Short Report

Germline mutation in \textit{BRAF} codon 600 is compatible with human development: \textit{de novo} p.V600G mutation identified in a patient with CFC syndrome


\textit{BRAF}, the protein product of \textit{BRAF}, is a serine/threonine protein kinase and one of the direct downstream effectors of Ras. Somatic mutations in \textit{BRAF} occur in numerous human cancers, whereas germline \textit{BRAF} mutations cause cardio-facio-cutaneous (CFC) syndrome. One recurrent somatic mutation, p.V600E, is frequently found in several tumor types, such as melanoma, papillary thyroid carcinoma, colon cancer, and ovarian cancer. However, a germline mutation affecting codon 600 has never been described. Here, we present a patient with CFC syndrome and a \textit{de novo} germline mutation involving codon 600 of \textit{BRAF}, thus providing the first evidence that a pathogenic germline mutation involving this critical codon is not only compatible with development but can also cause the CFC phenotype. \textit{In vitro} functional analysis shows that this mutation, which replaces a valine with a glycine at codon 600 (p.V600G), leads to increased ERK and ELK phosphorylation compared to wild-type \textit{BRAF} but is less strongly activating than the cancer-associated p.V600E mutation.

Conflict of interest

None.

Raf is a serine and threonine protein kinase and mediates the mitogen-activated protein kinase (MAPK) pathway, which is one of the many direct downstream cascades of Ras, a small G-protein [for review see (1)]. \textit{BRAF} is a well studied oncoprotein with somatic mutations in \textit{BRAF} occurring in approximately 8% of all human cancers (2). Somatic mutations in \textit{BRAF} have been reported at a high frequency in melanoma and thyroid cancer and at a lower frequency in colorectal and ovarian cancer. The majority of somatic \textit{BRAF} mutations result in missense substitutions found in, but not limited to, the glycine-rich loop and the activation segment.
p.V600G mutation in CFC syndrome

Germline BRAF mutations have been identified as one of the causes of cardio-facio-cutaneous (CFC) syndrome. CFC syndrome is one of the RASopathies and is caused by dysregulation of activation in the MAPK pathway [for review see (3)]. Heterozygous mutations in four of the known genes that encode proteins of the Ras/MAPK pathway have been associated with CFC syndrome: BRAF (4, 5), MEK1 and MEK2 (4) and KRAS (5). The phenotypic features associated with CFC syndrome include characteristic facies, short stature, and cardiac, ectodermal, gastrointestinal, ocular and musculoskeletal abnormalities [for review see (6)]. Variable neurologic and cognitive abnormalities are universally present and include hypotonia, motor delay, speech delay and/or learning disability (7). To date, neither the common cancer-associated BRAF p.V600E mutation nor any other amino acid substitution of the BRAF V600 codon has been reported in an individual with CFC syndrome.

Here, we present a young female with CFC syndrome who harbors a de novo germline mutation involving codon 600 of BRAF. Functional analysis of the novel p.V600G missense mutation shows that this mutation is activating in vitro, albeit to a lesser degree than the cancer-associated p.V600E mutation.

Materials and methods

Clinical report

A Caucasian female was seen for clinical genetic evaluation due to a history of developmental delay. She was conceived via in vitro fertilization by a healthy 33-year-old G2P0 mother and a healthy 32-year-old father. Following an uncomplicated pregnancy and delivery at 33 weeks due to preterm labor, she stayed in the neonatal intensive care unit for 3 weeks for feeding difficulties. Her medical history was notable for gastroesophageal reflux, spherocytic anemia that resolved, truncal hypotonia, joint hypermobility, sinus infections, allergies, recurrent otitis media, bilateral exotropia, eczema, and slow-growing hair requiring only occasional haircuts. Her developmental milestones were globally delayed, and she required Early Intervention as well as speech, occupational, and physical therapies. She had a normal EEG and cranial MRI. Psychoeducational evaluation revealed cognitive abilities in the low average range. The family history was non-contributory.

Physical examination (Fig. 1) revealed weight and height below the 5th centile, head circumference at the 25th–50th centile, a tall forehead with bitemporal narrowing, sparse and curly scalp hair, very sparse eyebrows, pale blue irides, mild exotropia, slightly overfolded superior ear helices, broad and depressed nasal root and bridge, coarse facies, slight depression of the mid-lower sternum, flat and thin toenails, dry skin with hyperkeratotic
Champion et al.

papules, several pigmented nevi, and hypotonia with associated joint laxity. The neck was neither broad nor webbed, and no abnormalities of the spine, heart, abdomen, genitalia, or extremities were appreciated. An electrocardiogram was normal, and an echocardiogram revealed a patent foramen ovale with a trivial left to right shunt seen only by color doppler. An abdominal ultrasound showed mild renal asymmetry. A high resolution blood chromosome analysis, subtelomere analysis by multiplex ligation-dependent probe amplification (MLPA), and sequencing of selected exons of PTPN11 (exons 2–4, 7, 8, and 13) were all normal.

Sequencing and analysis

Sequencing of the genes known to cause CFC syndrome was performed for the proband as part of a research protocol approved by the Institutional Review Board of Self Regional Healthcare (Greenwood, SC). Parental informed consent was obtained for the studies. Subsequent parental testing was performed on a clinical diagnostic basis. Genomic DNA extracted from the proband’s peripheral blood leukocytes (FlexiGene DNA kit; Qiagen, Valencia, CA) was used to amplify all coding exons and intronic flanking regions of BRAF (NM_004333.2), MEK1 (NM_002755.2), MEK2 (NM_030662.2) and KRAS (NM_004985.3; NM_033360.2) (Supporting Table S1). Genomic DNA extracted from the proband’s buccal epithelial cells and the parents’ peripheral blood leukocytes was used for targeted analysis of BRAF exon 15. Bidirectional sequencing was performed using the Big Dye Terminator Cycle Sequencing Kit v3.1 on a 3730 automated sequencer (Applied Biosystems, Foster City, CA). Data were analyzed using Sequencher® 4.5 DNA sequence assembly software (Gene Codes Corporation, Ann Arbor, MI). Functional analysis of detected sequence alterations was performed using two different programs: Sorting Intolerant From Tolerant (SIFT; http://blocks.fhcrc.org/sift/SIFT.html) and PMUT (http://mmb2.pcb.ub.es:8080/PMut/), which are web-based tools for the annotation of pathological mutations in proteins (8–12). The identified novel alterations were also screened against mutation and SNP databases NCBI (www.ncbi.nlm.nih.gov/SNP), Ensembl (http://uswest.ensembl.org/index.html), COSMIC (www.sanger.ac.uk/genetics/CGP/cosmic) and the Human Gene Mutation Database (HGMD; www.hgmd.cf.ac.uk/ac/validate.php). Control samples included 50 healthy individuals (100 alleles) and 60 CFC individuals (120 alleles).

Plasmids

Human BRAF cDNA was a kind gift from Dr Martin McMahon and was cloned into a pcDNA3 vector with a Flag-tag at the N-terminus. The BRAF c.1799 T → G transversion was introduced using Quick-Change Site-Directed Mutageneis (Stratagene, La Jolla, CA) and verified by direct sequencing.

Transient transfections, kinase assay and Western blot analysis

Human embryonic kidney (HEK) 293T cells were seeded the day before transfection in six-well dishes. The cells were transfected, in three independent experiments, with 2 μg total plasmid DNA and 5 μl of Lipofectamine 200 (Life Technologies, Carlsbad, CA) according to manufacturer’s instructions. In addition to the CFC p.V600G BRAF mutant, HEK 293T cells were transiently transfected with the following control plasmids: wild-type BRAF, pcDNA3 empty vector, kinase active BRAF mutant p.S365A (13), the common cancer mutant p.V600E (2), the known kinase-impaired CFC BRAF mutant p.E501G, and the most common CFC BRAF mutant p.Q257R (4). Cells were serum-starved (0.5% fetal bovine serum) and, 24 h later, lysed in buffer containing Protease and Phosphatase Inhibitor cocktails (Sigma, St Louis, MO). Expression levels of BRAF-flag, total ERK and phosphorylated ERK (pERK) were analyzed by Western blot. To further explore the downstream kinase activity of the novel BRAF p.V600E mutant, kinase assays were performed in triplicate using the p44/42 MAP Kinase Assay Kit (Cell Signaling Technology, #9800, Danvers, MA) according to the manufacturer’s protocol with the following minor substitutions: the immunoprecipitation beads (#3510) and the anti-ELK-1 antibody (#9186). Anti-flag antibody (F1804) was purchased from Sigma–Aldrich (St Louis, MO), pERK (E-4) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and p44/42 MAP Kinase antibody (total ERK, #9102) was purchased from Cell Signaling Technology (Danvers, MA). Quantification of the kinase assay was performed using the freely available NIH ImageJ software according to instructions provided on the software’s website (http://rsb.info.nih.gov/ij/). Three independent kinase assay blots were quantified, and the relative intensity of the phospho-ELK-1 level compared to that of wild-type BRAF was calculated and plotted for each mutant (Fig. 3b). The standard error of the mean (SEM) is represented by the error bars in Figure 3b.
Results

Bidirectional sequencing of all of the coding regions of \textit{BRAF}, \textit{MEK1}, \textit{MEK2}, and \textit{KRAS} using the proband’s lymphocyte genomic DNA revealed a heterozygous T → G transversion (c.1799 T → G) in exon 15 of \textit{BRAF} (Fig. 2). This predicts a valine to glycine substitution at amino acid 600 (p.V600G) and was not found in 50 healthy individuals (100 alleles) or in 60 individuals with CFC syndrome (120 alleles). DNA from buccal epithelium also harbored the mutation, suggesting that mosaicism is unlikely. Targeted analysis of parental DNA was normal indicating that the mutation occurred \textit{de novo} (Fig. 2). Sequence analysis also identified an intronic \textit{MEK2} alteration in the proband (c.920-43G → A), which was also present in the proband’s unaffected father, suggesting that this change is a benign variant (data not shown).

Both the SIFT and PMUT programs predicted that BRAF p.V600G would be a deleterious substitution. To corroborate this prediction, HEK 293T cells were transiently transfected with appropriate control plasmids and BRAF mutant plasmid. We found that the BRAF p.V600G mutant had increased ERK phosphorylation compared to the level induced by empty vector, wild-type BRAF and the CFC kinase-impaired mutant control (Fig. 3a). In addition, the level of ERK phosphorylation induced by BRAF p.V600G was less than that of the known cancer-associated BRAF p.V600E mutant and the BRAF p.S365A control.

Discussion

We have identified the first reported germline missense mutation in the well-characterized, cancer-causing \textit{BRAF} codon 600 (p.V600G) in a child with CFC syndrome. The proband has characteristic craniofacial dysmorphia, ectodermal anomalies, exotropia, gastroesophageal reflux, postnatal growth deficiency, and hypotonia resulting in gross motor delay. She does not have a cardiac abnormality, as seen in approximately 80% of individuals with CFC syndrome. While the majority of CFC individuals are reported to have moderate to severe intellectual impairment (7), our proband has only a mild degree of cognitive impairment. Currently at age 7, she is in good general health and making steady developmental progress. She is repeating regular kindergarten with resource help.

However, the phospho-ERK level was similar to, if not slightly less than, the level of ERK phosphorylation induced by the most common CFC mutant, BRAF p.Q257R. The kinase assay confirmed these results. Phosphorylated ELK-1 of the cancer-associated p.V600E mutant was much stronger in activity than the CFC p.V600G and p.Q257R mutants (Fig. 3a,b), indicating that the CFC mutants are active, but not to the same elevated level of the cancer mutant p.V600E. These results indicate that the p.V600G BRAF CFC mutant protein is a hypermorph, but is less active than the common cancer-causing p.V600E mutant \textit{in vitro}.

![Fig. 2. Electropherograms of BRAF exon 15 sequencing. The proband has a nucleotide 1799 T → G transition (c.1799 T → G) causing a heterozygous missense substitution p.V600G in exon 15. This mutation was detected in leukocytes (a) as well as in buccal epithelial cells (b), suggesting that the mutation is not mosaic. The p.V600G mutation was not detected in the proband’s mother or father, indicating that the mutation is \textit{de novo} (c, d).](image-url)
Champion et al.

Fig. 3. Functional characterization of the novel BRAF p.V600G mutant. Human embryonic kidney 293T cells were transiently transfected in triplicate with the BRAF p.V600G mutant plasmid and control plasmids. ERK phosphorylation was assayed in triplicate by Western blotting using phospho-specific antibodies (a). Downstream ELK-1 phosphorylation was assessed in triplicate by kinase assays (a). Quantification of the kinase assays was performed using NIH Image J software (b). The bar graph in panel (b) shows the relative intensities of the phospho-ELK-1 levels for different mutations as compared to that of wild-type BRAF. A total of three independent blots were quantified. The standard error of the mean (SEM) is represented by the error bars. The p.V600G BRAF mutant protein had increased ERK phosphorylation compared to the level induced by empty vector, wild-type BRAF and the known CFC kinase-impaired control. The phospho-ERK and phospho-ELK-1 levels of the p.V600G mutant appeared comparable to those of the common CFC p.Q257R BRAF mutant protein. However, the p.V600G BRAF mutant protein was less active than the common cancer p.V600E mutant as indicated by the levels of phospho-ERK and phospho-ELK-1. This indicates that p.V600G is hypermorphic, but to a lesser extent than the common cancer-associated mutant p.V600E. Flag-tagged BRAF served as a marker for transfection efficiency and total ERK served as a loading control.

and receives occupational and speech therapies. She has been started on medication for symptoms of attention deficit disorder.

In vitro functional analysis of this CFC mutant protein by Western blotting shows that p.V600G is a hypermorphic, but not to the kinase activity level of the cancer-associated p.V600E mutation. p.V600E is the most commonly reported BRAF mutation in cancer, although other codon 600 substitutions, including p.V600G, have been reported, but at a much reduced frequency [www.sanger.ac.uk/genetics/CGP/cosmic; (14–16)]. However, mutations involving BRAF codon 600 have never been reported in individuals with CFC syndrome. It is unclear whether this is simply a chance occurrence or whether codon 600 in particular may play a more crucial role in BRAF function than other residues more commonly mutated in patients with CFC syndrome.

There is evidence to suggest that alteration of codon 600 may be poorly tolerated in development. Conditional knock-in of the p.V600E mutation in mice using the Cre/Lox system results in embryonic lethality (17). Directed expression of the p.V600E mutation in the somatic tissues of mice (spleen and liver) causes a proliferative disorder and bone marrow failure leading to death by 4 weeks of age. Furthermore, expression of endogenous BRAF p.V600E in primary mouse embryonic fibroblasts is sufficient to induce several
hallmarks of transformation, including increased proliferation, loss of contact inhibition, and morphological transformation, suggesting that *BRAF* mutation may be a critical event in the development of many human tumors.

The patho-biochemistry of the *BRAF* V600 codon alteration is well documented in cancer. Oncogenic p.V600E mutations are presumed to mimic phosphorylation within the activation segment of the *BRAF* kinase domain, thereby overriding its dependence on Ras-mediated phosphorylation and allowing cells to proliferate autonomously (2). The p.V600E mutation within the *BRAF* kinase domain disrupts the hydrophobic interaction between the glycine-rich loop and the activation segment causing destabilization of the inactive conformation of *BRAF*. It has been shown in *vitro* that the p.V600E mutant has between a twofold and a 700-fold increase in kinase activity when compared to wild-type *BRAF* (4, 18).

Unlike other RASopathies such as Noonan syndrome (NS), Costello syndrome (CS) and neurofibromatosis type 1 (NF1), it is unclear whether individuals with CFC are at an increased risk for malignancies. The benign and malignant neoplasms that are observed in CS, NS and NF1 have not been reported in CFC syndrome. Acute lymphoblastic leukemia has been reported in two CFC individuals with *BRAF* mutations (19, 20) and one individual with a *MEK2* mutation (21). One individual with a *MEK1* mutation developed hepatoblastoma while on immunosuppressive therapy (22). Consequently, as the functional differences between cancer-associated *BRAF* mutations and those that result in CFC syndrome are not well understood, it is difficult to predict whether the presence of the *BRAF* p.V600G mutation in the germline of the patient described here will lead to an increased risk for malignancies.

In summary, we have identified a *de novo* pathogenic p.V600G *BRAF* mutation in an individual with CFC syndrome. To our knowledge, this is the first report of a germline mutation involving codon 600 of *BRAF*. The discovery of this mutation in our patient establishes that disruption of codon 600 is compatible with human development and causes CFC syndrome. However, the rarity of germline mutations affecting codon 600 is not completely clear. As this individual, along with the reported cohort of individuals with CFC syndrome, is still relatively young, it is not apparent whether individuals with CFC syndrome in general or only those with mutations in specific *BRAF* codons may have an increased risk of cancer.

**Supporting Information**

The following Supporting information is available for this article:


**References**