

Adrenal Medullary Hyperplasia Is a Precursor Lesion for Pheochromocytoma in MEN2 Syndrome^{1,2}

Esther Korpershoek^{*,3}, Bart-Jeroen Petri^{*,†,3}, Edward Post^{*}, Casper H.J. van Eijck[†], Rogier A. Oldenburg[‡], Eric J.T. Belt[†], Wouter W. de Herder[§], Ronald R. de Krijger^{*} and Winand N.M. Dinjens^{*}

*Department of Pathology, Erasmus MC Cancer Institute, University Medical Center Rotterdam, Rotterdam, The Netherlands; [†]Department of Surgery, Erasmus MC Cancer Institute, University Medical Center Rotterdam, Rotterdam, The Netherlands; [‡]Department of Clinical Genetics, Erasmus MC Cancer Institute, University Medical Center Rotterdam, Rotterdam, The Netherlands; [§]Department of Internal Medicine, Erasmus MC Cancer Institute, University Medical Center Rotterdam, Rotterdam, The Netherlands

Abstract

Adrenal medullary hyperplasias (AMHs) are adrenal medullary proliferations with a size < 1 cm, while larger lesions are considered as pheochromocytoma (PCC). This arbitrary distinction has been proposed decades ago, although the biological relationship between AMH and PCC has never been investigated. Both lesions are frequently diagnosed in multiple endocrine neoplasia type 2 (MEN2) patients in whom they are considered as two unrelated clinical entities. In this study, we investigated the molecular relationship between AMH and PCC in MEN2 patients. Molecular aberrations of 19 AMHs and 13 PCCs from 18 MEN2 patients were determined by *rearranged during transfection (RET)* proto-oncogene mutation analysis and loss of heterozygosity (LOH) analysis for chromosomal regions 1p13, 1p36, 3p, and 3q, genomic areas covering commonly altered regions in RET-related PCC. Identical molecular aberrations were found in all AMHs and PCCs, at similar frequencies. LOH was seen for chromosomes 1p13 in 8 of 18 (44%), 1p36 in 9 of 15 (60%), 3p12-13 in 12 of 18 (67%), and 3q23-24 in 10 of 16 (63%) of AMHs, and for chromosome 1p13 in 13 of 13 (100%), 1p36 in 7 of 11 (64%), 3p12-13 in 4 of 11 (36%), and 3q23-24 in 11 of 12 (92%) of PCCs. Our results indicate that AMHs are not hyperplasias and, in clinical practice, should be regarded as PCCs, which has an impact on diagnosis and treatment of MEN2 patients. We therefore propose to replace the term AMH by micro-PCC to indicate adrenal medullary proliferations of less than 1 cm.

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Abbreviations: AMH, adrenal medullary hyperplasia; LOH, loss of heterozygosity; MEN2, multiple endocrine neoplasia type 2; PCC, pheochromocytoma; RET, rearranged during transfection proto-oncogene

Address all correspondence to: Winand N. M. Dinjens, PhD, Department of Pathology, Erasmus MC Cancer Institute, University Medical Center Rotterdam, P.O. Box 2040, 3000 CA Rotterdam, The Netherlands.

E-mail: w.dinjens@erasmusmc.nl

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³These authors contributed equally to this work.

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Introduction

Pheochromocytomas (PCCs) are tumors that originate from adrenal medulla chromaffin cells and occur with an incidence of 1 to 2 per 100,000. PCCs are generally benign and only 10% of cases is metastatic. The clinical impact of PCCs is far beyond the issue of malignancy since the majority of PCCs produce catecholamines, which can cause severe life-threatening situations such as myocardial infarctions and cerebrovascular accidents [1]. Up to 25% of PCCs occur in the context of hereditary cancer syndromes. These include multiple endocrine neoplasia type 2 (MEN2) caused by germ-line activating *rearranged during transfection (RET)* proto-oncogene mutations, von Hippel-Lindau disease due to inactivating *von Hippel-Lindau* gene mutations, neurofibromatosis type 1 as a result of *neurofibromatosis type 1* gene inactivation, and the PCC-paranglioma syndrome resulting from inactivation of the *succinate dehydrogenase-A, -B, -C, -D, or -AF2* genes [2–4]. Other genes that have recently been associated with inherited PCCs are *TMEM127, KIF1B, PHD2, MAX, HIF2a, and FH* [5–10]. In total, mutations in all genes described above account for more than 50% of the PCCs and paragangliomas, the latter being tumors of chromaffin cells that arise outside the adrenal gland and, if located in the thorax or abdomen, generally also produce catecholamines [11].

Most syndrome-related PCCs are associated with *RET* mutations, which cause the autosomal dominant MEN2 syndrome. Fifty percent of patients with MEN2 develop PCC, which in up to 80% of patients occur bilaterally. Half of the bilateral lesions are diagnosed synchronously, but contralateral PCC can occur after an interval of many years [12]. The *RET* proto-oncogene encodes a transmembrane receptor tyrosine kinase involved in regulation of cell proliferation and apoptosis. Activating mutations in *RET* transform this proto-oncogene into an oncogene with constitutive activation of the receptor as a result. The activating germ-line mutation in the *RET* gene in patients with MEN2 is a prerequisite though insufficient to develop PCC and additional somatic molecular aberrations have to occur in adrenal medullary cells to initiate PCC development [13,14].

Some patients with MEN2 present with adrenal medullary hyperplasia (AMH), which is considered as a benign generally non-catecholamine-overproducing lesion. In contrast, PCCs are usually characterized by catecholamine overproduction and are potentially malignant. The distinction between AMH and PCC is currently based on the diameter of the lesion: An AMH is defined as a nodular and/or diffuse lesion of the adrenal medulla with a size of maximally 1 cm in diameter, whereas a larger lesion is considered as a PCC [1,15,16]. This definition has been put forward more than three decades ago by Carney et al. [17] and has been adopted by the Armed Forces Institute of Pathology [18].

By definition, a hyperplasia is a non-neoplastic lesion with an increased number of normal cells in normal arrangement in an organ or tissue, while a PCC is a neoplastic lesion composed of cancer cells. Most AMH cases are found in known hereditary PCC syndrome patients as a result of surveillance. Non-hereditary AMH is extremely rare and generally only diagnosed after catecholaminergic symptoms mimicking PCC [1,19–21].

Early genetic aberrations that occur in sporadic as well as in hereditary PCC, especially in MEN2, include loss of chromosomal arms 1p (82–100%), 3p (24–31%), and 3q (41–52%) [22–24]. The high frequencies of these chromosomal aberrations indicate that they might be causally related to PCC tumorigenesis, but tumor suppressor genes on these chromosomes are yet still to be found. In addition, allelic imbalance of the *RET* gene, in favor of the mutant allele, has been described in

MEN2-related neoplasia [25]. Currently, PCC and AMH are considered as two different clinical entities and their distinction is based on the lesional size. However, molecular evidence for this clinical distinction of AMH and PCC is lacking since data on molecular aberrations in AMH have not been reported to date. In the present study, we investigated the biologic relationship between AMH and PCC by determining molecular abnormalities in these lesions. Hence, 32 adrenal lesions (19 AMHs and 13 PCCs) from 18 patients with MEN2 were investigated for loss of chromosomes 1p13, 1p36, 3p, and 3q by loss of heterozygosity (LOH) analysis using highly polymorphic microsatellite markers. In addition, *RET* gene allelic imbalance was investigated with the mutation as an allele-specific marker.

Material and Methods

Tumor Samples

For several decades, at the Erasmus MC, the therapy of choice for MEN2 patients with elevated catecholamine levels has been bilateral adrenalectomy. In the resected adrenals, nodular and/or diffuse medullary lesions of variable size were found. The generally used size criterion of 1 cm, put forward by Carney et al. [17] and described in the Armed Forces Institute of Pathology guidelines [18], was used in the differential diagnosis of AMH and PCC.

Thirty-two histologically proven adrenal medullary lesions were investigated, comprising 19 AMHs and 13 PCCs, from 18 patients from 10 different MEN2 families, all with a known germ-line *RET* gene mutation (Table 1). Of the 18 patients, 14 underwent bilateral and 4 underwent unilateral adrenalectomy. All adrenal tumors were benign and were collected between 1982 and 2005. The routine formalin-fixed and paraffin-embedded tissue specimens were retrieved from the files of the Department of Pathology, Erasmus MC, following approval of the experimental design and protocols by the Institutional Medical Ethics Committee.

From each case, lesional and normal DNA was isolated from formalin-fixed and paraffin-embedded tissues by manual microdissection of tissue fragments comprising more than 80% lesional cells and 100% normal cells, respectively (Supplementary Figure 1). The isolated tissue fragments were digested by standard detergent-proteinase K treatment and DNA

Table 1. Clinical and Tumor Data of 32 Tumors from 18 Patients with MEN2.

| Pt | Family | M/F | m/b | Age | Mutation RET | L (cm) | R (cm) | MTC | PHP |
|----|--------|-----|-----|-----|--------------|--------|--------|-----|-----|
| 1 | A | F | b | 20 | pCys 634 Arg | 7 | 0.4 | + | - |
| 2 | A | M | b | 55 | pCys 634 Arg | 0.7 | - | + | + |
| 3 | B | F | b | 32 | pCys 634 Arg | - | 0.7 | + | + |
| 4 | B | M | b | 42 | pCys 634 Arg | 0.6 | 0.4 | + | + |
| 5 | B | F | b | 18 | pCys 634 Arg | 0.2 | 0.7 | + | + |
| 6 | B | F | b | 24 | pCys 634 Arg | 0.9 | 0.7 | + | - |
| 7 | B | F | b | 50 | pCys 620 Arg | <1 | <1 | + | - |
| 8 | C | F | b | 52 | pCys 634 Arg | 2 | 0.8 | + | - |
| 9 | C | F | b | 38 | pCys 634 Arg | 4 | 1.6 | + | + |
| 10 | C | F | b | 38 | pCys 634 Arg | 0.8 | - | + | - |
| 11 | C | F | b | 51 | pCys 634 Arg | 0.9 | 4 | + | + |
| 12 | D | F | b | 29 | pCys 634 Arg | 4 | 0.8 | + | + |
| 13 | E | F | b | 29 | pCys 634 Arg | 5 | 3 | + | - |
| 14 | F | M | b | 26 | pCys 611 Tyr | 0.8 | 3 | + | + |
| 15 | G | M | b | 23 | pMet 918 Thr | 2.5 | 0.6 | + | - |
| 16 | H | F | b | 51 | pCys 634 Arg | - | 1.5 | + | - |
| 17 | I | F | b | 34 | pCys 634 Arg | 0.2 | 0.8 | + | - |
| 18 | J | F | b | 25 | pCys 634 Arg | 4.2 | 2 | + | + |

Pt, patient; M/F, male/female; m/b, malignant/benign; Age, years; L, tumor size in left adrenal gland; R, tumor size in right adrenal gland; MTC, medullary thyroid carcinoma; PHP, primary hyperparathyroidism.

Table 2. Microsatellite Markers, Chromosomal Localization, Primer Sequences, and Approximate Product Sizes of the Eight Markers in Four Regions.

| | Marker | Localization | Forward 5'→3' | Reverse 5'→3' | Size (bp) |
|---|---------|--------------|--------------------------|--------------------------|-----------|
| 1 | D1S252 | 1p13.1 | AGCTTTTACTCTTAACCTATTCAT | TCATTAATACACATGTTCTCTGC | 107 |
| 2 | D1S2881 | 1p13.2 | ATCTGCCAGCCCATGA | CAAACAGAGCCTGGCA | 188 |
| 3 | D1S2885 | 1p36.11 | ATCTGCCAGCCCATGA | CAAACAGAGCCTGGCA | 127 |
| 4 | D1S234 | 1p36.11 | GCCCAGGAGGTTGAGG | CTGTAATTCAAGCCTGCCTT | 142 |
| 5 | D3S3681 | 3p12.3 | GTGAGAACCATTGGGGCAG | CCCTGACAGATAGCTCGCC | 226 |
| 6 | D3S3551 | 3p13 | AACGGAGTCCCACATAAA | CTTTGTCTGTATTGCTAATGAACC | 142 |
| 7 | D3S3694 | 3q23 | AGTGTCCATCAACATGGG | GAATCCTGTTATTTGTGCGA | 162 |
| 8 | D3S1569 | 3q24 | GCACCTTGGCTTACCTTCTA | CTTTAAGAACCTTCAACTGTCC | 234 |

was obtained after phenol/chloroform extraction and ethanol precipitation. Relevant characteristics and clinical data are summarized in Table 1.

LOH Analysis

All DNA samples were analyzed for allelic imbalances of chromosomes 1p13, 1p36, 3p, and 3q using highly polymorphic microsatellite markers, as summarized in Table 2. Polymerase chain reaction (PCR) amplification of paired lesional and normal DNA was performed in reaction mixtures of 15 µl. Each reaction contained 50 to 100 ng of template DNA, 0.02 mM dATP, 0.2 mM dTTP, dGTP, dCTP each, 0.8 µCi α32P-dATP, 20 pmol of each primer, 1.5 mM MgCl₂, 10 mM Tris-HCl, 50 mM KCl, and 1 unit of Taq DNA polymerase (Amplitaq Gold; PerkinElmer, Norwalk, CT). An initial denaturation step at 94°C for 5 minutes was followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 60 seconds, and extension at 72°C for 60 seconds. A final extension step was carried out at 72°C for 10 minutes. PCR products of tumor and normal DNA from each patient were diluted 1:1 in 10 µl of loading buffer (95% formamide, 20 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue) and loaded onto a denaturing 6% polyacrylamide gel. Electrophoresis was carried out at 60 W for 90 minutes. The gels were dried and exposed to X-ray film overnight at -80°C. Results were scored by two independent investigators. In addition, part of the LOH markers was investigated by PCR with fluorescence-labeled primers (Invitrogen, Paisley, United Kingdom) for 28 cycles with an annealing temperature of 60°C, and amplified products were analyzed, along with LIZ 500 size standard (Applied Biosystems, Foster City, CA), using capillary electrophoresis on an ABI 3130-XL genetic analyzer (Applied Biosystems).

RET Gene Allelic Imbalance Analysis

From eight PCCs and eight AMHs, tumor DNA was investigated for *RET* gene allelic imbalance by PCR amplification of tumor and matched normal DNA fragments containing the *RET* mutation. The PCR products were bidirectionally cycle sequenced on an ABI 3130-XL genetic analyzer (Applied Biosystems). Data were analyzed using GeneMarker software (SoftGenetics LLC, State College, PA).

Results

Nineteen AMHs and 13 PCCs from 18 patients with MEN2 were investigated for LOH on chromosomal regions 1p13, 1p36, 3p12-13, and 3q23-24. In all cases, there was sufficient DNA to perform LOH analysis on the four different loci with two highly polymorphic markers per locus. Representative LOH and retention of heterozygosity results are summarized in Tables 3 and 4. In each of the investigated 19 AMHs and 13 PCCs, at least one marker demonstrated unequivocal LOH indicating that the DNA was retrieved from a high percentage of lesional cells. LOH (of at least one marker per locus) in the AMH cases was found for 1p13 in 8 of 18 (44%), for 1p36 in 9 of 15 (60%), for

3p12-13 in 12 of 18 (67%), and for 3q23-24 in 10 of 16 (63%) of the informative cases. In the PCCs, LOH was detected at 1p13 in 13 of 13 (100%), 1p36 in 7 of 11 (64%), 3p12-13 in 4 of 11 (36%), and 3q23-24 in 11 of 12 (92%) of informative tumors. From 14 patients, bilateral lesions were investigated, and in 12 of these cases, the paired proliferations demonstrated different LOH profiles (example illustrated in Figure 1, *left panel*; case 14 different LOH pattern in the bilateral lesions). A total of 128 loci was investigated (4 loci in 32 samples), and only from 14 loci (1p13 in case 14 L; 1p36 in case 4 L, 4R, 8 L, 8R, 9 L, and 14 L; 3p12-13 in case 1 L, 4R, and 8 L; 3q23-24 in case 1 L, 1R, 17 L, and 17R), no information on possible LOH could be obtained. The lack of LOH information from both markers in each of these 14 loci was due to the fact that the markers were non-informative or no data were obtained.

RET gene allelic imbalance was investigated in 16 tumors by sequencing of the germ-line *RET* mutation. In one patient (case 9), allelic imbalance, in favor of the mutant *RET* allele, was found in the bilateral PCCs (Figure 1, *right panel*).

Discussion

AMHs and PCCs are adrenal medullary non-neoplastic and neoplastic proliferations, respectively. Both lesions are frequently found in patients with MEN2, considered unrelated and discriminated by lesional size (smaller or ≥1 cm, respectively). Because AMHs generally do not produce catecholamines whereas PCCs do, and PCCs can progress to malignancy, treatment of AMH and PCC is different. As a result, PCCs are almost always surgically resected, whereas AMHs are usually only subjected to follow-up. The diagnostic distinction between AMH and PCC solely on the basis of the size of the lesion is biologically doubtful and has been questioned by several researchers [15,16,26]. To obtain more information on the possible molecular relationship between these lesions, we investigated MEN2-related AMHs and PCCs.

Genetic abnormalities in PCC have been studied extensively over the last decades, although understanding of the molecular pathogenesis of this tumor is still limited. DNA-based analyses, including LOH and comparative genomic hybridization, have identified several distinct chromosomal regions frequently lost in PCC, including chromosomes 1p, 3p, and 3q [22–24,27,28]. In particular, loss of 1p has been suggested to be an early event and occurs at a frequency of 82% to 86% of PCC. Although chromosomal regions of minimal overlapping loss have been described at 1p13 and 1p36, relevant tumor suppressor genes have not been detected to date, which is also the case for regions on chromosomes 3p and 3q [27–29]. In the present study, MEN2-related PCCs and nodular and/or diffuse AMHs were screened for the presence of chromosomal aberrations frequently found in MEN2-related and sporadic PCCs. Frequent LOH at 1p13, 1p36, 3p12, and 3q24 was found both in the 19 AMH and 13 PCC cases. In addition, amplification of the mutated *RET* allele was found in the AMH and contralateral PCC of one patient (case 12), a molecular aberration described exclusively in PCCs [25].

Table 3. Results of LOH Analyses.

| Pt | Family | Left/ Right | Size (cm) | Tumor | 1p13.1 | 1p13.2 | 1p36.11 | 1p36.11 | 3p12.3 | 3p13 | 3q23 | 3q24 |
|----|--------|----------------|--------------|------------------|--------|---------|---------|---------|---------|---------|---------|---------|
| | | | | | D1S252 | D1S2881 | D1S2885 | D1S234 | D3S3681 | D3S3551 | D3S3694 | D3S1569 |
| 1 | A | L | 7.0 | PCC | ■↓ | x | | x | NI | x | NI | x |
| | | R | 0.4 | AMH ^N | | x | | x | NI | ■↑ | NI | x |
| 2 | A | L | 0.7 | AMH ^D | ■↑ | x | ■↑ | | ■↓ | ■↑ | ■↓ | ■↑ |
| 3 | B | R | 0.7 | AMH ^N | | | ■↓ | | ■↓ | | ■↓ | |
| 4 | B | L | 0.6 | AMH ^M | ■↑ | x | x | x | x | ■↑ | x | ■↑ |
| | | R | 0.4 | AMH ^N | ■↑ | x | x | x | x | x | x | |
| 5 | B | L | 0.2 | AMH ^D | | NI | | | ■↑ | ■↑ | ■↓ | ■↑ |
| | | R | 0.7 | AMH ^D | | NI | | | ■↑ | ■↑ | | |
| 6 | B | L | 0.9 | AMH ^D | | ■↓ | ■↓ | ■↓ | ■↓ | ■↓ | NI | |
| | | R | 0.7 | AMH ^D | ■↓ | ■↓ | | ■↓ | ■↓ | ■↓ | NI | |
| 7 | B | L | u | AMH ^N | | | | | ■↑ | ■↑ | | |
| | | R | u | AMH ^N | | | | | ■↑ | ■↑ | | |
| 8 | C | L | 2.0 | PCC | ■↑ | ■↑ | x | x | x | x | NI | ■↓ |
| | | R | 0.8 | AMH ^D | | ■↑ | x | x | | ■↓ | NI | ■↑ |
| 9 | C | L | 4.0 | PCC | ■↓ | | x | NI | | ■↓ | ■↑ | ■↓ |
| | | R | 1.6 | PCC | ■↓ | | ■↓ | NI | | ■↑ | ■↓ | ■↓ |
| 10 | C | L | 0.8 | AMH ^N | | NI | ■↓ | | | ■↓ | ■↑ | |
| 11 | C | L | 0.9 | AMH ^N | ■↑ | | NI | NI | | | ■↓ | ■↓ |
| | | R | 4.0 | PCC | ■↓ | | NI | NI | | | ■↓ | ■↑ |
| 12 | D | L | 4.0 | PCC | ■↑ | x | ■↑ | ■↓ | | ■↑ | ■↓ | ■↓ |
| | | R | 0.8 | AMH ^N | | x | | ■↓ | | | ■↓ | x |
| 13 | E | L | 5.0 | PCC | NI | ■↓ | x | ■↑ | NI | | ■↓ | ■↓ |
| | | R | 3.0 | PCC | NI | ■↓ | ■↓ | ■↑ | NI | ■↑ | ■↑ | ■↑ |
| 14 | F | L | 0.8 | AMH ^M | NI | x | x | x | NI | ■↑ | x | ■↓ |
| | | R | 3.0 | PCC | NI | ■↑ | x | | NI | ■↑ | ■↓ | ■↑ |
| 15 | G | L | 2.5 | PCC | ■↓ | ■↑ | | | | | ■↑ | ■↑ |
| | | R | 0.6 | AMH ^M | ■↑ | ■↓ | | ■↑ | | | ■↓ | ■↓ |
| 16 | H | R | 1.5 | PCC | | ■↓ | ■↑ | ■↑ | | NI | NI | |
| 17 | I | L | 0.2 | AMH ^N | | NI | x | ■↓ | | NI | NI | NI |
| | | R | 0.8 | AMH ^M | | NI | ■↓ | ■↓ | | NI | NI | NI |
| 18 | J | L | 4.2 | PCC | ■↓ | ■↑ | NI | ■↓ | | | ■↑ | NI |
| | | R | 2.0 | PCC | ■↑ | ■↓ | NI | ■↑ | | | ■↓ | NI |

Pt, patient; L, left adrenal gland; R, right adrenal gland; histologic growth patterns: N, nodular; D, diffuse; M, mixed nodular and diffuse. ■↑, Loss of upper allele; ■↓, loss of lower allele; NI, not informative; x, no data; u, unknown.

These results strongly suggest a molecular relationship between AMH and PCC. In 12 of 14 bilateral cases, the genomic aberrations were different in paired lesions indicating that the bilateral lesions are independent entities. The observed LOH frequencies in PCCs are comparable to several published series [22–24,27,28]. The percentage of loss of 1p and 3q is slightly less in the AMH series than in the PCC, which may be related to the accumulation of genetic abnormalities during tumor progression in the latter group. However, the differences are small and they may also be related to chance, which may be the case for the difference observed for loss of 3p between AMH (67%) and PCCs (36%).

The current therapy of choice for unilateral catecholamine-producing PCC patients, including MEN2 patients, is total unilateral adrenalectomy. In case a contralateral PCC occurs later in life, a cortex-sparing adrenalectomy is preferred. If a patient presents with synchronous bilateral PCC, the larger PCC will be resected by total adrenalectomy and the smaller will be treated by cortex-sparing adrenalectomy. In general, unilateral cortex-sparing adrenalectomies are preferred, because of the increased recurrence risk with cortex-

sparing surgery, while preventing morbidity and mortality due to adrenocortical insufficiency, occurring in patients with bilateral adrenalectomy [12,30–32]. Our results indicate that surgical treatment in patients with MEN2 may need to be more “aggressive”. In addition to surgical resection of PCCs, AMHs should be considered for surgical removal as well. Such an approach may have the advantage of allowing cortex-sparing surgery, with less chance of recurrence compared to cortex-sparing surgery in PCC, and may also lead to a lower risk of developing bilateral PCC than thus far reported [30]. Another advantage of early surgery for AMH is the fact that they are often asymptomatic, which gives a lower chance for pre-operative and intra-operative hypertensive crises.

In this study, we have shown that all MEN2-related AMH lesions, nodular and/or diffuse, have genomic alterations identical to MEN2-associated PCCs, at similar frequencies. Therefore, AMHs should be regarded as PCC precursor lesions and not as non-neoplastic hyperplasias. This is in line with other studies in which the value of the size-based distinction between AMHs and PCCs has been questioned and even considered as an academic difference rather than a relevant clinical distinction [15,16,26].

As a consequence of these findings, we propose that the size criterion for the distinction between AMH and PCC should be abolished in patients with MEN2, and we recommend to replace the term AMH by micro-PCC for nodular and/or diffuse adrenal medullary lesions of less than 1 cm. In addition, treatment by cortex-sparing adrenalectomy should be considered in case of a synchronous or metachronous contralateral micro-PCC.

Table 4. Overall LOH Results: AMHs versus PCCs.

| Locus | AMH (%) | PCC (%) |
|-------|---------|---------|
| 1p13 | 44 | 100 |
| 1p36 | 60 | 64 |
| 3p | 67 | 36 |
| 3q | 63 | 92 |

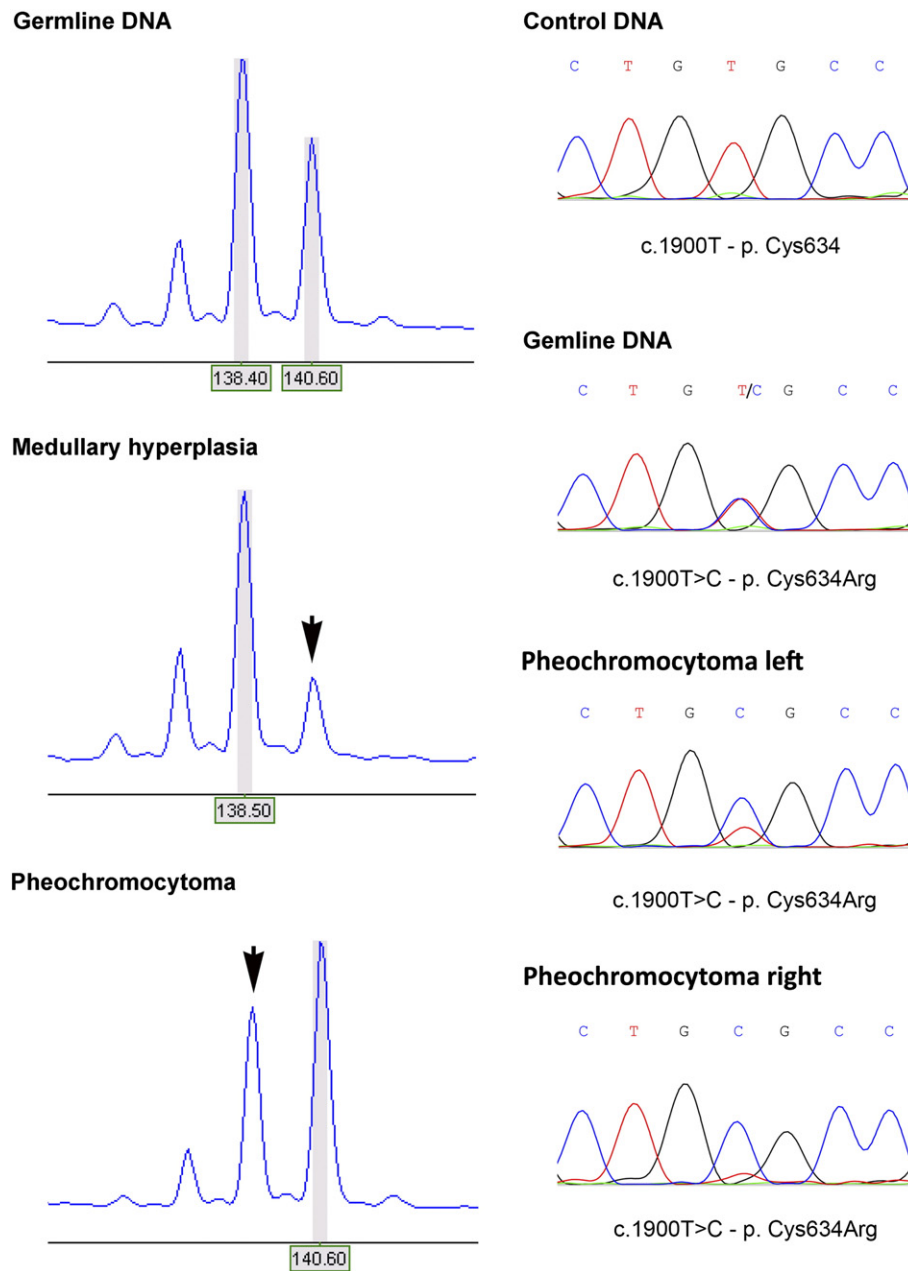


Figure 1. Left panel displays the LOH patterns of germ-line DNA (upper), PCC DNA (middle), and AMH DNA (lower) of case 14, for chromosome 3q24. Loss is indicated by the arrow. Note that the PCC and AMH show different LOH patterns. Right panel shows the sequences of a healthy control DNA (control) and the germ-line DNA, PCC DNA, and AMH DNA of case 9. Germ-line DNA displays the p. Cys634Arg, T>C mutation in an equal ratio, whereas both PCC DNAs show a higher signal of the mutation (C) compared to the wild-type signal (T), indicating amplification of the mutant allele.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.neo.2014.09.002>.

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