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MicroRNA expression profiling in benign (sporadic and hereditary) and recurring adrenal pheochromocytomas

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MicroRNAs are involved in the pathogenesis of several tumors, however, there have been no data on microRNA expression in pheochromocytomas to date. The objective of our study was to perform microRNA expression profiling in sporadic and hereditary benign, and recurring adrenomedullary tumors. Furthermore, the applicability of formalin-fixed paraffin-embedded tissue samples for the analysis of microRNA expression in pheochromocytomas was examined. MicroRNA expression data of three matched frozen and formalin-fixed paraffin-embedded samples were correlated. A total of 21 formalin-fixed paraffin-embedded samples (sporadic benign, multiple endocrine neoplasia 2, von Hippel-Lindau disease, sporadic recurring) were subjected to microRNA expression profiling using microarrays. MicroRNAs with significant differences in expression were validated and sample sizes were extended including tumors from neurofibromatosis type 1 patients by real-time quantitative reverse-transcription PCR ($n = 33$). MicroRNA target prediction was carried out by TargetScan and MicroCosm Targets. Pathway analysis of targets was performed by Ingenuity Pathway Analysis and DIANA mirPath. Furthermore, microRNA expression profiles of a malignant pheochromocytoma and a pair of primary and recurrent tumors were studied by TaqMan Human MicroRNA Cards. MicroRNA expression correlated well between frozen and formalin-fixed paraffin-embedded samples (70–92%). Microarray analysis revealed 16 significantly differentially expressed microRNAs. Five of these were validated by real-time RT-PCR. miR-139-3p, miR-541 and miR-765 were significantly differentially expressed between sporadic benign and von Hippel-Lindau-related pheochromocytomas. Significantly higher expression of miR-885-5p and miR-1225-3p was found in multiple endocrine neoplasia type 2 and sporadic recurring pheochromocytomas, respectively. Pathway analysis revealed the possible involvement of Notch- and G-protein-coupled receptor signaling in tumor recurrence. MicroRNA expression profiles in the primary recurrent and recurring malignant comparisons have been similar. In conclusion, we have proved that formalin-fixed paraffin-embedded samples can be used for the analysis of microRNA expression in pheochromocytomas. MicroRNA expression patterns differ between various sporadic, hereditary and recurring tumors and miR-1225-3p may be useful for identifying recurring pheochromocytomas.
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MicroRNAs (miR, miRNA) have been implicated in the pathogenesis of several tumors. Different expression of miRNAs has been described in various endocrine neoplasms and miRNA expression patterns can be of great relevance for the establish-

ment of malignancy in tumors in which the histological analysis is difficult (eg follicular tumors of the thyroid and adrenocortical tumors).[1,2]

Pheochromocytomas are rare catecholamine-secreting tumors occurring in approximately 1–20 per million people per year in the general population.[3,4] Severe complications including hypertensive crisis, stroke and myocardial infarction are associated with pheochromocytomas. Surgical removal is the treatment of choice. Most pheochromocytomas are benign, but both local tumor recurrence and rarely malignancy (7–13% of sporadic adrenal pheochromocytomas)[5] may occur. Pheochromocytoma metastases may develop many years, even decades after the removal of the primary tumor.[6] The histological analysis of pheochromocytomas is not reliable for the establishment of malignancy: according to the definition by the World Health Organization, malignancy of pheochromocytomas is based on the detection of metastases.[4] Ki-67 and S100 have been suggested as possible immunohistochemical markers of malignancy, but their reliability is a matter of debate.[7,8]

Pheochromocytomas are unique among other tumors, because 25–30% of them arise due to germ-line mutations related to hereditary endocrine tumor syndromes.[6,9] Four major syndromes are known to be associated with pheochromocytomas: (1) multiple endocrine neoplasia type 2, (2) neurofibromatosis type 1, (3) von Hippel-Lindau disease and (4) hereditary paraganglioma syndromes. Mutations of the *RET* (rearranged during transfection), *NF1*, *VHL* and *SDH* (succinate dehydrogenase) *B*, *C* and *D* genes, respectively, are responsible for these syndromes. Mutations of the *SDHAF2* (*SDH5*) gene have been recently identified in patients having hereditary paraganglioma syndrome 2.[10] Several studies including reports on mRNA expression profiling revealed two major pathways of pheochromocytoma pathogenesis: (1) activation of the Ras oncogene pathways (multiple endocrine neoplasia type 2 and neurofibromatosis type 1), (2) activation of hypoxia-inducible factor 1α (HIF1α)-related neoangiogenesis pathways (von Hippel-Lindau and paraganglioma syndromes).[11]

To the best of our knowledge, there have been no reports on the expression of miRNAs in these tumors to date. Therefore, in this study, we have performed miRNA expression profiling of various groups of human adrenal pheochromocytomas including sporadic benign, multiple endocrine neoplasia type 2-related, von Hippel-Lindau disease-associated and neurofibromatosis type 1-related benign, and sporadic recurring tumors. Furthermore, miRNA expression profiles of a metastatic pheochromocytoma and a pair of primary and recurrent tumors have also been studied. Our study has focused exclusively on adrenal pheochromocytomas, therefore *SDH* gene mutation-related hereditary paraganglioma syndromes predisposing patients to mostly extraadrenal pheochromocytomas[4] have not been included. Owing to the rarity of these neoplasms, the retrieval of sufficient quantities of fresh or immediately snap-frozen tumor samples is difficult. We have therefore examined and proved the applicability of formalin-fixed paraffin-embedded archived tissue samples for the analysis of miRNA expression in pheochromocytomas.

Materials and methods

Patients and Tissues Samples

Altogether 34 patients with 35 adrenal pheochromocytomas were involved in this study: 9 patients with sporadic benign pheochromocytomas, 8 patients with multiple endocrine neoplasia type 2-related pheochromocytomas, 6 patients with von Hippel-Lindau disease-associated pheochromocytomas, 5 patients with neurofibromatosis type 1-associated pheochromocytomas, 5 patients with sporadic recurring adrenomedullary tumors and 1 patient with a metastatic/malignant pheochromocytoma. Hereditary pheochromocytomas occurring in unrelated families were examined. The characteristics of patients are summarized in Table 1.

Diagnoses were set according to current clinical guidelines, histological analysis and the results of genetic testing. Patients were followed up for 5–7 years after surgery. In more recent cases, pheochromocytomas were only included in the benign group if tumor diameter was below 5 cm,[12] no metastasis was found and Ki-67 immunostaining was not suggestive of malignancy. Recurrent pheochromocytoma was defined as a histologically verified tumor at the same localization as the surgically removed first tumor. We have focused on the first tumors, but we have also examined the miRNA expression profiles in a pair of a primary recurring and recurrent tumors from the same patient.

Formalin-fixed paraffin-embedded tissue samples were collected from pathological archives. The age of formalin-fixed paraffin-embedded tissue blocks used for RNA isolation ranged from 0.2 to 14.6 years. Three tumors (one sporadic benign, one multiple endocrine neoplasia type 2 and one sporadic recurring) were available as both snap-frozen and formalin-fixed paraffin-embedded samples, and have been used for correlation studies. The study was approved by the ethical committee of the Hungarian Health Council.

Mutation Analysis

Mutation analysis of the *RET*, *VHL*, *SDHB*, *SDHC* and *SDHD* genes has been performed by direct sequencing of DNA extracted from peripheral blood leukocytes, as previously described.[13,14] Pheochromocytomas were only regarded as sporadic benign or sporadic recurring, if germ-line mutation in any

Table 1 Characteristics of patients

	Sporadic benign	*Sporadic recurring*	*Multiple endocrine neoplasia type 2*	*von Hippel-Lindau disease*	*Neuro-fibromatosis type 1*	*Malignant*
Number of patients	9	5	8	6	5	1
Female/male	6/3	2/3	6/2	3/3	4/1	1/0
Age at surgery for the primary tumor (years), mean ± s.d.	44.8 ± 18.1	46.0 ± 15.3	38.8 ± 10.2	44.8 ± 20.8	38.7 ± 16.6	54.4
Largest tumor diameter (if available) (cm), mean ± s.d.	5.2 ± 1.2 ($n=6$)	6.7 ± 4.1 ($n=5$)	4.8 ± 2.1 ($n=7$)	4.1 ± 2.4 ($n=5$)	5.3 ± 1.5 ($n=3$)	13 ($n=1$)
Metastases (synchronous)	—	—	—	—	—	liver
Period until recurrence (years) (Mean ± s.d.)	—	4.25 ± 2.8	—	—	—	—

Abbreviation: s.d.: standard deviation.
Altogether 34 patients with 35 adrenal pheochromocytomas have been involved.

candidate pheochromocytoma gene has been excluded. The diagnosis of neurofibromatosis type 1 was excluded by clinical examination. For the mutations found in multiple endocrine neoplasia type 2 and von Hippel-Lindau disease patients, see Supplementary Table 1. We have found no *SDH* gene mutation in our patients.

RNA Isolation

Total RNA isolation was carried out after 100% xylene deparaffinization from altogether five slices of formalin-fixed paraffin-embedded tissue sections with 15 μm thickness, by Ambion RecoverAll Total Nucleic Acid Isolation Kit (Applied Biosystems/Ambion, Austin, TX, USA) according to the manufacturer's protocol. If required, extended deparaffinization and protease digestion at 50°C was performed.

Total RNA extraction from fresh-frozen pheochromocytoma samples was performed after tissue homogenization with Qiagen miRNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) completed with optional on-column DNase digestion carried out by RNase-Free DNase Set (Qiagen GmbH).

RNA quality and quantity was determined by NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), whereas RNA integrity was verified by Agilent 2100 Bioanalyzer System (Agilent Technologies Inc., Santa Clara, CA, USA). RNA samples extracted from fresh-frozen tissues were only subjected to downstream applications when RNA integrity number exceeded 7.0.

miRNA Expression Profiling

miRNA expression profiling was performed on 21 formalin-fixed paraffin-embedded (6 sporadic benign, 5 multiple endocrine neoplasia type 2, 5 von Hippel-Lindau disease, 5 sporadic recurring) and 3 fresh-frozen pheochromocytoma tissue samples by 8 × 15K Agilent Human miRNA Microarray Rel12.0 (Agilent Technologies Inc.) containing probes for 955 miRNAs.

Total RNA (100 ng) was dephosphorylated, denaturated and Cyanine3-pCp ligated according to the manufacturer's instructions using miRNA Complete Labeling and Hyb Kit (Agilent Technologies Inc.). Labeled RNA was purified on Micro Bio-Spin P-6 Columns (Bio-Rad Laboratories, Hercules, CA, USA) and hybridized to Agilent Human miRNA Microarray Rel12.0 (Agilent Technologies Inc.) according to the manufacturer's instructions.

After washing microarray slides, array scanning and feature extraction was performed with default scenario by Agilent DNA Microarray Scanner and Feature Extraction Software 9.5.3 (Agilent Technologies Inc.). Total gene signal normalization at the 75th percentile of raw signal values and baseline transformation at the median of each array was performed by GeneSpring software 10.1 (Agilent Technologies Inc.) following Agilent's recommendation. Before statistical analysis of microarray data, flag (100% present in at least one group) and fold change (fold change >2) filters were applied by GeneSpring software 10.1. Flag-filtered (100% present or marginal in each group) and normalized microarray data were used for selection of housekeeping miRNAs with low interarray standard deviation in signal intensities. For correlation studies between formalin-fixed paraffin-embedded and frozen samples no interarray normalization and filter were applied as recommended by the manufacturer and described by Zhang *et al.*[15]

Quantitative Reverse-Transcription PCR Validation

Five miRNAs with the highest significant differences in expression and highest fold change revealed by miRNA microarray experiments were selected for further validation and sample size extension including tumors from neurofibromatosis type 1 patients by quantitative real-time reverse-transcription PCR (qRT-PCR). TaqMan MicroRNA

Assays were used as follows: hsa-miR-139-3p (002313), hsa-miR-541 (002201), hsa-miR-765 (002643), hsa-miR-885-5p (002296), hsa-miR-1225-3p (002766), hsa-miR-324-3p (002161), hsa-miR-320b (002844) and RNU48 (001006) (all materials from Applied Biosystems, Foster City, CA, USA). Total RNA (10 ng) was reverse transcribed by MicroRNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR was performed using TaqMan Fast Universal PCR Master Mix on a 7500 Fast Real-Time PCR System according to the manufacturer's protocol. All samples were run in triplicate.

Normalized signal levels for each miRNA were calculated using comparative cycle threshold method (ddCT method)[16] relative to the mean of miR-324-3p and miR-320b following the manufacturer's instruction (SDS Program; Applied Biosystems).

Statistical Analysis

Correlation analysis between the raw total gene signals of the three matched formalin-fixed paraffin-embedded and frozen samples retrieved from miRNA microarray analysis was performed by SPSS 15.0 for Windows statistical software (SPSS Inc., Chicago, IL, USA). Spearman and Kendall τ nonparametric rank correlation coefficients were calculated between all three matched samples and between the means of frozen and formalin-fixed paraffin-embedded groups. Total gene signals derived from matched formalin-fixed paraffin-embedded and frozen tissues were plotted together by GeneSpring 10.1 software (Agilent Technologies Inc.).

Normalized and filtered microarray data were further subjected to housekeeping selection and statistical analysis was performed by Microsoft Office Excel 2003 (Microsoft Corp., Redmond, WA, USA), GeneSpring 10.1 (Agilent Technologies Inc.) and STATISTICA 8.0 (StatSoft Inc., Tulsa, OK, USA) software applications. Identification of miRNAs differentially expressed in microarray analysis was carried out by one-way ANOVA ($P<0.01$) followed by Tukey's honestly significant difference test.

qRT-PCR data were subjected to one-way ANOVA ($P<0.05$) followed by Scheffé's and Fisher's least significant difference (Fisher's LSD) *post hoc* tests. Statistical analysis was carried out by STATISTICA 8.0 (StatSoft Inc.). Potential biomarker miRNAs appropriate for distinguishing recurring pheochromocytomas were tested by receiver operating characteristics (ROC) analysis (SPSS 15.0 for Windows).

miRNA Target Prediction and Pathway Analysis

Computationally predicted mRNA targets of the significantly differentially expressed miRNAs among the groups studied were identified by TargetScan Release 5.1 (http://www.targetscan.org)[17] and MicroCosm Targets version 5 (http://www.ebi.ac.uk/enright-srv/microcosm).[18]

The outputs of these target prediction algorithms were merged and all predicted targets (including overlapping targets) were revealed by Microsoft Visual FoxPro 9.0 (Microsoft Corp.) developer tool using the annotation database downloaded from the Ensembl homepage (www.ensembl.org).[2,19]

Targets of significantly differentially expressed miRNAs were subjected to pathway analysis in all pair-wise comparisons (von Hippel-Lindau disease *vs* sporadic benign, von Hippel-Lindau disease *vs* sporadic recurring, von Hippel-Lindau disease *vs* neurofibromatosis type 1, multiple endocrine neoplasia type 2 *vs* von Hippel-Lindau disease, multiple endocrine neoplasia type 2 *vs* sporadic benign, multiple endocrine neoplasia type 2 *vs* sporadic recurring, multiple endocrine neoplasia type 2 *vs* neurofibromatosis type 1, sporadic recurring *vs* sporadic benign). Union of mRNA targets predicted by the aforementioned algorithms and revealed by Microsoft Visual FoxPro 9.0 was analyzed by Ingenuity Pathway Analysis (IPA) 8.5 software (Ingenuity Systems, Redwood City, CA, USA; www.ingenuity.com) to identify canonical pathways in pheochromocytomas potentially modified by miRNAs. DIANA mirPath software[20] analysis was also performed using DIANA-MicroT-4.0 and TargetScan 5 prediction algorithms.

miRNA Expression Profiling of a Malignant Pheochromocytoma and a Pair of Primary Recurrent Pheochromocytomas by TaqMan Human miRNA Cards

We have used TaqMan Human MicroRNA 'A' Cards version 2.0 (Applied Biosystems) for the analysis of miRNA expression pattern of a malignant pheochromocytoma, and a pair of primary and recurrent pheochromocytomas from the same patient.

Total RNA isolation from the formalin-fixed paraffin-embedded samples and quality control was carried out as above. Altogether 75 ng total RNA was reverse transcribed by TaqMan MicroRNA Reverse Transcription Kit using Megaplex RT Primers Human Pool 'A'. Downstream cDNA preamplification reactions were prepared using Megaplex PreAmp Primers Human Pool 'A' according to the manufacturer's instructions (Applied Biosystems). qRT-PCR reactions were run on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Normalized signal levels for each miRNA were calculated by the ddCT method[16] relative to miR-324-3p (miR-320b is not represented on the array) following the manufacturer's instructions (SDS Program; Applied Biosystems). miRNA expression profiles were compared in primary *vs* recurrent and the malignant *vs* primary tumor comparisons. Expression values (2^{-dCT}) of all 384 miRNAs were correlated by SPSS 15.0 for Windows statistical software (SPSS Inc.). Spearman and Kendall τ nonparametric rank correlation coefficients were calculated.

As TaqMan Human MicroRNA 'A' Cards, version 2.0, do not include miR-765 and miR-1225-3p, these were measured in separate real-time qRT-PCR reactions along with miR-139-3p, miR-541, miR-885-5p, miR-324-3p, miR-320b and RNU48. Real-time qRT-PCR experiments were performed and normalized signal levels for each miRNA were calculated as above detailed. qRT-PCR results of the malignant sample were compared to the five experimental groups whereas the data of the recurrent pheochromocytoma sample were compared with the primary tumor of the same patient.

Results

RNA Integrity and miRNA Expression Correlation between Matched Frozen and Formalin-Fixed Paraffin-Embedded Samples

Total RNA quality was found to be superior in frozen samples (RNA integrity number: 7.7–9.0). RNA degradation in formalin-fixed paraffin-embedded samples (RNA integrity number: 2.0–4.0) turned out to be similar to previous findings on formalin-fixed paraffin-embedded samples from other tissues[15,21] (Supplementary Figure 1).

Three pairs of frozen and formalin-fixed paraffin-embedded samples derived from the same tumor tissues were subjected to miRNA expression profiling. Microarray results were correlated by nonparametric analysis. Spearman's rank correlation coefficient ranged from 0.7 to 0.93, whereas the correlation coefficient calculated by the Kendall's τ method turned out to be 0.58–0.85. Good correlation (Spearman's coefficient 0.86; Kendall's τ 0.72) was found between the means of total gene signals in frozen and formalin-fixed paraffin-embedded groups ($n = 3$) as well. Correlations between formalin-fixed paraffin-embedded and frozen samples are represented on scatter plots (Figure 1).

MiRNA Expression Profiling in Formalin-Fixed Paraffin-Embedded Samples

Having proven the applicability of formalin-fixed paraffin-embedded samples for pheochromocytoma miRNA profiling, 21 formalin-fixed paraffin-embedded samples were subjected to miRNA microarray analysis. All results are accessible at Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo) under accession number GSE21767.

Following flag filtering of microarray results, 478 miRNAs (50% of all miRNAs represented on the microarrays) have been found to be detectable in at least one experimental group ('present' or 'marginal'

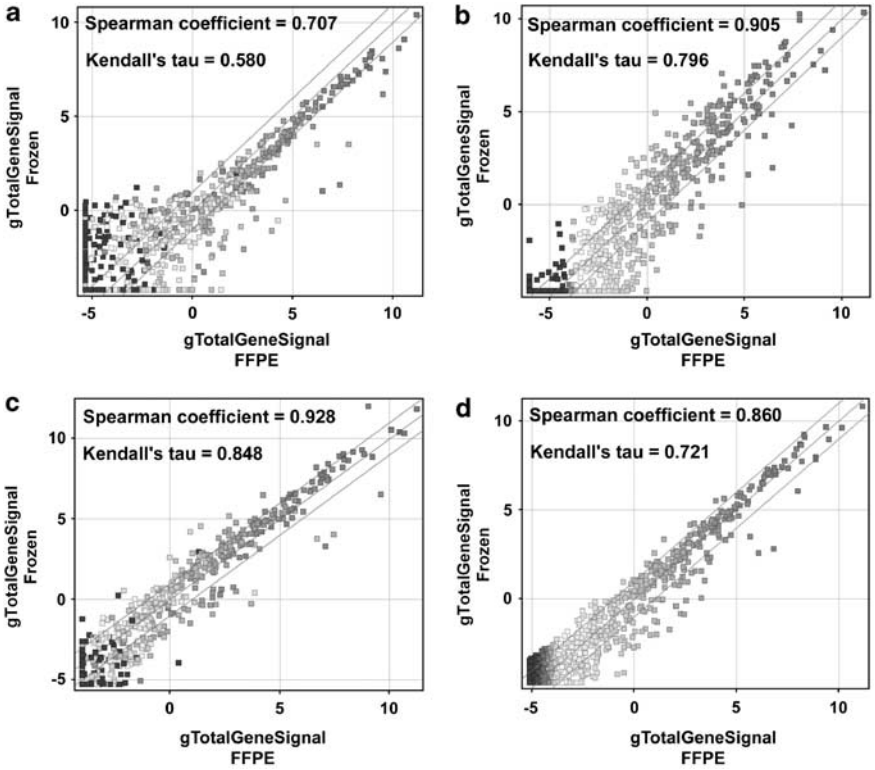

Figure 1 Correlation of miRNA microarray data for matched frozen and formalin-fixed paraffin-embedded samples. Spearman's and Kendall's τ nonparametric rank correlation coefficients were calculated using raw total gene signals retrieved from paired frozen and formalin-fixed paraffin-embedded samples derived from the same pheochromocytoma tumor tissue. Scatter plots of sporadic benign (**a**), sporadic recurring (**b**) and multiple endocrine neoplasia type 2-related (**c**) pheochromocytoma sample pairs, and correlation for the means of frozen and formalin-fixed paraffin-embedded groups ($n = 3$) (**d**) are shown.

flag in all samples of the group). Comparison of the four microarray groups based on the expression of detectable miRNAs (>2-fold change) is represented on the heat-map (Figure 2). Both miRNAs and the experimental groups were classified by hierarchical clustering based on the similarities in their expression profiles. Regarding the strength of miRNA expression patterns, recurring pheochromocytomas differed most from other groups.

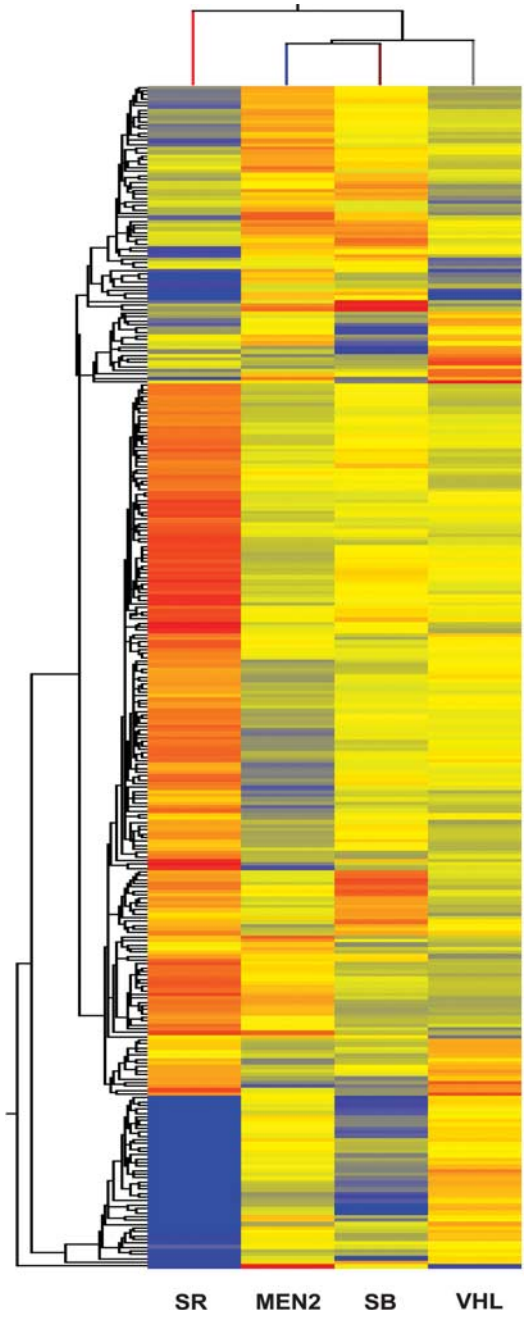

Figure 2 Hierarchical clustering of the four experimental groups based on their miRNA expression patterns. Following flag (100% 'present' or 'marginal' in at least one experimental group) and fold change (fold change>2) filtering of microarray results, 309 miRNAs were subjected to hierarchical clustering using GeneSpring software. Normalized signal intensities of these miRNAs were clustered after mean centering them for each miRNA and the experimental groups. Both miRNAs and the groups were clustered by centroid linkage method calculating with Euclidean distance. Overexpression is represented with red, whereas underexpression is indicated with blue. SB: sporadic benign, MEN2: multiple endocrine neoplasia type 2, VHL: von Hippel-Lindau disease, SR: sporadic recurring.

Statistical analysis of miRNA microarray data performed by GeneSpring 10.1 software revealed 14 miRNAs with significant differences in expression ($P<0.01$) among the experimental groups studied (>2-fold change). In a separate analysis carried out with STATISTICA 8.0 statistical software (StatSoft Inc.) using one-way ANOVA with the same parameters, 16 miRNAs differed significantly ($P<0.01$; Supplementary Table 2). A total of 11 miRNAs overlapped between these two significant miRNA sets. Two significantly differentially expressed miRNAs in the sporadic benign von Hippel-Lindau disease, one in sporadic benign-sporadic recurring, one in von Hippel-Lindau disease multiple endocrine neoplasia type 2, eleven in sporadic recurring von Hippel-Lindau disease and five in sporadic recurring multiple endocrine neoplasia type 2 comparison have been revealed. The comparison of sporadic recurring tumors to all other benign samples showed 12 miRNAs with significant differences in expression.

MiRNA microarray results were also used to select potential housekeeping miRNAs applicable for downstream qRT-PCR normalization. miR-324-3p and miR-320b have been identified as the most appropriate housekeeping miRNAs based on the low interarray standard deviation of their normalized total gene signals. These two miRNAs were previously described as suitable miRNA housekeeping genes in a large-scale miRNA expression analysis performed on various normal human tissues.[22]

qRT-PCR Experiments

Five miRNAs have been selected for further qRT-PCR validation and sample size extension including neurofibromatosis type 1 pheochromocytomas ($n=33$): miR-139-3p, miR-541, miR-765, miR-885-5p and miR-1225-3p. miR-324-3p, miR-320b and a generally applied small nucleolar RNA, RNU48 have been tested as housekeeping genes. As the cycle threshold (C_t) values of miR-324-3p and miR-320b showed the least standard deviation among the potential housekeeping miRNAs studied, the mean cycle threshold values of these two miRNAs were calculated and used for normalization in each sample.

Significant differences in expression for all selected miRNAs could be verified by qRT-PCR (Figure 3). Significantly higher expression of miR-541, miR-139-3p and miR-765 could be detected in von Hippel-Lindau disease samples compared with sporadic benign pheochromocytomas. Significant overexpression of miR-885-5p in multiple endocrine neoplasia type 2-associated pheochromocytomas

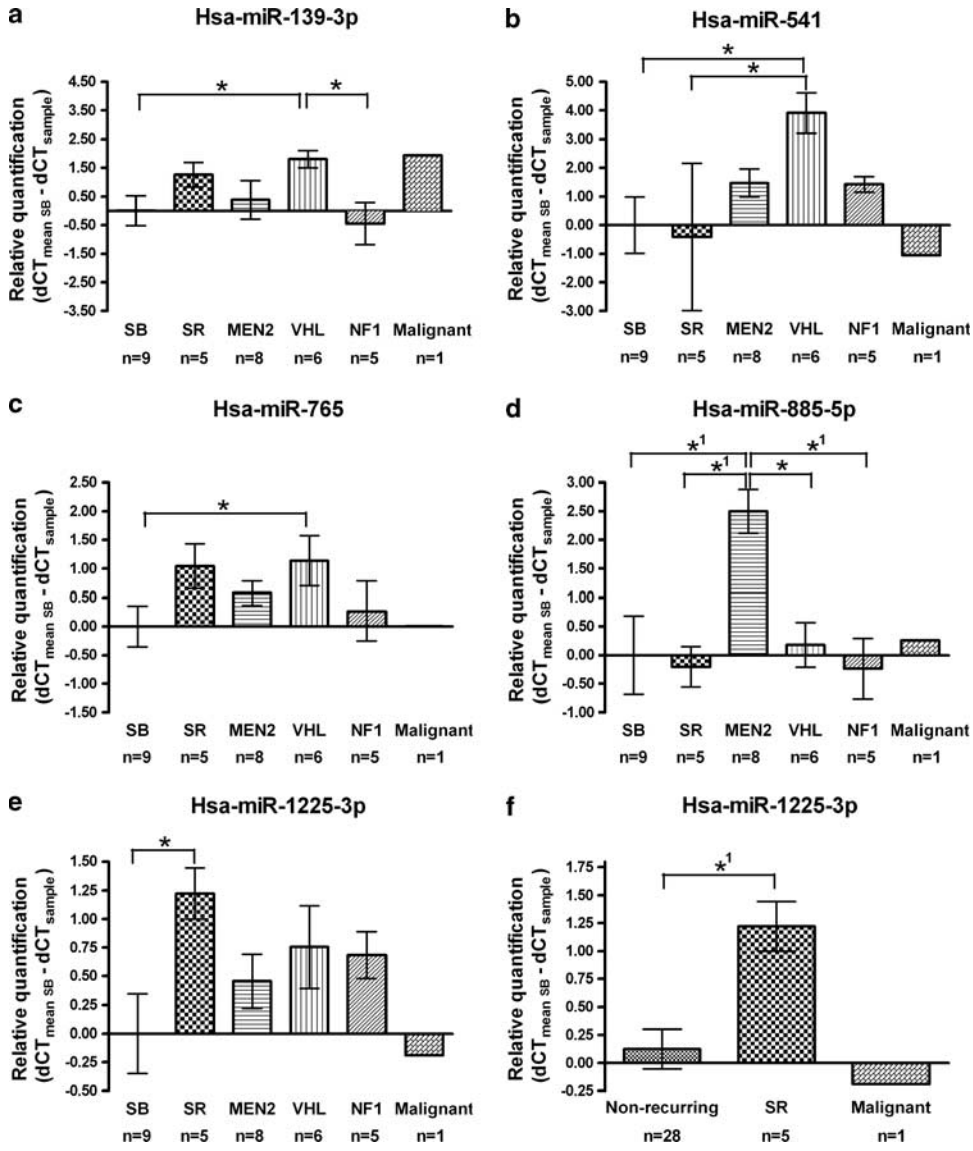

Figure 3 Significantly differentially expressed miRNAs validated by quantitative real-time RT-PCR. Results are represented by ddCT (cycle threshold) values relative to the mean of sporadic benign samples (mean ± s.e.m.). *$P<0.05$ (*post hoc* test: Fischer's LSD; [1]*post hoc* test: Fischer's LSD + Scheffé's). Expression of miR-139-3p (**a**), miR-541 (**b**) and miR-765 (**c**) was significantly overexpressed in von Hippel-Lindau disease-related pheochromocytomas. Overexpression of miR-885-5p (**d**) is characteristic for multiple endocrine neoplasia type 2 pheochromocytomas. miR-1225-3p was significantly upregulated in sporadic recurring pheochromocytomas relative to sporadic benign (**e**), and to nonrecurring (**f**) samples. SB: sporadic benign, MEN2: multiple endocrine neoplasia type 2, VHL: von Hippel-Lindau disease, NF1: neurofibromatosis type 1, SR: sporadic recurring.

has been observed relative to sporadic benign, sporadic recurring, von Hippel-Lindau disease- and neurofibromatosis type 1-related tumors. Expression of miR-1225-3p has been significantly higher in sporadic recurring relative to benign tumors, whereas, significantly lower expression of miR-541 has been found in sporadic recurring relative to von Hippel-Lindau disease tumors. It is important to note that the expression of RNU48 that is widely used as an internal control in miRNA studies has been significantly different between multiple endocrine neoplasia type 2- and neurofibromatosis type 1-related pheochromocytomas. RNU48 is therefore inappropriate for the study of miRNA expression in pheochromocytomas (Figure 4).

We have tested the dCT value of miR-1225-3p in the five experimental groups ($n=33$) as a potential biomarker characteristic for tumor recurrence by ROC analysis. Setting the cutoff value of $dCT_{miR\text{-}1225\text{-}3p}$ to 1.16 (diagnosis is nonrecurring pheochromocytoma if dCT is $\geqslant 1.16$), a pheochromocytoma sample can be regarded as not prone to recurrence with 80% specificity and 61% sensitivity.

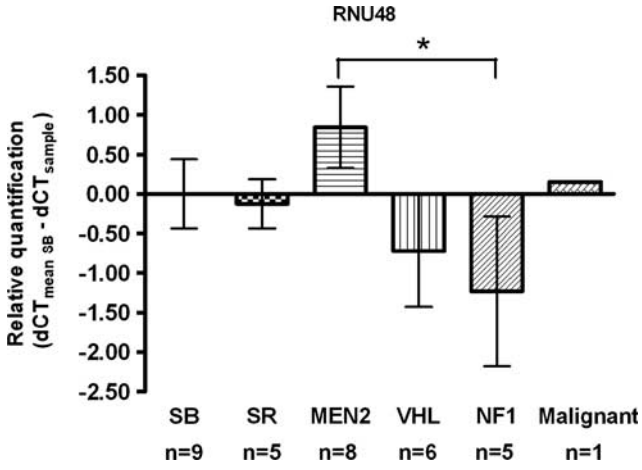

Figure 4 Expression of RNU48 (results of qRT-PCR analysis). Results are represented by ddCT (cycle threshold) values relative to the mean of sporadic benign samples (mean ± s.e.m.). $^{*}P<0.05$ (*post hoc* test: Fischer's LSD; [1]*post hoc* test: Fischer's LSD + Scheffé's). SB: sporadic benign, MEN2: multiple endocrine neoplasia type 2, VHL: von Hippel-Lindau disease, NF1: neurofibromatosis type 1, SR: sporadic recurring.

MiRNA Target Prediction and Pathway Analysis

Computational target prediction of the five significantly differentially expressed miRNAs has been performed with two widely applied algorithms: TargetScan Release 5.1 and MicroCosm Targets Version 5. Target prediction for miR-1225-3p has only been performed with the TargetScan algorithm, as it is not yet included in the other. Without using score threshold and site conservation filter, altogether 13 530 miRNA-mRNA target interactions could be identified for the five miRNAs. Among these predictions, 10 511 and 4323 have been given by TargetScan and MicroCosm, respectively. A total of 638 interactions have been predicted by both algorithms.

mRNA targets of significantly differentially expressed miRNAs in all pair-wise comparisons have been subjected to pathway analysis to decipher the function of predicted miRNA targets in biological processes and to identify pathogenetic pathways potentially modified by posttranscriptional regulation.

Among the top canonical pathways potentially affected by the significantly differentially expressed miRNAs, 'Wnt/β-catenin signaling' in von Hippel-Lindau disease sporadic recurring comparison and 'Myc-mediated apoptosis signaling' in multiple endocrine neoplasia type 2 *vs* any other pheochromocytoma group comparison was noteworthy. In the sporadic recurring pheochromocytoma group, 'Notch signaling' (Figure 5) and 'G-protein-coupled receptor signaling' have been established that may be posttranscriptionally inhibited by miR-1225-3p. Pathway analysis of miRNA target mRNAs performed with DIANA mirPath software has revealed partially concordant pathways. For detailed IPA and DIANA results, see Supplementary Tables 3 and 4, respectively.

MiRNA Expression Profiling of a Malignant Pheochromocytoma and a Pair of Primary Recurrent Pheochromocytoma

MiRNA expression profiles determined by TaqMan Human MicroRNA Arrays version 2.0 were compared in primary *vs* recurrent and malignant *vs* primary recurring tumor comparisons. Expression values correlated strongly in both comparisons. Spearman's rank correlation coefficient turned out to be 0.97 whereas Kendall's τ was 0.87 between the primary and the recurrent tumor derived from the same patient. In malignant *vs* primary tumor comparison the Spearman's coefficient was found to be 0.94 and the Kendall's τ was 0.81. Of all investigated miRNAs, 18 and 19% were undetectable in both tumors in primary *vs* recurrent and primary *vs* malignant comparisons, respectively. MiRNAs expressed in only one tumor of paired comparisons are presented in Supplementary Table 5. MiRNAs expressed in both tumors and showing the greatest expression differences are listed in Tables 2 and 3.

Expression of the five significantly differentially expressed miRNAs has been studied by single qRT-PCR experiments in the malignant and the recurrent pheochromocytoma sample as well. In the primary recurrent comparison, miR-765, miR-885-5p and miR-1225-3p showed 2.5-, 1.1- and 1.3-fold overexpression in the recurrent tumor, respectively, whereas underexpression of miR-139-3p (0.8-fold) and miR-541 (0.6-fold) was observed relative to the primary tumor. Expression data of miR-139-3p, miR-541 and miR-885-5p have been similar between the single malignant tumor examined and the mean of sporadic recurring tumors, whereas the expression of miR-1225-3p has been considerably higher in sporadic recurring tumors (Figure 3).

Discussion

To the best of our knowledge, this is the first report on miRNA expression profiling in sporadic, hereditary and recurring human adrenomedullary tumors to date. Because pheochromocytomas are rare neoplasms,[4,6] collection of fresh or frozen tumor tissues as gold standard starting materials for gene expression studies is difficult. In this study, miRNA expression profiling has been performed on total RNA samples extracted from archived formalin-fixed paraffin-embedded blocks. Gene expression (mRNA) analysis of RNA extracted from archived formalin-fixed paraffin-embedded samples is a great scientific challenge, because RNA recovery and quality is significantly reduced by formalin fixation due to chemical modifications of nucleic acids, RNA

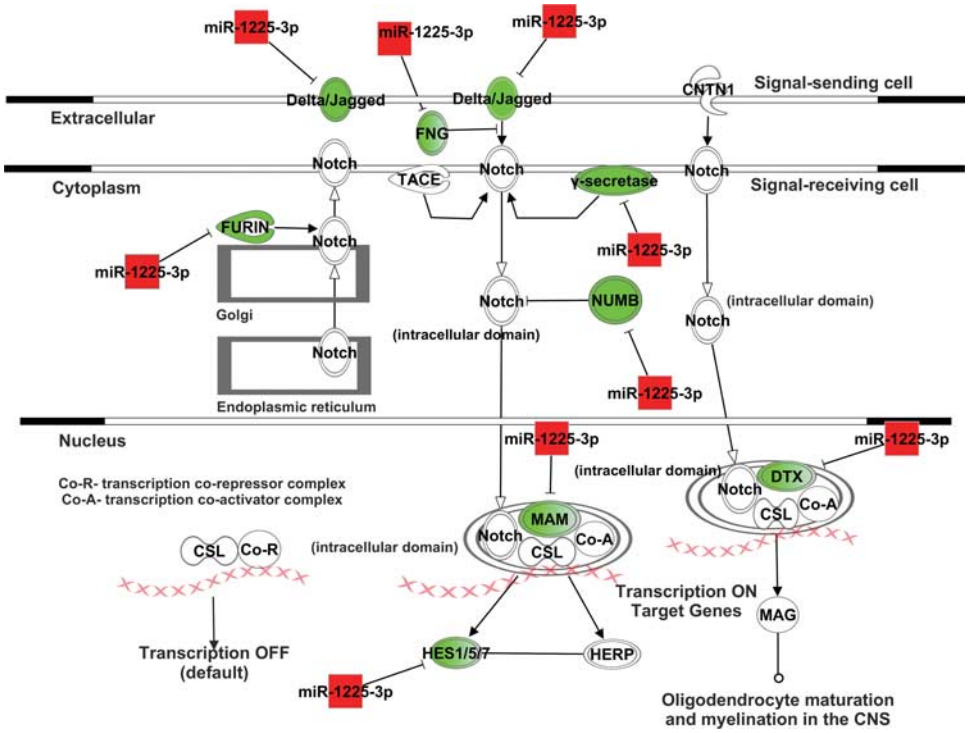

Figure 5 'Notch signaling' as one of the top canonical pathways potentially affected by microRNA-based posttranscriptional repression in sporadic recurring pheochromocytomas (Ingenuity Pathway Analysis). Lines between the proteins represent known interactions. Red rectangles indicate miR-1225-3p overexpressed in recurring pheochromocytomas, green: potentially repressed target mRNAs.

Table 2 Results of TaqMan Human MicroRNA Card experiments for the comparison of a primary and recurrent pheochromocytoma sample derived from the same patient (age at surgery for the primary tumor: 24.3 years, time until recurrence: 8.75 years)

Overexpressed miRNAs[a]	*Fold change*	*Underexpressed miRNAs*[a]	*Fold change*
hsa-miR-876-5p	23.86	hsa-miR-520f	0.01
hsa-miR-200a	16.17	hsa-miR-217	0.02
hsa-miR-519e	7.51	hsa-miR-515-3p	0.02
hsa-miR-380	7.12	hsa-miR-503	0.04
hsa-miR-544	6.88	hsa-miR-642	0.04
hsa-miR-369-3p	6.34	hsa-miR-193b	0.05
hsa-miR-153	6.04	hsa-miR-504	0.08
hsa-miR-542-3p	4.89	hsa-miR-886-5p	0.09
hsa-miR-889	4.84	hsa-miR-183	0.10
hsa-miR-190	4.63	hsa-miR-215	0.12

The top 10 miRNAs over- or underexpressed in the recurrent tumor relative to the primary one are indicated.
[a]The indicated miRNAs are detectable in both groups of comparison.

Table 3 Results of TaqMan Human MicroRNA Card experiments for the comparison of a primary recurring and a malignant/metastatic pheochromocytoma sample

Overexpressed miRNAs[a]	*Fold change*	*Underexpressed miRNAs*[a]	*Fold change*
hsa-miR-202	314.62	hsa-miR-431	5.86E-06
hsa-miR-518d-3p	214.53	hsa-miR-541	0.01
hsa-miR-508-3p	205.88	hsa-miR-18b	0.02
hsa-miR-376b	151.11	hsa-miR-34c-5p	0.03
hsa-miR-509-5p	87.33	hsa-miR-888	0.03
hsa-miR-885-5p	25.37	hsa-miR-891a	0.04
hsa-miR-449a	20.75	hsa-miR-410	0.04
hsa-miR-135a	18.96	hsa-miR-433	0.04
hsa-miR-33b	12.51	hsa-miR-136	0.05
hsa-miR-449b	8.19	hsa-miR-210	0.05

The top 10 miRNAs over- or underexpressed in the malignant tumor relative to the primary recurring one are indicated.
[a]The indicated miRNAs are detectable in both groups of comparison.

fragmentation, and formation of cross-links between RNA and proteins. Owing to their small size, miRNAs are less prone to degradation,[15,23] therefore, formalin-fixed paraffin-embedded samples are more suitable for miRNA than for mRNA expression analyses. Storage up to 7 years did not cause significant deterioration of miRNA quality, but older samples may exhibit loss of small RNA quality due to oxidation and fixation in nonbuffered formalin.[24] Formalin-fixed paraffin-embedded samples have already been successfully used for miRNA expression profiling in various tissues,[15,25,26] but there have been no data on their applicability for miRNA expression analysis in pheochromocytomas. We have observed good correlation between miRNA microarray results retrieved from three matched frozen and formalin-fixed paraffin-embedded samples derived from the same pheochromocytoma tissues. The correlation coefficients have been similar to previous reports studying formalin-fixed paraffin-embedded samples from other tissues.[15,25,26] These findings confirm the applicability of archived formalin-fixed paraffin-embedded blocks for miRNA expression analysis of human pheochromocytoma samples.

Our principal objective has been to compare the miRNA expression patterns of sporadic benign, sporadic recurring, hereditary multiple endocrine neoplasia type 2-related and von Hippel-Lindau disease-associated pheochromocytomas using high-throughput miRNA microarray analysis. By hierarchical clustering based on miRNA expression patterns, sporadic recurring pheochromocytomas have been most definitely separated from the other groups. This observation might be important from two aspects: (1) sporadic recurring pheochromocytomas may harbor distinct miRNA-regulated pathogenic routes; (2) miRNA markers might be used for the differentiation of recurring and nonrecurring pheochromocytomas. Statistical analysis of microarray data revealed 16 significantly differentially expressed miRNAs among the experimental groups, and we have successfully validated all five miRNAs selected for further qRT-PCR analysis including tumor samples from neurofibromatosis type 1 patients. Unfortunately, there are only very few data on the biological relevance of these five miRNAs in other tissues.

We have found miR-139-3p, miR-541 and miR-765 to be significantly overexpressed in von Hippel-Lindau disease-related pheochromocytomas relative to sporadic benign group.

miR-139 was previously described to be downregulated in primary squamous cell carcinoma of the tongue,[27] parathyroid cancer and adenoma,[28] and gastric cancer.[29] Two mature forms of miR-139 have been cloned: miR-139-3p processed from the 3′ arm and miR-139-5p processed from the 5′ arm of the stem loop sequence. In contrast with the mostly observed underexpression of miR-139 (and miR-139-5p) in various tumors, miR-139-3p has been significantly overexpressed in von Hippel-Lindau disease-related pheochromocytomas. As miRNAs function in a tissue-specific manner, the same miRNA might be overexpressed or underexpressed in different tumors.[2] The biological activity of miR-139-3p is unclear. The transcriptional factor FoxO1 (Forkhead box O1) involved in the regulation of signaling pathways coupled to growth factors and hormones was shown to be translationally repressed by miR-139,[30] but interaction between FoxO1 and miR-139-3p has not been described yet and there are no data linking FoxO1 to pheochromocytoma pathogenesis.

miR-541 and miR-765 have also been found to be significantly overexpressed in von Hippel-Lindau disease-related pheochromocytomas relative to sporadic benign samples. Moreover, miR-541 has been significantly upregulated in von Hippel-Lindau disease pheochromocytomas relative to sporadic recurring pheochromocytomas. Only very few data are available on these two miRNAs. miR-765 might target neurotrophin-3 receptor *(NTRK3*, also known as *TRKC).*[31] In the PC12 rat pheochromocytoma cell line expressing neurotrophin receptors, neurotrophin-3 (*NTF3*) activates the Erk1/2 and Erk5 pathways,[32] and induces moderate neurite overgrowth and neuron-like differentiation of PC12 cells.[33] Overexpression of miR-765 and the consequent *NTRK3* underexpression might thus be hypothesized to be involved in pheochromocytoma pathogenesis. Further studies are required to confirm this hypothesis.

The overexpression of miR-885-5p in multiple endocrine neoplasia type 2-related pheochromocytomas relative to sporadic benign, sporadic recurring, von Hippel-Lindau disease- and neurofibromatosis type 1-related pheochromocytomas is noteworthy. Overexpression of miR-885-5p may thus be regarded as characteristic for pheochromocytomas harboring *RET* protooncogene mutations. Overexpression of the mature miRNA processed from the 3′ arm of the stem-loop miR-885 (miR-885-3p) has been previously reported in malignant mesothelioma tissues.[34] The relative underexpression of miR-885-5p in von Hippel-Lindau disease-related pheochromocytomas may be linked to the frequent loss of chromosome 3p25.3. Loss of 3p or that of whole chromosome 3 could be detected in 94% of von Hippel-Lindau disease pheochromocytomas.[35] Among the hypoxia-responsive/angiogenesis genes previously described to be significantly underexpressed in multiple endocrine neoplasia type 2 relative to von Hippel-Lindau disease pheochromocytomas,[11] laminin subunits (*LAMB4*, *LAMA5*, *LAMC2*), collagene type 4 (*COL4A5*) and proline 4-hydroxilase α-1 precursor (*P4HA1*) could be identified to be targets potentially downregulated by miR-885-5p by our *in silico* target prediction approach.

Computational target prediction of the three miRNAs overexpressed in von Hippel-Lindau disease pheochromocytomas (miR-139-3p, miR-541

and miR-765) has also revealed several possible mRNA targets that may be involved in the hypoxia-associated pathway. These include the *VHL* gene itself and HIF1α subunit inhibitor (*HIF1AN*), hypoxia *HIF2α* and vascular endothelial growth factor that are all associated to the HIF1α pathway.

The major practical relevance of our study is related to the possible miRNA marker of tumor recurrence. Expression of miR-1225-3p has been significantly elevated in sporadic recurring pheochromocytomas compared with the benign tumors. This miRNA is located to the chromosome 16p13.3 in the neighborhood of miR-940 that was also found to be significantly overexpressed in sporadic recurring pheochromocytomas by microarray analysis. As there has been no appropriate assay available from the manufacturer due to the unique composition of miR-940, it could not be validated by qRT-PCR. The gain of 16p region in malignant pheochromocytomas has been previously described.[36] As the histological analysis of pheochromocytomas for the establishment of malignancy and for the prediction of tumor recurrence is unreliable, miR-1225-3p might be of clinical importance for selecting patients with potentially recurring pheochromocytomas. Patients harboring tumors overexpressing miR-1225-3p should be regularly screened. Further studies including larger numbers of tumors will be necessary to confirm the usefulness of this miRNA marker.

To highlight the potential pathogenetic role of significantly differentially expressed miRNAs in various subtypes of pheochromocytomas and to represent biological processes potentially modified by posttranscriptional regulation, *in silico* predicted miRNA-mRNA interactions have been identified, and pathway analysis of targets has been performed. Pathway analysis has revealed 'Notch signaling' (Figure 5) and 'G-protein-coupled receptor signaling' among the top three canonical pathways potentially downregulated by miR-1225-3p in sporadic recurring tumors in comparison with sporadic benign pheochromocytomas. Transcriptomic studies representing targets of miRNA-based regulation are pivotal for the correct interpretation of miRNA findings. There have been six previous studies[11,37–41] on gene expression profiling involving malignant pheochromocytomas to date. The studies of Dahia *et al.*,[11] Björklund *et al.*[37] and the most recent study of Waldmann *et al.*[41] included only two, three and five malignant pheochromocytomas, respectively. Brouwers *et al.*[38] examined 20 malignant tumors along with 70 sporadic and syndromic benign tumors: Gene Ontology Analysis of benign and malignant tumor comparison revealed the relevance of signal transduction changes. The significant gene sets established in two recent large-scale studies by Thouënnon *et al.*[39] and Suh *et al.*[40] are quite different, which indicates the need for further, large-scale gene expression profiling studies.

In the study by Suh *et al.* 'Notch1 signaling' and 'G_{α}-12 pathway' was found to be significantly enriched in sporadic benign relative to malignant pheochromocytomas by Gene Set Enrichment Analysis.[40] The activation of 'Notch signaling' acts in a cell-specific manner, as it can be responsible for carcinogenesis by inhibiting differentiation, whereas in the nervous system it induces oligodendrocyte maturation and myelinization.[42] The valproic acid-induced activation of Notch1 pathway in PC12 rat pheochromocytoma cell line was associated with differentiation and growth inhibition.[43] 'G-protein-coupled receptor signaling' is also involved in several biological processes (eg cell growth, differentiation, synaptic transmission, hormone release and actions, cell migration) in a cell-specific manner.[44] Activation of 'c-Myc-mediated pathway' has also been revealed by Gene Set Enrichment Analysis[40] in malignant pheochromocytomas. In our analysis, dysregulation of this pathway can be found in sporadic recurring pheochromocytomas compared with multiple endocrine neoplasia type 2-associated tumors. These findings might suggest that common pathomechanisms are involved in the development of metastases and tumor recurrence.

It is of interest to note that in the studies of Brouwers *et al.* and Thouënnon *et al.*, majority of genes were found to be underexpressed in malignant relative to sporadic benign pheochromocytomas.[38,39] Among the downregulated mRNAs of Thouënnon's study, we have identified *in silico* predicted targets of miR-1225-3p overexpressed in recurring tumors eg ankyrin-1 (*ANK1*), protein kinase C-ε (*PRKCE*), nuclear receptor interacting protein 2 (*NRIP2*).

It must be underlined, however, that the miRNA-affected pathways revealed in this study are only bioinformatically predicted and therefore these should be experimentally validated. To confirm the biological existence of miRNA-evoked posttranscriptional inhibition in pheochromocytomas, parallel miRNA and mRNA expression profiling on the same samples, proteomics analysis and further *in vitro* validation should be performed.[2,19] Unfortunately, formalin-fixed paraffin-embedded tissues used in our study do not allow all of these downstream applications, therefore, fresh or frozen tissues should be proposed for further validation.

The findings of our miRNA expression profiling studies on a single malignant and a pair of primary recurrent pheochromocytomas cannot be generalized. It is interesting to note that the profiles in the primary recurring recurrent and even that of the primary recurring malignant comparisons are similar. There are some data on the relevance of the top-ranking differentially expressed miRNAs (Tables 2 and 3) in these comparisons in other tumors: eg overexpression of miR-200a and underexpression of miR-217 have been described in pancreatic cancer[45,46] and slight overexpression of the top-ranking miR-202 in primary recurring-malignant comparison has been found in papillary thyroid cancer.[47] Expression of miR-1225-3p, however, has been

different in the recurring tumors and the malignant sample. This might indicate that beside the similarities established by the pathway analysis, there are also differences between pheochromocytoma recurrence and malignancy on the miRNA level.

In conclusion, to our knowledge this is the first report on miRNA expression profiling in different subgroups of human pheochromocytomas. We have proven the applicability of total RNA retrieved from formalin-fixed paraffin-embedded blocks for downstream miRNA microarray applications in pheochromocytoma samples. Significant differences in miRNA expression have been identified including characteristic markers for hereditary pheochromocytomas and recurring tumors. Based on the results of our bioinformatics analysis, posttranscriptional repression of 'Notch signaling' and 'G-protein-coupled receptor signaling' can have pathogenetic relevance in tumor recurrence. These pathways may represent novel pathways in pheochromocytoma pathogenesis.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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