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Original article

Investigation of gene dosage imbalances in patients with Noonan syndrome using multiplex ligation-dependent probe amplification analysis

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ABSTRACT

The RAS-MAPK syndromes are a group of clinically and genetically related disorders caused by dysregulation of the RAS-MAPK pathway. A member of this group of disorders, Noonan syndrome (NS), is associated with several different genes within the RAS-MAPK pathway. To date, mutations in *PTPN11*, *SOS1*, *KRAS*, *RAF1* and *SHOC2* are known to cause NS and a small group of patients harbour mutations in *BRAF*, *MEK1* or *NRAS*. The majority of the mutations are predicted to cause an up-regulation of the pathway; hence they are gain-of-function mutations. Despite recent advances in gene identification in NS, the genetic aetiology is still unknown in about ¼ of patients.

To investigate the contribution of gene dosage imbalances of RAS-MAPK-related genes to the pathogenesis of NS, a multiplex ligation-dependent probe amplification (MLPA) assay was developed. Two probe sets were designed for seven RAS-MAPK-syndrome-related candidate genes: *PTPN11*, *SOS1*, *RAF1*, *KRAS*, *BRAF*, *MEK1* and *MEK2*. The probe sets were validated in 15 healthy control individuals and in glioma tumour cell lines. Subsequently, 44 NS patients negative for mutations in known NS-associated genes were screened using the two probe sets. The MLPA results for the patients revealed no gene dosage imbalances. In conclusion, the present results exclude copy number variation of *PTPN11*, *SOS1*, *RAF1*, *KRAS*, *BRAF*, *MEK1* and *MEK2* as a common pathogenic mechanism of NS. The validated and optimised RAS-MAPK probe sets presented here enable rapid high throughput screening of further patients with RAS-MAPK syndromes.

1. Introduction

The autosomal dominant disorders Noonan syndrome (NS), cardiofacio-cutaneous (CFC), Costello and LEOPARD syndromes, Neurofibromatosis type 1 and Neurofibromatosis type 1-like syndrome/Legius syndrome display overlapping clinical features and are commonly denoted as RAS-MAPK syndromes [29]. NS (OMIM 163950) is characterized by cardiac defect, short stature and facial features such as hypertelorism, down-slanting palpebral fissures, and short/webbed neck [1,29]. The RAS-MAPK syndromes are clinically variable, e.g. NS is associated with skeletal defects, failure to thrive and cryptorchidism among other features [1].

These syndromes share a common pathogenesis, dysregulation of the RAS-MAPK pathway [29], a pathway that has previously been mainly associated with cancer [26]. Today, several genes within the pathway have been identified in the genetic aetiology of the RAS-MAPK syndromes [29]. NS is genetically heterogeneous, where mutations in *PTPN11* are the cause in about 50% of patients. Of the remaining NS cases, approximately 13% are associated with *SOS1*, another 3–17% with *RAF1*, less than 2% are caused by mutations in *KRAS* and less than 5% have mutations in *SHOC2* [5,29]. In addition, a few patients harbouring mutations in *BRAF* (~2%), in *MEK1* and recently in *NRAS* have also been described [6,15,17,20,24].

In CFC, the major gene is *BRAF* (75%), followed by *MEK1*, *MEK2* and *KRAS* [29]. Also, mutations in the *SOS1* gene have been reported in a few CFC patients [14,17]. LEOPARD syndrome is caused by mutations in *PTPN11* and *RAF1* [29]. Other components of the RAS-MAPK pathway are involved in the other RAS-MAPK syndromes, such as *HRAS*, which causes Costello syndrome, *NF1* which is responsible for Neurofibromatosis type 1 and *SPRED1*, which is associated with the newly identified disorder Legius syndrome [29]. The majority of the mutations involved in the RAS-MAPK syndromes increase RAS-MAPK signalling. Likewise, the somatic mutations identified in several of the genes in this pathway are also gain-of-function mutations that generally are more potent than the constitutional mutations [26,29].

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Despite the recent advances in gene identification in these syndromes, the aetiology is still unknown in about 15–30% of NS patients [5]. Because there are important phenotypic differences among the RAS-MAPK syndromes, such as an increased risk for malignancy in Costello syndrome, Neurofibromatosis type 1 and to a lesser extent NS and a more pronounced mental retardation in CFC and Costello, further molecular investigations are required to facilitate the diagnosis of remaining patients. Gene dosage imbalances of the RAS-MAPK genes have recently been identified both in cancer [8,9,18] and in the RAS-MAPK syndromes where large duplications involving the *PTPN11* gene have hitherto been found in two patients with an NS phenotype [7,28]. The genetic aetiology behind NS in these patients suggested that it could be caused by gene dosage imbalances in a minority of patients [7,28]. In addition, approximately 5–10% of patients affected by Neurofibromatosis type 1 are associated with whole-gene deletions of *NF1* [10,12]. Therefore, to assess the possibility of copy number variations as a pathogenic mechanism associated with RAS-MAPK syndromes, the *PTPN11*, *SOS1*, *KRAS*, *BRAF*, *RAF1*, *MEK1* and *MEK2* genes were investigated using an in-house designed MLPA assay in a mutation-negative cohort consisting of 44 NS patients.

2. Patients and methods

2.1. Patients

The clinical investigations and genetic analyses were performed according to the guidelines in the Declaration of Helsinki and were approved by the ethics committee at Uppsala University.

The study included 44 individuals with a clinical diagnosis of NS. Of the 44 patients, 17 were referred to the Uppsala University Hospital in Sweden and were clinically re-evaluated as described previously [17]. The additional 27 patients were recruited from Catholic University Leuven, in Belgium and were examined by EL. All patients were negative in a mutational screening of *PTPN11* (exons 2, 3, 4, 7, 8, 12, 13), *SOS1* (all exons), *RAF1* (all exons), *KRAS* (all exons), *BRAF* (exons 6, 11, 12, 14, 15), *MEK1* (exons 2, 3), *MEK2* (exons 2, 3, 7) and *SHOC2* (for the S2G mutation [5]). In addition, the 17 Swedish patients were screened for mutations in remaining exons of *PTPN11*, *BRAF*, *MEK1* and *MEK2* (as previously described [17] and unpublished results) with a negative result.

2.2. Methods

Genomic DNA was extracted from peripheral blood leukocytes from patients and from 15 healthy control individuals using standard procedures. A pool of genomic DNA from female blood donors was obtained from Promega (cat.no. G1521). DNA from two glioma cell lines (2987MG and 251MG) [19] was extracted using standard procedures.

2.2.1. MLPA

Two different synthetic probe sets (Table 1) were designed according to the recommendations by MRC-Holland (http://www.mlpa.com/). Probe set 1 targets *PTPN11* (NM_002834), *RAF1* (NM_002880) and *SOS1* (NM_005633) with two probes for each gene and *KRAS* (NM_004985) with one probe. Probe set 2 targets *BRAF* (NM_004333), *MEK1* (NM_002755) and *MEK2* (NM_030662) with two probes for each gene and *KRAS* with one probe. Five control probes targeting the genes *PXDN*, *SIP1*, *GCH1* and *MYT1L* were used in each probe set. The synthetic probe sets were used with the reagents of the MLPA EK-kit (MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer's instructions. In the MLPA reactions, 100 ng genomic DNA was used. The MLPA reaction was performed as previously described [12,25]. The PCR products were analysed using an ABI 3130XL Genetic Analyzer (Applied Biosystems). As internal size standard ROX-500 (ABI, Warrington, UK) was used.

2.2.2. MLPA data analysis

Data analysis was performed using GeneMarker v 1.75 (SoftGenetics, USA). Peak height was used in the analysis and threshold values were set at <0.75 for deletions and >1.25 for duplications. In all normalization analyses, five samples from a pool of genomic DNA from female blood donors were used. To determine the quality of the run of each sample, the ratio of the five internal control probes was used, where a ratio within 0.8 and 1.2 was accepted.

3. Results

To investigate the presence of gene dosage imbalances in patients with NS, a synthetic MLPA strategy was developed for analysing seven RAS-MAPK-syndrome-related genes. Two probe sets were designed, probe sets 1 and 2, targeting *PTPN11*, *SOS1*, *KRAS*, *RAF1*, *BRAF*, *MEK1* and *MEK2*. Variable performance of the smallest probe in a probe set has been reported, and inclusion of a pilot probe in the probe set, shorter than the smallest probe of interest, has been recommended [2]. Therefore pilot probes were included in each of the present probe sets. Initially, both probe sets were validated by analysing 15 control individuals (Fig. 1) to evaluate variability and reliability of the probes. In the validation experiments, the average and the standard deviation (SD) were calculated for each normalized probe. The probe was not considered reliable if SD $>$ 10%. This validation revealed that all probes were reliable, thus had a SD less than 10% (Fig. 1). The validation experiment also included two glioma cell lines with gains of *RAF1* (251MG) and *BRAF* (2987MG) (detected by Affymetrix 250K Nsp SNParray, data not shown). The results of the MLPA analysis showed that the gains of *BRAF* and *RAF1* were reliably detected by the probes targeting these genes in the probe sets (Fig. 2). The cell line 2987MG also contained a gain encompassing *MEK2* in some cells as detected by the array analysis. This mosaicism regarding the *MEK2* gain was also visible using the MLPA probe sets and as expected the average ratio was lower than for the gains of *BRAF* and *RAF1* (Fig. 2).

DNA from the 44 patients were analysed in duplicate using the two probe sets for the seven RAS-MAPK-syndrome-related genes. The ratios of the five internal control probes were within the acceptable range of all the runs, hence the results were considered reliable. To reduce the possibility of false-negative results in the analysis a threshold of <0.75 and >1.25 was used for deletions and duplications, respectively [3]. The MLPA results revealed no gene dosage imbalance of any of the examined genes in the 44 patients.

4. Discussion

Despite the recent advances in gene identification in NS, the genetic aetiology is still unknown in a substantial number of patients. Thus further investigations are justified. The association with dysregulation of the RAS-MAPK pathway has raised the question of whether additional genes located in this pathway or closely associated with the pathway might play a role in the pathogenesis of the NS patients with unknown aetiology. However, recent reports concerning screening of *MRAS*, *HRAS*, *DUSP6*, *CSK*, *PTPN6*, *PAG1*, *SOS2*, *ARAF*, *ERK1*, *ERK2* and functional domains of *NF1* [4,13,16,20,21,23,27] have hitherto not identified any mutations within these genes. Here, we present the development of an MLPA assay to investigate the contribution of gene dosage imbalances of seven RAS-MAPK-syndrome-related genes to the pathogenesis of RAS-MAPK syndromes. During the recent years, copy number variations in the human genome have been shown to contribute to the phenotypic diversity among humans as well as to