CASE REPORT

Comparative genomic hybridization studies in tumours from a patient with multiple endocrine neoplasia type 1

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Abstract

Objective: To identify genetic changes, other than the MEN1 gene, that might be involved in the tumorigenesis and progression of multiple endocrine neoplasia type 1 (MEN1)-related tumours.

Methods: We used comparative genomic hybridization (CGH) and loss of heterozygosity (LOH) to study tumours from various sites in a patient with MEN1.

Results: Gain of genetic material was not found. Frequent losses of genetic material were found in chromosomes 1, 4, 5, 6, 9, 11 and 18. Besides the chromosome 11 where the MEN1 gene is located, the other regions are known to harbour important tumour suppressor genes.

Conclusions: These results suggest the involvement of other cancer-related genes in the tumorigenesis and progression of MEN1 tumours that warrant further investigations.

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Introduction

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant cancer syndrome characterized by tumours of the parathyroid glands, endocrine entero-pancreas, and anterior pituitary gland. In addition, other tumours such as carcinoids, lipomas, adrenocortical and thyroid tumours may occur as part of the syndrome (1).

Loss of heterozygosity (LOH) studies and linkage analysis localized the MEN1 gene to chromosome 11q13 (2). The gene has recently been cloned (3) but its function has not yet been established. Nevertheless, the gene has been considered to be a tumour suppressor gene, because of the frequent findings of loss of wild-type alleles in the MEN1 gene region in MEN1-related tumours and their sporadic counterparts (4, 5). These findings are compatible with Knudson’s two-hit mutation theory (6) suggesting the following sequence of events: MEN1 patients have the germline MEN1 mutation (first hit) in all tissues but would acquire a tissue-specific somatic mutation (second hit) that abolishes the functions of the gene, resulting in tumour formation. However, in view of the natural history of MEN1-related tumours, the genetic profiles of MEN1-related tumours must be more complex. For example, the MEN1-related parathyroid tumours never, and pituitary tumours rarely, undergo malignant transformation, whereas pancreatic and gastrointestinal endocrine tumours frequently develop into malignant tumours and even metastases. These clinical findings would suggest the involvement of other cancer-related genes or other genetic changes constituting the multi-step tumour progression theory (7). However, such genetic information is not available to date. We have used comparative genomic hybridization (CGH) (8) to carry out a genome search for genetic changes in tumours from various sites, both primary and metastatic, in a patient with MEN1. The changes sought were either in the form of copy number increase, thus indicating overexpression of oncogenes, or of copy number decrease, indicating the inactivation of tumour suppressor genes. The latter was further substantiated by LOH studies using microsatellite markers.

Patient and methods

Patient

In 1988, at the age of 50 years, the patient underwent biochemical screening because of her family history of MEN1, and was found to have primary hyperparathyroidism. The patient eventually underwent a subtotal parathyroidectomy and partial thymectomy. In 1990, a small 4-mm polypoid tumour was found in the stomach
at an endoscopy; a biopsy sample was not taken from the tumour, but a specimen from elsewhere in the stomach showed a chronic gastritis with moderate atrophy. The duodenal biopsies were normal. A gastroscopy was performed 16 months later because the patient had anaemia, and a gastric tumour with a diameter of 5 cm was found just below the cardia. Another 8-mm tumour was noted in the descending duodenum. Bilateral adrenal tumours 1 cm in diameter and two hepatic carcinoid tumour metastases with diameters of 19 and 24 mm were detected separately by abdominal ultrasound and confirmed by fine-needle aspiration biopsy. The gastric tumour was excised and three hepatic segments containing metastases were resected. One coeliac lymph node was found to have metastases. Further examinations showed that the patient’s serum prolactin concentration was increased (91.5 μg/l) and a 5-mm pituitary adenoma was found by pituitary magnetic resonance imaging. Her serum oestradiol was less than 0.05 nmol/l and serum follicle-stimulating hormone (95 U/l) and luteinizing hormone (41 U/l) were increased. Other endocrine biochemical markers were normal. The pituitary tumour was treated with quinagolide 75 μg/day and no increase in the tumour size was observed. At subsequent autopsy, multiple carcinoid tumours of the stomach, up to 3 cm in diameter, along with multiple metastases to the liver and peritoneal carcinomatosis were found. Bilateral ovarian tumours with diameter of 5 cm each, were considered metastatic. Small carcinoid tumours of the bronchi, pancreas and small bowel, with diameters ranging from 5 mm to 2 cm, were considered to be separate primary tumours. No residual adenoma was found at autopsy. Linkage analysis using polymorphic microsatellite markers confirmed the linkage to MEN1 in the family, which included, in all, 12 clinically affected members. Among them, the most common presentation is hyperparathyroidism; two siblings of the patient also have gastrointestinal carcinoid tumours.

**Tumour DNA preparation**

Tumours from the parathyroids, adrenals, stomach, pancreas, bronchus, ileum, liver, ovary, lymph nodes and skin were studied. The parathyroid glands showed benign primary nodular chief cell hyperplasia and the adrenal glands showed cortical clear-cell adenoma. Carcinoids tumours were identified in the stomach, pancreas, bronchi and throughout the gastrointestinal tract. Metastases of primary stomach carcinoid were found in the liver, ovary, epigastric and mediastinal lymph nodes, the peritoneal cavity and the skin.

Tumour tissue was analysed by morphological point counting. For each tumour, the amount of neoplastic cells in relation to stromal cells were recorded, as well as total amount of neoplastic tissue in specimen. In cases where the amount of neoplastic tissue exceeded 90%, five to ten 5 μm thick sections were cut with a sterilized blade for DNA extraction. In cases where the amount of neoplastic tissue was under 90%, two to four 20 μm thick sections were cut onto glass slides, and tumour tissue was dissected according to the map based on haematoxylin–eosin stained sections. High-molecular weight DNA was extracted from the paraffin-embedded specimens using standard methods. DNA extraction and haplotype analysis in the family had been reported previously (9).

**LOH analysis**

LOH studies were performed on tumours from the parathyroid, adrenal gland, stomach, pancreas, bronchus, ileum, liver, ovary, epigastric lymph node and skin as described previously (9, 10), using the following microsatellite markers: D11S480, D11S4155 and D11S1889 in the region of MEN1. Microsatellite markers from the regions of loss from the CGH studies were used to verify the results.

**CGH studies**

CGH was performed as described previously (8, 11) in tumours from the parathyroid, adrenal gland, stomach, liver, ovary and epigastric lymph node. Tumours from the pancreas, bronchus, ileum and skin were not studied, as there was not sufficient DNA. Briefly, tumour DNA samples and normal DNA were labelled with fluorescein isothiocyanate (FITC)–dUTP (Dupont, Boston, MA, USA) and Texas Red–dUTP (Dupont), respectively, using nick translation. In cases in which the quantity of DNA was insufficient, the DNA was amplified and labelled by degenerate oligonucleotide primed-PCR (DOP-PCR) (12). It was then mixed with unlabelled human Cot-1 blocking DNA (GIBCO-BRL, Gaithersburg, MD, USA) and Texas Red–dUTP (Dupont), and hybridized to the normal denatured lymphocyte metaphase slides. After hybridization at 37 °C for 72 h, the slides were washed three times in 50% formamide–2×SSC (pH 7), twice in 2×SSC, and once in 0.1% NP40 (pH 8) at 45 °C, and in distilled water at room temperature, for 10 min each. After air drying, the slides were counterstained with 4,6-diamidino-2-phenylindole (DAPI) 0.1 μg/ml in an antifade solution (Vectorshield, Vector Laboratories, Burlingame, CA, USA).

**Digital image analysis**

Three single-colour images (DAPI, FITC, and Texas Red fluorescence) were collected from each metaphase spread, using a Nikon SA epifluorescence microscope and Xillix charge-coupled-device camera (Xillix Technologies Corp., Vancouver, BC, Canada) interfaced to a Sun LX workstation (Sun Microsystems Computer Corp., Mountain View, CA, USA). Four to six three-colour digital images were collected from each hybridization. Relative DNA sequence copy number changes
were detected by analysing the hybridization intensities of tumour and normal DNAs along the length of all chromosomes in each metaphase spread, as described previously (11). The absolute fluorescence intensities were normalized so that the average green:red ratio of all chromosomes in each metaphase was 1.0. The final results were plotted as a series of green:red ratio profiles and corresponding standard deviations for each human chromosome from p-telomere to q-telomere. Interpretation of CGH results was carried out as described previously (12): basically, eight ratio profiles (four separate metaphases) were averaged for each chromosome, to reduce noise; green:red ratios >1.15 were considered as gains of genetic material, and ratios <0.85 were considered losses.

Results
LOH studies of the MEN1 region in 11q13 were carried out in all tumours and LOH was detected in all except those in ileum, bronchus and adrenal tumour. A summary of the DNA copy number changes detected by CGH is presented in Fig. 1. No gain of genetic material was found in any of the tumours. Loss of genetic material was found in all tumours studied. The regions of loss were further confirmed by LOH studies using microsatellite markers; representative results are shown in Fig. 2. In the primary tumours of parathyroid, adrenal and stomach, and in metastases in the liver and lymph nodes, loss of 9p11pter was found. Frequent losses of chromosomes 1p, 2q, 3p, 4q, 5, 6, 9p, 11 and 18 were also found in the stomach tumour and the metastases in liver, ovary and lymph nodes. In the parathyroid nodular hyperplasia, besides the LOH of the MEN1 region, additional loss of chromosomes 2, 4, 5, 6, 8p, 9p, 10p, 11q and 18 was found. There was no histological feature suggestive of malignancy.

Discussion
The clonal evolution or multi-step process of tumorigenesis was borne out by molecular findings in neoplastic cells of different stages. As exemplified in the genetic model for colorectal cancer, the normal colon tissue acquires early genetic change involving mutations of the adenomatous polyposis coli (APC) gene and progress into hyperproliferative epithelium, adenoma, carcinoma and metastasis via additional genetic changes. These changes involve K-ras, DCC and p53 genes and further accumulation of genetic abnormalities (7). In MEN1 tumorigenesis, we expect a similar model, with the MEN1 mutation as the early genetic event. With the recent cloning of the MEN1 gene, it is timely to look for those genetic loci other than the MEN1 gene that might be involved in the tumorigenesis and progression of the tumours. A good way to start this search is by the use of comparative genomic hybridization (CGH) (8), which offers a reliable and effective screening of the entire tumour genome for variations in DNA sequence copy number. However, it is important to bear in mind that such variations in tumours can be either causal or consequential effects; nevertheless, if the findings are consistent, they can point directly to the location of these cancer-related genes (13).

In our patient’s tumours, no gain of genetic material was found. The losses of genetic material found in all tumours suggest that the genes that are possibly involved are putative tumour suppressor genes. In all tumours except those from ileum, bronchus and adrenal gland, LOH of 11q13 encompassing the MEN1 gene was found using MEN1-linked microsatellite markers. It is very likely that those tumours without LOH do have microscopic interstitial deletions, but that these were beyond the limit of detection of the present method. The LOH found in the other tumours represents the second-hit mutation of the MEN1 gene. These first (germline) and second (somatic) hits of mutation must have constituted the initial event in the tumorigenesis of MEN1 tumours. The second common region with loss of genetic material, which was present...
in every tumour except that of ovary, was 9p11pter. Although this region is large, two important cancer-related tumour suppressor genes are known to be located there: multi tumour suppressor 1 (MTS1) (14) and multi tumour suppressor 2 (MTS2) (15), which are cyclin-dependent kinase inhibitors (16, 17). Previously, studies of sporadic parathyroid and adrenal tumours have shown loss of genetic material in chromosome 9 (18, 19).

Histologically, the tumours from the ovary, lymph nodes and liver most probably represent metastases of the aggressive carcinoid tumour arising from the stomach. We therefore analysed the primary tumours and metastases to look for additional genetic changes. This multi-step process seemed to be supported by our findings. A number of common regions of loss in these metastatic tumours were found in the following chromosomes: 1p, 3p, 4q, 5, 6, 18. Chromosome 1p is known to harbour putative tumour suppressor genes associated with neuroblastomas, phaeochromocytomas, melanoma and carcinomas of the liver, breast and colon (20) although, to date, none of these gene(s) has been cloned. The 3p region is known to harbour tumour suppressor genes for renal cell carcinoma, non-small-cell lung carcinoma and nasopharyngeal carcinoma. One of them, the von Hippel–Lindau (VHL) tumour suppressor gene, encodes a 213 amino acid protein that negatively regulates the transcription elongation factor, elongin (21, 22), but has recently been shown to suppress the expression of vascular endothelial growth factor at a post-transcriptional level (23). The inactivation of VHL gene will thus lead to the loss of this suppression. In chromosomes 4q and 6, regions of frequent loss of heterozygosity have been found in cancer of the oesophagus, endometrium and breast (24–26). Chromosomes 5 and 8 are known to contain tumour suppressor genes such as APC, DCC, MADR2 and DPC4, which have been shown to be involved in colon and pancreatic cancers (27, 28).

The findings in the parathyroid glands with primary nodular hyperplasia were surprising and were verified by LOH studies. Any errors arising from mislabelling or handling of the sample were excluded. Despite the amount of genetic changes in the parathyroid tumour, no morphological evidence of malignancy was found. These results suggest two possibilities: the changes are secondary phenomena associated with increased cell replication, or some of these genetic changes are tissue-specific – that is, they exert their transforming effects only in certain types of tissue and, in this context, only in endocrine enteropancreatic tumours and not in parathyroid ones. The latter explanation would thus justify the common clinical observation that MEN1-related parathyroid tumours have never been known to develop into malignancy, in contrast with endocrine enteropancreatic tumours, which not uncommonly result in metastatic disease.

MEN1-related pancreatic and gastrointestinal endocrine tumours cause great morbidity and mortality. Assessing the prognosis of these tumours on a basis of histopathological studies is usually not reliable (29, 30). Among the conventional histopathological parameters, the size of a carcinoid tumour has a better predictive value over the histological and cytological properties (31). Recently molecular diagnosis and staging have been developed for certain types of cancer (32, 33), but not in endocrine tumours of the enteropancreas. Our findings showed that these tumours do acquire more genetic changes than just the LOH of the MEN1 region. It will be most worthwhile to study a larger number of tumours, to correlate any of these genetic changes with size, stage and prognosis.

In summary, we present the first genomic search for genetic alterations in MEN1 tumours by CGH. It will be important to extend the study by including more MEN1-related and sporadic tumours to delineate these regions of interest further, in an effort to identify the genes that might be involved in their tumorigenesis and progression. It might also be worthwhile to analyse some of the known tumour suppressor genes in these regions in the MEN1 tumours.

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References


