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Genetics of neuroendocrine and carcinoid tumours

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Abstract

Neuroendocrine tumours (NETs) originate in tissues that contain cells derived from the embryonic neural crest, neuroectoderm and endoderm. Thus, NETs occur at many sites in the body, although the majority occur within the gastro-entero-pancreatic axis and can be subdivided into those of foregut, midgut and hindgut origin. Amongst these, only those of midgut origin are generally argentaffin positive and secrete serotonin, and hence only these should be referred to as carcinoid tumours. NETs may occur as part of complex familial endocrine cancer syndromes, such as multiple endocrine neoplasia type 1 (MEN1), although the majority occur as non-familial (i.e. sporadic) isolated tumours. Molecular genetic studies have revealed that the development of NETs may involve different genes, each of which may be associated with several different abnormalities that include point mutations, gene deletions, DNA methylation, chromosomal losses and chromosomal gains. Indeed, the foregut, midgut and hindgut NETs develop via different molecular pathways. For example, foregut NETs have frequent deletions and mutations of the *MEN1* gene, whereas midgut NETs have losses of chromosome 18, 11q and 16q and hindgut NETs express transforming growth factor-α and the epidermal growth factor receptor. Furthermore, in lung NETs, a loss of chromosome 3p is the most frequent change and p53 mutations and chromosomal loss of 5q21 are associated with more aggressive tumours and poor survival. In addition, methylation frequencies of *retinoic acid receptor*-β, *E-cadherin* and *RAS-associated domain family* genes increase with the severity of lung NETs. Thus the development and progression of NETs is associated with specific genetic abnormalities that indicate the likely involvement of different molecular pathways.

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Introduction

Neuroendocrine tumours (NETs) occur in tissues that contain cells derived from the embryonic neural crest, neuroectoderm and endoderm (Le Douarin 1988, Vinik 2001, Oberg 2002). Thus, NETs may occur at many sites in the body, although the majority occur within the gastro-entero-pancreatic axis (Table 1). Amongst these, more than 50% are carcinoid tumours, which are traditionally considered to be serotonin (5-hydroxytryptamine) secreting and argentaffin positive, and are sub-classified into foregut, midgut and hindgut tumours (Vinik 2001, Oberg 2002). The foregut tumours include carcinoids of the bronchus, lung, thymus, stomach, first portion of the duodenum and pancreas; the midgut tumours derive from the second portion of the duodenum, the jejunum, ileum, appendix and ascending colon; and the hindgut tumours derive from the transverse colon, the descending colon and rectum. However, it is important to note that the foregut and hindgut tumours are argentaffin negative and have a low content (or none) of serotonin. In view of this, the term carcinoid tumour is now reserved for only those midgut tumours that are serotonin secreting and the term NETs, together with the tissue of origin, is used for the other tumours (Oberg 1998). Thus, in this definition, the midgut carcinoid tumours represent a subset of NETs. NETs and carcinoid tumours usually occur as non-familial (i.e. sporadic) tumours. However, they may sometimes occur as part of familial syndromes such as multiple endocrine neoplasia type 1 (MEN1) (Oberg 1998) and neurofibromatosis type 1 (NF1) (Griffiths *et al.* 1987), although the occurrence of familial isolated midgut carcinoids is rare and has been reported only five times (Eschbach & Rivaldo 1962, Anderson 1966, Kinova *et al.* 2001, Oliveira *et al.* 2001, Pal *et al.* 2001). The familial occurrence of NETs is consistent with a genetic aetiology for NETs. This review will focus on the molecular genetic basis of NETs and carcinoid tumours, together with

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Table 1 NETs and their frequencies

Tumours	Percentage (%)
Carcinoids (all, i.e. foregut, midgut and hindgut NETs)	< 50
Pancreatic islet cell tumours	
Gastrinomas	25
Insulinomas	15
VIPomas	6
Glucagonomas	3
Others	< 1

a brief outline of the models for tumour development. However, it is important to note that NETs represent a heterogenous group of tumours, and that comparisons between different studies (Debelenko *et al*. 1997*a*, Dong *et al*. 1997, Onuki *et al*. 1999, Zhao *et al*. 2000, Ullmann *et al*. 2002), which have generally not used a World Health Organisation (Brambilla *et al*. 2001, Younossian *et al*. 2002) classification are therefore difficult.

Models of tumour development

The development of tumours may be associated with mutations or inappropriate expression of specific normal cellular genes, which are referred to as oncogenes (Thakker & Ponder 1988, Thakker 1993, 1994, Brown & Solomon 1997). Two types of oncogenes, referred to as dominant and recessive oncogenes, have been described. An activation of dominant oncogenes leads to transformation of the cells containing them, and examples of this are the chromosomal translocations associated with the occurrence of Burkitt's lymphoma and the activating mutations of the RET proto-oncogene in MEN type 2. In these conditions, the mutations, which lead to activation of the oncogene, are dominant at the cellular level, and therefore only one copy of the mutated gene is required for the phenotypic effect. However, in some inherited neoplasms, which may also arise sporadically, such as retinoblastoma (RB) and MEN1, tumour development is associated with two recessive mutations, which inactivate oncogenes, and these are referred to as recessive oncogenes. In the inherited tumours, the first of the two recessive mutations is inherited via the germ cell line and is present in all the cells. This recessive mutation is not expressed until a second mutation, within a somatic cell, causes loss of the normal dominant allele. The mutations causing the inherited and sporadic tumours are similar but the cell types in which they occur are different. In the inherited tumours, the first mutation occurs in the germ cell, whereas in the sporadic tumours both mutations occur in the somatic cell. Thus, the risk of tumour development in an individual who has not inherited the first germline mutation is much smaller, as both mutational events must coincide in the same somatic cell. In addition, the apparent paradox that the inherited cancer syndromes are due to recessive mutations but dominantly inherited at the level of the family is explained by the fact that, in individuals who have inherited the first recessive mutation, a loss of a single remaining wild-type (WT) allele is almost certain to occur in at least one of the large number of cells in the target tissue. This cell will be detected because it forms a tumour, and almost all individuals who have inherited the germline mutation will express the disease, even though they inherited a single copy of the recessive gene. This model involving two (or more) mutations in the development of tumours is known as the 'two-hit' or Knudson's hypothesis (Knudson 1971, 1974). The normal function of these recessive oncogenes appears to be in regulating cell growth and differentiation, and these genes have also been referred to as anti-oncogenes, tumour suppressor genes or gatekeeper genes (Vogelstein & Kinzler 1993, Kinzler & Vogelstein 1997). An important feature which has facilitated the investigation of these genetic abnormalities associated with tumour development is that the loss of the remaining allele (i.e. the second hit), which occurs in the same somatic cell and gives rise to the tumour, often involves a large scale loss of chromosomal material. This 'second hit' may be detected by a comparison of the DNA sequence polymorphisms in the leukocytes and tumour obtained from a patient, and observing a loss of heterozygosity (LOH) in the tumour. In addition to LOH, the second hit may involve smaller deletions that may be intragenic or point mutations, or aberrant methylation, which is an epigenetic silencing mechanism (Lengauer *et al*. 1997). Methylation of DNA, which is intrinsically utilised in X-chromosome inactivation and imprinting of parental genes (Momparler & Bovenzi 2000, Robertson & Jones 2000, Tycko 2000, Jones & Baylin 2002), involves the transfer of a methyl group, by a DNA methyltransferase, to the cytosine of a cytosine-guanine (CpG) dinucleotide. These CpG dinucleotides are often found as clusters, which are referred to as CpG islands (Bird 1986), and they may encompass the regulatory sequences of genes. Indeed, hypermethylation of such promoter regions of genes has been shown to be involved in silencing recessive oncogenes and subsequent tumour development. For example, the Von Hippel Lindau gene has been shown to be hypermethylated in sporadic clear cell renal carcinomas (Herman *et al*. 1994). Studies of methylation patterns of foregut, midgut and hindgut NETs have revealed that five genes (*p14*, *p16*, *MGMT*, *THBS1* and *RAR*β) are more frequently methylated in these NETs when compared with pancreatic neuroendocrine tumours (PETs), whilst two genes (*ER* and *COX2*) are equally methylated in all types of NETs, PETs and normal tissue (Table 2). Furthermore, dominant and recessive oncogenes have been shown to be involved in the development and progression of NETs (Table 3). The underlying mechanisms altering the functions of these genes may involve point mutations, chromosomal deletions (i.e. losses), or duplications (i.e. gains), amplifications or insertions. Chromosomal

Table 2 Methylation patterns in foregut, midgut and hindgut NETs

Gene	Methylation status[a]	Reference
Cyclin-dependent kinase inhibitor-2A (p16)	Colon > stomach > ileum > PETs	Chan *et al.* (2003)
Adenomatous polyposis of the colon (APC)	NSCLC > SCLC > AC and TC	Toyooka *et al.* (2001)
H-cadherin (CDH13)	NSCLC > SCLC > AC and TC	Toyooka *et al.* (2001)
O^6-methylguanine-DNA-methyl transferase (MGMT)	Duodenum = rectum > colon > PETs	Chan *et al.* (2003)
Retinoic acid receptor-β 2 (RARβ)	Stomach = rectum > ileum > normal tissue > PETs	Chan *et al.* (2003)
	SCLC > AC and TC	Toyooka *et al.* (2001)
E-cadherin (CDH1)	SCLC > AC and TC	Toyooka *et al.* (2001)
RAS-associated domain family 1A (RASSFIA)	SCLC > AC and TC > NSCLC	Dammann *et al.* (2001), Toyooka *et al.* (2001)
p14 (cyclin-dependent kinase inhibitor-2D)	Stomach = rectum > ileum > PETs	Chan *et al.* (2003)
Cyclo-oxygenase 2 (COX2)	Stomach = rectum > colon > ileum > normal tissue = PETs	Chan *et al.* (2003)
Oestrogen receptor (ER)	Stomach > ileum > PETs > duodenum = colon > normal tissue	Chan *et al.* (2003)
Thrombosporin 1 (THBS1)	Rectum > duodenum = stomach > colon > ileum > PETs	Chan *et al.* (2003)
Caspase 8 (CASP8)	SCLC > AC and TC > NSCLC	Shivapurkar *et al.* (2002)

[a]NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; AC, atypical lung carcinoid; TC, typical lung carcinoid.

Table 3 Dominant and recessive oncogenes altered in NETs

	Chromosomal location	**Reference**
Dominant oncogenes		
K-ras	12p12.1	Couce *et al.* (1999), Maitra *et al.* (2000)
N-ras	1p13.2	Arany *et al.* (1994)
Recessive oncogenes		
Fragile histidine triad (FHIT)	3p14.2	Kovatich *et al.* (1998), Onuki *et al.* (1999)
p16	9p21	Onuki *et al.* (1999)
APC	5p21	Levin *et al.* (1995), Petersen *et al.* (1997)
Mutated in colorectal cancer (MCC)	5p21	Levin *et al.* (1995), Petersen *et al.* (1997)
MEN1	11q13	Gortz *et al.* (1999), D'Adda *et al.* (1999*b*)
Succinate-ubiquinone oxidoreductase subunit D (SDHD)[a]	11q23	Kytola *et al.* (2002)
Transforming protein 53 (p53)	17p13.1	Lohmann *et al.* (1993*a*,*b*)
RB	13q14.1–q14.2	Harbour *et al.* (1988), Hiyama *et al.* (1993)

[a]SDHD is unlikely to be involved as the reported alterations are polymorphisms.

gains and losses in the tumour genome can be detected by the use of comparative genomic hybridisation (CGH), which is a competitive *in situ* hybridisation-based procedure that reveals relative gene copy aberrations (Fig. 1) (Kallioniemi *et al*. 1992, du Manoir *et al*. 1993). Changes in relative gene copy number detected using CGH, which may be associated with oncogene amplification or loss of tumour-suppressor gene function, deleted in one non-familial bronchial NET are shown in Fig. 2.

The *MEN1* gene and NETs

NETs are commonly seen in patients with MEN1, which is an autosomal dominant disorder that is characterised by the combined occurrence of tumours of the parathyroid, pancreatic islet cells (e.g. gastrinomas, insulinomas, vasoactive intestinal polypeptidomas (VIPomas), glucagonomas, pancreatic polypeptidomas (PPomas) and non-functioning tumours), and anterior pituitary (e.g. prolactinomas, somatotrophinomas, corticotrophinomas and non-functioning tumours) (Thakker 2000, Pannett & Thakker 2001). Other associated tumours may also arise in MEN1 patients and these include adrenal cortical tumours, bronchial NETs, thymic NETs, lipomas, collagenomas and angiofibromas (Trump *et al*. 1996). The *MEN1* gene on chromosome 11q13 represents a putative tumour suppressor gene, consistent with the Knudson model for tumour development (Knudson 1974, Pannett & Thakker 2001). The *MEN1* gene consists of 10 exons which span > 9 kb of genomic DNA (Chandrasekharappa *et al*. 1997, The European Consortium on MEN1 1997) (Fig. 3). The 1.83

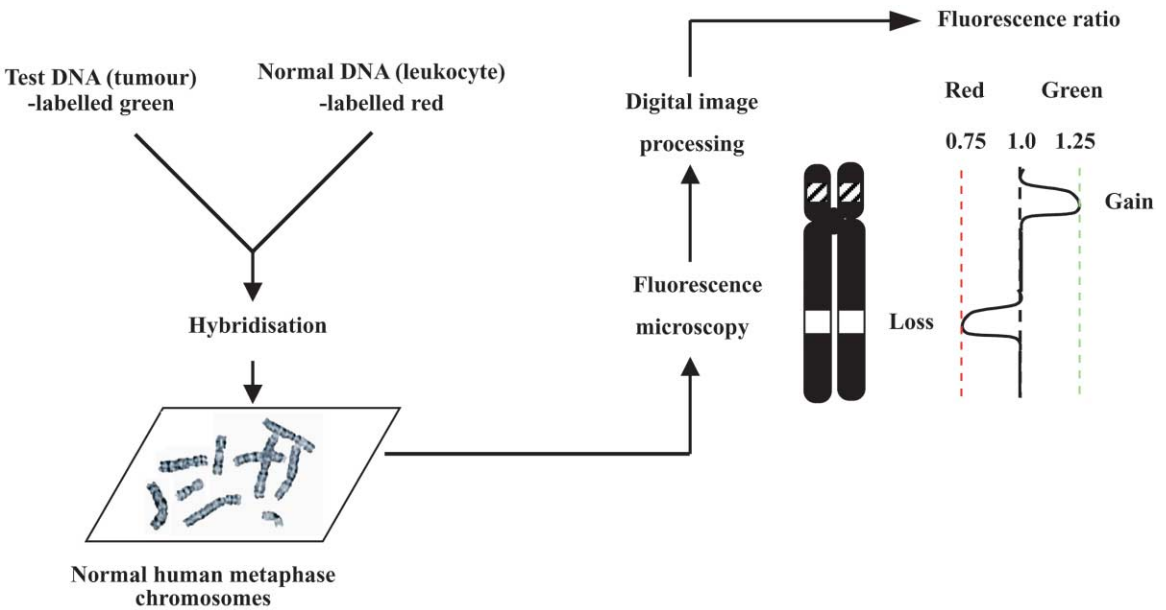

Figure 1 Differentially labelled test DNA and normal reference DNA are hybridised simultaneously to normal chromosome spreads. The hybridisation is detected with two different fluorochromes (green and red). Regions of gain (hatched boxes) due to duplications or amplifications of DNA sequences, or regions of loss (open boxes) due to deletions, are seen as changes in the ratio of the intensities of the two fluorochromes along the target chromosomes (Kallioniemi *et al.* 1993, du Manoir *et al.* 1993).

kb mRNA encodes a novel 610 amino acid protein referred to as MENIN (Chandrasekharappa *et al*. 1997). Over 600 germline mutations of the *MEN1* gene have been identified and these are scattered throughout the coding region and are of diverse types (Pannett & Thakker 1999, Turner *et al*. 2002). Thus, approximately 21% are nonsense, 44% are frameshift deletions or insertions, 9% are in-frame deletions or insertions, 7% are splice site mutations and 19% are missense mutations (Turner *et al*. 2002). Such germline MEN1 mutations are frequently associated with NETs and this is illustrated by family 7/03 (Fig. 4), in which affected members have a missense *MEN1* mutation and develop different NETs that include gastrinomas, insulinomas and lung NETs (Trump *et al*. 1996, The European Consortium on MEN1 1997). The majority of MEN1 tumours have LOH of chromosome 11q13 and a few have been shown to harbour somatic mutations consistent with the Knudson two-hit hypothesis (Pannett & Thakker 2001). In addition, LOH at the *MEN1* locus on chromosome 11q13 and somatic *MEN1* mutations have also been observed in sporadic NETs of foregut origin, thereby indicating a role for the *MEN1* gene in the aetiology of such tumours (Toliat *et al*. 1997, Zhuang *et al*. 1997*b*, Hessman *et al*. 1998, Shan *et al*. 1998, Wang *et al*. 1998, Fujii *et al*. 1999, Gortz *et al*. 1999, Mailman *et al*. 1999, Bergman *et al*. 2000, Goebel *et al*. 2000, Pannett & Thakker 2001). Thus, LOH at chromosome 11q13 has been shown in approximately 60% of sporadic foregut NETs and pancreatic islet-cell tumours, which include gastrinomas, insulinomas, VIPomas and glucagonomas (Jakobovitz *et al*. 1996, Debelenko *et al*. 1997*a*, Dong *et al*. 1997, Emmert-Buck *et al*. 1997, Toliat *et al*. 1997, D'Adda *et al*. 1999*b*). In addition, somatic *MEN1* mutations have, to date, been reported in 11 sporadic foregut NETs, excluding pancreatic NETs (Table 4 and Fig. 3) (Debelenko *et al*. 1997*a*, 2000, Baudin *et al*. 1999, Fujii *et al*. 1999, Gortz *et al*. 1999). The mutations consist of three nonsense mutations, six frameshift deletions and insertions, one missense mutation and one donor splice site mutation. The nonsense, frameshift and donor splice site mutations are predicted to lead to truncated MENIN proteins, and these and the form resulting from the missense mutation are likely to disrupt the functions of MENIN (see below) by altering its binding to interacting proteins (Fig. 5).

Function of MENIN

MENIN is predominantly a nuclear protein in non-dividing cells, but in dividing cells it is also found in the cytoplasm (Guru *et al*. 1998). The function of MENIN still remains to be elucidated, but it has been shown to interact with a number of proteins that are involved in transcriptional regulation and the control of genome stability. Thus, in transcriptional regulation, MENIN has been shown to interact with: (i) the activating protein-1 transcription factors JunD (Agarwal *et al*. 1999) and c-Jun (Yumita *et al*. 2003) to suppress Jun-mediated transcriptional activation; (ii) members of

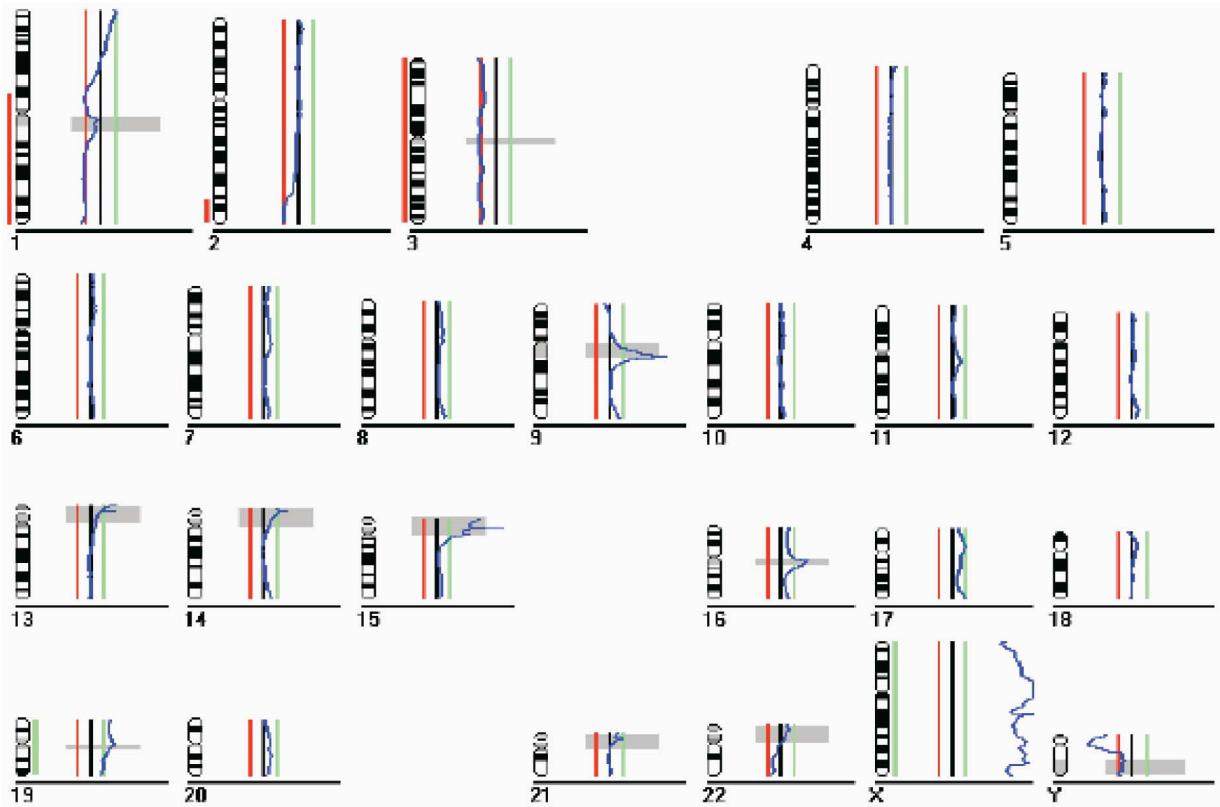

Figure 2 CGH analysis of a lung NET from a female patient 5 (see Table 5). The fluorescence ratio profiles, which indicate the chromosomal differences, are shown to the right of the chromosomes. The left-hand line represents a fluorescence ratio of 0.75 (i.e. loss), the centre line a ratio of 1.0 (i.e. normal) and the right-hand line a ratio of 1.25 (i.e. gain) (Fig. 1). The tumour DNA is from a female (46XX), and has been hybridised to metaphase chromosomes from a male (46XY), and hence an 'apparent' gain of the X chromosome (fluorescence ratio > 1.25) and an 'apparent' loss of the Y chromosome (fluorescence ratio < 0.75) are observed. In addition, 'apparent' gains are sometimes observed around the centromeric regions, which contain repetitive elements, e.g. chromosomes 9 and 6. Thus, the tumour reveals a 'true' loss of chromosomes 1q21-qter, 2q34-qter and 3, and a 'true' gain of chromosome 19.

the NF-κB family (e.g. p50, p52 and p65) of transcription regulators to repress NF-κB-mediated transcriptional activation (Heppner *et al*. 2001); (iii) members of the Smad family (Smad 3, Smad1/5 complex) to modulate the transforming growth factor-β (TGFβ) pathway (Kaji *et al*. 2001) and the bone morphogenetic protein-2-signalling pathways, respectively (Sowa *et al*. 2003); and (iv) the mouse placenta and embryonic expression (*Pem*) gene, which encodes a homeobox containing protein that plays a role in transcription (Lemmens *et al*. 2001). A role for MENIN in controlling genome stability has been reported because it interacts with: (i) a subunit of RPA2, which is a heterotrimeric protein required for DNA replication, recombination and repair (Sukhodolets *et al*. 2003); (ii) the tumour metastases suppressor NM23H1/nucleoside diphosphate kinase (Ohkura *et al*. 2001, Yaguchi *et al*. 2002), which induces GTPase activity; and (iii) the glial fibrillary acidic protein and vimentin (Lopez-Egido *et al*. 2002), which are involved in the intermediate filament network. The 11 somatic MEN1 mutations reported in the sporadic NETs (Table 4) are predicted to disrupt these protein interactions (Fig. 5) and hence the function of MENIN in regulating cell proliferation.

Other genes involved in NETs

Foregut NETs

The NETs that have been studied for genetic abnormalities are principally from the lung (bronchial), thymus, stomach and pancreas and each has the involvement of different genes.

Lung NETs

Lung NETs represent a broad spectrum of morphological types, ranging, in order of aggressiveness and malignant potential, from typical carcinoids (TC), atypical carcinoids

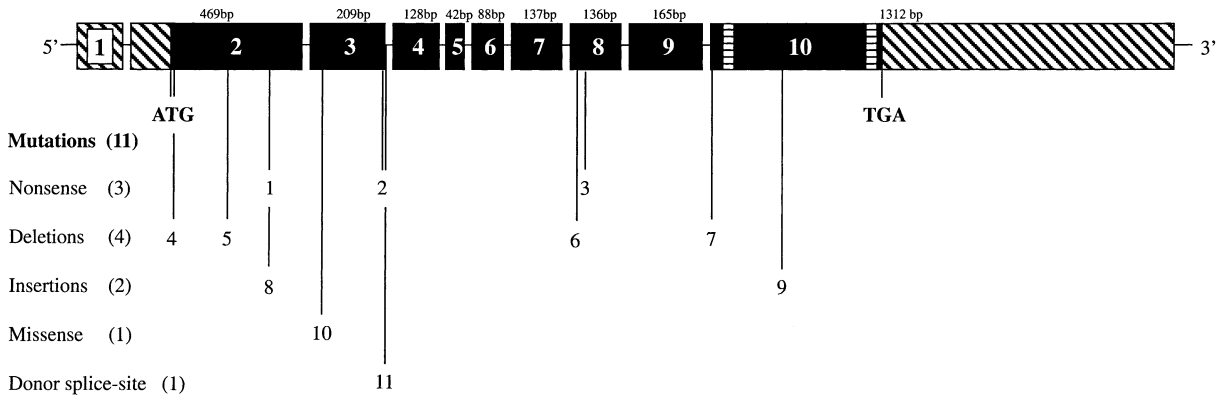

Figure 3 Schematic representation of the genomic organisation of the *MEN1* gene illustrating 11 somatic mutations found in non-familial, i.e. sporadic isolated (i.e. non-MEN1 patients) bronchial, thymic, gastric and duodenal NETs (see Table 4). The human *MEN1* gene consists of 10 exons that span > 9 kb of genomic DNA and encodes a 610 amino acid protein (Chandrasekharappa *et al.* 1997, The European Consortium on MEN1 1997). The 1.83 kb coding region is organised into 9 exons (exons 2–10) and 8 introns (indicated by a line but not to scale). The sizes of the exons (solid boxes) range from 42 to 1312 bp and those of the introns range from 41 to 1564 bp. The start (ATG) and stop (TGA) sites in exons 2 and 10 respectively, are indicated. Exon 1, the 5′ part of exon 2 and 3′ part of exon 10 are untranslated (indicated by hatched boxes). The locations of the two nuclear localisation sites, which are at codons 479 to 497, and 588 to 608 at the C-terminus, are represented by horizontally hatched boxes in exon 10. The sites of the 11 somatic mutations (three nonsense mutations, six frameshift mutations, one missense mutation and one donor splice site mutation) found in sporadic NETs are represented below the gene, and details of each of these are provided in Table 4.

(AC), higher grade LCNECs, to SCLCs. LOH involving chromosomes 3p, 5q21, 9p, 11q13 (*MEN1* gene, see above), 13q13 (*RB* gene) and 17p13 have been observed in lung NETs. These results of LOH studies are broadly confirmed by our CGH analysis of seven lung NETs that were all bronchial TCs, in which chromosome 3 loss was observed in two of the tumours and chromosome 9 loss was observed in one of the tumours (Table 5). Interestingly, our CGH studies reveal a gain of chromosome 19 in five of the lung NETs and these results suggest that amplification of genes on chromosome 19 may be important in the aetiology of foregut NETs. However, another study of 11 lung NETs found loss of chromosome 11q in 36% of tumours (Zhao *et al.* 2000). The other abnormalities that were found in approximately 10% of lung NETs included gains of chromosomes 5, 7, 8, 9q, 14q, 15q, 16q, 17 and 20q, and losses of chromosome 1p, 6q and 22q (Zhao *et al.* 2000). Further studies of such tumours are required to clarify the chromosomal abnormalities associated with these foregut NETs.

LOH at chromosome 3p is the most frequent change in lung NETs and has been observed in 40% of TC, 73% of AC, 83% of LCNEC and 85% of SCLC (Onuki *et al.* 1999). The *FHIT* locus at chromosome 3p14.2 has been particularly studied and LOH was observed in 23% of TC, 35%-50% of AC, 80% of LCNEC and 67% of SCLC, but no somatic mutations have been reported (Kovatich *et al.* 1998, Onuki *et al.* 1999). The frequency of LOH on chromosome 5q21, which is the location of the *APC*/*MCC* genes, increases with the severity of the tumour. Thus, LOH at chromosome 5q21 was observed in 0% of TC, 25% of AC, 46% of LCNEC and 86% of SCLC and was correlated with a poor survival in patients with lung NETs (Onuki *et al.* 1999). Furthermore, a meta-analysis using data from the literature revealed that LOH at chromosome 5q in lung neuroendocrine carcinomas (LCNEC and SCLC) was higher, at 75%, than in lung carcinoid tumours (TC and AC) in which the frequency was 1.4% (Ullmann *et al.* 2002). LOH at chromosome 9p21, which is the location of the *p16* locus, is also higher in carcinomas (53%) than in the carcinoid tumours (23%) (Onuki *et al.* 1999). LOH and point mutations of the *p53* locus on chromosome 17p13 were detected in 10% of TC (Lohmann *et al.* 1993*a*, Ramnani *et al.* 1999), 45% of AC (Hiyama *et al.* 1993, Couce *et al.* 1999), 72% of LCNEC (Lohmann *et al.* 1993*a*, Couce *et al.* 1999) and 90% of SCLC (Sameshima *et al.* 1992). Furthermore, these *p53* alterations in lung NETs were found to correlate with poor survival rates amongst patients (Onuki *et al.* 1999). LOH at the *RB* locus on chromosome 13q14.1-q14.2 also increased with the aggressiveness of the tumour such that there was a demarcation between the lung carcinoids (AC and TC) and the carcinomas (LCNEC and SCLC), thereby suggesting that different mechanisms may be involved in tumorigenesis in these two types of lung NETs (Onuki *et al.* 1999). Furthermore, two mutations of *K-ras*, which is a dominant oncogene that is aberrant in most human tumours including lung cancer (Nakano *et al.* 1984), have been found in atypical lung NETs (Couce *et al.* 1999). Overall,

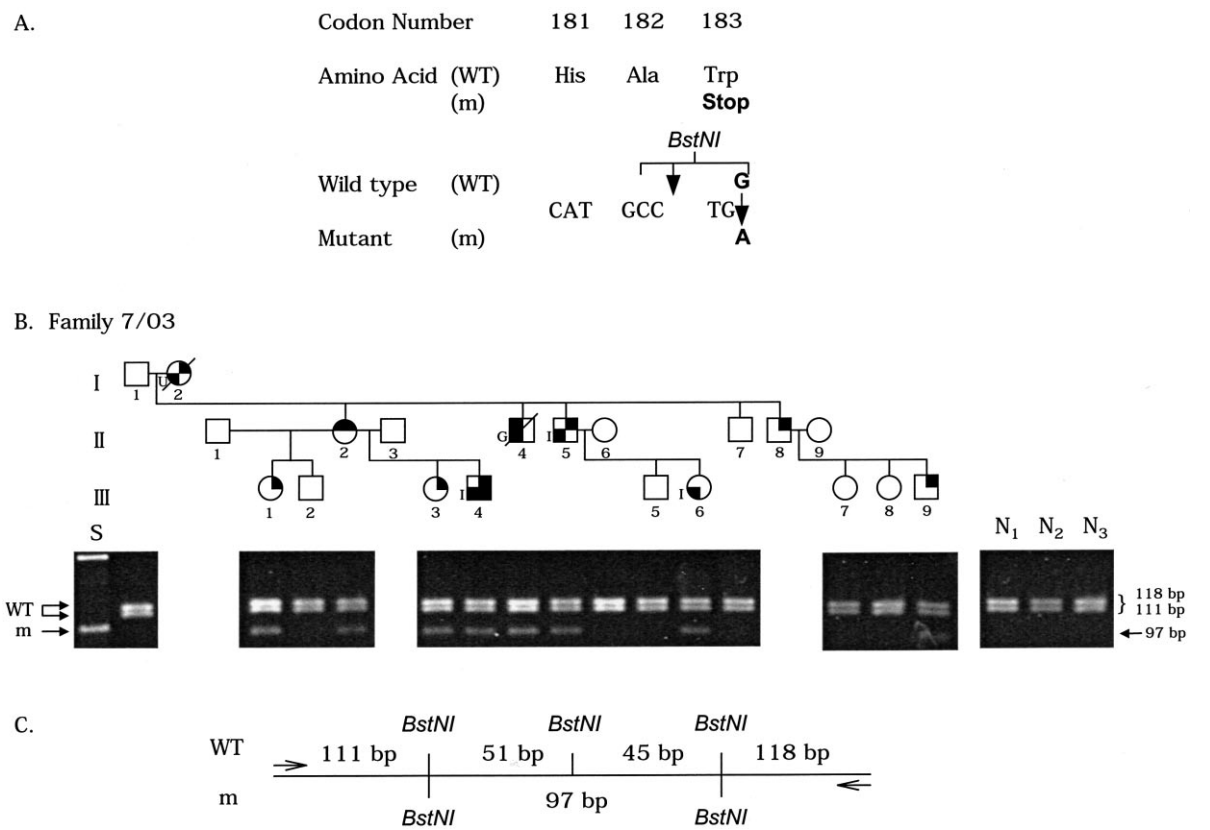

Figure 4 Detection of *MEN1* mutation (Trp183Stop) in exon 3 in family 7/03 by restriction enzyme analysis. DNA sequence analysis of individual II-5 revealed a G → A transition at the third nucleotide of codon 183. The transition results in the loss of a *BstNI* restriction enzyme site (CC/TGG) from the WT sequence (A), and this has facilitated the detection of the mutation in the other affected members (II.2, II.4, II.5, III.1, III.3, III.4, III.6 and III.9) of this family (B). The mutant (m) PCR product is 97 bp (C), whereas the WT products are 111, 118, 51 and 45 bp. The 51 and 45 bp products are not shown. The affected individuals are heterozygous (WT/m), and the unaffected family members (I.1, II.6, II.7, III.2, III.5, III.7 and III.8) and control unrelated normals (N1–N3) are homozygous for the WT allele (B). Individuals are represented as males (squares), females (circles), unaffected (open symbols), affected with parathyroid tumours (solid upper right quadrant), affected with corticotrophinoma (solid lower right quadrant), affected with pancreatic tumour (solid lower left quadrant; G, gastrinoma, I, insulinoma and U, unknown) and affected with lung NET (solid upper left quadrant). The positions of the size markers (S) at 100 bp and 200 bp are shown. Cosegregation of this mutation with MEN1 in family 7/03 and its absence from 110 alleles of 55 unrelated normal individuals (N1–N3 shown) was demonstrated, thereby indicating that it is not a common polymorphism. The clinical findings in this family, 7/03, with the Trp183Stop mutation have been updated from those previously reported as family 7/87 (Trump *et al.* 1996, The European Consortium on MEN1 1997).

these studies of lung NETs reveal four important findings: (i) LOH at chromosome 3p is the most frequent change in these tumours; (ii) LOH of the *MEN1* locus is also common; (iii) LOH and mutations of p53 cumulatively increase with severity of the tumour type; and (iv) LOH at 5q21, the location of the *APC* and *MCC* genes is correlated with a poor survival.

In addition to these studies of LOH, CGH and mutational analysis, lung NETs have also been investigated for silencing of genes by methylation. Genes that are frequently methylated in gastrointestinal malignancies were investigated in lung TCs, ACs, SCLCs and NSCLC, and the frequencies of methylation of the *RAR*β, *CDH1*, *RASSF1A* and *CASP8* genes were found to be higher in SCLC (45%, 55%, 80% and 35% respectively) than in TCs and ACs (20%, 25%, 55% and 18% respectively), although ACs seemed to have a higher frequency (71%) of *RASSF1A* methylation when compared with TCs (45%). However, methylation of the *RASSF1A*, *p16*, *APC* and *CDH13* genes was significantly higher in NSCLCs when compared with the lung NETs (TC, AC and SCLC) (Toyooka *et al.* 2001, Shivapurkar *et al.* 2002). These methylation differences between lung TCs, ACs, SCLCs and NSCLC reinforce the view that these tumours are fundamentally different and that the tumorigenic

Table 4 Somatic *MEN1* mutations in sporadic foregut NETs, excluding those of the pancreas[a]

Mutation type; number	Exon	Codon	Base change	Predicted effect[b]	Tissue	Reference
Nonsense						
1	Exon 2	108	*C*GA → *T*GA	ns, Arg108 Stop	Bronchial	Baudin *et al.* (1999)
2	Exon 3	209	*C*AG → *T*AG	ns, Gln209 Stop	Duodenal	Gortz *et al.* (1999)
3	Exon 8	393	*C*AA → *T*AA	ns, Gln393 Stop	Thymic	Fujii *et al.* (1999)
Deletion						
4	Exon 2	8–12	nt134del13bp	fs, ins 108 aa Stop	Bronchial	Debelenko *et al.* (1997*a*)
5	Exon 2	83–84	nt357del4p	fs, ins 34 aa Stop	Stomach[c]	Fujii *et al.* (1999)
6	Exon 8	372	nt1226delC	fs, ins 3 aa Stop	LCNEC[d]	Debelenko *et al.* (2000)
7	Exon 10	451	nt1461delG	fs, ins 7 aa Stop	Bronchial	Debelenko *et al.* (1997*a*)
Insertion						
8	Exon 2	108	nt134ins29bp	fs, ins 10 aa Stop	Bronchial	Gortz *et al.* (1999)
9	Exon 10	514	nt1650insC	fs, ins 44 aa Stop	Bronchial	Debelenko *et al.* (1997*a*)
Missense						
10	Exon 3	172	*G*AT → *T*AT	ms, Asp172Val	Bronchial	Gortz *et al.* (1999)
Donor splice site mutation						
11	Intron 3		nt764+3 (ag)	ds, fs, ins 18 aa Stop	Bronchial	Debelenko *et al.* (1997*a*)

[a]Somatic *MEN1* mutations have been found in sporadic pancreatic NETs, including gastrinomas (Zhuang *et al.* 1997*a*, Wang *et al.* 1998, Mailman *et al.* 1999, Goebel *et al.* 2000), insulinomas (Zhuang *et al.* 1997*a*, Shan *et al.* 1998, Pannett & Thakker 2001), VIPomas (Shan *et al.* 1998, Wang *et al.* 1998, Gortz *et al.* 1999) and glucagonomas (Hessman *et al.* 1998, Bergman *et al.* 2000). Midgut and hindgut NETs generally do not have *MEN1* mutations, though a missense mutation, Val53Ile, has been identified in one ileal NET (Gortz *et al.* 1999).
[b]Predicted effect: fs, frameshift mutation; ns, nonsense mutation; ms, missense mutation; ds, donor splice site mutation; ins, insertion; aa, amino acid.
[c]Gastric neuroendocrine carcinoma (Fujii *et al.* 1999).
[d]LCNEC, large cell neuroendocrine carcinoma. Mutations are numbered with reference to the *MEN1* cDNA sequence U93236 (GenBank).

process in each type evolves along different pathways with tumours of the same histological type having similar methylation patterns. Furthermore, methylation of the *death-associated protein kinase* and *APC* genes in lung cancers has also been shown to correlate with poor survival (Tang *et al.* 2000, Virmani *et al.* 2001). These studies are important as they may have a potential impact on the clinical management of patients, as treatment with demethylating agents, which results in a temporary restoration of gene expression, may represent a potential method of tumour therapy or chemoprevention (Momparler *et al.* 2000, Schwartsmann *et al.* 2000) in patients who have tumours with such methylated genes.

Thymic NETs

The genetic abnormalities in thymic NETs have not been as well characterised as the other foregut NETs. Twenty five per cent of reported thymic NETs are from MEN1 patients (Teh 1998), and LOH of the *MEN1* locus on chromosome 11q13 has not been reported in thymic NETs, though a somatic *MEN1* mutation has been reported in one thymic NET (Teh 1998, Fujii *et al.* 1999, Hessman *et al.* 2001). LOH of chromosome 1p has been reported in two thymic NETs (Teh *et al.* 1998), and our CGH results (Table 5) reveal the occurrence of deletions of chromosomes 3, 9p21-pter, and Y and a gain of chromosome 19p in a thymic NET from a male patient.

Gastric NETs

Gastric NETs are derived from the enterochromaffin-like (ECL) cell. Four main types referred to as ECL types I–III (ECL-I to -III) and neuroendocrine carcinoma are recognised depending upon their clinical and pathological characteristics (Rindi *et al.* 1993, Bordi 1995). ECL-I tumours, which are associated with atrophic gastritis and hypergastrinaemia, account for 70–80% of all ECLomas and consist of small, multiple tumours that are limited to the mucosa or submucosa, and rarely metastasise to regional lymph nodes. More than 85% of ECL-I tumours are monoclonal (D'Adda *et al.* 1999*a*). ECL-II tumours, which are associated with MEN1 and the Zollinger–Ellison syndrome, account for 6% of ECLomas and consist of multiple tumours that infiltrate to

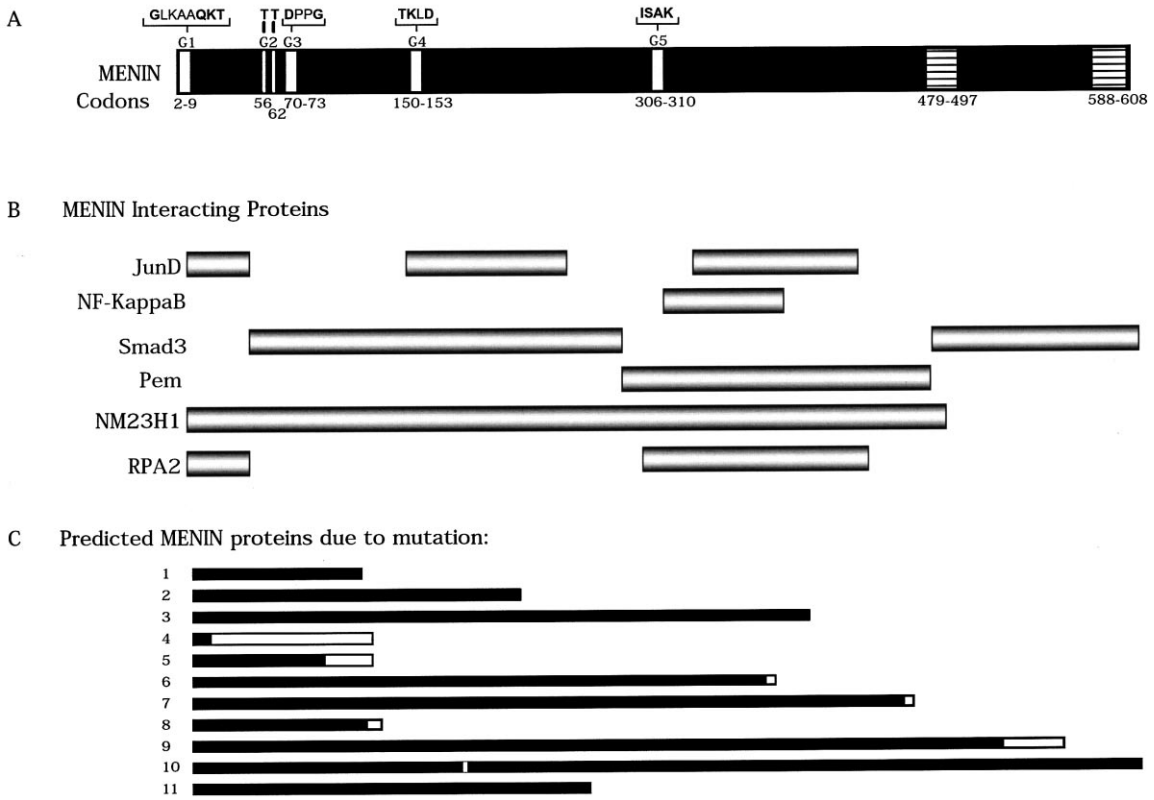

Figure 5 Schematic representation of the amino acid sequence of MENIN together with the regions that interact with other proteins. (A) MENIN has two nuclear localisation signals (horizontally hatched boxes) at codons 479–497 and 588–608 (Guru *et al.* 1998), and 5 putative GTPase sites (G1–G5) (open boxes), whose consensus sequences are shown above, with the conserved amino acid residues shown in bold (Yaguchi *et al.* 2002). (B) MENIN regions that likely interact with JunD (codons: 1–40, 139–242, 323–428) (Agarwal *et al.* 1999); nuclear factor-κB (NF-κB)) (codons: 305–381) (Heppner *et al.* 2001); small-mothers against decapentaplegic homology 3, Smad3 (codons: 40–278, 477–610) (Kaji *et al.* 2001); placenta and embryonic expression, Pem (codons: 278–476) (Lemmens *et al.* 2001); non-metastatic 23β human genes, NM23H1 (codons: 1–486) (Ohkura *et al.* 2001); and a subunit of replication protein A (RPA2) (codons 1–40, 286–448) (Sukhodolets *et al.* 2003) are indicated by the boxes with grey shading. (C) Truncated or other abnormal forms of MENIN that are predicted to result from the 11 somatic *MEN1* mutations reported in sporadic foregut NETs, excluding pancreatic neuroendocrine tumours, are represented by bars (normal sequence, solid bars and missense sequences, open bars).

Table 5 CGH analysis of eight NETs (seven lung TCs and a thymic NET)

Patient no.	Sex[a]	NET	Chromosomal losses[b]	Chromosomal gains
1	M	Lung TC[d]	11q12-pter, 11p13-pter	19
2	M	Lung TC	22q13-qter	13, 19
3	F	Lung TC		14, 17, 19, 20q, 22
4	F	Lung TC		5, 19
5	F	Lung TC	1p21-qter, 2q34-qter, 3	19
6	F	Lung TC	1q13-p31, 1q32-q41, 5, 4, 6q, 9p, 17p, X, 3q13-p14, 8q24-pter, 13q21-qter, 15q13-q21, 18q22-qter, 20p12-pter	8p23-qter, 17q
7[c]	M	Lung TC		19p, X
8[c]	M	Thymus	3, 9p21-pter, Y	19p

[a]Sex: M, Male; F, Female.
[b]p, Short arm of a chromosome; q, long arm of a chromosome; ter, telomeric.
[c]The lung nodule and the thymic NET were from the same patient.
[d]TC, typical carcinoid.

the submucosa. The prognosis for ECL-II tumours is generally good, even if they occasionally become aggressive (Rindi *et al*. 1993, Bordi 1995). ECL-III tumours, which are associated with hypergastrinaemia, account for 14–25% of ECLomas and usually consist of a single tumour that is often infiltrating the muscularis propia and serosa. ECL-III tumours, which may have LOH of the X chromosome (D'Adda *et al*. 1999*a*), frequently metastasise, and hepatic metastases occur in 50% of patients who have a poor prognosis. Neuroendocrine carcinomas, which are uncommon, are usually single, large, poorly differentiated and highly malignant, and LOH on chromosomes 8p, 11p, 12p, 13q, 15q and 17p has been reported in 82%, 50%, 50%, 50%, 58% and 57% of 15 such tumours respectively (Han *et al*. 2000).

LOH of the *MEN1* locus on chromosome 11q13 has been found in 48% of 25 ECL-I tumours (D'Adda *et al*. 1999*b*), 75% of 20 ECL-II tumours (Debelenko *et al*. 1997*b*) and both of two neuroendocrine carcinomas (D'Adda *et al*. 1999*b*). Somatic *MEN1* mutations have not been detected in type I and III ECLomas, but a 4 bp deletion at codon 83 of the *MEN1* gene that results in a frameshift and truncated protein (Table 4 and Figs 3 and 5) has been reported in one gastric neuroendocrine carcinoma (Fujii *et al*. 1999).

Pancreatic NETs

All pancreatic NETs from MEN1 patients have shown a loss (LOH) of chromosome 11. However, 30% of these tumours may also show LOH at chromosomes 3, 6, 8, 10, 18 and 21 (Hessman *et al*. 2001). In addition, LOH at chromosomes 3 and 6 is also observed in $>50\%$ and $>60\%$ of sporadic (i.e. non MEN1) pancreatic NETs respectively. The chromosome 3 losses particularly occur as late events, thereby suggesting that putative tumour suppressor genes on this chromosome may be involved in a more aggressive tumour phenotype (Barghorn *et al*. 2001, Guo *et al*. 2002). Furthermore, a point mutation of the *N-Ras* gene has been identified in a PET (Arany *et al*. 1994).

Midgut NETs (carcinoids)

In contrast to foregut NETs, which have frequent deletions and mutations of the *MEN1* gene, midgut NETs (i.e. carcinoids) rarely show involvement of the *MEN1* gene. However, CGH studies have shown losses of chromosome 11q in 22% of ileal and duodenal carcinoids, and this has involved the distal part of chromosome 11q, where the tumour suppressor gene *SDHD* is located (Kytola *et al*. 2002). Germline mutations of *SDHD* have been reported in families with hereditary head and neck paragangliomas linked to 11q23, and in families with adrenal and extra-adrenal phaeochromocytomas (Baysal *et al*. 2000, Gimm *et al*. 2000, Astuti *et al*. 2001*a*,*b*, Badenhop *et al*. 2001, Milunsky *et al*. 2001, Taschner *et al*. 2001). Two possible germline missense mutations (His50Arg and Gly12Ser) of the *SDHD* gene were reported in two sporadic midgut carcinoid tumours, which also had LOH at the *SDHD* locus on chromosome 11q23 (Kytola *et al*. 2002). There is debate about whether these changes are mutations or polymorphisms, particularly as Gly12Ser has been reported in a phenotypically normal patient (Gimm *et al*. 2000) and it has also been reported that His50Arg may represent a rare polymorphism. Furthermore, *SDHD* mutations have not been detected in 45 NETs of the lung, gastrointestinal, pancreatic and parathyroid (Perren *et al*. 2001), and thus it would seem unlikely that the *SDHD* gene is involved in midgut NETs which are likely to be due to other genes. Indeed CGH studies of midgut carcinoids have shown losses of chromosomes 9p, 18p and 18q in 21%, 38% and 33% respectively (Zhao *et al*. 2000, Tonnies *et al*. 2001), gains of chromosomes 17q and 19p in 57% (Tonnies *et al*. 2001) and LOH of chromosome 18 has been reported in 88% of midgut NETs (Lollgen *et al*. 2001). In addition, 22% of midgut tumours have losses of 16q21 and metastases show an accumulation of genetic abnormalities, which particularly involve a loss of chromosome 16q and a gain of chromosome 4p. Furthermore, a *K-ras* mutation, Gly12Asp, has been identified in an extra-hepatic bile duct NET (Maitra *et al*. 2000). These findings indicate that midgut and foregut NETs are likely to develop via different molecular pathways, although the genes involved at chromosomes 4p, 9p, 16q, 18p and 18q, in causing midgut carcinoids, remain to be identified. Appendiceal goblet-cell carcinoids may have yet another molecular pathway of development as up-regulation of cyclin D1 and p21 together with down-regulation of p16 have been reported (Kanthan *et al*. 2001, Oberg 2002).

Hindgut NETs

The genetic abnormalities in hindgut NETs have not been as well characterised as those in foregut and midgut NETs, and LOH of chromosome 18 has been identified in only one ascending colon NET (Lollgen *et al*. 2001). Rectal NETs that are >5 mm in size have been reported to express TGFα more frequently, and the epidermal growth factor (EGF) receptor was expressed in all lesions (Shimizu *et al*. 2000). It has therefore been proposed that TGFα and the EGF receptor may participate in an autocrine mechanism that regulates the growth of hindgut NETs, and hence these hindgut tumours are likely to develop via different molecular pathways from those of the foregut and midgut NETs (Shimizu *et al*. 2000).

Conclusion

NETs may occur as part of complex familial endocrine cancer syndromes such as MEN1 and NF1, although the majority occur as non-familial (i.e. sporadic) isolated tumours. Different genes are involved in the aetiology of each type of NET and different genetic abnormalities that

include point mutations, gene deletions, DNA methylation, chromosomal losses and chromosomal gains may be involved. Furthermore, the foregut, midgut and hindgut NETs develop via different molecular pathways. For example, foregut NETs have frequent deletions and mutations of the *MEN1* gene, whereas midgut NETs have losses of chromosomes 18, 11q and 16q, and hindgut NETs, express TGFα and the EGF receptor. In addition, in lung NETs, a loss of chromosome 3p is the most frequent change, and p53 mutations and chromosomal loss of 5q21 are associated with more aggressive tumours and poor survival. Moreover, methylation frequencies of *RAR*β, *CDH1* and *RASSF1A* genes increase with the severity of lung NETs. Thus the development and progression of NETs is associated with specific genetic abnormalities that indicate the likely involvement of different molecular pathways.

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