Hsa-mir31-3p Expression in FFPE tumor samples as a predictor of anti-EGFR response in patients with metastatic colorectal cancer

G. Manceau[1], S. Imbeaud[2], N. Mosakhani[3], R. Thiébaut[4], F. Liebaert[4], J-L. Van Laethem[5], P.Demetter[5], B. Chibaudel[6,7], T. André[6,7], P. Laurent-Puig[1,8]*

[1]UMR-S775, [2]UMR-S674, Paris Descartes; [3]Haartman Institute, University of Helsinki [4]Integragen; [5]Erasme University Hospital; [6]Gercor ; [7]Hôpital St Antoine; [8]Hopital Georges Pompidou

*Corresponding author: pierre.laurent-puig@parisdescartes.fr

Introduction

✓ In metastatic colorectal cancer (mCRC), KRAS mutations are associated with resistance to anti-EGFR antibodies. To identify markers that predict response to anti-EGFR antibodies in wild-type KRAS mCRC patients, we explored focused on miRNAs

✓ Previously, RNA extracted from fresh frozen (FF) samples from 43 patients chosen randomly from two separate training sets were analyzed by Illumina BeadChips. Eleven microRNAs were identified as being associated with progression free survival (PFS). All identified miRNA in both training sets were then tested using Taqman probes with only one, **hsa-miR-31-3p**, showing a significant association with PFS for both training sets.

✓ A Cox model and principal component analysis were performed on training set 1 allowing us to define two groups of patients having either a high or low risk of progression. The miR-31-3p expression threshold was then applied to training set 2 with the logrank test showing a significant difference between the low and high-risk groups. The model was confirmed on a validation set of patients (set 1a). A nomogram based on Cox's proportional hazards regression, modeling patients PFS probability and integrating BRAF status, was built using FF training set 1 and validated on FF training set 2 & FF validation set. The area under the curve (AUC) demonstrated the performance of the test with an AUC superior to 0.70 for all time thresholds tested.

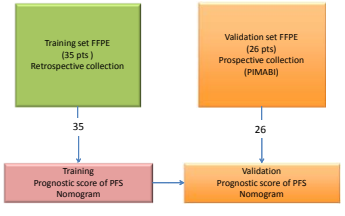
Patients

✓ Different cohorts of advanced CRC patients with FF or FFPE tissue samples were included in this study for a total of 132 patients, all wild-type for KRAS)

✓ Previous results on FF were done with 2 training set: a retrospective collection[1] of 33 patients refractory to FOLFOX or FOLFIRI regimen and considered refractory to the regimen associated with anti-EGFR or received panitumumab as a single agent and a prospective collection (performed in the CETRAS study CPP Ile-de-France 2 2007-03-01-RCB 2007-A00124-49 AFSSAPS A70310-31) of 19 patients refractory to FOLFIRI treated by anti-EGFR antibodies with or without irinotecan chemotherapy-based regimen.

✓ **FFPE Training set**: Retrospective series of pooled Belgian and Finnish[2] patients. All patients were refractory to FOLFIRI and/or FOLFOX regimen. (no: 173/13/03/02/09).

✓ **FFPE validation set:** 45 patients from a randomized phase II trial (NCT00655499) sponsored by the GERCOR. 26 samples were only available in FFPE and 16 were available either in FFPE and FF tissues and 3 were only available in FF tissues. All patients were treated with 3rd line therapy by a combination of irinotecan and panitumumab after progression with oxaliplatin and irinotecan chemotherapy based regimens.



[1] Laurent-Puig et al. 2009; [2]Mosakhani et al. 2012

Methods

MiR-31-3p expression analysis:

✓ For each FFPE tumor sample, 5 FFPE slides of 5µm thickness were scratched specifically in the tumor area and samples were placed in 160µl of deparaffinization Solution (Qiagen, Hilden, Germany). Total RNAs were extracted using the FFPE miRNeasy extraction kit (Qiagen) according to the manufacturer's instruction.

✓ Specific quantification of expression level of miRNA hsa-miR-31-3p was performed using specific TaqMan pre-designed assays on retrotranscribed RNA and a ABI7900HT Real-Time PCR System. Expression levels were normalized to the references snRNA RNU6B levels through the ΔΔCt method.

✓ Survival model prediction: MicroRNA expression-based predictor of survival risk group was calculated by combining a Cox proportional hazards model and a supervised principal component method.

Cell Culture :

✓ Three colorectal adenocarcinoma cell lines (HTB-37, CCL-222, CCL220-1; ATCC, Manassas, CA) were selected and utilized for cell culture studies. All cells were transfected with miRVanamiRNA (Ambion) mimic negative control or hsa-miR-31-3p miRVanamiRNA mimic. The transfection efficacy was demonstrated by a1,500 times average rise of hsa-miR-31-3p levels.

✓ Cells were harvested 24h hours after transfection and extraction of total RNA was done with miRNeasy extraction kit.

✓ To detect putative down and up regulation in response to hsa-miR-31-3ptransfection, expression signals of transfected cells were compared to controls using a modified paired t-test (with a moderated t statistic) from the R package eBayes. The tests were made for each of the 3 cell lines independently using the biological replicates of the two experimental conditions as pairs. A fold change fc>1.3 or fc< 0.7 with p<0.05 was retained. Each probeset with an associated signal satisfying the selection criterion in 2 out of the 3 cell lines was retained in the final list of candidate targets for hsa-miR-31-3p.

✓ A database integrating contemporary miRNA target predictions from 6 individual databases (PITA, picTar 5-way Targetscan, microRNA.org, MicroCosm and miRDB) was developed to determine miRNAs target or the miRNA that target a particular gene taking into account the number of miRNA prediction databases which predicted each miRNA/target relationship and the rank of this prediction.

Results

Figure 1: FFPE Training Set

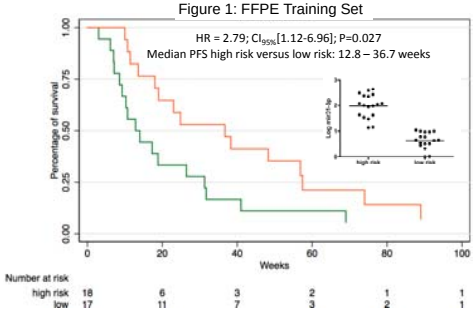
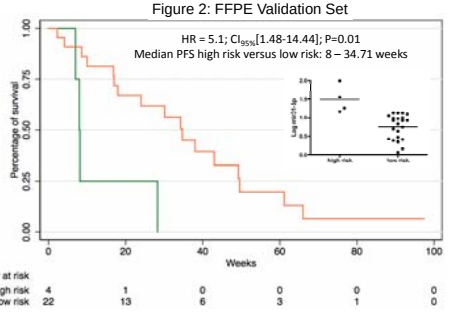


Figure 2: FFPE Validation Set



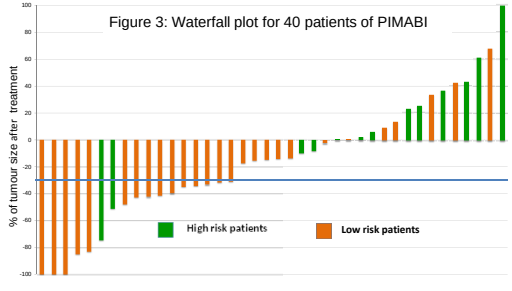
✓ FFPE Training set and FFPE Validation set were used to assess the prognostic value of the expression of miR-31-3p in FFPE samples. A hazard ratio for PFS of 2.79 [1.12-6.96] was observed for patients at low risk compared to patients at high risk (Fig.1). This threshold was validated in FFPE validation set: the PFS hazard ratio between low and high risk patients was 5.1 CI95%[1.48-14.44] (Fig.2).

✓ A nomogram for FFPE samples was established based on the training set and validated on all the FFPE samples from the validation set.

High risk patients Low risk patients

✓ A significant inverse correlation between the percentage variation of the RECIST criteria and the expression of miR-31-3p was found (r^2= 0.49 p=0.0035). This result is confirmed by the significant association between the risk status of the patients from the validation set determined by the level of the expression of miR-31-3p status and the percentage of variation of RECIST criteria (p=0.02, Kruskal-Wallis rank test) (Fig.3).

Figure 3: Waterfall plot for 40 patients of PIMABI



Research of miR-31-3p target genes

➢ Three CRC cell lines were transfected with hsa-miR-31-3p mimic or with a mimic control. Expression profile analysis of transfected cells allowed us to identify:

✓ 47 genes significantly down-regulated (fc<0.7;p<0.05),

✓ 27 genes significantly up-regulated by hsa-miR-31-3p (fc<1.3;p<0.05).

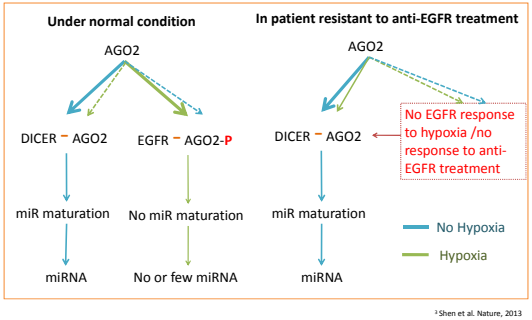
➢ 25 of the genes were predicted to be putative direct targets of hsa-miR-31- 3p in our prediction database and displaying a significant good rank (P<0.0001 for both by permutation test).

➢ The 25 putative direct and 27 indirect target genes were validated on qRT-PCR and out of these 52 genes, 45 displayed an expression level comparable to the level obtained in the array.

➢ Expression of these genes was analyzed in 39 tumor samples: a correlation between hsa-miR-31-3p expression pattern and PFS for 2 genes (p=0.02 and p=0.009 respectively). Interestingly, both genes displayed a negative correlation with hsa-miR-31-3p expression levels (-0.5;p=0.001; 0.3;p=0.04).

Discussion

- In the letter of Shen et al.[3] under hypoxia, AGO2 directly interact with and is phosphorylated by EGFR leading to a diminution of the interaction of AGO2 with DICER and resulting in a default of maturation of miRNA with long loop structure, decreasing the expression of the mature miRNA
- One of this long loop miRNA is mir-31, the precursor of miR-31-3p
- In the mCRC patients with poor prognosis: high expression of miR-31-3p and no response to anti-EGFR:
  - The absence of EGFR response could induced an increase expression of the mir-31 as well as the inhibition of EGFR by tyrosine kinase inhibitor
  - In the mCRC patients tumor samples, the high level expression of miR31-3p could be the witness of the absence of EGFR response to hypoxia and resistance to anti-EGFR therapy



[3] Shen et al. Nature, 2013

Conclusion

✓ We identified miR-31-3p, a microRNA which expression is associated with anti-EGFR response
✓ Results were obtained on FF samples and validated on FFPE samples
✓ These results were validated in samples from a phase II clinical trial (PIMABI; NCT00655499)
✓ In vitro experiments identify 25 putative direct target genes and 27 indirect target genes
✓ Tumor patients analysis confirm 2 direct target genes