EXPERIMENTAL STUDY

Multiple endocrine neoplasia type 1 gene expression is normal in sporadic adrenocortical tumors

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Abstract

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant disorder with neoplasia of the anterior pituitary, the parathyroid, the endocrine pancreas and other endocrine tissues including the adrenal cortex. The tumor-suppressor gene causing this disease was identified at the gene locus 11q13. We recently reported that adrenocortical carcinomas frequently show loss of heterozygosity (LOH) of 11q13, but do not contain point mutations within the MEN1-coding region. To investigate whether reduced gene expression (for example by mutations within the MEN1 promoter) may contribute to the tumorigenesis of sporadic adrenocortical tumors, 24 adrenocortical specimen were studied by Northern blot analysis. This series included six adrenocortical carcinomas, four cortisol-producing adenomas, six aldosterone-producing adenomas, three endocrine-inactive adenomas and six normal adrenal glands. The presence of LOH of 11q13 was investigated using five polymorphic microsatellite markers (D11S956, PYGM, D11S4939, D11S4946 and D11S987) close to the MEN1 gene. Poly-A mRNA was hybridized with a PCR-generated cDNA probe of the MEN1 gene, a cDNA of the former MEN1 candidate gene phospholipase (PLC) β 3 and a mouse β -actin cDNA for normalization. LOH of 11q13 was detected in five out of six carcinomas and two inactive adenomas, but in none of the hormone-producing adenomas. Compared with normal adrenals ($100 \pm 6.5\%$, mean $\pm$ S.E.M.) MEN1 mRNA in adrenocortical tumors was expressed in similar amounts (carcinomas $109 \pm 11\%$, cortisol-producing adenomas $131 \pm 10\%$, aldosterone-producing adenomas $113 \pm 13\%$, endocrine-inactive adenomas $111 \pm 2\%$) with the exception of one adrenocortical carcinoma with low MEN1 mRNA expression (66%). PLC β 3 mRNA expression showed a variable pattern without reaching significant differences between the groups. We conclude that since mRNA levels were unaltered in the majority of tumors, mutations of the MEN1 gene causing altered gene transcription is unlikely to be a major pathogenic factor of sporadic adrenocortical tumors.

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Introduction

Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant disorder with neoplasia of the anterior pituitary, the parathyroid, the endocrine pancreas and other endocrine tissues, including the adrenal cortex (1, 2). The tumor-suppressor gene causing this disorder was identified recently at gene locus 11q13 flanked by the microsatellite markers PYGM and D11S4936 (3, 4). Previous studies revealed involvement of this gene locus in sporadic endocrine tumors, for instance in tumors of the parathyroid gland (5–7), in anterior pituitary adenomas (8–11), endocrine pancreatic tumors (12–14), aldosterone-producing adenomas of the adrenal cortex (15) and sporadic carcinoid tumors of the lung (16).

Molecular mechanisms leading to adrenal neoplasms

are still not elucidated in detail. Our intention was to investigate whether the MEN1 gene plays a role in tumorigenesis of sporadic adrenocortical masses like adrenocortical carcinoma, aldosterone- and cortisol-producing adenomas and endocrine-inactive adenomas. We recently discovered loss of heterozygosity (LOH) of the MEN1 gene locus in all sporadic adrenocortical carcinomas and two out of 24 sporadic adenomas (17). Sequence analysis of these tumors showed no point mutations within the MEN1-coding region. To investigate whether reduced gene expression (for example by mutations of the MEN1 promoter) may contribute to tumorigenesis of sporadic adrenocortical tumors we investigated 24 adrenocortical tissues by Northern blot analysis. In these tumor samples we determined the transcription of the MEN1 gene and the former MEN1

candidate gene phospholipase C (PLC) $\beta 3$, which is located close to the MEN1 gene locus (18).

Patients and Methods

Patients

We used tissues of six adrenocortical carcinomas, four cortisol-producing adenomas, six aldosterone-producing adenomas, three non-producing adenomas and six normal adrenal glands. All tumor samples were negative for MEN1 gene mutations within the coding region, as reported earlier (17). All patients had given written informed consent. The study protocol was approved by the ethical committee of the University Hospital of Würzburg. The normal adrenal tissues were obtained after organs were removed from brain-dead patients for transplantation. The adrenal glands were snap frozen in liquid nitrogen immediately after excision and then stored at -80°C until processing. The diagnoses of the patients were confirmed by clinical and biochemical and morphological data following standard criteria.

RNA extraction

Poly-A mRNA was extracted from the tissues using a QIAquick mRNA Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The tissues were stored in liquid nitrogen until microdissection and extraction started. The amount of extracted mRNA was measured photometrically and degradation was excluded by electrophoresis on a 1.2% agarose gel.

Northern blot

Three micrograms mRNA and an RNA marker (G319 Promega, Madison, WI, USA) were mixed with loading buffer containing formamide and ethidium bromide, heated for 3 min at 95°C , chilled on ice and then loaded on a 1.2% agarose gel containing 6.6% formaldehyde. Electrophoresis was run for 13 h at 40 V. Blotting to a nylon membrane (QIABrane; Qiagen) was performed as an inversed gravity-assisted blot for 20 h as described elsewhere (19). The mRNA was crosslinked to the membrane by exposure to UV radiation.

Labeling and hybridization

A 1895 bp fragment of the MEN1 cDNA was amplified using primers 5'-CCGCCACTATTTCCAGGCTCTGCGGGGC-3' and 5'-GTAGTTCAGAGGCCCTTTGCGCTGCCGCTTG-3', kindly provided by C. Heppner (NIDDK, NIH, Bethesda, MD, USA). As a PLC $\beta 3$ probe, a 4.4 kb cDNA fragment of PLC $\beta 3$ spanning the entire coding mRNA sequence was used, as published previously (20). The cDNAs were labeled with [α - ^{32}P]CTP (Amersham Buchler, Braunschweig, Germany) using a random primed labeling kit (Boehringer, Mannheim, Germany)

following the manufacturer's protocol. Unincorporated nucleotides were separated from radiolabeled DNA probes by NucTrap purification columns (Stratagene, Heidelberg, Germany). Labeling was monitored with 5 μl of the probes in scintillation fluid in a beta-counter. The blots were pre-hybridized for 1 h on 68°C with 20 ml Quickhyb (Stratagene), hybridized with the labeled cDNA for 1 h at 68°C and washed twice with $1\times\text{SSC}$ and twice with $0.5\times\text{SSC}$ (each containing 0.1% SDS) for 15 min at 60°C . Kodak X-omat-DS films were exposed for 5–48 h at -80°C using intensifying screens. The blots were stripped and rehybridized with a mouse β -actin cDNA (Stratagene) for standardization. Scanning densitometry of the major signals (MEN1, 2.8 kb; PLC 1.9 kb) of the autoradiographic films was performed with a video camera and a Macintosh PowerMac 7100 computer-based image analysis system using the NIH Image Program (NIMH, NIH, Bethesda, MD, USA). The results were expressed as a percentage of the mean of the normal adrenal glands, after normalization for β -actin expression and after normalization for a control probe on every blot.

DNA extraction and LOH analysis

DNA was extracted from tissue and leukocytes using the Qiagen Blood and Cell Culture DNA Kit (Qiagen) following the manufacturer's protocol.

Primers for the polymorphic microsatellite markers D11S956, PYGM and D11S987 were identical to those published previously (21). PCR was performed in a total volume of 100 μl containing 30 pmol of each primer, 2.5 U TAQ polymerase (Perkin Elmer, Foster City, USA) and 100–400 ng DNA. The cycling program was as follows: initial denaturation at 95°C for 5 min, 30 cycles of 55°C for 1.5 min, 72°C for 1.5 min, 94°C for 1.5 min, final extension at 72°C for 5 min. Primer sequence for D11S4939 and D11S4946 and PCR conditions were used as published previously (22), with minor modifications. The PCR product was purified with chloroform, dried under vacuum, redissolved in 10 μl formamide and 10 μl H_2O and loaded onto a 6% polyacrylamide gel (Roth, Karlsruhe, Germany). Electrophoresis was performed at 150 V for 90 min. After staining with ethidium bromide for 10 min the amplification products were visualized on a UV screen. LOH was determined by comparing tumor DNA with a leukocyte DNA pattern. Reproducible results were obtained after repetition of each experiment at least twice.

Results

All tumors were informative for at least three markers tested. Five out of six carcinomas showed LOH of the centromeric markers D11S956 and two out of six revealed LOH of PYGM but none showed LOH of the intragenic markers D11S4939 and D11S4946 or the telomeric D11S987. LOH of D11S956 was detected in

Figure 1 LOH of the five polymorphic markers studied. ○ = retained heterozygosity, ● = LOH, □ = not informative. Patients 1–6 = adrenocortical carcinoma, 7–10 = cortisol-producing adenoma, 11–16 = aldosterone-producing adenoma, 17–19 = non-secreting adenoma.

two out of three, and LOH of PYGM in one out of three endocrine-inactive tumors respectively. None of the ten aldosterone- or cortisol-producing tumors showed LOH of any of the markers studied (Fig. 1).

MEN1 mRNA was expressed in roughly equal amounts (70–130%) in all tumors compared with normal adrenals ($100 \pm 7\%$, mean $\pm$ S.E.M.) with the exception of one carcinoma with LOH of the MEN1 locus showing a reduced expression of 66% and two adenomas showing an expression of 157% and 149% (Figs 2 and 3).

The mean of MEN1 mRNA expression was $109 \pm 11\%$ for the carcinomas, $131 \pm 10\%$ for the cortisol-producing adenomas, $113 \pm 13\%$ for aldosterone-producing adenomas and $111 \pm 3\%$ for non-producing adenomas compared with normal adrenals.

In contrast to MEN1 mRNA, PLC β 3 mRNA expression showed greater variability between tumor tissues (Figs 4 and 5). The carcinoma with LOH and decreased MEN1 mRNA levels showed non-detectable PLC β 3 mRNA expression, whereas the other carcinomas expressed PLC β 3 mRNA in amounts comparable to normal adrenal glands. Two aldosterone-producing adenomas without LOH of MEN-1 locus and with normal MEN-1 mRNA levels had decreased PLC β 3 mRNA expression of 51 and 53% respectively. Cortisol- and endocrine-inactive adenomas showed mRNA

concentrations comparable to that of normal adrenal tissue.

Discussion

For nearly a decade since the mapping of the MEN1 locus to 11q13 and the suggestion that it is a tumor-suppressor gene, efforts have been made to identify the gene responsible for this familial cancer syndrome. Recently, a positional cloning approach led to the identification of the MEN1 gene product, termed menin (3). The predicted 610 amino acid protein structure exhibited no apparent similarities to any previous known proteins. Immunofluorescence studies indicated that menin functions principally as a nuclear protein but may be found in the cytoplasm during cell division (23, 24). Further functional approaches revealed the transcription factor JunD as a direct menin-interacting partner (25).

Whereas the association of the MEN1 syndrome with adrenal tumors was reported to be a relatively uncommon feature with an incidence of adrenal tumors of 9.6% (26), other groups found adrenal lesions in 36–40% of MEN1 patients by computed tomography scan examination (1, 27, 28). Bilateral adrenal enlargement occurred in 21% of all patients. Pancreatic endocrine tumors with insulin–proinsulin excess were overrepresented in the

Figure 2 Northern blot after hybridization with MEN1 cDNA with a signal of 2.8 kb (upper panel) and a mouse β -actin cDNA for normalization (lower panel). Lanes 1–5 adrenocortical carcinoma, lanes 6–8 cortisol-producing adenoma. One carcinoma shows low MEN1 gene expression (66% compared with normal adrenal glands).

Figure 3 Northern blot after hybridization with PLC β 3 cDNA with signal at 4.4 kb (upper panel) and a mouse β -actin cDNA. Lanes 1–5 adrenocortical carcinoma, lanes 6 and 7 non-secreting adenomas. One carcinoma shows loss of PLC β 3 expression.

patients with adrenocortical involvement. Histopathology revealed diffuse and nodular cortical hyperplasia, adenomas, and rarely adrenocortical carcinomas (3–6% of MEN1 patients). The apparently benign adrenal enlargements were not associated with presently ascertainable biochemical disturbances in the hypothalamic–pituitary–adrenocortical axis, and they were without radiological signs of progression during follow-up. Whereas in this kindred, only the carcinomas were reported to exhibit adrenocortical hormone excess,

occasionally aldosterone-producing adenomas were reported to be coincident with MEN1 (2).

In a recently published manuscript, we investigated 31 sporadic adrenocortical tumors and two adrenocortical tumor cell lines for the presence of mutations of the MEN1 gene locus. LOH was found in five out of five carcinomas and two out of five non-secreting adenomas but no point mutations in the MEN1 gene-coding region could be detected by sequencing of the entire MEN1 gene (17). Kjellman *et al.* (29) and Görtz *et al.* (30)

Figure 4 Menin gene expression in the tumors, expressed as menin/ β -actin ratio (arbitrary units) and compared with normal adrenal glands as 100%.

Figure 5 PLCβ3 gene expression in the tumors, expressed as PLCβ3/β-actin ratio (arbitrary units) and compared with normal adrenal glands as 100%.

reported similar results with LOH of the 11q13 locus in eight out of eight adrenocortical carcinomas and two out of 14 adenomas, and nine out of 29 adrenocortical lesions respectively, but no point mutations in the MEN1 gene.

Nevertheless, if the MEN1 locus is an important tumor-suppressor gene in the adrenal gland, neoplasia may result from decreased gene expression due to epigenetic factors that could result in down-regulation of menin. Possible mechanisms leading to a decrease of MEN1 gene expression could include mutations affecting intronic sequences or the promoter region or splicing sites of the MEN1 gene. *In vivo*, such mutations have been detected in MEN1 patients by various groups. Therefore we investigated the expression of the MEN1 gene by Northern blot analysis after hybridization with specific cDNA probes.

One carcinoma with LOH of the flanking marker PYGM showed a slightly decreased MEN1 gene expression of 66%, whereas all other tumors had normal gene expression. Because the MEN1 gene is thought to be a tumor-suppressor gene following Knudson's two-hit model (31), a loss of gene expression of at least 50% would be expected, but none of our tumors showed loss to that degree, making mutations at the splice sides or in the promoter region unlikely. These data, together with the missing evidence of point mutations within the coding region (17, 29, 30), suggest that MEN1 gene mutations are not involved in tumorigenesis of sporadic

adrenocortical neoplasms, despite the high prevalence of LOH of 11q13.

In other sporadic endocrine tumors, mutations in the MEN1 gene are detected more frequently. Hessmann *et al.* (13) found LOH in 70% of malignant tumors of the endocrine pancreas with somatic mutations in three out of the 11 tumors tested. Zhuang *et al.* (14) detected LOH of 11q13 in 93% of sporadic gastrinomas and 50% of sporadic insulinomas with point mutations of the MEN1 locus in 33 and 17% respectively. In sporadic parathyroid tumors, LOH was found in 13 out of 45 tumors (6). Six of those tumors harbored mutations in the coding region of the MEN1 gene. Heppner *et al.* (5) reported LOH of 11q13 occurring in 13 out of 33 sporadic parathyroid tumors with mutations of the MEN1 gene in seven of the tumors tested. In sporadic pituitary adenomas, MEN1 mutations were detected less frequently: Tanaka *et al.* (9) found one case out of 31 pituitary adenomas with LOH of the MEN1 gene locus. This tumor also had a point mutation within the MEN1-coding region. Prezant *et al.* (10) detected one out of 45 adenomas with LOH of 11q13 without a MEN1 mutation, and Zhuang *et al.* detected four out of 39 tumors with LOH, two of them harboring a point mutation of the other allele (8).

Despite the fact that LOH of 11q13 was reported in a series of aldosterone-producing adenomas (15), none of the aldosterone- or cortisol-producing adenomas in our study showed comparable results. Our findings are in

accord with the results of Kjellman *et al.* (29) and Görtz *et al.* (30). Furthermore, Kjellman *et al.* (32) could not detect genetic alterations at 11q13 with comparative genomic hybridization in sporadic aldosteronomas. Taken together, these data make an involvement of the MEN1 gene in sporadic aldosterone- and cortisol-producing adenomas unlikely.

PLC β 3 plays an important role in initiating receptor-mediated signal transduction. Activation of PLC takes place in many cells as a response to stimulation by hormones, growth factors, and other ligands (33). Its functional role together with the mapping of the PLC β 3 locus to 11q13 made it a candidate gene for MEN1 (34), until further studies excluded mutations in this locus as the cause for tumorigenesis in MEN1 (35, 36). However, because of the involvement of PLC β 3 in signal transduction including the inositol-(1,4,5)-trisphosphate pathway, this gene is not excluded as being a tumor-suppressor gene in general. Loss of expression in a variety of non-familial endocrine tumors was reported for the PLC β 3 gene. In our series, the polymorphic marker located in the chromosomal region of the PLC β 3 gene and more centromerically to the MEN1 gene (18, 20) showed LOH in five out of six carcinomas and two out of three non-secreting adenomas. Major chromosomal alteration, as observed in adrenocortical carcinomas (29), raises the possibility of involvement of multiple gene loci and possibly different tumor-suppressor genes. Since the 11q13 locus is known to be a genetically unstable region, PLC β 3 seems to be a secondary event rather than a causative tumor-suppressor gene involved in the tumorigenesis of adrenal cancer. However, there is an increasing body of evidence for the existence of an additional tumor-suppressor gene at 11q13 based on findings of LOH of markers localized distally with retained heterozygosity at the MEN1 locus (37–39).

In conclusion, since MEN1 mRNA expression was normal and point mutations are excluded in all tumors tested, we conclude that the MEN1 gene does not play a major role in sporadic adrenocortical tumorigenesis. One carcinoma showed loss of expression of PLC β 3 mRNA and LOH at 11q13, so PLC β 3 might need further investigation.

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