Title page

Title: IMP dehydrogenase rods/rings structures in Acral Melanomas

Running Title: IMPDH-based RR in Acral Melanomas

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Significance:

Accessing malignancy of melanoma require a complex analysis of histopathology and in some instances, immunohistochemistry markers in the affected tissue. Here we proposed that the screening by indirect immunofluorescence of the IMPDH rods/rings structures in the tumor is a valuable cost effective tool to help differentiating ALM from Melanocytic Nevi, with >80% sensitivity and specificity, and also accessing malignancy of in acral melanoctic lesions. Follow-up studies may show the application of this test in the diagnosis of other types of melanomas, and possibly other types of aggressive tumors.

Keywords:

Melanoma, Cutaneous Malignant; Acral Lentiginous Melanoma; Melanocytic Nevi; IMP Dehydrogenase; Rods/rings structures; tissue micro array.

Main Document

IMP dehydrogenase rods/rings structures in Acral Melanomas

Abstract: Acral lentiginous melanoma (ALM) is a rare subtype of melanoma with aggressive behavior. IMPDH enzyme, involved in de novo GTP biosynthesis, has been reported to assemble into large filamentary structures called rods/rings (RR) or cytoophidium (cellular snakes). RR assembly induces a hyperactive state in IMPDH, usually to supply a high demand for GTP nucleotides, such as in highly proliferative cells. We investigate if aggressive melanoma tumor cells present IMPDH-based RR structures. Forty-five ALM paraffin-embedded tissue samples and 59 Melanocytic Nevi were probed with anti-IMPDH2 antibody. Both the rod and ring-shaped RR could be observed, with higher frequency in ALM. ROC curve analyzing proportions of RR-positive cells in ALM versus Nevi yielded a 0.88 AUC. Using the cutoff of 5.5% RR-positive cells, there was a sensitivity of 80% and specificity of 85% for ALM diagnosis. In ALM, 36(80%) showed RR frequency above the cutoff, being classified as RR-positive, compared to only 9(15%) of the Nevi ($p<0.001$). Histopathology showed that 71% of the RR-positive specimens presented Breslow thickness >4.0mm, compared to only 29% in the RR-Low/Negative ($p=0.039$). We propose that screening for RR structures in biopsy specimens may be a valuable tool helping differentiate ALM from Nevi and accessing tumor malignancy.

Keywords: Melanoma, Cutaneous Malignant; Acral Lentiginous Melanoma; Melanocytic Nevi; IMP Dehydrogenase; Rods/rings structures; tissue microarray.

a) Introduction

Acral lentiginous melanoma (ALM) is a rare subtype of melanoma mainly arising in areas of the skin not exposed to solar radiation, such as the palms, soles, and nail beds. ALM has an aggressive behavior and is the most common subtype of melanoma not related to solar radiation that is found in patients of Asian or African descent.

Inosine 5´-monophosphate dehydrogenase (IMPDH) enzyme is involved in de novo GTP biosynthesis pathway, catalyzing the rate-limiting step of converting the substrate IMP to XMP, which is immediately and sequentially converted into GMP, GDP and GTP. IMPDH is usually dispersed in the cytoplasm as tetramers and octamers. Recently, several groups have reported that IMPDH will assemble into large (3-10μm) filamentous structures, either as rod (snake-like) or as circular (donut-like rings) structures. Accordingly, these structures have been named rods and rings (RR) or cytoophidium (from the Greek, “cellular snakes”) (Carcamo et al., 2011; Chen et al., 2011; Keppeke, Calise, Chan, & Andrade, 2015). For the sake of clarity, we will name these structures as rods and rings in this manuscript. Our group has recently described that IMPDH assembling into RR results in an enzymatic hyperactive state, usually required to supply an increased consumption rate of GTP nucleotides, such as observed in highly proliferative cells (Chang et al., 2015; Keppeke et al., 2018). This is further supported by the observation of naturally occurring RR structures in pluripotent stem cells, known to maintain a very high proliferation rate (Keppeke et al., 2018).

A recent study has shown that another enzyme that also assembles into RR structures, Cytidine Triphosphate Synthase (CTPS), forms RR in cells from 28% out of 203 samples of human hepatocellular carcinoma (Chang et al., 2017). CTPS catalyzes the rate-limiting step in the de novo CTP synthetic pathway in which UTP is converted into CTP. Levels of the oncogenic heat shock protein 90 (HSP90) were higher in samples with a high proportion of CTPS-based RR (Chang et al., 2017). This suggests that the screening for the presence of CTPS-based RR could be a useful tool in the analysis of the metabolic status and prognosis of tumors.

Since IMPDH-based RR structures are naturally present in cells at high proliferative state and ALM tumors have an aggressive behavior, our goal was to study if melanoma tumor cells present IMPDH RR structures and if the presence of RR can help differentiating ALM from Melanocytic Nevi. We also correlated the presence of RR structures with other markers for tumor malignancy.

b) Materials e Methods

Tumor Samples

Forty-five specimens of ALM and 59 Melanocytic Nevi were obtained from the Department of Pathology, Escola Paulista de Medicina, Sao Paulo, Brazil. Samples were obtained in the period between 1996 and 2010 and reviewed by an experienced melanoma pathologist (G.L.). Histopathological prognostic factors were established according to the WHO protocol and guidelines of the Brazilian Society of Pathology (Castro et al., 2016; Castro et al., 2015; LeBoit, Burg, Weedon, & Sarasain, 2006). The study was approved by the ethics review board of the Federal University of Sao Paulo (CEP #1013/11), Brazil.

Tissue Microarrays, Indirect Immunofluorescence, Histopathology and Immunohistochemistry

Tissue microarrays (TMAs) were assembled with 1mm-thick sections for the melanoma specimens available. From the paraffin block of each primary ALM melanoma specimen, four different areas were harvested and analyzed as indicated in Figure 3C. Two different areas were harvested and analyzed for each Nevi specimen.

For the screening of RR structures by indirect immunofluorescence, we used a previously tested protocol adapted for paraffin-embedded tissue sections (Calise, Abboud, Kasahara, Morel, & Chan, 2018). After a procedure for antigen recovery in the tissue sections, the immunofluorescence reaction was performed using the rabbit polyclonal anti-IMPDH2 antibody (1:300 dilution, #12948-1-AP, Proteintech) as primary antibody and Cy™3-conjugated anti-Rabbit IgG donkey polyclonal antibodies (1:300, #711-165-152, Jackson ImmunoResearch) as secondary antibody. TMA slides were analyzed and images captured with an Axio Imager.M2 (Zeiss) fluorescence microscope.

Histopathology and immunohistochemistry data for a pre-defined set of markers (MYC, SCF, PTEN, KIT, BRAF and CYCLIN D1) were obtained from a previous study using the same set of samples (Carapeto et al., 2017).

Image analysis and statistics

Captured images with 400x magnification were analyzed for the quantification of cells (based in the Hoechst DNA staining) and the amount of cells presenting RR. At least two images (an average of ~400 cells) were analyzed per section. Four sections were analyzed for each ALM specimen and two sections for each Melanocytic Nevi specimen.

Classificatory variables (proportions) were compared with Chi-square test with Bonferroni's correction when necessary. For continuous variables, average ± Standard Deviation (SD) or Standard Error of the Mean (SEM) were used. Continuous variables were compared with Mann-Whitney's t test as no variable presented Gaussian distribution accessed with the "D'Agostino & Pearson omnibus normality test".

A receiver operating characteristic curve (ROC curve) analysis was used to plot proportions of RR-positive cells in ALM versus Nevi, with determination of the Area Under the Curve (AUC). The cutoff (% of RR-positive cells per TMA section) was defined based in Youden's Index to find best Sensitivity and Specificity values. A p value <0.05 was considered statistically significant. All statistical analyses were performed with GraphPad Prism software.

c) Results

Presence of RR structures in ALM versus Melanocytic Nevi

Clinical and pathological data referent to the 104 patients has been previously published (Carapeto et al., 2017). After labeling, sections were blindly screened for the presence of RR structures. Both the rod- and ring-shaped RR structures could be observed (arrows and arrowhead in Figure 1) although most of the structures were rod-shaped. Quantification of the proportion of RR-positive cells showed great variation across specimens, ranging from zero up to ~80% (Figure 2A and B), indicating heterogeneity in the capacity of RR structures assembly in different melanoma tumors.

We next compared the proportion of RR-positive cells in 45 ALM and 59 Nevi specimens. As we have observed a great heterogeneity in the distribution of imunohistochemistry markers among the different sections of each specimen (Carapeto et al., 2017), we used two alternative parameters to describe the frequency of RR-positive cells in specimens from ALM and Melanocytic Nevi: 1- average proportion of RR-positive cells in all sections of each specimen; 2- the highest proportion of RR-positive cells among the multiple sections in each specimen. In both scenarios, the proportion of RR-positive cells was higher in ALM compared to Nevi samples (Figure 2A and B). In scenario 1, a ROC analysis showed an AUC of 0.83 for the differentiation of ALM and Nevi. Setting the cutoff at 4.3% RR-positive cells, based in Youden's index, ALM was identified with a sensitivity of 76% and specificity of 85% (Figure 2C). In scenario 2, ROC analysis showed an AUC of 0.88 and ALM was identified with a sensitivity of 80% and specificity of 85% at the cutoff of 5.5% RR-positive cells (Figure 2D).

Next, we compared the proportions of RR-positive specimens in ALM versus Nevi based in the cutoff determined in the ROC curve analysis. With the cutoff at 4.3% RR-positive cells from scenario 1, 76% of specimens were RR-positive in the ALM group compared with only 15% in the Nevi group (Figure 2E). With the cutoff at 5.5% RR-positive cells from scenario 2, 80% of specimens were RR-positive in the ALM group versus 15% in the Nevi group (Figure 2F). Considering a cutoff of ≤1% of RR-positive cells in all sections for definition of RR-negative specimens, only 4 (9%) were RR-negative among the 45 ALM samples compared with 36 (61%) in the 59 Nevi samples (Figure 2G).

Altogether, these data indicate that the immunofluorescence analysis of the presence and proportion (%) of RR-positive cells is a valuable aid in differentiating ALM tumors from Melanocytic Nevi. In fact, both parameters showed good sensitivity and specificity in differentiating ALM from Nevi.

Heterogeneity of RR in ALM sections

In this and subsequent analyses, we will use the highest proportion of RR-positive cells among the multiple sections of each specimen and the cutoff of 5.5% of RR-positive cells to classify RR-positive samples. To evaluate whether the presence and frequency of RR-positive cells vary across the same tumor specimen, as previously demonstrated for the traditional imunohistochemistry markers (Carapeto et al., 2017), we analyzed the distribution of RR-positive cells in the four sections collected at different pre-determined regions for each ALM specimen (Figure 3). The proportion of RR-positive cells varied considerably among the sections from the same tumor sample. Among the 36 ALM samples classified as RR-positive (at least one section above the cutoff of 5.5% of RR-positive cells), 15 (42%) showed all sections with proportion of RR-positive cells above the cutoff. However, for 21 (58%) of the RR-positive samples, one, two or three sections had proportion of RR-positive cells bellow the cutoff (Figure 3A and B). This observation indicates heterogeneity in the expression of RR-positive cells across the tumor tissue and indicates that the proportion of RR-positive cells in ALM specimens should not be evaluated based in a single section from the tumor sample, but rather in multiple sections from different regions.

Presence of RR-positive cells versus histopathology and immunohistochemistry data

We used the histopathology and immunohistochemistry data referent to this set of ALM and Nevi specimens, as detailed in a previous study from our group (Carapeto et al., 2017), in order to further investigate the biological significance of RR structures in the tumor biology. We first correlated the presence of RR-positive cells in ALM specimens with Breslow thickness, ulceration, Clark's level and mitosis index, all valuable indicators and/or prognostic factors of malignancy (Figure 4A). In

the RR-positive group, 71% of the samples presented Breslow thickness >4.0mm, compared to only 29% in the RR-Low/Negative group ($p=0.039$). Other variables such as ulceration, Clark's level and classification of mitotic index as >3 per mm^2 or ≤3 per mm^2 did not differ between the RR-positive and RR-Low/Negative groups (Figure 4A). Breslow thickness and mitotic index averages were ~1.65 and ~2.37 times higher, respectively, in RR-positive specimens compared to RR-Low/Negative specimens, although the difference did not reach statistical significance (Figure 4B and C).

Protein expression for a pre-defined set of markers related to MAPK/AKT pathways (MYC, SCF, PTEN, KIT, BRAF and CYCLIN D1), that are some of the most relevant to melanoma, was determined by imunohistocemistry in the specimens. Quantification of immunohistochemical protein expression and classification as positive or negative for each marker was performed as previously described (Carapeto et al., 2017). Analyzing the distribution of RR positivity in the ALM specimens according to the presence of each immunohystochemical marker (Figure 4D), we found that RR positivity was not associated with the expression of any of the tested immunohystochemical markers, although some interesting observations could be made. For example, among the 35 RR-positive specimens, 29 presented MYC and 28 presented PTEN. Accordingly, RR positivity in the Nevi specimens was not associated with the expression of most markers, except SCF, were 37 (86%) RR-Low/Negative specimens were also negative for SFC, compare to 3 (43%) SCF negative in the RR-positive group ($p=0.008$) (Figure 4D). However, when analyzing this data, one should keep in mind the lower proportion of RR-Low/Negative in ALM specimens and the lower proportion of RR-positive in the Melanocytic Nevi group. Altogether, these data highlight that detection of RR structures could aid to the histopathology and immunohistochemistry markers in accessing malignancy in ALM-suspected samples.

d) Discussion

Although with considerable variability, most malignant tumor cells show uncontrolled and intense proliferation, resulting in high consumption of nucleotides. Guanosine nucleotide is not just a building block for nucleic acids, but also serves as energy carrier, when in the phosphorylated GTP form, to be used in many energy-requiring cellular processes, such as protein synthesis and phototransduction in the retina. Guanosine also participates in cell signaling, for example as part of the second messenger cyclic GMP. Thus cells with high proliferation rates need a constant supply of guanosine.

Previous reports demonstrated that stem cells and induced pluripotent stem cells, which have a very high proliferation rate, present RR structures naturally in vitro (Carcamo et al., 2011; Keppeke et al., 2018). We recently reported that IMPDH assembles into RR structures to boost its activity and supply the guanosine requirements to support intensive cell proliferation rates (Keppeke et al., 2018). Here, we show that RR structures may also be naturally present in other highly proliferative cell types, such as malignant melanomas. Since this is the first study to investigate IMPDH-based RR structures in tumor samples, the extension of this observation to other tumor types should be addressed in future studies.

It is worth mentioning that in vitro cultures of immortalized cancer-derived cell types usually do not naturally present a relevant proportion of RR structures, except when the de novo guanosine biosynthesis is disturbed by certain drugs, or when IMPDH is directly inhibited (Carcamo, Calise, von Muhlen, Satoh, & Chan, 2014). Of special interest, some conditions that promote RR structures in cultured cells may mimic the tumor environment. Amino acid deprivation, such as glutamine and serine, and also excess glycine are some of the conditions that promote RR assembly and may point to plausible mechanisms for RR structures assembly in ALM tumor samples, which characteristically present unbalanced nutrient pools within the tumor microenvironment (Calise et al., 2014; Calise et al., 2016).

We recently reported that most tissues of healthy mice do not present RR-positive cells, including stomach, liver, kidney, cardiac and skeletal muscle, brain and skin (Keppeke, Calise, Chan, & Andrade, 2019). However, we found RR structures in ~15% of cells from the spleen and pancreas of the healthy mice. We also showed that mice treated with the drug ribavirin, an inhibitor of IMPDH, present RR structures in all tissues analyzed, including the skin, where the overall percentage of RR-positive cells was 37% (Keppeke et al., 2019). This indicates that all kind of cells can potentially assemble RR structures under certain conditions. Here we observed that ~60% of Nevi specimens showed none or very low proportion of RR-positive cells (≤1%), being considered as negative samples for RR structures. Altogether, this indicates that healthy skin tissue tend not to present RR structures under natural conditions, unless the patient is ingesting RR-inducing drugs or some other relevant abnormality occurs in the cell metabolism, as in the case of melanoma.

Screening for RR structures as a diagnostic tool is not completely new. We have proposed a test (pending patent deposit BR 10 2016 009754 1) to screen for RR structures in patients' blood cells, such as PBMC, to track proper ingestion of RR-inducing drugs such as ribavirin, azathioprine and mycophenolate, among others (Keppeke, Prado, et al., 2016). This would help identifying non-adherence to the treatment in some diseases. RR structures are also important in the screening for anti-IMPDH autoantibodies (Keppeke et al., 2012). Using HEp-2 cells as substrate for autoantibody

screening in indirect immunofluorescence assay (IFA), one is able to determine the presence of autoantibodies against an array of cellular domains (Calise, Zheng, et al., 2018). Accordingly, by identifying RR structures, the HEp-2 IFA test allows prompt recognition of autoantibodies against IMPDH in the serum of hepatitis C virus-infected patients treated with Interferon-α plus ribavirin (Calise, Keppeke, Andrade, & Chan, 2015; Keppeke, Calise, Chan, & Andrade, 2016). As previously mentioned, CTPS-based RR structures are also present in many cancer types, including melanomas (Chang et al., 2017), indicating its potential as prognostic tool. Here, we propose the screening for IMPDH-based RR structures as an additional diagnostic and possible prognostic tool for ALM.

About 50 years ago, inhibition of IMPDH was proposed as anticancer therapy (Carter et al., 1969; Williams, Lively, DeLong, Cline, & Sweeny, 1968). Despite extensive efforts showing that many IMPDH inhibitors effectively suppress cancer cell proliferation in vitro and in vivo, no drug that inhibits IMPDH has been approved as anticancer therapy, mainly due to adverse effects upon high dose ingestion, lack of efficacy or inconsistent response in certain cancer types. A comprehensive review on the topic was recently published (Naffouje et al., 2019). The increased and heterogeneous frequency of RR structures assembly across diverse sections of ALM specimens indicates that IMPDH activity within tumor microenvironment is increased and under complex regulation. Therefore, considering that RR assembly potentiates IMPDH activity, drugs that interfere with IMPDH RR-assembly should be interesting to test as anticancer therapy, especially in RR structure-positive tumors like ALM, which indicates their high demand for GTP.

Heterogeneity within and between tumors has been described in different tumor types and organs (Bonin & Stanta, 2019; Burrell, McGranahan, Bartek, & Swanton, 2013; Carapeto et al., 2017; Comodo-Navarro et al., 2019; de Souza et al., 2012; Harbst et al., 2016). We have recently reported that acral melanomas are heterogeneous at the level of gene mutation (*BRAF mutation* and *KIT mutation)* and regarding immune-expression of several cell-cycle proteins (Carapeto et al., 2017; Comodo-Navarro et al., 2019; Fernandes et al., 2019). Using a TMA collection of well-characterized ALM specimens, we now demonstrate that IMPDH RR structures are present increased in ALM, but there is heterogeneity in the frequency of RR-positive cells across different sections of a given ALM specimen. This feature must be taken into account, should labeling of these structures be used for prognosis and treatment purposes.

Specimens used in this study were, in general, thick melanomas, due to the technical requirements for TMA assembly. Nonetheless, we have demonstrated that melanomas bear an increased proportion of IMPDH RR structures, an additional tool towards the diagnosis of malignancy. Further studies are warranted to evaluate the occurrence of IMPDH RR structures in thin

melanomas (< 1.0 mm in thickness) and in borderline lesions as a potential diagnostic and prognostic tool. Similarly, the clinical behavior in long-term follow-up of tumors with and without RR structures should be investigated.

e) Conclusion

Associated to histopathology and immunohistochemistry information, screening for IMPDH RR structures in melanomas may be a valuable additional tool to help differentiating ALM from Nevi, and accessing malignancy of the tumor.

Acknowledgments:

G.D.K. is supported by grant #2017/20745-1, from São Paulo Research Foundation (FAPESP).

G.L. is supported by grant # 2017/10547-8, from São Paulo Research Foundation (FAPESP).

Authorship contributions:

G.D.K. conceived the study, designed and performed the experiments, analyzed and interpreted data, and wrote the original draft of the manuscript; D.B. and M.F. interpreted data and wrote the original draft of the manuscript; A.N.C., D.P.G. collected and analyzed morphological and clinical data; L.C interpreted immuno-histochemical data; F.C.L.C. designed, collected, analyzed and interpreted immuno-histochemical and morphological data. L.E.C.A. and G.L. conceived the study, designed the experiments, and critically reviewed and edited the manuscript.

Conflict of Interest Statement:

The authors declare no conflicts of interest.

References

Bonin, S., & Stanta, G. (2019). Pre-analytics and tumor heterogeneity. *N Biotechnol, 55*, 30-35. doi:10.1016/j.nbt.2019.09.007

Burrell, R. A., McGranahan, N., Bartek, J., & Swanton, C. (2013). The causes and consequences of genetic heterogeneity in cancer evolution. *Nature, 501*(7467), 338-345. doi:10.1038/nature12625

Calise, S. J., Abboud, G., Kasahara, H., Morel, L., & Chan, E. K. L. (2018). Immune Response-Dependent Assembly of IMP Dehydrogenase Filaments. *Front Immunol, 9*, 2789. doi:10.3389/fimmu.2018.02789

Calise, S. J., Carcamo, W. C., Krueger, C., Yin, J. D., Purich, D. L., & Chan, E. K. (2014). Glutamine deprivation initiates reversible assembly of mammalian rods and rings. *Cell Mol Life Sci, 71*(15), 2963-2973. doi:10.1007/s00018-014-1567-6

Calise, S. J., Keppeke, G. D., Andrade, L. E., & Chan, E. K. (2015). Anti-rods/rings: a human model of drug-induced autoantibody generation. *Front Immunol, 6*, 41. doi:10.3389/fimmu.2015.00041

Calise, S. J., Purich, D. L., Nguyen, T., Saleem, D. A., Krueger, C., Yin, J. D., & Chan, E. K. (2016). 'Rod and ring' formation from IMP dehydrogenase is regulated through the one-carbon metabolic pathway. *J Cell Sci, 129*(15), 3042-3052. doi:10.1242/jcs.183400

Calise, S. J., Zheng, B., Hasegawa, T., Satoh, M., Isailovic, N., Ceribelli, A., . . . Committee, I. A. S. (2018). Reference standards for the detection of anti-mitochondrial and anti-rods/rings autoantibodies. *Clin Chem Lab Med, 56*(10), 1789-1798. doi:10.1515/cclm-2017-1152

Carapeto, F. C. L., Comodo, A. N., Germano, A., Guimaraes, D. P., Barcelos, D., Fernandes, M., & Landman, G. (2017). Marker Protein Expression Combined With Expression Heterogeneity is a Powerful Indicator of Malignancy in Acral Lentiginous Melanomas. *Am J Dermatopathol, 39*(2), 114-120. doi:10.1097/DAD.0000000000000635

Carcamo, W. C., Calise, S. J., von Muhlen, C. A., Satoh, M., & Chan, E. K. (2014). Molecular cell biology and immunobiology of mammalian rod/ring structures. *Int Rev Cell Mol Biol, 308*, 35-74. doi:10.1016/B978-0-12-800097-7.00002-6

Carcamo, W. C., Satoh, M., Kasahara, H., Terada, N., Hamazaki, T., Chan, J. Y., . . . Chan, E. K. (2011). Induction of cytoplasmic rods and rings structures by inhibition of the CTP and GTP synthetic pathway in mammalian cells. *PLoS One, 6*(12), e29690. doi:10.1371/journal.pone.0029690

Carter, S. B., Franklin, T. J., Jones, D. F., Leonard, B. J., Mills, S. D., Turner, R. W., & Turner, W. B. (1969). Mycophenolic acid: an anti-cancer compound with unusual properties. *Nature, 223*(5208), 848-850. doi:10.1038/223848a0

Castro, L. G., Bakos, R. M., Duprat Neto, J. P., Bittencourt, F. V., Di Giacomo, T. H., Serpa, S. S., . . . Gontijo, G. (2016). Brazilian guidelines for diagnosis, treatment and follow-up of primary cutaneous melanoma - Part II. *An Bras Dermatol, 91*(1), 49-58. doi:10.1590/abd1806-4841.20164715

Castro, L. G., Messina, M. C., Loureiro, W., Macarenco, R. S., Duprat Neto, J. P., Di Giacomo, T. H., . . . Gontijo, G. (2015). Guidelines of the Brazilian Dermatology Society for diagnosis, treatment and follow up of primary cutaneous melanoma--Part I. *An Bras Dermatol, 90*(6), 851-861. doi:10.1590/abd1806-4841.20154707

Chang, C. C., Jeng, Y. M., Peng, M., Keppeke, G. D., Sung, L. Y., & Liu, J. L. (2017). CTP synthase forms the cytoophidium in human hepatocellular carcinoma. *Exp Cell Res, 361*(2), 292-299. doi:10.1016/j.yexcr.2017.10.030

Chang, C. C., Lin, W. C., Pai, L. M., Lee, H. S., Wu, S. C., Ding, S. T., . . . Sung, L. Y. (2015). Cytoophidium assembly reflects upregulation of IMPDH activity. *J Cell Sci, 128*(19), 3550-3555. doi:10.1242/jcs.175265

Chen, K., Zhang, J., Tastan, O. Y., Deussen, Z. A., Siswick, M. Y., & Liu, J. L. (2011). Glutamine analogs promote cytoophidium assembly in human and Drosophila cells. *J Genet Genomics, 38*(9), 391-402. doi:10.1016/j.jgg.2011.08.004

Comodo-Navarro, A. N., Fernandes, M., Barcelos, D., Carapeto, F. C. L., Guimarães, D. P., Moraes, L. d. S., . . . Landman, G. (2019). Intratumor Heterogeneity of KIT Gene Mutations in Acral Lentiginous Melanoma. *Am J Dermatopathol*. doi:10.1097/DAD.0000000000001475

de Souza, C. F., Xander, P., Monteiro, A. C., Silva, A. G., da Silva, D. C., Mai, S., . . . Jasiulionis, M. G. (2012). Mining gene expression signature for the detection of pre-malignant melanocytes and early melanomas with risk for metastasis. *PLoS One, 7*(9), e44800. doi:10.1371/journal.pone.0044800

Fernandes, M., Barcelos, D., Comodo, A. N., Guimaraes, D. P., Carapeto, F. C. L., Cardili, L., . . . Landman Ap, G. (2019). Acral Lentiginous Melanomas Harbour Intratumor Heterogeneity in BRAF Exon 15, With Mutations Distinct From V600E/V600K. *Am J Dermatopathol, 41*(10), 733-740. doi:10.1097/DAD.0000000000001418

Harbst, K., Lauss, M., Cirenajwis, H., Isaksson, K., Rosengren, F., Torngren, T., . . . Jonsson, G. (2016). Multiregion Whole-Exome Sequencing Uncovers the Genetic Evolution and Mutational Heterogeneity of Early-Stage Metastatic Melanoma. *Cancer Res, 76*(16), 4765-4774. doi:10.1158/0008-5472.CAN-15-3476

Keppeke, G. D., Calise, S. J., Chan, E. K., & Andrade, L. E. (2015). Assembly of IMPDH2-based, CTPS-based, and mixed rod/ring structures is dependent on cell type and conditions of induction. *J Genet Genomics, 42*(6), 287-299. doi:10.1016/j.jgg.2015.04.002

Keppeke, G. D., Calise, S. J., Chan, E. K., & Andrade, L. E. (2016). Anti-rods/rings autoantibody generation in hepatitis C patients during interferon-alpha/ribavirin therapy. *World J Gastroenterol, 22*(6), 1966-1974. doi:10.3748/wjg.v22.i6.1966

Keppeke, G. D., Calise, S. J., Chan, E. K. L., & Andrade, L. E. C. (2019). Ribavirin induces widespread accumulation of IMP dehydrogenase into rods/rings structures in multiple major mouse organs. *Antiviral Res, 162*, 130-135. doi:10.1016/j.antiviral.2018.12.017

Keppeke, G. D., Chang, C. C., Peng, M., Chen, L. Y., Lin, W. C., Pai, L. M., . . . Liu, J. L. (2018). IMP/GTP balance modulates cytoophidium assembly and IMPDH activity. *Cell Div, 13*, 5. doi:10.1186/s13008-018-0038-0

Keppeke, G. D., Nunes, E., Ferraz, M. L., Silva, E. A., Granato, C., Chan, E. K., & Andrade, L. E. (2012). Longitudinal study of a human drug-induced model of autoantibody to cytoplasmic rods/rings following HCV therapy with ribavirin and interferon-alpha. *PLoS One, 7*(9), e45392. doi:10.1371/journal.pone.0045392

Keppeke, G. D., Prado, M. S., Nunes, E., Perazzio, S. F., Rodrigues, S. H., Ferraz, M. L., . . . Andrade, L. E. (2016). Differential capacity of therapeutic drugs to induce Rods/Rings structures in vitro and in vivo and generation of anti-Rods/Rings autoantibodies. *Clin Immunol, 173*, 149-156. doi:10.1016/j.clim.2016.10.004

LeBoit, P. E., Burg, G., Weedon, D., & Sarasain, A. (2006). Pathology and Genetics of Skin Tumours. In P. Kleihues & L. H. Sobin (Eds.), *World Health Organization Classification of Tumours* (3rd ed., Vol. 6). Lyon, France: IARC Press.

Naffouje, R., Grover, P., Yu, H., Sendilnathan, A., Wolfe, K., Majd, N., . . . Sasaki, A. T. (2019). Anti-Tumor Potential of IMP Dehydrogenase Inhibitors: A Century-Long Story. *Cancers (Basel), 11*(9). doi:10.3390/cancers11091346

Williams, R. H., Lively, D. H., DeLong, D. C., Cline, J. C., & Sweeny, M. J. (1968). Mycophenolic acid: antiviral and antitumor properties. *J Antibiot (Tokyo), 21*(7), 463-464. doi:10.7164/antibiotics.21.463

Figure legends

Figure 1. RR structures in melanoma specimens. After antigen recovery, sections were labeled with anti-IMPDH2 antibody (red) and Hoechst (blue). **A**, **B** and **C** are representative images of specimens that present RR structures in high proportion. **D**, representative image of rings. **E**, higher magnification of rods. Most of the observed structures are rods (arrows in **A**, **B**, **C** and **E**), although rings (arrowhead in **D**) could also be observed. **F**, representative image of a negative sample without RR structures.

Figure 2. Presence of RR in ALM compared to Nevi. Mean proportion of RR-positive cells in all sections of each specimen in (**A**, **C** and **E**). Highest proportion of RR-positive cells among the multiple sections of each specimen (**B**, **D**, **F** and **G**). **A** and **B,** proportions of RR-positive cells were higher in ALM compared to Nevi group. **C** and **D,** ROC curve analysis for ALM versus Nevi. #Cutoff (% of RR-positive cells per section) defined based in Youden's Index, aiming optimal sensitivity and specificity to differentiate ALM from Nevi. **E** and **F**, comparison of the proportions of RR-positive specimens classified according to the ROC curve cutoff in ALM versus Nevi group. **G**, Samples were rated as RR-negative (≤1% of RR-positive cells in all sections), RR-Low positive (≥2 and <5.5% of RR-positive cells) and RR-positive (≥5.5% RR-positive cells). *** p≤0.001.

Figure 3. Heterogeneity of RR in ALM specimens. A, proportion of RR-positive cells among the four sections of each of the 45 ALM specimens. **B,** 36 ALM specimens were classified as RR-positive because at least one section had proportion of RR-positive cells above the 5.5% cutoff: in 21(58%), one, two or three other sections had proportion of RR-positive cells bellow the cutoff**. C,** positions of the TMA/sections obtained for each tumor specimens.

Figure 4. Histopathology and immunohistochemistry data. A, histopathology characteristics of RR-positive and RR-Low/Negative in ALM specimens based in the 5.5% cutoff. A significantly higher proportion of RR-positive specimens was observed in specimens with Breslow >4.0 mm. **B** and **C**, Breslow thickness and mitosis index were ~1.65 and ~2.37 times higher, respectively, in RR-positive ALM specimens compared to RR-Low/Negative specimens, although the comparison did not reach statistical significance (p=0.097 and p=0.110, respectively). Error bars mean SEM. **D**, distribution of RR-positive and RR-Low/Negative specimens in ALM and Melanocytic Nevi according to the expression of immunohistochemistry markers (*n* indicate the number of samples with data available). In **A** and **D**, for continuous variables, average ± SD is shown, and for categorical variables, proportion and number of patients are shown in brackets.