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TITOLO DELLA RELAZIONE

Identification of miRNA signature in primary GIST and matched metastases as potential novel therapeutic approach

Gastrointestinal stromal tumors (GIST) are the most common mesenchymal tumors of the gastrointestinal tract. Originally a poorly defined pathologic entity, after the identification of KIT or PDGFRA mutations as the event driving tumorigenesis, GIST have emerged as distinct oncogenetic disease [1]. KIT/PDGFRA mutations occur in about 85-90% of cases, whereas the remaining 10-15% cases are referred to as KIT/PDGFRA wild-type (WT) GIST. The discovery of KIT/PDGFRA mutations has led to the introduction of the tyrosine kinase receptor inhibitor (TKI) imatinib. Imatinib, which binds KIT or PDGFRA, and prevents their downstream cascades, has emerged as a paradigm for targeted therapies [2]. MiRNAs are a highly conserved group of short noncoding RNAs (~22 bp) playing a pivotal role in post-translational regulation of gene expression, and are critical regulators of oncogenic pathways [3]. One of the main difficulties hindering advances in cancer treatment is the dynamic molecular evolution of tumors across the course of disease. Tumors may evolve in time during tumor progression and therapy, as well as in space, across different tumor clones and within metastases. The dynamics of tumor evolution is individual for each patient, shaped by intrinsic genetic factors along with extrinsic selective forces, such as cancer therapy. Most comprehensive studies, aimed at profiling the molecular players involved in cancer, were based on the primary tumor samples. However, the molecular evolution toward recurrence is the key to understanding the processes responsible for the formation of metastases. Ideally, to understand tumor evolution at the individual level, for any single patient, analysis of molecular

changes in tumors, at different progression stages should be performed. Multiple studies have been conducted to investigate the role of genes and their products in metastases [4]. Recently, miRNAs have been found to be attractive candidates as novel biomarkers for different types of cancer as they display specific expression patterns and can be detected in tissues, or body fluids [5]. They have emerged as important regulators of the metastatic process [6]; however, GIST metastasis-related miRNAs and their biological roles remain to be identified. The ability to modulate miRNA expression and activity in vivo through miRNA mimics or antimiRs provides an opportunity for the development of innovative therapeutic approaches to cancer; various strategies have been investigated in preclinical development to replenish tumour suppressive miRNAs (using miRNA mimics) or to suppress oncomiRs (using antimiRs) [7]. We hypothesize that expression modulations in individual patients throughout the course of their disease can directly identify miRNA sets differentially expressed at the various stages of the disease, associated with disease progression and drug resistance. In view of these considerations, we aim to evaluate the expression miRNA profile in primary GIST and in the corresponding matched metastasis. Indeed, among the many possibilities currently investigated, the use of cellular miRNAs as therapeutic agents is one of the most promising strategy from a clinical point of view. Because a single miRNA could potentially affect several clinically relevant targets, artificially increasing or decreasing the expression level of a given miRNA offers interesting therapeutic perspectives.

With the aim to identify a miRNA signature, which can help to pinpoint changes in miR expression during progression from the primary tumor to specific distant sites, we will analyze miRNAs expression levels in primary tumor tissues and in 2 matched distant metastases from the same surgical procedure. The second step will consist in the validation of the significant results in an extend cohort of GIST through TaqMan assay approach.

Material and Methods

In collaboration with the Hospital Vall d'Hebrón, Passeig de la Vall d'Hebrón (Spain) and with the Università Cattolica (Rome), we collected a total of 26 GIST samples, of which eight were primary tumors and 18 were metastasis. Four GIST primary samples were involved in the initial discovery step. In the first part of the project we focused on primary tumors miRNA profiles.

Total DNA and RNA were isolated from FFPE (sections >10 µm) by RecoverAll total Nucleic Acid Isolation Kit for FFPE (Ambion). During RNA isolation, ath-159a - a non human miRNA – was added. For miRNA profiles, 10 ng of total RNA were reverse trascribed to cDNA, using TaqMan Advanced miRNA cDNA synthesis kit (Applied Biosystems). This kit is specific for detection and quantification of mature human miRNAs in biological samples. The cDNAs were then amplified using the Universal miR-Amp Primers and Master Mix to uniformly increase the amount of cDNA

for each target, maintaining the relative differential expression levels. MiRNA profiles were analyzed for four GIST cases; specifically, we analyzed the miRNA expression levels in four primitive samples and, for each ones, in two matched metastasis. The cDNA was loaded into the TaqMan arrays, evaluating the expression of almost 800 different miRNAs, and run in a 7900HT Fast PCR system (Applied Biosystems). MiRNA data were analyzed with SDS RQ Software version 2.4; miRNAs with Ct values ≥ 35 were considered as not expressed and excluded from further analysis.

Results

During this first part of the project, DNA and RNA were isolated from all the samples.

First, to confirm the primary mutation, each DNA was sequenced for KIT exon 11; in addition, to exclude the appearance of secondary mutations, exons 13, 14, 17 and 18 were analyzed. We confirmed all the GIST harboured an alteration on exon 11; we did not detect additional base changes on different exons. Table 1 summarizes the mutations detected in GIST cases.

Table 1. Primary mutations detected in GIST cases

Name	Location	Size (cm)	Mitosis/50HPF	Mutation
Case # 1	Stomach	21	18	KIT Ex 11 p.K550_558del
Case # 2	Small bowel	14	44	KIT Ex 11 p.Y568_L576>CV
Case # 3	Small bowel	9.5	14	KIT Ex 11 p.W557R
Case # 4	Stomach	45	36	KIT Ex 11 p. p.551_M552>L

Second, we tested the quality of RNA isolation procedure and of cDNA retrotranscription using single Taqman assays for ath-159a, a non-human spike-in, and miR-16-5p. We verified that all the samples showed similar Ct values and we could proceed with the miRNA profile.

The global miRNA profiles for GIST primary tumors were run. Pool A and pool B arrays were analyzed, for a total of 754 miRNA evaluated. The number of amplified miRNA was around 350.

As expected, the miRNA profiles in these 4 GIST samples were very similar.

Indeed, to avoid confounding variables, we selected GIST cases with the same KIT mutant exon, given that the location of KIT mutation is associated with the prognosis of the disease.

We grouped the four GIST cases in two subgroups taking into account dimension (size < or > 20 cm) or the mitotic index.

With regard to the size, we identified 12 miRNA differentially expressed between the two classes ($p<0.05$).

The 12 miRNA are reported in table 2 and showed in figure 1.

Table 2. MiRNA differentially expressed between the two classes (size cm <20 or >20)

Detector	pvalue
miR-93-5p	0.00753
miR-653-5p	0.00767
miR-200b-3p	0.0108
let-7e-5p	0.0143
miR-513c-5p	0.0270
miR-15b-3p	0.0274
miR-339-3p	0.0277
miR-151b	0.0320
miR-193a-5p	0.0366
miR-338-3p	0.0383
miR-146a-5p	0.047694
miR-130b-5p	0.0480

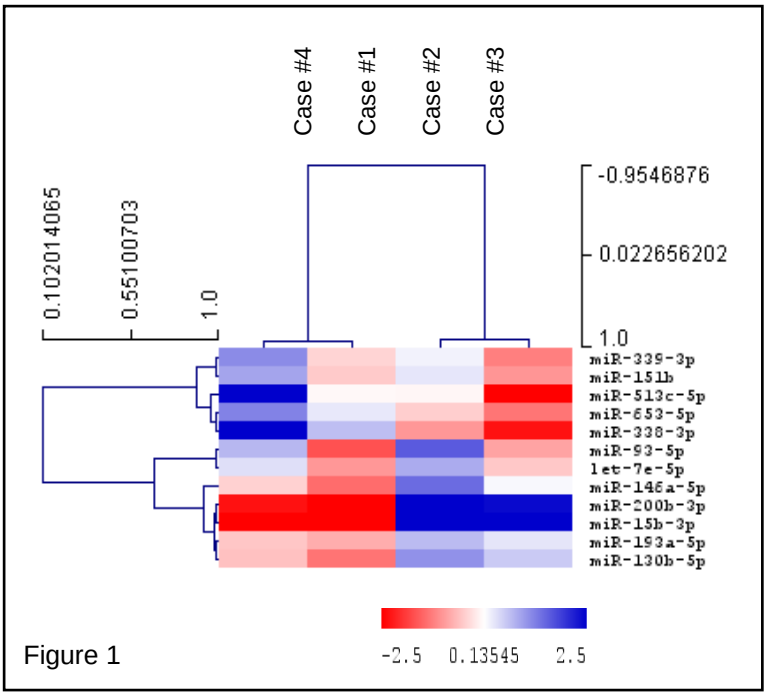

Figure 1

Regarding the mitotic index, we identified 45 miRNA differentially expressed between the two classes.

The miRNA differentially expressed ($p<0.05$) are reported in table 3 and figure 2 shows the expression levels.

Table 3. MiRNA differentially expressed between the two classes (Mitotic index/50HPF <35 or >35)

Detector	Pvalue	Detector	pvalue
miR-539-5p	0.001229	miR-431-3p	0.029445
miR-499a-5p	0.005464	miR-425-5p	0.029828
miR-543	0.006187	miR-323a-3p	0.03089
miR-145-5p	0.008051	miR-337-5p	0.033006
miR-410-3p	0.012021	miR-136-3p	0.033169
miR-17-5p	0.013086	miR-361-5p	0.033468
miR-409-3p	0.013114	miR-487b-3p	0.033505
miR-450a-5p	0.013414	miR-656-3p	0.03399
miR-100-5p	0.013748	miR-377-5p	0.034624
miR-487a-3p	0.015762	miR-28-3p	0.035911
miR-485-3p	0.019384	miR-1197	0.039163
miR-379-5p	0.019449	miR-28-5p	0.039295
miR-1248	0.022836	miR-1247-5p	0.041355
miR-10a-5p	0.023237	miR-548e-3p	0.043174
miR-134-5p	0.024062	miR-99b-5p	0.043761
miR-29a-3p	0.026409	miR-758-3p	0.04442
miR-532-3p	0.027096	miR-433-3p	0.044712
miR-148a-3p	0.027572	miR-598-3p	0.046243
miR-411-5p	0.027625	miR-369-3p	0.046548
miR-15a-5p	0.028286	miR-655-3p	0.046685
miR-454-3p	0.028494	let-7f-2-3p	0.04747
miR-551b-3p	0.028736	let-7g-3p	0.04848
miR-103a-2-5p	0.029277		

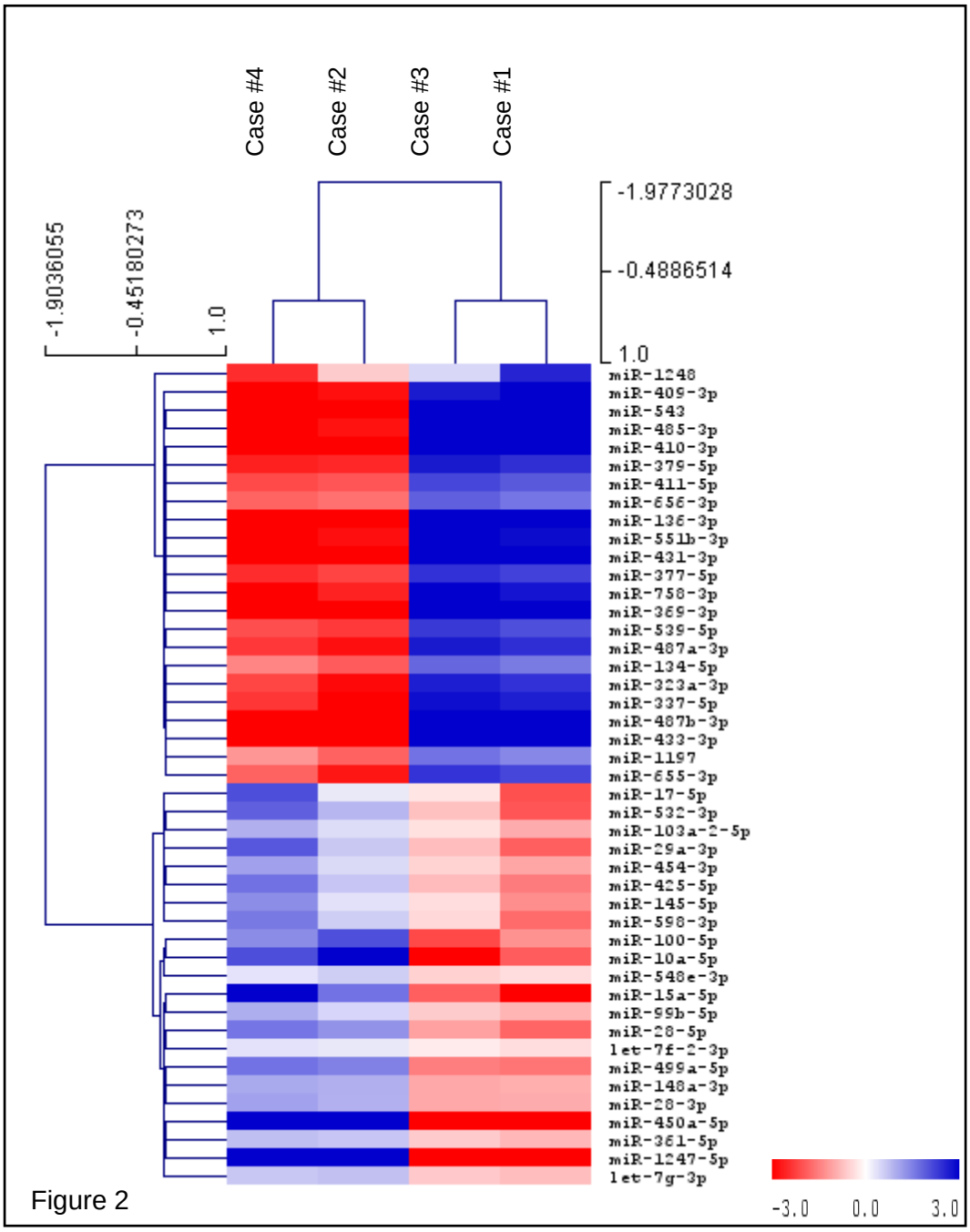

Figure 2

This first analysis highlighted the tumor size and, mostly, mitotic index are associated with peculiar miRNA signatures.

In the second part of the project, miRNA profiles of metastasis samples will be analyzed.

References

[1] Corless et al, Nat Rev Cancer, 2011

[2] Ravegnini et al, Int J Mol Sci, 2015

[3] He et al. Nat Rev Genet, 2004

[4] Lee et al, BMC Med Genomics, 2013

[5] Lu et al, Nature, 2005

[6] Seven et al; Future Oncol, 2014

[7] Slack et al, Nat Rev, 2017

La Società Italiana di Farmacologia dichiara che i dati personali comunicati dal Socio sono trattati in conformità alle disposizioni del D. Lgs. 196/2003 ed alla normativa comunitaria secondo quanto indicato specificamente nell'informativa privacy reperibile sul sito internet della Società all'indirizzo https://sif-website.s3.amazonaws.com/uploads/attachment/file/240/Informativa_Privacy_SIF_Generica.pdf che il Socio, con la sottoscrizione del presente Contratto, dichiara di aver compiutamente visionato, compreso e accettato.

Data

18/05/2018

Firma