MicroRNA dysregulation in the tumor microenvironment influences the phenotype of pancreatic cancer

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Cellular interactions in the tumor microenvironment influence neoplastic progression in pancreatic ductal adenocarcinoma. One underlying mechanism is the induction of the prognostically unfavorable epithelial–mesenchymal-transition-like tumor budding. Our aim is to explore the expression of microRNAs implicated in the regulation of tumor budding focusing on the microenvironment of the invasive front. To this end, RNA from laser-capture-microdissected material of the main tumor, tumor buds, juxta-tumoral stroma, tumor-remote stroma, and non-neoplastic pancreatic parenchyma from pancreatic cancer cases with ($n=7$) and without ($n=6$) tumor budding was analyzed by qRT-PCR for the expression of a panel of miRNAs that are known to be implicated in the regulation of epithelial–mesenchymal transition, including miR-21, miR-183, miR-200b, miR-200c, miR-203, miR-205, miR-210, and miR-217. Here we show that at the invasive front of pancreatic ductal adenocarcinoma, specific microRNAs, are differentially expressed between tumor buds and main tumor cells and between cases with and without tumor budding, indicating their involvement in the regulation of the budding phenotype. Notably, miR-200b and miR-200c were significantly downregulated in the tumor buds. Consistent with this finding, they negatively correlated with the expression of epithelial–mesenchymal-transition-associated E-cadherin repressors ZEB1 and ZEB2 in the budding cells ($P<0.001$). Interestingly, many microRNAs were also dysregulated in juxta-tumoral compared to tumor-remote stroma suggesting that juxta-tumoral stroma contributes to microRNA dysregulation. Notably, miR-200b and miR-200c were strongly downregulated while miR-210 and miR-21 were upregulated in the juxta-tumoral *vs* tumor-remote stroma in carcinomas with tumor budding. In conclusion, microRNA targeting in both tumor and stromal cells could represent a treatment option for aggressive pancreatic cancer.
Modern Pathology (2017) **30,** 1116–1125; doi:10.1038/modpathol.2017.35; published online 26 May 2017

Our understanding of the contribution of the tumor microenvironment to cancer in general and pancreatic ductal adenocarcinoma in particular has been broadened based on recent data.[1–6] Many of the cellular interactions within the tumor microenvironment support enhanced growth and dissemination of cancer cells.[2–6] Especially, the emerging role of stromal cells in the establishment of a tumor-permissive environment has opened a great potential for therapeutic intervention. In pancreatic ductal adenocarcinoma, which is characterized by dismal prognosis and a rich stromal component, targeting of the tumor microenvironment is increasingly becoming an attractive therapeutic option, because of lack of efficacy of standard chemotherapy.

Especially at the invasive front of aggressive gastrointestinal cancers, including pancreatic cancer, tumor cells are represented by a population of dedifferentiated cancer cells with epithelial–mesenchymal-transition-like features and dissociative growth, coined tumor budding cells.[7–10] Epithelial–mesenchymal-transition-like tumor budding is a strong and independent prognostic factor in pancreatic cancer.[9,10] The microenvironment surrounding tumor buds is therefore especially interesting, since it probably has a distinguished role in supporting tumor budding cells, promoting their migration, angioinvasion, and metastatic potential. However, the molecular mechanism underlying tumor budding remains currently unclear.

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Received 27 December 2016; revised 20 March 2017; accepted 20 March 2017; published online 26 May 2017

MicroRNAs are a class of non-coding RNAs involved in key cellular processes such as proliferation, apoptosis, and differentiation.[11,12] These small (~18–25 nucleotides) RNAs may bind to target mRNAs by simple Watson–Crick base-pairing to repress their proper posttranscriptional maturation process. It is thought that individual microRNAs can target multiple genes implicated in diverse cellular functions, suggesting that microRNAs play important roles in a wide variety of cellular processes. Because of their role in gene regulation at the posttranscriptional level, microRNAs are potential clinical biomarkers for human cancers as well as targets for molecular therapy.[13–15] Restoration or repression of microRNA expression and activity is very promising in managing cancer, and many studies on pre-clinical models have demonstrated the feasibility and efficacy of microRNA-based therapy.[15–17]

Here we aim at investigating the expression profile of specific microRNAs originating from different cell populations within the tumor microenvironment of the invasive front of pancreatic ductal adenocarcinoma with special focus on epithelial–mesenchymal-transition-associated microRNAs. Therefore, the expression of these microRNAs was analyzed in cases with and without tumor budding in microdissected material from the main tumor mass, the tumor budding cells (in cases with budding), the juxta-tumoral and tumor-remote stroma, and the matched normal pancreatic parenchyme. We show that some of these microRNAs are differentially regulated between normal tissue and tumor, between main tumor cells and budding cells, as well as between juxta-tumoral and tumor-remote stroma, suggesting a crosstalk between tumor cells and their surrounding stromal environment through microRNAs.

Material and methods

Patients and Tissues

Histomorphological data from 120 non-pretreated pancreatic ductal adenocarcinomas, surgically resected between 2002 and 2010, were reviewed from the corresponding hematoxylin and eosin stained slides, while clinical data were obtained from corresponding reports. Clinicopathological information included age, gender, tumor diameter, number of positive lymph nodes, and total number of lymph nodes collected, TNM stage (8th Edition, 2017),[18] perineural, blood vessel and lymphatic invasion, and resection margin status (R-status) (Supplementary Table 1). The use of the material was approved by the local Ethics committee of the University of Bern.

Construction of Tissue Microarrays

Tissue microarrays were built as previously described.[19] To reduce bias due to heterogeneity, at least four tumor punches from the tumor center and the invasive tumor front of each patient was included on this array.

Immunohistochemistry

Tissue microarray blocks were cut at 4 μm and sections were de-waxed and re-hydrated in dH_2O. Sections were stained immunohistochemically for E-cadherin (1:200; Dako, Glostrup, Denmark), ZEB1 (1:200; Sigma-Aldrich, St Louis, CA, USA), and ZEB2 (1:400; Sigma-Aldrich). Antigen retrieval was performed with Tris-HCl, pH 9 for 30 min at 95 °C. Antibody testing and staining protocols have been established and staining was performed by an automated Leica Bond RX System with the Bond Polymer Refine Kit (with DAB as chromogen) and Bond Polymer Refine Red Detection Kit for the double staining (Leica Biosystems, Newcastle, UK). Immunohistochemistry was evaluated by estimating the percentage of positive cells per punch. In the case of multiple tumor punches per localization, the average protein expression was calculated across all punches from the same localization. Evaluation was performed blinded to clinical end points.

Assessment of Tumor Budding

Tumor budding, defined as detached single cells or clusters of less than five cells, has been evaluated in the course of another project.[10] Briefly, whole-tissue sections of each case underwent immunohistochemistry for pancytokeratin staining (1:100, clone AE1/AE3, Monosan, Uden, the Netherlands) and were evaluated for tumor budding using a 10-in-10 approach: The 10 densest hotspots of tumor budding were identified and buds were counted at high magnification (×40, 0.55 mm^2). The average number of buds per case was obtained. Using a receiver operating characteristic curve approach, a cutoff score of 10 buds on average was identified as most discriminatory for survival. Cases with an average of >10 buds were classified as 'high' budders; those with ≤ 10 buds were defined as 'low' budders (Supplementary Figure 1).[9]

MicroRNAs Expression Analysis

Tumor specimens (seven cases with and six cases without tumor budding) underwent laser capture microdissection to obtain tissue from the main tumor mass, the tumor buds as well as the juxta-tumoral and the tumor-remote stroma (at least 0.5 cm away from the tumor). The cases were randomly selected among our high-grade budding and non-budding tumor specimens. To better visualize tumor buds, a monoclonal antibody directed against pancytokeratin (1:100, clone MNF116, Dako, Glostrup, Denmark) was used. All 13 cases underwent immunohistochemistry using a high salt protocol, which protects

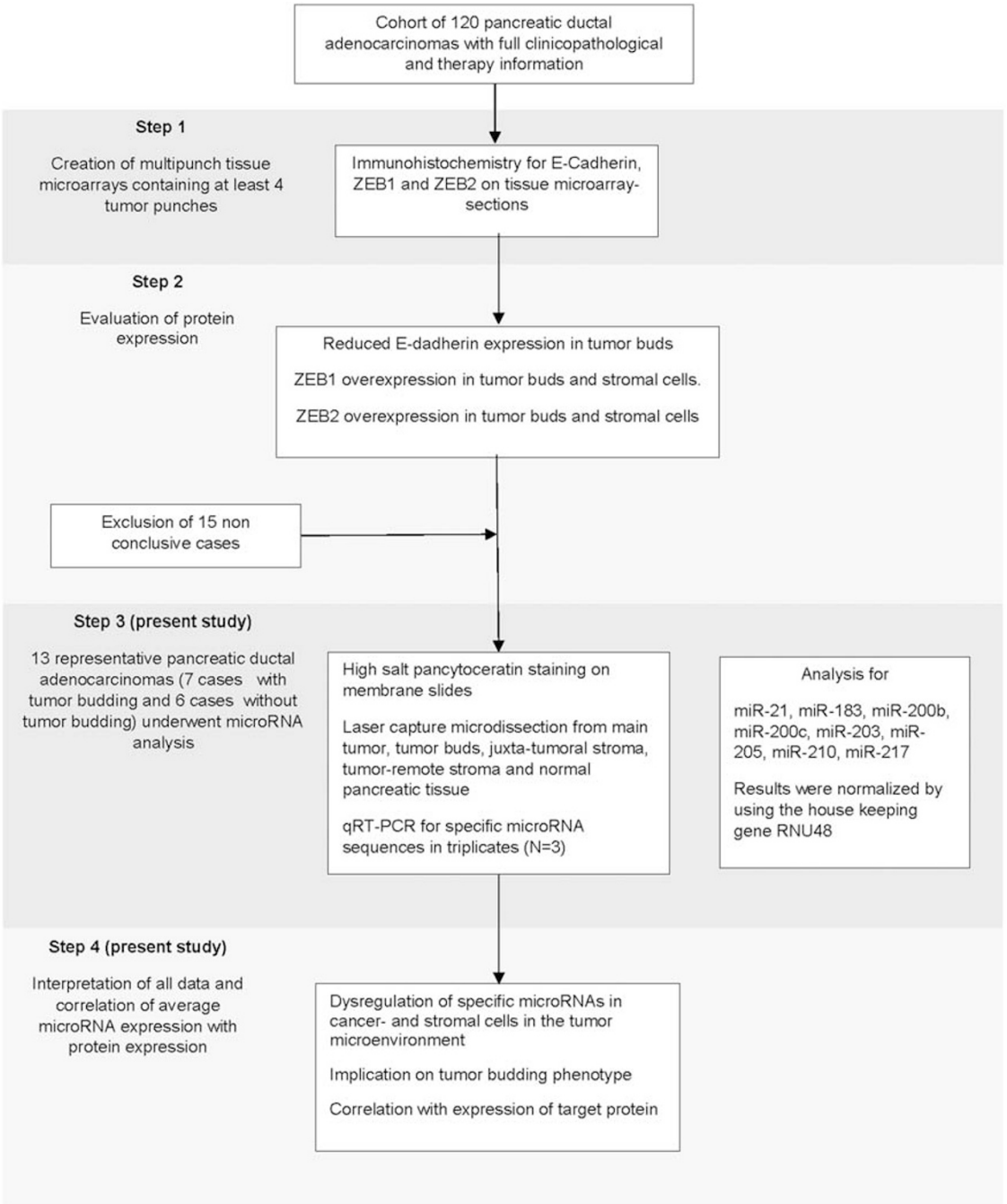

Figure 1 Study design.

microRNAs from degradation by RNases.[19] RNA was extracted and subjected to a demodification protocol using RecoverAll Total Nucleic Acid Isolation kit (AM1975, Life Technologies) as described.[20] A measure of 120 ng t-RNA was added as carrier RNA and final elution was performed in 30 μl ddH_20. Accurate microRNA expression analysis was performed by pre-amplification following the manufacturer's recommendation, 1:3 dilution of the pre-amplified product, followed by qRT-PCR using TaqMan technology (Life Technologies). We confirmed that pre-amplification has no significant influence on the relative expression of the analyzed micrRNAs (data not shown). Results are shown relative to the expression of the housekeeping gene RNU48 ($N=3$).

Analyses were carried out using SAS V9.2 (The SAS Institue, NC, Cary). P-values <0.05 were considered statistically significant.

The study design is shown in Figure 1. The study was designed to comply with the REMARK guidelines for tumor marker prognostic studies.[21]

Results

Epithelial–Mesenchymal-Transition-Associated Markers

Expression of the epithelial–mesenchymal-transition-associated markers E-cadherin, ZEB1 and ZEB2, in the tumor buds has been analyzed for the needs of a previous study.[19] Briefly, pancreatic cancer cases with absent or low density of tumor budding showed preservation of the membranous E-cadherin and markedly reduced nuclear ZEB1 and ZEB2. On the contrary, cases with high density of tumor buds at the invasive front showed a reduced membranous expression of E-cadherin with almost complete loss of protein expression in the tumor buds (Supplementary Figure 1). In addition, an increased nuclear ZEB1 and ZEB2 expression was observed, especially in the budding cells and the stromal cells surrounding them.

Expression Analysis of MicroRNAs in the Main Tumor and Tumor Buds

The expression of a panel of microRNAs that are known to be implicated in the regulation of epithelial–mesenchymal transition in pancreatic cancer, including miR-21, miR-183, miR-200b, miR-200c, miR-203, miR-205, miR-210, and miR-217, was analyzed by qRT-PCR from RNA of laser-capture-microdissected material including the main tumor, tumor buds, and matched normal tissue (Figure 2).

Mir-200 family members miR-200b, miR-200c, and miR-205 are known regulators of E-cadherin repressors ZEB1 and ZEB2.[22–27] In cases with tumor budding, miR-200c and miR-205 were significantly downregulated both in the main tumor and the tumor buds compared to normal tissue ($P < 0.001$), while miR-200b was downregulated in the tumor buds compared to the main tumor ($P < 0.05$). On the contrary, in cases without budding both miR-200c and miR-200b were overexpressed in the tumor cells, while no dysregulation of miR-205 was observed.

Interestingly, qRT-PCR analysis revealed that miR-203 and miR-200b gave rise to a similar expression profile and were downregulated in the tumor buds compared to the main tumor of cases with budding, without being downregulated in the main tumor itself. miR-183, another microRNA that is able to target ZEB1 (ref. 28), was upregulated in all tumors independently of tumor buddng.

In addition, miR-21 and miR-210 were overexpressed in the tumor cells (including tumor budding cells), compared to normal ($P < 0.001$) in all cases independently of tumor budding. However, miR-21 showed an aggravated upregulation in the tumor buds compared to main tumor in cases with budding. MiR-217 was significantly downregulated in the tumor cells compared to normal, in all cases ($P < 0.0001$) independently of tumor budding. The average expression level and range of the examined microRNAs in the several cell populations are depicted in Table 1 (cases with tumor budding) and Table 2 (cases without tumor budding).

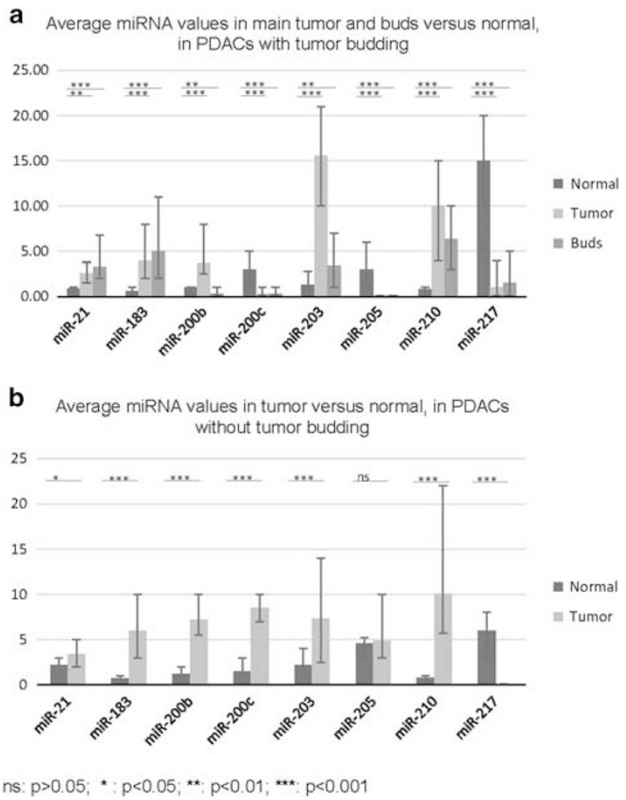

Figure 2 Expression of miRNAs in the tumor cells, in pancreatic ductal adenocarcinomas with (**a**) and without (**b**) tumor budding, in comparison with non-neoplastic pancreatic parenchyma (normal).

Correlation of Expression of ZEB1 and ZEB2 with Analyzed MiRNAs

To analyze if microRNAs are able to induce epithelial–mesenchymal transition by targeting *ZEB1* and/or *ZEB2* genes, we assessed whether the expression of these microRNAs are correlated inversely with the expression of ZEB1 and ZEB2 proteins. To this end, serial tissue microarray sections were analyzed by IHC for ZEB1 and ZEB2 proteins and results were compared to the microRNA expression. According to our findings,[19] the expression of both proteins was induced in the tumor buds in cases with tumor budding. A negative correlation was found between miR-200c, miR-200b, and miR-205 levels and ZEB1 and ZEB2 expression by the tumor budding cells ($P < 0.001$ and $P < 0.005$, respectively) (Figure 3 and Supplementary Figure 2), suggesting that ZEB1 and ZEB2 are potential targets of these microRNAs, but further studies are necessary to clarify this hypothesis.

Table 1 Pancreatic ductal adenocarcinomas with epithelial–mesenchymal-transition-like tumor budding

Tissue	*MiR-21*	*MiR-183*	*MiR-200b*	*MiR-200c*	*MiR-203*	*MiR-205*	*MiR-210*	*MiR-217*
Normal	0.9 (0.5–1)	0.6 (0.1–1)	1 (0.1–1)	3 (1–5)	1.3 (1–2.5)	3 (1–6)	0.8 (0.1–1)	15 (10–20)
Tumor	2.6 (1.5–3.8)	4 (2–8)	3.7 (1.5–8)	0.3 (0.1–1)	15.6 (10–22)	0.1 (0.1–0.1)	10 (4–15)	1 (0.1–4)
Tumor buds	3.3 (2–6.8)	5 (2–11)	0.3 (0.1–1)	0.3 (0.1–1)	3.4 (1–7)	0.1 (0.1–0.1)	6.4 (3–10)	1.5 (0.1–5)
Juxta-tumoral stroma	2.5 (1.2–5)	1.4 (0.8–4)	0.2 (0.1–0.3)	0.4 (0.2–1)	2.3 (1–8)	4 (2–10)	5.5 (1.8–10)	11.8 (5.5–20)
Tumor-remote stroma	0.9 (0.5–1)	0.5 (0.1–1)	0.75 (0.1–1)	3.9 (1.5–10)	0.5 (0.2–2)	4.3 (2–10)	0.2 (0.1–1)	15 (10–20)

Average values and range of examined microRNAs in the different tissue areas.

Table 2 Pancreatic ductal adenocarcinomas without epithelial–mesenchymal-transition-like tumor budding

Tissue	*MiR-21*	*MiR-183*	*MiR-200b*	*MiR-200c*	*MiR-203*	*MiR-205*	*MiR-210*	*MiR-217*
Normal	2.2 (1–3)	0.7 (0.1–1)	1.2 (1–2)	1.5 (1–3)	2.2 (1–5)	4.6 (1–8)	0.8 (0.1–1)	6 (3–8)
Tumor	3.4 (2–5)	6 (3–10)	7.2 (5.5–10)	8.5 (7–10)	7.3 (2.5–14)	4.9 (3–10)	10.1 (5.5–22)	0.1 (0.1–0.1)
Juxta-tumoral stroma	4 (2–7)	1.6 (1–4)	4.2 (1–9)	3 (1–5)	2 (1–4.5)	5.3 (2.5–20)	1.5 (1–2)	3 (1–4)
Tumor-remote stroma	2.4 (1–4)	0.8 (0.2–2)	0.8 (0.1–1)	1.5 (1–3)	1.2 (1–1.5)	4.3 (1.5–10)	1.2 (0.2–1.5)	6 (2–10)

Average values and range of examined microRNAs in the different tissue areas.

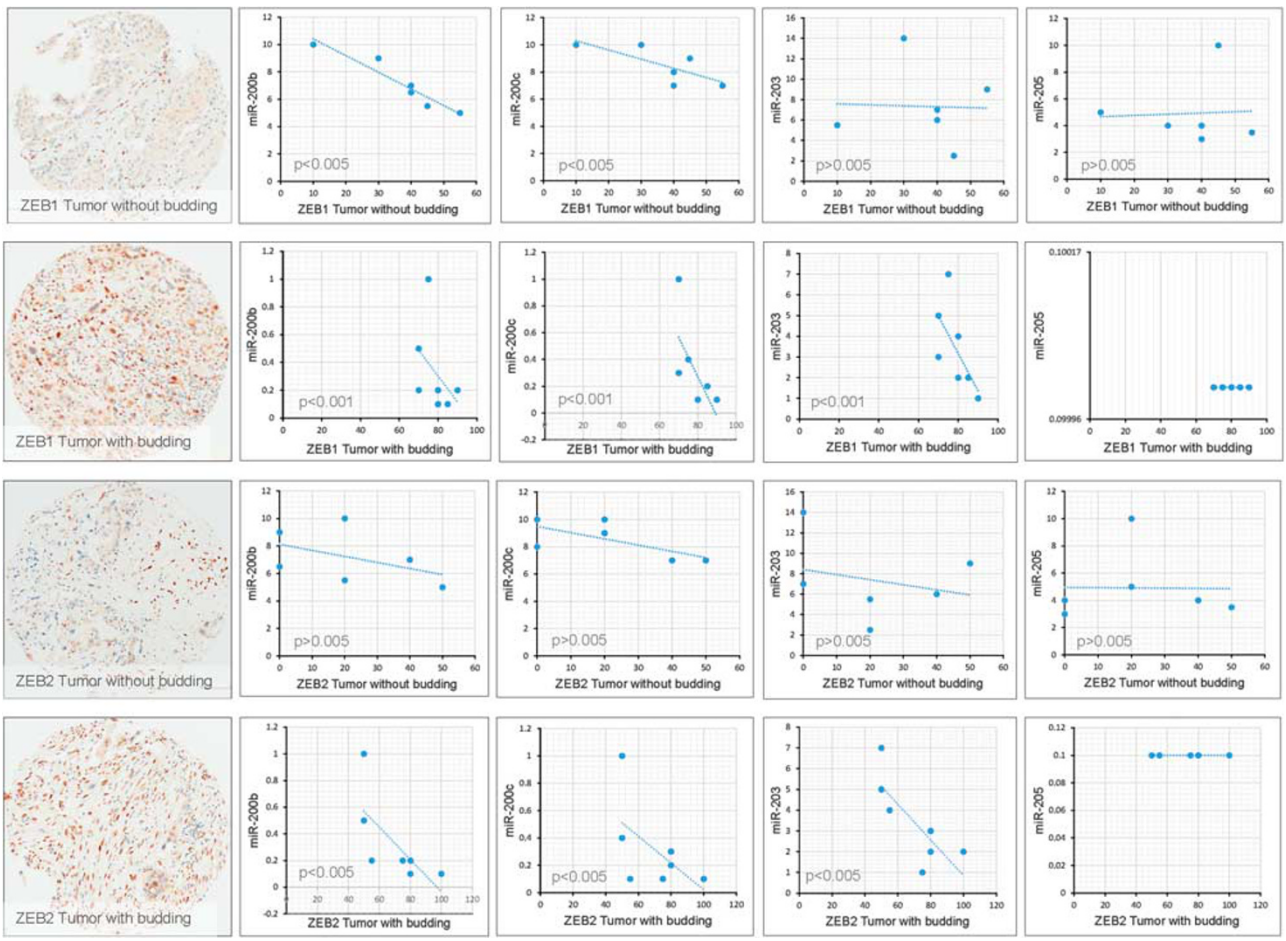

Figure 3 Correlation between ZEB1 and microRNA expression in the tumor cells of cases without (first row) and with tumor budding (second row), as well as between ZEB2 and microRNA expression in the tumor cells of cases without (third row) and with tumor budding (fourth row), concerning miR-200b, miR-200c, miR-203, and miR-205.

Dysregulation of MicroRNAs in the Stromal Cells. Juxta-tumoral and tumor-remote stromal cells were also compared for microRNA expression. Interestingly, many of the microRNAs were differentially regulated in the juxta-tumoral stromal cells compared to the tumor-remote stroma. MiR-200c and miR-200b were downregulated in the juxta-tumoral stroma compared to tumor-remote stroma ($P < 0.001$)

in cases with tumor budding and upregulated in cases without tumor budding compared to tumor-remote stroma (Figure 4).

MiR-210 showed increased expression in juxta-tumoral stromal cells compared to tumor-remote stroma only in cases with tumor budding, while miR-21, miR-183, and miR-203 were found to be upregulated in the juxta-tumoral stroma in all cases, independently of the presence of tumor budding. MiR-217 was underexpressed in juxta-tumoral stroma in all cases independently of tumor budding, while miR-205 was not found to be dysregulated in the stromal cells in any of our cases. No microRNA dysregulation was found in the tumor-remote stroma (Figure 4).

In conclusion, our results suggest that tumor and stromal cells communicate via microRNAs. To assess if this communication network affects the expression of target genes, ZEB1 and ZEB2 were analyzed in juxta-tumoral and tumor-remote stromal cells. A negative correlation was found between miR-200b and miR-200c expression and ZEB1 and ZEB2 expression in the juxta-tumoral stromal cells ($P < 0.005$) (Figure 5 and Supplementary Figure 3).

A graphical representation of the results is depicted in Figure 6.

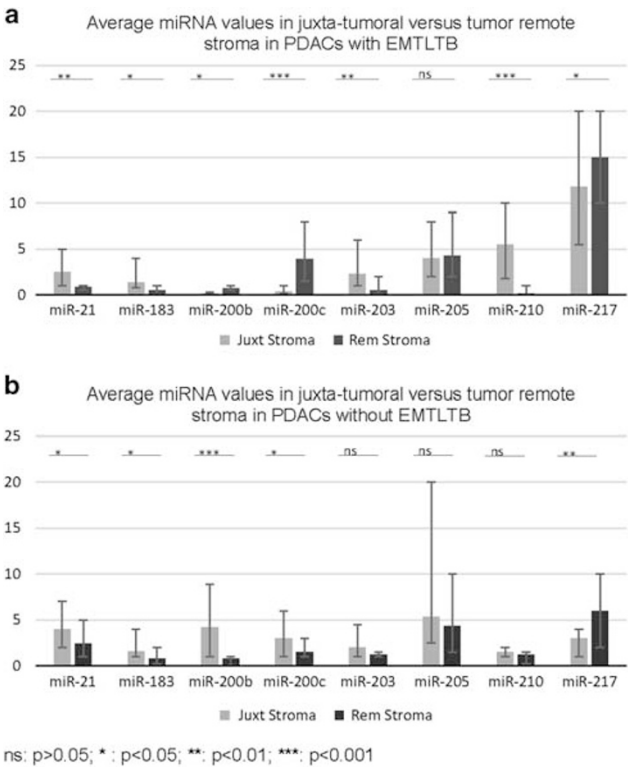

Figure 4 Expression of microRNAs in the juxta-tumoral stromal cells, in pancreatic ductal adenocarcinomas with (**a**) and without (**b**) tumor budding, in comparison with tumor-remote stroma.

Discussion

Here we show that specific microRNAs are differentially regulated between the main tumor cells of cases with and without epithelial–mesenchymal-transition-like tumor budding and between main tumor and tumor budding cells of cases with tumor budding. An inverse correlation in the expression of target genes involved in epithelial–mesenchymal transition was observed for some of these microRNAs, suggesting that they are directly implicated in the induction of the prognostically unfavorable tumor budding phenotype.

Among the most interesting microRNAs in this regard are the miR-200 family members, miR-200b, miR-200c, and miR-205. This functionally related microRNA family has been shown to play a crucial role in cancer through its strong suppressive effects on cell transformation, proliferation, migration, invasion, tumor growth, and metastasis.[29–31] Especially, overexpression of miR-200c has been shown to inhibit chemoresistance, invasion, and colony formation of human pancreatic cancer stem cells in pancreatic cancer cell lines.[32] In accordance to this, we find a marked downregulation of miR-200c and miR-205 in all tumor cells in cases with tumor budding, suggesting that loss of miR-200c and miR-205 expression and abrogation of their suppressive role on migration, taking place already in the main tumor, is a critical step for the formation of tumor buds. Moreover, in cases with tumor budding, miR-200b was significantly reduced in tumor buds compared to main tumor, suggesting that functionally related microRNAs act in a concerted action to induce tumor bud formation. Interestingly, the expression of miR-200b and miR-200c correlated negatively with the expression of ZEB1 and ZEB2 in the tumor microenvironment of pancreatic cancer. A negative feedback loop between ZEB proteins and microRNA-200 family has been described in various carcinoma models[33–35] and is considered to constitute the molecular basis for stabilization of either the epithelial or the mesenchymal state in the context of epithelial–mesenchymal transition.[36] Our findings suggest that this mechanism is also important for tumor bud formation in pancreatic cancer.

On the basis of bioinformatics target prediction (http://www.targetscan.org), miR-203 is able to bind to ZEB1 and ZEB2, but experimental evidence is still missing. In the present study, miR-203 and miR-200b give rise to a similar expression pattern in pancreatic ductal adenocarcinomas with or without tumor budding. Consistent with this finding, both microRNAs are predicted to bind to the same target genes. In the literature, there are contradicting results concerning miR-203 with some studies supporting a tumor promoting and others a tumor suppressive role.[37,38] However, it has also been suggested that miR-203 has a suppressing role on epithelial–mesenchymal transition, by interfering with the TGFb-SMAD3 pathway.[39] In our study, miR-203 expression showed no consistent correlation with

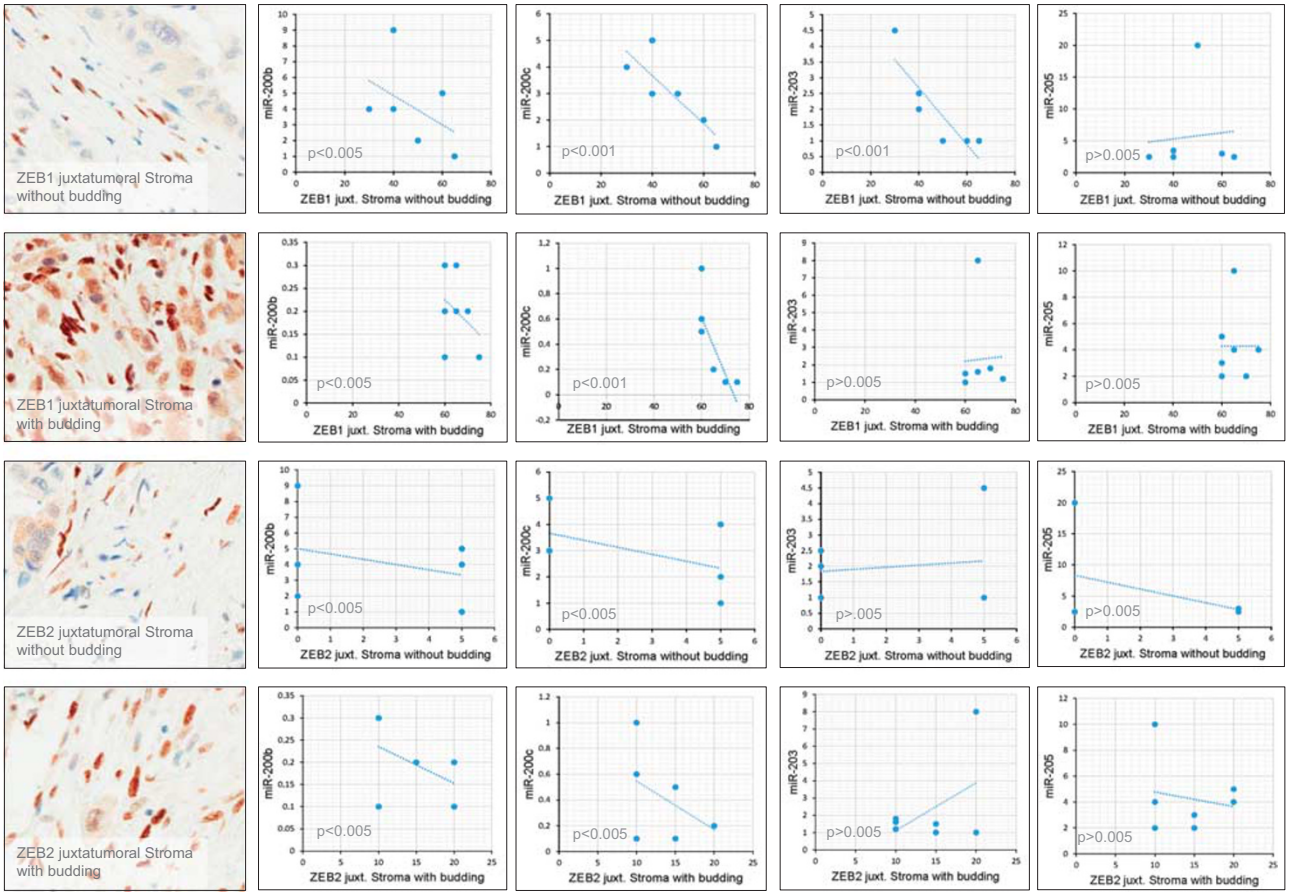

Figure 5 Correlation between ZEB1 and microRNA expression in the juxta-tumoral stromal cells of cases without (first row) and with tumor budding (second row), as well as between ZEB2 and microRNA expression in the juxta-tumoral stromal cells of cases without (third row) and with tumor budding (fourth row), concerning the microRNAs miR-200b, miR-200c, miR-203, and miR-205.

ZEB1 or ZEB2 expression, suggesting that it may affect epithelial–mesenchymal transition independently of ZEB1/ZEB2.

MiR-21 is known to promote epithelial–mesenchymal transition,[40–43] while miR-183 suppresses apoptosis and promotes proliferation,[44–46] functions that are necessary for the survival and further growth of the tumor buds. According to this, an aggravated upregulation of miR-21 and miR-183 was seen in the tumor buds compared to main tumor. However, both microRNAs were overexpressed in the tumor cells independently of tumor budding. MiR-217 was found to be downregulated in all tumor cells independently of tumor budding, consistent with its known function as a tumor-suppressing microRNA.[47,48]

In addition, our findings suggest that microRNAs are involved in the crosstalk between tumor and stromal cells, which is increasingly believed to be involved in the neoplastic progression of pancreatic cancer.[2–6] Indeed, communication between cancer and stromal cells via microvesicles containing microRNAs may represent one way by which tumor cells can modify their microenvironment. This is particularly important for the regulation of further tumor growth at the microenvironment of the invasive front. Aggressive pancreatic cancer cases show at the invasive front high density of dissociative growing cells with epithelial–mesenchymal-transition-like features (tumor buds). Tumor buds have reduced E-cadherin expression and are surrounded by stromal cells overexpressing the E-cadherin repressors ZEB1 and ZEB2.[19] In these cases, we show that miR-200b and miR-200c are significantly downregulated not only in the tumor buds, but also in the juxta-tumoral stromal cells, this being correlated with the high-level expression of ZEB1 and ZEB2. On the contrary, pancreatic cancer cases with an upregulation of miR-200c and miR-200b in the juxta-tumoral stroma show reduced expression of E-cadherin repressors ZEB1 and ZEB2. These tumors show a retained E-cadherin expression and absence or low density of tumor buds at the invasive front. Our findings in human pancreatic cancer material confirm previous findings from *in vitro* and mice experiments[49,50] highly suggesting that the balance between E-cadherin expression by the tumor cells on one hand and the expression of E-cadherin repressors by the stromal cells on the other hand is finely tuned by the expression of miR-200 family in the

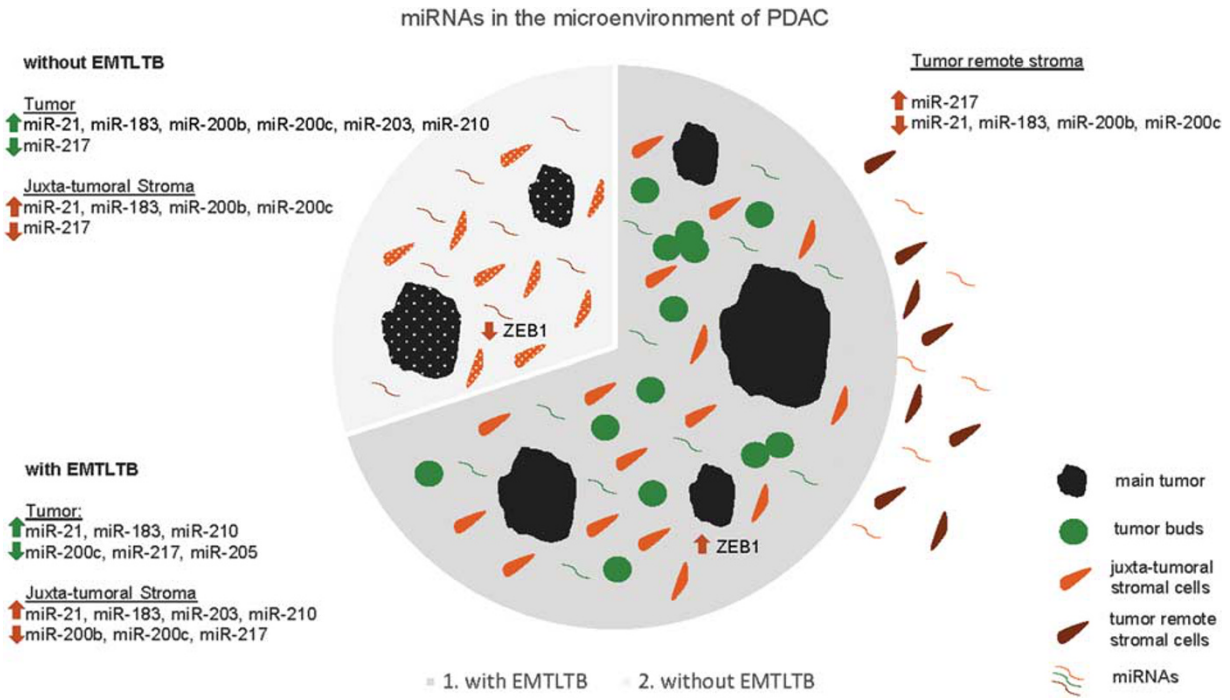

Figure 6 Graphic representation of the differences between pancreatic ductal adenocarcinomas with (**a**) or without (**b**) tumor budding.

tumor microenvironment. Moreover, stromal cells seem to regulate the expression of the tumor- and epithelial–mesenchymal-transition-promoting miR--210, exhibiting an upregulation in cases with and a downregulation in cases without tumor budding. Our results are consistent with a previous study showing that stellate cells induced miR-210 upregulation in co-culture experiments with Panc-1 cells, while inhibition of miR-210 expression decreased migration and expression of SNAIL-1, and increased the membrane-associated expression of β-catenin in cancer cells.[51] All these findings imply that stromal cells actively influence the differential microRNA expression within the tumor microenvironment in pancreatic cancer.

Furthermore, miR-21 and miR-183 were upregulated and miR-217 was downregulated in the juxta-tumoral stromal cells in all cases, analogous to their expression profile in the tumor cells. We could recently show that increased levels of miR-21 in stromal cells were associated with the loss of it's target protein PTEN, especially at the microenvironment of the invasive front of pancreatic ductal adenocarcinoma, which correlated with increased metastatic potential.[52] MiR-205 was not dysregulated in the stromal cells in agreement with recently published work depicting miR-205 as a purely epithelial microRNA in pancreatic cancer.[53]

This study has several major findings: analysis is based on a well-characterized cohort of pancreatic cancer cases and is designed in a hypothesis-driven approach employing advanced techniques for accurate examination of microRNA levels, while microRNA analysis is performed on human pancreatic cancer tissue reflecting the genuine state of the tumor microenvironment. Moreover, the expression of microRNAs was examined on material from several areas of importance, including non-neoplastic populations, main tumor, tumor buds, and stromal cells, which were separately analyzed to achieve a comprehensive picture of the microRNA levels in the tumoral and non-tumoral microenvironment.

In conclusion, our findings provide evidence for the dysregulation of specific microRNAs in the tumor microenvironment of pancreatic ductal adenocarcinoma, which may have an impact on the tumor budding phenotype. Since tumor budding is a powerful prognostic factor for pancreatic cancer, microRNAs regulating tumor bud formation can be regarded as attractive therapeutic targets. Moreover, by emphasizing the role of the stromal cells in tumor progression, our results support the concept of novel approaches of therapeutic intervention focusing on the interactions between cancer and stromal cells.

Acknowledgments

This project was supported by the Bernese Cancer League, the Werner and Hedy Berger-Janser Foundation, and the Foundation for Clinical-Experimental Tumor-Research. The funders had no involvement in the study design; in the collection, analysis, and interpretation of the data; in the writing of the report; and in the decision to submit the paper for publication.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)