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Overexpression of Dicer predicts poor survival in colorectal cancer

C. Faber [*], *D. Horst* [a], *F. Hlubek*, *T. Kirchner*

Institute of Pathology, University of Munich (LMU), Germany

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ABSTRACT

Aims: The RNASE III endonuclease Dicer is one of the key enzymes of microRNA biogenesis. The influence of Dicer-expression in tumour cells on the prognosis of patients with several cancers has been studied with controversial results among different cancer types. To date no one has examined the effect of this biomarker on survival in colorectal carcinoma. Thus, we aimed to study the influence of Dicer expression on survival in colorectal cancer.
Methods: We performed immunohistochemical analyses on formalin-fixed paraffin embedded (FFPE) cancer tissue with an antibody against the Dicer protein. Tumour material from 237 cases was available from patients with colorectal adeonocarcinomas with moderate differentiation (G2) and without evidence of lymph-node (N0) or distant metastasis (M0). Sixty-four cases were in T2 and 173 in T3 stages. A tissue microarray (TMA) was constructed with each tumour in triplicate. Each tumour was assigned to a scoring scale of 0–3, depending on the cytoplasmatic expression of Dicer. A Kaplan–Maier analysis was performed and the log-rank test was used for significance levels by using SPSS v.17 software.
Results: The expression of Dicer in colorectal carcinoma shows a strong association with poor survival (cancer specific survival = CSS, $p < 0{,}001$) as well as with reduced progression free survival (PFS, $p < 0{,}001$). In the group with no Dicer staining there was no recorded relapse (0/15) compared with 10/18 relapses in the group with the strongest staining of Dicer.
Conclusions: Strong expression of the central microRNA biosynthesis enzyme Dicer predicts poor prognosis in patients with colorectal cancer. This is in line with investigations on prostate cancer. Contradictory, in breast, lung and ovary cancer Dicer has been shown to be a marker of good prognosis. Further studies on the cellular functions of Dicer need to address these issues.

1. Introduction

Dicer is one of the key enzymes of microRNA (miRNA) biogenesis, acting on the processing of transcribed precursor microRNAs.[1] Mature miRNAs are small RNAs of 22–25 bp that control protein expression by interfering with several target mRNAs, resulting in mRNA degradation or repression of translation by transcript specific interactions. Thus, they act as global regulators of gene expression in various cell types. As miRNAs recognise their target-mRNAs with an imperfect sequence complementarity, they influence the expression of a large set of genes. Not surprisingly, a disturbed production

* *Corresponding author*: Address: Pathologisches Institut der LMU München, Thalkirchner Str. 36, 80337 Munich, Germany. Tel.: +49 89 2180 73710; fax: +49 89 2180 99 73710.
E-mail address: c.faber@lmu.de (C. Faber).
[a] Current address: Dana-Farber Cancer Institute, Harvard Medical School, Boston, USA.
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of miRNAs can have serious consequences for a cells fate, with altered miRNA expression being observed in a broad range of human diseases such as cancer and immunologic diseases.

Table 1 – Patient details show the highly homogenous study population with all 237 patients having moderately differentiated adenocarcinoma of the colon or rectum (G2) in T2 or T3 stage without metastatic disease (N0, M0).

All patients	237	(100.0%)
Age (median 69 years)		
⩽50	14	(5.9%)
>50	223	(94.1%)
Sex		
Female	106	(44.7%)
Male	131	(55.3%)
Stage		
pT2	64	(27.0%)
pT3	173	(73.0%)
Metastases		
pN0, pM0	237	(100.0%)
Grading		
G2	237	(100.0%)

The expression of Dicer directly influences the biosynthesis of all microRNAs, severely affecting the cells gene expression pattern. If any of the key components of miRNA biogenesis is knocked down by RNA interference, mature miRNAs are significantly reduced.[1] The influence of expression on prognosis has been studied in several cancers with controversial results among different cancer types. Patients with ovarian cancer and a reduced expression of Dicer in the tumour cells showed a poor prognosis.[2] Similarly, in lung and breast cancers decreased Dicer levels were also a marker of poor prognosis.[2,3] Conversely, neoplasia of the prostate show an upregulation of Dicer in invasive adenocarcinoma. These increased Dicer levels in invasive tumours furthermore correlate with established clinicopathological parameters such as nodal status or the presence of distant metastases.[4] Regarding colorectal cancer, an immunohistochemical study by Chiosea et al. found a dysregulation of Dicer in 65% of 147 examined colorectal carcinomas compared to the corresponding normal mucosa. However, no correlation with TNM staging parameters could be established in this study.[5]

In colorectal carcinoma, the second most common cancer in the world, several microRNAs are dysregulated, showing associations with tumour subtypes, UICC stage and prognosis.[6] However, the influence of Dicer expression on survival, as one of the key enzymes for the biogenesis of micro-RNAs, has not been investigated to date. Thus, we aimed to study

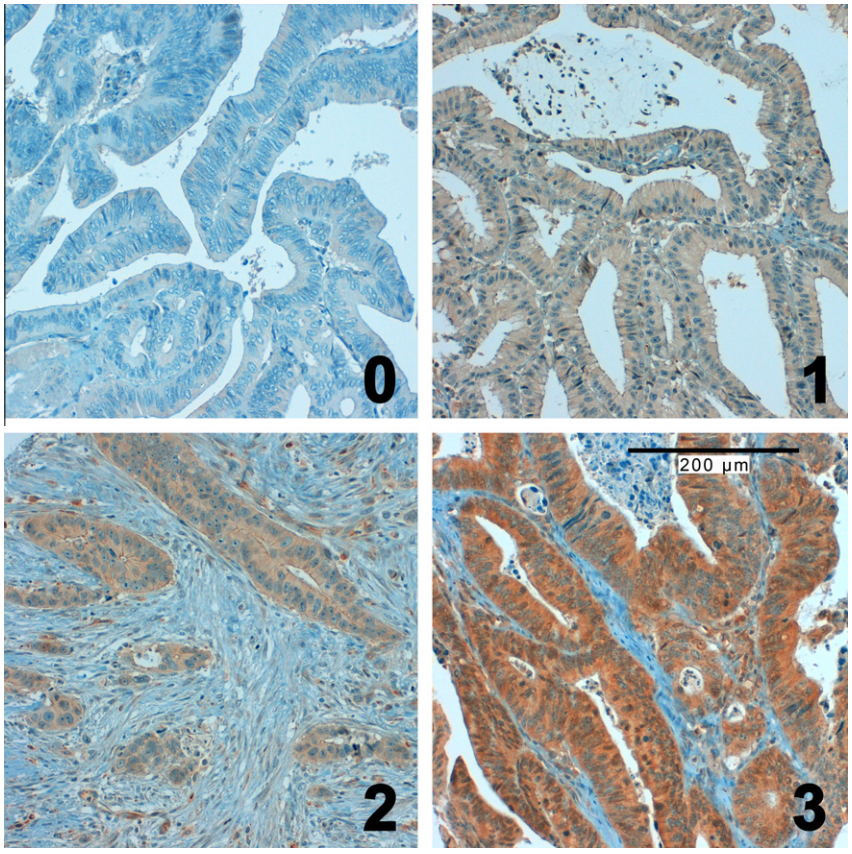

Fig. 1 – Different staining intensities of Dicer in colorectal adenocarcinomas. The images show four different tumour samples with different cytoplasmatic staining intensities against the Dicer-protein assessed by immunohistochemistry (IHC). The IHC-score of 0, 1, 2 or 3 is shown at the lower right corner of each image. The examples are representative for the whole set of samples.

the influence of Dicer expression on survival in colorectal cancer.

2. Methods

2.1. Patient characteristics

Our case collection included tumour specimens of 237 patients with moderately differentiated adenocarcinoma (G2) of the colon or rectum in T2 ($n = 64$) or T3 ($n = 173$) stage and without lymph node or distant metastasis (N0 and M0) from patients who underwent intentionally curative surgical resection at the Ludwig–Maximilians-Universität München. Consecutive paraffin material was chosen from the archive of the Institute of Pathology and used when adequate tumour tissue was available. Thirty-one patients (13.1%) died from colorectal cancer in a mean follow-up period of 5.8 years. Fifty-one patients (21.5%) had a progress of colorectal cancer, i.e. local recurrence or distant metastasis in a mean follow-up time of 5.4 years. Cancer-specific survival (CSS) and progression-free survival (PFS) were calculated from the date of primary surgical resection to the date of colon cancer associated death or to the date of recorded cancer progression. Patient details are summarised in Table 1. The study complied with the guidelines of the local ethics committee.

2.2. Tumour tissue and follow-up data

All samples were received from patients who had intentionally curative surgical resection of colorectal cancer between 1994 and 2004 at the Ludwig–Maximilians-University Munich. Follow up data were available from the Tumour register München. Only tumours with T-categories T2 or T3 and no metastases at the time of diagnosis were used (nodal or distant; N0,M0). All samples were moderately differentiated (G2 according to World Health Organization).

2.3. Tissue microarray (TMA) construction and immunohistochemistry

A paraffin tissue microarray (TMA) was constructed containing tissue from the tumour centre in triplicates, as described previously.[7] Briefly, sections of 5 μm of formalin fixed, paraffin embedded (FFPE) tissue samples stained with haematoxylin–eosin were used to define representative areas of viable tumour tissue. Needle core-biopsies of 1.0 mm were taken from the corresponding areas of the paraffin-embedded tumour blocks using a tissue arraying instrument (Beecher Instruments, Sun Prarie, WI, USA). These needle-core biopsies were then placed in recipient paraffin array blocks at defined coordinates. Three probes of each tumour were taken from central tumour areas. The cores in the paraffin block were incubated for 30 min at 37 °C to improve adhesion between cores and paraffin of the recipient block.

For immunohistochemical staining, 5 μm sections from each paraffin block were stained with antibodies reacting with Dicer (Anti-DICER1, 1:75, Sigma–Aldrich, Hamburg, Germany). Staining was performed on a Ventana Benchmark XT autostainer with the XT ultraView DAB Kit (Ventana Medical Systems, Illkirch, France). All slides were counterstained with Haematoxylin (Vector Laboratories). Isotype and system controls were performed and did not yield a positive result.

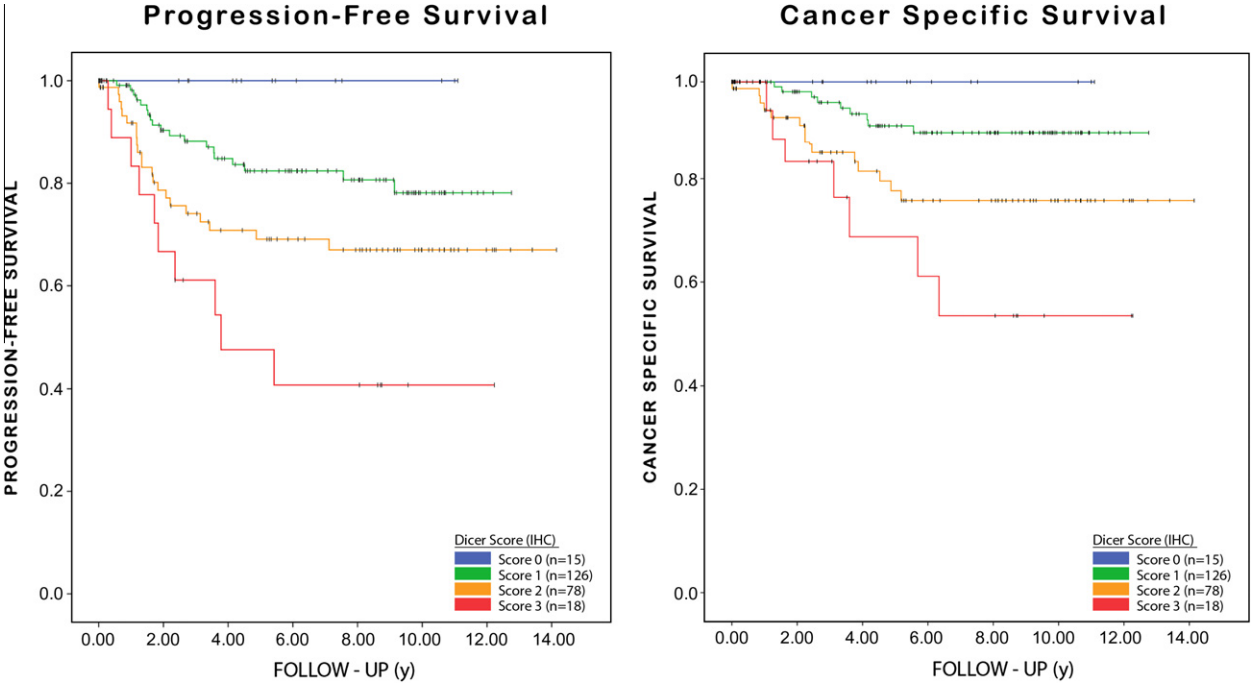

Fig. 2 – Kaplan–Meier survival analysis comparing 237 colorectal cancer patients with different Dicer staining intensities of the tumour. The graph on the left shows the effect on progression-free survival (PFS), the graph on the right shows the effect on cancer-specific survival (CSS). Colorectal carcinomas with higher Dicer expression show a significantly shorter survival than those with lower Dicer expression scores. The differences are highly statistically significant with *p*-values of 0.0003 (PFS) and 0.0004 (CSS) in the log-rank test. The number of patients per group is indicated in the lower right corner of the graphs.

Table 2 – Expression of Dicer in colorectal cancer according to immunohistochemical staining graded from 0 (no cytoplasmatic staining) to 3 (strong cytoplasmatic staining) and correlation with survival and disease status.

	Number of tumours (%)		Dicer 0	Dicer 1	Dicer 2	Dicer 3
Survival status						
Tumour related deaths	31	(13%)	0	9	15	7
Alive	206	(87%)	15	117	63	11
Fraction died	0.13		0.00	0.07	0.19	0.39
Disease status						
Tumour progress	51	(22%)	0	19	22	10
Tumour free	186	(78%)	15	107	56	8
Fraction relapsed	0.22		0.00	0.15	0.28	0.56

Sections were evaluated independently and blinded to outcome data by a pathologist three times. The staining of Dicer was scored from 0 to 3, considering only the cytoplasmatic reaction (Figure 1).The mean of all three values was then calculated and each sample was assigned to one of the four different IHC-score categories (IHC-score 0–3).

2.4. *Statistical analysis*

Kaplan–Meier analysis was applied to estimate cancer-specific survival, different groups were compared with the log-rank test. Multivariate analysis was conducted with the Cox regression model. Statistical procedures were analysed with SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered statistically significant.

3. Results

Stained sections of tissue microarrays (TMA) of 237 tumours were graded for their cytoplasmatic immunohistochemical staining intensity (IHC-score) against the Dicer protein from 0 (no staining) to 3 (strong staining intensity, Figure 1). No staining (IHC-score 0) and strong staining intensity (IHC-score 3) was observed in 15 and 18 samples, respectively. Most tumours showed either a low (IHC-score 1, 126 cases) or a moderate (IHC-score 2, 78 cases) cytoplasmatic staining intensity of Dicer. In full sections of normal colonic mucosa as well as in full sections of colorectal carcinoma, Dicer expression was limited to the epithelium.

The Kaplan–Meier survival analysis shows a strong correlation of high Dicer expression levels with a shorter progression-free survival (PFS) as well as with poor survival (cancer specific survival, CSS, Figure 2). Both findings are highly significant with *p*-values of 0.0003 for PFS and 0.0004 for CSS (log-rank test).

The strong expression of Dicer is robustly associated with poor survival (cancer specific survival, CSS) as well as with shorter progression-free survival (PFS). As shown in Table 2, there was no tumour related death or tumour relapse in the group with no Dicer staining (IHC-score 0). On the other hand, a stronger Dicer expression is associated with an increase in deaths as well as in the number of relapses of 39% and 56%, respectively, for the subgroup with the highest Dicer-Score (IHC-score 3).

For the Cox multivariate analysis only few variables were left due to the highly homogenous study population regarding established histopathological parameters, such as T, N, M and G status. For the remaining variables there was a trend to a

Table 3 – Multivariate analysis of cancer-specific and progression-free survival. The Dicer expression has been grouped into a no/low staining intensity group (IHC-score 0 + 1) and a moderate/strong staining intensity group (IHC-score 2 + 3) for the multivariate analysis.

	Cancer-specific survival			Progression-free survival		
	HR	95% CI	*p*	HR	95% CI	*p*
Age (median 69 years)						
⩽69	1			1		
>69	1.59	(0.76–3.32)	0.22	1.14	(0.65–2.01)	0.65
Sex						
Male	1			1		
Female	1.07	(0.51–2.22)	0.86	1.18	(0.66–2.10)	0.57
Stage						
pT2	1			1		
pT3	1.38	(0.55–3.48)	0.49	1.18	(0.61–2.30)	0.62
Dicer expression						
low (IHC Score 0–1)	1			1		
high (IHC Score 2–3)	3.58	(1.62–7.90)	0.002	2.50	(1.40–4.45)	0.002

higher chance of cancer-related death or relapse in the older age group (>69 years), in the T3 vs. T2 group and nearly no difference regarding sex. However, all these differences were non-significant. On the other hand, for Dicer expression we found statistically highly significant differences with hazard ratios of 3.58 ($p = 0.002$) and 2.50 ($p = 0.002$) for PFS and CFS, respectively, by comparing the no/low expression group (IHC-scores 0 and 1) and the moderate/strong expression group of tumours (IHC-scores 2 and 3, Table 3). Furthermore, there were no differences between males and females regarding Dicer expression (data not shown).

4. Discussion

We examined formalin-fixed, paraffin embedded tumour tissues from 237 patients with moderately differentiated colorectal cancer without metastatic disease (T2 or T3, N0, M0) for the expression of Dicer, a key enzyme of microRNA biogenesis. We found a strong correlation of Dicer expression and poor survival (CSS) as well as disease progression (PFS), showing a highly significant, linear distribution in Kaplan–Meier analysis (each $p < 0.001$).

A severe dysregulation of certain miRNAs in cancer is now widely accepted and has been extensively studied, especially in colorectal cancer.[6] Early reports suggested generally lower miRNA levels in cancer cells compared to corresponding normal tissue and lower levels in poorly differentiated compared to well differentiated tumour tissues.[8] The same was observed in several cell lines.[9] However, subsequent studies challenged this perspective of a global underexpression of miRNAs in tumour cells and report a global increase instead.[10,11] Following functional experiments revealed that by targeting Dicer and Drosha by short hairpin RNAs, cells can be driven towards transformation.[12] Thus, several studies were performed on different cancer types to elucidate the role of Dicer and Drosha in carcinogenesis and their impact on prognosis. In our study, the poor prognosis in tumours expressing high Dicer levels in colorectal adenocarcinoma is in accordance with results reported from prostate adenocarcinoma, where Dicer expression was increased in carcinomas with higher clinical stage, lymph node status and Gleason score, all established prognostic parameters.[4] Conversely, in large studies on breast, lung and ovarian cancer low expression of Dicer predicted poor survival.[2,3] In one smaller study on colorectal cancer, no correlation with clinicopathological parameters (TNM) could be established.[5] Precursor lesions of lung[13] and prostate carcinoma[4] both showed an increase in Dicer expression compared to normal mucosa. Thus, a transient increase of Dicer expression in precursor lesions might precede a differential expression in invasive carcinomas depending on the tissue of origin.

The cause for these controversial results between cancers of different origin and for the increased aggressiveness of highly Dicer-expressing tumour cells in colorectal cancer is not clear. As described above, aggressive tumours are thought to have decreased total miRNA levels, contributing to their poor differentiation. This would suggest a decreased rather than an increased expression of Dicer in aggressive cancers. Thus, a simple concept of over- or underexpression in cancer in general does obviously not apply in the case of Dicer. A recent study sheds new light upon the maturation of miRNAs. *Chelouf*i et al. report a Dicer-independent way of maturation, at least for some miRNAs. They show that miR-451 is processed by Ago, an Argonaute protein without the need for Dicer.[14] Thus, alternative pathways of miRNA-biogenesis apart from Dicer exist and might be an explanation for conflicting data from total miRNA and Dicer levels. This assumption is in line with siRNA-mediated knock-down experiments of Dicer and two other miRNA biogenesis enzymes, where mature microRNAs, though significantly smaller amounts are still detectable.[1]

However, Dicer has additional functions apart from miRNA processing that might contribute to malignant transformation. Recently, Dicer has been shown to be essential to maintain CpG promoter island hypermethylation in human colorectal cancer cell lines.[15] This might be of relevance, as hyper- as well as hypo-methylation of several genes is a frequent phenomenon in colorectal cancer and Dicer could promote or at least maintain these oncogenic events.[16] One group of frequently epigenetically silenced tumour suppressors in colorectal cancer is the gene product of the family of secreted frizzled related genes (SFRPs). They are able to antagonise the Wnt-β-catenin pathway, even in the presence of activating downstream mutations, frequently observed in colorectal cancer.[17] Investigations on breast cancer cell lines, which are depleted from Dicer by siRNA knockdown, show a cell cycle arrest in G1 Phase and down-regulation of miR-21.[18] MiR-21 is known to be upregulated in colorectal cancer and shows a strong association with poor prognosis.[19] Several lines of evidence furthermore point to an important role of Dicer in the maintenance of centromeric heterochromatin structure and centromeric silencing, as these basic cellular processes are severely disturbed in Dicer-deficient mouse embryonic stem cells.[20] This link between RNAi and chromatin structure is further supported from data produced by Dicer knock-out in fission yeast.[21,22]

The regulation of Dicer expression is poorly understood. Recently, Wiesen and coworkers have shown that Dicer expression is inhibited by type-I-interferons and by certain cellular stress factors. Conversely, Interferon-γ was able to induce Dicer expression.[23] On the other hand, Dicer itself is a target of microRNA-mediated regulation. The miRNA let-7 inhibits the expression of Dicer, representing a negative feedback-loop on overall miRNA production.[24] Thus, a deficiency of this negative feedback loop could contribute to overexpression of Dicer. Furthermore, genomic mutations in the gene for Dicer might be a reason for altered expression. However, this hypothesis was tested in two previous studies in ovarian and lung cancers and did not explain differences in expression levels.[2,3]

Summarised, we report for the first time the strong prognostic influence of Dicer in resected colorectal cancer. Our study adds new data to the variable expression patterns of Dicer in different tumour types. Due to these partly conflicting results among different tumour types, further studies are warranted to clarify the role of the microRNA machinery in carcinogenesis.

Conflict of interest statement

None declared.

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