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MicroRNAs and Rectal Cancer

Miroslav Svoboda[1] and Ilona Kocakova[2]
[1]*Oncobios, Brno,*
[2]*Masaryk Memorial Cancer Institute, Brno*
Czech Republic

1. Introduction

Spontaneous rectal cancers usually arise as a consequence of somatic mutation of the APC gene followed by other mutations (K-ras mutation, DCC inactivation and p53 gene mutation), well-known today as the adenoma-to-carcinoma sequence (Kinzler & Vogelstein, 1996). This sequence covers most spontaneous rectal cancers (80%). However mutation(s) in DNA repair genes; the MSH1, MSH2, PMS1, PMS2 are also involved in certain fraction of rectal tumours, leading to microsatellite instability (Kim et al., 2006). Today about seventy different mutations, including important oncogenes and tumour suppressor genes, are known to be present in various colorectal cancers (Sjoblom, 2008). Colorectal cancers also exhibit changes in DNA methylation with hypermethylation of CpG islands and hypomethylation of oncogenes (Kang, 2007). The mutated cancer genotype is associated with changed expression in many genes, as has been demonstrated by powerful microarray analysis and Real Time PCR technology. It is now well known that mutations and changed DNA methylation pattern, as well as changes of mRNA transcription, are accompanied by changes of expression in certain microRNAs.

2. Background information

2.1 Therapy of rectal cancer

Surgical excision is the primary treatment. However locally advanced rectal cancer (LARC, T3,T4,N0, or TX, N1, N2) needs supportive pre-operative and postoperative therapy. This therapy combines pre-operative linear accelerator irradiation and chemotherapy with fluoropyrimidines, such as 5-fluorouracil or capecitabine. Postoperative therapy is based on adjuvant treatment with further doses of fluoropyrimidines combined with biological treatment where appropriate. (for details, see Lee et al., 2008). Supportive therapy is necessary for downsizing and downstaging of LARC tumours before surgery. Downsizing and downstaging during pre-operative treatment increases the frequency of operations in which the sphincter is saved (Lee et al., 2008). Moreover, this pre-operative treatment may also lower the risk of cancer dissemination during surgery. Seventy to seventy-five percent of patients react with some downstaging and downsizing of rectal tumours following chemoradiotherapy before surgery. However, only about 30% of patients exhibit substantial downstaging and downsizing tumour response and only 10-20% of them exhibit complete tumour eradication through this pre-operative procedure (Kim, 2007). The reasons for these

differences in tumour response are not yet well understood. It is widely known that irradiation or anticancer drug treatment of cell lines causes extensive changes in gene expression as well as changes in certain microRNAs, and that differences in responsiveness of cell lines to irradiation and drug treatment are dependent on individual genetic background and the presence of certain mutations in certain oncogenes or tumour suppressor genes. However information is very limited concerning molecular events associated with tumour response to therapy in vivo.

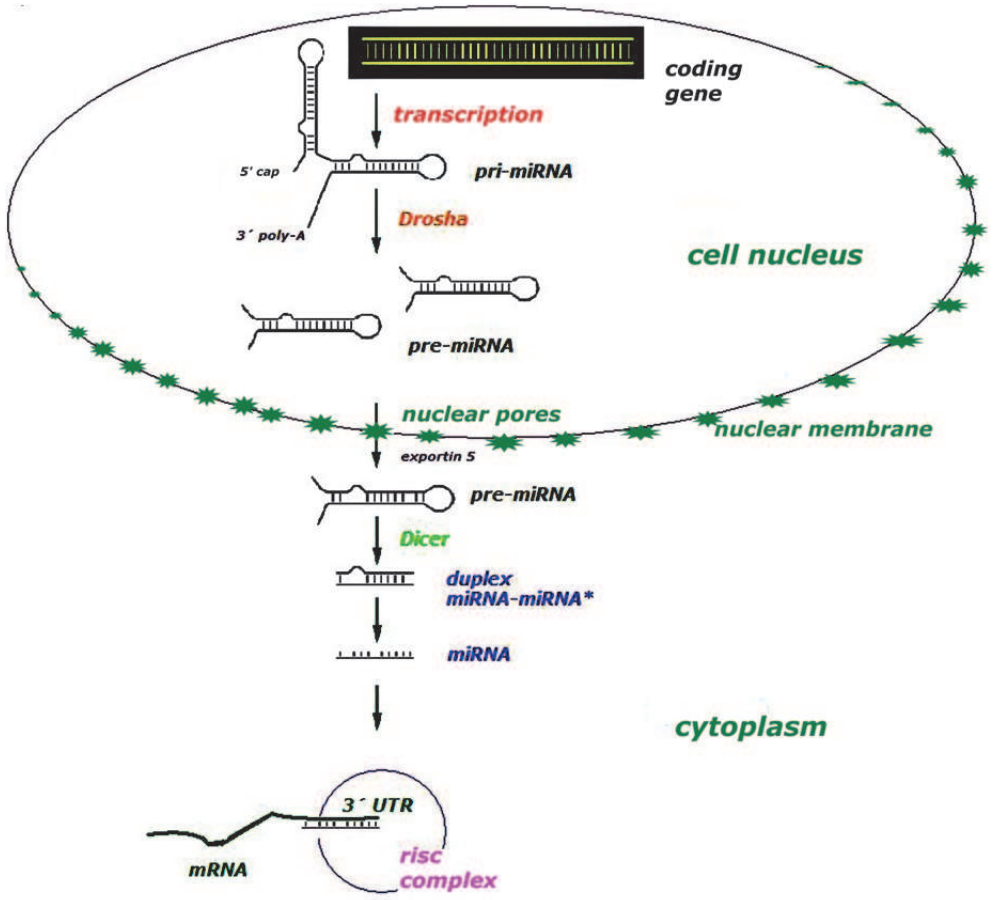
2.2 MicroRNA- basic information

MicroRNAs, also known as miRNAs, are small regulatory molecules (19-25 nucleotides long) that play an important role at the post-translational level of gene regulation (Ambros, 2001). MicroRNAs are widespread molecules, present in all eukaryotic organisms studied to date, including fungi, green plants and animals. MicroRNAs were first described in Western literature in 1993 and were found to play an irreplaceable regulatory role in the spatiotemporal development of the nematode worm *Caenorhabditis elegans* (Lee, 1993). Some 1800 different microRNAs and their sequence variants have been discovered in humans to date. Mature molecules are processed from primary transcripts (pri-miRNAs) that are 1000 nucleotides or more long (Winter et al., 2009). Primary microRNA transcripts originate either at intergenic locations or from intronic sequences of certain actively transcribed genes. If located at intronic sites, microRNAs may be transcribed both as sense and antisense sequences of these actively transcribed genes. Primary transcripts are processed in the nucleus by the specific nuclease Drosha to form approx. 70-nucleotide-long double-stranded pre-miRNAs (see also Scheme 1. for details). These pre-mature microRNA molecules are transported through nuclear pores of the nuclear membrane via the exportin 5 complex to the cytoplasm. Once within the cytoplasm, pre-miRNAs are processed by nuclease Dicer to form 19-25-nucleotide long, double-stranded molecules of sense miRNA and antisense miRNA*. Single stranded microRNAs finally bind to an RNA-silencing protein complex (known as RISC) and target complementary sequences present at the 3' end of the mRNA molecules. If a complex of target mRNA-miRNA-RISC is generated, translation inhibition of mRNA occurs. In humans approximately one-third of mRNA coding genes also contain target sites for one or more of several different types of microRNA. The same target sequence for a given microRNA may be present in mRNAs transcribed from many different genes.

Thus one type of microRNA may regulate many different genes simultaneously. Moreover, since several target sites for different microRNAs may be present in one mRNA and its gene, one gene can be regulated by several microRNAs. Thus post-translational regulation of gene expression by microRNAs is a very complex process; it is not yet fully understood.

2.3 MicroRNAs as regulatory molecules

MicroRNAs were originally discovered as important regulators of spatiotemporal development in the nematode worm *Caenorhabditis elegans* (Slack and Ruvkun, 1997) and were thought to have a canalisation function (i.e. phenotype stabilizing) in the organism (Hornstein and Shomron, 2006). Other authors later found that microRNAs may also have a buffering function in the regulation of gene expression (Cui and Yu, 2007). It is accepted today that microRNAs may play both the above roles (Wu et al., 2009). MicroRNAs are integrated into many regulatory circuits influencing cell cycle progression, genome maintenance, apoptosis and differentiation (Ambros, 2004; Re et al., 2009).



Scheme 1. MicroRNA processing.

2.4 MicroRNAs and exosomes

The term "exosome" has become somewhat ambiguous over time. It was originally applied to the extrachromosomal DNA elements mediating non-Mendelian inheritance of certain traits in the fruit fly *Drosophila melanogaster* (Fox et al., 1970). Later the term "exosome complex" came to designate the supermolecular aggregates responsible for RNA degradation in eukaryotic cells (Mitchell, 1997). Finally, since the 1980's, the term "exosome vesicles" or "exosomes" came to be consistently applied to the membrane vesicles that are exported from the cytoplasmic membrane of mammalian cells to the surrounding milieu (Trams et al., 1981). Any further mention of exosomes in this paper refers to this third meaning of the term. Exosomes may function as tools of intercellular communication (Simons et al., 2009). They may influence such an important processes as immunity responses (Lee et al., 2011). Moreover, since exosomes are exported to the bloodstream, they may transfer information to cells that are distant from the site at which the exosomes

themselves are produced in the body. Thus something like "long distance calls" may occur by means of exosome extravasation by one kind of cell at one body site and exosome intravasation to other cells at a second site, and vice versa. Exosomes may transport not only proteins, but also mRNAs, DNAs and microRNAs. It has been demonstrated that the information carried can be exploited by cells that intravasate exosomes. Intravasated mRNA can be translated to a functional product, while transferred microRNA may inhibit translation of target proteins in cells to which exosome microRNAs have been admitted(Keller et al., 2011). Apart from the establishment of exosomes as a new platform for intercellular communication, exosomes can serve as a diagnostic tool, since cancer cells extravasate a different spectrum of microRNAs compared to normal cells (Keller et al., 2011). Moreover, microRNAs in exosomes secreted to the blood are relatively stable (Wittman et al., 2011). It is well proven that plasma microRNA profiles from cancer patients have different spectra of microRNAs compared to microRNA profiles from healthy people (Kosaka et al., 2011). However, multi-centre studies are required to investigate the clinical diagnostic validity of results obtained to date, since the majority of the studies have involved relatively small numbers of clinical samples, usually from fewer than a hundred patients.

2.5 MicroRNA expression profiles in cancer

It is widely accepted that microRNA expression profiles are different in all types of cancer when compared with non-tumourous tissue counterparts studied to date, including for example sarcoma, glioma, carcinoma and haematological malignancies (Volinia, 2006) MicroRNAs actively involved in carcinogenesis operate by inhibiting tumour suppressor genes or by activation of cellular proto-oncogenes. Both the suppressing role and the activating role are most frequently mediated by the inhibitory role of microRNAs in translation of target mRNAs containing complementary sequences. Thus the first mode of miRNA action (suppression) is mediated directly, while the second mode, i.e. proto-oncogene activation, must take place indirectly through negative feedback, inhibiting translation of certain proto-oncogene suppressors. However, some microRNAs may well activate target genes by an as-yet-undisclosed mechanism (Iwasaki and Tomari, 2009).

2.6 Changes of microRNA expression in rectal cancer

Several microRNAs exhibit specific differences of expression levels in rectal and colon cancer when compared with healthy or non-tumourous tissue. Colorectal cancers show decreased levels of miR-143, miR-145 and Let-7a-1 microRNAs (Michael, 2003; Akao, 2006). These microRNAs are known to function as tumour suppressors since they inhibit expression of the known cellular proto-oncogenes c-myc and K-ras (Akao, 2006). Levels of these microRNAs are also lowered in other cancers, including haematological malignancies (Akao, 2007). A further microRNA, miR-21, acts as an oncogene, since it inhibits apoptotic processes and induces cancer cell proliferation (Si, 2007). This microRNA is significantly overexpressed in higher states of colon and rectal cancers and higher miR-21 levels are associated with worse prognosis (Schetter, 2008). Recently miR-95 was found to be overexpressed in approximately 50% of CRC tumours (Huang, 2011). This microRNA promotes proliferation by direct repression of sorting nexin 1 (Huang, 2011). Nowadays, several dozen different microRNAs are known to exhibit changed expression levels in association with CRC (Volinia, 2006; Bandres, 2006).

2.7 Potential role of microRNAs in modulating anticancer drug and radiation response

Drug resistance and the comparative impact of radiation has been fairly frequently studied in cancer cells in vitro (Bandres, 2007; DiGennaro, 2009). However, our knowledge of the molecular events that take place in response to anticancer drugs and radiation in human tumours in vivo is very limited, and this is even more true of microRNA expression changes induced by these events. It has been demonstrated that several microRNA levels are significantly changed in response to 5-fluorouracil in CRC cell lines in vitro (Rossi, 2007). MicroRNAs miR-27a a miR-451 have been found to stimulate expression of multidrug resistance protein MDR1, thus increasing resistance to several anticancer drugs in vitro (Zhu, 2008). Two further important microRNAs, miR-181b and Let-7g, have been found to be involved in responses to the S-1 anticancer drug in colon cancer cells (Nakajima, 2006). Several studies have also been dedicated to the role which 5-fluorouracil therapy may play in the induction of microRNA level changes in clinical samples of cancers. It has been disclosed that 5-fluorouracil therapy induces changes in several microRNAs in gastric cancer (Takagi, 2009) and breast cancer (Salter, 2008). One of our previously-published papers addressed the induction of miR-125b and miR-137 in rectal cancer in response to pre-operative chemoradiotherapy (Svoboda et al., 2008). A German research group has recently noted that miRNAs are returned to normal levels after successful pre-operative chemoradiotherapy and subsequent surgery of locally advanced rectal cancer (Drebber et al., 2011).

2.8 MicroRNAs as prognostic and predictive markers

This subject has recently been reviewed by (Dong et al., 2011). The expression levels of several microRNAs are associated with the TNM state of rectal cancer and might be used for prognosis. MicroRNA miR-21 is upregulated in rectal cancer and higher levels are associated with node positivity, metastasis, and poor survival (Kulda 2010, Schetter 2008, Slaby 2007). High miR-21 stromal expression levels are associated with short disease-free intervals in stage II colorectal cancer patients (Nielsen et al., 2011). MicroRNAs miR-143 and miR-145 are downregulated in rectal cancer. Lower levels are related to large tumour sizes and to disease-free intervals (Slaby et al., 2007; Motoyama et al., 2009; Wang et al., 2009). MiR-31 and miR-106a are upregulated in CRC and reflect tumour states (Bandres et al., 2006; Schetter 2008). Several microRNAs have also been found to be associated with tumour response to therapy or response of cell lines to anticancer drugs. Patients who responded to fluoropyrimidine S-1 showed lower levels of miR-181b and Let-7g. However neither microRNA was associated with survival (Nakajima 2006). MiR-215 increased resistance of cancer cell lines to methotrexate and tomudex (Song 2010). We have previously noted that microRNAs miR-125b and miR-137 are upregulated in response to pre-operative chemoradiotherapy, and higher levels of expression have been associated with worse response to therapy (Svoboda 2008). Various modalities of X-irradiation may give rise to different microRNA expression in vitro (Ahmed 2009). Ragusa suggested that microRNAs let-7b, let 7e and miR-17-3p might be potential predictors of cetuximab resistance (Ragusa et al 2010). MicroRNAs are embedded in exosomes in blood plasma. Since molecules embedded in exosomes are relatively stable for a period of time (up to several days), microRNA expression may simply be monitored from patients' blood (Ng 2009).

3. Aims of the study

The aim of this study was to test the possible involvement of the miR-21, miR-125b, miR-137 and miR-145 in tumour responses to standard pre-operative capecitabine chemoradiotherapy. A further aim was to evaluate the possibility of using mentioned microRNAs as predictors of antiacancer drug response or as prognostic markers.

4. Patients and methods

4.1 Patients

Patients aged 33-76 years, median age being 59, 31 man and 12 woman, ECOG performance status of 0-2, who had histologically confirmed locally advanced rectal adenocarcinoma (LARC) without distant metastases, stages II-III (cT3 - cT4, cN0, cM0 or T2 –T4, cN+,cM0) according to lUCC (Wittekind, 2002) were included in the study. The Ethics Committee of the Masaryk Memorial Cancer Institute approved the treatment protocol. All patients gave written informed consent.

4.2 Methods

Preoperative capecitabine was administered orally, at a dose of 825 mg/m^2 twice a day, two hours prior to radiotherapy for approximately 5.5 weeks from the first to the last day of radiotherapy. Radiation therapy was given in conventional fractionation in locally curative dosage. The daily fraction dose was 1.8 Gy, applied in five days per week up to cumulative dose of 45 Gy, boosting up to 50,4 Gy, during the period of 5.5 weeks. The standard total rectal resection or amputation (Faerden, Naimy et al. 2005), leaving tumor-free resection margins including total mesorectal excision (TME) was performed within the 6th week after completion of radiotherapy. Clinical cTNM stage (preceding a therapy) was based on the endorectal ultrasonography, CT and colonoscopy. Pathological examination after surgery involved the former tumor-bearing area and its macroscopic and microscopic description. The tumor response to therapy was investigated microscopically. Our department of pathology has routinely been using tumor regression (TRG 1-5) criteria adapted to colon cancer (Bouzourene et al., 2002). Tumor biopsies (1-3 mm^3) were taken before starting therapy and again after two-week therapy. Tumor samples were immersed immediately in RNA Later solution (Quiagen GmbH, Germany). The RNAs from bioptic samples were isolated by the standard Trizol method (Chomczynski 1993). RNAs were quantified using Eppendorf spectrophotometer (Eppendorf, Germany). Quality of RNA was tested by standard denaturing electrophoresis. The microRNA levels in pre-treatment and treatment samples were determined by means of stem-loop RT-Real Time PCR and TaqMan detection (Chen, Ridzon et al. 2005). Reverse transcription of cDNA was performed using gene-specific primers, TaqMan MicroRNA Reverse Transcription Kit and 10 ng RNA according to TaqMan MicroRNA Assay Protocol. Stem-loop RT primer (50nM), 1x RT buffer, 10mM dNTP each, RNase inhibitor 0.19ul, MultiScribe reverse transcriptase 1ul, water and RNA were mixed in 15ul final reaction volume and incubated for 30 min at 16^{o}C, 30 min at 42^{o}C, 5 min at 85^{o}C, cooled and kept at 4^{o}C. Real Time PCR mix contained 10ul TaqMan Universal Master Mix No Amp Erase UNG, 1ul 20x Assay Mix from TaqMan MicroRNA Assay Kit (both from Applied Biosystems, Foster City, USA), RT product 1.33ul and water in final volume of 20ul.Real Time PCR was performed on Applied Biosystems 7000 instrument in a

96- well optical plate under following conditions: 95oC 10min initial denaturation, 40 cycles of 95oC for 15s and 60oC for 40s. RNU6B RNA was used as an reference endogenous control. The threshold cycle CT was determined using default instrument settings. Adjacent non-tumorous mucosa before treatment was used as a calibrator.

4.3 Data analysis

We used comparative C_T method approach ($2^{-\text{delta delta Ct}}$) for the calculation of relative miRNA expression (Applied Biosystems User Bulletin #2, P/N 4303859). Expression of miRNA was related to RNU6B RNA as an endogenous active reference. The data before starting therapy were designated as a control group versus a sample group representing data two weeks after starting therapy. Standard statistical analyses were calculated using MedCalc and Statistica version 7 software.

5. Results

5.1 Clinical data

Table 1 summarizes data of patients under study. Forty- three patients were recruited. Nine patients exhibited recurrent disease within follow-up period. Eight of them died. One patient died from comorbidity. All recurrent diseases occured within the three-years period after surgery.

Patients	Attribute	%	Value range
man/woman	31/12	74/26	
Age (median and range)			59 (33-76)
Patients undergoing surgery	42	98	
Number of recidives	9	21	
Median follow-up (months) Local recidives	 2	 5	49
Median disease-free period to local recidive			23 (10-36)
Distant metastases	7	17	
Median disease-free period to distant metastase recurrence			19 (10-58)
Secondary malignities	0	0	
Number of deaths	9	21	
Deaths owing to cancer recurrence	8	19	
a) local	2	5	
b) distant	6	14	
Comorbidities	1	2	
Postoperative complications	0	0	

Table 1. Basic clinical data of recruited patients.

5.2 Non-parametric distribution of statistical data

Statistical analysis of microRNA expression levels determined by standard comparative C_T method shows non-parametric distribution of data (Shapiro-Wilk and Lilliefors tests). We therefore used non-parametric testing for all data (Wilcoxon paired test and Mann-Whitney-U-test).

5.3 MicroRNA expression levels

Our results show that median levels of miR-21, miR-125b and miR-145 were upregulated two weeks after starting therapy. Expression level of miR-137 is not included since we already published its upregulation (Svoboda et al., 2008).

miR21	No.samples	Median	95% confidence interval		p Mann-Whitney U-test (two- sided)	p Wilcoxon (paired, two-sided)
before	42	9,318	0,057	26,173	0,1129	0,0464
two weeks	35	16,450	5,637	29,651		(N=35)

a) miR-21 induction

Mann-Whitney U-test (two- sided)	No.samples	Median	95% confidence í interval		p
miR125b before	42	0,463	0,045	5,618	0,03054
miR125b , two weeks	35	1,173	0,129	8,282	

b) miR-125b induction

Mann-Whitney U-test (two- sided)	No.samples	Median	95% confidence interval		p
miR145 before	42	0,145	0,078	1,279	0,000001
miR145 , two weeks	35	1,661	0,483	9,383	

c) miR-145 induction

Table 2. a,b,c. Induction of microRNA expression by the preoperative chemoradiotherapy.

MicroRNAs exhibited extensive intertumoral level variability both before treatment and in samples taken two weeks after starting therapy (see 95% confidence intervals in Tables 3.,4.). The observation of frequent upregulation after starting therapy may support our initial hypothesis that miRNA levels tend to change to normal levels after efficient tumor destruction as both miR125b and miR137 are known to be down-regulated either in CRC lines or colorectal and breast carcinomas (Iorio, Ferracin et al. 2005). Nevertheless, miR-21 is upregulated in most colorectal cancers and functions as an oncogene (Nielsen et al., 2011).

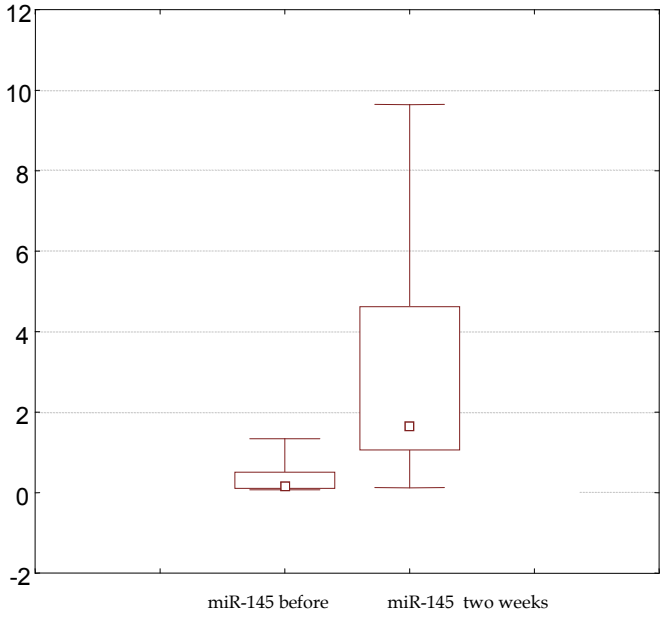


Table 3. Variability of microRNA miR-145 expression levels before and two weeks after starting preoperative chemoradiotherapy

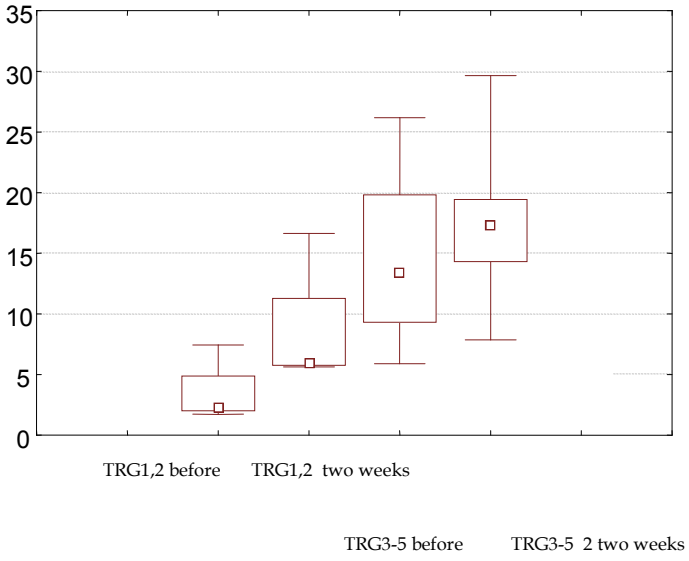


Table 4. a) Box-plot graph of the relative expression of miR-21. Role of tumour regression grade TRG.

Therefore, we investigated whether the changes of miRNA levels are reflected within immediate tumor responses and downstaging. As table 2 shows., miR-21 is upregulated in rectal cancer two weeks after starting preoperative chemoradiotherapy. Moreover, there are statistically significant differences between responsive (TRG1,2) and non-responsive group (TRG 3-5) before starting therapy and different ypT stages respectively (tables 4. and 5.).

Mann-Whitney U-test (two-sided)	Median relatve expression	95% confidence interval		p
miR21 TRG1,2 before	2,312	1,731	7,438	0,014
miR21 TRG3-5 before	13,404	5,897	26,173	
miR21 TRG1,2 two wks.	5,849	5,637	16,641	0,077
miR21 TRG3-5 two wks.	17,749	7,863	29,651	

Table 4. b) Median levels of relative miR-21 expression. Role of tumour regression grade TRG.

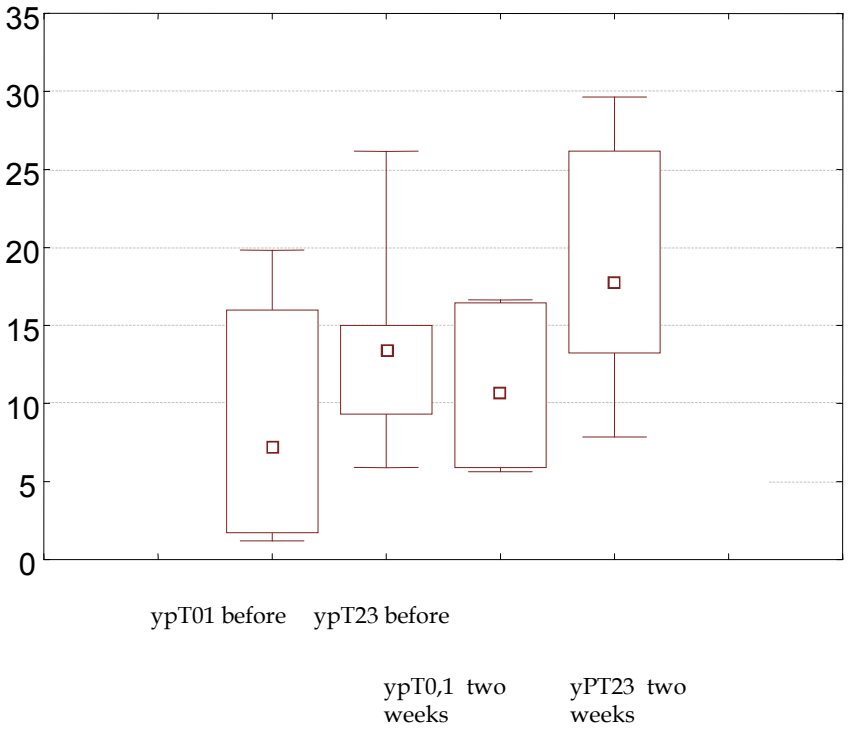
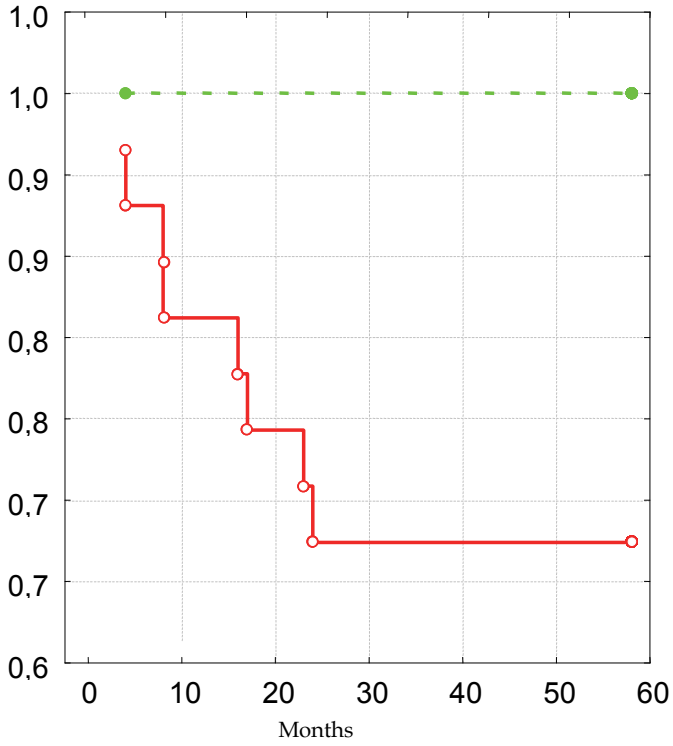


Table 5. a) The Box-plot graph of relative miR-21 expression. Role of ypT.

Mann-Whitney U-test (two-sided)	Patients ypT0,1	Patients ypT2,3	p
ypT0,1 vs. ypT2,3 before	16	26	0,1088
ypT0,1 vs. yPT2,3 two weeks	10	25	0,0185

Table 5. b) Median levels of relative miR-21 expression. Role of ypT.



Log-Rank Test p = ,15754

Table 6. Kaplan-Meyer graph of disease-free survival. Red line: patients with high-level miR-21 tumours suffer from recurrent disease. Green line: patients with low-level miR-21 tumours. Median level of relative miR-21 expression is the cut-off value discriminating between high-level miR-21 tumours and low-level miR-21 tumours respectively.

Although 125b is upregulated in all ypT groups, the highest and the only statistically significant change is observed in the group ypT3 patients (no downstaging). It is well known that T3/4 stage or node involvement is usually associated with worse prognosis than T0-T2, N0. Therefore, higher induction of miR125 is associated with a worse prognosis.

miR-125b	Patients	Median expression	95% confidence interval		p (Wilcoxon paired)
TRG12 before	25	0,393	0,092	2,761	0,005
TRG12 two weeks	21	0,905	0,329	9,646	(N=21)
TRG3-5 before	17	0,694	0,188	1,414	0,059
TRG 3-5 two weeks	14	1,131	0,189	3,204	(N=14)

Table 7. a) Dependence of induced miR-125b levels on the tumour regression grade. Wilcoxon paired test

Mann-Whitney U- test	TRG1-2	TRG3-5	p
TRG12 vs TRG3-5 before	25	17	0,109
TRG12 vs TRG3-5 two weeks	21	14	0,391

Table 7. b) Dependence of induced miR-125b levels on the tumour regression grade. Comparison of different TRG groups.

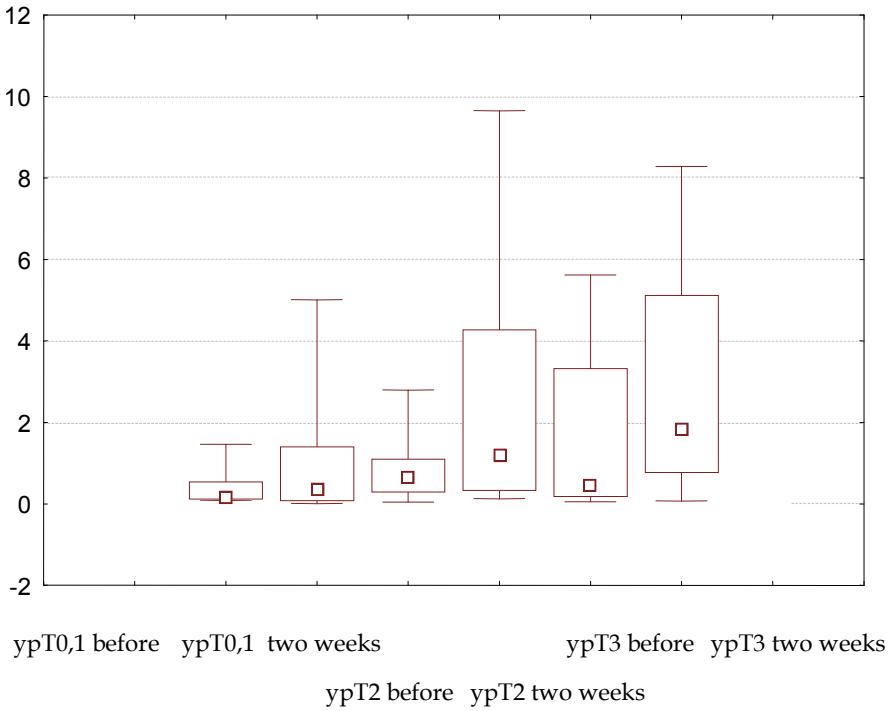


Table 8. a) Dependence of induced miR-125b levels on the tumour state ypT. Box-plot graph.

There are profound differences of miR125b levels between ypT0,1, and ypT3 patient groups after starting therapy (Table 8). Patients with low stage tumours have lower miRNA induction than patients with more advanced cancers. These results tell us that we should use carefully term oncogene or tumour suppresor in connection with certain miRNAs. MicroRNA miR125b is downregulated in several cancers and may be therefore considered a tumor suppressor from this point of view. However, in this study we show no downstaging and less regression (bad response) in the tumors with the highest upregulation of miR125b level two weeks after starting therapy. Non-responding tumors exhibited induction of miR125b level close to and above normal levels of adjacent non-tumorous mucosa.

miR-125b	Patients	Median	95% confidence interval		p Wilcoxon (paired, two-sided)
ypT0,1 before	16	0,158	0,092	1,464	0,4446
ypT0,1 two weeks	10	0,362	0,011	5,011	
ypT2 before	14	0,662	0,045	2,796	0,0843
ypT2 two weeks	13	1,186	0,129	9,646	
ypT3 before	12	0,463	0,054	5,618	0,0164
ypT3 two weeks	12	1,828	0,073	8,282	

Table 8. b) Dependence of induced miR-125b levels on the tumour state ypT. Wilcoxon paired test.

Mann-Whitney U-test (two-sided)	Patients ypT0,1	Patients ypT2 ypT3	p
ypT0,1 vs pT2 before	16	14	0,1223
ypT0,1 vs pT2 two weeks	10	13	0,1375
ypT0,1 vs pT3 before	16	12	0,1971
ypT0,1 vs pT3 two weeks	10	12	0,0295

Table 8. c) Dependence of induced miR-125b levels on the tumour state ypT. Comparison of different ypT states by Mann-Whitney two-sided test.

Our results show that miR137 is significantly upregulated in both responder groups (Table 9.a). However there is no association of miR137 induction with tumour response (Table 9.b). Interestingly, on the contrary to the above-mentioned miR125b although upregulated, miR137 in tumors never reached the original median value of normal tissue. We therefore speculate that low miR137 levels may be important to maintain tumour state.

miR-137	Patients	Median	Confidence interval		p (Wilcoxon paired)
TRG1,2 beforer	25	0,037	0,003	0,688	0,027
TRG1,2 two weeks	21	0,162	0,012	0,766	(N=21)
TRG3-5 before	17	0,035	0,006	0,646	0,006
TRG3-5 two weeks	14	0,301	0,005	0,655	(N=14)

Table 9. a) Dependence of induced miR-137 levels on the tumour regression grade. Wicoxon paired test.

Mann-Whitney U-test (two-sided)	Patients TRG1,2	Patients TRG3-5	p
TRG12 vs. 3-5 before	25	17	0,538
TRG12 vs. 3-5 two weeks	21	14	0,373

Table 9. b) Comparison of miR-137 levels between responders and non-responders. Mann-Whitney two-sided test.

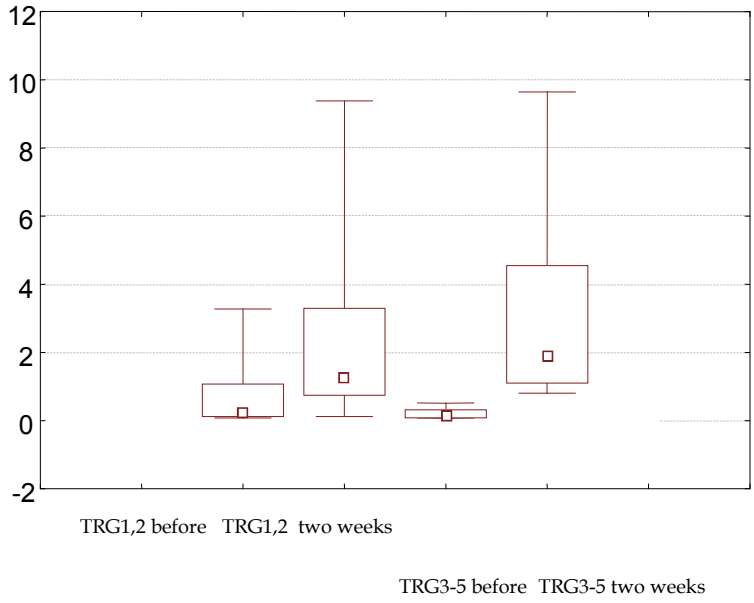


Table 10. a) Dependence of induced miR-145 levels on the tumour state ypT.

MicroRNA miR-145 is significantly upregulated both in responders and non-responders respectively (Tables 10 a,b). Moreover, miR-145 has significantly higher expression in tumors from responders before therapy (Table 10 c). Similar effect we can observe in the ypT state groups: patients with ypT0,1 tumours (better prognosis) have higher miR-145 levels. This is in accordance with known tumour-supressive role of miR-145.

miR-145	Valid N	Median expresion	95% confidence interval		p (Wilcoxon paired t.)
TRG1,2 before	25	0,226	0,078	3,279	0,0021
TRG1,2 two weeks	21	1,248	0,126	9,383	(N=21)
TRG3-5 before	17	0,115	0,075	0,518	0,0003
TRG3-5 two weeks	14	1,886	0,812	9,646	(N=14)

Table 10. b) Dependence of induced miR-145 levels on the tumour regression grade.

Mann-Whitney U-test	Patients TRG 1,2	Patients TRG 3-5	p (two sided)
TRG 1,2 vs.3-5 before	25	17	0,013
TRG1,2 vs. 3-5 two weeks	25	17	0,274

Table 10. c) Dependence of induced miR-145 levels on the tumour regression grade. Comparison of diffferent TRG groups.

miR-145	Valid N	Median	95% confidence interval		p Wilcoxon (paired, two sided test)
ypT0,1 before	16	0,226	0,111	1,338	0,0004
ypT0,1 two weeks	10	1,586	0,483	9,383	(N=10)
ypT2 before	14	0,132	0,078	3,279	0,1361
ypT2 two weeks	13	1,227	0,126	9,646	(N=13)
ypT3 before	12	0,133	0,075	0,518	0,0010
ypT3 two weeks	12	1,621	0,812	4,691	(N=12)

Table 10. d) Dependence of induced miR-145 levels on the tumour state ypT.

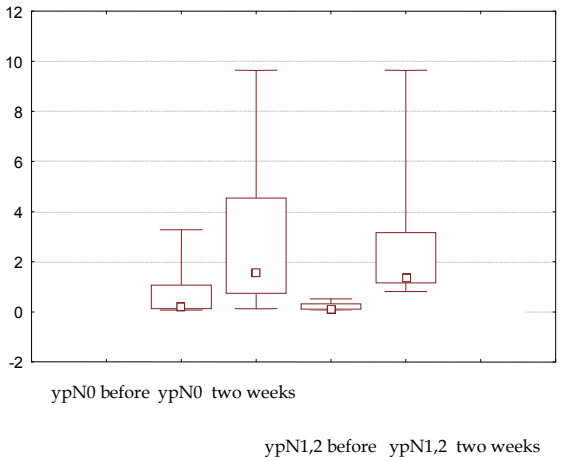


Table 11. a) Dependence of pre-therapeutic and induced miR-145 levels on the node state pN. Box-plot graph.

miR-145	Patients	Median	95% confidence interval		p Wilcoxon (paired, two sided t.)
ypN0 before	25	0,226	0,075	3,279	0,0010
ypN0 two weeks	23	1,586	0,126	9,646	(N=23)
ypN1,2 before	17	0,115	0,078	0,518	0,0003
ypN1,2 two weeks	12	1,357	0,812	9,646	(N=12)

Table 11. b) Dependence of pre-therapeutic and induced miR-145 levels on the node state pN .

Mann-Whitney U-test (two-sided)	Patients pN0	Patients pN1,2	p
ypN0 vs. ypN1,2 before	25	17	0,0283
ypN0 vs. ypN1,2 two weeks	23	12	0,8196

Table 11. c) Dependence of pre-therapeutic and induced miR-145 levels on the node state pN. Comparison of ypN0 and ypN1,2 groups.

We have also investigated the role of node involvement. Table 11 shows that miR-145 levels of ypN0 patients differ significantly from ypN1,2 before starting therapy. The upregulation of miR-125b was published previously (Svoboda, 2008).

6. Discussion

MicroRNAs play an important part in the regulation of many important cellular processes and target approximately a third of expressed genes. It is therefore reasonable to assume

that microRNAs would be influenced by such a massive cell-destructive process as pre-operative chemoradiotherapy. This pre-operative treatment degrades the proliferative potential of many cancer cells and leads to extensive tumour regression in many patients (Bouzourene et al., 2002). Among the microRNAs we have been investigating, miR-21 exhibits the highest difference in expression levels considered in terms of response to therapy. While tumours that respond well contain low miR-21 levels, non-responders have high miR-21 levels (median level as cut-off value discriminating between low and high levels). It is crucial to note that these differences are already pronounced in samples taken from tumours before starting therapy ($p=0.014$). Therefore, miR-21 is not only a known prognostic factor, but it may also be used also as a predictor of tumour response to pre-operative chemoradiotherapy. This is not a surprising fact, since miR-21 is a known anti-apoptotic and pro-proliferative factor and is currently recognized as an oncogene (Zhang et al., 2008). We also show in our preliminary data that high miR-21 levels might be associated with short disease-free survival and recurrent disease, as may be seen on the Kaplan-Meyer graph (Table 6). Patients with high-level miR-21 tumours suffer from recurrent disease while patients with low-level miR-21 tumours are all disease-free within the five-year follow-up period. However since only a small number of patients has been monitored to date, statistical significance according to log-rank test remained only $p=0.15$ and more patients must be recruited in order to obtain statistically valid data. Epithelial-to-mesenchymal transition (EMT) is a primary event leading to the prometastatic behaviour of cancer cells (Gregory et al., 2008). TGF-beta-induced EMT leads to upregulation of miR-21 in a model system of human keratinocytes in vitro (Zavadil et al., 2007). Induction of miR-21 leads to pro-invasive behaviour in breast cell lines in vitro and metastasizing of tumours related to those lines in animals in vivo (Zhu et al., 2008). This effect is mediated by miR-21 inhibition of tropomyosin 1 activity (Zhu et al., 2008). Tropomyosin 1 is a tumour suppressor. MiR-21 also inhibits PDCD4 and maspin, further important regulators: (Zhu et al., 2008). Our data are in accordance with these in vitro findings, since high levels of miR-21 are associated with recurrent and refractory disease in our study. MicroRNA miR-125b is an ortholog of Lin-4 microRNA of the worm *Caenorhabditis elegans* (Ambros, 2003). High levels of this microRNA prolong the lifespan of the worm, probably by influencing the insulin metabolic pathway (Boehm, 2006). High levels of miR-125b give rise to similar effects in rectal cancer: tumours with highly-induced miR-125b survive chemoradiotherapy intervention and are refractory, while low-MiR-125b-level tumours are partially or completely destroyed. On the basis of the analogy with the nematode worm and of our findings, we suggest that miR-125b supports mechanisms necessary for cell survival that are undoubtedly initiated as an adaptation to the chemical and radiation stress induced as a consequence of preoperative chemoradiotherapy. On the other hand, miR-125b is known to suppress proto-oncogenes ERBB2 and ERBB3 expression in vitro (Scott et al., 2007). ERBB2 and ERBB3 are known pro-metastatic and pro-proliferative factors. We speculate that this opposite effect of miR-125b may co-exist in parallel with the previously-mentioned effect and may provide a base for explanation of the fact that, while only 30-40% of tumours are extensively shrinked, the frequency of metastasis and recurrent disease is lower in patients who have undergone preoperative chemoradiotherapy versus patients who did not in the past when this treatment modality was not yet established (Lee, 2008). The level of miR-137 is frequently suppressed in glioblastoma and CRC (Silber et al., 2008). This is caused by aberrant methylation of CpG islands near coding genes (Kozaki, 2008). Here, the observed induction of miR-137 is in accordance with the fact that this microRNA suppresses G_0 to G_1

transition (Silber et al., 2008). Suppression of cell growth is a general process accompanying chemoradiation treatment. Tables 9 a,b show that miR-137 levels are upregulated in all tumours despite TRG. We may therefore expect that miR-137 induction is a part of the general process of adaptation to chemical and radiation stress. However therapy-induced miR-137 levels never achieve their original levels, i.e. those present in non-cancerous tissue counterpart. We may therefore assume that miR-137 might be a tumour suppressor and that lower miR-137 expression helps to maintain the transformed phenotype. This is also supported by the finding that miR-137 directly targets carboxy-terminal binding protein I (CtBPI) to inhibit epithelial-to-mesencyhmal transition and induce apoptosis in melanoma cells (Deng et al., 2011). We therefore speculate that, although miR-137 does not contribute to an immediate effect of tumour regression, it may lower later cancer recurrence by inhibiting epithelial-to-mesenchymal transition processes. The relevance of this speculative construction is also supported by the fact that transfection of pre-miR-137 (a microRNA precursor molecule) stops proliferation and induces differentiation in glioblastoma cells in vitro (Silber et al., 2008). MicroRNA miR-145 is downregulated in many cancers, including carcinomas of the bladder, lung and stomach (Takagi et al., 2009; Cho et al., 2009; Ichimi, 2009). Levels of this microRNA are also downregulated in CRC (Wang&Zhou, 2009). It is recognised as a tumour suppressor, supported by the fact that transfected miR-145 precursors inhibit the growth of lung cancer cells in vitro and also inhibit the growth of MCF-7 breast cancer-derived cells (Cho et al., 2009). Moreover, miR-145 upregulation induces apoptosis in MCF-7 cells (Wang& Bian et al., 2009). According to our results, miR-145 is upregulated after starting pre-operative chemoradiotherapy. In the light of the tumour suppressor role, we assume that upregulated miR-145 participates in vivo (in vitro model analogy) in the inhibition of cancer cell growth. Our results show that microRNA miR-145 levels before starting therapy well reflect therapy outcome. Therefore, miR-145 may be used as a predictor of response to pre-operative chemoradiotherapy. This accords with the recent finding of German authors (Drebber et al., 2011).

7. Conclusion

MicroRNAs miR-21, miR-125b, miR-137 and miR-145 all display up-regulation of expression induced by preoperative chemoradiotherapy of locally advanced rectal cancer. Among microRNAs we have been investigating, miR-21 and miR-145 exhibit the highest differences in expression levels considered in terms of response to therapy. MiR-21 as well as miR-145 may be used as potential predictive and prognostic markers.

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Notice: both authors participated equally.

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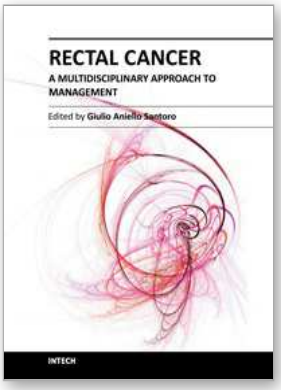

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Dramatic improvements in medicine over the last few years have resulted in more reliable and accessible diagnostics and treatment of rectal cancer. Given the complex physiopathology of this tumor, the approach should not be limited to a single specialty but should involve a number of specialties (surgery, gastroenterology, radiology, biology, oncology, radiotherapy, nuclear medicine, physiotherapy) in an integrated fashion. The subtitle of this book "A Multidisciplinary Approach to Management" encompasses this concept. We have endeavored, with the help of an international group of contributors, to provide an up-to-date and authoritative account of the management of rectal tumor.

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Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821