Gene and MicroRNA Expression Are Predictive of Tumor Response in Rectal Adenocarcinoma Patients Treated With Preoperative Chemoradiotherapy

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Preoperative chemoradiotherapy (pCRT) followed by surgery is the standard treatment for locally advanced rectal cancer (LARC). However, tumor response to pCRT is not uniform, and there are no effective predictive methods. This study investigated whether specific gene and miRNA expression are associated with tumor response to pCRT. Tissue biopsies were obtained from patients before pCRT and resection. Gene and miRNA expression were analyzed using a one-color microarray technique that compares signatures between responders (R) and non-responders (NR), as measured based on tumor regression grade. Two groups composed of 38 "exploration cohort" and 21 "validation cohort" LARC patients were considered for a total of 32 NR and 27 R patients. In the first cohort, using SAM Two Class analysis, 256 genes and 29 miRNAs that were differentially expressed between the NR and R patients were identified. The anti-correlation analysis showed that the same 8 miRNA interacted with different networks of transcripts. The miR-630 appeared only with the NR patients and was anti-correlated with a single transcript: *RAB5B*. After PAM, the following eight transcripts were strong predictors of tumor response: *TMEM188*, *ITGA2*, *NRG*, *TRAM1*, *BCL2L13*, *MYO1B*, *KLF7*, and *GTSE1*. Using this gene set, an unsupervised cluster analysis was applied to the validation cohort and correctly assigned the patients to the NR or R group with 85.7% accuracy, 90% sensitivity, and 82% specificity. All three parameters reached 100% when both cohorts were considered together. In conclusion, gene and miRNA expression profiles may be helpful for predicting response to pCRT in LARC patients.

Colorectal cancer (CRC) is one of the principal causes of cancer mortality in the world. Approximately 30% of CRC cases are designated as rectal cancer, and approximately 40% of them are locally advanced (LARC) (Siegel et al., 2014). Preoperative chemoradiotherapy (pCRT) is the standard treatment for patients with LARC (Agostini et al., 2014). The response to pCRT varies from none to complete (Rullier et al., 2001; Rodel et al., 2003) and only the responding patients seem to have an outcome improvement (Maas et al., 2010). To spare the exposure to toxic and inefficient therapy of non-responder patients and to quickly proceed with surgery, it is clinically relevant to determine early predictors of tumor response. Many clinical, metabolic, and imaging tools have been evaluated

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as predictors; however, the performance of these methods for predicting the histopathological response is poor (Nutt et al., 2003; Amthauer et al., 2004; Denecke et al., 2005; Gearhart et al., 2006; Molinari et al., 2013; Crotti et al., 2015).

Controversial issues and poor predictability of tumor response were also experienced with the following biological markers: epidermal growth factor receptor (EGFR), thymidylate synthase (TS), bcl-2/bax, cyclooxygenase (COX-2), p53, Ki-67, p21, and serum carcinoembryonic antigen (CEA) (Spolverato et al., 2011).

Drug sensitivity in chemotherapy is thought to be attributable to variations in the underlying genetic cancer traits. Gene expression signatures have a great potential for predicting outcomes, and they may be superior to conventional clinical and pathological approaches (van de Vijver et al., 2002; Gordon et al., 2003; Nutt et al., 2003; Parissenti et al., 2007). Some studies on the prediction of treatment response have been based on mRNA profiling of LARC patients, but no consensus has been obtained for the list of predictive genes (Ghadimi et al., 2005; Kim et al., 2007; Rimkus et al., 2008).

microRNAs (miRNAs) are non-coding RNAs that negatively regulate gene expression at the post-transcriptional level by cleavage and/or translational repression of their mRNA targets. miRNA dysregulation is believed to promote malignant behavior of tumors because they control biological processes that are implicated in carcinogenesis (Chen, 2005; VandenBoom et al., 2008; D'Angelo et al., 2015). Depending on the target genes, they can either be considered as tumor suppressors or oncogenes. Aberrant expression of miRNAs has been shown in various types of cancer such as breast, brain, lung, pancreas, thyroid, and colon cancer, as well as hematological malignancies.

miRNAs have been extensively studied to determine their role as predictive markers in cancer cell lines or in metastatic CRC but in LARC patients the data are still poor (Aslam et al., 2009; Agostini et al., 2010; Perilli et al., 2014; Vicentini et al., 2014; D'Angelo et al., 2016). In this study, we investigated the whole spectrum of transcriptional signatures of tumor and normal tissues of LARC patients analyzing the differences between responders (R) and non-responders (NR) to pCRT, before applying chemoradiotherapy. This study was aimed at investigating whether specific gene and miRNA expression profiles might be associated with rectal cancer response to pCRT and improving the information about the responsive biomarkers in LARC patients.

Materials and Methods

Patients

The study included consecutive patients treated in the Department of Surgery, University of Padua, Italy and in the Centro di Riferimento Oncologico of Aviano, Italy. All of the patients fulfilled the following criteria: histologically confirmed primary adenocarcinoma of the rectum, tumor within 12 cm of the anal verge on proctoscopic examination, clinical stage (Edge and Compton, 2010) cT3-4 and/or N0-2, resectable disease, age $\geq$18 years, Karnofsky Performance Status $\geq$60%, and written informed consent. CEA level was also determined. After baseline staging, all of the patients were treated with radiotherapy (total dose of at least 45 Gy to the entire pelvis at 1.8 Gy daily, five times per week) concomitantly with chemotherapy (5-Fluorouracil [5-FU] intra-venous or capecitabine, with or without oxaliplatin). Surgery was planned 6–8 weeks after the completion of pCRT.

Pathological assessment and definition of tumor response

Standardized histological examination of the surgical specimens was performed according to the American Joint Committee on Cancer (AJCC) classification (Edge and Compton, 2010).

Histologic tumor response to pCRT was assessed according to the modified five-grade tumor regression (TRG) classification of Mandard (Mandard et al., 1994). For the purpose of this study, patients were subdivided into responders (TRG 1–2) and non-responders (TRG 3–5).

Tissue samples and RNA extraction

Endoscopic tumor and normal adjacent rectal biopsies were collected from each patient before pCRT, according to a standard protocol approved by the local ethics committee (Comitato Etico per la Sperimentazione—Azienda Ospedaliera di Padova). Briefly, each patient signed an informed consent for the use of these samples for research purposes. All clinical investigation has been conducted according to the principles expressed in the Declaration of Helsinki. All biopsies were preserved in the Tissue Bank of the 1st Surgical Clinic—University of Padua. All biopsies underwent standardized histopathological examination based on hematoxylin-eosin staining of 5-μm frozen sections. Tumor specimens with $\geq$60% malignant cells were considered for the experiment. Total RNA extraction was performed using TRIZOL® Reagent (Invitrogen, Carlsbad, CA) following standard procedures. Samples with RIN $>$6.5 (RNA 6000 Series Nano Chips) and samples enriched for small nucleic acid fragments with a percentage $<$35% (Agilent Small RNA Kit) were selected for the microarray analysis.

Gene expression analysis

Total RNA from tumor and normal tissue were analyzed using a microarray technique (Agilent, Santa Clara, CA) with the Whole Human Genome Oligo microarray platform 4 $\times$ 44 K (V1); 1 μg of each sample of total RNA was labeled using the Agilent One-Color Microarray-Based Gene Expression Analysis kit (Quick Amp Labelling, Agilent Technologies), linearly amplified, labeled, and hybridized. Microarrays were read with the Agilent DNA Microarray scanner, and images were analyzed with Feature Extraction 10.5.1.1. The data were filtered and normalized using Moltiplicatively Detrended and Quantile methods. Expression datasets were compiled according to the standards proposed by the Microarray Gene Expression Data Society.

MicroRNA expression analysis

We used the human miRNA microarray platform Rel 12.0 (V3) manufactured by Agilent SurePrint Technology containing 866 human and 89 human viral miRNA probes. Only the first cohort's samples were used for this analysis; 100 ng of each RNA sample was directly labeled. The data were filtered and normalized using the cyclic Loess method.

Statistical analysis of the expression data

The statistical analysis was performed with TMEV 4_5_1. Hierarchical clustering analysis was performed using the complete linkage method and Euclidean distance or Pearson's correlation. The differential gene expression between the responders and non-responders was determined using SAM (Significance Analysis of Microarrays) Two Class, FDR (False Discovery Rate) 0%. Class and gene prediction analysis was performed using the PAM (Prediction Analysis of Microarrays, http://www-stat.stanford.edu/~tibs/PAM). The association with biological annotation terms (Gene Ontology Terms and Pathways) was detected using DAVID (Database for Annotation, Visualization, and Integrated Discovery) tool (http://david.abcc.ncifcrf.gov/). For the integrative analysis of miRNA target predictions of differentially expressed miRNA and gene expression data, the MAGIA (miRNA and genes integrated analysis) tool (http://gencomp.bio.unipd.it/magia) was used. We used a parametric linear correlation measure (Pearson) and

selected only those that were anti-correlated less than −0.3 as functional. miRNA-mRNA anti-correlations were visualized using Cytoscape software package (Cline et al., 2007).

Real-time quantitative PCR (qPCR)

To validate miRNA expression, 10 ng of total RNA for each sample, three miRNA specific primers for target (hsa-miR-630, hsa-miR-638, and hsa-miR-7) and three for references (RNU48, RNU44, RNU6B) were used (TaqMan® MicroRNA Inventoried Assays, Applied Biosystems, Foster City, CA). qPCR was performed on an 7500 Real Time PCR System (Applied Biosystems). Data analysis was performed using the REST software 2009.

Results

Patient, tumor, and treatment characteristics

Of the 59 patients enrolled in the study, 38 were included in the first "exploration" cohort and 21 in the second "validation" cohort. There were no significant differences between the two cohorts regarding patient, treatment, and tumor characteristics (Table I). The median tumor distance from the anal verge was 6 cm (range 2–11). The majority of the patients were clinically staged as cTNM III (n = 55). A radical tumor resection was achieved in 52 patients, and the remaining 7 patients underwent full-thickness local excision, and consequently, the status of the pathological lymph nodes remained indeterminate. Following the TRG classification, 32 patients were considered NR (54%) and were considered 27 R (46%), 12 (20%) of them showed a pathological complete response (TRG 1).

TABLE I. Patient, treatments, and tumor characteristics of 59 local advanced rectal cancer patients

	First cohort (N = 38)[a]	Second cohort (N = 21)[a]	Total (N = 59)[a]
Age			
Median (range), years	64 (43–79)	66 (31–79)	64 (31–79)
Sex			
Male	29 (76)	16 (76)	45 (76)
Female	9 (24)	5 (24)	14 (24)
CEA			
≤5 ng/ml	32 (84)	18 (86)	50 (85)
>5 ng/ml	6 (16)	3 (14)	9 (15)
cTNM			
II	2 (5)	2 (10)	4 (7)
III	36 (95)	19 (90)	55 (93)
RT dose			
Median (range), Gys	50.4 (45–55)	50.4 (46–56)	50.4 (45–56)
ChT			
Cape	18 (47)	10 (47)	28 (47)
Cape + Oxa	13 (34)	9 (43)	22 (37)
5-FU	3 (8)	1 (5)	4 (7)
5-FU + Oxa	4 (11)	1 (5)	5 (9)
Surgery			
LAR	29 (76)	16 (76)	45 (76)
APR	4 (11)	3 (14)	7 (12)
LE	5 (13)	2 (10)	7 (12)
ypT			
0	8 (21)	5 (24)	13 (22)
1	4 (11)	4 (19)	8 (14)
2	8 (21)	5 (24)	13 (22)
3	16 (42)	7 (33)	23 (39)
4	2 (5)	0 (0)	2 (3)
TRG			
1	8 (21)	4 (19)	12 (20)
2	8 (21)	7 (33)	15 (25)
3	16 (42)	7 (33)	23 (39)
4	5 (13)	3 (14)	8 (14)
5	1 (3)	0 (0)	1 (2)

LAR, low anterior resection; APR, abdominoperineal resection; LE, local excision; ypT, pathological T stage after neoadjuvant treatment; TRG, tumor regression grade; CEA, carcinoembryonic antigen; cTNM, clinical tumor node metastasis stage; RT, radiotherapy; ChT, concomitant chemotherapy; Cape, capecitabine; Oxa, oxaliplatin; 5-FU, 5-fluorouracil; (%) unless stated otherwise.
[a]Data are expressed as N.

Gene expression profiling: patient exploration cohort

To identify the molecular signatures of responsiveness to pCRT, we analyzed gene expression profiles from the first 46 rectal cancer biopsies: 38 from tumor and 8 from normal tissues. Among the 38 tumor samples, 22 were classified as NRT and 16 as RT. Among the normal tissues, four were from NRN and four from RN patients. In the first unsupervised cluster analysis, tumor and normal samples were correctly clustered, whereas the NR and R groups did not. Using SAM Two Class analysis, no differentially expressed genes between NR and R were detected.

Hence, the ratio between tumor and the mean of normal sample expression (e.g., $RT_i/MeanRN$ and $NRT_i/MeanNRN$) was considered for further analyses. Also in this case, the first unsupervised cluster analysis did not correctly cluster the NR and R groups. However, using SAM Two Class analysis, 256 transcripts were found to be differentially expressed (DE) between NR and R with a FDR of 0%. These transcripts, in the cluster analysis, separate exactly into two responsive classes (Fig. 1A); 188 of these are up-regulated and 68 down-regulated in NR in comparison with R (Supplementary Table SI). To investigate the cellular components of discriminating genes, we performed the analysis of Gene Ontology categories using the DAVID bioinformatics' tool. "Nuclear envelope" (6 transcripts) and "Plasma membrane" (34 transcripts) showed a higher proportion of DE transcripts than other categories (Table II). Instead, for biological processes, we found six categories reported in Table II, and the most interesting were "Transmembrane transport," "Cell cycle," and "Apoptosis." In the pathway analysis, the most altered ($P \leq 0.01$) appear to be "Chemokine signaling" (six transcripts), "Pathways in cancer" (seven transcripts), "MAPK signaling" (seven transcripts), and "Axon guidance" (six transcripts). Moreover, 85 DE transcripts still have unknown functions, and 27 are non-coding RNAs.

We applied PAM to the expression data and found that only eight transcripts (*TMEM188*, *MYO1B*, *ITGA2*, *GTSE1*, *NRG1*, *KLF7*, *TRAM1*, and *BCL2L13*) discriminate between NR and R with a misclassification error of 0. Of them, five (*TMEM188*, *ITGA2*, *NRG1*, *TRAM1*, and *BCL2L13*) unexpectedly belong to the same category "Integral to membrane" of cellular components.

Gene expression profiling: patient validation cohort

To corroborate the significance of the genes found in PAM analysis, we evaluated the gene expression profiles of a new group of 34 LARC biopsies (validation cohort): 21 were taken from tumor (11 RT and 10 NRT) and 13 from normal (7 RN and 6 NRN) tissues. In these experiments, we considered only samples composed of ≥70% malignant cells. The expression data were treated as for the previous analysis. An unsupervised cluster analysis was performed only with the expression values of all probes of the 8 PAM discriminant transcripts (Fig. 1B). The samples clustered in two groups: NR and R. The accuracy of class prediction was 85.7% (18 of 21 correct calls) with three misclassification errors, two of which belonged to R, and one to NR. Considering NR as a test sample in this test set, the sensitivity was 90% and the specificity 81.8%. Accuracy, sensitivity, and specificity became 100% when we considered the two cohorts together (Fig. 1C).

The unsupervised cluster analysis was also applied to the samples of the validation cohort. All normal samples clustered together and separately from tumor samples. The cluster of

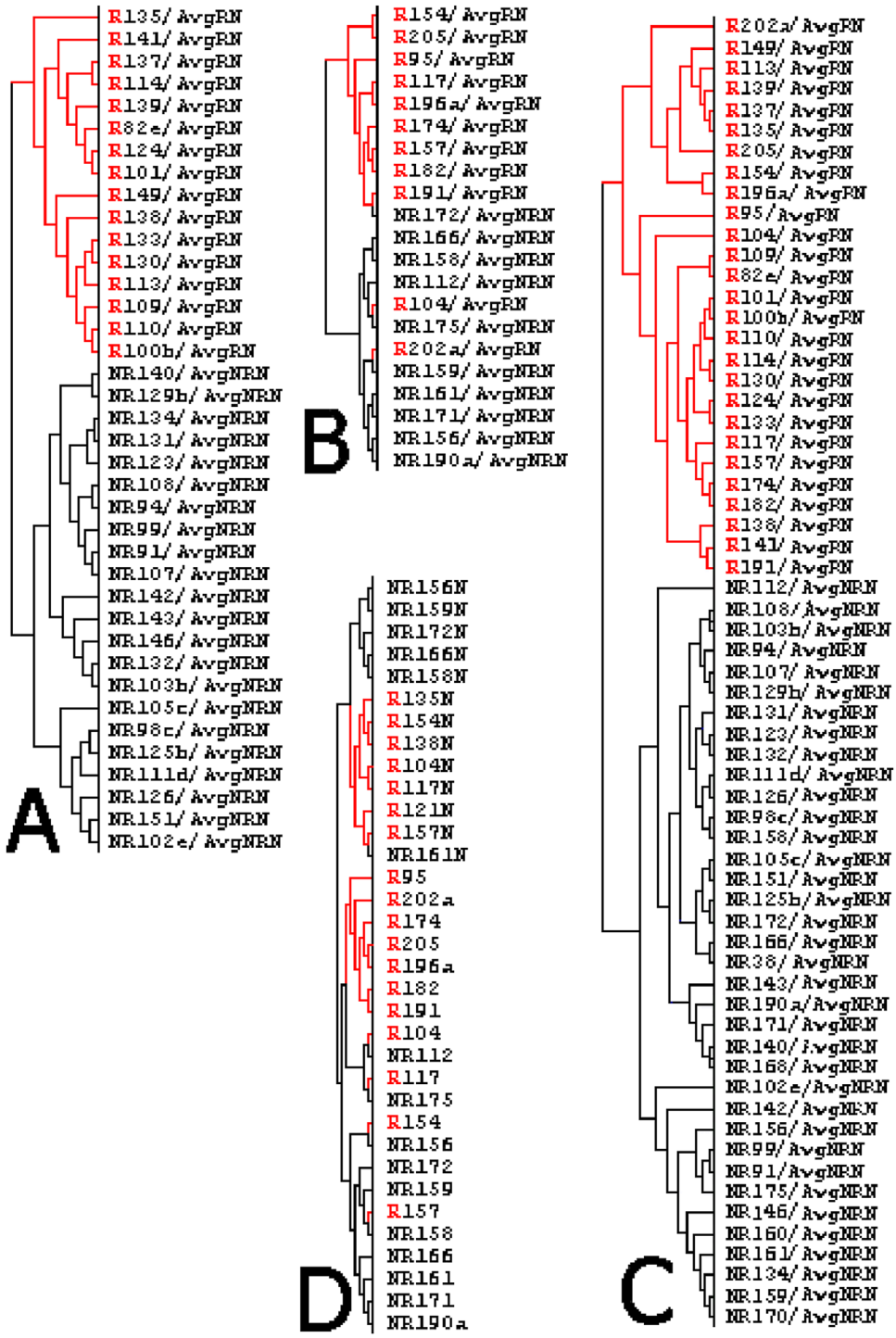

Fig. 1. Four unsupervised clustering A. (Euclidean distance, complete linkage) with 256 differentially expressed transcripts of the first cohort patients resulting by SAM. NR and R were clustered correctly into two groups. B and C (Pearson's correlation and Euclidean distance, respectively, complete linkage) with all probes of the eight discriminant PAM transcripts of (B) the second cohort patients and of (C) both cohorts. NR and R were correctly classified into two distinct groups except for three cases (one NR and two R) in B. D: (Pearson's correlation, complete linkage) with all of the second cohort's samples. Two-way clustering separated normal and tumor tissue: tumor cluster with three subgroups: NR, R, and a mixed of R-NR patients and normal cluster in NR and R. Red, up-regulated; green, down-regulated. In A, B, and C the expression values are considered as RT_i/MeanRN (RT, responder tumor; RN, responder normal) and NRT_i/MeanNRN (NRT, non-responder tumor; NRN, non-responder normal).

TABLE II. Gene Ontology analysis for cellular component and significant pathways of 256 differentially expressed transcripts in the first cohort's patients

Gene Ontology: cellular component
Nuclear envelope
Up-regulated
NPIP, LOC440353
Plasma membrane part
Up-regulated
ABCC2, DENND1A, LMO7, CGN, DST, MCF2L, RAPGEF2, ADD1, CACNB1, ENG, IGSF9, ITGA2, ITSN1, NRG1, PLXNB1, SLC12A7, SPRY4, TMEM16J, TM6SF1, LAMA5, TMPRSS3, TPCN1
Gene Ontology: biological process
Transmembrane transport
Up-regulated
ABCC2, NPIP, SCL12A7, SCL37A2, SCL5A11, TPCN1
Intracellular signaling cascade
Up-regulated
ARFRP1, DFFB, ERC1, MCF2L, RASEF, RASL11B, RAPGEF2, RAPGEF4, WNK, DGKD, DUSP8, HGF, IKBKB, ITSN1, MAPK7, NISCH, PLXNB1
Regulation of phosphate metabolic process
Up-regulated
RAPGEF4, DGKD, DUSP8, ENG, HGF, HNF4A, ITGA2, NRG1, SPRY4, ZFYVE28
Cell motion
Up-regulated
KLF7, ENG, ITGA2, LAMA5, PPARD, PLXNB1
Cell cycle
Up-regulated
CLIP1, DST, HGF, MAPK7
Apoptosis
Up-regulated
DFFB, MCF2L, ITSN1, MAPK7, NGEF, NISCH, PPARD, SCL5A11
Pathway analysis
Chemokine signaling
Up-regulated
IKBKB
Pathways in cancer
Up-regulated
HGF, IKBKB, ITGA2, LAMA5, PPARD
MAPK signaling
Up-regulated
MAPK7, IKBKB, DUSP8, CACNB1, RAPGEF2
Axon guidance
Up-regulated
SEMA4B, PLXNB1, NGEF, SRGAP1

tumor samples is divided instead in three subgroups: NRT, RT, and a combination of RT-NRT patients. Whereas, in the normal cluster, RN and NRN were correctly divided (Fig. 1D). Using SAM Two Class analysis with only the normal samples, 14 differentially expressed transcripts were found: 2 up- and 12 down-regulated (Table III). "Extracellular matrix" was the most enriched category of the cellular components with *MFAP5*, *FBLN5*, and *VCAN*, while *AGAP1* and *BLOC1S6* transcripts that are involved in "Vesicle trafficking" pathway.

miRNA expression profiling: patient exploration cohort

To extend the transcriptional analysis of LARC patients, we profiled with microarrays the miRNAs from the total RNA samples of the first patient cohort. Also in this case, the unsupervised cluster analysis correctly divided tumor from normal samples, but did not clearly separate NR and R groups. Then, the ratio with normal tissues was used. Using SAM Two Class analysis, 29 miRNAs were found DE between NR and R groups with a 0% FDR, 24 of them were down-regulated, and 5 were up-regulated. These miRNAs based on the cluster analysis separate into two responsive classes (Fig. 2); 24 of these were up-regulated, and 5 down-regulated. The expression values are reported in Supplementary Table SII.

miRNA data validation

Specific real-time qPCR assays were performed for hsa-miR-7, hsa-miR-630, and hsa-miR-638, by comparing the pathological and normal tissue of the same patients classified either in the R or in the NR group. Overall, the qPCR test was applied to eight pathological (four NR and four R) samples and eight normal (four NR and four R) samples. These experiments confirmed the results obtained with microarrays: hsa-miR-7 and hsa-miR-638 were, respectively, up- and down-regulated in both groups, whereas hsa-miR-630 was up-regulated in the NR group and down regulated in the R group (Supplementary Table SIII).

Anti-correlation analysis

To identify the transcripts that are most likely targeted by DE miRNAs between NR and R patient samples, we integrated 2118 mRNA (FDR 3.6%) and 29 miRNA (FDR 0%) significantly DE using MAGIA tool. Because miRNAs act as negative regulators, up-regulated miRNAs should be connected to down-regulated target mRNAs, and vice-versa. Two analyses were conducted after uploading the NR sample data separately from the R sample data. These analyses showed that the same eight miRNAs interacted with different networks of transcripts. The miR-630 appeared only with NR patients and was anti-correlated with a single transcript: *RAB5B* (Figs. 3 and 4).

The topology of the regulatory networks showed that miR-939 and miR-638 (up-regulated in NR samples but down-regulated in R) result in the highest number of anti-correlations with DE mRNAs: 173 transcripts in the NR group (47 shared by

TABLE III. List of differentially expressed genes between NR and R normal tissue (FDR = 0%)

Gene name	Systematic name	Description	Log_2 NR/R	Mean NR	Mean R	SAM score
ENST 00000340301	ENST 00000340301	mRNA for FLJ00286 protein	**1.1**	7.6	6.4	5.4
AGAP1	NM_133446	Centaurin, gamma-like family, member 1	**1.1**	9.7	8.6	5.5
MFAP5	NM_003480	Microfibrillar associated protein 5	**−1.4**	7.3	8.7	−6.8
BLOC1S6	NM_012388	Pallidin homolog	**−1.1**	7.0	8.1	−5.2
ZNF24	NM_006965	Zinc finger protein 24	**−0.8**	10.3	11.0	−4.9
C1orf52	NM_198077	Chromosome 1 open reading frame 52	**−0.6**	8.1	8.7	−4.6
CPA3	NM_001870	Carboxypeptidase A3	**−2.2**	7.5	9.7	−4.6
FBLN5	NM_006329	Fibulin 5	**−0.9**	7.3	8.2	−4.5
CENPL	NM_033319	Centromere protein L	**−1.1**	3.6	4.7	−4.5
ING3	NM_019071	Inhibitor of growth family, member 3	**−0.6**	9.3	9.9	−4.5
VCAN	NM_004385	Versican	**−1.0**	7.7	8.7	−4.5
RPN1	NM_002950	Ribophorin I	**−0.8**	9.5	10.3	−4.5
A_24_P480464	A_24_P480464	Unknown	**−0.8**	12.8	13.6	−4.4
RP11-78J21.1	NM_001011724	Heterogeneous nuclear ribonucleoprotein A1-like, 1	**−0.8**	13.4	14.3	−4.3

R, responders; NR, non-responders; SAM, significance analysis of microarrays.

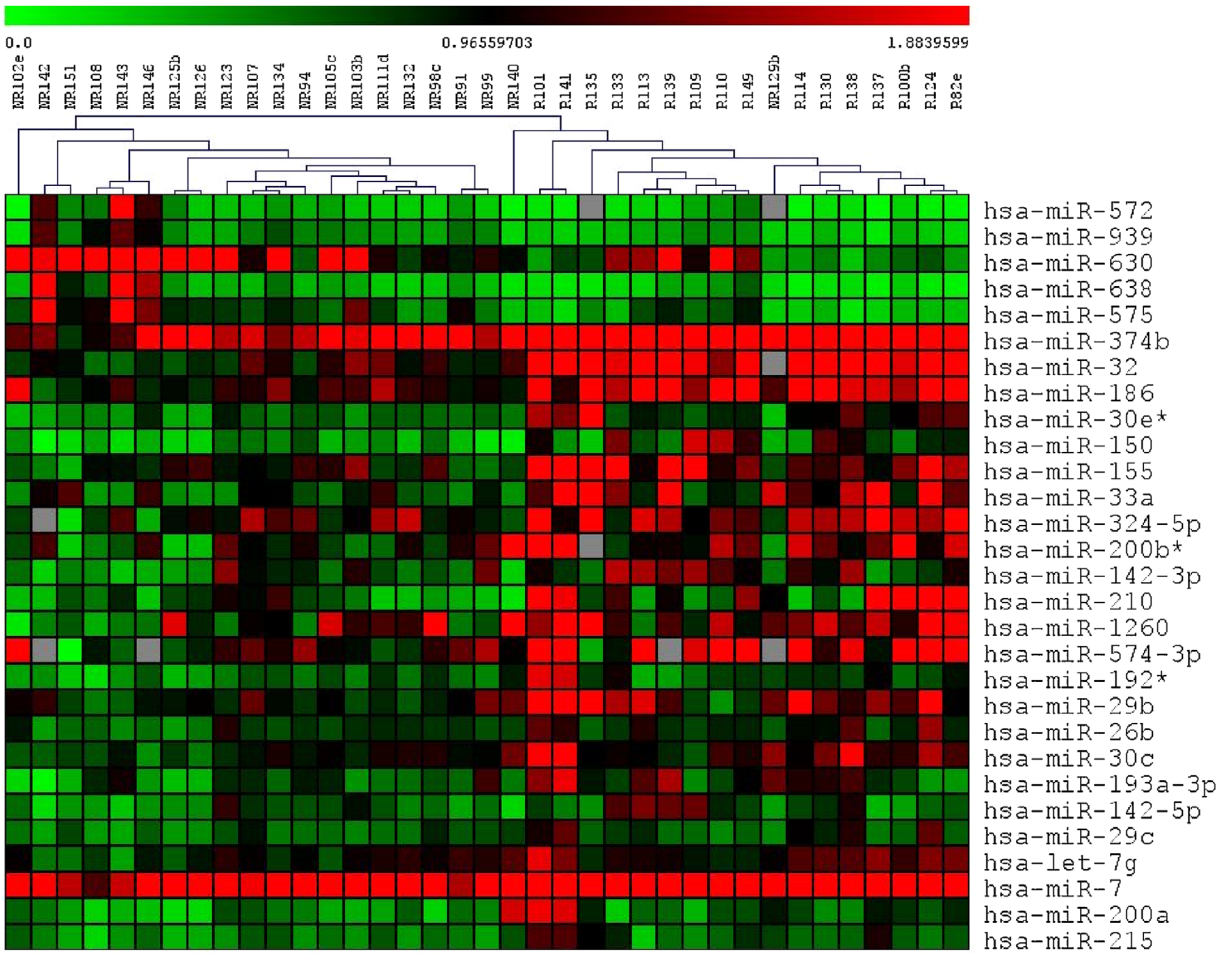

Fig. 2. Data from 29 differentially expressed miRNAs separated in two correct groups the NR and R patients in the unsupervised clustering (Euclidean distance, complete linkage).

both miRNAs) and 163 in the R group (30 shared by both miRNAs). The complete list of miRNA and anti-correlated transcripts is reported in the Supplementary Tables SIV and SV. These were classified according to DAVID by pathway enrichment analysis using KEGG (Kyoto Encyclopedia of Genes and Genomes). We found that the "Chemokine signaling," "Axon guidance," and "Insulin signaling" pathways were enriched in NR networks while only "Adherens junction" pathway was enriched in R.

Data registration

All expression data have been registered in Gene Expression Omnibus (GEO) database, ID: GSE68204.

Discussion

Several investigators have used gene or miRNA expression profiling to analyze tumor response to pCRT in colon and rectal cancer. While most studies used tumor cell lines (Mariadason et al., 2003; Arango et al., 2004; Shimizu et al., 2005) or biopsies from patients with primary advanced (Del Rio et al., 2007) or metastatic (Khambata-Ford et al., 2007) CRC, few studies reported findings on LARC patients. Ghadimi et al. (2005) analyzed 30 LARC biopsies, identifying 54 differentially expressed genes. Kim et al. (2007) found 95 predictive genes by analyzing 46 biopsies, while Rimkus et al. (2008) identified 42 genes analyzing 43 biopsies. There is a similar scenario for miRNA expression: the majority of studies are on colon cancer lines (van de Vijver et al., 2002; Gordon et al., 2003; Nutt et al., 2003; Parissenti et al., 2007; Spolverato et al., 2011), and only two (Kim et al., 2007; Rimkus et al., 2008) reported on LARC patients. With different sensitivity and specificity, the following miRNAs were found to be involved in the tumor response: miR143 (Borralho et al., 2009), miR-192/miR-215 (Boni et al., 2010), miR-140 (Song et al., 2009), miR-31 (Wang et al., 2010), miR-34 (Akao et al., 2011), miR-21 (Valeri et al., 2010), miR-622/miR-630 (Scarpati et al., 2012), and miR-223/miR-142-3p (Hotchi et al., 2013).

Unfortunately, no data overlap was found in predictive gene and miRNA lists among these various studies (Agostini et al., 2015a). This lack of concordance could be ascribed to several factors: differences in the tumor content of patient specimens, studied populations, chemotherapy regimen, microarray platforms, definitions of responders, and the analytical tools used to analyze the signatures. Herein, we decided to investigate the expression profile rationing the tumor with the surrounding normal tissue and analyzing concurrently the

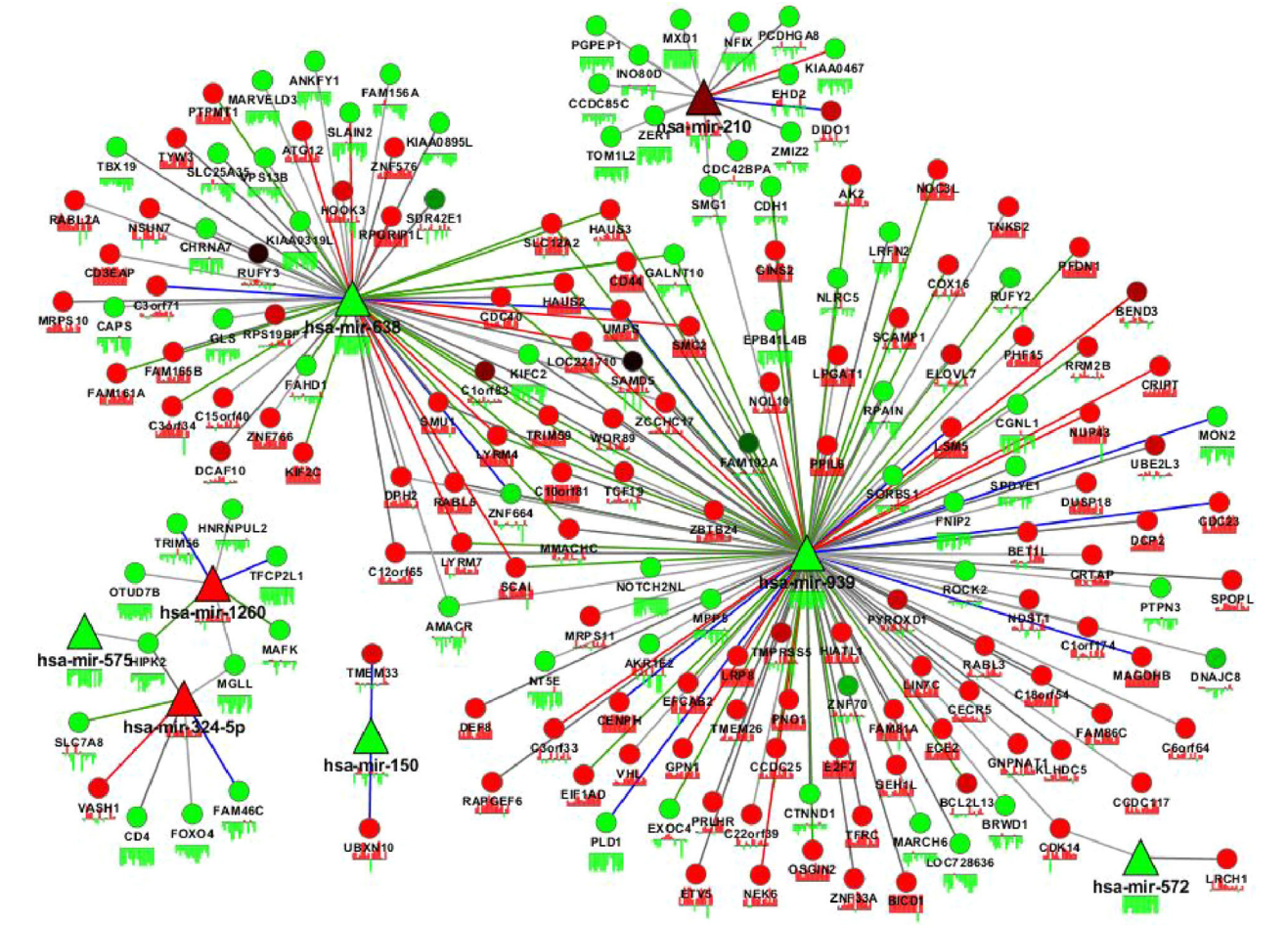

Fig. 3. Visualization of functional miRNA-mRNA anti-correlation in responders patients. Each feature are represented as red, up-regulated; green, down-regulated. Below each node are drawn as "heat strips" the expression values of each patient. The edge color varies according to the value of anti-correlation from least to most anti-correlated: gray-red-green-blue, respectively.

mRNA and miRNA gene signatures with new informatics tools. In addition, we validated the transcripts determined as predictors in a second cohort of patients increasing the percentage of tumor cells.

We found 256 transcripts differentially expressed between R and NR; 8 of them were strong predictors of tumor response, as well as the 29 miRNAs that resulted in differentially expression between the R and NR groups.

Our gene expression analysis highlights many transcripts belonging to the category of plasma membrane and nuclear envelope genes. This is interesting because their products may be involved in the regulation of drug access. For example, *ABCC2* was found to be up-regulated in NR. This protein is a member of the superfamily of ATP-binding cassette (ABC) transporters. ABC proteins transport various molecules across extra- and intra-cellular membranes. ABCC2 is involved in multi-drug resistance due to its role in excretion processes (Jemnitz et al., 2010; Sissung et al., 2010). Recently, Cecchin et al. (2011) found that *ABCC2-1249G>A* polymorphism is associated with a better tumor response in rectal cancer patients who underwent pCRT.

Another interesting finding is the up-regulation of UMP Synthase (UMPS) only in R patients both of the first and second cohorts. UMPS plays a crucial role in 5-FU metabolism, in fact the main mechanism of 5-FU activation is its conversion to fluorouridine monophosphate (FUMP), either directly by UMPS or indirectly via fluorouridine (FUR) with consequent RNA and/or DNA damage (Longley et al., 2003). The enzymes of the indirect pathway (UPP1 and UCK1) have been found to be down-regulated in NR patients. The up-regulation of UMPS enhances the effect of 5-FU on other cancer cell lines (Taomoto et al., 2006). A reasonable hypothesis is that the intracellular 5-FU is transported out across ABCC2 transmembrane protein in NR patients, while it is rapidly metabolized causing the death by damage to DNA and RNA in R patients.

Analyzing the gene expression data obtained from the tumor and its corresponding normal tissue, both in NR and in R, we found the classical pathways of CRC: apoptosis, p53, or PI3K-AKT or Wnt signaling, or DNA damage repair, colorectal cancer (data not shown). These pathways disappear when comparing the two classes of patients because there is the same transcripts deregulation. Other pathways emerge instead, likely typical of responsiveness to pCRT: chemokine signaling (most likely involved in radiation response) (Nagtegaal et al., 2005), pathways in cancer, MAPK signaling, axon guidance.

After PAM analysis, eight discriminating transcripts were found in the first set of LARC biopsies: *TMEM188*, *ITGA2*, *NRG*,

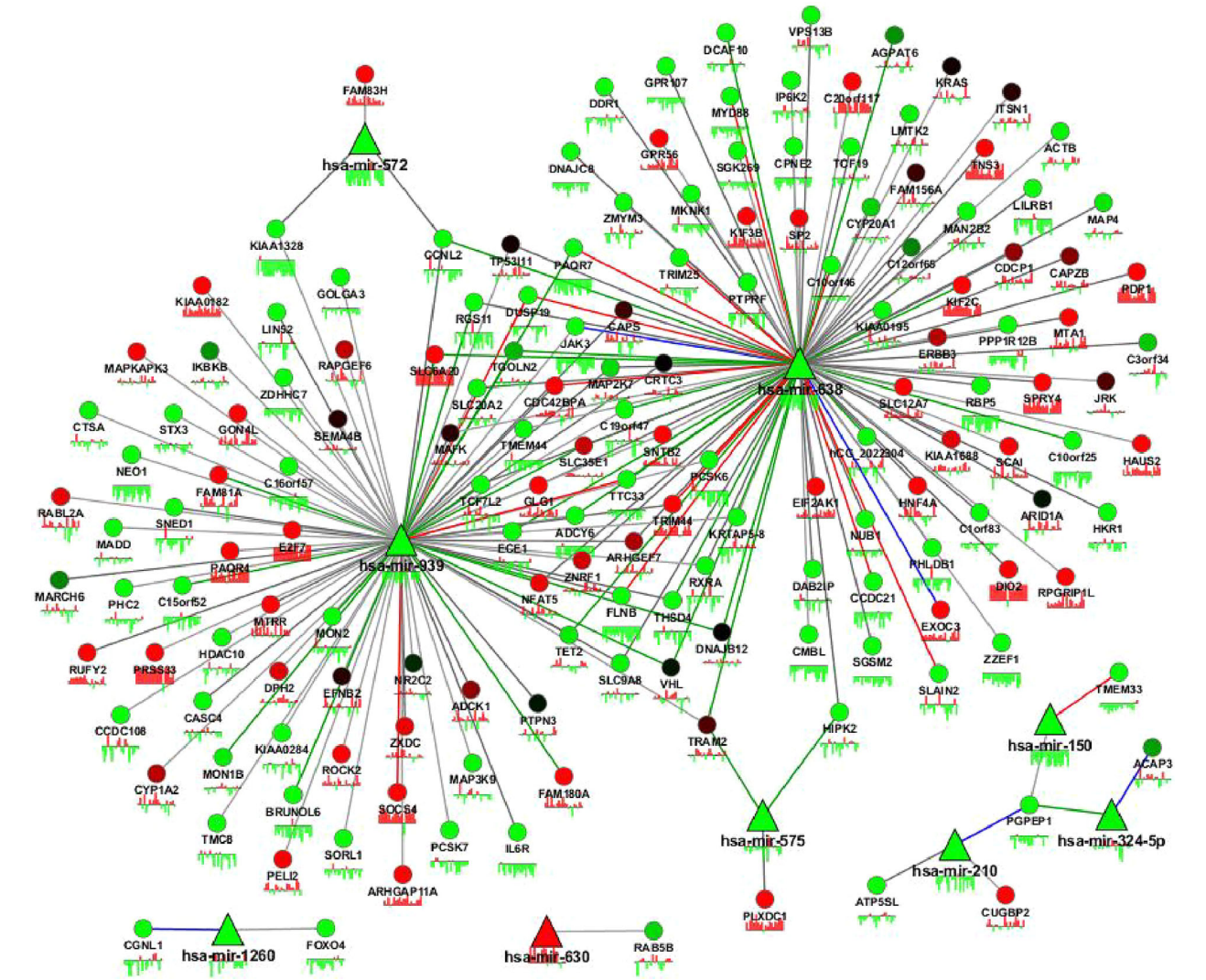

Fig. 4. Visualization of functional miRNA-mRNA anti-correlation in non-responders patients. Each feature are represented as red, up-regulated; green, down-regulated. Below each node are drawn as "heat strips" the expression values of each patient. The edge color varies according to the value of anti-correlation from least to most anti-correlated: gray-red-green-blue, respectively.

TRAM1, *BCL2L13*, *MYO1B*, *KLF7*, and *GTSE1*. These findings were validated in an independent cohort with an overall accuracy of class prediction of 85.7%, sensitivity of 90%, and specificity of 81.8%. Accuracy, sensitivity, and specificity reaches 100% when the analysis is performed in the two cohorts together. The majority of 8 PAM transcripts code for integral membrane proteins. Particularly interesting are *GTSE1* and *BCL2L13*, both down-regulated in NR. GTSE1 plays a specific role after DNA damage, regulating the cell arrest in G_2 and controlling the triggering of the p53-dependent apoptotic program (Monte et al., 2003). The *BCL2L13* (or *BCL-rambo*) over-expression induces apoptosis mediated by the activation of Caspase-3. *BCL2L13* is a known prognostic factor in other cancers such as childhood leukemia (Holleman et al., 2006). *NRG* and *KLF7* encode proteins that regulate cell proliferation, differentiation and survival.

Among the 29 miRNAs found to be differentially expressed in the present study, 8 are already known in CRC (Corte et al., 2012): miR-7, let7g, miR-30c, miR-29b, miR-192*, miR-215, miR-32, and miR-33a. MiR-630 and miR-142-3p have already been described in miRNA studies for response prediction in LARC patients (Scarpati et al., 2012; Hotchi et al., 2013) and miR-192/miR-215 in CRC cell lines (Boni et al., 2010). Although it has been demonstrated that miR-192/miR-215 target thymidylate synthase expression in CRC cell lines, their principal role in 5-FU resistance is the cell proliferation reduction by targeting cell cycle progression. Galluzzi et al. (2010) reported that miR-630 arrested A549 cells in the G_0–G_1 phase of the cell cycle too.

In this study, the web tool MAGIA (Sales et al., 2010) was used. The strength of this tool is based on the use of three different algorithms (PITA, miRanda, and Target Scan) for miRNA target prediction and in the integration of these information with miRNA and gene expression analysis. Furthermore, MAGIA reconstructs miRNA-gene bipartite regulatory networks of the best miRNA and mRNA putative interactions. The same eight miRNAs were found DE both in NR and R groups. miR-630 appeared only in NR and, based on

our analysis, is anti-correlated with *RAB5B*. This gene is a member of the RAS oncogene family, which plays a central role in CRC tumorigenesis. *RAB5B* acts in the biogenesis of endosomal and lysosomal compartments (Hirota et al., 2007). Furthermore, this gene is a candidate marker for early stage and malignant transformation in melanocytes (Meije et al., 2002). We found that the "Chemokine signaling," "Axon guidance," and "Insulin signaling" pathways were enriched in NR networks while only "Adherens junction" pathway was enriched in R.

We are conscious of the limits of the present work, first of which is the small sample size that is a recurrent characteristic of similar study-sets (Ghadimi et al., 2005; Kim et al., 2007; Rimkus et al., 2008; Agostini et al., 2015b). Moreover, the population is uniform (all the patients are from North-East of Italy) and could affect the gene and miRNA selection. It could be interesting to test the expression pattern that we found in a different population (as North American or Asiatic for example), to verify the reliability of our results.

In conclusion, we propose as significant prognostic factors for response to treatment in LARC patients the *TMEM188*, *ITGA2*, *NRG*, *TRAM1*, *BCL2L13*, *MYO1B*, *KLF7*, and *GTSE1* transcripts. Using this gene set, we were able to establish a model to predict response to pCRT with a sensitivity of 90% and a specificity of 82%. In the future, the analysis of these transcripts may be used to avoid the exposure to toxic and inefficient therapy for NR LARC patients.

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