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The prognostic importance of miR-21 in stage II colon cancer: a population-based study

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BACKGROUND: Despite several years of research and attempts to develop prognostic models a considerable fraction of stage II colon cancer patients will experience relapse within few years from their operation. The aim of the present study was to investigate the prognostic importance of miRNA-21 (miR-21), quantified by *in situ* hybridisation, in a unique, large population-based cohort.

PATIENTS AND METHODS: The study included 764 patients diagnosed with stage II colon cancer in Denmark in the year 2003. One section from a representative paraffin-embedded tumour tissue specimen from each patient was processed for analysis of miR-21 and quantitatively assessed by image analysis.

RESULTS: The miR-21 signal was predominantly observed in fibroblast-like cells located in the stromal compartment of the tumours. We found that patients expressing high levels of miR-21 had significantly inferior recurrence-free cancer-specific survival (RF-CSS): HR = 1.26; 95% CI: 1.15–1.60; $P < 0.001$. In Cox regression analysis, a high level of miR-21 retained its prognostic importance and was found to be significantly related to poor RF-CSS: HR = 1.41; 95% CI: 1.19–1.67; $P < 0.001$.

CONCLUSION: The present study showed that increasing miR-21 expression levels were significantly correlated to decreasing RF-CSS. Further investigations of the clinical importance of miR-21 in the selection of high-risk stage II colon cancer patients are merited.

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Colon cancer represents a major challenge all over the Western World (Parkin *et al*, 2005). The incidence has been increasing for decades but screening holds the promise of a shift in distribution towards earlier stages (Gross *et al*, 2006). Surgery is a cornerstone in the treatment, but with operation alone a considerable fraction of patients will experience tumour recurrence, calling for adjuvant chemotherapy. Stage II (no lymph node metastases or distant metastases) patients represent about 25% of all cases and they have a 5-year overall survival (OS) of 73–85%, when surgically resected (O'Connell, 2004).

There is a general agreement on postoperative chemotherapy for stage III colon cancer patients, but there is still a debate on this approach for patients with stage II tumours. Meta-analyses, reviews and international guidelines agree that adjuvant chemotherapy should be offered to a subgroup of patients with high risk of recurrence, including T4 and T3 tumours with lymphovascular invasion, high malignancy grade, perforation and/or inadequate lymph node sampling (Benson III *et al*, 2004; Figueredo *et al*, 2008; Kopetz *et al*, 2008; Bastos *et al*, 2010; Gangadhar and Schilsky, 2010; Lombardi *et al*, 2010). Interestingly, the recent CALBG 9581 study of 24 000 stage II patients from the Medicare Registry found no improvement in 5-year survival in patients receiving adjuvant chemotherapy, irrespective of a division of

patients into two groups with high- or low-risk characteristics (O'Connor *et al*, 2011). These results question the current clinical practice and underline the urgent need of new and reliable prognostic and predictive markers in stage II colon cancer.

MicroRNAs (miRNAs) comprise an abundant class of endogenous, small non-coding RNAs, 18–25 nucleotides in length, which regulates protein synthesis at the post-transcriptional level. MicroRNAs are highly conserved in sequence between distantly related organisms, indicating their participation in essential biological processes (Carrington and Ambros, 2003). Abnormal expression of cancer-related miRNAs is characterised by increased or decreased expression levels compared with their levels in the corresponding normal tissue. The miRNA-21 (miR-21) is consistently upregulated in many types of cancers including cancer of the colon (Slaby *et al*, 2007; Schetter *et al*, 2008), breast (Yan *et al*, 2008; Qian *et al*, 2009), lung (Markou *et al*, 2008), stomach (Chan *et al*, 2008) and glioblastoma (Gabriely *et al*, 2008). Among the reported mRNA targets of miR-21 are PTEN, PDCD4, Spry-1 and NF1B, suggesting that miR-21 is involved in regulation of apoptosis, proliferation and migration (Meng *et al*, 2007; Asangani *et al*, 2008; Gabriely *et al*, 2008; Yao *et al*, 2011). Additionally, miR-21 levels seem to increase with increasing stage of the tumour in, for example, colon and breast cancer, suggesting an important role of miR-21 in cancer invasion and dissemination (Slaby *et al*, 2007; Yan *et al*, 2008; Qian *et al*, 2009). These data taken together strongly suggest miR-21 as a potential prognostic marker in early-stage cancer such as stage II colon cancer. Studies have found high levels of miR-21 in colorectal cancer patients to be

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an indicator of poor prognosis, but so far none have been population based (Slaby *et al*, 2007; Schetter *et al*, 2008; Nielsen *et al*, 2011).

Most studies quantifying miRNAs for identification of differential expression have been based on microarrays (Lim *et al*, 2005), real-time PCR (RT-PCR) (Bandres *et al*, 2006) and deep sequencing (Ferdin *et al*, 2010). These quantitative miRNA expression analysis platforms are strong quantitative tools with a high level of precision and reproducibility and have been used to identify valuable biomarker candidates. However, important clinical information may be lost when focal miRNA expression is masked by non-cancerous tissue present in the samples, such as normal mucosa, submucosa and muscularis externa, mucous and necrotic tissue (Bandres *et al*, 2006). Additionally, RNA extraction from formalin-fixed paraffin-embedded (FFPE) tissue blocks is both time-consuming and suboptimal. Current pathological practice is based on FFPE tissue blocks, and the vast majority of archival material is preserved as such. Therefore, new methods should be considered. Recently, it has been reported that *in situ* hybridisation (ISH) using locked nucleic acid (LNA) modified DNA probes, raise the potential of evaluating tissue morphology and to quantitatively assess the miR-21 signal (Nielsen *et al*, 2011; Rask *et al*, 2011). The study by Nielsen *et al* also addressed the prognostic information of miR-21 expression and found that high miR-21 level was associated with poor disease-free survival. However, the patient material was limited (Nielsen *et al*, 2011).

The aim of the present study was to further investigate the prognostic importance of miR-21, quantified by ISH, in a unique, large, homogeneous, population-based cohort consisting of all patients with stage II colon cancer diagnosed and treated exclusively by surgical resection in Denmark in the year 2003.

PATIENTS AND METHODS

Patients

The study population was retrieved by a search in the nationwide registry administered by the Danish Colorectal Cancer Group (DCCG). This database contains prospectively collected surgical and pathological data. The search identified all surgically treated patients diagnosed with stage II colon cancer in 2003 ($N = 764$). Patients dying within 30 days from their operation were excluded ($N = 66$). All but two hospitals participated in the study and hence patients from those hospitals were not included ($N = 56$), thus creating a highly representative cohort (93%) of the Danish population of stage II colon cancer patients in 2003. Furthermore, there were the following exclusions: tumour samples missing in archives ($N = 23$); patients being categorised wrongly as stage II colon cancer ($N = 14$); patients receiving adjuvant chemotherapy ($N = 16$); lack of sufficient tissue for analysis ($N = 7$); analysis exclusion criteria regarding number of images acquired <8 ($N = 41$) and TR values $<20\,000$ ($N = 21$), see below. Thus, 520 patients were included in the statistical analysis. The study was approved by the Regional Scientific Ethical Committee of Southern Denmark according to Danish law. Postoperatively, patients were followed by the treating departments according to local guidelines. The patients were not routinely referred to a Department of Oncology. Thus, the majority of data on recurrence and death were obtained through Danish registries linked to the Danish social security number given to every Danish citizen at birth and to all ethnic immigrants.

The national registry holding all pathology reports in Denmark was used to identify patients with pathologically verified recurrence. Patients experiencing other malignancies were also identified using this registry and subsequently censored from survival analysis on the date of their new cancer diagnosis. To identify patients who had been in contact with the Department

of Oncology or who had died, the national registry recording status on all Danish citizens as to their contact to the healthcare system and date of death was used. Patients with non-pathologically verified recurrence and patients receiving adjuvant chemotherapy were found by evaluating patient charts in the Departments of Oncology. A search in the 'Cause of Death Registry' identified patients registered with colon cancer as the cause of death. Follow-up was closed at 1 January 2010. From the study population of 520 patients, we identified the following: 206 patients had died and 73 patients had experienced disease recurrence. According to the registries, 90 patients had died from colon cancer, only 53 of these patients were also identified as having disease recurrence and in the analysis of recurrence-free cancer-specific survival (RF-CSS) events on the remaining 20 patients were added giving 110 events. The date registered was the first coming event of either disease recurrence or death from colon cancer. We also identified 16 patients who had received adjuvant chemotherapy and 23 patients who had been diagnosed with another cancer.

Pathology

One FFPE tissue block representing the invasive front of the tumour of each patient was collected from the Departments of Pathology in Denmark. Information on T-category, malignancy grade, nerve- and vascular-invasion, and the number of lymph nodes assessed was obtained from the pathology reports. Patients were scored as 'not assessed' if the pathological feature was not described. Data on localisation, tumour perforation, and tumour fixation was obtained from the DCCG registry and based on the surgeons' reports.

In situ hybridisation

In situ hybridisation was performed essentially as described previously (Nielsen *et al*, 2011). In brief, the single 6-μm section from each tumour was processed in a Tecan Evo (Männedorf, Switzerland) automated ISH instrument. Predigestion with proteinase-K (15 μg ml^{-1}) was performed at 37°C for 8 min. After prehybridisation for 15 min in hybridisation buffer (Exiqon, Vedbaek, Denmark), double-digoxigenin labelled 22'mer LNA probe specific for full-length miR-21 (Exiqon) was incubated at 30 n$_M$ for 1 h at 59°C. SSC stringent washes were performed at 59°C. Probe was detected with alkaline phosphatase-conjugated Fab fragments diluted 1:800 (Roche, Mannheim, Germany), followed by 1 h incubation with NBT-BCIP substrate (Roche). Sections were dehydrated and mounted using Eukitt (VWR, Herlev, Denmark). All reagents, including the proteinase-K, the DIG-labelled probe and the anti-DIG antibody were from the same batch and prepared identically each day. All steps in the Tecan were controlled regarding volumes, temperature and time.

Image analysis

Quantification of relative expression levels was obtained essentially as described earlier (Nielsen, 2012). In brief, the tumour area was identified on overview images and digitally selected as a region of interest. The areas containing normal mucosa and submucosa and ulcerating areas were not included. Within the selected area, 25 random images were collected per slide using a $\times 20$ objective. Exposure of sample images was strictly controlled and locked at 6.993 ms with RGB (red–green–blue) values at 170–180 in non-stained blank areas. Sample images with staining artifacts were discarded, as were also images with $<10\%$ cancer cells in the image field. Thus, all images contained both cancer cells and stromal cells. A Bayesian pixel classifier to discriminate blue (B, ISH signal), red (R, nuclear counter stain), purple (P, blue ISH signal overlaying nuclear stain) and tissue background was designed based on three images from three different tumours that

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represented variations in blue and red colour. The following parameters were obtained for each sample image; B, R, P, total blue ($TB = B + P$), total red ($TR = R + P$) and $TBR = TB/TR$.

Series of 50 slides were analysed and included two control slides in each series to obtain a precision estimate of the measurements. The series also included replicates of adjacent sections obtained from three tumours ($CV < 49\%$). The variation of TBR between individuals was 215%, indicating that the precision of the measurements was sufficiently high to justify statistical analyses (25).

A test run, using four sections from each of 12 tumours was performed to adjust the probe concentration and the hybridisation temperature for optimal signal intensity.

Statistical analysis

Overall survival was defined as time from surgery until death from any cause. Recurrence-free cancer-specific survival (RF-CSS) was defined as the time from surgery until documented tumour recurrence or death from colon cancer, death from other causes was censored and also patients diagnosed with other malignancies were censored from the date of their new cancer diagnosis. Survival was estimated according to the Kaplan–Meier method, and the log-rank test was used to test for differences among groups. The Cox regression model was used to adjust for known prognostic factors; Gender, age, T-category, malignancy grade, localisation, perforation, fixation and number of lymph nodes (dichotomized into above and under 12). The proportional hazard assumption was tested for all variables used in the Cox model, using Schoenfeld residuals. All statistical calculations were carried out using the STATA 11.0 (StataCorp LP, College Station, TX, USA), and all statistical tests were two-sided with a significance level of 0.05.

By visual inspection of the relevant qq-plots, the log-transformed miR-21 values (TB and TBR) followed normal distributions.

RESULTS

Patient characteristics

Patient characteristics are presented in Table 1. The majority of the patients were female patients with a medium differentiated T3 tumour. Location of the tumours was evenly distributed between left (left flexure, descendent, sigmoid) and right colon (coecum, ascendant, right flexure, transverse). Owing to the low number and uncertainty of the low malignancy grade classification, tumours of low and medium malignancy were pooled together in the survival analysis. No significant associations were found between miR-21 expression and patient characteristics.

Localisation of miR-21 ISH signal

The miR-21 ISH signal intensity and prevalence varied considerably within the tumours and between patients, and it was predominantly seen in fibroblastic stromal cells (Figure 1C). We found that miR-21 was predominantly expressed in fibroblast-like cells in the stromal compartment of the tumour. In a smaller fraction of the samples (10–20%) some miR-21-positive cancer cells were also seen (Figure 1F), and in these cases only sub-populations of cancer cells were miR-21 positive. The miR-21-positive cancer cells also included budding cancer cells. The intensity varied from patient to patient and locally within the tumour slide. The miR-21 expression pattern was characterised by intense expression in the tumour area in contrast to the normal mucosa, where weakly stained mononuclear cells were observed.

Table I Patient characteristics of the study population (all excluded patients have been subtracted)

Parameter	Number of patients
Study population	520
Gender	
Male	231 (44.4%)
Female	289 (55.6%)
Age (years)	
Median	71.9
Range	33–95
T-category	
3	454 (87.3%)
4	66 (12.7%)
Malignancy grade	
Low	34 (6.5%)
Medium	391 (75.2%)
High (including mucinous, signet)	95 (18.3%)
Localisation[a]	
Right	269 (51.7%)
Left	251 (48.3%)
Tumour perforation	45 (8.7%)
Tumour fixation	93 (17.9%)
Lymph nodes	
Median	11.3
Range	0–40
Nerve invasion	
Yes	34 (6.5%)
No	360 (69.2%)
Not assessed	126 (24.2%)
Vascular invasion	
Yes	30 (5.8%)
No	372 (71.5%)
Not assessed	118 (22.7%)

Information on T-category and malignancy grade was obtained from HE-stained sections. Nerve and vascular invasion was obtained from the pathology reports and was scored 'not assessed' when the histopathological feature was not described. [a]Left = left flexur, descendens, sigmoideum; right = coecum, ascendens, right flexur, transversum.

Quantification of miR-21 ISH signal

To quantify the ISH signal in the tissue sections, systematic random non-overlapping image fields were compiled from each tumour section. A mean of 16 image fields (range: 8–26) were compiled from each tumour. If the number of image fields was less than eight for one tumour section, the patient was excluded from the statistical analyses. All image fields that passed the inclusion criteria (see Patients and Methods) were processed with a pixel classifier that discriminated the blue ISH signal (B), the red nuclear stain (R), the blue signal located over red nuclear stain (P) and all other tissue structures (see Figures 1C and F). Total blue area ($TB = B + P$), total red area ($TR = R + P$) and total blue area per total red area ($TBR = TB/TR$) were obtained of which the TB and TBR values represent estimates of miR-21 expression, and TR the overall nuclear density. Sections cut too thin, samples fixed inadequately and tumours with poor cell density (prevalent in mucinous lesions) generally had low TR, and cases with TR $< 20\,000\,\mu m^2$ were excluded, see patients paragraph. As expected, substantial variation of both TB and TBR was observed; TB range $= 23.61$–$185\,274\,\mu m^2$; TBR range $= 0.0001814$–13.085, as
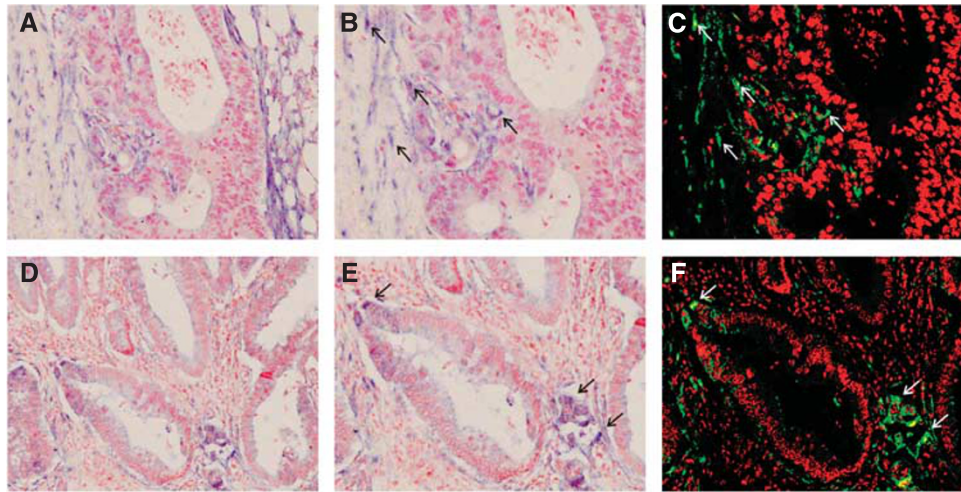
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Figure 1 Localisation and image analysis of miR-21 *in situ* hybridisation signal in colon cancer. Paraffin sections from two representative colon cancers (**A**–**C** and **D**–**F**) stained for miR-21 using LNA-based *in situ* hybridisation. The images, **A** and **D**, are selected random images obtained with a $\times 20$ objective. A selected area in **A** and **D** has been enlarged in **B** and **E**, and is presented after colour segmentation in **C** and **F**. The miR-21 *in situ* hybridisation signal (blue, examples indicated by arrows in **B** and **C**) is seen in tumour-associated stromal fibroblast-like cells (**B**, arrows) in virtually all colon tumours, whereas miR-21 signal in cancer cells (**E**, arrows) was less frequently seen. For quantitation of the *in situ* hybridisation signal, the blue and purple stain in **A**/**B** and **D**/**E** are translated into green and yellow pixels in **C** and **F**. The nuclear red counterstain is likewise translated into red and the tissue background into black in **C** and **F**. Bars are 50 μm.
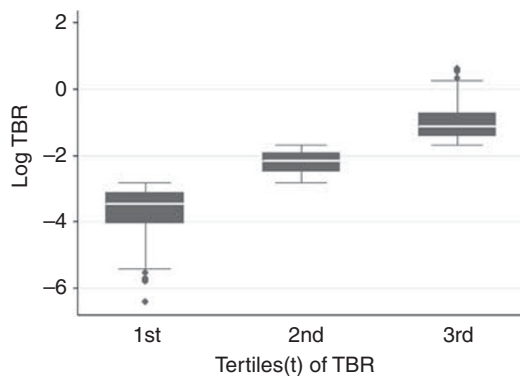

Figure 2 Box-plot of miR-21 expression levels in patients divided into tertiles of log(TBR). Substantial variation of both TB and TBR was observed; TB range = 23.61–185 274 μm^2; TBR range = 0.0001814–13.085.

illustrated in Figure 2 where patients were divided into tertiles according to their mean log(TBR) value.

The prognostic value of miR-21

Results from the log-rank analysis of mean log(TBR) are listed in Table 2. Patients expressing high levels of miR-21 (high mean log(TBR)) had significantly inferior RF-CSS (HR = 1.26; 95% CI: 1.15–1.60; $P < 0.001$), and the same applied to mean log(TB) (HR = 1.12; 95% CI: 1.0–1.42; $P = 0.05$). Proportional hazard assumptions were tested for all variables. Results from the multiple Cox regression are outlined in Table 3 and included age, gender, T-category, malignancy grade, localisation, tumour perforation, tumour fixation and number of lymph nodes. Mean log(TBR) was found to be an independent predictive marker of poor RF-CSS (HR = 1.41; 95% CI: 1.19–1.67; $P < 0.001$), and the same applied to high levels of mean log(TB) ($P = 0.018$). In addition to mean log(TBR), the following parameters were found to be predictive of a poor outcome regarding RF-CSS in the Cox regression analysis: male gender ($P = 0.033$), T4-category ($P < 0.001$), and tumour perforation ($P < 0.001$). In the Cox regression analysis of OS, the following parameters were found to be prognostic of a poorer outcome; increasing age ($P < 0.001$), male gender ($P < 0.001$) and

Table 2 Log-rank test on OS and RF-CSS related to log-transformed mean values of log(TBR)

	OS		RF-CSS	
	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
Mean log(TBR)	1.05 (0.94–1.18)	0.38	1.26 (1.15–1.60)	**<0.001**

Abbreviations: OS = overall survival; RF-CSS = recurrence-free cancer-specific survival. *P* value <0.001 is indicated in bold.

Table 3 Cox regression analysis on OS and RF-CSS related to log-transformed mean log(TBR), including gender, T-category, malignancy grade, localisation, tumour perforation, tumour fixation and number of lymph nodes

	OS		RF-CSS	
	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
Mean log(TBR)	1.08 (0.97–1.22)	0.17	1.41 (1.19–1.67)	**<0.001**
Age	1.06 (1.04–1.07)	**<0.001**	1.02 (1.00–1.04)	0.055
Male gender	1.81 (1.36–2.42)	**<0.001**	1.54 (1.04–2.29)	0.033
T4-category	1.59 (1.09–2.32)	0.017	2.51 (1.58–3.97)	**<0.001**
Left localisation	0.93 (0.70–1.25)	0.64	1.04 (0.69–1.55)	0.87
Perforation	1.57 (0.99–2.49)	0.056	2.69 (1.56–4.63)	**<0.001**
Fixation	1.41 (0.98–2.04)	0.064	1.41 (0.86–2.32)	0.17
Lymph nodes ⩾12	1.03 (0.78–1.37)	0.84	0.93 (0.64–1.38)	0.74
High malignancy grade	1.24 (0.87–1.77)	0.24	1.54 (0.95–2.50)	0.08

Abbreviations: CI = confidence interval; OS = overall survival; RF-CSS = recurrence-free cancer-specific survival. *P* values <0.001 are indicated in bold.

T4-category ($P = 0.017$). We did not find any significant relation between miR-21 expression and OS.

Vascular and nerve invasion were not described in a large fraction of the patients (22.7% and 24.2%, respectively). Thus, the Cox regression analysis was only performed with respect to these two pathological features in the limited subsets of patients. However, high levels of mean log(TBR) was consistently found

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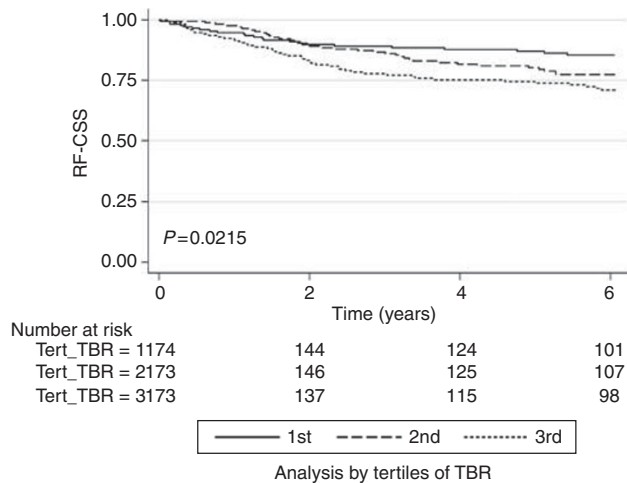
Figure 3 Kaplan–Meier plot illustrating recurrence-free cancer-specific survival (RF-CSS) of the patients divided into tertiles of log(TBR). Patients with mean log(TBR) values in the upper tertile had significantly shorter RF-CSS (HR = 1.92; 95% CI: 1.19–3.09; P = 0.008) compared with patients with mean log(TBR) values in the lower tertile.

to be significantly related to poor RF-CSS (HR = 1.52; 95% CI: 1.23–1.88; $P < 0.001$). The same applied to log(TB) ($P = 0.008$).

To illustrate the effect of increasing mean log(TBR), the population cohort was stratified into tertiles according to their mean log(TBR) value. As illustrated in Figure 3, patients with mean log(TBR) values in the upper tertile had significantly shorter RF-CSS (HR = 1.92; 95% CI: 1.19–3.09; $P = 0.008$) compared with patients with mean log(TBR) values in the lower tertile.

DISCUSSION

At present, the decision on adjuvant chemotherapy for stage II colon cancer patients is based on clinical and pathological markers of risk, which are inadequately informative in most of the patients, and better methods are certainly needed (Tournigand and de Gramont, 2011). For several years, new prognostic markers of significant importance have been a central topic in cancer research with the perspective of individualised treatment.

Most studies have been retrospective and included small numbers of highly selected patients. The risk of such studies being false positive is very high, and it has become evident that most biomarker studies have failed verification in new independent patient series (Locker *et al*, 2006). However, the present study included nearly all stage II patients in a population of 5 million people and can thus be considered unbiased with regard to selection.

The present study shows that increasing mean log(TB) (representing the relative miR-21 expression level) and mean log(TBR) (representing values normalised to the nuclear density) are significantly related to decreasing RF-CSS. These results thus confirm the prognostic importance of miR-21 independently from prognostic markers, proven to be inadequate, for example, T-category, malignancy grade, tumour perforation, tumour fixation, invasion of nerves and invasion of veins. Our results are in agreement with the previous study by Nielsen *et al* (2011) reporting that high miR-21 levels determined by ISH in 130 cases of stage II colon cancer is correlated with poor disease-free survival. The findings were based on a sample cohort as part of the RANX study and contained patient samples collected from 1991 to 1993 (Nielsen *et al*, 2011). They also found a significant impact on OS. In contrast, we found no prognostic value of miR-21 in analyses of OS. At the cut-off point set to 1 January 2010, 116 patients had died from other causes than colon cancer, whereas

110 patients had experienced relapse or had died from colon cancer. This indicates that at least half of stage II colon cancer patients die from causes other than colon cancer. Hence, OS analyses will be diluted, calling for endpoint analyses focusing on recurrence of the disease.

Our results are also in keeping with previous studies using PCR to quantify miR-21 in CRC tissue. Schetter *et al* (2008) studied the profile of the miRNA expression in paired tumour and normal tissue samples from 84 patients with colon cancer (29 stage II patients) as a training set and paired samples from another 113 patients with colon cancer (37 stage II patients) as a validation set. Among the 37 miRNAs that were differentially expressed, miR-21 was selected for validation. They found that high expression of miR-21 was significantly associated with poor prognosis, independent of T-category, age, sex and tumour location (Schetter *et al*, 2008). Slaby *et al* (2007) also reported that miR-21 upregulation in CRC patients was associated with lymph node positivity and development of distant metastasis based on their study of 29 patients.

In this study, the majority of tumours expressed the miR-21 ISH signal in fibroblast-like stromal cells. However, miR-21-positive cancer cells were also found in a small fraction of the tumours. Previous studies using identical probes to visualise miR-21 report the same distribution (Nielsen *et al*, 2011; Rask *et al*, 2011), while other groups using different probes describe miR-21-positive tumour cells in the majority of the tumours (Schetter *et al*, 2008; Yamamichi *et al*, 2009). The reason for the discrepancies could be explained by cross-reaction of the probe to 'similar sequences' allowed under suboptimal hybridisation conditions as well as different reagents employed to detect the labelled probe. However, it can not be excluded that a different probe could influence the results. It is noteworthy that miR-21 demonstrates prognostic impact despite the fact that it is found in the stroma cells and not in the tumour cells. Explanations are speculative but it can not be excluded that signals from stroma to the tumour cells, including paracrine signalling, influence the behaviour of the tumour.

Because of the exclusion criterion that each slide should be evaluated by at least eight images, 41 patients were excluded. This is most likely explained by the fact that we collected the tumour block representing the invasive front from each patient. Therefore, some of the blocks had small tumour content and as the software did not allow image fields to overlap, fewer images were available for analysis.

Using image quantified ISH we were able to assess miR-21 on archival FFPE tissue in this unique population. The results showed that increasing mean log(TBR) is an independent prognostic marker of poor RF-CSS in a stage II colon cancer population consisting of nearly all patients diagnosed with this disease in Denmark in the year 2003. Our findings strongly suggest that miR-21 measured by ISH is a robust and valuable indicator of adverse prognosis in stage II colon cancer. The results call for evaluation together with other variables to generate a fully specified prognostic index, pointing out the individuals, in this group of patients, in need of adjuvant chemotherapy. Eventually, such an index should be tested in a prospective, randomised trial setting for conclusive evaluation of the clinical impact.

Conflict of interest

SJ and BSN held a full-time employment at the time of analysis for an entity (name of entity: Exiqon A/S) having a commercial interest in the subject matter under consideration in the manuscript. The remaining authors declare no conflict of interest.

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