MICRORNAS AS MOLECULAR MARKERS OF GLIOBLASTOMA MULTIFORME

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The aim of this project was to unravel the role that miR-221 and miR-222, of which we had already demonstrated the specific differential expression in glioblastoma *multiforme* compared to normal brain, play in the control of cell proliferation, with the ultimate goal to provide new insights in the molecular basis of cancer. The results of our research allowed to identify an important molecular target for miRNA-221 and miR-222, highly expressed in glioblastoma multiforme tissues and cell lines, and to precisely recognize the mRNA regions responsible for this regulation.

As a first approach to identify genes that could be targets of miR-221/222 we used some recently developed miRNA target prediction algorithms that optimize sequence complementarity using position-specific rules and rely on strict requirements of interspecies conservation. We critically compared the results obtained by using three algorithms available on-line (Targetscans; Pictar; MiRBase), and we selected the 3'UTR of p27kip1 mRNA, that presents many putative sites for MiR-221/222.

To validate the bioinformatics prediction expression analysis of p27kip1, a protein inhibitor of cell cycle progression, a Western blot analysis was performed in glioblastoma human cell lines and glioblastoma tissues, showing an inverse correlation between miR-221/222 expression and amount of p27kip1.

Our objective was then to identify the miRNA recognition elements located in the 3'UTRs of the target mRNA. We "isolated" the 3'UTR region from p27kip1 gene context, and artificially inserted it into a luciferase reporter system used to clearly evaluate the effects of microRNA expression on the reporter protein expression. Luciferase activity was compared to that of analogous reporters with point substitutions disrupting the predicted target sites. A specific negative effect on luciferase activity was observed in the presence of miR-221/222, demonstrating a functional interconnection between the microRNA and the predicted target p27kip1.

Knock-down of miR-221/222 in glioblastoma cells where they commonly are up-regulated drastically increased the endogenous expression of p27kip1, while the up-regulation of the two microRNAs reduced p27kip1 amount. These results confirm our previous results that miR-221 and miR-222 are a very significant microRNA pair, possibly representing a molecular signature of glioblastoma, and demonstrate that p27kip1, a basic modulator of cell cycle progression whose knock down is very often associated with glioblastoma oncogenesis, is one major target of miR221/222 (Galardi *et al*., 2007; le Sage *et al*., 2007).

Our research also focused on the characterization of the specific expression of microRNAs in primary cell lines derived from glioblastoma patients' samples. We studied microRNA-specific expression in distinct cell subpopulations derived from glioblastoma samples. Two distinct cell preparations were produced from each patient's sample: one of them grows as a population of adherent cells (GBM-AC), while the other one shows a "neurosphere-like" growth (GBM-NS).

The latter, presenting a typical "stem-like" phenotype, such as neurospheres growth itself, together with the high tumorigenicity of *in vivo* xenografts, very closely resembles the recently described "cancer stem cells", proposed to be at the origin of several types of human malignancies. In our experimental setting, for each glioblastoma patient we obtained both the adherent cells (GBM-AC) and the neurospheres (GBM-NS), thus providing a precious source for the search of the hypothetical markers of "tumor stemness". We have utilized three pairs of GBM-AC and GBM-NS cell lines, derived from three glioblastoma patients; from each cell line we have purified total RNA, that has been analyzed by microarray for the expression of the whole panel of known human microRNAs. After the validation of the most significant microarray data *via* Northern blot, we confirmed that a specific group of 12 microRNAs strongly differentiates GBM-ACs from GBM-NSs; among these microRNAs, four are significantly down-regulated in GBM-NSs and the other eight are over-expressed. Moreover, when we analyzed microarray data by applying a slightly lower stringency, we recognized, among overexpressed microRNAs, some molecules that have been previously shown to play an "oncogenic" role or at least to be involved in the oncogenesis of several humans tumors.

Our present experimental efforts are dedicated to unravel if these microRNAs can play a determinant role as promoters of the "stem tumorigenicity" also in glioblastoma, as they do in other tumors: we have cloned the pre-miR precursor molecules of the most significant GBM-NS overexpressed microRNAs into plasmid expression vectors, with which we have transfected GBM-AC cells. If our prediction is correct, the ectopic overexpression of these microRNAs in GBM-AC cells should enhance their tumorigenicity, that we will measure *in vitro* by calculating their proliferation potential and the potential acquisition of the growth in suspension (neurospheres) rather than in adherence.

In summary, the results of our research have led to the identification of p27kip1 as a key target molecule mediating the oncogenic role of miR-221/222 in glioblastoma cell lines and tumor samples. Most importantly, we have also identified a set of differentially expressed microRNAs whose expression allows to discriminate between GBM-ACs and GBM-NSs, thus providing the experimental basis for the identification of microRNA markers of the "stem" cell population in glioblastomas. These data represent a first step towards the possible future use of microRNAs as diagnostic tools or even as therapeutic targets in glioblastoma *multiforme*.

Publications of the project

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