MEN1 Gene Mutation and Reduced Expression Are Associated with Poor Prognosis in Pulmonary Carcinoids

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Context: MEN1 gene alterations have been implicated in lung carcinoids, but their effect on gene expression and disease outcome are unknown.

Objective: To analyse MEN1 gene and expression anomalies in lung neuroendocrine neoplasms (NENs) and their correlations with clinicopathologic data and disease outcome.

Design: We examined 74 lung NENs including 58 carcinoids and 16 high-grade neuroendocrine carcinomas (HGNECs) for MEN1 mutations (n=70) and allelic losses (n=69), promoter hypermethylation (n=65), and mRNA (n=74) expression. Results were correlated with disease outcome.

Results: MEN1 mutations were found in 7/55 (13%) carcinoids and in 1 HGNEC, mostly associated with loss of the second allele. MEN1 decreased expression levels correlated with the presence of mutations (P=0.0060) and was also lower in HGNECs than carcinoids (P=0.0024). MEN1 methylation was not associated with mRNA expression levels. Patients with carcinoids harbouring MEN1 mutation and loss had shorter overall survival (P=0.039 and P=0.035, respectively), and low MEN1 mRNA levels correlated with distant metastasis (P=0.00010) and shorter survival (P=0.0071). In multivariate analysis, stage and MEN1 allelic loss were independent predictors of prognosis.

Conclusion: Thirteen percent of pulmonary carcinoids harbour MEN1 mutation, associated with reduced mRNA expression and poor prognosis. Also in mutation-negative tumours, low MEN1 gene expression correlates with an adverse disease outcome. Hypermethylation was excluded as the underlying mechanism.

Pulmonary neuroendocrine neoplasms (NENs) include well differentiated carcinoids, classified into typical (TCs) and atypical (ACs) subtypes, with a relatively favorable prognosis (1, 2), and aggressive high-grade neuroendocrine carcinomas (HGNECs), ie, large cell neuroendocrine carcinoma (LCNEC) and small cell lung cancer (SCLC).

Lung carcinoids occur in 5% of patients with multiple endocrine neoplasia (MEN) type 1 syndrome with MEN1 germline mutations (3). MEN1 mutations have been reported in approximately 16% of sporadic lung carcinoids.
(4–6), but are exceptional in HGNECs (7, 8). Allelic losses of the MEN1 locus are detected more frequently (~36%) than gene mutations (5), suggesting that alternative mechanisms might lead to MEN1 gene inactivation. So far, the association of MEN1 gene alterations with clinical follow-up has not been reported in lung NENs.

Here we report a comprehensive analysis of MEN1 alterations, methylation status and transcriptional expression in 74 lung NENs. Results are compared with tumor characteristics and clinical outcome.

Materials and Methods (detailed in Supporting Information)

Tissue samples
Seventy-four sporadic lung NENs (34 TCs, 23 ACs, 1 unclassified carcinoid, 9 LCNECs, 6 SCLCs and 1 mixed LCNEC-SCLC), including 71 with follow-up (median 52 months) were studied. Institutional review board approval for the study was obtained by each contributing center following local ethical requirements. Nine neuroendocrine cell lines were included for MEN1 mRNA expression analysis.

MEN1 mutational analysis
The entire coding region and splice junctions of MEN1 were analyzed on tumor and matched normal DNA by Sanger sequencing, as described (9) (Supplementary Table 1A).

MEN1 deletion analysis
Fluorescent in situ hybridization on frozen sections was performed using the MEN1 locus BAC-probe CTD-222019, and centromere probes for chromosomes 1, 11, 3 and 7 as described (10).

Quantitative real-time PCR (QRT-PCR)
QRT-PCR was performed using mRNA on MEN1, menin interactors JUND and MLL, and menin targets HOXC8, P18, P21, P27, using primers listed in Supplementary Table 1B. Expression levels were normalized to ACTB and CYP2A housekeeping genes as described (11).

Methylation-Specific PCR (MSP) and next-generation sequencing (NGS)
MEN1 promoter methylation analysis was performed on all cases by nested MSP (Supplementary Table 1C) and by NGS on Methyl-CpG-binding Domain (MBD)-immunoprecipitated DNA of 10 primary carcinoids and carcinoid cell lines NCI-H720 and NCI-H727 (focusing on the MEN1 gene).

Statistical analysis
Correlations between MEN1 data and follow-up were determined using IBM SPSS Statistics (v20.0.0; IBM) setting the P-values cut-off for significance at \( \leq 0.05 \). Differences in mean MEN1 mRNA levels were determined by Student’s t-test, and the optimal cut-off point was determined using area under the ROC-curve analysis. Associations between clinical and molecular parameters were calculated using logistic and linear regression, Fisher’s exact or \( \chi^2 \) tests, as appropriate. Associations between relative expression levels of MEN1 and other genes were calculated using Pearson’s correlation.

Survival curves were created by the Kaplan-Meier method and compared using the log-rank test. The prognostic value of age at diagnosis, histopathology, sex, smoking status, tumor diameter and MEN1 mutation, deletion and gene expression were tested in univariate and multivariate settings using Cox-regression. Only variables with a \( P \)-value < 0.10 in univariate analyses were included in multivariate analyses.

Results
MEN1 gene mutations and deletions are more frequent in ACs
MEN1 mutations were searched in 70 tumors, including 7 previously published cases (5). Eight cases (11%) showed nonsynonymous somatic alterations: 2/32 TCs (6%), 5/23 ACs (22%) and 1/7 LCNECs (14%). Mutations included three small deletions, three single nucleotide substitutions and two insertions (Supplementary Tables 2 and 3). Patient 35 showed the same MEN1 mutations in the primary tumor and two distant metastases removed eight and ten years later.

Ploidy status and MEN1 deletion were analyzed by FISH in 69 tumors (Table 1B and Figure 1A-C). Most carcinoids were diploid whereas HGNECs were more frequently polysomic. Among carcinoids, 8/57 (14%, 1 TC and 7 ACs) showed MEN1 allelic loss. In HGNECs, MEN1 was deleted in 3/12 cases (25%). MEN1 deletion was more frequent in ACs than in TCs (\( P = .0060 \)), and was significantly associated with presence of mutations in both the total group of NENs (\( P = .0022 \)) and in carcinoids (\( P = .0063 \)) (Supplementary Table 3).

MEN1 gene expression is heterogeneous in lung NENs
Quantitative RT-PCR performed on all 74 NENs showed that relative MEN1 expression levels in lung NENs ranged from 0.031 to 0.85, generally lower than exocrine pancreas (mean 0.58; Figure 1D) but higher than normal lung (mean 0.078). A gradual decrease in mean MEN1 expression values was apparent from TCs to ACs and HGNECs (Figure 1D-E). Similarly, MEN1 expression levels in SCLC NCI-H69 and LCNEC NCI-H460 cell lines were lower than in lung carcinoid NCI-H720 and NCI-H727 cells (Figure 1D). MEN1 gene expression levels were inversely correlated to the presence of mutations and deletions (Supplementary Table 3) in the complete group of lung NENs (\( P \leq .0060 \)) and carcinoids (\( P \leq .025 \)).
Table 1. Univariate and multivariate analysis of clinical data and MEN1 parameters correlated with outcome of patients with pulmonary neuroendocrine neoplasms (NENs)

<table>
<thead>
<tr>
<th>A) Lung NENs</th>
<th>B) Carcinoids</th>
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<tr>
<td><strong>Univariate</strong></td>
<td><strong>Multivariate</strong></td>
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<tr>
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<td>P-value</td>
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<tr>
<td>MEN1 gene expression</td>
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</tbody>
</table>

*Only parameters displaying a P-value <0.05 are included in multivariate analysis and in the final model all P-values needed to be <0.05.

Abbreviations used: NEN: neuroendocrine neoplasm; NI, not included in multivariate analysis; NS, not significant

No correlation between MEN1 methylation and gene expression

As low MEN1 expression levels were present in tumors with neither MEN1 mutation nor deletion, we analyzed MEN1 promoter methylation by MSP. No methylation-specific product was seen in 65 primary NENs, cell lines and 15 normal lungs from noncancer patients. NGS in 10 primary carcinoids and lung carcinoid cell lines NCI-H720 and NCI-H727 showed some methylation signals in MEN1 promoter region and gene body, but not at the locus used for MSP analysis (Supplementary Figure 1). No methylation signals were found in CpG-islands. There were no differences in methylation between samples with high and low MEN1 mRNA levels.

Deregulation of MEN1 is associated with metastatic disease and poor prognosis

MEN1 gene mutations and deletions and decreased gene expression were associated with the presence of distant metastasis in both the total group of NENs and the carcinoid subgroup, but were not related to sex, age and smoking status (Supplementary Table 4). In particular, MEN1 mRNA levels were significantly lower in patients with distant metastasis and/or patients who died from their tumor, both in the entire group (0.162 vs. 0.318, P = 0.00080) and within the carcinoid subgroup (Figure 1E).

Univariate survival analysis showed in both the complete group of NENs and the carcinoid subgroup that age, histopathology, stage, presence of MEN1 deletions and decreased gene expression were significantly associated with higher risk of death (Table 1). At multivariate survival analysis, histopathology and decreased MEN1 gene expression in lung NENs overall, and stage and MEN1 deletion in the carcinoid subgroup retained statistical significance. Kaplan-Meier survival analysis confirmed that the 15-year overall survival of lung carcinoids was significantly shorter in patients with MEN1 mutation (Figure 1F), decreased mRNA levels (Figure 1G) and deletion (P = .035).

Menin interactors and target genes

Within the complete set of NENs, MEN1 gene expression was correlated with that of its interactors JUND (P = .016) and MLL (P = .026) but not of its targets HOXC8, P18, P21 and P27. Significant associations between MEN1 deletion and low HOXC8 expression (P = .010) and low P18 gene expression (P = .017) were also found. MEN1 mutational status and menin protein expression were not associated with mRNA levels of interactors and downstream targets.

In the complete group of NENs, as well as within carcinoids, lower levels of P18 expression (P = .0074 and P = .0011, respectively) were associated with lower 15-year survival.

Discussion

Our study on pulmonary NENs shows that the presence of MEN1 mutation and/or a lower MEN1 gene expression is associated with distant metastasis and lower 15-year survival, in both the entire cohort of NENs and lung carcinoids considered separately.

MEN1 mutations were found in 13% (7/55) of carcinoids, in line with previous reports (4, 5), with most mutations occurring in ACs. All mutations were of the loss of function type, and most mutated cases also displayed deletion of the second MEN1 locus. Only 1/15 HGNECs was mutated, confirming that MEN1 mutations do not play a major role in HGNECs (7, 8).

The large majority of neoplasms with wild-type MEN1 gene had high mRNA levels. However, not all cases with low mRNA levels harbored mutations or deletions, indicating that other mechanisms might unbalance MEN1 ex-
expression in these tumors. No hypermethylation was seen at CpG-islands within the MEN1 promoter at MSP-analysis, while methylated regions outside the CpG-island were identified by MBD-sequencing. However, such methylation was not associated with reduced MEN1 mRNA levels, suggesting that MEN1 hypermethylation unlikely underlies MEN1 downregulation.

MEN1 gene alterations and reduced expression were significantly associated with tumor stage, presence of distant metastases, and patient survival. At multivariate analysis, decreased MEN1 gene expression was an independent predictor of disease outcome in both the complete group of NENs and in the group of carcinoids alone. This finding seems specific for lung NENs since it is not observed in pancreatic NENs (10, 14).

The mechanisms underlying the role of MEN1 gene loss of function and the acquisition of clinical aggressiveness in lung carcinoids deserves further investigation. We identified correlations between MEN1 and JUND/MLL mRNA levels, but not with menin targets P18, P21 and P27, suggesting that lung-specific mechanisms might be active, such as the E-cadherin/β-catenin pathway which interacts with menin and is frequently deregulated in lung NENs (7, 15, 16).

In conclusion, our comprehensive overview of MEN1 molecular alterations in lung carcinoids demonstrates a significant association between MEN1 gene deregulation and clinical aggressiveness in lung carcinoids and postulates different mechanisms of gene regulation in well and poorly differentiated NENs.

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**Figure 1.** MEN1 gene alterations and expression in lung neuroendocrine neoplasms, and association with survival. A-C: Fluorescence in situ hybridization for the MEN1 locus (in red), showing a case with a MEN1 mutation but retention of two copies of the MEN1 gene (A), and two cases with MEN1 mutation and associated loss of the MEN1 locus (B and C), the latter in the presence of two copies of chromosome 7 (centromeric probe of chromosome 7 in green). D: Scatterplots showing the relative mRNA expression values of MEN1 for typical carcinoids (TC), atypical carcinoids (AC), large cell neuroendocrine carcinomas (LCNEC), small cell lung cancers (SCLC), neuroendocrine (NE) cancer cell lines and normal tissues, compared to the geometric mean of the expression levels of the housekeeping genes ACTB and CYPA. The horizontal bars represent the mean expression values. E: Differences between the mean values ± SD of MEN1 gene expression levels between the total group of carcinoids (TC/AC) and NE carcinomas (LCNEC/SCLC) (left; 95% confidence interval (CI) [CI] –0.175/-0.0400) and of carcinoid patients (TC/AC) with a favorable (Fav) and a poor disease outcome (right; 95% CI 0.0423/0.257). F-G: Kaplan-Meier analyses depicting 15-year overall survival in the group of carcinoids according to the presence of MEN1 gene mutations (F, dotted line) or MEN1 gene expression levels below the cut-off point of 0.136 (G, dotted line).
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