bjh short report

Germ-line mutation of the *NRAS* gene may be responsible for the development of juvenile myelomonocytic leukaemia

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Summary

We report the case of a child with clinical and haematological features indicative of juvenile myelomonocytic leukaemia (JMML). The patient showed dysmorphic features: high forehead, bilateral epicanthal folds, long eyebrows, low nasal bridge and slightly low-set ears. A 38G>A (G13D) mutation in exon 1 of the *NRAS* gene was first demonstrated on peripheral blood cells, and then confirmed on granulocyte-macrophage colony-forming units. The same mutation was also found in buccal swab, hair bulbs, endothelial cells, skin fibroblasts. This case suggests for the first time that constitutional mutations of *NRAS* may be responsible for development of a myeloproliferative/myelodysplastic disorder in children.

Keywords: JMML, RAS, PTPN11, Noonan.

Juvenile myelomonocytic leukaemia (JMML) is a rare, clonal myeloproliferative/myelodysplastic disorder of infancy, accounting for 2–3% of haematological malignancies of childhood and characterised by overproduction of myeloid-monocytic cells that infiltrate haematopoietic and non-haematopoietic tissues (Emanuel *et al*, 1996; Niemeyer *et al*, 1997). JMML patients present with monocytosis, often with dysplastic features, leucocytosis, anaemia and thrombocytopenia; liver and, in particular, spleen enlargement, as well as lymph-node and skin involvement, are common findings. The clinical course of the disease is generally extremely aggressive, the median survival time of patients who are not given allogeneic hematopoietic stem cell transplantation (HSCT) being less than 1 year (Niemeyer *et al*, 1997). Indeed, although occasional long-term survivors without transplantation have been reported (Niemeyer *et al*, 1997; Matsuda *et al*, 2007) allogeneic HSCT is the only accepted curative therapy for this disease (Locatelli *et al*, 2005).

Myeloid progenitors of patients with JMML show a characteristic hypersensitivity to granulocyte-macrophage colony stimulating factor (GM-CSF) (Emanuel *et al*, 1991, 1996). This hypersensitivity is due to abnormalities of the

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RAS-RAF-MAP (mitogen-activated protein) kinase signalling pathway, which is pathologically activated by mutations of *RAS*, *NF1* and *PTPN11* genes. In detail, approximately 30–35% of children affected by JMML show somatic mutations in *PTPN11*, 25–30% in either *NRAS* or *KRAS*, another 10–15% of children having a clinical diagnosis of neurofibromatosis type 1 (Flotho *et al*, 2007). The mutations involving these genes are, usually, mutually exclusive.

We report the case of a child with haematological and clinical features suggestive of JMML in whom a heterozygous germ-line Gly13Asp activating-mutation of *NRAS* was present; to the best of our knowledge this is the second reported case of a *NRAS* germ-line mutation.

Case report and methods

A male infant developed fever and marked hepato-splenomegaly at 2 months of age. JMML was diagnosed at 7 months of age, according to the European Working Group on Myelodysplastic Syndrome in Childhood (EWOG-MDS) criteria (Niemeyer *et al*, 1998), based on the presence of leucocytosis (31×10^9/l), absolute monocytosis ($4{\cdot}8 \times 10^9$/l), modest thrombocytopenia (130×10^9/l), less than 20% bone marrow (BM) blasts, spontaneous growth of granulocyte-macrophage colony-forming units (CFU-GM) from peripheral blood (PB), and presence of a 38G>A (G13D) mutation in exon 1 of *NRAS*. Myeloid and erythroid precursors were evident on PB smear. The search for bcr/abl fusion transcript was negative, and the karyotype on BM and PB cells was normal (46, XY). A search for locating a suitable unrelated donor was started and no cytotoxic therapy was administered.

At the last evaluation, when the patient was 37 months old, the haematological and clinical situation was stable, with absolute leucocyte, monocyte and platelet counts of 19×10^9/l, $1{\cdot}7 \times 10^9$/l and 170×10^9/l, respectively. At that time, hepato-splenomegaly persisted; the patient weighed 13·9 kg (25–50th centile), height was 88 cm (5–10th centile), while head circumference was 51 cm (75–90th centile). Both parents showed height at the 25–50th centile and head circumference above the 75th centile.

In addition to short stature and relative macrocephaly, the patient showed some dysmorphic features (not present in the parents) including a high forehead, bilateral epicanthal folds, long eyebrows, low nasal bridge, slightly low-set ears and two *cafè-au-lait* spots. Echocardiography showed normal findings in repeated controls. Overall General Quotient (G.Q. = 97·6), as tested through the Griffith Development Scales, was within normal limits, with lower scores on performance tasks: locomotor 126; personal-social 114; hearing and language 84; hand coordination 84; performance 68.

PB mononuclear cells (PBMC) were isolated by Ficoll-hypaque (Lymphoprep, Nycomed Pharma AS, Oslo, Norway) density gradient centrifugation by standard method. Some of the PBMCs were used to generate an Epstein-Barr Virus-induced B-lymphoblastoid cell line (B-LCL), phytohaemagglutinin (PHA)-activated T-lymphoblastoid cells and endothelial cells according to previously reported methods (Comoli *et al*, 2002).

Erythroid burst-forming units (BFU-E) and CFU-GM derived colonies were grown in a classical clonogenic assay as previously reported (Massa *et al*, 2005). Long term culture initiating cells (LTC-IC) assays were performed as previously described (Rocci *et al*, 2007). DNA was prepared using QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany), according to manufacturer's instructions, from the following sources: PBMC, buccal swab, hair bulbs, B-LCL, PHA-activated T-lymphoblastoid cells, BFU-E, CFU-GM, LTC-IC, endothelial cells and cultured skin fibroblasts.

Results and discussion

The patient showed clinical and haematological findings indicative of JMML; the 38G>A (G13D) mutation in exon 1 of *NRAS* was first demonstrated in PBMC and then confirmed in CFU-GM derived colonies. Mutation analysis was extended to *PTPN11* (exons 3 and 13) (Tartaglia *et al*, 2005), and *KRAS* (exons 1 and 2) (Jongmans *et al*, 2005) with normal results.

The presence of dysmorphic features prompted us to extend mutation analysis to the above-mentioned cell types. All DNA sources examined consistently showed the presence of the 38G>A mutation (Fig 1A, B). Clonal subcultures from cultured fibroblasts were started from 1 to 5 cells and all the six clones examined demonstrated the presence of the mutation. The polymerase chain reaction (PCR) product of exon 1 from the original fibroblast culture was then cloned in bacterial TOP10 competent cells (TA Cloning® kit; Invitrogen, Carlsbad, CA, USA); both wild type and mutated sequences were observed (Fig 1C, D). These results clearly demonstrated that the 38G>A mutation was a germ-line (constitutional) mutation found in all patient's cells examined and occurred *de novo*, as it was not evidenced in the parents.

Acquired somatic mutations in *PTPN11*, *NRAS*, *KRAS* and *NF1* are associated with the development of JMML. Patients with neurofibromatosis type 1 are prone to develop JMML if a subsequent somatic inactivation of the remaining normal allele leads to homozygous inactivation of both *NF1* alleles. Some patients with Noonan Syndrome (NS), a heterogenous disorder defined by short stature, facial dysmorphia, cardiac defects, skeletal defects, mental retardation and bleeding diathesis (van der Burgt, 2007), carry constitutional mutations of *PTPN11* or *KRAS*, as well as of other genes, including *SOS1* and *RAF1*. JMML or a JMML-like clinical picture showing spontaneous remission, have been observed in NS (van der Burgt, 2007; Flotho *et al*, 2007).

No germ-line mutation had been described for *NRAS* until Oliveira *et al* (2007) described a 49-year-old male with a clinical diagnosis of autoimmune lymphoproliferative syndrome (ALPS) carrying a heterozygous, germ-line, G13D activating mutation in *NRAS*. The patient showed a peculiar defect in cytokine withdrawal-induced apoptosis due the

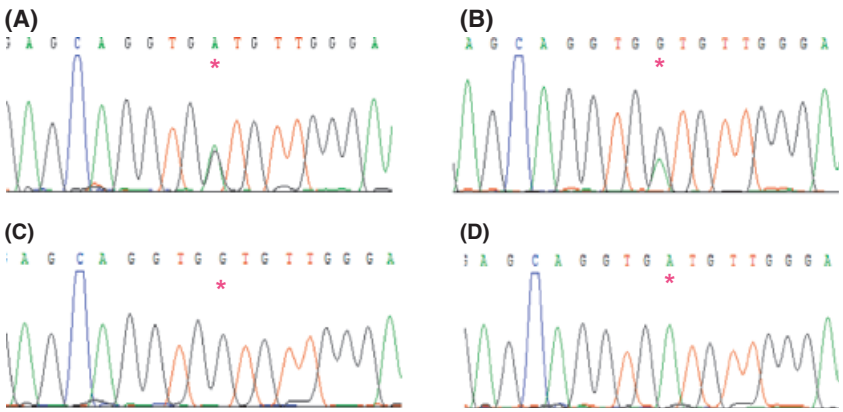

Fig 1. Electropherogram showing the 38G>A mutation found in: (A) PBMC sample and (B) clonal subculture from cultured fibroblasts. Panels (C) and (D) show the sequence obtained after cloning the PCR product of exon 1 from the original fibroblast culture in bacterial TOP10 competent cells [(C) wild type sequence; (D) mutated sequence].

NRAS mutation, but no data as to the presence of dysmorphic features or as to abnormalities of neurological development were provided. So far, our patient, who carries the same mutation, has not shown any haematological alterations related to ALPS, and the study of Fas-mediated apoptosis gave normal results (data not shown).

Despite some minor but clearly evident dysmorphisms, our child did not fit the criteria included in the most recent scoring system for NS (van der Burgt, 2007). Moreover, it is noteworthy that, while constitutional mutations of *KRAS* have been reported in NS patients, no patient was found to carry germ-line mutations of *NRAS*.

The constitutional mutation of *NRAS* found in our patient was already reported to occur as a somatic mutation in children with confirmed diagnosis of JMML (Matsuda *et al*, 2007; Flotho *et al*, 2008). We cannot exclude that some of these patients apparently carrying the somatic mutation may have a germ-line one. In any case, our patient provides evidence that germ-line mutations may occur also for *NRAS*, and that they may be associated with different haematological disorders in children and adults. Our case also emphasises the concept that an accurate search for dysmorphic features should be performed in any patient showing clinical and haematological features suggestive of JMML, as an indication of the presence of constitutional mutations.

Our patient has so far benefited from a stable haematological and clinical situation for more than 2 years after diagnosis. This could be explained by the fact that he was diagnosed below the age of 2 years, a variable associated with a less aggressive course (Niemeyer *et al*, 1997) and a better chance of benefiting from allogeneic HSCT (Locatelli *et al*, 2005). Some authors also suggested that patients with specific *RAS* mutations may have spontaneously improving disease (Matsuda *et al*, 2007), although other studies contradicted this hypothesis (Flotho *et al*, 2008). We cannot exclude that also germ-line mutations of *NRAS per se* may be associated with a less aggressive course. If true, the intriguing question of why the same mutation, either germ-line or somatically acquired, is associated with different degrees of clinical severity, remains open and should be addressed in further studies.

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