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Characterization of High-grade Neuroendocrine Tumors of the Lung in Relation to *menin* Mutations

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It has been suggested that mutations in the *menin* gene play a role in the development of multiple endocrine neoplasia type 1 (MEN1)-associated and of sporadic forms of low- and intermediate-grade neuroendocrine tumors of the lung. In the present study, eight tumor specimens of large cell neuroendocrine carcinoma (LCNEC) and 13 of small cell lung cancer (SCLC), which represent a high-grade category of neuroendocrine tumors, were examined for the potential involvement of *menin* alterations as well as for the expression of various neuroendocrine markers and p53 and Rb abnormalities. All specimens expressed multiple neuroendocrine markers as expected and almost invariably carried p53 and Rb alterations. Unexpectedly, however, mutations in the *menin* gene were not detected in any of the high-grade neuroendocrine tumors examined. We thus conclude that *menin* mutations do not play a crucial role in the pathogenesis of high-grade subsets, in contrast to their suggested significant role in the development of low- and intermediate-grade subsets. Interestingly, loss of heterozygosity (LOH) in the *menin* gene appeared to be more prevalent in LCNEC (50%) than in SCLC (22%), suggesting a possible distinction between SCLC and LCNEC.

Key words: Large cell neuroendocrine tumor — Small cell lung cancer — Carcinoid tumor — Multiple endocrine neoplasia type 1 — Menin

Neuroendocrine tumors of the lung represent a wide spectrum of morphologic types, ranging from typical carcinoids (TC) with low malignant potential to small cell lung cancer (SCLC) with the most rapid and disseminated growth. In a four-category scheme for classification of neuroendocrine tumors proposed by Travis *et al*.,[1] lower-grade neuroendocrine tumors with uniform morphology and minimal or no mitotic activity are termed TCs and have excellent prognosis, while atypical carcinoid (AC) tumors are defined as a more malignant variant. The remaining two types of tumors with neuroendocrine morphological characteristics but manifesting marked cellularity, abundant mitotic activity, and prominent necrosis are designated as high grade and are subdivided into either large cell neuroendocrine tumor (LCNEC) or SCLC, depending on cell size and nuclear morphology.

Debelenko *et al*. recently reported that two of eight sporadic TCs and three of four sporadic ACs carried somatic mutations in the *menin* gene,[2] which had originally been cloned as a tumor suppressor gene responsible for multiple endocrine neoplasia type 1 (MEN1).[3,4] MEN1 is an autosomal dominant familial cancer syndrome characterized by parathyroid, enteropancreatic and anterior pituitary tumors as well as other tumor types including carcinoids of the lung.[5] For this reason, germline *menin* mutations mean extreme risk of occurrence of lung carcinoids in the MEN1 family,[3,4,6] while acquisition of somatic *menin* mutations appears to play a role in the development of sporadic lung carcinoids.[2] In general, genetic alterations at a given locus are more prevalent in higher-grade than in lower-grade tumors, and show an association with biologically more malignant behavior within a spectrum of tumors.[7] In view of the higher prevalence of *menin* mutations in AC than in TC,[2] *menin* alterations could be expected to be present even more frequently in high-grade neuroendocrine tumors of the lung, i.e., LCNEC and SCLC.

In the present study, we examined eight LCNEC and 13 SCLC tumor specimens for the potential involvement of *menin* alterations in the pathogenesis of high-grade neuroendocrine tumors of the lung. In addition, we immunohistochemically examined LCNEC and SCLC tumors for the presence of neuroendocrine marker expression and other genetic changes, including p53 and Rb abnormalities, to make our study more comprehensive.

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MATERIALS AND METHODS

Histologic examination Histologic slides were independently reviewed by two pathologists (Y.Y. and W.D.T.), and were classified as SCLC or LCNEC according to the modified criteria proposed by Travis *et al*.[8] DNAs were then extracted from the tumor specimens, which had been stored at −80°C until use. In addition, DNAs of three MEN1-associated tumors (one insulinoma of the pancreas and two parathyroid tumors) were extracted from archival, formalin-fixed, paraffin-embedded tissues.

Polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) analysis The entire coding sequence and exon-intron junctions of the *menin* gene were examined by means of PCR-SSCP analysis, as described previously.[9] Oligonucleotide primer sequences and conditions for PCR amplification are given in Table I. Following PCR amplification in the presence of [^{32}P]-dCTP, PCR products were electrophoretically separated on 6% non-denaturing polyacrylamide gels both at 4°C without glycerol and at room temperature in the presence of 5% glycerol. Distinctly shifted bands were excised from the PCR-SSCP gels, amplified by PCR, polished and then cloned into the *Eco*RV site of pBluescript SKII(−) (Stratagene, La Jolla, CA), and the resultant plasmid DNAs prepared from multiple clones were sequenced. PCR products of the corresponding normal lung DNAs were also subjected to PCR-SSCP and sequencing analyses.

Immunohistological examination Immunohistochemical examination was carried out by applying the standard avidin-biotin-peroxidase complex method, as described previously.[10] The antibodies used were: anti-CD56 monoclonal antibody (mAb) (123C3) (Zymed, South San Francisco, CA); anti-synaptophysin polyclonal antibody (Dako, Copenhagen, Denmark); anti-chromogranin A polyclonal antibody (Dako); anti-p53 mAb (DO-7) (Dako); anti-Rb (3H9) mAb (Medical and Biological Laboratories, Nagoya); and anti-Ki-67 (MIB1) mAb (Medical and Biological Laboratories). Antigens were retrieved, except for synaptophysin and chromogranin A, by means of microwave treatment in citrate buffer (pH 6.8).

RESULTS

The present study identified eight cases as LCNEC and 13 as SCLC (Table II). In addition to the morphologic examination, immunohistological analysis of the expression of neuroendocrine markers and tumor suppressor proteins was also performed in a total of 19 cases. The majority of the cases showed expression of both CD56 and synaptophysin, while expression of chromogranin A was detected in seven of the 17 cases examined. Aberrant p53 expression was present in seven of eight LCNEC and in six of eight SCLC, while all but one LCNEC tumor lacked the Rb protein. These immunohistochemical findings were consistent with the neuroendocrine morphologies of the tumors examined and their highly aggressive nature.

Eight LCNEC and 13 SCLC tumor specimens were then examined for the presence of *menin* mutations with the aid of 12 overlapping sets of PCR primers, which covered the entire coding exons and adjacent splicing junctions. A distinct mobility shift was detected in the analysis of fragment 10 in a single case (case 2, Fig. 1). Subsequent sequence analysis revealed that the mobility shift in this case was due to a nucleotide substitution at codon 503 (G<u>G</u>C to G<u>A</u>C), which resulted in an amino acid substitution of aspartic acid for glycine (Table III). This change was also detected in normal lung DNA of case 2. Results of an additional screening of 143 normal lung DNAs indicated that none of them exhibited the same mobility shift as case 2, which suggests that it may represent either a

Table I. Oligonucleotide Primers and PCR Conditions Used for PCR Amplification of the *menin* Gene

Sense primer	Antisense primer	Annealing temperature (°C)	Glycerol (%)
F1: gtggaaccttagcggacc	R1: cacgaagcccagcaccaa	60	10
F2: ggacctggtgctcctttc	R2: cgaggatagagggacagg	60	15
F3: tctatgcccgcttcaccg	R3: ttcccacctactgggctc	60	15
F4: cacagaggaccctctttc	R4: atgaaggggacaaggctg	63	15
F5: ggtgggccatcatgagac	R5: ccattggctcagccctca	63	10
F6: cctgttccgtggctcata	R6: ctgcaccctccttagatg	63	10
F7: ctgaggatcctctgcctc	R7: gaaaggacaggctgcagg	63	15
F8: agaccccactgctctcac	R8: ctggagctccagcctttc	63	10
F9: cctgtgccctctgctaag	R9: ccagacctctgtgcagct	63	10
F10: agttccagccactggccg	R10: ttcagggcctcgggctgt	63	10
F11: aagcctcctgggactgtc	R11: gatcttggtggccaccag	60	15
F12: gaagggcatgaaggagct	R12: acaagcggtccgaagtcc	60	15

Table II. Immunohistological Characterization of High-grade Neuroendocrine Tumors

Case	Histology	NE[a] differentiation			TSG[b] status		Proliferation index	
		CD56	Syn[c]	ChgA[d]	p53	Rb	Mitosis	MIB1 (%)
1	LCNEC	++[e]	++[e]	+[e]	+[f]	−[g]	76[h]	55
2	LCNEC	++	++	−	+	−	70	43
3	LCNEC	++	++	−	+	−	80	57
4	LCNEC	+	−	−	+	−	65	78
5	LCNEC	+	+	+	−	+	21	17
6	LCNEC	++	++	++	+	−	24	16
7	LCNEC	++	++	++	+	−	72	52
8	LCNEC	+	−	−	+	−	45	31
9	SCLC	ND[i]	ND	ND	ND	ND	ND	ND
10	SCLC	++	+	+	+	−	81	62
11	SCLC	++	+	−	+	−	76	58
12	SCLC	ND	ND	ND	ND	ND	92	ND
13	SCLC	−	+	−	ND	ND	79	ND
14	SCLC	++	ND	ND	−	−	120	59
15	SCLC	++	++	−	+	−	ND	76
16	SCLC	ND	ND	ND	ND	ND	ND	ND
17	SCLC	++	++	++	+	−	68	44
18	SCLC	+	+	−	+	−	98	58
19	SCLC	++	++	−	−	−	48	49
20	SCLC	++	+	−	−	−	59	48
21	SCLC	++	++	+	+	−	64	42

a) NE, neuroendocrine.
b) TSG, tumor suppressor gene.
c) Syn, synaptophysin.
d) ChgA, chromogranin A.
e) ++, strongly positive; +, positive; −, negative.
f) +, >10% positivity; −, <10% positivity.
g) +, presence of positive cells; −, absence of positive cells.
h) Mitotic figures per 10 high power fields.
i) ND, not determined.

very rare polymorphism or a germline mutation. In addition, we observed mobility shifts in fragments 1, 9 and 11 at relatively high frequency. Sequence analysis showed the presence of nucleotide substitutions in intron 1, exon 9 and exon 10, which corresponded to common polymorphisms known to exist within the examined genomic regions in the Japanese population (Table III).[11, 12]

To ascertain that our PCR-SSCP analysis had sufficient sensitivity, we also analyzed three MEN1-associated tumors, all of which showed distinct mobility shifts (Fig. 1), thus suggesting the presence of mutations in the *menin* gene. Sequence analysis revealed that these distinct mobility shifts were the result of a missense mutation at codon 45 in the case of MEN1-2, a nonsense mutation at codon 312 in MEN1-3, and a 1-bp deletion mutation at codons 397–398 in MEN1-1 (Table IV). It should be noted that the MEN1-associated germline mutation in MEN1-1, which has not been reported previously in the Japanese population,[11–13] was found to result in the elimination of both of the two nuclear localization signals in the C-terminus. This finding supports the notion that nuclear localization is important for the function of menin.[14] Two other identified germline mutations were identical to the changes previously reported in independent Japanese MEN1 families,[11] suggesting that they might share common founders.

DISCUSSION

The present study was initiated to gain an insight into the molecular pathogenesis of high-grade neuroendocrine lung tumors. The pathological classification itself of pulmonary neuroendocrine tumors is often difficult and needs to be executed with care,[15] while extensive studies of this tumor type have been hampered by the rarity of LCNEC. The 13 SCLC and eight LCNEC analyzed in the present study were defined by strict application of the modified diagnostic criteria proposed by Travis *et al.*[8] in 1998 to ensure reproducibility of the results obtained for this complex subject.

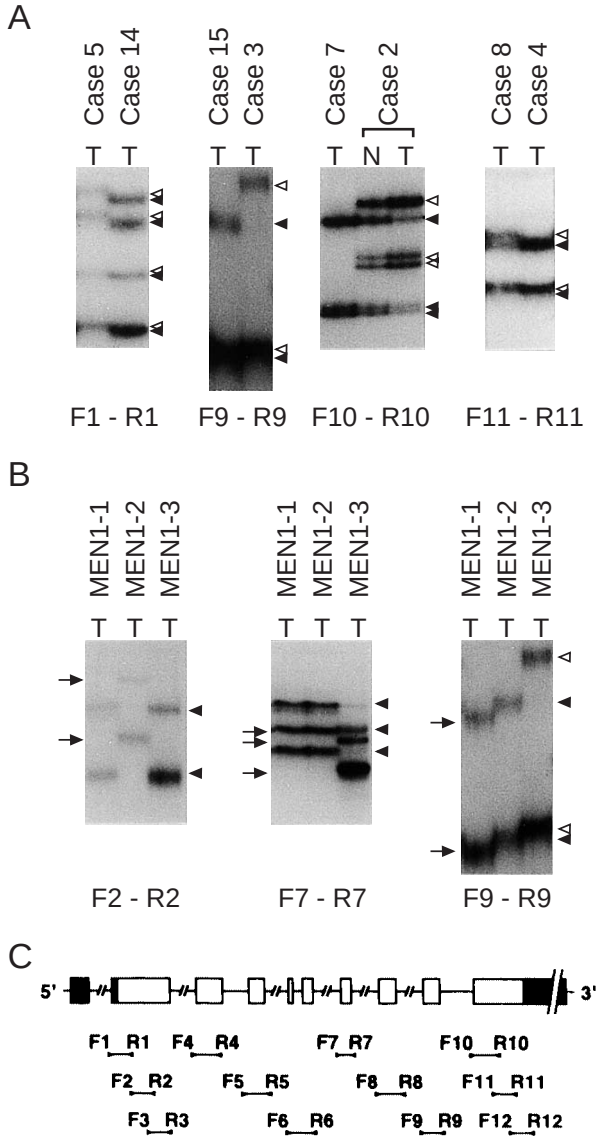

Fig. 1. PCR-SSCP analysis of the *menin* gene in high-grade neuroendocrine tumors (A) as well as in MEN1-associated tumors (B). A distinct rare mobility shift is seen in fragment 10 in both normal and tumor DNAs of case 2. Mobility shifts representing common polymorphisms in fragments 1, 9 and 11 are also shown (data not shown for normal DNAs). *Menin* mutations are clearly detectable in all of the three MEN1-associated tumors. Arrows, mutant alleles; arrowheads, polymorphic alleles. Open box, open reading frame; solid box, 5′ and 3′ untranslated regions. Schematic illustration of the location of the PCR primers used is shown below C.

In general, genetic alterations present in less malignant subsets are more prevalent in higher-grade subsets of the same tumor spectrum,[7] and this appears to be true also in neuroendocrine tumors of the lung. Abnormal p53 expression[16–18] and Rb inactivation[16, 19, 20] were previously reported to be rare in low- and intermediate-grade neuroendocrine tumors. In contrast, and consistent with previous notions,[16–20] we observed very frequent alterations in these tumor suppressor genes in both LCNEC and SCLC high-grade neuroendocrine tumors. Inactivation of the *menin* gene has been reported to be present in low- and intermediate-grade neuroendocrine lung tumors.[2] Interestingly, however, the present study yielded unexpected results for the frequencies of *menin* mutations, i.e., mutations in the *menin* gene were not detected in 21 high-grade neuroendocrine tumors. The present finding thus indicates that in contrast to the previously reported high incidence in lower-grade neuroendocrine tumors,[2] *menin* mutations are rare, if present at all, in high-grade neuroendocrine tumors, although epigenetic down-regulation of *menin* remains to be studied in such tumors. It can be assumed that our PCR-SSCP analysis was sufficiently sensitive for the detection of nucleotide substitutions in the *menin* gene, since it could detect all common polymorphisms known in the Japanese population.[11, 12] In addition, germline mutations, including a previously unreported one in the case of MEN1-1, were detected in all of the MEN1-associated tumors examined here. As to the extremely rare (1 in 328) allele identified in case 2 (LCNEC), we note that this substitution has never been reported in the Japanese population thus far, although it remains to be determined whether this rare allele reflects a germline mutation resulting in the impairment of menin functioning. We thus conclude that *menin* mutations do not play a crucial role in the pathogenesis of high-grade subsets, in contrast to the significant role in the development of low- and intermediate-grade subsets as stated by Debelenko *et al*.[2]

The present findings consequently imply that, from the genetic point of view, LCNEC and SCLC may be regarded as a distinct subgroup within the spectrum of neuroendocrine tumors of the lung, while TC and AC form the other subgroup. Using polymorphisms within the coding region of the *menin* gene, we could detect LOH at 11q13 in only two of nine (22%) SCLC (data not shown), which is consistent with the previously reported results of a comparative genomic hybridization analysis, [21, 22] although the observed low incidence contradicts the results reported by Onuki *et al*.[23] Our finding is unlikely to be the result of contamination with normal cells, since we could clearly detect LOH on 3p in all but one SCLC tumor (unpublished observation). Although the number of LCNEC studied was not sufficiently large to draw any definitive conclusions, it is interesting to note that LOH in the *menin* gene was present in three of six (50%) LCNEC cases heterozygous for the polymorphisms of the *menin* gene, suggesting a possible distinction between SCLC and LCNEC. In this regard, it has been suggested that an as yet unidentified tumor suppressor gene may reside in the region telomeric

Table III. Sequence Variations Identified in Neuroendocrine Tumors

Location		Change		Allele frequency
Exon/Intron	Codon	Nucleotide	Amino acid	
Very rare polymorphism or germline mutation?				
Exon 10	503	GGC	Gly	0.997
		GAC	Asp	0.003[a)]
Common polymorphisms				
Intron 1	NA[b)]	ccggcttgccttgcag	NA	0.548
		gcggcttgccttgcag	NA	0.452
Exon 9	418	GAC	Asp	0.714
		GAT	Asp	0.286
Exon 10	541	GCA	Ala	0.714
		ACA	Thr	0.286

a) Only a single allele was detected in 328 alleles examined.
b) NA, not affected.

Table IV. Germline Mutations Identified in Japanese MEN1 Cases

Case	Origin	Location		Change	
		Exon	Codon	Nucleotide	Amino acid
MEN1-1	Parathyroid	9	397–398	ACCCAG to ACCAGA	Frameshift
MEN1-2	Pancreas	2	45	GAG to GGG	Glu to Gly
MEN1-3	Parathyroid	7	312	TAC to TAG	Tyr to Stop

to the *menin* locus, based on an analysis of parathyroid tumors[24)] and gastrinomas[25)] occurring in MEN1 families as well as of both MEN1-associated and sporadic endocrine tumors.[26, 27)]

In summary, accumulating evidence including the present observations suggests the presence of both homogeneity and heterogeneity within the spectrum of neuroendocrine tumors of the lung. Further studies are warranted to better understand the biology and the molecular pathogenesis of this interesting type of lung tumor.

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