Gene and microRNA expression predictive of tumour response in patients treated with preoperative chemoradiotherapy for rectal cancer
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RIASSUNTO

Introduzione

Il trattamento standard del cancro rettale localmente avanzato è rappresentato dalla radiochemioterapia preoperatoria (pRCT) seguita dalla chirurgia. Tuttavia, la risposta alla pRCT non è uniforme e non esiste oggi un metodo efficace per predire la risposta tumorale alla pRCT. L'identificazione di pazienti non responsivi alla pRCT potrebbe evitare l'esposizione alla radio- e chemioterapia, trattamenti non scevri da affetti avversi; inoltre l'identificazioni delle risposte complete potrebbe selezionare pazienti candidati ad un trattamento chirurgico meno invasivo. Questo studio si propone di associare profili di espressione genica e di microRNA (miRNA) al grado di risposta tumorale in pazienti affetti da cancro rettale e sottoposti a pRCT.

Materiali e metodi

Pazienti consecutivi affetti da carcinoma rettale medio-basso localmente avanzato e candidati quindi a pRCT, sono stati sottoposti a biopsie tissutali multiple prima dell'inizio della pRCT. Tutti i pazienti sono stati quindi sottoposti a pRCT standard seguita dalla resezione chirurgica del tumore. Tutti i pezzi operatori sono stati analizzati da uno stesso team di patologi ed in modo standardizzato. Le biopsie con una percentuale di cellule tumorali $\geq 50\%$ sono state considerate idonee per gli esperimenti.

Previo isolamento del RNA, l'espressione genica e di miRNA è stata valutata con tecnica microarray one color (Agilent®). I dati ottenuti sono stati quindi normalizzati sia intra- che inter-array, filtrati e infine clusterizzati. L'espressione genica e di miRNA è stata comparata tra responders (R) e non responders (NR) in base al grado di regressione tumorale (TRG) valutato dal patologo al momento dell'esame del pezzo operatorio. Una validazione dei dati ottenuti con i microarray è stata fatta mediante PCR quantitativa (qPCR).

Risultati

Sono stati considerati per lo studio 38 pazienti, 16 (42%) R (TRG1-2) e 22 (58%) NR (TRG3-5). Nonostante una prima unsupervised cluster analisi non abbia separato nettamente i due gruppi di pazienti, usando il programma SAM (Significance Analysis of Microarray) two class, 256 trascritti sono risultati differenzialmente espressi tra NR e R (188 sovra- e 68 sotto-espressi). Usando PAM (Prediction Analysis for Microarray), 12 trascritti erano fortemente predittivi di risposta tumorale. SAM two class ha permesso inoltre di evidenziare 30 miRNA differenzialmente espressi tra NR e R (24 sovra- e 6 sotto-espressi). Analisi di anti-correlazione, mediante MAGIA (miRNA and genes integrated analysis), hanno rilevato gli stessi 8 miRNA sia nel gruppo NR che R, ad eccezione di miR-630, sovra-espresso solo nei NR. La validazione mediante qPCR del gene ABCC2 e di miR-7, miR-182, miR-200a, miR-630, miR-638 e miR-1300 ha confermato i risultati dell'analisi microarray.

Conclusioni

I profili di espressione genica e di miRNA pre-trattamento sembrano essere utili alla predizione di risposta tumorale alla pRCT in pazienti affetti da cancro del retto, tuttavia sono necessari ulteriori studi di validazione per confermare questi risultati e per il loro utilizzo nella pratica clinica.
SUMMARY

Background
Preoperative chemoradiotherapy (pCRT) followed by surgery is the standard treatment for locally advanced rectal cancer (LARC). However, the response to pCRT is not uniform, and there is no effective method to predict tumour response to pCRT. Identification of patients not responsive to pCRT could avoid useless exposure to radiation or chemotherapy which is associated with adverse effects. Moreover, the identification of pathological complete response could select patients candidated to a more preserving surgery. The aim of this study is to investigate whether gene and micro-RNA (miRNA) expression profiling is associated with rectal cancer response to pCRT.

Materials and methods
Tissue biopsies were obtained from patients with mid-low LARC, before pCRT. All the patients underwent standard pCRT followed by resection. All surgical specimens underwent standardized histopathological examination. The biopsies with ≥50% of cancer tissue were considered for the experiment. Gene and miRNA expression was analyzed using one color microarrays technique (Agilent®), after RNA isolation. The data were normalized intra- and inter-array, filtered and then clustered. Gene and miRNA expression was compared between responders (R) and non responders (NR) as measured by histopathological tumour regression grade (TRG). Validation of microarrays data was made by quantitative PCR (qPCR).

Results
Thirty-eight patients, 16 (42%) R (TRG1-2) and 22 (58%) NR (TRG3-5), were considered. Using SAM (Significance Analysis of Microarrays) two class, 256 genes were found differentially expressed between NR and R (188 over- and 68 down-expressed). Performing PAM (Prediction Analysis for Microarray), 12 genes were strongly predictive of tumour response. Using SAM two class, 30 miRNAs were found differentially expressed between NR and R (24 over- and 6 down-expressed). Anti-correlation analyses, using MAGIA (miRNA and genes integrated analysis), revealed the same 8 miRNAs both in NR and R group, except for miR-630, over-expressed only in NR group. ABCC2 gene, miR-7, miR-182, miR-200a, miR-630, miR-638, and miR-1300 were validated by qPCR, confirming the data obtained by microarray analysis.

Conclusions
Pre-treatment gene and miRNA expression profiling may be helpful to predict response to pCRT in LARC. Further analyses to confirm these findings are required.
BACKGROUND

Colorectal cancer (CRC) is the third most common cancer diagnosis among both genders, with an estimated 663,600 new cases and 320,600 deaths per year worldwide\(^1\). Approximately 20% of CRCs are distal to the rectosigmoid junction and designated as rectal cancer. It is a significant health problem in Italy, accounting 20,500 new male cases and 17,300 new female cases per year\(^2\).

Regarding the mid-low locally advanced rectal cancer (LARC), studies show that preoperative chemoradiation (pCRT) significantly improves local control and reduces toxicity profiles compared to post-operative CRT with a similar survival rate\(^3\)-\(^7\). Furthermore, the ability to achieve a pathological complete response (pCR) after pCRT is correlated with improved survival, decreased local recurrence and higher rate of sphincter-preserving surgery\(^8\),\(^9\). However, although pCR rates of 20-25% can be obtained, more than one third of patients do not respond at all or show a poor response to the treatment\(^10\).

The survival and the prognosis of patients depend on the stage of the tumour at the time of detection. Unfortunately more than 57% of them have regional or distant cancer spread at the time of diagnosis\(^11\). Despite significant advances in the management of CRC, the overall survival for advanced and metastatic disease has only little changes over the past 20 years. Five-year overall survival rate is about 64%, with 90% 5-year survival in localized disease and 10% 5-year survival for patients with metastases\(^12\). Therefore, predicting the potential aggressiveness of a primary tumour could help in improving patient’s survival, identifying those who should receive pre- and post-operative CRT. Due to the not uniform response to adjuvant therapies, exposure to radiation or chemotherapy could be spared to patients with a priori resistant tumour and surgery could be scheduled without delay. Moreover, the identification of those patients with a complete response after pCRT could be useful to select candidates to a more preserving surgery (e.g. local excision of the residual scar or “wait and see” approach)\(^13\).

In this scenario it is necessary to find predictive markers of response to pCRT. Many clinical, metabolic and radiologic tools have been evaluated as predictors of tumour response to pCRT, however these methods often do not correlate with histopathologic response. Therefore, many potential additional markers, such as epidermal growth factor receptor (EGFR), thymidylate synthase (TS), bcl-2/bax,
cyclooxygenase (COX)-2, p53, Ki-67, p21 and serum carcinoembryonic antigen (CEA) have been studied, but the predictive value of most of these genes is low and controversial\textsuperscript{14}. Recent advances in expression genomics by DNA microarray have made possible to analyze tens of thousands of genes at a time and have shown that expression profile of cancer cells may be used to discriminate responders and non responders to pCRT\textsuperscript{15,16}. On the other hand small regulatory RNAs have gained tremendous interest in cancer research. MicroRNAs (miRNAs) are non-coding RNA molecules, 18-25 nucleotides in length, which regulate the expression of their target genes and play an important role in the control of biological processes, such as cellular development, differentiation, proliferation, apoptosis and metabolism. miRNAs are involved in tumour biology too, including oncogenesis, progression, invasion, metastasis and angiogenesis. Moreover miRNAs have been recently demonstrated to be potential markers of tumour response for rectal cancer after pCRT\textsuperscript{17,18}.

\textbf{AIM}

The aim of this study is to investigate whether the genetic signature, gene and miRNA, in pretherapeutic biopsy specimens of LARC, is able to predict histopathological response to pCRT.
MATERIALS AND METHODS

Patients and tissue samples

The study encompassed consecutive patients treated in the Department of Surgery, Gastroenterology and Oncology of the University of Padua, Italy and in the Centro di Riferimento Oncologico of Aviano, Italy. All the patients were involved in one of the following two ongoing randomized, multicenter, phase III clinical trials: INTERACT and STAR. The patients fulfilled the following criteria: histological confirmed primary adenocarcinoma of the rectum, tumour within 12 cm from the anal verge by proctoscopic examination, clinical stage\textsuperscript{19} cT3-4 and/or N0-2, resectable disease, age ≥18 years, Karnofsky Performance Status\textsuperscript{20} ≥60%, and provision of written informed consent. The staging procedures, whose goal was to define the cTNM, were performed by digital visit, rectal/colonoscopy, transrectal ultrasound (TRUS), pelvic magnetic resonance imaging (MRI), and multislice computed tomography (CT). Baseline carcinoembryonic antigen (CEA) level was also determined. After pretherapeutical staging all the patients were treated with pCRT. Patient involved in the INTERACT trial had a total dose of 45 Gy to the whole pelvis at 1.8 Gy daily, 5 times per week; patients in the XELOX-RT arm got a boost of 5.4 Gy delivered to the mesorectum to a total dose of 50.4 Gy plus a concomitant chemotherapy with Xeloda and Oxaliplatin; patients in the XEL-ACRT arm got a boost of 10 Gy to the mesorectum, at 1 Gy for fraction to a total dose of 55 Gy plus a concomitant chemotherapy with Xeloda alone. The patients involved in the STAR trial were treated as follow: 50.4 Gy at 1.8 Gy daily, 5 times per week plus 5-Fluorouracil (5-FU) or 50.4 Gy plus 5-FU and Oxaliplatin. The restaging was performed 4-5 weeks after the completion of pCRT with the same clinical/instrumental exams used for baseline staging.

Surgery was planned 6-8 weeks after the completion of pCRT. The choice of operative procedure was at the discretion of the surgeon: low anterior resection (LAR) or abdominal perineal resection (APR) with standard lymphadenectomy up to the origin of the inferior mesenteric artery and total mesorectal excision (TME). In case of patients with a major clinical response (yT0-T1), documented with MRI, TRUS, and/or proctoscopy, a local excision was permitted, using either a conventional transanal local excision or the transanal endoscopic microsurgery (TEM).
Pathological assessment and definition of tumour response

Standardized histological examination of the surgical specimens was done according to the American Joint Committee on Cancer (AJCC) guidelines\(^9\). In particular, the histologic tumour response to chemoradiotherapy was assessed according to the modified tumour regression classification of Mandart et al.\(^{21}\) for the oesophageal cancer (Figure 1). They stratified Tumour Regression Grade (TRG) in five grades: TRG 1 (complete regression) showed absence of residual cancer and fibrosis extending through the different layers of the oesophageal wall; TRG 2 was characterized by the presence of rare residual cancer cells scattered through the fibrosis; TRG 3 was characterized by an increase in the number of residual cancer cells, but fibrosis still predominated; TRG 4 showed residual cancer outgrowing fibrosis; TRG 5 was characterized by absence of regressive changes.

For the purpose of this study, patients were subdivided in responders (R, TRG 1-2) and non responders (NR, TRG 3-5).

Tissue samples and extraction of RNA

Endoscopic tumour and normal rectal biopsies were collected from each patient before the beginning of pCRT, according to a standard protocol approved by the local ethics committee. Briefly, each patient signed an informed consent for use these samples for research purposes; at least 4 micro-biopsies or 1 macro-biopsy both from tumour and normal mucosa have been taken and immediately frozen in liquid nitrogen, and stored at -80°C in the Institutional BioBank.

All biopsies underwent standardized histopathological examination by Haematoxylin-eosin stain on 5 µm frozen sections and tumour specimens with ≥50% malignant cells were considered for the experiment.

Total RNA extraction, from at least 2 micro-biopsies, was performed using TRIZOL® Reagent (Invitrogen, Carlsbad, CA) following standard procedures from each endoscopic biopsy by sections of 20 µm thick. Total RNA was preserved in a final volume of 20 µl of DEPC water at -80°C with 1 µl RNase Inhibitor (RNaseOUT Recombinant, 40 U/µl, Invitrogen). RNA quantity was measured on an ND-1000 spectrophotometer (NanoDrop Technologies by Celbio, Italy) and quality was assessed by capillary electrophoresis with Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). 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 microarray analysis.

**Gene expression analysis**

RNAs extracted were analyzed using microarrays technique (Agilent) with Whole Human Genome Oligo microarray platform 4X44K (V1); 1 µg of each sample of total RNA was labeled with Agilent One-Color Microarray-Based Gene Expression Analysis (Quick Amp Labeling, 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 with Multiplicatively Detrended and Quantile methods.

**microRNA expression analysis**

We used Human miRNA microarray platform Rel 12.0 (V3) manufactured with Agilent SurePrint Technology containing 866 human and 89 human viral miRNA probes. The same samples used for gene expression experiments were used also for this analysis. 100 ng of each RNA sample were directly labeled with Agilent Cyanine3-pCp reagent and hybridized using the miRNA Agilent Technologies protocol. Microarrays were scanned and the images were analyzed. The data were filtered and normalized with cyclic Loess method.

**Statistical analysis of expression data**

The statistical analysis was performed with TMEV 4_5_1. Hierarchical clustering analysis was performed with complete linkage method and Euclidean distance. The differential gene or miRNA expressions between R and NR were found by SAM (Significance Analysis of Microarrays) Two Class. Class and gene prediction analysis was performed with PAM (Prediction Analysis for Microarray). For the integrative analysis of target predictions of miRNA and gene expression data a new web tool was used: MAGIA (miRNA and genes integrated analysis) with Pearson Correlation.
Real-time quantitative PCR

Real-time quantitative PCR (qRT-PCR) was carried out using the SYBR® Green chemistry with GeneAmp 5700 Sequence Detection System (Applied Biosystems) to validate ABCC2 gene expression.

To validate miRNA expression, single-stranded cDNA from 10ng of total RNA samples were synthesized using MultiScribe™ MuLV reverse transcriptase (50 U/µl) (TaqMan® MicroRNA Reverse Transcription Kit, Applied Biosystems) and miRNA-specific primers (TaqMan® MicroRNA Assays). The process was carried out in three steps (30’ at 16°C, 30’ at 42°C, and 5’ at 85°C) and cDNA was stored at-20°C.

The reactions of qPCR were carried out in triplicates in a final volume of 20 µl in 2X TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems). miRNA-specific probes for 5 target miRNA (hsa-miR-630, hsa-miR-1300 v13, hsa-miR-200a, hsa-miR-638 and hsa-miR-7) and 3 references (RNU48, RNU44 RNU6B) were considered for the experiments (TaqMan® MicroRNA Inventoried Assays, Applied Biosystems).

qPCR was performed on an ABI 7900 HT Fast Real Time PCR instrument (10 min. at 95°C followed by 40 cycles of 15 sec. 95°C and 1 min. 60°C). Cycle threshold values (Ct) were determined using the SDS software of the 7900 HT (version 2.2.2.).

Data analysis were done using the REST software 2009 (version 2.0.13 QIAGEN GmbH).
RESULTS

Patient characteristics

To identify molecular signatures of responsiveness to pCRT, we analyzed gene expression and miRNA expression profiles from 38 patients affected by rectal adenocarcinoma. Clinical data of the 38 patients are shown in Table 1. There was a prevalence of males (29 males vs 9 females) and the median age was 64 years. The median tumour distance from the anal verge was 7 cm. The majority of patients were clinically staged as cTNM III (n=36), while the remaining two patients were staged as cTNM II. The baseline CEA level was ≥ 5 ng/ml only in 6 out of 38 patients.

The treatment characteristics are listed in Table 2. A radical tumour resection was achieved for all patients, except for those (n=5) who underwent local excision. For these 5 patients, even though radical margins have been achieved, pathological lymph node status remained indeterminate. The most frequent surgical procedure was LAR with TME (76%).

As shown in Table 3, based on ypT stage we observed 12 (32%) good responders (ypT0-1), 18 (47%) non-responders (ypT3-4), and 8 (21%) intermediate responders (ypT2). Based on the TRG classification, there were 16 (42%) responders (TRG 1-2) and 22 (58%) non-responders (TRG 3-4-5). Eight patients (21%) showed a pathological complete response (ypT0 or TRG 1).

Gene expression

For this study, a total of 46 mRNA samples, 38 from tumour and 8 from normal rectal tissue biopsies, respectively, have been considered. All the 46 arrays have been normalized both intra- and inter-array, Figure 2 shows the data before and after normalization. After filtering also for background, a total of 26,330 probes have been considered idoneous for the analyses.

Using TMEV 4_5_1 tool, a first unsupervised cluster analysis was done. Although tumour and normal samples correctly clustered, R and NR groups did not clusterized, neither with complete linkage method nor with Euclidean distance. Using SAM Two Class, no differentially expressed genes between R and NR were found.

Thus, the ratio between tumour sample expression and the mean of normal samples expression has been considered for further analyses.
Also in this case, a first unsupervised cluster analysis did not correctly clusterized R and NR groups. However, using SAM Two Class, 256 genes were found differentially expressed between NR and R with a FDR of 0%. As shown in Figure 3, 188 of these genes were over-expressed and 68 down-expressed, respectively, in NR. Performing PAM, 12 genes were strongly predictive of tumour response, with a misclassification error near 0 (Figure 4).

miRNA expression
The same samples used for gene expression analysis were considered also for this study. In particular, the ratio between tumour sample expression and the mean of normal samples expression was measured. After the filtering process, a total of 159 miRNA probes were idoneous for the analyses.

Also in this case, a first unsupervised cluster analysis correctly clusterized tumour and normal samples, but did not R and NR groups.

Using SAM Two Class, 30 miRNAs were found differentially expressed between NR and R with a FDR of 0%. As shown in Figure 5, 24 of these genes were over-expressed and 6 down-expressed, respectively, in NR. Performing PAM, we did not find miRNAs strongly predictive of response.

Anti-correlation analysis
For an integrative analysis of target predictions, MAGIA tool has been used with data obtained from the present study. Interestingly, the same 8 miRNAs are present both in the R and NR interaction networks, as shown in Figure 6, except for miR-630, over-expressed only in NR group.

Data validation
For the following transcripts and miRNAs, qRT-PCR was performed: ABCC2, miR-7, miR182, miR-200a, miR-630, miR-638, and miR-1300, confirming the expression levels obtained with microarray experiments. In particular miR-7, miR182, and miR-200a resulted down regulated in NR group, while miR-630 and miR-1300 were up regulated in the same group in comparison to R group.
DISCUSSION

The response of locally advanced rectal cancers to pCRT varies tremendously. Although a good response to preoperative treatment is associated with favorable outcome, molecular markers that allow therapy stratification are still lacking\textsuperscript{22-24}. The identification of differentially regulated genes and miRNAs could therefore contribute to understand better the underlying mechanisms of rectal cancer and its response to pCRT, in order to individualize therapy.

This study showed that microarray technology is applicable to rectal cancer patients; in particular, gene and miRNA expression profiling of pre-treatment biopsies may be useful for predicting response to pCRT. We found 256 transcripts differentially expressed between responders and non responders, 12 of them were strongly predictive of tumour response. At the same time, 30 miRNAs resulted differentially expressed between responders and non responders.

Among the differentially expressed genes, ABCC2 arises, over-expressed in non responders. The protein encoded by this gene is a member of the superfamily of ATP-binding cassette (ABC) transporters. ABC proteins transport various molecules across extra- and intra-cellular membranes. ABC genes are divided into seven distinct subfamilies (ABC1, MDR/TAP, MRP, ALD, OABP, GCN20, White). This protein is a member of the MRP subfamily which is involved in multi-drug resistance. This protein is expressed in the canalicular (apical) part of the hepatocyte and functions in biliary transport. Anticancer drugs, such as vinblastine, are substrates of these proteins; therefore, this protein appears to contribute to drug resistance in mammalian cells. Several different mutations in this gene have been observed in patients with Dubin-Johnson syndrome (DJS), an autosomal recessive disorder characterized by conjugated hyperbilirubinemia. In a recent work\textsuperscript{25}, Cecchin and Coll. found that ABCC2-1249G>A polymorphism is associated with a better tumour response in rectal cancer patients who underwent pCRT. Several chemotherapeutic agents (raltitrexed, platinum derivatives, irinotecan, gefitinib) represent a substrate for ABCC2, supporting a possible enhanced exposure of patients carrying the defective polymorphism to the therapeutic effect of the drugs. In the present study, the over-expression of wild type ABCC2 gene was associated with resistance to pCRT.
The majority of the 12 genes strongly predictive of response (found by PAM in the present work) were membrane transporters; particularly interesting are BCL2L13 and PITX2, both over-expressed in responders. BCL2L13 (or BCL-rambo) encodes a mitochondrially-localized protein with conserved B-cell lymphoma 2 homology motifs. Over-expression of the encoded protein results in apoptosis, mediated by the activation of Caspase-3. This is the first study in which an over-expression of BCL2L13 is associated with CRC and, in particular, with a better tumour response in rectal cancer patients treated with pCRT.

PITX2 gene encodes a member of the RIEG/PITX homeobox family, which is in the bicoid class of homeodomain proteins. The encoded protein acts as a transcription factor and regulates procollagen lysyl hydroxylase gene expression. This protein controls cell proliferation in a tissue-specific manner and is involved in morphogenesis. PITX2 is also a downstream effector of wnt/\beta\text{-}catenin signaling and seems to play a role in the pathogenesis of CRC. A recent work\textsuperscript{26} found that PITX2 expression is significantly related to the biological behavior of CRC cells and appears to be correlated with clinical survival. This is the first report that correlates PITX2 expression to tumour response in rectal cancer patients treated with pCRT.

Among the 30 miRNAs found differentially expressed in the present study, miR-7, miR-32, and miR-630 are particularly interesting. Bioinformatic predictions suggest that the human EGFR mRNA 3'-untranslated region contains three miR-7 target sites, which are not conserved across mammals. In Drosophila photoreceptor cells, miR-7 controls EGFR signaling and promotes photoreceptor differentiation. Other targets of miR-7 are insulin-like growth factor 1 receptor (IGF1R), PIK3CD, E(spl) gene family, and Pak1 (cancer cells). c-Fos is also a target of miR-7b in mice. Multiple roles and targets of miR-7 as well as its expression pattern were linked to regulatory mechanisms and pathogenesis in glioblastoma, breast cancer and other types of cancers, as well as in schizophrenia and visual abnormalities. Inhibition of the motility, invasiveness, anchorage-independent growth, and tumourigenic potential of highly invasive breast cancer cells through the introduction of miR-7 suggests a strong therapeutic potential of miR-7. In a recent work\textsuperscript{27}, miR-7 was identified to be significantly downregulated in CRC by miRNA expression array, and acts as a tumour suppressor in CRC. miR-7 exerts its role inhibiting directly the oncogenic protein Yin Yang 1 (YY1), resulting in differential regulations of the functional units in p53 and wnt pathways with significant impact on cancer development. The same Authors revealed that YY1 was associated
with poor survival of CRC patients. In the present study, for the first time, an up-regulation of miR-7 is associated with better response to pCRT for rectal cancer.

In a recent work\textsuperscript{28}, Yu and Coll. found that miR-32 over-expression was associated with human colon cancer stem cells (SW1116csc) and colon cancer cells proliferation. Moreover, Kheirelseid and Coll.\textsuperscript{29} found a miRNA predictor of complete response to pCRT for rectal cancer, based on a six-step model; miR-32 was one of these six miRNAs. Also in the present study, the over-expression of miR-32 was associated with a better response (TRG1-2).

Also miR-630 has been described in CRC\textsuperscript{30}. In particular, a recent work\textsuperscript{31} found a specific 14 miRNAs signature predictive of tumour response for rectal cancer treated with pCRT. miR-630 was one of these 14 miRNAs. Moreover, Galluzzi and Coll.\textsuperscript{32} demonstrated that miR-630 was upregulated by non-small cell lung cancer A549 cells in response to cisplatin. In the present study, an over-expression of miR-630 was associated with tumour resistance to pCRT.

Interestingly, performing anti-correlation analysis with MAGIA, the same 8 miRNAs were found both in responder and non responder groups. Among these, miR-638 has been yet implicated in gastric cancer, leukemia, and colorectal liver metastases\textsuperscript{33}. miR-630 appeared only in non responders, anti-correlated with RAB5B gene. This is a member of RAS oncogene family, which play a central role in colorectal cancer tumourigenesis. It acts in protein transport, probably in vesicular traffic. This is the first report in which RAB5B is associated to rectal cancer response to pCRT, possibly under the regulation of miR-630.

Although these results are preliminary, they are encouraging and microarray technology seems to be useful to find gene and miRNA expression profiling predictive of tumour response to pCRT in rectal cancer. However, this study has several limits. First, the low sample size do not permit to draw firm conclusion. Since complex phenotypes, such as tumour responsiveness to chemoradiotherapy, likely do not depend on the alteration or deregulated expression of single genes, high-throughput technologies have emerged as a central tool in deciphering the molecular basis of this clinically important phenotype because they offer the possibility to identify genomic differences between two groups of patients. However, due to the high number of observed genomic features, it represents a nontrivial task to determine which of these features are actually relevant, and this kind of analysis generally requires a high number of patients.
Another limitation is the few number of genes/miRNAs validated by qRT-PCR and the lack of validation of these results in a new prospective set of patients (ongoing study). Moreover, all cases of our series are from a small area of Northern Italy. Considering the genetic variability across different populations, it should be useful to perform the same analyses in a new cohort of patients possibly from another geographical area out of Europe.

Several investigators have used gene or miRNA expression profiling to analyze the genetics of rectal cancer response to pCRT\textsuperscript{29,31,34-43}. Although construction of the "best" predictive test in terms of clinical usefulness is desirable, the previously reported gene/miRNA signatures have differed considerably in terms of gene composition, with only few genes/miRNAs overlapping between different studies. This lack of concordance could be attributed to several factors, including differences in the tumour contents, studied populations, chemotherapy regimens, microarray platforms, definitions of responders, and the analytical tools used to generate the signatures. The current microarray predictors are not robust enough for clinical utility in rectal cancer at this point because of these limitations. However, considering the promising data and usefulness of gene profiling in breast cancer\textsuperscript{44}, the microarray analysis of gene/miRNA expression profiling could still have the potential to improve the management of patients with locally advanced rectal cancer. An higher number of patients analyzed for confident and accurate prediction would be required in future research. Furthermore, the candidate genes and miRNAs included in the predictor sets should be carefully validated by an alternative approach, and selection of the best predictive test is required in terms of ensuring the clinical usefulness of such a strategy. The final hurdle is the requirement of the extensive validation of predictive classifiers in an independent large number of patients or in prospective clinical trials.
CONCLUSIONS

Although the microarray analysis of individual tumours represents a promising approach to predict the responsiveness to pCRT in patients with rectal cancer, no optimal predictive gene expression signatures have been yet identified. Much larger studies using homogeneous cohorts of patients and the extensive validation of predictive classifiers in prospective clinical trials will be required before they can be incorporated into future clinical practice.

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REFERENCES


## TABLES

### Table 1. Clinical characteristics (N=38)

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<td>2</td>
<td>95</td>
</tr>
<tr>
<td>3</td>
<td>36</td>
<td>5</td>
</tr>
</tbody>
</table>

a.v.: anal verge; CEA: carcinoembryonic antigen; cTNM: clinical TNM stage.

### Table 2. Treatment characteristics (N=38)

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>%</th>
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</thead>
<tbody>
<tr>
<td><strong>RT dose</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range), Gys</td>
<td>50.4</td>
<td>(45-55)</td>
</tr>
<tr>
<td>Xeloda</td>
<td>18</td>
<td>47</td>
</tr>
<tr>
<td>Xeloda + Oxaliplatin</td>
<td>13</td>
<td>34</td>
</tr>
<tr>
<td>5-FU</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>5-FU + Oxaliplatin</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td><strong>Type of surgery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAR</td>
<td>21</td>
<td>55</td>
</tr>
<tr>
<td>RAR</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td>APR</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>Local excision</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td><strong>Interval pCRT-surgery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range), days</td>
<td>50</td>
<td>(34-108)</td>
</tr>
</tbody>
</table>

RT: radiotherapy; ChT: chemotherapy; 5-FU: 5-Fluorouracil; LAR: low anterior resection, RAR: anterior resection of rectum; APR: abdominoperineal resection; pCRT: preoperative chemoradiotherapy.
Table 3. Response to treatments (N=38)

<table>
<thead>
<tr>
<th>ypT</th>
<th>N</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>42</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>21</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TRG</th>
<th>N</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>16</td>
<td>42</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

ypT: pathological T stage after neoadjuvant treatments; TRG: tumour regression grade.
FIGURES

Figure 1. Tumour Regression Grade (TRG)

Figure 2. Gene expression - data normalization, a) before and b) after the process
Figure 3. Gene expression - SAM Two Class
**Figure 4.** Gene expression - PAM

1: responders group (R)
2: non responders group (NR)
Figure 5. miRNA expression - SAM Two Class
Figure 6. Anti-correlation analysis in a) R and b) NR groups