Next-generation panel sequencing identifies *NF1* germline mutations in three patients with pheochromocytoma but no clinical diagnosis of neurofibromatosis type 1

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Abstract

Objective: Our objective was to improve molecular diagnostics in patients with hereditary pheochromocytoma and paraganglioma (PPGL) by using next-generation sequencing (NGS) multi-gene panel analysis. Derived from this study, we here present three cases that were diagnosed with *NF1* germline mutations but did not have a prior clinical diagnosis of neurofibromatosis type 1 (NF1).

Design: We performed genetic analysis of known tumor predisposition genes, including *NF1*, using a multi-gene NGS enrichment-based panel applied to a total of 1029 PPGL patients. We did not exclude genes known to cause clinically defined syndromes such as NF1 based on missing phenotypic expression as is commonly practiced.

Methods: Genetic analysis was performed using NGS (TruSight Cancer Panel/customized panel by Illumina) for analyzing patients' blood and tumor samples. Validation was carried out by Sanger sequencing.

Results: Within our cohort, three patients, who were identified to carry pathogenic *NF1* germline mutations, attracted attention, since none of the patients had a clinical suspicion of NF1 and one of them was initially suspected to have MEN2A syndrome due to co-occurrence of a medullary thyroid carcinoma. In these cases, one splice site, one stop and one frameshift mutation in *NF1* were identified.

Conclusions: Since phenotypical presentation of NF1 is highly variable, we suggest analysis of the *NF1* gene also in PPGL patients who do not meet diagnostic NF1 criteria. Co-occurrence of medullary thyroid carcinoma and PPGL was found to be a clinical decoy in NF1 diagnostics. These observations underline the value of multi-gene panel NGS for PPGL patients.

European Journal of
Endocrinology
(2018) 178, K1–K9

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Introduction

Pheochromocytomas and paragangliomas (PPGL) are rare tumors derived from the adrenal medulla or extra-adrenal sympathetic and parasympathetic ganglia (1). Most of the tumors occur sporadically, but PPGLs can also be caused by genetic predisposition in association with hereditary syndromes such as multiple endocrine neoplasia type 2 (MEN2), von Hippel–Lindau disease (VHL) and neurofibromatosis type 1 (NF1) (1, 2). Furthermore, hereditary PPGLs have been identified as independent paraganglioma syndromes 1–5 caused by germline mutations in succinate dehydrogenase (*SDH*) genes (*SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*) (3, 4). Rare germline mutations of the transmembrane encoding *TMEM127* gene and MYC-associated factor X (*MAX*) gene as well as the Krebs cycle-related genes *MDH2*, *FH*, *IDH1* and *PHD2* (4, 5, 6, 7, 8, 9) were also found to cause hereditary PPGL. It is now generally accepted that at least 30% of all PPGLs are caused by underlying germline mutations (1, 10). In children and adolescents with PPGL, germline mutations are present in up to 80% of cases (11, 12).

NF1 (OMIM # 162200) (von Recklinghausen's disease) is a clinically well-characterized autosomal dominant disorder occurring in 1 per 3500 individuals (13). The condition is caused by loss-of-function mutations in *NF1*, a tumor suppressor gene located on chromosome 17q11.2 (13, 14, 15). About 50% of patients have *de novo* spontaneous mutations that, if postzygotic, can give rise to a mosaic phenotype (16). Sequencing *NF1*-related tumors frequently reveals alterations of both alleles, usually one germline mutation and one acquired mutation or LOH of the wild-type allele (1, 13, 17).

Although there is a high variability in phenotypic expression of NF1 even in patients with the same mutation, the clinical diagnosis is routinely based on internationally valid diagnostic criteria (18, 19). Pheochromocytomas occur in approximately 5–7% of NF1 patients, representing a considerably higher annual incidence than in the general population (2–8/million people) (14, 20). Vice versa, somatic mutations in NF1 have been reported as the most common genetic lesion in PPGLs, with *NF1* mutations being identified in up to 20–25% of sporadic pheochromocytomas (21, 22, 23). However, occurrence of PPGL is not included in the diagnostic criteria for NF1 and as a result, it is common practice to exclude NF1 as a differential diagnosis for patients with PPGL, who do not meet clinical NF1 criteria (18, 24).

Almost all pheochromocytomas that develop due to an underlying *NF1* germline mutation produce

metanephrine and normetanephrine (25). NF1 patients with pheochromocytomas are not at increased risk of malignant transformation compared to those without a known genetic cause (12% vs 10%) (3, 14), but in 14% of cases bilateral pheochromocytomas are observed. Average age of onset has been reported to be 42 years (1).

Here, we report on three cases of pathogenic germline *NF1* mutations identified in patients with pheochromocytomas who did not have a prior diagnosis of NF1. In all three cases, family history was described as inconspicuous with neither pheochromocytoma nor NF1-related symptoms reported in any of the family members. The patients were identified within a cohort of 1029 PPGL patients who received genetic screening by targeted NGS. In one of these patients, no signs of NF1 could be detected even by retrospective clinical examination. In the second case, retrospective examination revealed subtle signs of NF1 that initially had not been noted. Co-occurrence of a medullary thyroid carcinoma (MTC) had, however, led to the strong suspicion of an underlying MEN2A syndrome, side-tracking from the diagnosis of NF1. The third patient was unavailable for a retrospective clinical examination, but according to her physicians, no signs of NF1 were present in the patient or her family.

Subjects and methods

Clinical report patient 1

The female patient was first diagnosed with a pheochromocytoma of the right adrenal gland at 39 years of age and consequently adrenalectomy was performed in 1994. In 2008, at the age of 53 years, disease recurred on the right side and examinations additionally revealed a synchronous pheochromocytoma of the left adrenal gland, as was histologically and immunohistochemically confirmed in the course of treatment. The patient presented with episodes of palpitations, sweating and flushes and had been diagnosed with hypertension. A detailed clinical description of the patient was published in 2011 (26). Initial biochemical testing showed mildly elevated urinary outputs of metanephrine at 384 µg/day (upper cutoff: 236 µg/day) and normetanephrine at 904 µg/day (upper cutoff: 605 µg/day) as well as moderately elevated plasma concentrations of metanephrine (170 pg/mL) (upper cutoff: 84 pg/mL) and normetanephrine (594 pg/mL) (age-specific upper cutoff: 166 pg/mL), but

normal plasma catecholamines. There was no evidence of metastasis. At this point the patient was first introduced to genetic counseling.

Clinical assessment revealed multiple soft, partly pedunculated fibromas (fibroma molle) at the patients back and upper abdomen as well as few hemangiomas present mainly at the patients legs, each measuring only a few millimeters in diameter. Furthermore, the patient had macrocephaly with a head circumference of 58.5 cm (>97 percentile, +2.35z). Since the presence of skin lesions and macrocephaly are indicative of phacomatosis syndromes such as Cowden syndrome or NF1, dermatologic and ophthalmologic assessment regarding symptoms of the neurofibromatosis spectrum as well as biopsy and histologic evaluation of the cutaneous fibromas were initiated. However, the patient did not exhibit café-au-lait spots, axillary or inguinal freckling or Lisch nodules. Neither did she show optic glioma or any neurofibromas.

Family history revealed no other relatives with PPGs or other tumors associated with NF1 or Cowden syndrome. The only other family member with cancer was a niece, who had developed melanoma at the age of 30 years. No skin lesions similar to those of the index patient were reported in the family. The patient also stated that she had two healthy daughters and four healthy grandchildren (Fig. 1A). However, so far none of these family members was available for clinical examination regarding NF1-related features.

Importantly, besides macrocephaly and multiple pheochromocytomas, the patient did not show any other tumors or characteristics related to NF1 at the age of 53 years. The patient also did not report NF1-associated symptoms in any other of her family members. Since she did not fulfill any of the clinical diagnostic criteria for NF1, this syndrome was excluded as a differential diagnosis and therefore the *NF1* gene was initially not tested in 2011 (18, 26).

Clinical report: patient 2

A second, male patient was incidentally diagnosed with biochemically positive bilateral pheochromocytomas at 56 years of age. He did not report any palpitations, flushes, episodes of sweating or headache or any other symptoms associated with catecholamine excess except for high blood pressure. Biochemical testing revealed elevated urinary outputs of metanephrine at 926 µg/day (upper cutoff: 320 µg/day), normetanephrine at 3188 µg/day (upper cutoff: 390 µg/day), epinephrine at 124 µg/day

(upper cutoff : 27 µg/day), norepinephrine at 823 µg/day (upper cutoff: 97 µg/day) and dopamine at 653 µg/day (upper cutoff: 500 µg/day). The patient's past medical history included a prostatic adenocarcinoma that had been treated by radical prostatectomy two years earlier. In the course of diagnostics, a sonography revealed a thyroid nodule that was confirmed to be a MTC. Family history revealed that the patient's father had died from thyroid cancer at the age of 55 years (Fig. 1B). There was no information available, however, about the histologic type of thyroid cancer. Based on these clinical findings, MEN2A syndrome was suspected, and clinical examination was limited to the relevant aspects of this disease.

However, after the diagnosis of NF1 was made on a molecular level, a careful examination by a medical geneticist revealed 6 café-au-lait spots, mainly located on the patient's chest and in the gluteal region, with the largest one measuring 6×2 cm. He was further diagnosed with axillary and inguinal freckling and Lisch nodules. Two lesions on the patient's arm and popliteal fossa, which were primarily diagnosed as fibromata molle, were histologically re-categorized as neurofibromas. Furthermore, the patient had macrocephaly with a head circumference of 60 cm (>97 percentile, +1.98z). An axial MRI scan showed intra-arachnoidal neurofibromas in segments T8 to L1 as well as extra-arachnoidal neurofibromas located in segments T11 to L1. A cranial MRI did not show any specific signs of NF1.

Clinical report patient 3

A third, female patient was diagnosed with hypertension up to 240/150 mmHg at 22 years of age. An abdominal ultrasound was performed, showing a tumor of the left adrenal that was classified as a pheochromocytoma by CT and MIBG scans. While high plasma concentrations of normetanephrine (4567 pg/mL) (upper age-specific cutoff: 112 pg/mL), metanephrine (532 pg/mL) (upper cutoff: 84 pg/mL) and methoxytyramine (164 pg/mL) (upper cutoff: 16 pg/mL) were detected, the patient did not show any further evidence of catecholamine excess, except for high blood pressure. Molecular diagnosis of NF1 was established by panel sequencing. Consequent re-evaluation of the patients' records, including both her past medical history and information on family history, did not reveal any NF1-related symptoms. The only notable feature was the patient's short body height of 150 cm. The patient did not, however, consent to further medical examinations regarding subtle NF1 symptoms.

Figure 1

Pedigrees of the families of patient 1 (A) and patient 2 (B). (A) According to patient 1, none of her family members suffered from any symptoms related to NF1. There was no further information available on the father's family side. (B) Note the patient's father who also suffered from medullary thyroid carcinoma.

Molecular analyses

Informed consent for has been obtained from each patient prior to genetic diagnostics. Stepwise routine diagnostic Sanger sequencing of *SDHA*, *SDHB*, *SDHC*, *SDHD*, *MAX*, *TMEM127*, *RET*, *VHL* and *PTEN* supplemented by MLPA (multiplex-ligation assay) was performed using the first patient's blood between 2011 and 2014. When NGS technology became available for routine use, we analyzed DNA isolated from paraffin-embedded tumor tissue of the recurrent pheochromocytoma of the right adrenal gland (patient 1) using the TruSight Cancer gene panel (Illumina, San Diego, CA, USA) targeting the coding exons of 94 genes relevant in hereditary cancer syndromes, as described earlier (27, 28, 29).

The second patient was suspected to have MEN2A syndrome, based on the rare co-occurrence of bilateral pheochromocytomas and MTC and therefore the initial molecular diagnostics was done by Sanger sequencing of the *RET* gene using DNA derived from the pheochromocytoma. Since no mutation could be detected, DNA derived from the thyroid carcinoma was analyzed with regards to somatic mutations in *RET*, *NRAS*, *KRAS* and *MERTK*. When again the analysis did not confirm a mutation, a panel-based NGS approach, using

a customized panel including all PPGL-associated genes, was applied to the pheochromocytoma-derived DNA (30).

With no clinical suspicion of NF1, the third patient was initially tested for mutations by Sanger sequencing as well and in this case only the *SDH*, *RET* and *VHL* genes were targeted. When no mutation could be detected, panel sequencing was initiated due to the early age of PPGL onset and large tumor size (6×5×6 cm) in this patient. Validation of the identified *NF1* mutations was performed by Sanger sequencing. DNA from all three patients' blood samples.

Results

Patient 1

Using NGS a pathogenic splice-site mutation in *NF1*: NM_000267.3:c.6084+1G>A was revealed in a homozygous state in pheochromocytoma tumor tissue (Fig. 2A). The same mutation had already been described as being pathogenic by De Luca and coworkers (31). The variant was proven to be present in the patient's blood in heterozygous state and validated in homozygous state in tumor tissue by Sanger sequencing (Fig. 2B and C). Other

Figure 2

Patient 1: (A) NGS data generated by sequencing tumor-derived DNA. The homozygous splice-site mutation c.6084+1G>A is marked in gray. Forward reads are displayed in green, backward reads are displayed in red. (B) Sanger sequencing confirmed the mutation to be present in heterozygous state in blood and (C) in a homozygous state in the tumor. Patient 2: (D) NGS data generated by sequencing tumor-derived DNA. The heterozygous deletion c.7207_7207del is marked in gray. (E) Sanger sequencing confirmed the mutation to be present in heterozygous state in blood. (F) While sequencing data did not indicate LOH in tumor tissue, a SNP array performed from tumor-derived DNA indicated loss of the long arm of chromosome 17 (17q-), including the *NF1* gene located on 17q11.2. Patient 3: (G) Sequencing data generated from blood-derived DNA from patient 3. The heterozygous nonsense mutation c.7846C>T is marked in green. (H) The sequencing results were validated by Sanger sequencing.

variants identified on chromosome 17 were also found in a homozygous state in tumor tissue. Therefore, loss of heterozygosity in *NF1* was ascertained to be the underlying mechanism of pathogenesis in this patient's tumor. In line with the framework for variant interpretation in PPGL outlined in the Consensus Statement on NGS-based diagnostic testing of PPGL, the variant was classified as a class 5 pathogenic mutation (10).

Patient 2

Since Sanger sequencing of *RET*, *HRAS*, *KRAS* and *MERTK* using MTC-derived DNA did not identify any pathogenic mutations, a panel-based NGS approach was used for analyzing DNA derived from the pheochromocytoma. By this, a heterozygous pathogenic germline mutation was revealed in *NF1*: NM_000267.3: c.7206_7207del (p.His2402Glnfs*4) (Fig. 2D). In this mutation, a deletion of two bases leads to a frameshift and a subsequent truncation of the protein, thereby impeding protein

function. While the variant has never been reported in literature, it is not listed in the ExAc database (<http://exac.broadinstitute.org/>), which comprises over 66 000 healthy individuals. In the ClinVar database (<https://www.ncbi.nlm.nih.gov/clinvar/>), the variant is listed as a pathogenic mutation. With regards to the truncating mutation mechanism in the middle of the protein (exon 29 of 58) the variant was therefore also regarded as a class 5 pathogenic mutation. The mutation could also be verified to be present in the germline by Sanger sequencing (Fig. 2E) and was further validated in heterozygous state in the MTC as well. While sequencing data did not show any evidence of a loss of heterozygosity or a somatic, compound heterozygous variant in *NF1* in both tumors, a SNP-array performed from pheochromocytoma-derived DNA indicated possible monoallelic loss of the q-arm of chromosome 17, where *NF1* is located (Fig. 2F). This discrepancy might be explained by contamination of tumor tissue with normal cells.

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Patient 3

Analyzing DNA extracted from the patient's blood and applying the NGS approach revealed a heterozygous nonsense mutation in *NF1*: NM_000267.3:c.7846C>T, p.Arg2616* (Fig. 2G). In this case, transition from C to T at position 7846 leads to preliminary chain termination at codon 2612 within exon 45. The variant has been described before in a patient with typical symptoms of NF1 including cutaneous involvement and plexiform neurofibromas (32) and was therefore also classified as class 5 pathogenic variant. The variant was validated by Sanger sequencing using blood-derived DNA (Fig. 2H). In this case, no tumor tissue was available for genetic testing.

Discussion

NF1 is one of the largest genes, comprising 360kb and 63 exons. Consequently, sequencing *NF1* has long been a laborious and expensive task (13). For this reason, diagnosis of NF1 is commonly based on clinical criteria only and does not routinely include validation on a molecular basis. To date, healthy individuals from families with known NF1 have not routinely been sequenced for *NF1* mutations. The same is true for individuals with PPGL, as they typically do not get tested for *NF1* mutations if clinical criteria for NF1 are not met (24, 33). Today, however, with cost-effective NGS-sequencing methods at our hands, *NF1* can easily be included in routine molecular diagnostics for patients with PPGL. We thereby identified pathogenic *NF1* mutations in three PPGL patients who would otherwise not have caught our attention as being suspicious for NF1.

We therefore propose to include the *NF1* gene into routine diagnostics for PPGL patients even if there are no clinical symptoms suggestive of NF1. Analyzing tumor-derived DNA further broadens the spectrum of diagnostic possibilities providing the opportunity to also diagnose mutations in a mosaic state, which could ultimately lead to a milder phenotype. These propositions are in line with the recommendations summarized in the Consensus Statement on NGS-based diagnostic testing of PPGLs, where targeted gene panels are characterized as the gold standard in the diagnostics of this highly heterogeneous disease (10).

Our second patient showed typical but subtle clinical signs of NF1 in retrospective clinical examination. A similar case was reported in 2006, where the patient also had Lisch nodules, café-au-lait spots and axillary freckling, which were not recognized by several physicians. Comparable

to our case the clinical diagnosis of NF1 was not made until genetic testing revealed a pathogenic *NF1* mutation (34). It seems, therefore, that routine clinical examination performed in the context of PPGL diagnostics does not suffice NF1 detection and occurrence of PPGL should prompt a specific and detailed clinical examination for subtle signs of this syndrome.

In our patient, the co-occurrence of a MTC additionally constituted a clinical decoy for MEN2A syndrome. This is of special interest since, to the best of our knowledge, the co-occurrence of pheochromocytoma, MTC and hyperparathyroidism in NF1 patients has been reported only three times in the literature (35, 36, 37). To exclude a somatic mutation in *RET* as an independent cause for MTC development in this patient, analysis of tumor-derived DNA for *RET* mutations was performed. Since no somatic *RET* mutation was detected and with this being the fourth patient described with a mutation in *NF1* and occurrence of pheochromocytomas as well as MTC, we propose NF1 should be considered in pheochromocytoma patients showing the full spectrum of MEN2A syndrome if no mutation can be identified in the *RET* gene. However, it cannot be excluded that the occurrence of an MTC in patient 2 was a sporadic event independent of the NF1 germline mutation.

Although we could identify the underlying *NF1* germline mutations in the PPGL patients described here using NGS, it is nevertheless important to consider the limitations of the technique (38). Current panel sequencing is limited to the coding exons and adjacent intronic regions of target regions. Therefore splice-site mutations can be detected while deep intronic variants may be missed. Additionally, larger genomic rearrangements (translocations, deletions and inversions) cannot robustly be detected by NGS. Also, while the approach in general is better at detecting mosaicism than Sanger sequencing, low-grade mosaicism may still be missed.

NF1 is known to be phenotypically heterogeneous even in patients carrying the very same mutation. Considering that we identified an *NF1* germline mutation in a patient who did not fulfill the diagnostic NF1 criteria, as well as in a patient in whom the NF1 symptoms were overlooked during initial clinical examination, it seems possible that the condition has long been underdiagnosed. Using NGS panel diagnostics for PPGL patients might reveal that a higher percentage of these tumors than originally presumed in the literature are due to *NF1* germline mutations. This assumption is supported by the fact that somatic *NF1* mutations have frequently been identified in PPGL (39). Vice versa, it has lately

been proposed that occurrence of PPGLs in NF1 patients might also have been underestimated, since in the past, biochemical testing was only recommended if patients developed hypertension. With increasing recognition of normotensive cases of the tumor in NF1 (40, 41) and findings of pheochromocytomas in 13% of patients with NF1 at autopsy (14, 35), it is now being advocated that patients with NF1 mutations should be screened for PPGLs at least once every three years (35, 40, 41). In line with these findings of asymptomatic or inconspicuous PPGL in NF1 patients, both our second and third patient did not report any symptoms of catecholamine excess except for hypertension (40). The genotype–phenotype correlation in our patients is consistent with earlier reports of PPGL in *NF1* mutation carriers where pheochromocytomas producing metanephrine and normetanephrine were predominantly observed (25). Therefore, surveillance measurements for PPGL in NF1 patients could be planned in accordance with these findings, specifically targeting catecholamine producing PPGL. It is noteworthy that, while it has been reported that the majority of NF1-related pheochromocytomas occur unilaterally, two of our patients had bilateral, and in the case of patient 1, even recurrent pheochromocytomas (35, 40). This, again, emphasizes the broad phenotypic spectrum of NF1 underlining the need to consider NF1 as a differential diagnosis both in unilateral and bilateral pheochromocytomas. We recommended close follow-up examinations for our patients with regard to PPGL as well as surveillance for other NF1-related symptoms. Additionally, we offered predictive *NF1* mutation testing to healthy relatives. So far, however, none of the relatives consented to genetic testing.

In summary, identifying pathogenic *NF1* mutations in three PPGL patients unsuspecting for NF1 led us to conclude that ideally sequencing of *NF1* should be included in routine PPGL diagnostics. With NGS currently advancing to be the predominant diagnostic approach for PPGL (42), inclusion of the *NF1* gene in targeted sequencing and analytic algorithms seems to be a reasonable approach. We additionally suggest sequencing of tumor tissue since mosaicism might not be detected in blood samples. Additionally, we recommend thorough examination specifically for NF1 symptoms in all patients with pheochromocytomas by an experienced geneticist. The three cases presented here show the importance of keeping an open mind even if clinical stigmata do not provide the full clinical evidence of NF1 and, in addition, the importance of being wary of clinical decoys (e.g. MTC). Knowledge of the underlying NF1 mutations prompted us

to adjust preventive measures for our patients and their family members and to offer predictive testing to healthy relatives.

Declaration of interests

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this case report.

Funding

This work was supported by the Deutsche Forschungsgemeinschaft (grant numbers RI 2684/1-1; KL 2541/2-1) and the Fondo de Investigaciones Sanitarias (project PI14/00240), co-financed by FEDER.

Acknowledgements

The authors would like to thank the patients and their families for participating in this work. They further thank Alexander Krüger and Eva Gerlach for excellent technical support.

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Received 28 August 2017

Revised version received 13 November 2017

Accepted 20 November 2017