**SUBPROJECT 1:** Genetic heterogeneity of tumor cells: identification and functional characterization of biomarkers for therapeutic purposes in cancer.

**Principal Investigator:** *Wilson Araújo da Silva Jr*

## Abstract

Tumor heterogeneity is a natural process of evolution of tumor cells, with singular consequence in response to different therapies [1] [2] [3]. In the era of personalized medicine gives much importance to the concept that a tumor is a unique disease in each patient. In a recent study, Gerlinger et al. (2012) [4] demonstrated that within a primary tumor mass this uniqueness extends to other regions of the tumor and its metastases. The autors examined the concept of intratumoral heterogeneity in patients diagnosed with renal clear cell cancer. Analysis of global sequencing identified a deletion in the VHL gene (commonly mutated in this disease) present in all regions studied, including metastases. Interestingly, it was only possible to connect a particular region of the patient with metastases. What drew most attention was that the different regions and the metastases have distinctly enriched genes with good or poor prognosis. The 110 genes that conferred a signature for this type of cancer, related to better prognosis were enriched in the primary tumor and metastasis, and poor prognosis genes were enriched in other areas of the primary tumor. In search of new therapeutic targets, noncoding RNAs have attracted the attention of researchers because of its role in regulating biological processes involved in developing cancer. The non-coding, small RNAs (miRNAs, piRNAs), medium (PASRs) or long (long non-coding RNAs - lncRNAs) [5] [6] [7] regulate various molecular, genetic and cellular processes linked to tumor progression which include: chromosome dosage compensation, modification of chromatin structure, transcription and translation, splicing, cell differentiation, integrity of cell structures, cell cycle, intracellular trafficking, reprogramming of stem cells and response to heat-shock (Mattick and Clark , 2011; Esteller M., 2011) [8] [9]. The role of noncoding RNAs in regulating biological processes linked to tumorigenesis opens a new front in the identification of biomarkers for diagnosis, prognosis, and potential therapeutic targets for cancer treatment. On this proposal we intend to apply Next Generation Sequencing (NGS) technology to investigate the genetic and epigenetic changes that confer selective advantage to tumor cells, with the potential to be used as genetic markers for diagnosis and prognosis as well as therapeutic targets.

**Goals**

Explore the genetic heterogeneity of the tumor cell subpopulations, as well as characterizing the expression profile of RNAs coding and non-coding, with special attention to long microRNAs and regulatory RNAs. The functional characterization of these molecules assist in the identification of potential therapeutic targets

**Specific Goals**

*1. Identification of mRNAs, lncRNAs and miRNAs enriched in tumor tissues and tumor cell lines;*

*Data from RNA-Seq data (transcriptome) of melanoma (cell lines), recurrent glioblastoma, gastric tumor and metastatic tumors of bone and brain will be examined. Additionally, data from RNA-Seq published by TCGA will be analyzed for in silico validation of selected transcripts.*

*2. Characterization of the genetic and epigenetic heterogeneity of tumor cell subpopulations;*

*The subpopulations of tumor cells will be isolated and collected by flow cytometry with cell type-specific markers. Then, a pool of each cell type will be processed in C1 Single Cell ™ Auto Prep System platform for preparation of RNA-Seq and exoma libraries and of each single cell subpopulation.*

*3. functional study;*

*The transcripts selected as a potential therapeutic target will be functionally characterized using tumor cell lines with approaches of siRNA, shRNA and CRISPR/Cas9, followed by the evaluation of biological processes such as cell proliferation, apoptosis, migration, invasion and repair of DNA damage.*

*4. Therapeutic Approach.*

*After functional validation, in the case of a lncRNA, we can use the approach of AntagoNAT (Natural Antisense Transcripts - NATs) transcripts or ATFs (Artificial Transcription Factors). To the miRNAs, the antagomir is the most appropriate approach to cancer treatment. The three types of targets will be tested in animal model and tumor cell lines. Once proven the efficiency of the target, the next step will be the development of nanostructures for its controlled and selective release in animal model.*

**Implementation schedule**

| Specific goals | Semesters | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| 1 | | | | | | | | | | | | |
| 2 | | | | | | | | | | | | |
| 3 | | | | | | | | | | | | |
| 4 | | | | | | | | | | | | |

**Researchers**

Anelisa Ramão
Cleidson de Padua Alves
Houtan Noushmehr
Valeria Valente
Israel Tojal da Silva
Josane de Freitas Sousa
Valtencir Zucolotto
Wilson Araújo da Silva Jr

**Associated Researchers**

Carlos Gilberto Carlotti Jr
Luciano Neder Serafini
Vanessa da Silva Silveira
Daniela Pretti da Cunha Tirapelli
Valtencir Zucolotto

**Postgraduate students**

Ádamo Davi Diogenes Siena
Carlos R Oliveira
Jessica Rodrigues Plaça
Rodolfo Bortolozo Serafim
Bruna Rodrigues Muys
Isabela Ichihara de Barros
Luiza Ferreira Araújo
Simon Coetzee

**Technical Staff**

| | |
|---|---|
| Adriana Aparecida Marques | Greice Andreotti de Molfetta |
| Ana Julia Machry | Kamila Chagas Peronni Zueli |
| Anemari Ramos Dinardi Baccarin | Marcelo Gomes |

**References**


[1] Marusyk A, Polyak K. Tumor heterogeneity: causes and consequences. Biochim Biophys Acta. 2010 Jan;1805(1):105-17

[2] Durrett R, Foo J, Leder K, Mayberry J, Michor F. Intratumor heterogeneity in evolutionary models of tumor progression. Genetics. 2011 Jun;188(2):461-77.

[3] Burrell RA, McGranahan N, Bartek J, Swanton C. The causes and consequences of genetic heterogeneity in cancer evolution. Nature. 2013 Sep 19;501(7467):338-45.

[4] Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, Martinez P, Matthews N, Stewart A, Tarpey P, Varela I, Phillimore B, Begum S, McDonald NQ, Butler A, Jones D, Raine K, Latimer C, Santos CR, Nohadani M, Eklund AC, Spencer-Dene B, Clark G, Pickering L, Stamp G, Gore M, Szallasi Z, Downward J, Futreal PA, Swanton C: Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med. 2012 Mar 8;366(10):883-92.

[5] A long non-coding RNA signature in glioblastoma multiforme predicts survival. Neurobiol Dis. 2013 Oct; 58:123-31.

[6] CHEETHAM, S. W. et al. Long noncoding RNAs and the genetics of cancer. British journal of cancer, v. 108, n. 12, p. 2419–25, 25 jun. 2013.

[7] SÁNCHEZ, Y.; HUARTE, M. Long non-coding RNAs: challenges for diagnosis and therapies. Nucleic Acid Ther. 2013 Feb;23(1):15-20.

[8] CLARK, M. B.; MATTICK, J. S. Long noncoding RNAs in cell biology. Seminars in cell & developmental biology, v. 22, n. 4, p. 366–76, jun. 2011.

[9] Esteller, M. Non-coding RNAs in human disease. Nature Reviews Genetics 12, 861-874.

**SUBPROJETO 1**: Análise da heterogeneidade genética das células tumorais: identificação e caracterização funcional de biomarcadores para fins terapêuticos em câncer.

**Pesquisador Responsável:** *Wilson Araújo da Silva Jr*

## Introdução

A heterogeneidade tumoral é fruto da evolução e adaptação das células tumorais, prejudicando sobremaneira a melhor resposta as diferentes terapias. Na era da medicina personalizada dá-se muita importância ao conceito de que um tumor é uma doença única em cada paciente. Em estudo recente, Gerlinger et al. (2012) demonstraram que dentro de uma massa tumoral primária esta singularidade se estende a outras regiões do tumor e suas metástases. Neste trabalho os pesquisadores examinaram o conceito de heterogeneidade intratumoral em pacientes diagnosticados com câncer renal de células claras. Análise de sequenciamento global identificou uma deleção no gene VHL (comumente mutado nessa doença) presente em todas as regiões estudadas, inclusive nas metástases. Interessantemente, foi possível conectar apenas uma região em particular com as metástases do paciente. O que chamou mais atenção foi que as diferentes regiões e as metástases eram distintamente enriquecidos com genes de bom ou péssimo prognóstico. Dos 110 genes que conferiam uma assinatura para este tipo de câncer, os relacionados com bom prognóstico estavam enriquecidos nas metástases e no tumor primário, e os genes de péssimo prognóstico estavam enriquecidos nas demais áreas do tumor primário. Na busca de novos alvos terapêuticos, os RNAs não-codificadores tem despertado atenção dos pesquisadores dados o seu papel na regulação de processos biológicos inerentes ao desenvolvimento câncer. Os RNAs não-codificadores, pequenos (miRNAs, piRNAs), médios (PASRs) ou longos (long non-coding RNAs - lncRNAs), regulam diversos processos moleculares, genéticos e celulares ligados a progressão tumoral, que incluem: compensação da dosagem cromossômica, modificação da estrutura da cromatina, transcrição e tradução, splicing, diferenciação celular, integridade de estruturas celulares, ciclo celular, tráfego intracelular, reprogramação de células-tronco e resposta ao heat-shock (Clark e Mattick, 2011; Esteller M., 2011). Conhecer o papel dos RNAs não-codificadores na regulação de processos biológicos ligados a tumorigênese abre uma nova frente de identificação de biomarcadores de diagnóstico, prognóstico, e potenciais alvos terapêuticos para o tratamento do câncer. A presente proposta usará dados de Next Generation Sequencing (NGS) para investigar as alterações genéticas e epigenéticas que conferem vantagem seletiva as células tumorais, com potencial de ser usado como marcadores genéticos de diagnóstico e prognóstico, assim como alvos terapêuticos.

**Objetivo Geral**

Analisar a heterogeneidade das células tumorais caracterizando as populações isoladas quanto ao perfil de expressão de RNAs codificadores e não-codificadores, com especial atenção aos microRNAs e os RNAs longos reguladores. A caracterização funcional dessas moléculas contribuirá para a identificação de alvos com potencial terapêutico

**Metas**

*1. Identificação de mRNA, lncRNAs e miRNAs enriquecidos em tecidos tumorais e linhagem de células tumorais*

Será analisado dados de RNA-Seq (transcriptoma) de melanoma (linhagens celulares), glioblastoma recorrente, tumor gástrico e de tumores metastáticos de osso e cérebro. Adicionalmente, dados de RNA-Seq publicados pelo TCGA serão analisados para validação in silico dos transcritos selecionados.

*2. Caracterização da heterogeneidade genética e epigenética das subpopulações de células tumorais.*

As subpopulações de células tumorais serão isoladas e coletadas por citometria de fluxo com marcadores específicos à cada tipo celular. Em seguida, um pool de cada tipo celular será processado na plataforma C1™ Single-Cell Auto Prep System para preparação das bibliotecas de RNA-Seq e exoma de 96 células únicas de cada subpopulação.

*3. Estudo funcional.*

Os transcritos selecionados como potencial alvo terapêutico serão caracterizados funcionalmente usando ensaios in vitro com linhagens de células tumorais com abordagens de siRNA, shRNA e CRISPR/Cas9, seguido da avaliação de processos biológicos como: proliferação celular, apoptose, migração, invasão e reparo de danos no DNA.

*4. Abordagem terapêutica.*

Após a validação funcional dos transcritos selecionados como potenciais alvos, quando se tratar de um lncRNA as abordagens de AntagoNAT (Natural Antisense Transcripts – NATs) ou ATFs (Artificial Transcription Factors). No caso do alvo ser um miRNA, a abordagem usando antagomir é a mais indicada no tratamento do câncer. Os três tipos de alvos serão testados em linhagens tumorais e modelo animal. Uma vez comprovada a eficiência do alvo, o passo seguinte será o desenvolvimento de sistemas nanoestruturados para liberação controlada e seletiva de antagomir, ATFs e antagoNATs em modelo animal.

**Cronograma de execução referente a seis anos de projeto**

| Metas | Semestres | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| 1 | | | | | | | | | | | | |
| 2 | | | | | | | | | | | | |
| 3 | | | | | | | | | | | | |
| 4 | | | | | | | | | | | | |

**Pesquisadores:**

Anelisa Ramão

Cleidson de Padua Alves

Houtan Noushmehr

Israel Tojal da Silva

Josane de Freitas Sousa

Valeria Valente

**Pesquisadores Colaboradores:**

Carlos Gilberto Carlotti Jr

Daniela Pretti Cunha Tirapelli

Luciano Neder Serafini

Valtencir Zucolotto

**Referências**


Van Meir, EG. et al. Exciting New Advances in Neuro-Oncology: The Avenue to a Cure for Malignant Glioma. CA Cancer J Clin 2010; 60; 166-193.

Brower JV et al. MicroRNAs in cancer: Glioblastoma and glioblastoma cancer stem cells. Neurochem Int. 2014 Jun 14. pii: S0197-0186(14)00141-7.

A long non-coding RNA signature in glioblastoma multiforme predicts survival. Neurobiol Dis. 2013 Oct; 58:123-31.

Li X et al. Two mature products of MIR-491 coordinate to suppress key cancer hallmarks in glioblastoma. Oncogene. 2014 Apr 21.

Correa P, Houghton J: Carcinogenesis of Helicobacter pylori, Gastroenterology 2007, 133:659-672.

Correa P, Piazuelo MB: The gastric precancerous cascade, J Dig Dis 2012, 13:2-9

Correa P: A human model of gastric carcinogenesis, Cancer Res 1988, 48:3554-3560.

Danaei G, Vander Hoorn S, Lopez AD, Murray CJ, Ezzati M: Causes of cancer in the world: comparative risk assessment of nine behavioural and environmental risk factors, Lancet 2005, 366:1784-1793 3.

Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, Martinez P, Matthews N, Stewart A, Tarpey P, Varela I, Phillimore B, Begum S, McDonald NQ, Butler A, Jones D, Raine K, Latimer C, Santos CR, Nohadani M, Eklund AC, Spencer-Dene B, Clark G, Pickering L, Stamp G, Gore M, Szallasi Z, Downward J, Futreal PA, Swanton C: Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med. 2012 Mar 8;366(10):883-92.

Goldenring JR, Nam KT, Wang TC, Mills JC, Wright NA: Spasmolytic polypeptide-expressing metaplasia and intestinal metaplasia: time for reevaluation of metaplasias and the origins of gastric cancer, Gastroenterology 2010, 138:2207-2210, 2210 e2201.

Goldenring JR, Nomura S: Differentiation of the gastric mucosa III. Animal models of oxyntic atrophy and metaplasia, Am J Physiol Gastrointest Liver Physiol 2006, 291:G999-1004.

Lee HJ, Nam KT, Park HS, Kim MA, Lafleur BJ, Aburatani H, Yang HK, Kim WH, Goldenring JR: Gene expression profiling of metaplastic lineages identifies CDH17 as a prognostic marker in early stage gastric cancer. Gastroenterology, 2010, 139(1):213-25.e3.

Nam KT, Lee HJ, Mok H, Romero-Gallo J, Crowe JE, Jr., Peek RM, Jr., Goldenring JR: Amphiregulin- deficient mice develop spasmolytic polypeptide expressing metaplasia and intestinal metaplasia, Gastroenterology 2009, 136:1288-1296.

Nam KT, Lee HJ, Sousa JF, Weis VG, O'Neal RL, Finke PE, Romero-Gallo J, Shi G, Mills JC, Peek RM, Jr., Konieczny SF, Goldenring JR: Mature chief cells are cryptic progenitors for metaplasia in the stomach, Gastroenterology 2010, 139:2028-2037 e2029.

Schmidt PH, Lee JR, Joshi V, Playford RJ, Poulsom R, Wright NA, Goldenring JR: Identification of a metaplastic cell lineage associated with human gastric adenocarcinoma, Lab Invest 1999, 79:639-646.

Sousa JF, Ham AJ, Whitwell C, Nam KT, Lee HJ, Yang HK, Kim WH, Zhang B, Li M, Lafleur B, Liebler DC, Goldenring JR: Proteomic Profiling of Paraffin-Embedded Samples Identifies Metaplasia-Specific and Early-Stage Gastric Cancer Biomarkers, Am J Pathol. 2012, 181(5):1560-72.

Yoshizawa N, Takenaka Y, Yamaguchi H, Tetsuya T, Tanaka H, Tatematsu M, Nomura S, Goldenring JR, Kaminishi M: Emergence of spasmolytic polypeptide-

expressing metaplasia in Mongolian gerbils infected with Helicobacter pylori, Lab Invest 2007, 87:1265-1276.

Esteller, M. Non-coding RNAs in human disease. Nature Reviews Genetics 12, 861-874.

SÁNCHEZ, Y.; HUARTE, M. Long non-coding RNAs: challenges for diagnosis and therapies. Nucleic Acid Ther. 2013 Feb;23(1):15-20.

WAHLESTEDT, C. Targeting long non-coding RNA to therapeutically upregulate gene expression. Nat Rev Drug Discov. 2013 Jun;12(6):433-46.

CHEETHAM, S. W. et al. Long noncoding RNAs and the genetics of cancer. British journal of cancer, v. 108, n. 12, p. 2419–25, 25 jun. 2013.

CLARK, M. B.; MATTICK, J. S. Long noncoding RNAs in cell biology. Seminars in cell & developmental biology, v. 22, n. 4, p. 366–76, jun. 2011.

INCA. Prevenção e controle do câncer: normas e recomendações do INCA. v. 48, n. 3, p. 317–32, 2002.

LI, R. et al. Long Non-Coding RNA BANCR Promotes Proliferation in Malignant Melanoma by Regulating MAPK Pathway Activation. PloS one, v. 9, n. 6, p. e100893, 26 jan. 2014.

MACKIE, R. M.; HAUSCHILD, A.; EGGERMONT, A. M. M. Epidemiology of invasive cutaneous melanoma. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO, v. 20 Suppl 6, p. vi1–7, ago. 2009.

MAZAR, J. et al. The functional characterization of long noncoding RNA SPRY4-IT1 in human melanoma cellsOncotarget, 2014.

MILLER, A. J.; MIHM, M. C. Melanoma. New England Journal of Medicine, v. 355, n. 1, p. 51–65, 6 jul. 2006.