Microsatellite instability and loss of heterozygosity at the *MEN1* locus in lung carcinoid tumors: A novel approach using real-time PCR with melting curve analysis in histopathologic material

DIMITRA VAGELI[1], ZOE DANIIL[2], JUBRAIL DAHABREH[3], ELENI KARAGIANNI[4],
TRIANTAFILLOS LILOGLOU[5], GEORGE KOUKOULIS[1] and KONSTADINOS GOURGOULIANIS[2]

[1]Department of Pathology, [2]Respiratory Medicine Department, Medical School, University of Thessaly, Larissa;
[3]Thoracic Surgery Department, [4]Department of Pathology, Athens Medical Centre, Athens, Greece;
[5]Roy Castle Lung Cancer Programme, University of Liverpool Cancer Research, Liverpool, UK

Abstract. The possible causes and genetic mechanisms of pulmonary carcinoid tumor development are unclear. In this study, we examined genetic alterations at the *MEN1* locus in archival material from 15 pulmonary carcinoids. We employed, for the first time in this setting, real-time PCR with melting curve analysis in order to identify loss of heterozygosity (LOH) or microsatellite instability (MI) in two polymorphic markers (PYGM, D11S449) at the *MEN1* locus and one additional marker (D11S906) of a putative oncosuppressive region distal to the *MEN1* gene. Sequencing data were available in a selected subset of tumors in order to verify the reliability of real-time PCR analysis. We observed LOH at PYGM in 38% of the cases and MI in 13.3% of the cases. Our data indicate that real-time PCR with melting curve analysis is a reliable technique for LOH and MI detection and indicate that genetic errors at the *MEN1* locus but also distal to it may be involved in the development of sporadic pulmonary carcinoid tumors.

Introduction

The *MEN1* gene was discovered in 1997 by Chandrasekharappa *et al* (1). The *MEN1* locus has been mapped to chromosome 11q13 by establishing linkage to PYGM (2) while additional linkage analysis placed the *MEN1* locus within a region flanked by D11S1883 and D11S449 (3,4). Loss of hetero-zygosity (LOH) studies attributed tumor suppressing function to the *MEN1* gene region, telomerically to PYGM (5-7). Other LOH studies suggest the presence of another tumor suppressor gene close to the *MEN1* gene (8,9).

Alterations of the *MEN1* gene, including allelic deletions and somatic mutations, have been documented in a significant number of endocrine neoplasms (2,10-16). In pulmonary carcinoids, an interesting subset of endocrine tumors, similar *MEN1* alterations have been reported and considered to be involved in the development of these tumors (17-23).

The possible existence of genomic instability in the *MEN1* region has not been extensively investigated, although few studies have indicated its presence (24,25). Microsatellite instability (MI), as an indicator of genetic polymorphism, has received attention in a single report (25).

In the current study, we employed a real-time PCR method using melting curve analysis in histopathological material from 15 lung carcinoid tumors in order to identify genetic alterations close or distal to the *MEN1* tumor suppressor region. We used probes for three polymorphic markers: PYGM, D11S449 at the *MEN1* region and D11S906 distal to *MEN1* on chromosome 11q.

Materials and methods

Histopathological specimens. Tumor specimens were collected from the archives of the Pathology Departments of the University Hospital of Larissa, and the 'Medical Centre' Hospital in Athens, Greece. In each case, formalin-fixed and paraffin-embedded tumor and non-neoplastic (normal) tissue samples were available. The pathology of all tumor specimens was reconfirmed indicating >70% tumor cells. All 15 samples were typical carcinoids (TC) (Table II). Two patients exhibited positive lymph nodes at pathology including metastasis, 1 exhibited positive lymph node without metastasis, and 2 had metastasis without positive lymph nodes.

Sample preparation and DNA extraction. Tissue sections, 8-10 μm in thickness, were prepared from paraffin blocks of each tumor and normal tissue sample. Sections were deparafinized using a xylene/EtOH procedure by Wright and Manos (26). DNA was extracted from the tissue sections using the Puregene® Cell and Tissue extraction kit, Gentra, following the manufacturer's instructions. Genomic DNA

Correspondence to: Dr Dimitra Vageli, Department of Pathology, Medical School, University of Thessaly, 22 Papakiriazi Str., 41222 Larissa, Greece
E-mail: vagelidim@med.uth.gr

Table I. Oligonucleotide sequences of primers.

|	Primer name	Sequence	Annealing temperature (˚C)
Marker			
PYGM	PYGM.CA3'-2	GCT GTC AGG TAG CAA CTG AC	59
	PYGM.CA5'	CTA GCA GAG TCC ACC TAC TG	
D11S449	C1219-CA	CCT CGT GCT GGA ATG GGC TC	62
	C1219-GT	CAG GAA GTG TTA AGA GGC TGG	
D11S906	AFM107xc7a	AGC TGG GCA CCG ATA GTA GT	59
	AFM107xc7m	GCA CAG GCA AAG ANG AGG TA	
Housekeeping gene			
ß-globin	S-b-globin	5'-GGA AAA TAG ACC AAT AGG CAG-3'	61
	A-b-globin	5'-ACA CAA CTG TGT TCA CTA GC-3'	
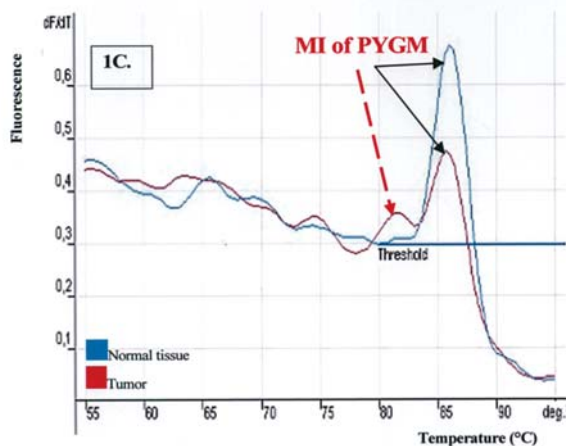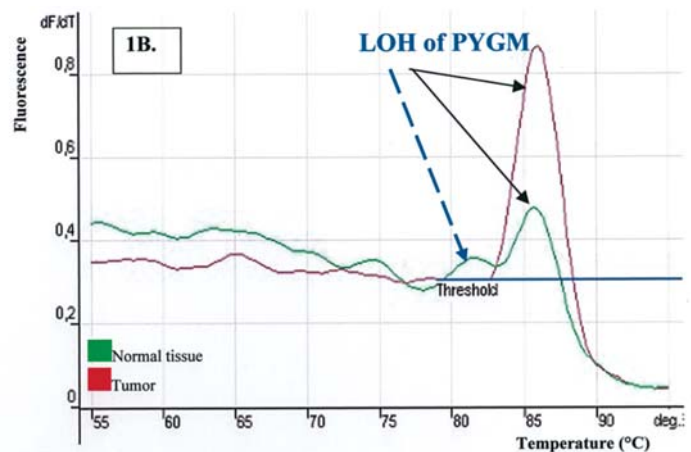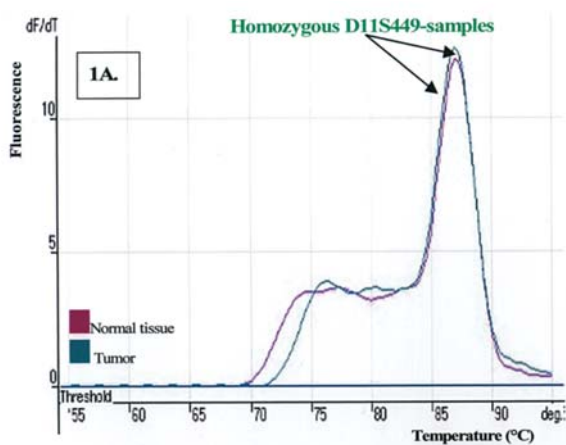
Figure 1. Detection of genetic alterations of MEN1 locus, by real-time PCR - melting curve analysis in DNA pulmonary carcinoid samples. (A) Detection of homozygosity at D11S449 polymorphic site. Homozygosity was shown by a single melting peak. Note that the homozygous samples, normal (green) and tumor (purple) showed a similar melting temperature peak. (B) Detection of LOH at PYGM polymorphic site. Heterozygous normal tissue (green) (two melting peaks) had lost an allele (blue arrow) in tumor tissue (red). (C) Detection of MI at PYGM polymorphic marker. Homozygous normal tissue (blue) [one melting peak (black arrow)] was changed by MI since in the tumor tissue (red) appeared a new melting peak (red arrow) that was not present in the non-neoplastic sample.

samples were stored at -20˚C. The quality of DNA was checked by electrophoresis (1.3% agarose gel) and DNA concentration was assessed spectrophotometrically ($A_{260/280}$).

PCR analysis. Amplification of the *ß-globin* gene was performed in order to qualify and normalize the amount of DNA in each sample. The primers of the *ß-globin* gene were 5'-GGA AAA TAG ACC AAT AGG CAG-3' (sense oligo) and 5'-ACA CAA CTG TGT TCA CTA GC-3' (antisense oligo). The PCR product was 250 bp and the annealing temperature was 61˚C. The primers used for the amplification of the

PYGM, D11S449 and D11S906 markers have previously been referred (Table I). PCR reactions were performed in a 25-μl reaction volume and 100 ng of genomic DNA, 400 μM dNTPs, 20 pmoles of each forward and reverse primers, 0.2 units of Taq DNA polymerase (Invitrogen®) and 10X PCR buffer (Invitrogen) with 1.5 mM $MgCl_2$. The DNA was amplified for 30-35 cycles. 10-15 μl of the PCR products were electrophoresed for 6 h on a 10% (19:1) polyacrylamide gel in 150 V and viewed by silver-staining. Interpretation of the allelic imbalance was undertaken using an image analysis system (Bio-Profil®, UVP Image Analysis Software, Vilbert Lourmat).

Table II. Real-time PCR analysis of polymorphic markers at *MEN1* locus.

Case	Sex	Age	Histology	Positive nodes at pathology[a]	Metastasis[b]	*MEN1* 11q13 PYGM	D11S449	11q14.3-21 D11S906	1 or 2 markers[c]
1	M	58	TC			LOH	NI	HR	•
2	M	65	TC	-		HR	NI	HR	
3	M	70	TC	-		LOH	NI	LOH	••
4	F	68	TC	-		NI	NI	MI	•
5	F	53	TC	-		NI	NI	MI	•
6	M	38	TC	-	Mx	NI	NI	NI	
7	F	30	TC	+		HR	NI	MI	•
8	F	29	TC	-	-	MI	NI	MI	••
9	F	26	TC	+	M	HR	NI	LOH	•
10	M	45	TC	-	-	MI	NI	-	•
11	F	27	TC	-	-	LOH	NI	MI	••
12	F	11	TC	-	Mx	NI	NI	NI	
13	F	73	TC	+	Mx	HR	NI	NI	
14	M	40	TC	-		NI	NI	-	
15	M	29		-		NI	NI	HR	

[a]Positive lymph nodes at pathology (+); no lymph node metastasis reported (-); ND, no data. [b]Metastasis (M); TC, typical carcinoids. [c]Each one of the polymorphic markers that showed a genetic alteration is indicated with a dot (•). Two dots (••) indicate that two markers were affected.

Real-time PCR analysis. Real-time PCR with melting curve analysis was performed for three polymorphic markers: PYGM, D11S449 at the *MEN1* region and D11S906 distal to *MEN1* on chromosome 11q. The sequences of previously published primers (3,20) were used for the amplification of the three markers (Table I). In addition, primers for the amplification of a housekeeping gene (*ß-globin*) were used for reaction quality control reaction purposes. Real-time PCR reactions were performed by RG-3000 (Corbett Research Rotor Gene™). All samples were run in duplicate and two non-template-controls (NTC) were included in the reactions. PCR amplifications and melting curve analysis were repeated two times. Each reaction volume was 20 µl and contained 100 pg of genomic DNA, 0.6 µM each of forward and reverse primers, and SYBR®-Green qPCR SuperMix-UDG (2X) (Invitrogen) containing SYBR-Green I fluorescent dye, 60 U/ml Platinum® Taq DNA polymerase, 40 mM Tris-HCl (pH 8.4), 100 mM KCl, 6 mM $MgCl_2$, 400 µM dGTP, 400 µM dATP, 400 µM dCTP and 400 µM dUTP, 40 U/ml uracil-DNA glycosylase (UDG) and stabilizers. The conditions of reactions were as follows: 50˚C for 2 min, 95˚C for 2 min, 35 cycles of 95˚C for 15 sec (not acquiring), annealing temperature for each set of primers (Table I) for 20 sec (not acquiring) and 72˚C for 20 sec (acquiring for SYBR). Continuously melting curve analysis performed ramping 55-95˚C (rising by 1˚C each step) and finishing at 72˚C for 5-10 min. Before the experiment started, we calibrated (acquiring for SYBR) at 72˚C.

Data analysis. Following completion of the amplification, melting curve analysis was performed. Bins were created for each peak. The melting temperature peak of each marker was determined by using control DNA samples. Threshold level and temperature threshold were useful when there was noise of the signal at low temperatures. Full report was saved for each sample. PCR product specificity was confirmed on a 2% agarose gel.

Analysis of a PCR-amplified DNA sample showed one melting peak for homozygous patients (Fig. 1A) and two melting peaks for heterozygous patients (Fig. 1B and C). In LOH, only one of the two melting peaks remains in the tumor sample (Fig. 1C). Microsatellite instability (MI) was identified when, in a tumor tissue sample, a new melting peak was created which was not observed in the paired normal sample from the same patient (Fig. 1C).

LOH threshold calculation. In order to detect LOH with high specificity, we measured the interassay variability by repeated reactions of normal samples (27). In brief, we assayed seven normal samples five times each and calculated the allelic ratio variation among the reactions of each sample. We then calculated the 95% reference range (mean ± 2 x SD) to 0.71-1.27. Thus, during specimen evaluation, any specimen (T/N) value found in both reactions outside this region was scored as LOH with a false positive probability of 2.5×10^{-4}.

Sequencing analysis. Sequencing data were available in a group of samples. The PCR products were purified with QIAquick® PCR purification kit (Qiagen). Nucleotide sequences were determined by direct sequencing (www.Lark.com.) and analyzed with chromas Sequencer program (http://www. technelysium.com.au/chromas.html).

Results

Real-time PCR and melting curve analysis. The melting curve analysis at the PYGM locus revealed that 3 out of 8 (38%) informative (heterozygous) samples displayed LOH (Tables II

Table III. Genetic alterations at the *MEN1* locus using melting curve analysis.

No Case (T/N samples)	Melting peak* D11S906											Melting peak PYGM								M. peak D11S449
	A	B	C	D	E	F	G	H	I	J	K	A	B	C	D	E	F	G	H	A
	78	79	80	82	83	84	86	87	88	89	90	77	78	80	81	82	84	85	86	87
1T						84				89								85		87
1N						84				89			78					85		87
2T	78				83								78						86	87
2N	78				83								78						86	87
3T		79															84			87
3N		79		82									78				84			87
4T			80		83												84			87
4N	78				83												84			87
5T						84	86												86	87
5N				82		84													86	87
6T									88										86	87
6N									88										86	87
7T				82					88							82			86	87
7N				82												82			86	87
8T					83				88			77							86	87
8N	78				83										81				86	87
9T				82									78					85		87
9N				82				87					78					85		87
10T																82			86	87
10N																			86	87
11T					83				88										86	87
11N					83						90					82			86	87
12T						86													86	87
12N						86													86	87
13T						86							78						86	87
13N						86							78						86	87
14T																			86	87
14N																			86	87
15T							86			89									86	87
15N							86			89									86	87

*Melting peaks of polymorphic markers (PYGM, D11S906, D11S449); allele of each locus (A, B, C etc.) and the corresponding melting peak temperatures; genetic alterations at the *MEN1* locus: LOH (yellow), MI (orange), LOH and MI (orange and yellow); genotyping, heterozygous samples (green) and homozygous (blue).
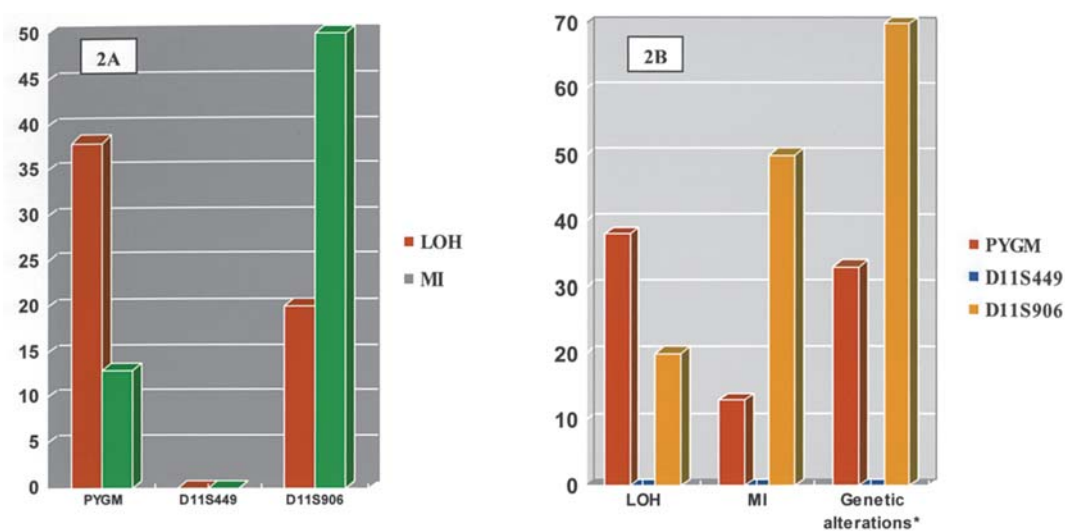

Figure 2. (A) Percentages of cases with genetic alteration for PYGM, D11S449 and D11S906 polymorphic markers in lung carcinoid tumors. LOH, loss of heterozygosity; MI, microsatellite instability. (B) Genetic alterations of *MEN1* polymorphic markers in lung carcinoid tumors. LOH, loss of heterozygosity; MI, microsatellite instability; genetic alterations*, total percentage of cases with genetic alterations (LOH or MI) in those markers.

and III; Figs. 2A and 3B). In addition, 2 out of 15 total (13.3%) samples showed microsatellite instability at the PYGM site (Table II, Figs. 2A and 3C). Unfortunately, none of the 15 samples analyzed for the DS11449 marker was informative

(Tables II and III; Figs. 2A and 3A). The melting curve analysis for the D11S906 locus revealed LOH for this locus in 2 out of 10 (20%) informative samples (Table III, Fig. 2A) while 5 out of 10 (50%) samples showed microsatellite
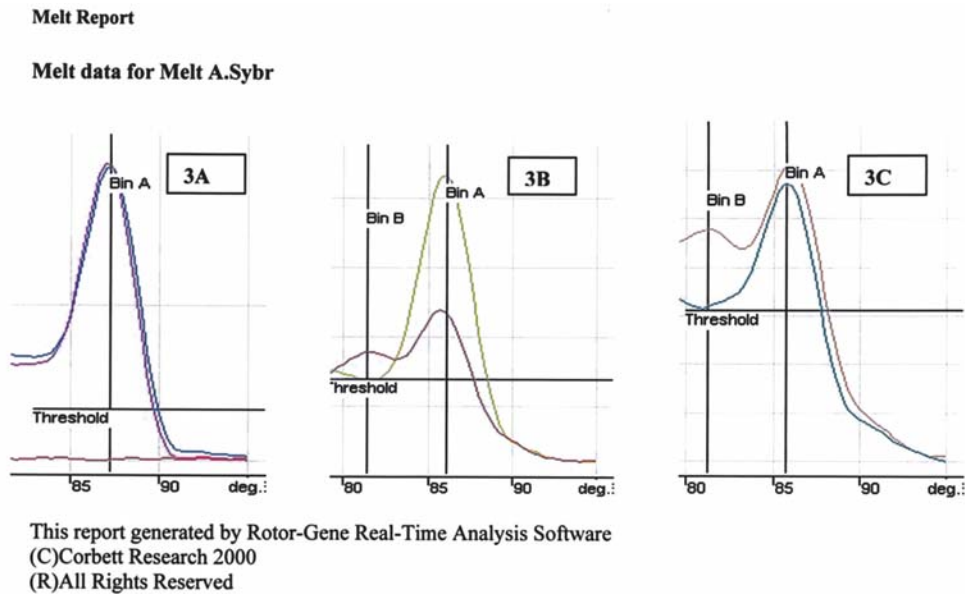
Melt Report

Melt data for Melt A.Sybr

This report generated by Rotor-Gene Real-Time Analysis Software
(C)Corbett Research 2000
(R)All Rights Reserved

Figure 3. (A) Homozygous sample for D11S449 marker. Blue: 697T-D-SG, 87.2˚C (112 bp) (Bin A). Purple: 697N-D-SG, 87.2˚C (112 bp) (Bin A) melting peak. Red: NTC (non-template control). (B) LOH of PYGM locus. Yellow: 2405T-pygmSG, 86.1˚C (180 bp) (Bin A). Red: 2405N-pygmSG, 82˚C (171 bp) (Bin B), 85.8˚C (180 bp) (Bin A). (C) MI of PYGM locus. Orange: 2696T-pygmSG, 82˚C (171 bp) (Bin B), 86˚C (180 bp) (Bin A). Green: 2696N-pygmSG, 86˚C (180 bp) (Bin A).
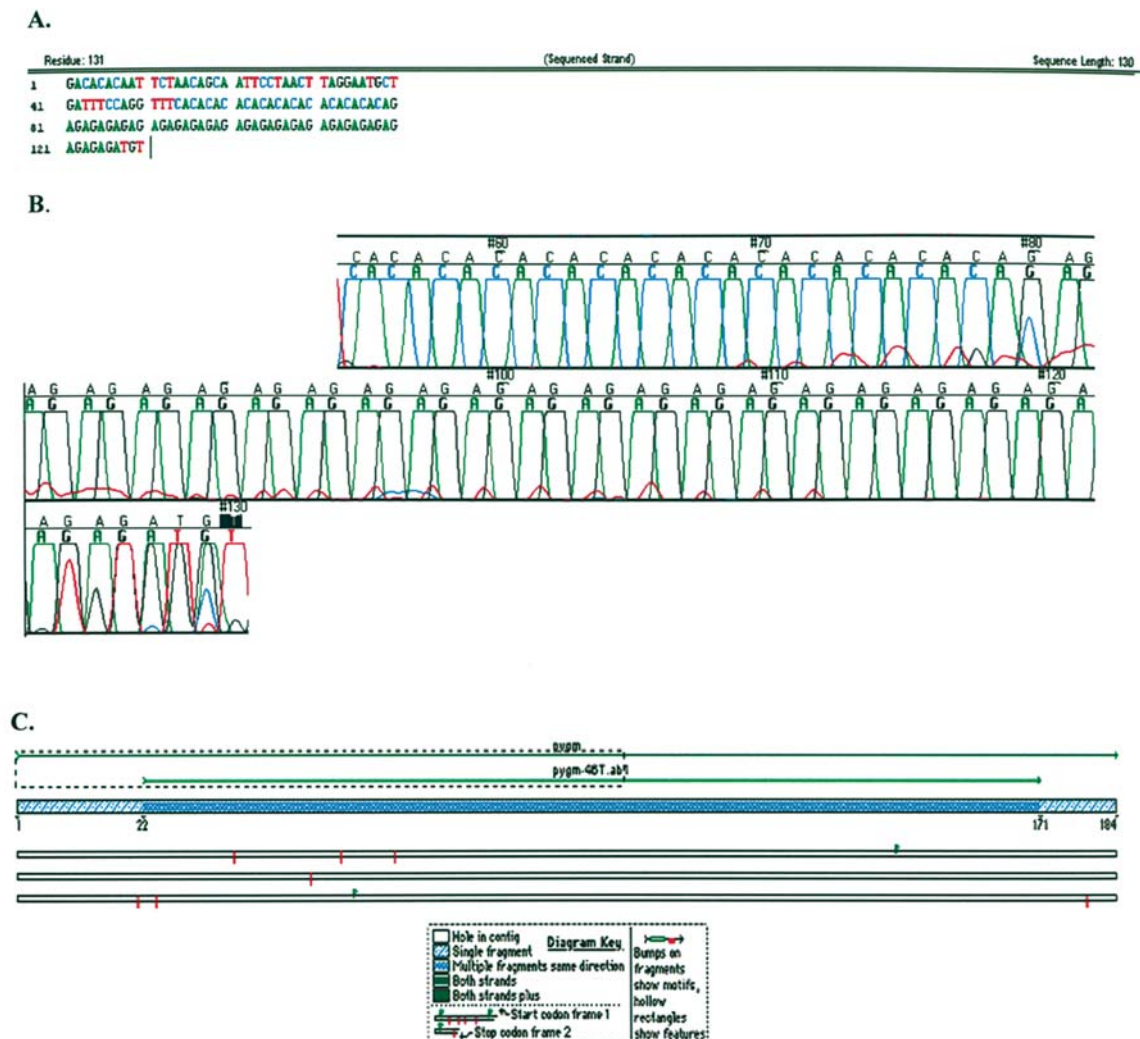

Figure 4. Representative sample of sequencing with reverse primer of PYGM marker. There is loss of a (GA)$_5$ dinucleotide repeat [wild-type contains (GA)$_{29}$]. (A) A portion of the sample sequence. (B) Chromatograms of dinucleotide repeats sequence. (C) Comparison between wild-type and sample sequences.

Table IV. Sequences of polymorphic markers at the *MEN1* locus.

```
Pygm
                                                         c agagtccacc tactg ttagt gacacacaat
         56161 tctaacagca attcctaact taggaatgct gatttccagg ttcacacaca cacacacaca
         56221 cacacacaga gagagagaga gagagagaga gagagagaga gagagagaga gagagagaga
         56281 gagagat gtc agttgctacc tgacagc


D11S449
                                                  cctcgtgc tggaatgggc tc taacacct
          6481 acacagacac atacagacac acacagacac atacagacac acatacacac gcatcagaga
          6541 ctgc ccagcc tcttaacact tcctg


D11S906(afm)

             1 agctgggcac cgatagtagt nggtaatccc tgctactcag naggctgagg tgggagaatc
            61 acttgaacct gggagncana ggttgcagta agccgaggtc acgttattgt actccggcct
           121 ggacgacaga gcaagactct gtctcaaaca cacacacaca cacacacaca cacacacaca
           181 atcagtgtat atacacacac aaaaggacac aagactgaaa gnatatgcaa cagaaaatta
           241 atagtaaatg catctcagaa ttatatttat tttttc tac ctcntctttg cctgtgc
```

The sequences of primers are boxed.

instability at the D11S906 site (Table III, Fig. 2A). Sequencing analysis in a subset of specimens confirmed the repeat changes in all samples with detected MI (Table IV, Fig. 4).

In summary, genetic alterations of the *MEN1* gene were found in 45% of the cases for the PYGM marker while 70% of the cases exhibited a genetic alteration in the D11S906 site (Fig. 2B). Moreover, 33% of the cases exhibited a genetic alteration (LOH or MI) in two polymorphic markers (Table III, Fig. 2B). No correlation was observed between LOH/MI and any of the clinicopathological data available.

Real-time PCR and PCR analysis data. PYGM analysis with real-time PCR showed LOH in 3 patients, which was in agreement with PCR analysis data. Moreover, real-time PCR revealed microsatellite instability in 2 patients that had not observed.

D11S449 analysis with real-time PCR was in agreement with PCR analysis for this locus. D11S906 analysis with real-time PCR showed LOH in 2 patients, which was in agreement with PCR analysis. Moreover, MI in the D11S906 locus was observed in 2 patients by PCR analysis while real-time PCR and melting curve analysis revealed three more cases that were affected by MI.

Discussion

Alterations of the *MEN1* gene occur in a great variety of endocrine tumors and they are considered also to be involved in the pathogenesis of pulmonary carcinoids (17-24). Specifically, allelic deletions and mutations of the *MEN1* gene have been reported in lung carcinoid tumors, including those developing sporadically in the absence of MEN1 syndrome (17,20,24). These alterations appear to be important in cancer pathogenesis and to affect additional genes telomerically to the *MEN1* gene (8,9).

Several previous reports have focused on PYGM, a highly informative marker of the *MEN1* gene (19,24,25). The application of conventional PCR has revealed LOH in a significant number of predominantly sporadic pulmonary carcinoid cases (17,19,20,24). Our findings are, generally, in agreement with those previously reported (17) as we detected LOH in 38% of the studied cases. It is possible that the application of real-time PCR with melting curve analysis offers some increased sensitivity in the detection of LOH. Real-time PCR has been previously shown to be an efficient method for the detection of MI (28-30). In this study, we employed real-time PCR using SYBR-Green I dye and melting curve analysis and we showed that this is a different version of the method employed for MI detection. Genomic or microsatellite instability in the PYGM region has not been investigated extensively to date as there seems to be a single report of genomic instability associated with high poly-morphism of the PYGM locus (25). In our study, microsatellite instability of the same locus was detected in 13.3% of the cases by real-time PCR with melting curve analysis comparing tumor samples with normal tissue. D11S449 was also not extensively analyzed in pulmonary carcinoids. There is one previous report of LOH in 4 of the 8 studied cases (17). In our study, this marker did not prove do be polymorphic at all as all the examined samples were homozygous.

We also analysed D11S906, which is located telomerically to the *MEN1* gene and is a locus that has not been analyzed intensely in sporadic lung carcinoids, despite preliminary indications that it may label a locus with oncosuppresive activity (8,20,31). LOH at D11S906 was previously shown in >50% of the cases (20,24) while MI has been reported in a single case (24). We confirmed that LOH is frequent and we demonstrated, for the fist time, that the D11S906 region shows significant genetic instability, as we found MI in 50% of our cases.

Multiple genetic alterations of *MEN1* markers were found (Fig. 2) in certain samples. This is in agreement with previous studies indicating that, in sporadic lung carcinoids, there are multiple genetic alterations concentrated at the 11q locus, with a significant proportion of concurrent alterations of various genetic markers (17,20,24,31).

Of course, loss of the oncosuppresive function of *MEN1* would not be the only mechansim for the development of sporadic lung carcinoids (32). It has been suggested that there is an independent locus with oncosuppressive activity, on 11q telomerically to the *MEN1* gene (8). We have noticed that D11S906 showed frequent existence of allelic loss or MI. These findings could be due to inactivation of another putative oncosuppressive gene in the 'neighborhood' of *MEN1*.

In this study, we have applied for the first time, to our knowledge, a real-time PCR and melting curve analysis to assess LOH and MI in polymorphic markers at the *MEN1* region, a technique which has not been applied previously in the analysis of pulmonary carcinoids. We demonstrated the reliability of our method, comparing its reproducibility with PCR analysis, and calculated the LOH thresholds based on the measured interassay variability to avoid false positives with a high probability. We also verified the MI cases by sequencing.

In conclusion, our findings suggest that real-time PCR with melting curve analysis can be applied feasibly and reliably in order to reveal genetic alterations such as LOH and MI. In addition, these findings further support previous findings implicating the *MEN1* gene and a neighboring putative onco-suppressive gene in the development of sporadic pulmonary carcinoids, even when they arise outside of the context of MEN1 syndrome.

References

1. Chandrasekharappa SC, Guru SC, Manickam P, Olufemi SE, Collins FS, Emmert-Buck MR, Debelenko LV, Zhuang Z, Lubensky IA, Liotta LA, Crabtree JS, Wang Y, Roe BA, Weisemann J, Boguski MS, Agarwal SK, Kester MB, Kim YS, Heppner C, Dong Q, Spiegel AM, Burns AL and Marx SJ: Positional cloning of the gene for multiple endocrine neoplasia-type 1. Science 276: 404-407, 1997.
2. Larsson C, Skogseid B, Oberg K, Nakamura Y and Nordenskojoid M: Multiple endocrine neoplasia type 1 gene maps to chromosome 11 and is lost in insulinoma. Nature 332: 85-87, 1988.
3. Courseaux A, Grosgeorge J, Gaudray P, *et al*; European Consortium on MEN1: Definition of the minimal MEN1 candidate area based on a 5-Mb integrated map of proximal 11q13. Genomics 37: 354-365, 1996.
4. Manickam P, Guru SC, Debelenko LV, *et al*: Eighteen new polymorphic markers in the multiple endocrine neoplasia type 1 (MEN1) region. Hum Genet 101: 102-108, 1997.
5. Emmert-Buck MR, Lubensky IA, Dong Q, Manickam P, Guru SC, Kester MB, Olufemi SE, Agarwal S, Burns AL, Spiegel AM, Collins FS, Marx SJ, Zhuang Z, Liotta LA, Chandrasekharappa SC and Debelenko LV: Localization of the multiple endocrine neoplasia type I (MEN1) gene based on tumor loss of heterozygosity analysis. Cancer Res 57: 1855-1858, 1997.
6. Lubensky IA, Debelenko LV, Zhuang Z, Emmert-Buck MR, Dong Q, Chandrasekharappa S, Guru SC, Manickam P, Olufemi SE, Marx SJ, Spiegel AM, Collins FS and Liotta LA: Allelic deletions on chromosome 11q13 in multiple tumors from individual MEN1 patients. Cancer Res 56: 5272-5278, 1996.
7. Byström C, Larsson C, Blomberg C, Sandelin K, Falkmer U, Skogseid B, Öberg K, Werner S and Nordenskjöld M: Localization of the *MEN1* gene to a small region within chromosome 11q13 by deletion mapping in tumors. Proc Natl Acad Sci USA 87: 1968-1972, 1990.
8. Chakrabarti R, Srivatsan ES, Wood TF, Eubanks PJ, Ebrahimi SA, Gatti RA, Passaro E Jr and Sawicki MP: Deletion mapping of endocrine tumors localizes a second tumor suppressor gene on chromosome band 11q13. Genes Chromosomes Cancer 22: 130-137, 1998.
9. Iwasaki H: A possible tumor suppressor gene for parathyroid adenomas. Int Surg 81: 71-76, 1996.
10. Friedman E, De Marco L, Gejman PV, Norton JA, Bale AE, Aurbach GD, Spiegel AM and Marx SJ: Allelic loss from chromosome 11 in parathyroid tumors. Cancer Res 52: 6804-6809, 1992.
11. Debelenko LV, Emmert-Buck MR, Zhuang Z, Epshteyn E, Moskaluk CA, Jensen RT, Liotta LA and Lubensky IA: The multiple endocrine neoplasia type I gene locus is involved in the pathogenesis of type II gastric carcinoids. Gastroenterology 1139: 773-781, 1997.
12. Bale AE, Norton JA, Wong EL, Fryburg JS, Maton PN, Oldfield EH, Streeten E, Aurbach GD, Brandi ML, Friedman E, *et al*: Allelic loss on chromosome 11 in hereditary and sporadic tumors related to familiar multiple endocrine neoplasia type 1. Cancer Res 51: 1154-1157, 1991.
13. Zhuang Z, Ezzat SZ, Vortmeyer AO, Weil R, Oldfield EH, Park WS, Pack S, Huang S, Agarwal SK, Guru SC, Manickam P, Debelenko LV, Kester MB, Olufemi SE, Heppner C, Crabtree JS, Burns AL, Spiegel AM, Marx SJ, Chandrasekharappa SC, Collins FS, Emmert-Buck MR, Liotta LA, Asa SL and Lubensky IA: Mutations of the *MEN1* tumor suppressor gene in pituitary tumors. Cancer Res 57: 5446-5451, 1997.
14. Heppner C, Kester MB, Agarwal SK, Debelenko LV, Emmert-Buck MR, Guru SC, Manickam P, Olufemi SE, Skarulis MC, Doppman JL, Alexander RH, Kim YS, Saggar SK, Lubensky IA, Zhuang Z, Liotta LA, Chandrasekharappa SC, Collins FS, Spiegel AM, Burns AL and Marx SJ: Somatic mutation of the *MEN1* gene in parathyroid tumours. Nat Genet 16: 375-378, 1997.
15. Hai N, Aoki N, Matsuda A, Mori T and Kosugi S: Germline *MEN1* mutations in sixteen Japanese families with multiple endocrine neoplasia type1 (MEN1). Eur J Endocrinol 141: 475-480, 1999.
16. Kawamura J, Shimada Y, Komoto I, Okamoto H, Itami A, Doi R, Fujimoto K, Kosugi S and Imamura M: Multiple endocrine neoplasia type 1 gene mutations in sporadic gastrinomas in Japan. Oncol Rep 14: 47-52, 2005.
17. Debelenko LV, Brambilla E, Agarwal SK, Swalwell JI, Kester MB, Lubensky IA, Zhuang Z, Guru SC, Manickam P, Olufemi SE, Chandrasekharappa SC, Crabtree JS, Kim YS, Heppner C, Burns AL, Spiegel AM, Marx SJ, Liotta LA, Collins FS, Travis WD and Emmert-Buck MR: Identification of *MEN1* gene mutations in sporadic carcinoid tumors of the lung. Hum Mol Genet 6: 2285-2290, 1997.
18. Dong Q, Debelenko LV, Chandrasekharappa SC, Emmert-Buck MR, Zhuang Z, Guru SC, Manickam P, Skarulis M, Lubensky IA, Liotta LA, Collins FS, Marx SJ and Spiegel AM: Loss of heterozygosity at 11q13: analysis of pituitary tumors, lung carcinoids, lipomas, and other uncommon tumors in subjects with familial multiple endocrine neoplasia type 1. J Clin Endocrinol Metab 82: 1416-1420, 1997.
19. Onuki N, Wistuba II, Travis WD, Virmani AK, Yashima K, Brambilla E, Hasleton P and Gazdar AF: Genetic changes in the spectrum of neuroendocrine lung tumors. Cancer 85: 600-607, 1999.
20. Petzmann S, Ullmann R, Klemen H, Renner H and Popper HH: Loss of heterozygosity on chromosome arm 11q in lung carcinoids. Hum Pathol 32: 333-338, 2001.
21. Ulmann R, Petzmann S, Klemen H, Fraire AE, Hasleton P and Popper HH: The position of pulmonary carcinoids within the spectrum of neuroendocrine tumors of the lung and other tissues. Genes Chromosomes Cancer 34: 78-85, 2002.
22. Debelenko LV, Swalwell JI, Kelley MJ, Brambilla E, Manickam P, Baibakov G, Agarwal SK, Spiegel AM, Marx SJ, Chandrasekharappa SC, Collins FS, Travis WD and Emmert-Buck MR: *MEN1* gene mutation analysis of high-grade neuro-endocrine lung carcinoma. Genes Chromosomes Cancer 28: 58-65, 2000.
23. Oliveira AM, Tazelaar HD, Wentzlaff KA, Kosugi NS, Hai N, Benson A, Miller DL and Yang P: Familial pulmonary carcinoid tumors. Cancer 91: 2104-2109, 2001.
24. Jakobovitz O, Nass D, De Marko L, Barbosa AJ, Simoni FB, Rechavi G and Friedman E: Carcinoid tumors frequently display genetic abnormalities involving chromosome 11. J Clin Endocrinol Metab 81: 3164-3167, 1996.
25. Iwasaki H, Stewart PW, Dilley WG, Holt MS, Steinbrueck TD, Wells SA Jr and Donis-Keller H: A minisatellite and a micro-satellite polymorphism within 1.5 kb at the human muscle glycogen phosphorylase (PYGM) locus can be amplified by PCR and have combined informativeness of PIC 0.95. Genomics 13: 7-15, 1992.

26. Wright DK and Manos MM: Sample preparation from paraffin-embedded tissues. In: PCR Protocols: A Guide to Methods and Applications. Innis MA, Gelfand DH, Sninsky JJ and White TJ (eds). Academic Press, San Diego, CA, pp153-158, 1990.
27. Liloglou T, Maloney P, Xinarianos G, Fear S and Field JK: Sensitivity and limitations of high throughput fluorescent microsatellite analysis for the detection of allelic imbalance. Application in lung tumours. Int J Oncol 16: 5-14, 2000.
31. Görtz B, Roth J, Krähenmann A, *et al*: Mutations and allelic deletions of the *MEN1* gene are associated with a subset of sporadic endocrine pancreatic and neuroendocrine tumors and not restricted to foregut neoplasms. Am J Pathol 154: 429-436, 1999.
32. Marx SJ: Molecular genetics of multiple endocrine neoplasia types 1 and 2. Nat Rev Cancer 5: 367-375, 2005.