**Multiple Endocrine Neoplasia Type 1: clinical correlates of *MEN1* gene methylation**

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**Abstract**

Multiple Endocrine Neoplasia Type 1 (MEN 1) has marked severity variation between individuals with the same mutation. To investigate any relationship between promoter methylation and clinical features, blood and tissue samples were collected from 16 members of the Tasman 1 MEN 1 kindred carrying a common splice site mutation and 7 patients with sporadic MEN 1. Methylation at 39 CpGs in the *MEN1* promoter were assessed in formalin-fixed paraffin embedded parathyroid tissue. Clinical disease severity markers included age at first parathyroid operation, parathyroid hormone level and corrected serum calcium levels. Six patients with sporadic hyperparathyroidism were used for comparison.

Minimal methylation was observed in all patients across CpG sites 1 – 23. In contrast, hypermethylation was observed at CpG sites 24 – 31 in MEN 1 patients, a pattern not observed in patients with non-MEN 1 parathyroid disease. Mean methylation at sites 24 – 31 was significantly correlated with age at first parathyroid operation ($r$ = 0.652, *p* = 0.041). A permutation test, utilising the mean correlation coefficient ($r$ = -0.401) revealed a possible association between relative PHPT severity and methylation score for each significant CpG site (*p* = <0.103). This novel study reveals evidence supporting a possible association between altered *MEN1* promoter methylation and clinical severity of disease.

***Key words:*** methylation; MEN 1; parathyroid; familial

**1. Introduction**

Multiple endocrine neoplasia type 1 (MEN 1) is an autosomal dominant condition associated with the development of hyperplasia in the parathyroid, pancreas, upper gastrointestinal tract, adrenal and pituitary glands [1](#_ENREF_1). Primary hyperparathyroidism (PHPT) resulting in hypercalcaemia develops in over 95% of *MEN1* mutation carriers [2](#_ENREF_2). Patients with MEN 1 require lifelong surveillance for a range of benign and malignant tumours [3](#_ENREF_3). Co-morbidities such as cardiovascular disease and renal failure in particular are thought to result from the adverse metabolic profile associated with early onset of parathyroid hyperplasia [3](#_ENREF_3), [4](#_ENREF_4).

Parathyroid hyperplasia usually manifests in the second decade of life and is identified through serial screening for elevation of serum calcium in the context of non-suppressed parathyroid hormone (PTH) levels [5](#_ENREF_5). Hypercalcaemia is known to stimulate gastrin secretion, worsening the hypergastrinaemia from gastro-duodenum G cell hyperplasia associated with this syndrome [6](#_ENREF_6), [7](#_ENREF_7). Optimal treatment for patients with established PHPT involves subtotal parathyroidectomy followed by lifelong monitoring for recurrent hyperparathyroidism [8](#_ENREF_8).

Tasmania has a five to ten times higher prevalence of MEN 1 due to an *MEN1* founder splice-site mutation (NM\_130799.2:c.446-3 C > G heterozygous, rs377461506) associated with the large *Tasman 1* pedigree [4](#_ENREF_4), [9](#_ENREF_9), [10](#_ENREF_10). To our knowledge, no studies have investigated the impact of this mutation on protein function. The ClinVar entry for this SNP reports it to be of uncertain significance [11](#_ENREF_11). Using the CADD framework this mutation was given a score of 14.91, indicating that it is likely to be deleterious [12](#_ENREF_12). Individuals carrying the mutation display wide variability in disease phenotype, from minimal to malignant disease or early onset of PHPT. This observation has led to a search for disease modifying factors, including speculation that epigenetic modifications occur in MEN 1 [1](#_ENREF_1). Located on chromosome 11q13, *MEN1* is composed of ten exons with an 1830 bp region coding for the protein product, menin. The *MEN1* gene behaves as a tumour suppressor and menin, a transcription regulator, interacts with a range of proteins and exhibits a ubiquitous expression profile [13](#_ENREF_13), [14](#_ENREF_14).

CpG islands are often associated with regulatory regions of genes and are a common site of altered DNA methylation [15](#_ENREF_15). DNA methylation can affect gene transcription in two ways; either by preventing transcription factor binding or recruitment of chromatin modifying proteins. Currently, there is strong interest in the description of key genes regulated by altered methylation in tumour development and their potential as predictors of clinical outcomes [16](#_ENREF_16).

Whilst the *MEN1* promoter contains a large CpG island, few studies have examined promoter methylation in diseased tissue. Previous evidence has shown that there is no abnormal methylation in 19 CpG loci in the *MEN1* CpG island in pancreatic and small bowel neuroendocrine tumours (NETs) [17](#_ENREF_17). Starker and colleagues (2011) assessed the DNA methylome of 51 benign and malignant parathyroid tumours. The following genes were reported as having altered methylation; *CDKN*, *RASSF1A*, *APC*, *WT1* and *RB1,* however no altered methylation was reported for *MEN1* [18](#_ENREF_18). A study in pulmonary carcinoids also found no difference in *MEN1* promoter methylation levels between samples with high and low *MEN1* mRNA expression [19](#_ENREF_19).

Detailed examination of the *MEN1* gene reveals that the CpG rich regions in the promoter extend beyond the CpG island previously examined. These additional regions lie in previously identified regulatory elements of the gene [20](#_ENREF_20), [21](#_ENREF_21). A significant advance in epigenomics was the discovery that tissue-specific DNA methylation occurs within CpG ‘shores’, rather than in CpG islands, and can have a significant impact on gene expression [22](#_ENREF_22). Gene expression has been shown to be closely tied to methylation of shores through DNA methyltransferase (DNMT) knockout experiments, supporting a functional role for these differentially methylated regions (DMRs) [22](#_ENREF_22).

In this study we hypothesise that altered methylation of *MEN1* promoter regions may contribute to the tissue specific manifestation of MEN 1, specifically in endocrine tissues such as the parathyroid. We also investigate the possibility that differential methylation may contribute to the differing clinical phenotype in patients with the same mutation, as observed in the Tasman 1 kindred.

**2. Materials and Methods**

**2.1. Study design**

Archival formalin fixed paraffin embedded (FFPE) tissue from parathyroidectomies, carried out as part of routine MEN 1 patient management at the Royal Hobart Hospital (RHH), were used following approval from the Human Research Ethics Committee (H0006838 MEN1, Tasmania). A total of 54 samples from 23 patients with MEN 1 or sporadic PHPT were included in our study. Multiple parathyroid FFPE blocks were available for some patients, resulting in a total of 46 parathyroid samples for MEN1 mutation carriers. FFPE parathyroid tissue from a further six patients, (*N* = 8 blocks), with sporadic PHPT were included for comparison.

The following clinical parameters were collected, with blood results at a time point as close to the operation as possible; age at PHPT diagnosis, age at parathyroid resection, parathyroid hormone level, ionised calcium level (ICa) and corrected calcium level (CCa). For each patient, a measure of relative disease severity was calculated by expression of normalised CCa/PTH ratio. This value was expressed as 10-Cca/PTH so that higher ratios indicate relatively more severe PHPT.

Matched peripheral blood samples for patients 1, 2, 4, 6, 8, 9 and 10 were collected during routine practice and were available for methylation analysis. Blood samples from two normal unaffected controls were also analysed.

**2.2. DNA isolation and bisulphite conversion**

DNA was extracted from FFPE samples using the Qiagen ‘QIAamp DNA FFPE Tissue Kit’. Five 10 μM sections were processed with xylene and proteinase K according to the manufacturers’ instructions. DNA from blood control samples was isolated using a Nucleon BACC3 Genomic DNA Extraction Kit (RPN-8512, GE Healthcare, UK), according to the manufacturer’s instructions. The Zymo Research ‘EZ DNA Methylation Kit™’ (D5002, USA) was used to bisulphite convert unmethylated cytosines as per the manufacturer’s instructions.

2.3. **FFPE tumour genotyping**

DNA of 50 samples from 19, (*N* = 16 MEN 1 patients, *N* = 3 sporadic), individuals were genotyped for the *MEN1* mutation. These represented 38 and 12 parathyroid samples from operation 1 and 2 respectively. DNA from FFPE tumour tissue was quantified and 20 ng of DNA was used in the PCR amplification. Primers covering the Tasman 1 Kindred mutation (rs377461506) are shown below and produced a single 260 bp product.

F: 5’ AAGGGATGGAGGGATAGTGG 3’

R: 5’ ACACTACCCAGGCATGATCC 3’

Reactions were completed using 0.8 μL of each primer, 5 μL of MyTaq™ HS Mix (Bioline) and 20 ng of DNA in a 10 μL reaction volume. Reaction conditions are provided in Supplementary Table 3. The PCR product was purified using AmpureXP (Beckmann Coulter™) and sequenced using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Cat#: 4337455) (Life Technologies, USA). Sequencing reactions were purified using CleanSEQ (Beckmann Coulter™) and analysed on an ABI 3500.

**2.4. Bisulphite DNA Sequencing**

Two primer sets were used to amplify target areas of the *MEN1* promoter region. The first set amplified a 291 bp region, covering CpG sites 1 – 23, hereafter referred to as ‘region 1’. This primer was designed to extensively cover a previously described area enabling comparison with the existing literature [23](#_ENREF_23).

F1: 5’ GGTTTGAAGGGAAGGGTTAATT 3’

R1: 5’ CAAACCCCAAAAAAAATCCTAAC 3’

The second primer pair amplified a 296 bp region further upstream of region 1 covering an additional 16 CpG sites, 24 – 39. This amplicon is hereafter referred to as ‘region 2’.

F2: 5’ GTATATGTATATATATATAAAATTAG 3’

R2: 5’ CTTTAACTAAATATCAATATCTATTAAC 3’

Candidate primer pairs were generated using ‘MethPrimer’ [24](#_ENREF_24). To test primer specificity, sequences were compared with the human genome using BLASTn [25](#_ENREF_25). A CpG map displays the amplified sequences and their relative positions in the promoter region of the *MEN1* gene (Figure 1). To follow the sequence data presented in this paper, the position +1 is attributed to the nucleotide 1832 (accession no. U93237) which is described as the putative transcription initiation site (TIS) [26](#_ENREF_26). For region 1, GoTaq® Green Master Mix and 25 ng of bisulphite converted DNA was amplified using the Veriti Thermal Cycler (Life Technologies, USA) and cycling conditions used are described in Supplemental Table 1. For region 2, MyTaq™ HS Mix and 25 ng of converted DNA was amplified under the conditions described in Supplementary Table 2.Amplified fragments were cloned into the pGEM-T vector according to the manufacturer’s instructions using the Promega pGEM®-T Easy Vector System I (A1360, Promega, USA). Tenpositive clones were selected for follow up sequencing. Isolated inserts were sequenced using Big Dye Terminator v3.1 Cycle Sequencing Kit (Cat#: 4337455) (Life Technologies, USA) in tandem with SP6 reverse primer (5’ ATTTAGGTGACACTATAG 3’) at 3.3 μM. Sequences were generated using an ABI Prism 310 Genetic Analyser (Applied Biosystems, USA) and Sequencher (Version: 4.10.1, Gene Codes Corporation, USA) was used for analysis. Fine-scale methylation diagrams were created using BiQ Analyser [27](#_ENREF_27).

**2.5. Statistical analysis**

CpG site modification was identified by comparison with the original DNA sequence and was recorded as either a C or a T following bisulphite conversion. The percent methylation for each CpG site was then calculated as the number of methylated cytosines divided by the total number of sequenced clones at that site: (methylC/total clones) X 100. The means and standard deviation (SD) for methylation scores for all samples were calculated using the ‘average’ and ‘stdev’ functions in Microsoft Excel 2010 or generated using column statistics in Prism 6.0c. Error bars reported are the standard error of the mean (SEM). A two-way ANOVA was used to test for statistical significance in methylation levels between different patient groups for each CpG site in each region. Following a two-way ANOVA that reported significance, a Tukey’s Honestly Significant Difference (HSD) or Šídák multiple comparisons test was used to identify differentially methylated sites and generate confidence intervals. Methylation results for a sample/patient were not included in the final analysis if fewer than six clonal sequences were available or if the conversion rate for non-CpG cytosines was low.

An XY-plot and simple linear regression, created in Prism 6.0c, was used to represent average methylation and clinical severity. A two-tailed Pearson’s correlation coefficient ($r$) was calculated for percentage methylation at operation 1 and severity at significant CpG sites, corrected for age. The average correlation ($r$ = -0.401) across these sites was calculated, and a permutation test, with 1000 permutations, was used to assess whether there was a significant relationship between severity and methylation, (HA : $\overbar{r}$ ≠ 0).

**3. Results**

**3.1. Clinical characteristics**

The average age of diagnosis was 34.4 years (18.5 – 50.9) and 40.7 years (18.2 – 63.2) for familial and sporadic cases respectively. The average age at first parathyroid operation was 35.6 and 42.1 years respectively. The average severity score was 6.64 and 6.88 for Tasman 1 MEN1 carriers and sporadic cases respectively.

**3.2. Genotyping FFPE tumour DNA**

To examine whether there was evidence for loss of the wild-type allele in these samples, we sequenced DNA extracted from archived FFPE tissues as previously described. The *MEN1* mutation (CG) was detected in all Tasman 1 family members as expected. Forty-two DNA samples demonstrated no loss, however for individuals 2, 6, 8, 9 and 10, loss of the wild-type (CG>GG) allele was detected in a single pathology sample. Interestingly, individual 9 showed loss of the wild-type allele between surgical interventions. These results are shown in Supplementary Table 6.

**3.3. Promoter methylation in parathyroid tissue**

The *MEN1* gene promoter, individual CpG loci, previously identified regulatory regions (Figure 1A) and sequences amplified are shown in Figure 1B. CpG loci were given a score of ‘1’ or ‘0’ where a methylated cytosine was present or absent following clone sequencing. Methylation was then calculated for each CpG site giving a percentage similar to previous studies [28](#_ENREF_28). CpGs 1 – 23 and 24 – 39 are in region 1 and region 2 respectively.

Region 1 showed overall low levels of methylation and little difference in pattern between samples across all 23 CpG sites in the island (Figure 1C). In contrast, sites 24 – 39 which lie distal to sites 1 – 23, were methylated at a much higher level in parathyroid samples. The average methylation levels across these sixteen sites ranged from 13 – 92%. Comparison of average methylation per locus in samples from MEN 1 individuals (Figure 1D) versus sporadic patients (Figure 1E-F) revealed that CpG sites 25, 27 and 28 had significantly higher methylation in MEN 1 patients when compared with sporadic patients. A non-significant trend of hypermethylation in MEN 1 patients continues in CpG loci 26, 29 – 33 (site 26 and 29 report adjusted *p*-values = 0.06 and 0.056 respectively (Figure 2A). A breakdown of methylation levels for CpG loci 24 – 39 in both operations is shown in Figure 2B. A summary of the adjusted *p*-values for each comparison is shown in Table 1.

**3.4. Association of clinical parameters with methylation**

The correlation coefficient was calculated for MEN 1 patient severity index against the average methylation level per relevant CpG site (24 – 31). The mean of these coefficients was$ r$ = -0.401, suggestive of a negative association between methylation and severity (Supplementary Table 4), however this relationship was not significantly different from zero using a permutation test, (*p* = 0.17) and when adjusted for patient age, *p* = 0.45. A plot of the linear regression of 10-Cca/PTH (severity) versus average methylation across sites 24 – 31 is shown in Figure 3, $r$2 = 0.271. Correlation analysis was also calculated for sporadic hyperparathyroidism cases, with a mean $r $= -0.09 (*p* = 0.527), indicating a possible inverse relationship between methylation and severity of parathyroid disease (Supplementary Table 5).

In order to further explore a possible association between clinical correlates of disease severity and methylation, the correlation between age of each individual at parathyroid operation and average methylation across CpG sites 24 – 31 was examined (Figure 4). A significant association between these measures was observed (*p* = 0.0407) with an $r$2 = 0.4262 and 95% CI = 0.03921 - 0.9089. A permutation test was used to further examine this relationship*.* The average correlation between methylation and age at parathyroid operation (CpG sites 24 – 31) was $\overbar{r}$ = 0.4999 (*p* = 0.045).

**3.5. Differential methylation at the *MEN1* promoter in whole blood**

Negligible methylation was observed in region 1 and relatively high methylation across CpG sites 24 – 34 in region 2 (Supplementary Figure 1). Significantly lower methylation in MEN 1 patient whole blood was reported for CpG site 31 when compared against both the control and sporadic patient blood. Although no other significant differences were found, confidence intervals and an XY-plot of the data suggest a non-significant trend towards low methylation levels in CpG sites 24 – 33 in MEN 1 patient blood samples compared to both the control and sporadic patient samples indicating that MEN1 gene methylation differences may also be evident in whole blood (Supplementary Figure 2).

 **4. Discussion**

Analysis of an extended region of the *MEN1* promoter region revealed variable levels of methylation, with significantly higher levels of methylation at CpG loci 24 – 31 within region 2. We report little methylation in the first 23 CpG pairs of the CpG island consistent with findings previously reported [17](#_ENREF_17), [23](#_ENREF_23). Analysis of further loci (region 2) in MEN 1 parathyroid tissue indicated that CpG sites 25, 27 and 28 were significantly hypermethylated when compared to the same CpG sites from sporadic parathyroid samples. Methylation analysis of the CpG sites in region 2 has not been previously targeted by bisulphite sequencing, although there are studies reporting parathyroid tumour methylation utilising the Illumina 27K array [18](#_ENREF_18), [29](#_ENREF_29).

There is evidence that the area of the *MEN1* gene encompassed by region 2 may be involved in regulation and therefore hold functional importance. Gene reporter assays reveal that the promoter region -985 to -437 contains inhibitory elements involved in the regulation of the *MEN1* promoter (region 2: -721 to -426) [20](#_ENREF_20), [21](#_ENREF_21). Additionally, a marked decrease in luciferase activity is observed following the removal of the region 437 to -64, supporting its importance in promoter activation [21](#_ENREF_21). It is possible that specific areas or individual CpG sites of the *MEN1* promoter region differentially influence gene expression rather than the overall methylation status of the promoter.

The positive association of methylation with the CCa/PTH ratio of relative disease severity supports the hypothesis that methylation may hinder transcription factor binding in the *MEN1* promoter region, leading to increased gene expression and a milder form of disease. As mentioned above, previous studies have reported that the promoter region with sites 24 – 31 functions as a repressor in the regulation of menin. Therefore, increased methylation may interfere with binding of factors involved in repression of *MEN1*. Using the online prediction tool PROMO, ten different transcription factors were predicted within the DNA fragment with varying confidence [30](#_ENREF_30), [31](#_ENREF_31). We identified five transcription factors that encompassed CpG sites with altered methylation. Of interest were, E2F-1, RPR-β and AhR. However, the regulatory roles of these transcription factors in *MEN1* expression remain to be addressed directly.

Loss of heterozygosity (LOH) was examined in 50 different tumours and LOH was demonstrated in six of these. It has previously been reported that a significant proportion of sporadic or familial MEN 1 parathyroid tumours show LOH [32](#_ENREF_32). Immunohistochemical staining of parathyroid sections also showed no evidence for expression changes associated with LOH (data not shown). However, amino acids 139 – 242 encompass one of three regions in menin required for interaction with JunD, a functional component of the AP1 transcription factor complex [33](#_ENREF_33). Therefore, the splice-site mutation described may disrupt JunD binding [34](#_ENREF_34). It remains to be examined if the methylation reported in this study was observed in the mutated or the wild-type allele and what implications this might have in variable disease severity. We also note that sample size and access to normal parathyroid tissue was limited in this study. A power study that simulated data (5000 simulations per size) by sampling with replacement from 15 familial and sporadic patients indicated that approximately 40 patients with similar correlation patterns would be required to observe a *p* < 0.05.

**4.1. Conclusion**

In conclusion adetailed examination of the MEN1 gene promoter has provided the first evidence that DNA methylation of eight CpG sites in the *MEN1* promoter may influence biochemical severity of hyperparathyroidism. Further studies targeting this region as well as global methylome studies may advance our understanding of epigenetic changes in MEN 1.

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**Author Contributions**

RDP did laboratory experiments, statistical analyses and interpretation, prepared the manuscript, and participated in study design. LP participated in study design, manuscript preparation and provided clinical expertise. JM provided expertise in laboratory experiments, assisted with data analysis and interpretation and critical manuscript review. RT provided expertise in statistical analyses and manuscript preparation. AH provided expertise in study design, data analyses and manuscript preparation. JD provided expertise in study design, laboratory analyses and manuscript preparation and critical review. JB provided clinical expertise including patient selection, interpretation and collection of clinical measures and assisted with manuscript preparation.

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**Figure Legends**

**Figure 1. CpG site map of MEN1 promoter with amplicons. A:** CpG site map (vertical red lines, to scale) for the MEN1 promoter and exon 1. Exon 1 is underlined in blue (+455/+757) and approximately 1200 bp (+455/-1250) of the promoter region is underlined in green. Green vertical lines indicate forward and reverse primers. **B:** Region 1 and 2 sequences with SNPs in green, CpG pairs numbered in red, TIS in blue and primers underlined. CpG sites are numbered 5’ to 3’. **C – F** shows fine-scale ‘maps’ of CpGs, columns represent individual CpG sites where methylated = (●), unmethylated = (○) or uninformative = (X). Rows represent clones sequenced for each sample. **C:** Region 1 results (CpG 1:23) for MEN1 patient 1, *operation 1*. **D:** Region 2 (CpG 24:39) results for MEN1 patient 1, *operation 1*. **E:** Region 2 results for a sporadic MEN1 parathyroid sample with histologically normal appearance. **F:** Region 2 results for sporadic patient 18 with hyperplastic appearance.

**Figure 2. A:** **Plot of the mean percentage methylation for each CpG site in region 2.** Error bars are standard error of the mean (SEM). MEN1 = patients that carry known familial MEN1 mutation (*N* = 46), sporadic (wild-type) = patients with PHPT but are non-carriers of mutation (*N* = 8). **B:** **Plot of the mean percent methylation for each CpG site in region 2: MEN1 vs. Sporadic.** Error bars are SEM. MEN1 (Op1) = MEN1 *operation 1* (*N* = 34), MEN1 (Op2) = MEN1 *operation 2* (*N* = 12), Sporadic (norm\_histo) = sporadic disease with histologically normal tissue (*N* = 2) and Sporadic (abnorm\_histo) = sporadic disease with hyperplastic tissue (*N* = 6). (See Table 1 for accompanying significance results.)

**Figure 3. Average methylation vs. severity.** Plot of linear regression of the mean percent methylation for CpG sites 24 – 31 in *operation 1* parathyroid tissue with matched patient severity ratio. Each data point represents an individual patient. Confidence intervals are shown by dotted lines.

**Figure 4.** Plot of themean percent methylation of CpG sites 24 – 31 in *operation 1* parathyroid tissue with matched patient age in years at *operation 1*. Each point represents a patient as indicated by the corresponding number.

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| --- |
| **Table 1.** Summary of adjusted p-values for Tukey’s multiple comparisons |
| *CpG site* | *Comparison* | *p* |
| 24 | MEN1 (Op2) vs. Sporadic (norm\_histo) | 0.02 |
| 25 | MEN1 (Op1) vs. Sporadic (norm\_histo) | 0.01 |
|  | MEN1 (Op2) vs. Sporadic (norm\_histo) | 0.0005 |
|  | MEN1 (Op2) vs. Sporadic (abnorm\_histo) | 0.03 |
| 26 | MEN1 (Op1) vs. Sporadic (norm\_histo) | 0.02 |
| 27 | MEN1 (Op1) vs. Sporadic (norm\_histo) | 0.008 |
|  | MEN1 (Op2) vs. Sporadic (norm\_histo) | 0.003 |
| 28 | MEN1 (Op1) vs. Sporadic (norm\_histo) | 0.03 |
|  | MEN1 (Op2) vs. Sporadic (norm\_histo) | 0.003 |
| 29 | MEN1 (Op1) vs. Sporadic (norm\_histo) | 0.009 |
|  | MEN1 (Op2) vs. Sporadic (norm\_histo) | 0.001 |
| 30 | MEN1 (Op2) vs. Sporadic (norm\_histo) | 0.004 |
| 31 | MEN1 (Op2) vs. Sporadic (norm\_histo) | 0.02 |
| 32-39 | All | ns |