Expression analysis of potentially *MEN1*-targeting microRNAs in sporadic and MEN-1 syndrome associated parathyroid adenomas and hyperplasias

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BACKGROUND AND AIM

Primary hyperparathyroidism (PHPT) is a frequent endocrinopathy (incidence: 1/1000/year), especially among postmenopausal women. Autonomous hyperfunctioning of the parathyroid glands leads to elevated levels of parathyroid hormone (PTH) and hypercalcaemia resulting in e.g. osteoporosis and nephrolithiasis. At histological examinations, hyperfunctioning parathyroid glands have benign lesions (adenomas 80%, hyperplasias 15-20%) in the vast majority of the cases, malignant carcinomas are rare (1%). Although sporadic in the 95% of the cases, certain familial syndromes are associated with PHPT. Among these, multiple endocrine neoplasia type 1 (MEN-1, caused by germline mutations of the menin-coding *MEN1* gene) is the most frequent. Additionally, recent whole exome sequencing studies on sporadic PHPT tissues identified heterozygous *MEN1* mutations to be the sole main driver mutation in tumorigenesis, occuring in ~35% of the cases [1].

Moreover, microRNAs (miRNAs) were also proposed to contribute to menin silencing *via* RNA interference and therefore accelerating tumourigenesis. Hsa-miR-24 has already been confirmed to target the 3' untranslated region of *MEN1* mRNA and elevated levels of this miRNA was detected in MEN-1 associated adenomas lacking loss of heterozigosity (LOH) of *MEN1* [2]. Therefore, elevated levels of hsa-miR-24 might represent Knudson's "second hit" in sporadic parathyroid tumourigenesis.

Our aim was to determine if there is a difference in expression of potentially *MEN1*-targeting microRNAs between MEN-1 syndrome associated and sporadic PHPT tissues.

METHODS

Immunohistochemical analysis of menin (Abcam, ab2605, 1:100 dilution) and Ki67 was performed in 16 MEN-1 associated and 41 sporadic PHPT tissues. *In silico* analysis using 5 different miRNA target prediction algorithms (Diana microT 3.0, miRWalk, microCosm Targets, PicTar, TargetScan) was performed to detect microRNAs potentially targeting *MEN1*. RNA was isolated from formalin-fixed, paraffin-embedded PHPT tissues using Ambion RecoverAll Total Nucleic Acid Isolation Kit (Life Technologies).

MiRNA expression analysis of 6 chosen microRNAs was performed using predesigned TaqMan probes for quantitative PCR on an Applied Biosystems Fast 7500 qRT-PCR instrument. Germline *MEN1* mutation status was determined by Sanger sequencing. Statistical analysis was performed using IBM SPSS Statistics software. In all comparisons $p < 0.05$ was considered statistically significant.

Figures and tables

	MEN-1 associated	sporadic
N	16	41
sex (female, %)	81.3	82.9
age (years)	34.9 ± 3.58	53.6 ± 2.39
largest diameter (mm)	16.7 ± 2.0	20.7 ± 1.47
mitosis (/10 HPF)	0.0 ± 0.0	0.0 ± 0.0
Ki67 index (%)	0.5 ± 0.3	0.3 ± 0.1
menin (cytoplasm)	0.69 ± 0.15	0.59 ± 0.1
menin (nucleus)	0.0 ± 0.0	1.5 ± 0.2
PTH (pg/ml)	131 ± 13.7	250 ± 55
Ca^{2+} (mmol/l)	2.79 ± 0.06	2.84 ± 0.06

Table 1 – Characterization of investigated PHPT tissues. Data are given as mean ± S.E. Statistics: Student's independent samples' T-test. Statistically significant differences are highlighted with yellow letters on blue background.

patients	*MEN1* mutation	Mutation type
1	E8: c.1270delA	deletion
2	E8: c.1270delA	deletion
3	E6: c.904C>G, p.Leu301Arg	missense
4	E6: c.904C>G, p.Leu301Arg	missense
5	E6: c.904C>G, p.Leu301Arg	missense
6	E10: c.1657insC	insertion
7	E10: c.1657insC	insertion
8	E1: c.358_360delAAG	deletion
9	E1: c.231C>A, p.Tyr77STOP	nonsense
10	E2: c359del4bp	deletion
11	E2: c.202_206dupGCCCC (5bp)	insertion
12	E8: c1003C>A, p.Cys354STOP	nonsense
13	E4: c.668T>C, p.Leu223Pro	missense
14	E1: c168delC	deletion
15	E9: c.1177C>T, p.Gln393Stop	nonsense
16	I9: c.-2A>C	splice mutation

Table 2 – Germline *MEN1* mutations found in 16 patients with MEN-1 syndrome. Patients harbouring the same mutation are close relatives and are displayed with same background colours.

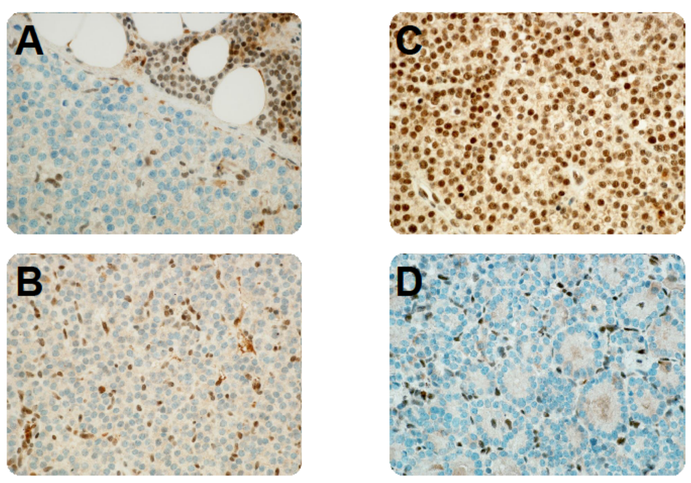

Figure 1 – Representative slides of immunohistochemical analysis of menin in MEN-1 associated (Panel A: MEN-1 adenoma, Panel B: MEN-1 hyperplasia) and sporadic (Panel C: adenoma with intensive presence of nuclear menin, Panel D: adenoma lacking nuclear menin presence) PHPT tissues. 600× magnification.

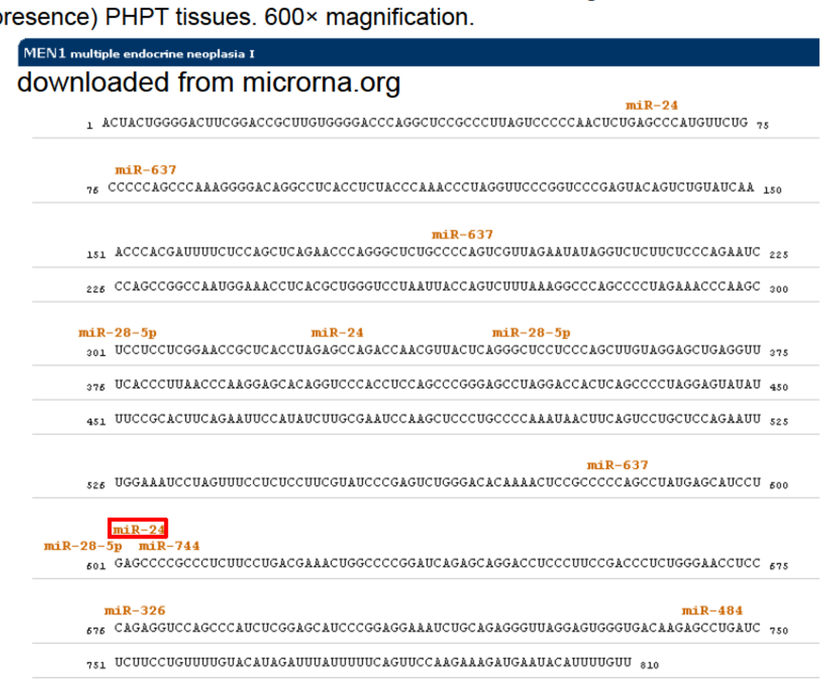

Figure 2 – 3' UTR of MEN1 mRNA with predicted binding sites of the 6 chosen miRNAs. Red frame highlights binding site for hsa-miR-24 confirmed earlier [2].

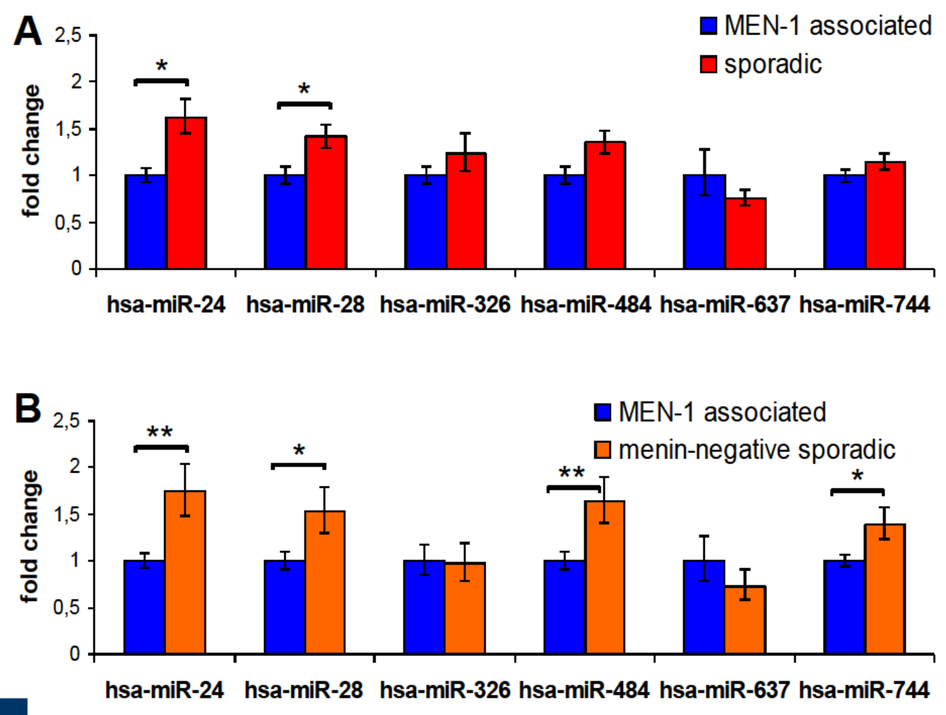

Figure 3 – MiRNA expression changes between MEN-1 associated and sporadic (Panel A) and MEN-1 associated and menin-negative sporadic (Panel B) PHPT tissues. Data are given as mean ± S.E, normalized to MEN-1 associated PHPT tissues. Statistics: Student's independent samples' T-test. Statistically significant differences are highlighted with asterisks (* $p<0.05$; ** $p<0.01$).

	MEN-1 associated	Menin-negative sporadic
N	16	12
sex (female, %)	81.3	75.0
age (years)	34.9 ± 3.58	50.1 ± 2.96
Largest diameter (mm)	16.7 ± 2.02	24.2 ± 2.54
Mitosis (/10 HPF)	0.0 ± 0.0	0.0 ± 0.0
Ki67 index (%)	0.5 ± 0.3	0.1 ± 0.1
PTH (pg/ml)	131 ± 13.7	355 ± 173
Ca^{2+} (mmol/l)	2.79 ± 0.06	2.81 ± 0.07

Table 3 – Characterization of MEN-1 associated and menin-negative sporadic PHPT tissues. Data are given as mean ± S.E. Statistics: Student's independent samples' T-test. Statistically significant differences are highlighted with yellow letters on blue background.

RESULTS

16 MEN-1 syndrome associated and 41 sporadic PHPT tissues were analyzed. MEN-1 associated PHPT occurred at earlier age (Table 1). Upon immunohistochemical analysis, all MEN-1 associated as well as 12/41 (29,3%) sporadic PHPT tissues lacked nuclear menin (Table 1, Figure 1). Mutations of various types were found in MEN-1 associated patients (Table 2).

Upon *in silico* analysis, 6 miRNAs (hsa-miR-24, hsa-miR-28, hsa-miR-326, hsa-miR-484, hsa-miR-637 and hsa-miR-744 – all of these were predicted by at least two algorithms) were chosen for further investigations (Figure 2).

MiRNA expression profiling revealed that hsa-miR-24 and hsa-miR-28 levels are elevated in sporadic compared to MEN-1 associated PHPT tissues (Figure 3, Panel A). Upon comparing MEN-1 associated and menin-negative sporadic PHPT tissues, this alteration strengthened and two other miRNAs – hsa-miR-484 and hsa-miR-744 – displayed elevated expression in menin-negative PHPT tissues (Figure 3, Panel B).

Further analysis revealed that menin-negative PHPT tissues were larger compared to MEN-1 associated PHPT tissues. (Table 3).

CONCLUSIONS

Lack of nuclear menin presence in MEN-1 associated and sporadic PHPT tissues confirms the tumour suppressor nature of menin. MiRNAs hsa-miR-24, hsa-miR-28, hsa-miR-484 and hsa-miR-744 may be involved in the tissue-specific downregulation of menin, contributing to sporadic parathyroid tumourigenesis.

References

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Endocrine tumours

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