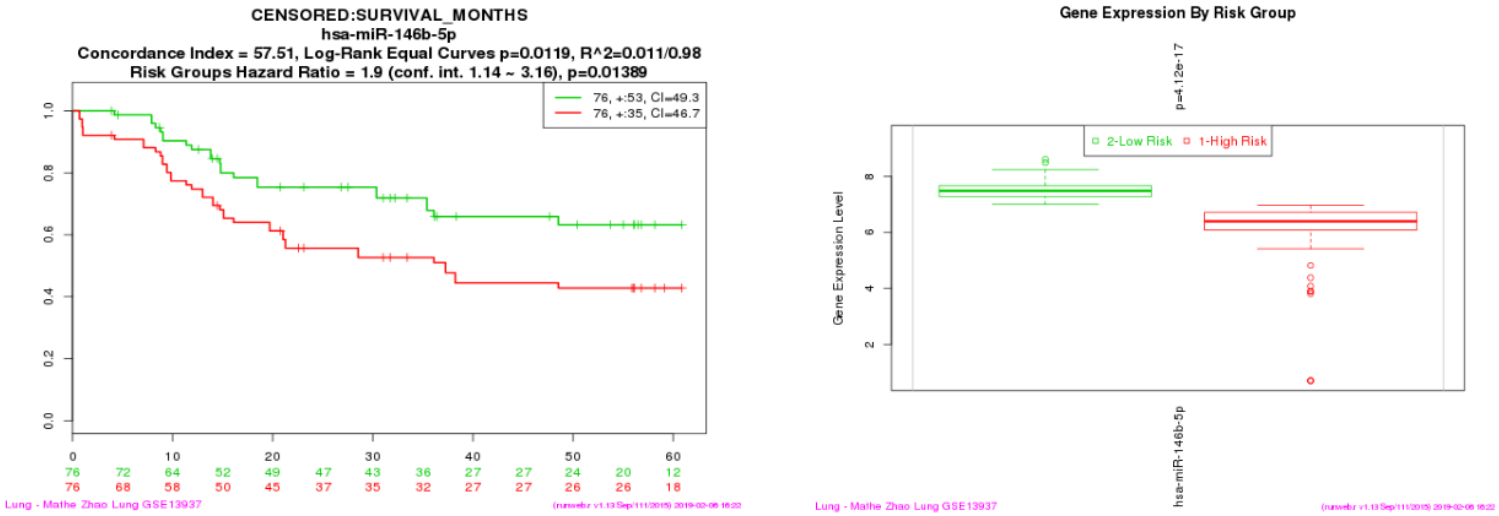


Figure 5. Venn Diagram depicting the total number of deregulated miRNAs (black), down-regulated (green) and up-regulated (red) in tumor, metastasis and in common to both sample types.



Supplementary Figure 1. Kaplan-Meier survival analysis. The down-regulation of miR-146b-5p was associated with poor survival of patients with esophagus adenocarcinoma (left panel). Expression levels of the miRNA for low and high risk group patients are shown in the right panel.

Tables

Table 1. Validation datasets.

Public dataset/ Accession ID	Sample size	Platform
Deng *et al*. 2014 GSE58600	23 lung typical carcinoid tumors 23 normal lung tissues	Illumina Human v2 MicroRNA expression beadchip
Yoshimoto *et al*. 2017 GSE77380	25 lung typical carcinoid tumors 3 lung atypical carcinoid tumors 12 normal lung tissues	Agilent-031181 Human_miRNA_V16.0_Microarray (miRBase release 16.0 miRNA ID version)

Table 2. Commonly deregulated miRNAs in typical carcinoid tumor and metastasis.

	Tumor		**Metastasis**	
miRNA ID	**FC**	***P-value***	**FC**	***P-value***
hsa-let-7b-5p	0,067	0,001	0,002	0,001
hsa-let-7e-5p	0,355	0,016	0,036	0,002
hsa-miR-126-3p	0,060	0,019	0,047	0,018
hsa-miR-146b-5p	0,063	0,004	0,360	0,036
hsa-miR-16-5p	0,384	0,038	0,161	0,015
hsa-miR-186-5p	0,250	0,049	0,061	0,005
hsa-miR-24-3p	0,378	0,029	0,074	0,004
hsa-miR-26a-5p	0,038	0,001	0,014	0,002
hsa-miR-26b-5p	0,175	0,032	0,058	0,018
hsa-miR-29a-3p	0,156	0,002	0,111	0,005
hsa-miR-30c-5p	0,018	0,001	0,018	0,003
hsa-miR-320a	0,314	0,021	0,076	0,006
hsa-miR-342-3p	0,367	0,015	0,209	0,011
hsa-miR-494-3p	0,030	0,011	0,055	0,008
hsa-miR-660-5p	0,210	0,022	0,030	0,006
hsa-miR-411-5p	66,128	0,015	17,968	0,031

FC: fold change

Table 3. Validated miRNAs in two external datasets.

Public data set/Accession ID	**miRNA ID**	**log FC**	***p-value***
Deng *et al*. 2014 GSE58600	hsa-let-7b-5p*	-0.745	1.23e-06
	hsa-miR-126-3p*	-0.273	6.98e-03
	hsa-miR-146b-5p*	-0.702	7.08e-06
	hsa-miR-320a-3p*	-0.337	8.75e-04
	hsa-miR-411-5p*	2.521	1.33e-13
Yoshimoto *et al*. 2017 GSE77380	hsa-let-7b-5p*	-1.45	6.16e-03
	hsa-miR-126-3p*	-4.14	1.10e-04
	hsa-miR-146b-5p*	-5.33	6.16e-03
	hsa-let-7b-5p**	-1.451	1.22e-01
	hsa-miR-126-3p**	-2.922	7.62e-02

miRNAs were validated using two publicly available datasets (Deng *et al*. Cell & Bioscience. 2014;4:1–10 and Yoshimoto *et al*. Neuroendocrinology. 2017;106:47–57).
FC: fold change.
* indicates miRNAs deregulated in typical carcinoids *vs*. normal
** indicates miRNAs deregulated in atypical carcinoids *vs*. normal.

Capítulo 2

microRNA expression profiles and prognostic value in typical and atypical lung carcinoid tumors

microRNA expression profiles and prognostic value in typical and atypical lung carcinoid tumors

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Abstract

Lung carcinoid tumors are a subtype of neuroendocrine neoplasia. Typical and atypical lung carcinoids are rare subtypes with widely unknown molecular alterations. MicroRNAs (miRNAs) are potent gene expression regulators that control important cellular processes and have been shown as clinically applicable biomarkers in several cancer types. We analyzed annotated miRNA sequences in the human genome (2,578 miRNAs) in typical (N=5) and atypical (N=2) lung carcinoids using the GeneChip™ miRNA 4.0 platform. Significantly deregulated miRNAs ($p<0.01$ and FDR<0.05) were identified compared to histologically normal lung tissues (9 miRNAs in typical and 20 miRNAs in atypical). These miRNAs were further used to investigate their target genes and pathways, using bioinformatics tools. We found that miRNAs regulate target genes involved in the regulation of the FcƐ receptor I (*FCƐRI*), *PDGF* and *NGF* signalling via *TRKA* from the plasma membrane in typical carcinoids. In atypical histology, miRNAs regulate target genes with roles in immune system response and neuronal pathways. In addition, a subset of miRNAs were associated with survival in an independent, publicly available next generation sequencing dataset from lung adenocarcinoma (N=195 patients) profiled by The Cancer Genome Atlas. Differences in miRNA profiles between the histological subtypes of lung carcinoids are important to understand their molecular features and pathophysiology of these tumors. Our data contribute to the identification of miRNAs that may be clinically relevant as diagnostic and prognostic biomarkers, as well as pathways that may be potentially targeted in cancer cells to improve patient treatment.

Key-words: carcinoid tumors, microRNAs, bioinformatics, pathways.

Introduction

Neuroendocrine tumors of the lung comprise ~25% of all primary lung tumors and are classified, histologically, in four subtypes: well-differentiated, low grade typical carcinoid (TC) (~2% of all lung neoplasia); well differentiated, intermediated-grade atypical carcinoid (AC) (<1%); poorly differentiated, high-grade large cell neuroendocrine carcinoma (~3%) and poorly differentiated, high grade small cell lung carcinoma (~20%) (1,2). Disease diagnosis is based on histological features, mitotic index and the evidence of necrosis (3). Typical carcinoid tumors have less than 2 mitoses per 2 mm^2 and absence of necrosis, while the atypical carcinoids have 2-10 mitoses per 2 mm^2 and/or presence of necrosis (3).

Differentially expressed microRNAs were reported in typical *vs*. atypical lung carcinoids (4–10); however miRNA target genes and pathways and their potential role as biomarkers are not well explored. miRNAs may have a potential role as biomarkers useful for subtype classification as well as in carcinoid development and progression, since these tumors have a low frequency of genetic alterations, suggesting that epigenetics is likely involved (11).

miRNAs are small non-coding RNAs with ~18-22 nucleotides in length and act as post-transcriptional regulators of gene expression. miRNAs bind to the 3' untranslated region (3'UTR) of target messenger RNA (mRNA) molecules, blocking translation. miRNAs are known to have a key role as modulators of several cellular processes such as cell growth and differentiation, metabolism, embryonic development, proliferation, and apoptosis. Therefore, deregulated miRNA expression is associated with oncogenesis. miRNAs have been suggested as potential biomarkers for diagnosis, prognosis and treatment in many cancers (12).

We analyzed global miRNA expression in lung typical and atypical carcinoids with the goal of evaluating whether miRNA profiles were distinct and targeted different genes and

pathways in these different histological disease subtypes. By computational in silico analyses, we were able to identify miRNA-mRNA networks and pathways, which may represent an opportunity for development of improved treatment strategies for patients who are not eligible to curative surgery.

Material and Methods

Ethics approval

This study was approved by the Research Ethics Board of the Faculty of Medicine (FMB), São Paulo State University (UNESP), under the REB # 1.908.978.

Patients and samples

Formalin-fixed, paraffin-embedded (FFPE) tissues were obtained from five typical and 2 atypical carcinoid tumors from patients treated at Botucatu Clinical Hospital, a public teaching hospital at UNESP. Patients with typical carcinoids were all female, non-smokers, mean age was 50.8 years (range 34-77) and tumors did not have lymph node metastasis. Patients with atypical carcinoids were a 52 year old female and a 71 year old male, both smokers. The male patient had one metastatic lymph node.

FFPE samples were cut (10 sections of 10 μm each) for needle microdissection using the stereo microscope Leica EZ4 (Leica Microsystems, Wetzlar, Germany) before RNA extraction, in order to isolate tumor cells. An expert lung pathologist marked the tumor areas

on the H&E-stained sections. A polled RNA sample containing 8 histologically normal lung tissues was used as a reference to calculate relative miRNA expression.

RNA extraction

RNA was extracted using the RecoverAll Total Nucleic Acid Isolation Kit for FFPE tissues (Ambion/Thermo Fisher), according to the manufacturer's instructions.

Global miRNA expression analysis

130 ng of total RNA was used for sample preparation, labelling and hybridization to the GeneChip™ miRNA 4.0 Assay (Affymetrix/Thermo Fisher Scientific). Quality control (QC) assessment was performed according to the manufacturer's recommendations and all samples passed the QC criteria. miRNA expression profiles were obtained in carcinoid tumors and the pooled normal lung reference sample, for a panel of 2,578 miRNAs including endogenous controls. The protocol followed the manufacturer's instructions. Data were analyzed using the Transcriptome Analysis Console (Affymetrix/Thermo Fisher Scientific). miRNA expression profiles of typical and atypical carcinoids were analyzed separately, relative to the normal control. We first identified statistically significantly deregulated miRNAs with $p<0.05$, in each tumor subtype. In order to select the most significantly deregulated miRNAs for downstream target gene prediction and pathways analyses, we further applied a more stringent filtering criteria of $FC\geq 2$ and $p<0.01$ and $FDR<0.05$.

Computational analyses

miRNA target genes were predicted using *microRNA Data Integration Portal* (mirDIP) (http://ophid.utoronto.ca/mirDIP/) (13) and miRNA-target gene interactions were validated using miRTarBase (14). In addition, ToppGene Suite (https://toppgene.cchmc.org/) (15) was used to identify statistically enriched pathways.

Survival analysis

In order to verify whether any combination(s) of miRNAs were associated with survival, Kaplan Meier analysis was performed for deregulated miRNAs using *SurvMicro* (16) tool. We tested identified miRNAs against the publicly available The Cancer Genome Atlas (TCGA) datasets of Lung Adenocarcinoma (N= 195 patients) and Lung Squamous Cell Carcinoma (N=142) profiled by next generation sequencing in the Illumina HiSeq platform.

Results

Deregulated miRNA expression in typical and atypical carcinoid tumors

A total of 150 and 108 miRNAs were significantly deregulated ($p<0.05$) in typical and atypical carcinoids, respectively. Of these, 42 miRNAs were commonly altered in both tumor types, 108 miRNAs being exclusively deregulated in typical and 66 miRNAs exclusively deregulated in atypical histology (Supplementary Table 1). A reduced number of miRNAs were identified as highly significantly altered using stringent filtering of FC≥2 and $p<0.01$

and FDR<0.05; 8 miRNAs were under- and 1 was over-expressed in typical carcinoids (**Table 1**) and 8 miRNAs were under- and 12 were over-expressed in atypical carcinoids (**Table 2**).

miRNA target genes in typical carcinoids modulate FcƐ receptor I, PDGF and NGF signalling via TRKA

mirDIP analysis resulted in 1,805 predicted miRNA-mRNA interactions, with 1,576 of these being validated on miRTarBase. Validated interactions include genes that play roles in 56 significantly enriched pathways (FDR<0.05) (Supplementary Table 2). Among them, 19 genes were involved in FcƐ receptor I (FCƐRI) pathway, 17 genes in PDGF signalling, and 17 other genes in NGF signalling via TRKA from the plasma membrane.

miRNA target genes in atypical carcinoids regulate adaptive and innate immune system response and neuronal pathways

mirDIP analysis resulted in 4,019 predicted interactions, with 3,438 interactions validated on miRTarBase. Validated interactions include genes that play roles in 245 significantly enriched pathways (FDR<0.05) (Supplementary Table 3). Among them, 55 genes were involved in the innate immune system response, 40 in adaptive immune system response, 36 in axon guidance, 34 in NGF signalling, and 31 genes play roles in NGF signalling via TRKA from the plasma membrane.

miRNA signatures are associated with poor survival of patients with lung adenocarcinoma (TCGA dataset)

Although we tested both datasets of lung adenocarcinoma and squamous cell carcinoma, we found statistical correlation with survival for patients with lung adenocarcinoma only. In this dataset, a 4-miRNA expression signature (hsa-miR-1261/hsa-miR-3148/hsa-miR-595/hsa-miR-375) identified in typical lung carcinoids was found to be associated with survival. All miRNAs except miR-375 were under-expressed in typical carcinoids and showed lower expression in lung adenocarcinoma patients with poor survival (**Figure 1A**).

In addition, using miRNAs identified in atypical lung carcinoids, we found that a subset of 5 miRNAs (hsa-miR-520g/hsa-miR-520h/hsa-miR-941-1/hsa-miR-941-3/hsa-miR-941-4) was associated with worse survival of patients with lung adenocarcinoma. All miRNAs were under-expressed in atypical carcinoids and have significantly lower expression in the high-risk patient group (**Figure 1B**).

Discussion of Main Findings

We identified differences in miRNA expression profiles of typical and atypical carcinoid lung tumors. Previous studies have reported genetic (17–19), epigenetic (11) and miRNA changes in lung carcinoids (4,5,7–9). To the best of our knowledge, ours is the only study to date, which examined a much larger number of miRNAs in a high throughput platform containing 2,578 miRNAs annotated on miRBase (20), compared to previously published reports. In addition, our study was performed with stringent sample preparation criteria, by using needle microdissection to select and analyze miRNA expression in samples containing nearly pure cell populations of at least 95% tumor cells. By applying these

methods, we were able to identify distinct molecular pathways significantly enriched in typical and atypical tumors.

In typical carcinoids, the 9 significantly altered miRNAs play roles in the regulation of genes involved in immune response via the FcЄ receptor I (*FCЄRI*), a receptor expressed in surface of human cells, including monocytes, mast cells and eosinophils. FCЄRI has high affinity and binds to Immunoglobulin E (IgE), activating transcription factors and leading to secretion of cytokines and chemokines. In addition, FCЄRI signalling activates RAS and MAPKs (21), which are known to play roles in lung tumorigenesis. Notably, it has been suggested that mast cells expressing high levels of FCЄRI are key for the efficacy of anti-tumor IgE therapeutics, being able to improve the retention of anti-IgE at tumor sites (22). These findings may represent an opportunity for development of alternative therapeutic strategies to benefit patients with lung carcinoids and who are not eligible to surgery.

Platelet-derived growth factor (PDGF) signalling is known to control several cellular processes such as differentiation, migration, proliferation, and survival, acting by binding to its tyrosine kinase receptor (23). Recently, computational methods were applied to investigate the expression of PDGF family members PDGFA, PDGFB, PDGFC and PDGFD and the receptors PDGFRA and PDGFRB in 7,616 samples from 16 different cancer types using public data retrieved from The Cancer Genome Atlas and the Human Protein Atlas (24). Their study showed that solid tumors showed gene expression alterations in PDGF/PDGFR members and a potential role for PDGF signalling mechanisms were suggested as associated with the tumor microenvironment. These data indicate a crosstalk between tumor cells and the microenvironment mediated by PDGF alterations.

Interestingly, we identified striking differences in the number and type of miRNA alterations in atypical *vs*. typical carcinoids, with the atypical histology having a much higher

number of over-expressed miRNAs. Deregulated miRNAs in atypical carcinoids were found to mainly target genes involved in immune system regulation and neuronal pathways, among other mechanisms of transcriptional control.

Considering that the immune system can contribute to either prevent or promote tumor progression, it is important to understand the interaction between immune and cancer cells within the tumor microenviroment (25,26), as well as the regulatory mechanisms that may affect these interactions, such as miRNA deregulation. Indeed miRNAs expressed by cancer cells have been described to modulate immune system function and immune escape associated with the tumorigenesis process (27).

Dendritic cells are important in innate immune system activation, since they act as antigen-presenting cells, however this function may be blocked during tumor cell growth. Dendritic cells activated by tumors showed a deficiency in several pathways such as MHC class II family, chemokines, cytokines, NF-κB and STAT3 signalling (28).

Lung cancer cells are able to produce several immune suppressive molecules and inhibitory receptors, regulating negatively the immune system, especially for tumor-specific T lymphocytes (29,30). These characteristics support tumor cell proliferation and cancer progression (29).

The nerve growth factor (NGF) is a type of neurotrophin which binds in receptors of tyrosine kinase (TRKA, TRKB and TRKC). TRKA is specifically activated by NGF. The expression of NGF was reported in typical and atypical carcinoids, however, their specific role has not been elucidated. It has been suggested that NGF signalling promotes cancer cell growth and survival through expression of TRK receptors (31).

The binding between NGF and TRK activates a series of cellular pathways related to proliferation, differentiation, survival and apoptosis, via RAS/ERK, PI-3 kinase and AKT (32). This signalling also activates the angiogenesis in epithelial ovarian cancer (33).

Distinct miRNA subsets were associated with poor survival of patients with lung adenocarcinoma. The importance of this finding relates to the ability of lung carcinoids to form lymph node or distant metastasis, directly impacting patient prognosis (34). Patients with typical and atypical lung carcinoids may experience locally recurrent disease, and metastasis has been observed in up to 20% and 40% of patients, respectively (35).

Typical and atypical carcinoids of the lung are rare tumors and there is little information about their alterations and molecular characteristics, as well the role of miRNAs in the tumorigenesis process. We identified two different subsets of significantly deregulated miRNAs in typical and atypical carcinoids, which modulate the expression of target genes in different pathways. These findings show that typical and atypical lung carcinoids have different altered miRNAs. These data contribute to biomarker identification with diagnostic applications. More importantly, our data are novel since we showed that miRNAs regulate distinct tumorigenesis pathways in the different tumor histologies. Innate and adaptive immune response pathways are attractive for the development of novel therapeutics for patients. Therefore, our study contributes to the current literature regarding the molecular classification of these tumors, and may impact clinical management of patients with lung carcinoid tumors.

Acknowledgements

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References

1. Travis WD, Brambilla E, Nicholson AG, Yatabe Y, Austin JHM, Beasley MB, et al. The 2015 World Health Organization Classification of Lung Tumors: Impact of Genetic, Clinical and Radiologic Advances Since the 2004 Classification. J Thorac Oncol [Internet]. 2015;10(9):1243–60. Available from: http://dx.doi.org/10.1097/JTO.0000000000000630
2. Rekhtman N. Neuroendocrine Tumors of the Lung: An update. Arch Pathol Lab Med. 2010;134(11):1628–38.
3. Righi L, Gatti G, Volante M, Papotti M. Lung neuroendocrine tumors: Pathological characteristics. J Thorac Dis. 2017;9(Suppl 15):S1442–7.
4. Di Fazio P, Maass M, Roth S, Meyer C, Grups J, Rexin P, et al. Expression of hsa-let-7b-5p, hsa-let-7f-5p, and hsa-miR-222-3p and their putative targets HMGA2 and CDKN1B in typical and atypical carcinoid tumors of the lung. Tumor Biol [Internet]. 2017;39(10):1–8. Available from: https://www.ncbi.nlm.nih.gov/pubmed/?term=29017393
5. Yoshimoto T, Motoi N, Yamamoto N, Nagano H, Ushijima M, Matsuura M, et al. Pulmonary Carcinoids and Low-Grade Gastrointestinal Neuroendocrine Tumors Show Common MicroRNA Expression Profiles, Different from Adenocarcinomas and Small Cell Carcinomas. Neuroendocrinology. 2017;106(1):47–57.
6. Demes M, Aszyk C, Bartsch H, Schirren J, Fisseler-Eckhoff A. Differential miRNA-Expression as an adjunctive diagnostic tool in neuroendocrine tumors of the lung. Cancers (Basel). 2016;8(4):1–9.
7. Rapa I, Votta A, Felice B, Righi L, Giorcelli J, Scarpa A, et al. Identification of MicroRNAs Differentially Expressed in Lung Carcinoid Subtypes and Progression. Neuroendocrinology [Internet]. 2015;101(3):246–55. Available from: https://www.ncbi.nlm.nih.gov/pubmed/?term=25791280

8. Deng B, Molina J, Aubry MC, Sun Z, Wang L, Eckloff BW, et al. Clinical biomarkers of pulmonary carcinoid tumors in never smokers via profiling miRNA and target mRNA. Cell Biosci [Internet]. 2014;4(35):1–10. Available from: https://www.ncbi.nlm.nih.gov/pubmed/?term=25105010

9. Mairinger FD, Ting S, Werner R, Walter RFH, Hager T, Vollbrecht C, et al. Different micro-RNA expression profiles distinguish subtypes of neuroendocrine tumors of the lung: results of a profiling study. Mod Pathol an Off J United States Can Acad Pathol Inc [Internet]. 2014;27(12):1632–40. Available from: http://dx.doi.org/10.1038/modpathol.2014.74

10. Lee HW, Lee EH, Ha SY, Lee CH, Chang HK, Chang S, et al. Altered expression of microRNA miR-21, miR-155, and let-7a and their roles in pulmonary neuroendocrine tumors. Pathol Int. 2012;62(9):583–91.

11. Fernandez-cuesta L, Peifer M, Lu X, Sun R, Ozretić L, Seidal D, et al. Frequent mutations in chromatin-remodeling genes in pulmonary carcinoids. Nat Commun [Internet]. 2014;3518(5):1–17. Available from: https://www.ncbi.nlm.nih.gov/pubmed/?term=10.1038%2Fncomms4518

12. Saliminejad K, Khorram Khorshid HR, Soleymani Fard S GS. An overview of microRNAs : Biology , functions , therapeutics , and analysis methods. J Cell Physiol. 2018;1–15.

13. Tokar T, Pastrello C, Rossos AEM, Abovsky M, Hauschild AC, Tsay M, et al. MirDIP 4.1 - Integrative database of human microRNA target predictions. Nucleic Acids Res [Internet]. 2018;46(D1):D360-70. Available from: https://www.ncbi.nlm.nih.gov/pubmed/?term=29194489

14. Chou CH, Shrestha S, Yang CD, Chang NW, Lin YL, Liao KW, et al. MiRTarBase update 2018: A resource for experimentally validated microRNA-target interactions.

Nucleic Acids Res [Internet]. 2018;46(D1):D296–302. Available from: https://www.ncbi.nlm.nih.gov/pubmed/?term=29126174

15. Chen J, Bardes EE, Aronow BJ, Jegga AG. ToppGene Suite for gene list enrichment analysis and candidate gene prioritization. Nucleic Acids Res [Internet]. 2009;37(SUPPL. 2):305–11. Available from: https://www.ncbi.nlm.nih.gov/pubmed/?term=19465376

16. Aguirre-Gamboa R, Trevino V. SurvMicro: Assessment of miRNA-based prognostic signatures for cancer clinical outcomes by multivariate survival analysis. Bioinformatics. 2014;30(11):1630–2.

17. Asiedu MK, Thomas CF, Dong J, Schulte SC, Khadka P, Sun Z, et al. Pathways impacted by genomic alterations in pulmonary carcinoid tumors. Clin Cancer Res. 2018;24(7):1691–704.

18. Simbolo M, Mafficini A, Sikora KO, Fassan M, Barbi S, Corbo V, et al. Lung neuroendocrine tumours: deep sequencing of the four World Health Organization histotypes reveals chromatin-remodelling genes as major players and a prognostic role for TERT, RB1, MEN1 and KMT2D. J Pathol. 2017;241(4):488–500.

19. Asiedu MK, Thomas CF, Tomaszek SC, Peikert T, Sanyal B, Sutor SL, et al. Generation and sequencing of pulmonary carcinoid tumor cell lines. J Thorac Oncol [Internet]. 2014;9(12):1763–71. Available from: http://dx.doi.org/10.1097/JTO.0000000000000339

20. Kozomara A, Griffiths-Jones S. MiRBase: Annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res [Internet]. 2014;42(D1):68–73. Available from: https://www.ncbi.nlm.nih.gov/pubmed/?term=24275495

21. Abramson J, Pecht I. Regulation of the mast cell response to the type 1 Fcε receptor. Immunol Rev. 2007;217(1):231–54.

22. Oldford SA, Marshall JS. Mast cells as targets for immunotherapy of solid tumors. Mol Immunol. 2014;63(1):113–24.

23. Noskovičová N, Petřek M, Eickelberg O, Heinzelmann K. Platelet-derived growth factor signaling in the lung: From lung development and disease to clinical studies. Am J Respir Cell Mol Biol. 2015;52(3):263–84.

24. Bartoschek M, Pietras K. PDGF family function and prognostic value in tumor biology. Biochem Biophys Res Commun. 2018;503(2):984–90.

25. Liu X, Wu S, Yang Y, Zhao M, Zhu G, Hou Z. The prognostic landscape of tumor-infiltrating immune cell and immunomodulators in lung cancer. Biomed Pharmacother. 2017;95:55–61.

26. Heuvers ME, Aerts JG, Cornelissen R, Groen H, Hoogsteden HC, Hegmans JP. Patient-tailored modulation of the immune system may revolutionize future lung cancer treatment. BMC Cancer. 2012;12(580):1–12.

27. Eichmüller SB, Osen W, Mandelboim O, Seliger B. Immune Modulatory microRNAs Involved in Tumor Attack and Tumor Immune Escape. J Natl Cancer Inst. 2017;109(10):1–14.

28. Li R, Fang F, Jiang M, Wang C, Ma J, Kang W, et al. STAT3 and NF-κB are Simultaneously Suppressed in Dendritic Cells in Lung Cancer. Sci Rep. 2017;7(March):1–11.

29. Guo W, Liu S, Zhang X, Chen Y, Qian R, Zou Z, et al. The coexpression of multi-immune inhibitory receptors on T lymphocytes in primary non-small-cell lung cancer. Drug Des Devel Ther. 2017;11:3367–76.

30. Zhang L, Wang J, Wei F, Wang K, Sun Q, Yang F, et al. Profiling the dynamic expression of checkpoint molecules on cytokine-induced killer cells from non-small-cell lung cancer patients. Oncotarget. 2016;7(28):43604–15.

31. Ricci A, Graziano P, Mariotta S, Cardillo G, Sposato B, Terzano C, et al. Neurotrophin system expression in human pulmonary carcinoid tumors. Growth Factors. 2005;23(4):303–12.

32. Hoyle GW. Neurotrophins and lung disease. Cytokine Growth Factor Rev. 2003;14(6):551–8.

33. Retamales-Ortega R, Oróstica L, Vera C, Cuevas P, Hernández A, Hurtado I, et al. Role of nerve growth factor (NGF) and miRNAs in epithelial ovarian cancer. Int J Mol Sci. 2017;18(3).

34. Hendifar AE, Marchevsky AM, Tuli R. Neuroendocrine Tumors of the Lung: Current Challenges and Advances in the Diagnosis and Management of Well-Differentiated Disease. J Thorac Oncol [Internet]. 2017;12(3):425–36. Available from: http://dx.doi.org/10.1016/j.jtho.2016.11.2222

35. Chong CR, Wirth LJ, Nishino M, Chen AB, Sholl LM, Kulke MH, et al. Chemotherapy and irradiation for locally advanced and metastatic pulmonary carcinoid tumors. Lung Cancer. 2014;86(2):241–6.

Figure

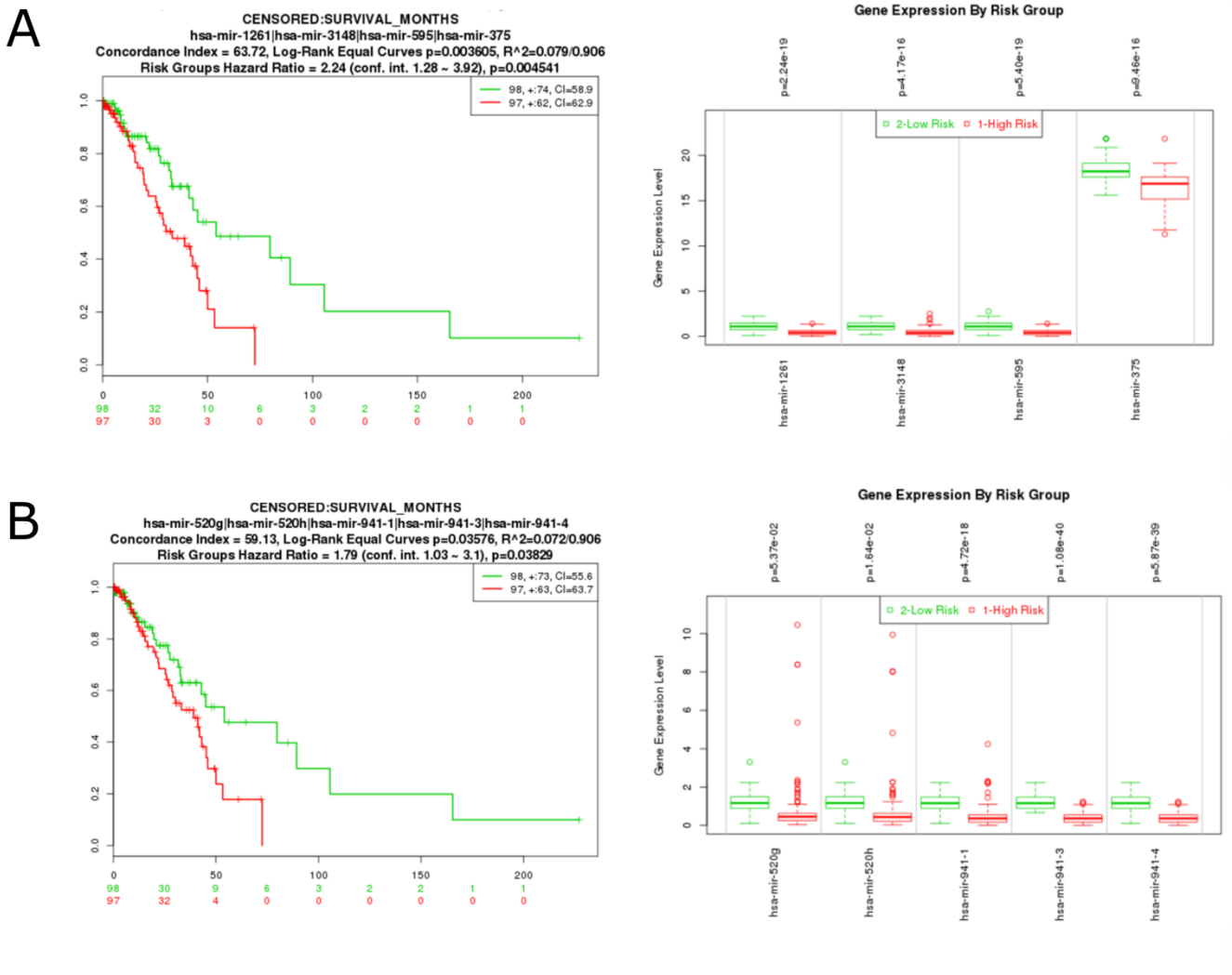

Figure 1. Kaplan-Meier survival analysis of two distinct miRNA subsets identified in typical (A) and atypical (B) lung carcinoids (left panels). These miRNA subsets were correlated with poor survival of patients with lung adenocarcinoma (TCGA dataset). miRNA expression levels for low and high risk group patients are shown in the right panels, for typical and atypical tumors, respectively.

Tables

Table 1. Significantly deregulated miRNAs in typical carcinoid tumors compared to normal lung tissues.

miRNA ID	Fold Change	*p-value*	FDR
hsa-miR-1261	-3	5,46E-05	0,0259
hsa-miR-27a-5p	-7,41	0,0001	0,0398
hsa-miR-3064-5p	-7,04	0,0001	0,0455
hsa-miR-3148	-4,44	9,04E-05	0,0398
hsa-miR-4481	-4,18	1,00E-05	0,0146
hsa-miR-595	-4,19	3,82E-05	0,0259
hsa-miR-7152-3p	-3,58	0,0001	0,0398
hsa-miR-92a-1-5p	-2,94	4,72E-05	0,0259
hsa-miR-375	43,51	5,41E-05	0,0259

Table 2. Significantly deregulated miRNAs in atypical carcinoid tumors compared to normal lung tissues.

miRNA ID	Fold Change	*p-value*	FDR
hsa-miR-1287-5p	-2,05	1,02E-17	1,55E-15
hsa-miR-452-5p	-2,3	8,69E-18	1,55E-15
hsa-mir-520g	-2,5	7,85E-18	1,55E-15
hsa-mir-520h	-2,5	7,85E-18	1,55E-15
hsa-mir-941-1	-2,11	9,70E-18	1,55E-15
hsa-mir-941-2	-2,11	9,70E-18	1,55E-15
hsa-mir-941-3	-2,11	9,70E-18	1,55E-15
hsa-mir-941-4	-2,11	9,70E-18	1,55E-15
hsa-miR-1237-5p	2,57	0,0009	0,0074
hsa-miR-1323	2,51	3,58E-17	1,55E-15
hsa-miR-1909-3p	2,06	1,94E-16	2,07E-15
hsa-miR-330-3p	7,01	0,0037	0,0307
hsa-miR-4433b-3p	2,5	0,0019	0,0158
hsa-miR-4498	3,5	5,24E-17	1,55E-15
hsa-miR-4739	2,01	0,0059	0,0483
hsa-mir-6729	2,48	7,91E-18	1,55E-15
hsa-miR-6787-5p	2,17	0,0056	0,0463
hsa-miR-6803-5p	2,68	0,0013	0,0106
hsa-miR-6813-5p	6,49	0,0015	0,0128
hsa-miR-6815-5p	2,33	8,53E-18	1,55E-15

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: PERFIL DE EXPRESSÃO DE MICRORNAS e ANÁLISE COMPUTACIONAL DE VIAS MOLECULARES MODULADAS POR MICRORNAS EM TUMOR CARCINÓIDE DE PULMÃO

Pesquisador: ANA LAURA SENEDA

Área Temática:

Versão: 1

CAAE: 63732616.0.0000.5411

Instituição Proponente: Departamento de Cirurgia e Ortopedia

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.908.978

Apresentação do Projeto:

Segundo os autores O carcinoma pulmonar de células pequenas é menos frequente, porém é muito mais agressivo que o de células não pequenas. Apresenta quatro classificações: (1) tumor carcinoide típico, (2) tumor carcinoide atípico, (3) carcinoma de grandes células de alto grau e (4) carcinoma de células pequenas. Os atípicos têm maior probabilidade de recorrência ou aparecimento de metástases. Devido à baixa incidência, muito pouco se conhece sobre as vias metabólicas que atuam na tumorigênese desse tipo de carcinoma. Nos últimos anos, moléculas responsáveis pela regulação da expressão gênica, como os microRNAs (miRNAs) têm sido associados à tumorigênese. Os miRNAs são pequenas fitas de RNAs (~18-12 nucleotídeos de comprimento) que não são transcritos, cuja função é se ligar à extremidade 3' não traduzida (3' UTR) do RNA mensageiro (mRNA), inibindo a tradução ou degradação do mRNA, sendo indicados como candidatos ideais a biomarcadores diagnósticos, prognósticos e como alvos terapêuticos potenciais no câncer. Esse projeto compreenderá a análise da expressão global dos 2.588 miRNAs mapeados no genoma humano e com dados de anotação de sequências, disponíveis no miRBase. Após, esse perfil de expressão global será validado em amostras independentes, identificando (in silico) as vias moleculares envolvidas. Os RNAs serão extraídos e sua expressão serão analisados na plataforma GeneChip® miRNA 4.0 Array (Affymetrix), sendo aqueles significativamente (p 0,05)

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alterados, validados em amostras independentes, por meio de Reação em Cadeia da Polimerase Quantitativa em Tempo Real (RT-QPCR). Ferramentas de bioinformática serão utilizadas para predizer os mRNAS-alvos potencialmente regulados por esse miRNAs desregulados e validados. Além disso, os genes regulados por miRNAs serão identificados (in silico) em suas respectivas proteínas para análise de interações entre proteínas e entre mutações. Esses dados serão correlacionados aos dados do paciente (idade, sexo, fatores de risco, dados histopatológicos) por meio de métodos estatísticos incluindo a descrição de variáveis categóricas e variáveis contínuas pelo teste de Mann-Whitney e o teste exato de Fisher. Espera-se contribuir para o desenvolvimento de novas estratégias diagnósticas e terapêuticas para pacientes com essas neoplasias.

Objetivo da Pesquisa:

- Objetivo Primário: Determinar o perfil global de expressão de miRNAs e identificar (in silico) os mRNAs-alvo regulados pelos miRNAs e vias moleculares potencialmente condutoras da tumorigênese em tumores carcinoides do pulmão.

- Objetivo Secundário: . Validar alterações na expressão de miRNAs em amostras independentes.

Avaliação dos Riscos e Benefícios:

- Método - O perfil de expressão global de miRNAs será determinado em 2 amostras de tecido tumoral primário do subtipo carcinoide (grupo treinamento) e será validado em outras 4 amostras de tumor carcinoide (grupo validação). As amostras serão provenientes de pacientes tratados no Hospital de Clínicas da FMB - UNESP. Portanto, utilizaremos um total de 6 amostras de tecido emblocadas em parafina e disponíveis no Departamento de Patologia da FMB – UNESP. As amostras serão obtidas em colaboração com o patologista (Dr. Julio Defaveri), colaborador dessa pesquisa e após a extração do RNA (ver abaixo), serão armazenadas na Unidade de Pesquisa Experimental (UNIPEX) até o seu uso para análise de miRNAs. Os dados dos pacientes (demográficos; idade e sexo), fatores de risco, dados histopatológicos e de seguimento dos pacientes serão coletados pelo grupo clínico-cirúrgico (Drs. Erica Hasimoto, Daniele Castâneo, Antônio Castâneo). Não haverá identificação dos pacientes; utilizaremos códigos numéricos para a manutenção da confidencialidade. Os autores justificam que o pequeno número de amostras se dá devido à raridade desses tumores.

- Extração do RNA das amostras de tecido tumoral: O RNA das amostras de tumor será extraído utilizando o RecoverAll Total Nucleic Acid Isolation - Kit (Ambion/Life Technologies), o qual é otimizado para a extração de RNA de tecidos fixados em formalina e embebidos em parafina (FFPE). A quantidade e a qualidade do RNA serão determinadas utilizando o equipamento NanoDrop 8000 (Thermo Scientific). A expressão dos miRNAs significativamente (p 0,05) alterados serão validados em amostras independentes (N = 4 tumores do tipo carcinoide). A metodologia de validação da expressão de miRNAs será a Reação em Cadeia da Polimerase Quantitativa em Tempo Real (RT-QPCR). Os miRNAs identificados como significativamente desregulados (Obj. 1) e validados (Obj. 2) serão utilizados para análises subsequentes de bioinformática, para predição dos mRNAs-alvo potencialmente regulados por esses miRNAs.

- Riscos: Não há riscos envolvidos.

- Benefícios: Os resultados esperados incluem a identificação e validação de miRNAs em tumores carcinoides do pulmão, os quais constituem um grupo de tumores raros. Além disso, a identificação de mRNAs-alvo dos miRNAs identificados, os quais podem estar associados a vias moleculares importantes no desenvolvimento e progressão do tumor carcinoide. Direções futuras incluem a elaboração de estudos funcionais para a determinação do papel de vias moleculares reguladas por miRNAs no desenvolvimento e progressão desses tumores. Estudos como este podem contribuir para o desenvolvimento de novas estratégias diagnósticas e terapêuticas para pacientes com essas neoplasias.

Comentários e Considerações sobre a Pesquisa:

Trata-se de projeto de mestrado da autora Ana Laura Seneda com a orientação da Profa Dra. Patrícia Pintor dos Reis, do Departamento de Cirurgia e Ortopedia da FMB e colaboração dos Professores Júlio De Faveri, Antônio José Maria Castâneo, Erica Nishida Hasimoto, Daniele Cristina Castâneo, cujo objetivo é determinar o perfil global de expressão de miRNAs em tumores carcinoides do pulmão. Considerando que existe uma escassez de dados sobre alterações genéticas e epigenéticas associadas ao desenvolvimento e progressão de tumores carcinoides do pulmão, e que a identificação de vias moleculares condutoras da tumorigênese é de grande importância para determinar terapêuticas mais precisas para pacientes com câncer, estudos como este são fundamentais.

O projeto está escrito de forma clara e apresenta todos os dados de identificação dos autores e

serviços a que estão vinculados, a descrição dos objetivos do estudo, bem como, as referências bibliográficas sustentam as justificativas e importância do tema abordado. É um projeto com condições de realização.

Considerações sobre os Termos de apresentação obrigatória:

Os autores solicitam dispensa do TCLE devido ao fato de que todos os pacientes (6) a serem incluídos na pesquisa apresentam perda de seguimento, ou foram a óbito, não estando em acompanhamento no Hospital de Clínicas de Botucatu.

Conclusões ou Pendências e Lista de Inadequações:

O projeto é claro bem escrito, o cronograma está adequado, a justificativa para dispensa do TCLE é pertinente, portanto o projeto apresenta todas as condições e autorizações necessárias para sua realização.

Considerações Finais a critério do CEP:

Projeto de Pesquisa APROVADO, deliberado em reunião ORDINÁRIA do CEP de 06/02/2017, sem necessidade de envio à CONEP.

O CEP, no entanto, solicita aos pesquisadores que após a execução do projeto em questão, seja enviado para análise o respectivo “Relatório Final de Atividades”, o qual deverá ser enviado via Plataforma Brasil na forma de “NOTIFICAÇÃO”.

OBS: LEMBRAMOS QUE A PRESENTE PESQUISA SOMENTE PODERÁ SER INICIADA APÓS DIA 06/02/2017 – DATA DA APROVAÇÃO DO CEP.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_792083.pdf	21/12/2016 11:30:52		Aceito
Folha de Rosto	Plat_Brasil_Ana_Laura_Seneda.pdf	21/12/2016 11:29:05	ANA LAURA SENEDA	Aceito
Outros	Esclarecimento_Coorientador.pdf	20/12/2016 17:40:06	ANA LAURA SENEDA	Aceito
Declaração de Instituição e Infraestrutura	2612_Ana_Laura_Seneda.pdf	14/10/2016 13:52:19	ANA LAURA SENEDA	Aceito

Continuação do Parecer: 1.908.978

Projeto Detalhado / Brochura Investigador	PROJETO_MESTRADO_AnaLaura_Setembro_14_2016.docx	14/10/2016 13:51:28	ANA LAURA SENEDA	Aceito
Outros	CEP_AnaLaura.pdf	12/09/2016 12:22:41	ANA LAURA SENEDA	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	Dispensa_TCLE_assinada_AnaLaura.pdf	12/09/2016 11:56:18	ANA LAURA SENEDA	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

BOTUCATU, 06 de Fevereiro de 2017

Assinado por:
SILVANA ANDREA MOLINA LIMA
(Coordenador)