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Identification of differentially expressed microRNA in parathyroid tumors

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Abstract

Background—The molecular factors that control parathyroid tumorigenesis are poorly understood. In the absence of local invasion or metastasis, distinguishing benign from malignant parathyroid neoplasm is difficult on histologic examination. We studied the miRNA profile in normal, hyperplastic, and benign and malignant parathyroid tumors to better understand the molecular factors that may play a role in parathyroid tumorigenesis and that may serve as diagnostic markers for parathyroid carcinoma.

Methods—MiRNA arrays containing 825 human microRNAs with 4 duplicate probes per miRNA were used to profile parathyroid tumor (12 adenomas, 9 carcinomas, and 15 hyperplastic) samples normalized to 4 reference normal parathyroid glands. Differentially expressed miRNA were validated by real-time quantitative TaqMan PCR.

Results—One hundred and fifty six miRNAs in parathyroid hyperplasia, 277 microRNAs in parathyroid adenoma, and 167 microRNAs in parathyroid carcinomas were significantly dysregulated as compared to normal parathyroid glands (FDR < 0.05). By supervised clustering analysis, all parathyroid carcinomas clustered together. Three miRNAs (miR-26b, miR-30b and miR-126*) were significantly dysregulated between parathyroid carcinoma and parathyroid adenoma. Receiver operative characteristic curve analysis showed mir-126* was the best diagnostic marker, with an area under the curve of 0.776. MiRNAs are differentially expressed in parathyroid neoplasms.

Conclusions—Most miRNA are downregulated in parathyroid carcinoma while in parathyroid hyperplasia most miRNA are upregulated. MiRNA profiling shows distinct differentially expressed miRNAs by tumor type which may serve as helpful adjunct to distinguish parathyroid adenoma from carcinoma.

Keywords

Parathyroid Carcinoma; MicroRNA; Parathyroid Neoplasm; Hyperparathyroidism; Parathyroid Adenoma; Parathyroid Hyperplasia; Parathyroid tumors

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Introduction

Hyperparathyroidism is characterized by hypersecretion of parathyroid hormone. Hyperparathyroidism may be primary, secondary or rarely tertiary. Primary hyperparathyroidism occurs as a result of inappropriate parathyroid hormone secretion from enlarged parathyroid gland(s). Primary hyperparathyroidism is caused by a parathyroid adenoma (85%), parathyroid hyperplasia involving 4 glands (10%), double parathyroid adenomas (2–5%), or rarely due to parathyroid carcinoma (<1%)¹. Secondary hyperparathyroidism occurs as a response usually due to low serum calcium levels associated with renal failure. In tertiary hyperparathyroidism, once the secondary cause of hyperparathyroidism is resolved there is continued autonomous hypersecretion of parathyroid hormone from an enlarged parathyroid gland.

Most parathyroid tumors in primary hyperparathyroidism are sporadic but approximately 5% are associated with autosomal dominant familial syndromes: Multiple Endocrine Neoplasia types 1 (MEN 1; OMIM #131100) and 2A (MEN 2A; OMIM #171400), Familial Isolated Hyperparathyroidism (OMIM #145000), and Hyperparathyroidism-Jaw Tumor Syndrome (HPT-JS; OMIM #145001)^{2–4}. Parathyroid tumors are heterogeneous and histologic classification of tumors is often difficult. The pathologic distinction of parathyroid adenoma from carcinoma is also difficult in the absence of obvious local invasion and distant metastasis or disease recurrence^{5, 6}. Moreover, if not grossly enlarged, parathyroid glands can also be difficult to histologically classify as hyperplastic³.

Several responsible germline genetic changes associated with primary hyperparathyroidism in familial syndrome have been identified (*menin* in MEN 1, *RET* in MEN 2A, *CDC73/HRPT2* in HPT-JS) but most cases of hyperparathyroidism are sporadic^{2, 7}. Several genetic changes have been implicated in a subset of sporadic parathyroid tumors. A chromosomal rearrangement of the cyclinD1 gene to the parathyroid hormone gene locus occurs, and cyclin D1 is overexpressed in up to 40% of sporadic parathyroid adenomas⁸. Recently, mutation in the tumor suppressor gene *HRPT2* has also been identified in sporadic parathyroid carcinoma and a small subset of parathyroid adenomas⁶. Both the calcium sensing receptor (*CaSR*) and vitamin D receptor (*VDR*) may also play a role in parathyroid tumorigenesis^{4, 9}.

MicroRNAs (miRNA, miR) are short, 19–22 nucleotides, non-coding RNAs. They account for 1% of the genome, and play a role in cellular processes such as apoptosis, proliferation and differentiation^{10, 11}. MiRNAs are conserved across species and their expression is highly specific for tissue type. MiRNA regulate gene expression through mRNA degradation, translational modulation, and or gene silencing^{10, 12}. Approximately, 30% of the genome is regulated by miRNA. In general, miRNAs are downregulated in most carcinoma and can function as either tumor suppressor or oncogene^{10, 12}.

MiRNA profiling in several human malignancies have shown that such an approach may identify miRNAs with a role in tumor cell biology, to classify tumor subtypes, and to identify diagnostic and prognostic markers¹⁰. To further understand the molecular mechanisms involved in parathyroid tumorigenesis and, thus, improve clinical diagnosis of patients with primary hyperparathyroidism, we performed miRNA gene expression profiling in 40 parathyroid tumor samples (9 parathyroid carcinomas, 12 parathyroid adenomas, 15 parathyroid hyperplasia, with 4 reference normal parathyroid glands).

Methods

Patients and Parathyroid tissue samples

Parathyroid tissue samples including clinical and histopathologic data were obtained for 40 patients with approval of the Committee on Human Research at the University of California, San Francisco. Nine parathyroid carcinoma, 12 parathyroid adenoma and 15 parathyroid hyperplasia were obtained from 40 patients who had primary hyperparathyroidism. The 4 normal parathyroid gland samples were obtained from biopsy specimens at the time of neck exploration for parathyroidectomy. Cases of parathyroid carcinoma had Schantz and Castleman's histologic criteria and all cases had local invasion, recurrence and or distant metastasis⁶.

RNA extraction and Microarray preparation

Total RNA was extracted from fresh frozen tissue. At the time that tumor samples were sectioned for RNA extraction, representative portions of the tissue were examined by H & E histology. The quality of total RNA was determined with the Agilent 2100 Bioanalyzer and all samples had a RNA integrity number ≥ 7.0 . MiRNA microarray profiling was done using the miRCURY LNA array version 11.0 (Exiqon). This array contains 7,720 probes, 3,300 of which represent 825 human miRNAs with 4 duplicate probes per miRNA. One μg of total RNA for each sample and pooled normal reference were labeled with Hy3 and Hy5 fluorescent label, respectively, using the miRCURY LNA Array power labeling kit (Exiqon), as described by the manufacturer. The Hy3-labeled samples and an Hy5-labeled reference RNA sample were mixed pair wise and hybridized to the miRCURY LNA array. The miRCURY LNA array microarray slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Inc.), and the image analysis was carried out using the ImaGene 7.0 software (BioDiscovery, Inc.).

Quantitative reverse transcriptase-PCR

MiRNAs were validated using quantitative reverse transcriptase-PCR. An adjusted p-value controlling for false discovery rate (FDR) < 0.01 was used to identify miRNAs that were differentially expressed between adenoma and carcinoma. Thirteen out of the 24 miRNA were commercially available. Quantitative reverse transcriptase-PCR was used to quantify miRNA levels [hsa-miR-26b (assay# 000407), hsa-miR-27a (assay#000408), hsa-miR-27b (assay# 000409), hsa-miR 30b (assay # 000602), hsa-miR-28 (assay# 000411), hsa-miR-34a (assay# 000426), hsa-miR-100 (assay# 000437), hsa-miR-126 (assay# 000450), hsa-miR-126* (assay# 000451), hsa-miR-145 (assay# 000467), hsa-miR-423-3P (assay# 002626), hsa-let-7a (assay # 000377) and hsa-let-7f (assay# 000382)] using primers and probe purchased from Applied Biosystem (Foster City, CA). The gene expression pattern of these tumors was compared with a reference sample that consisted of a pooled sample of four normal parathyroid glands. Quantitative reverse transcriptase-PCRs (RT-PCR) for each sample were done in triplicate. Total RNA (10 ng for 10 μl reaction) was converted to cDNA using primers specific for each miRNA and the TaqMan MiRNA reverse transcription kit (4366597; Applied Biosystems), according to the manufacturer's instructions. All quantitative reverse transcriptase-PCRs were done using a 5' nuclease technique with specific Taqman Gene Expression Assays (Applied Biosystems) and Taqman Universal PCR Master Mix, NO AmpErase UNG (Applied Biosystems), on an ABI 7900HT Fast Real-Time PCR System. MiRNA expression level was expressed as the difference (ΔC_t) between cycle threshold (C_t) for the miRNA of interest and pooled normal samples.

Target analysis

We conducted a literature search and identified genes of interest in parathyroid carcinoma. The genes of interest were CDC73 (HRPT2, parafibromin), Rb1, Galectin 3 (Lgals 3), Ki-67 (MKI67), CCND 2, CASR or VDR^{3, 4, 8, 9}. We then used the miR-Ontology data base from Ferro Lab Data Mining and Informatics Group website that cross references Target Scan, miRanda and PicTar data base to search for potential targets¹³.

Statistical analysis

The microarray GPR files were loaded into R/Bioconductor using the marray package (Gentleman 2004). Flagged spots were removed from subsequent analysis and the remaining probes were used for normalization and subsequent analyses. The log₂ ratio of the intensity of Cy5 to Cy3 signals were calculated for each miRNA on every array (with no background subtraction) and normalized by print tip loess normalization^{14, 15}. Since individual miRNAs were represented by more than one probe on the array, the median of normalized log₂ ratio of the replicate probes (for those with more than one unflagged probe) was used as the value for the miRNA. The summarized log₂ ratios for each experiment were then used in moderated t-statistics and p-value calculation using the limma package in R/Bioconductor^{16, 17} with adjustment for false discovery rate using the Benjamini-Hochberg method. A linear model with one factor, tumor type, with three groups (Adenoma, Carcinoma and Hyperplasia) was established, followed by linear contrasts (Adenoma vs. Carcinoma, Adenoma vs. Hyperplasia, and Carcinoma vs. Hyperplasia). Adjusted p-value controlling for FDR using the Benjamini-Hochberg method were calculated. Unsupervised clustering analysis was performed using hierarchical clustering of the top (50,100 and 200) most variably expressed miRNAs based on the median absolute deviation using the Euclidean distance as a similarity metric. Supervised clustering analysis using the random Forest method in Bioconductor was performed using miRNAs that were uniquely differentially expressed between Adenoma and Carcinoma. The quantitative reverse transcriptase-PCR data was analyzed by using the XLSTAT statistical software. Pearson correlation was used to compare the array data with the real-time PCR data with $r > 0.65$ showing strong correlation. The Mann-Whitney U test was used to compare non-parametric data between tumor types. Receiver operative characteristic curves were constructed using the Wald two-tailed 95% confidence intervals with the Bamber continuity correction.

Results

MiRNA Cluster Analysis

We analyzed miRNA expression in 9 parathyroid carcinoma, 12 parathyroid adenoma and 15 parathyroid hyperplasia normalized to 4 reference pooled normal parathyroid glands. Unsupervised cluster analysis using the top 50 most variable miRNAs as defined by the median absolute deviation showed two main groups with modest clustering by tumor type Figure 1. Supervised cluster analysis using the random Forest method for differentially expressed miRNA that were uniquely dysregulated in adenoma versus carcinoma (FDR<0.05) showed complete clustering by tumor type Figure 2.

Differentially expressed miRNAs

We found that 277 miRNA were differentially expressed in parathyroid adenoma, 167 in parathyroid carcinoma and 156 in parathyroid hyperplasia as compared to normal (FDR<0.05) Table 1. We found some overlap in the differentially expressed miRNAs between the three tumor types as compared to pooled normal parathyroid samples but 50 miRNAs were unique to parathyroid carcinoma, 122 to parathyroid adenoma and 22 to parathyroid hyperplasia Figure 3A. The percentage of downregulated miRNAs unique to the

three tumor types were significantly higher for parathyroid carcinoma (58%) and parathyroid adenoma (50%) than for parathyroid hyperplasia (27%) ($p < 0.04$). We also found in parathyroid carcinoma there was greater fold change in the differentially expressed miRNAs. In parathyroid carcinoma, 11 miRNAs (22.0%) were downregulated by greater than 2-folds and 3 miRNAs (6.0%) were upregulated by greater than 2 folds. While for miRNAs dysregulated in parathyroid adenoma and parathyroid hyperplasia, only 1 miRNA for parathyroid adenoma was dysregulated by greater than 2 fold Table 1.

Because distinguishing between parathyroid adenoma and carcinoma is difficult by histology, a comparison of miRNA expression differences between these groups was also done. We identified 91 miRNA that were differentially expressed between adenoma and carcinoma Figure 3B. The majority of these miRNA were downregulated (58.2%) with nearly a fourth of the miRNA being downregulated by greater than 2 fold Table 2.

Validation of miRNA expression profile by TaqMan RT PCR

Using a $FDR < 0.01$ from our microarray data we identified 24 differentially expressed miRNA between adenoma versus carcinoma. Thirteen miRNAs were used to validate the miRNA array expression data by real-time quantitative PCR. For 3 out of 13 miRNA there was strong correlation with the array data (Table 2). Similar to our microarray data all 13 selected miRNA were downregulated in carcinoma compared to adenoma. Three of the 13 selected miRNAs were significantly downregulated between adenoma and carcinoma (miR-126*, miR-26b, miR-30b, $P < 0.05$) (Table 2). The area under the receiver operative characteristic curve was determined for the 13 differentially expressed miRNA. The miRNA with the highest accuracy was miR-126* ($AUC = 0.766$).

Target analysis for differentially expressed miRNAs

We conducted a comprehensive target search for the 24 differentially expressed miRNA between parathyroid adenoma and carcinoma that were used in the validation process. The search included genes identified through literature search of genes involved in parathyroid tumorigenesis. The genes of interest were CDC73/HRPT2, Rb1, Galectin 3 (Lgals 3), Ki-67 (MKI67), CCND 2, CASR and VDR^{3, 4, 8, 9}. We used the miR-Ontology data base that cross references Target Scan, miRanda and PicTar data base to search for potential targets¹³. In our search we were able to identify the CDC73 gene as a predicted target for miR-28. We further looked at the relationship between CDC73 expression and miR-28 using quantitative RT-PCR and immunohistochemistry for CDC73 protein expression³. We found no correlation between miR-28 expression and CDC73 expression in the parathyroid carcinoma samples (data not shown).

Discussion

In this study, we sought to determine if parathyroid neoplasm had distinct miRNA signature. We found that the miRNA expression profile by unsupervised cluster analysis showed clustering of most parathyroid carcinomas together. As expected, in the supervised cluster analysis there was complete segregation of parathyroid samples by tumor type (parathyroid adenoma versus parathyroid carcinoma). We further validated our results using RT-PCR. Approximately two-thirds (9 of 13) of the miRNA expression levels showed good correlation with the microarray data. All the validated miRNA had the same pattern of downregulation in carcinoma compared to adenoma. Furthermore, miR-126*, miR-26b and miR-30b were significantly differentially expressed between parathyroid adenoma and parathyroid carcinoma by quantitative RT-PCR. MiR-126* levels was the most accurate for differentiating between parathyroid carcinoma and parathyroid adenoma (71% sensitivity, 82% specificity).

Parathyroid carcinoma is rare and little is known about the genetic basis for it. Moreover, distinguishing between benign and malignant parathyroid tumors can be extremely difficult^{3, 5, 6, 18}. It is not rare for a patient with presumed benign disease to be diagnosed with parathyroid carcinoma after developing local recurrence or distant metastases¹⁹. Some histologic criteria for identifying parathyroid carcinoma (thick capsule, fibrous bands, nuclear pleomorphism, increased mitosis, capsular invasion and vascular invasion) are subjective and leave room for misdiagnosis⁵. In fact, most parathyroid carcinomas are diagnosed after recurrent disease develops. Some markers (galectin-3 and Rb overexpression, loss of CDC73/HRPT2) may be helpful for diagnosing parathyroid carcinoma. The absence of parafibromin (CDC73/HRPT2) staining has been reported to have 31% to 100% sensitivity and 41% to 100% specificity for distinguishing benign from malignant parathyroid tumors^{3, 20, 21}. The identification and use of adjunct markers to improve the accuracy of parathyroid carcinoma diagnosis may have several clinical implications. For example, those patients found to have histologic equivocal criteria for parathyroid carcinoma but suggestive of parathyroid carcinoma on marker analysis, for carcinoma, may have more close follow up or reoperation with more aggressive surgical resection if tumor margins are positive.

In recent years, there has been a great deal of advancement in our knowledge regarding the role of miRNA in tumorigenesis¹⁰. However, in the current literature there are no studies that have attempted to show the miRNA signature for parathyroid carcinoma, adenoma and hyperplasia. There has been one study which has evaluated miRNA expression profiling in a limited number of samples and parathyroid tumor types. Corbetta and colleagues found 17 miRNA differentially expressed between 4 parathyroid carcinoma samples compared to 2 normal parathyroid glands out of 362 miRNAs profiled²². In contrast to our findings, unsupervised hierarchical cluster analysis did not show separate clustering of parathyroid tumor types comparing the 6 samples. The investigators validated 4 miRNAs (miR-139, miR-296, miR-222 and miR-503) to be differentially expressed in 4 parathyroid carcinoma samples compared to 2 normal parathyroid gland samples. None of the miRNAs identified to be differentially expressed in their study were differentially expressed in our study. Our study design and array platform used was different than the study by Corbetta et al. as was the goal of our study²². We profiled a greater number of miRNAs among a greater number of parathyroid tumor types using a larger number of samples per histologic group, which were compared to reference pooled normal parathyroid glands. The strength of such an approach is it allowed us to determine if the miRNA profile was different among the different tumor types providing at least some clues to the miRNA signature of parathyroid tumor types, for what often can be a clinically and histologically heterogeneous group of tumor types. Thus, the clustering of most parathyroid carcinoma samples together by unsupervised analysis lends some support to a miRNA signature being present which is emphasized with the complete separation between parathyroid carcinoma and adenoma by supervised cluster analysis.

A formal diagnostic accuracy analysis of the significantly differentially expressed miRNAs between parathyroid adenoma and carcinoma showed one miRNA with good accuracy (miR-126*) by quantitative RT PCR. For these reasons, we believe our study results are unique and provide some insight into miRNA expression in parathyroid tumors and identify possible candidate miRNAs which may have a role in parathyroid tumorigenesis.

Again, by unsupervised clustering all but one parathyroid carcinoma grouped in two distinct clusters. For parathyroid hyperplasia and parathyroid adenoma there was no distinct pattern observed in the unsupervised clustering. One parathyroid adenoma and one parathyroid hyperplasia clustered with parathyroid carcinoma. Given the current limitations in the clinical and histologic classification of some parathyroid tumors, it is unclear if these

samples were misclassified as parathyroid adenoma and hyperplasia but only long term clinical follow up will be able to help clarify this issue.

We also conducted a target search for the 13 differentially expressed miRNA between carcinoma and adenoma (FDR<0.01) looking at genes implicated in parathyroid dysregulation as potential targets. The genes of interest were CDC73/HRPT2, Rb1, Galectin 3 (Lgals 3), Ki-67 (MKI67), CCND 2, CASR or VDR^{3, 4, 8, 9}. Our search identified CDC73 as a potential target for miR-28, but we did not find a relationship between miR-28 and CDC73 levels using RT PCR and immunohistochemistry. We also examined the chromosomal location of the 13 miRNAs and none of the target genes and miRNAs shared a common chromosomal location. The most common reported chromosomal imbalance in parathyroid carcinoma is loss of the 1q21-q32 region which is the coding region for CDC 73⁷. None of our miRNAs were located in that region. Another common site for chromosomal imbalance is at 9q33-qter, this imbalance is reported in up to 24% of tumor samples²³. This region is the same chromosomal region for miR-126* (9q 34.3). Kytola and colleagues reported this region to be a region of genomic gain by comparative genomic hybridization but miR-126* (the minor strand) and miR-126 (the major strand) were both downregulated in our carcinoma samples²³.

The 3 miRNAs (miR-126*, miR-30b, miR-26b) that were significantly dysregulated in carcinoma versus adenoma have been implicated in several malignancies^{24–29}. For example, the major strand of miR-126* has been implicated in non-small cell carcinoma^{30, 31}, progression of adenoma to carcinoma in colon cancer³² and some leukemia³³. Mir-126 is believed to regulate the epidermal growth factor-like domain 7 (EGFL7), which is involved in cellular migration and angiogenesis³¹. Also, an upregulation in miR-126 has been shown in vitro to decrease growth rate of cell lines from lung cancer origin³⁰.

In summary, miRNAs are differentially expressed in parathyroid neoplasms. Most miRNA are downregulated in parathyroid carcinoma while in parathyroid hyperplasia most miRNA are upregulated. MiRNA profiling shows distinct differentially expressed miRNAs by tumor type which may serve as helpful adjunct to distinguish parathyroid adenoma from carcinoma.

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Synopsis

A large number of miRNA are downregulated in parathyroid carcinoma while in parathyroid hyperplasia miRNA are upregulated. MiRNA profiling shows distinct differentially expressed miRNAs by tumor type which may serve as helpful adjunct to distinguish parathyroid adenoma from carcinoma.

Figure 1. Unsupervised cluster analysis of top 50 most variably expressed miRNAs as defined by median absolute deviation

Figure 2.
Supervised cluster analysis using differentially expressed miRNA between adenoma and carcinoma with FDR <0.05

A

B

Figure 3.

A) Venn diagram of differentially expressed miRNA as compared to normal by tumor type using a FDR < 0.05, B) Venn Diagram of differentially expressed miRNA as compared between tumor types using a FDR of < 0.1

Table 1

Differentially expressed miRNA by tumor type with FDR <0.05

Differentially Expressed miRNA by Tumor Type			
	Carcinoma n (%)	Adenoma n (%)	Hyperplasia n (%)
All Differentially Expressed miRNA	167	277	156
Down Regulated	85 (50.9)	131 (47.3)	72 (46.2)
> 2 fold	23 (13.8)	6 (2.2)	6 (3.8)
Up Regulated	82 (49.1)	146 (52.7)	84 (53.8)
>2 fold	12 (7.2)	6 (2.2)	3 (1.9)
Differentially Expressed miRNA Specific for Tumor Type	50 (29.9)	122 (44.0)	22 (14.1)
Down Regulated	29 (58.0)	61 (50)	6 (27.3)
> 2 fold	11 (22.0)	1 (0.8)	0 (0.0)
Up Regulated	21 (42.0)	61 (50.0)	16 (72.7)
>2 fold	3 (6.0)	0 (0.0)	0 (0.0)

Table 2

Correlation of miRNA expression between real-time quantitative TaqMan PCR and miRNA array for 13 differentially expressed miRNAs and fold difference

miRNA	Pearson Correlation Coefficient (r)	Fold difference (Adenoma vs. Cancer)	P value
hsa-miR-100	0.83	-1.8	0.22
hsa-miR-126	0.67	-2.2	0.10
hsa-miR-126 *	0.65	-1.2	0.04 *
hsa-miR-28	0.12	-1.8	0.19
hsa-miR-34a	0.12	-2.5	0.06
hsa-miR-27b	0.25	-1.7	0.14
hsa-miR-423-3P	0.29	-2.8	0.10
hsa-miR-27a	0.30	-1.7	0.33
hsa-let-7a	0.33	-1.8	0.15
hsa-miR-30b	0.41	-1.9	0.04 *
hsa-let-7f	0.44	-1.4	0.24
hsa-miR-26b	0.46	-2.3	0.04 *
hsa-miR-145	0.53	-2.1	0.33

* Statistically significant