REPORT
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LIQUID BIOPSY IN PEDIATRIC SARCOMAS: DECIPHERING THE PREDICTIVE POTENTIAL OF CIRCULATING TUMOR DNA AND OF TUMOR EXOSOMES FOR EARLY DETECTION OF RELAPSES

Dr Josep Roma Castanyer

Hospital Universitari Vall d'Hebron - VHIR Institut de Recerca Vall d'Hebron

Dr Francisco Javier Alonso García de la Rosa

Instituto de Investigaciones en Enfermedades Raras - Instituto de Salud Carlos III

1. Summary of the project

Background of the project: Sarcomas constitute a broad family of cancers, among which rhabdomyosarcoma (RMS), non-RMS soft tissue sarcomas, osteosarcoma, and Ewing sarcoma stand out due to their malignancy and relatively high incidence in pediatric patients. Due to their high complexity and biological variability, sarcomas have historically been poorly studied compared to more common cancers. In this sense, the development of new methods aimed at detecting specific relapse biomarkers may be crucial for establishing new risk assessment protocols for these neoplasms, especially in cases prone to relapse.

Main objective: Monitoring tumor response during and after treatment has led to advances in patient survival, but early non-invasive biomarkers are still lacking.

Methodology: In this project, two key extracellular components of cancer cells will be studied: cell-free circulating tumor DNA (ctDNA) and exosomes derived from the tumor in order to study their correlation with tumor burden.

Expected results: The development of these technologies associated with liquid biopsies will open new avenues for cancer biological markers, with the consequent potential for improving patient clinical management. The project's goal is to translate our previous experience in pediatric solid tumors and the optimization of new technologies into new monitoring protocols to allow for earlier detection of relapses in the short term. Early detection of these markers may allow for the anticipation of relapse diagnosis and, therefore, intensifying monitoring and/or initiating treatment earlier, thereby increasing the chances of survival for patients with pediatric sarcoma.

2. Results

The results have allowed us to demonstrate the significant clinical applicability that the technology for detecting circulating tumor DNA, developed thanks to this project, will have. The results are so promising that we are clear that this project does not end here, but rather, the achievement of this *La Marató de TV3* project

has been key to laying the cornerstone of a new system to assess tumor burden in pediatric sarcoma patients, which will not only be reflected in scientific publications, but we believe it will also change and improve the clinical monitoring of patients. Thus, we have demonstrated that the technology we have developed can assess (with a simple blood sample) the effectiveness of therapy, and above all, we have been able, in some patients, to detect relapse up to 5 months earlier than with conventional techniques. This is very complex in sarcomas (due to the high mutational variability they present, despite having a low mutation rate), and therefore, we believe that the findings are very important and will change some paradigms of monitoring and treatment of these patients. One of the goals we pursue is to be able to anticipate new treatments in cases of relapse, which could greatly increase the effectiveness of second-line treatments.

Thus, we have found cases where the 10 selected tumor markers completely disappear by the end of treatment and do not reappear, indicating that there is no sign of disease. In other cases, we have found patients where, at the end of first-line treatment, the disease remains active, and these are cases where cancer has progressed rapidly. In these cases, positivity indicates that the treatment has not been effective enough. We also have cases that responded well during initial therapy but had a subsequent relapse during follow-up, which we were able to detect up to 5 months in advance using the technology we have developed. It is clear that the system works and that it can have considerable clinical applicability. In the new stage of the project that is now beginning, the objective will be to increase the number of patients included in the study and evaluate their correlation with the clinic with a much larger cohort and even longer follow-ups.

On the other hand, significant progress has been made in basic research on the role of exosomes and how they can be used to detect biomarkers inside them. Thus, the project has focused on the molecular characteristics, especially the protein and miRNA profiles, of exosomes in the plasma of pediatric sarcoma patients with the aim of identifying prognostic and disease progression markers. Due to the scarcity of samples and patients with these tumors, much effort has been devoted to finding the best method of isolating exosomes from plasma. Thus, the purification of plasma vesicles was evaluated using exclusion chromatography columns. This method has allowed the isolation of homogeneous populations of

vesicles, eliminating contaminants such as albumin, which has been crucial for analyzing samples for proteomics. After optimizing the best molecular exclusion columns, these were used with control samples provided by the project's coordinating laboratory, which showed homogeneous results both in the protein present in the elutions and in the number of particles analyzed. Once the method of isolating the vesicles was selected, the molecular characterization of the proteins and miRNAs contained in them was undertaken. Thanks to this work, a list of proteins and miRNAs has been compiled that could be used as candidate biomarkers in later stages for disease evaluation. Finally, the system has also been optimized in liquid biopsy samples from pediatric sarcoma patients, provided by the coordinating center, and the vesicles present in these samples have been positively isolated.

In summary, we can affirm that the results obtained in the two subprojects point to a strong clinical potential to improve disease detection and carry out accurate and minimally invasive monitoring, either through circulating tumor DNA or through the biomarkers contained in the vesicles.

3. Relevance and potential future implications

The present project has a high component of clinical implications, it was already designed to have them, but the results we have obtained thanks to this support have turned the high potential that the project had at its beginning into a firm reality to advance towards better patient monitoring. This improved monitoring will bring an improvement in disease detection (with more sensitivity and specificity, and at the same time with a less invasive and relatively rapid procedure). In addition, earlier detection of the disease can lead to an earlier initiation of treatment and therefore increase its effectiveness, thus contributing to increasing survival rates. On one hand, we have been able to optimize the detection of circulating tumor DNA. We had many difficulties in finding the right way to do it, since sarcomas do not present mutations that are highly prevalent and can be used in all cases (and translocations are very difficult to find in blood), and with the system we have devised, we can track 10 specific DNA fragments of the tumor, each of which can be considered an independent biomarker. Tracking 10

biomarkers (instead of 1 as is usually done) provides a high robustness to the system. Thus, we have been able to detect patients who have relapsed, and we have been able to anticipate the detection of relapse through this system, up to 5 months. It has also served us to detect residual disease after treatment in some patients, patients who have progressed rapidly at the end of treatment. Therefore, we believe that our system can be key to evaluating the effectiveness of therapy and, therefore, the clinical decision could be made to prolong it until the tumor burden in blood is undetectable.

Exosome subproject: We have been able to define the procedure to isolate exosomes from patients' plasma. Due to limitations in obtaining samples from pediatric sarcoma patients, we considered it essential to experimentally determine the best possible procedure for studying these samples. We devoted a large part of the project to this objective, evaluating various commercial and non-commercial options. After these studies, we have clearly identified the best option, which will be used in future studies to identify progression biomarkers. We have identified expression patterns of proteins and miRNAs that could be related to prometastatic profiles in Ewing sarcoma cells. These results will allow us to analyze in future projects whether these expression patterns are present in plasma samples from patients and whether they correlate with disease development. We have laid the groundwork for the development of a new therapy in Ewing sarcoma, based on the use of exosomes and genetic editing tools. The experience gained in this project on exosome analysis has been fundamental to advance in the development of this therapy. In future projects, we will continue to advance in the development of this approach at the preclinical level.

In summary, liquid biopsy is here to stay, and projects like this one consolidate its use to improve patient monitoring and facilitate its future implementation as a clinical routine.

4. Scientific bibliography generated

Published articles that mention the present grant from the La Marató Foundation:

Dickkopf Proteins and Their Role in Cancer: A Family of Wnt Antagonists with a Dual Role. Irina Giralt, Gabriel Gallo-Oller, Natalia Navarro, Patricia Zarzosa, Guillem Pons, Ainara Magdaleno, Miguel F Segura, José Sánchez de Toledo, Lucas Moreno, Soledad Gallego, Josep Roma. Int J Mol Sci. 2021 Nov 29;22(23):12921. doi: 10.3390/ph14080810. PMID: 34451907.

Targeting the Hedgehog Pathway in Rhabdomyosarcoma. Zarzosa P, Garcia-Gilabert L, Hladun R, Guillén G, Gallo-Oller G, Pons G, Sansa-Girona J, Segura MF, Sánchez de Toledo J, Moreno L, Gallego S, Roma J.Cancers (Basel). 2023 Jan 24;15(3):727. doi: 10.3390/cancers15030727. PMID: 36765685

Integrin alpha9 emerges as a key therapeutic target to reduce metastasis in rhabdomyosarcoma and neuroblastoma. Navarro N, Molist C, Sansa-Girona J, Zarzosa P, Gallo-Oller G, Pons G, Magdaleno A, Guillén G, Hladun R, Garrido M, Segura MF, Hontecillas-Prieto L, de Álava E, Ponsati B, Fernández-Carneado J, Almazán-Moga A, Vallès-Miret M, Farrera-Sinfreu J, de Toledo JS, Moreno L, Gallego S, Roma J.Cell Mol Life Sci. 2022 Oct 11;79(11):546. doi: 10.1007/s00018-022-04557-y. PMID: 36221013.

Articles in preparation where the present grant from the La Marató Foundation will be mentioned:

"A new strategy based on cfDNA follow up to improve the detection of relapses in childhood sarcomas". Gallo-Oller G et al.