Clinical Report

Clinical Reports

The novel \textit{RAF1} mutation p.(Gly361Ala) located outside the kinase domain of the CR3 region in two patients with Noonan syndrome, including one with a rare brain tumor\textsuperscript{1}

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\textsuperscript{1} This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi:10.1002/ajmga.38569

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Abstract

Noonan syndrome is characterized by typical craniofacial dysmorphism, postnatal growth retardation, congenital heart defect, and learning difficulties and belongs to the RASopathies, a group of neurodevelopmental disorders caused by germline mutations in genes encoding components of the RAS-MAPK pathway. Mutations in the RAF1 gene are associated with Noonan syndrome, with a high prevalence of hypertrophic cardiomyopathy (HCM). RAF1 mutations cluster in exons encoding the conserved region 2 (CR2), the kinase activation segment of the CR3 domain and the C-terminus. We present two boys with Noonan syndrome and the identical de novo RAF1 missense variant c.1082G>C/p.(Gly361Ala) affecting the CR3, but located outside the kinase activation segment. The p.(Gly361Ala) mutation has been identified as a RAF1 allele conferring resistance to RAF inhibitors. This amino acid change favors a RAF1 conformation that allows for enhanced RAF dimerization and increased intrinsic kinase activity. Both patients with Noonan syndrome showed typical craniofacial dysmorphism, macrocephaly, and short stature. One individual developed HCM and was diagnosed with a disseminated oligodendroglial-like leptomeningeal tumor (DOLT) of childhood at the age of nine years. While there is a well-established association of NS with malignant tumors, especially childhood hemato-oncological diseases, brain tumors have rarely been reported in Noonan syndrome. Our data demonstrate that mutation scanning of the entire coding region of genes associated with Noonan syndrome is mandatory not to miss rare variants located outside the known mutational hotspots.

Introduction

Noonan syndrome (NS, MIM 163950) is a genetically heterogeneous autosomal dominant disorder caused by germline mutations in genes encoding proteins of the RAS/mitogen-activated protein kinase (MAPK) signalling pathway. NS is characterized by craniofacial dysmorphism, short stature, cardiovascular defects, cryptorchidism in males, and learning difficulties of varying degree (Romano et al., 2010; van der Burgt, 2007). The four major genes of this disorder are PTPN11 (Tartaglia et al., 2001), SOS1 (Roberts et al., 2007; Tartaglia et al., 2007), RAF1 (Pandit et al., 2007; Razzaque et al., 2007), and RIT1 (Aoki et al., 2013). Pathogenic mutations in KRAS (Schubbert et al., 2006), SHOC2
(Cordeddu et al., 2009), NRAS (Cirstea et al., 2010), CBL (Martinelli et al., 2010), RRAS (Flex et al., 2014), SOS2 and LZTR1 (Cordeddu et al., 2015; Yamamoto et al., 2015), PPP1CB (Gripp et al., 2016) and MRAS (Higgins et al., 2017) have rarely been reported in individuals with NS or a similar phenotype. Variants in the two genes RASA2 (MIM 601589) and A2ML1 (MIM 610627) have recently been described in few individuals with NS-like phenotypes (Chen et al., 2014; Vissers et al., 2015), however, these findings are still awaiting confirmation. NS and phenotypically related diseases constitute the group of RASopathies, developmental syndromes caused by increased signal flux through the RAS-MAPK pathway (Rauen, 2013).

Mutations in RAF1 account for 5-10% of NS-affected patients (Aoki, Niihori, Inoue, & Matsubara, 2016; Roberts, Allanson, Tartaglia, & Gelb, 2013). Pathogenic RAF1 alleles are associated with specific clinical features. For example, RAF1 mutation-positive individuals have a much higher incidence of hypertrophic cardiomyopathy (HCM) (73-83%) than is found in the overall population with NS (~20%). Similarly, short stature (60-82%), pectus deformity (57-70%), relative macrocephaly (76-94%), and multiple nevi, lentigines, or café au lait spots are frequently observed in patients with a RAF1 mutation (Kobayashi et al., 2010; Kouz et al., 2016; Roberts et al., 2013). RAF1 encodes the V-RAF1 murine leukemia viral oncogene homolog 1, which is a member of the mammalian RAF serine-threonine kinase family involved in the RAS-MAPK signalling pathway by activating RAS downstream targets, such as MEK and ERK (Lavoie & Therrien, 2015). The three family members ARAF, BRAF and RAF1 (also known as CRAF) share the three highly conserved regions CR1, CR2, and CR3 (Wellbrock, Karasarides, & Marais, 2004). NS-associated RAF1 alleles cluster in exons encoding the CR2, the activation domain of the CR3 domain and the C-terminus (Pandit et al., 2007). 79.2% of pathogenic RAF1 alleles affect residues within the 14ζ-3-3 recognition site of CR2, such as Arg256, Ser257, Ser259, Thr260, Pro261, Asn262 and Val263 (Kobayashi et al., 2010; Pandit et al., 2007; Razzaque et al., 2007; Sana et al., 2014); binding of RAF1 to 14ζ-3-3 is critical for its autoinhibition (Kubicek et al., 2002; Light, Paterson, & Marais, 2002). The second group of mutations (8.1%) affects the adjacent residues Ser612 and Leu613 located C terminally to the CR3 domain and the third group (7.9%) affects the two amino acid residues Asp486 and Thr491 within the activation segment of the kinase.
domain (Croonen et al., 2013; Hartill, Dillon, Warren, & Blyth, 2017; Hopper, Feinstein, Manning, Benitz, & Hudgins, 2015; Ko, Kim, Kim, & Yoo, 2008; Kobayashi et al., 2010; Pandit et al., 2007; Ratola et al., 2015; Razzaque et al., 2007; Sana et al., 2014; Schulz, Frober, Kraus, & Schneider, 2012). Only two amino acid substitution in CR3 have been described so far (4%): p.(Ser427Gly) was found in mother and son with NS as well as in an unrelated patient (Kobayashi et al., 2010; Zebisch et al., 2006) and p.(Glu478Lys) was found in one patient (Ezquieta et al., 2012). Greater RAF1 kinase activity was determined for amino acid substitutions located in CR2, CR3 and at the end of CR3, while mutations affecting residues 486 and 491 impaired kinase activity (Kobayashi et al., 2010; Pandit et al., 2007; Razzaque et al., 2007). Nevertheless, expression of the NS-associated RAF1 mutants Ser257Leu, Pro261Ser, Pro261Ala, Val263Ala and Leu613Val as well as Asp486Asn, Thr491Ile, and Thr491Arg resulted in robust ERK activation indicating increased and prolonged signalling through the RAS-MEK-ERK cascade (Pandit et al., 2007; Razzaque et al., 2007; Wu et al., 2012). In a recent study, both kinase-defective and kinase-activating RAF1 mutants were found to promote heterodimerization with BRAF providing evidence for a common pathogenic mechanism in this NS subtype (Wu et al., 2012).

Besides germline alterations in RAF1, somatic mutations have rarely (1%) been identified in human cancers. In contrast to the variants found in NS, cancer-associated missense mutations are spread over the entire RAF1 coding region (Maurer, Tarkowski, & Baccarini, 2011) (http://www.sanger.ac.uk/genetics/CGP/cosmic). In the past, mutation screening of RASopathy-related genes has often been limited to exons representing mutational hot spots, such as exons 7, 14 and 17 of the RAF1 gene (Ko et al., 2008; Tumurkhuu et al., 2010), possibly explaining clustering of RAF1 mutations in NS-affected individuals. Here we report two cases with a novel RAF1 amino acid substitution located outside CR2, the kinase domain of CR3 and the C-terminus.

Materials and Methods

Subjects and phenotyping
Clinical data and samples for the two individuals were obtained with informed consent of the patients’ parents/legal guardians, including written consent to use photographs in this report. Consent procedures were approved by the Ethics Committees of the Medical Chamber of Hamburg (reference number PV3802). Standardized phenotypic data was collected using the electronic questionnaire of the NSEuroNet database (www.nseuronet.com).

Sanger-sequencing based genetic testing for RASopathy-associated genes was performed in the two patients with NS covering all coding exons of \(\text{PTPN11}, \text{KRAS}, \text{NRAS},\) and \(\text{RIT1}\) as well as mutational hotspot exons of \(\text{SOS1}\) (exons 3-11, 13, 14, 16), \(\text{RAF1}\) (exons 7, 12, 14, 17), \(\text{BRAF}\) (exons 6, 11-17), \(\text{MAP2K1}\) (exons 2, 3, 6, 7), \(\text{MAP2K2}\) (exons 2, 3, 6, 7), \(\text{SHOC2}\) (exon 2), and \(\text{CBL}\) (exons 7-9) within \(\text{SHOC2}\) (exon 2), and \(\text{CBL}\) (exons 7-9) within a clinical diagnostic setting with no causative variants identified.

**Whole-exome sequencing (WES), multigene panel testing and data analysis**

DNA was isolated from leukocytes by standard procedures. DNA of patient 1 and his parents was used to perform targeted enrichment and massively parallel sequencing as described previously (Kortüm et al., 2015). Whole-exome enrichment was performed using Nextera® Exome Enrichment Kit (62 Mb) (Illumina, San Diego, USA) according to the manufacturer’s protocols. Captured libraries were loaded onto the HiSeq 2500 platform (Illumina). Trimmomatic was employed to remove adapters, low quality (phred quality score < 5) bases from the 3’ ends of sequence reads (Bolger, Lohse, & Usadel, 2014). Reads shorter than 36 bp were subsequently removed. Further processing was performed following the Genome Analysis Toolkit’s (GATK) best practice recommendations. Briefly, trimmed reads were aligned to the human reference genome (UCSC GRCh37/hg19) using the Burrows-Wheeler Aligner (BWA mem v0.7.12). Duplicate reads were marked with Picard tools (v1.141). GATK (v3.4) was employed for indel realignment, base quality score recalibration, calling variants using the HaplotypeCaller, joint genotyping, and variant quality score recalibration. AnnoVar (v2015-03-22) was used to functionally annotate and filter alterations against public databases (dbSNP138, 1000 Genomes Project, and ExAC Browser). Exonic sequence alterations (non-
synonymous, frameshift and synonymous) and intronic variants at exon-intron boundaries ranging from -10 to +10, which were clinically associated with unknown frequency in public databases were retained.

Multigene panel testing was performed for mutation screening in patient 2 using an Illumina Nextera® Rapid Capture Custom Kit (Illumina) for enrichment and an Illumina MiSeq system for analysis. This multigene panel included 44 genes related to RASopathies or overlapping disorders (Supplementary Table 1). The obtained sequence data was analyzed using the Illumina’s VariantStudio Data Analysis Software v2.2.1.

Variant validation

Sanger sequencing was performed to confirm the \textit{RAF1} variant in both patients and to test segregation of this variant in their healthy parents. Primer pair and PCR condition are available on request. The amplicon was directly sequenced using the ABI BigDye Terminator Sequencing Kit (Applied Biosystems, Darmstadt, Germany) and an automated capillary sequencer (ABI 3500; Applied Biosystems). Sequence electropherograms were analyzed using the Sequence Pilot software (JSI Medical Systems, Ettenheim, Germany). Mutation nomenclature refers to GenBank mRNA reference sequence NM_002880.3.

Case Reports

\textbf{Patient 1} (Australia) is an 11-year-old boy born to non-consanguineous healthy parents. Ultrasound during pregnancy showed polyhydramnios, macrosomia and a prominent tongue. At age 10 years, his occipitofrontal circumference was 53.8 cm (60\textsuperscript{th} centile), weight 24.7 kg (5\textsuperscript{th} centile), and height 114 cm (<1\textsuperscript{st} centile). He presented with thoracic deformity, short stature, relative macrocephaly, keratosis pilaris, and cryptorchidism. His craniofacial features were typical of Noonan syndrome (Figure 1A). At age 3 months, he was diagnosed with mild pulmonary stenosis and HCM, and treatment with atenolol was commenced. Echocardiography at age 18 months showed asymmetrical septal hypertrophy with dynamic subaortic stenosis and moderate left ventricular outflow
obstruction. There were never any clinical symptoms attributable to cardiomyopathy. Echocardiographic changes improved over time and by age 6 years the pulmonary valve appeared normal and the left ventricular outflow obstruction had resolved, although there was persistent mild septal hypertrophy. Atenolol was ceased at age 8 years, and cardiac findings have since remained stable. His gross motor development was delayed (unsupported walking at age 34 months), but his language development was normal. Intellect was not formally assessed, but he likely had a mild intellectual disability. He had prominent keratosis pilaris, sparse eyebrows, and multiple pigmented nevi. The clinical manifestations were highly suggestive of NS or a related disorder out of the RASopathy spectrum. At age 22 months, he was diagnosed with a cerebellar cyst leading to acute hydrocephalus treated with a ventriculo-peritoneal shunt. From age 4 years he developed a severe progressive kyphoscoliosis treated with serial plasters and brace. Between ages 6 and 8 three separate giant cell tumors were excised from the naso-maxilla. At age 9, he was diagnosed with an extensive leptomeningeal tumor affecting the spine and brain. Biopsies revealed a low grade glioma, and in conjunction with the radiologic findings, the histological presentation was regarded as consistent with disseminated oligodendroglial-like leptomeningeal tumor (DOLT) of childhood. In retrospect, it is likely that the DOLT had been present since early life and was the cause of the hydrocephalus and scoliosis. Treatment with carboplatin resulted in clinical improvement and radiological stabilization, but tumors progressed after carboplatin was ceased. He was then treated with second line therapy of vinblastine and bevacizumab.

As testing of mutational hotspot exons of NS-associated genes did not reveal a mutation in patient 1, we performed WES on him and his healthy parents. The heterozygous missense variant c.1082G>C/p.(Gly361Ala) in the gene \( \text{RAF1} \) was identified as the top candidate. This variant was absent in population databases (1000 Genomes Project, EVS, ExAC, and gnomAD browser), but was listed in dbSNP (rs397516813) and in the ClinVar database, which annotates this variant as “pathogenic/likely pathogenic” (SCV000061333.4, SCV000209024.8). The \( \text{RAF1} \) variant was validated in the patient’s DNA in the heterozygous state and was confirmed by Sanger-sequencing to be \text{de novo} (Figure 1A).
**Patient 2** is an 18-month-old boy born to healthy non-consanguineous parents. Pregnancy was unremarkable. Fetal karyotyping performed because of advanced maternal age (37 years) was normal. The boy had feeding difficulties in infancy, bilateral cryptorchidism and inguinal hernia. A mild valvular pulmonic stenosis was diagnosed at age 14 months. He also had persistent pericardial effusions without evidence of an infectious, autoimmune or malignant etiology. At age 18 months, his body length was 76 cm (1st centile) and his weight 9.5 kg (18th centile). He was macrocephalic (51 cm, 99.6 centile, +3 SDS). Facial features were compatible with a RASopathy. They included a broad prominent forehead, hypertelorism with downslanting palpebral fissures, broad philtrum, and low-set ears. His hair was curly with a high frontotemporal hairline. His motor and language development was mildly to moderately delayed. Brain MRI revealed mild ventricular dilatation and polymicrogyria was suspected.

As testing of mutational hotspot exons of NS-associated genes did not reveal a mutation, we analyzed a multigene panel covering the entire coding sequence of all RASopathy genes known at that time (all except for PPP1CB). The only variant that passed the filters was c.1082G>C/p.(Gly361Ala) in RAF1 (Figure 1A). The mutation was confirmed by Sanger-sequencing in the heterozygous state and was absent in parental DNA samples (Figure 1A). The RAF1 missense variant p.(Gly361Ala) has not been detected among 41 RAF1 mutation-positive individuals with NS in our cohort (Kouz et al., 2016) and in a cohort of more than 300 RASopathy-affected individuals who had multigene panel testing in a diagnostic setting (M. Zenker, personal communication).

**Discussion**

The NS-associated RAF1 mutations directly or indirectly result in hyperactivation of MEK-ERK related pathways, either by enhancing the kinase activity of RAF1 itself and/or through an increased formation of RAF1-BRAF heterodimers with an enhanced catalytic activity compared to RAF1 homodimers or monomers (Pandit et al., 2007; Wu et al., 2012). Antony et al. (2013) identified the RAF1 variant p.Gly361Ala as a RAF1 allele conferring resistance to RAF and MEK inhibitors. Transient expression of the RAF1\(^{Gly361Ala}\) mutant in HEK 293/T cells led to enhanced amount of phosphorylated
MEK and ERK indicating enhanced MEK-ERK signalling compared to cells ectopically expressing wild-type RAF1. An *in vitro* kinase assay showed modest steady-state kinase activity for the RAF1<sub>Gly361Ala</sub> mutant which was highly upregulated after treatment with BRAF inhibitor. Wild-type RAF1 and the other investigated BRAF inhibitor-resistant RAF1 mutants did not exhibit steady-state kinase activity. In addition, robust RAF1 homodimerization was observed for the RAF1<sub>Gly361Ala</sub> mutant accompanied by accumulation of this mutant protein. To test whether RAF1 dimerization is essential for enhanced MEK-ERK signalling, the p.Arg401His mutation was introduced which abolishes RAF1 homodimerization. Ectopic expression of the RAF1<sub>Gly361Ala/Arg401His</sub> double mutant revealed a reduced but constitutive MEK-ERK activation. Together with the results of the *in vitro* kinase assay these data demonstrate that the RAF1<sub>Gly361Ala</sub> mutant indeed has enhanced intrinsic kinase activity (Antony, Emery, Sawyer, & Garraway, 2013). In line with this, the analogous cancer-associated BRAF<sub>Gly468Ala</sub> mutant showed a strong activation of kinase activity (Wan et al., 2004). Further experiments demonstrated that interaction of RAF<sub>Gly361Ala</sub> with the scaffold protein 14-3-3ζ was diminished which may favor a RAF1 conformation permissive for enhanced dimerization and kinase activity (Antony et al., 2013). Mutations in RAF1 leading to elevated kinase activity and thereby enhanced ERK activation have been correlated with the development of HCM (Pandit et al., 2007; Wu et al., 2011), as observed in patient 1.

Cardiac defects in patients 1 and 2 carrying the same RAF1 variant were different: patient 1 had mild pulmonary stenosis and HCM, while patient 2 was diagnosed with mild valvular pulmonic stenosis. HCM is the leading cardiac anomaly in patients with RAF1 mutation (Gelb, Roberts, & Tartaglia, 2015; Kobayashi et al., 2010; Pandit et al., 2007), but its expression is known to be variable. Even among individuals with the most common RAF1 germline mutation p.(Ser257Leu), heart abnormalities can vary substantially. For example, in a series of eleven patients with this RAF1 change, eight had HCM, five pulmonic stenosis, four mitral valve anomaly, three atrial septal defect and two ventricular septal defect (Kobayashi et al., 2010). The pathophysiologic basis of the variability in cardiac phenotype, particularly HCM, in patients with the same RAF1 mutation is not yet understood.
As activation of the RAS-MAPK signalling pathway is one of the main contributors to cancer development, patients with germline mutations in RASopathy-associated genes are at increased tumor risk. Nevertheless, the empirical prevalence of cancer in NS is relatively low (Kratz, Rapisuwon, Reed, Hasle, & Rosenberg, 2011). For NS-affected individuals with pathogenic \textit{RAF1} alleles, the specific tumor risk is still unclear. Patient 1 had three separate giant cell tumors in the naso-maxilla. Giant cell tumors of the jaws and other bone/soft tissues have repeatedly been reported in patients with NS and mutations in \textit{PTPN11} and \textit{SOS1} (Eyselbergs et al., 2014; Karbach, Coerdt, Wagner, & Bartsch, 2012; Neumann et al., 2009; van den Berg et al., 2016). These tumors are often multilocular. To our knowledge, only one previously reported patient with NS and a mutation in \textit{RAF1} [c.1837C>G/p.(Leu613Val)] was suspected of having a giant cell tumor due to a conspicuous mandibular mass (Denayer et al., 2010). Our observation corroborates the view that giant cell tumors may occur as a rare complication in RASopathies of various genetic etiologies (Neumann et al., 2009). Whereas low grade gliomas have been reported in patients with pathogenic germline mutations in NS-associated genes other than \textit{RAF1} (Kratz et al., 2015; Kratz et al., 2011), the same brain tumor (DOLT) as identified in patient 1 has not yet been described in this context. Tumor association in patients with a germline \textit{RAF1} mutation is apparently low, as only one individual was reported to have Burkitt lymphoma [c.776C>T/p.(Ser259Phe)] (Cianci et al., 2013). In addition, two cases of therapy-related acute myeloid leukemia (AML) associated with apparent germline \textit{RAF1} mutations [p.(Ser427Gly) and p.(Ile448Val)] have been reported (Zebisch et al., 2006). While the boy with Burkitt lymphoma had a clear-cut NS phenotype (Cianci et al., 2013), clinical manifestations other than AML were not reported in the two adult individuals (Zebisch et al., 2006). As p.(Ile448Val) was found in six out of 246,082 alleles in the gnomAD browser and the \textit{RAF1} \textit{I448V} mutant did not show enhanced kinase activity and did not stimulate phosphorylation of MEK and ERK (Zebisch et al., 2006), the \textit{RAF1} allele p.(Ile448Val) likely is not associated with NS. In contrast, p.(Ser259Phe) and p.(Ser427Gly) were absent in the gnomAD browser database and both had already been associated with NS (Kobayashi et al., 2010; Pandit et al., 2007). Cancer-associated somatic mutations in \textit{RAF1} are also relatively rare. Only 171 entries (including 141 missense) are listed in the COSMIC database, in
comparison to over 47,000 for BRAF1 (Figure 1B). The low oncogenic potency of RAF1 is thought to be due to its low basal kinase activity compared with that of BRAF (Holderfield, Deuker, McCormick, & McMahon, 2014). RAF1 mutations have mostly been found in large bowel and skin cancer, but only once in glioma (COSMIC database). However, it is well established that rearrangements of RAF1 with for example SRGAP3, NFIA, ATG7 or QKI most likely lead to hyperactivation of RAF1 and therefore play a role as somatic events in pilocytic and low grade astrocytomas, or low grade gliomas (Jones et al., 2009; Phillips et al., 2016; Yde et al., 2016; Zhang et al., 2013). Development of two independent childhood tumors in patient 1 is very unusual for NS, but as a single observation it is still insufficient to support the hypothesis that the c.1082G>C/p.(Gly361Ala) mutation in RAF1 is a more significant tumor-predisposing mutation than other RAF1 alleles. The secondary events driving tumor development in NS are largely unknown. Loss of heterozygosity of the germline mutation has occasionally been described in NS-related leukemia (Cave et al., 2016), but this could be excluded in the brain tumor sample in patient 1 (data not shown).

In summary, we identified two NS-affected patients with the de novo missense mutation c.1082G>C/p.(Gly361Ala) in RAF1. Published functional data on the RAF1 Gly361Ala mutant convincingly demonstrate enhanced intrinsic kinase activity and increased MEK-ERK signalling indicating that the p.(Gly361Ala) mutation in RAF1 most likely is causative for NS in the two individuals reported here. We show that targeted gene panel sequencing and WES are the methods of choice over serial single-gene testing of mutational hotspot exons.

Acknowledgements

We are grateful to the families for participating in this study. This work was supported by grants from the Deutsche Forschungsgemeinschaft (DFG) (KU 1240/9-1 to K.K. and ZE 524/10-1 to M.Z.) and the BMBF (GeNeRARe, TP1 to M.Z., funding code 01GM1519A, and TP6 to K.K., funding code 01GM1519E).

Conflicts of interest
The authors of this manuscript have no conflicts of interest to declare.

References


Figure legend:
**Figure 1:** The *RAF1* c.1082G>C/p.(Gly361Ala) mutation in two Noonan syndrome-affected individuals and location and number of germline and cancer-associated *RAF1* missense variants. (A) Pedigree of both families with photograph of patient 1 showing typical facial features of Noonan syndrome. Partial sequence electropherogram shows the *RAF1* c.1082G>C variant in DNA of patients 1 and 2 in the heterozygous state. +: wild-type allele (c.1082G); M: mutant allele (c.1082C). (B) Schematic
representation of RAF1 structure. RAF1 has three conserved regions (CR1-3); amino acid positions are indicated. The amino acid sequence (one-letter code) of the CR2 domain is given above the domain. Amino acid substitutions (one-letter code) identified in patients with Noonan syndrome are indicated above the protein structure. Mutations cluster in CR2, CR3 and the C-terminus. The p.(Gly361Ala) variant identified in this study is indicated in bold. Below the protein structure, the number of cancer-associated missense variants in the three conserved regions (CR1-3) of both RAF1 and BRAF and in regions outside CR1-3 of the two proteins (other) is stated (from COSMIC database). CR: conserved region; CRD: cysteine-rich domain; RBD: Ras binding domain.
**Figure 1**

A

- **Patient 1**
  - +/+ M
  - c.1082G>C p.(G361A)

- **Patient 2**
  - +/+ M

B

- P261A/H/L/R/S/T
- T260R
- S259F/P/T
- S257K/L/P
- R256S
- QRQRTSTPPNVHM
- N262_V263delinsl
- N262I
- V263A/G
- S427G
- G361A
- E476K
- D486G/N
- T491I/R
- S612T
- L013V
- S648R

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Date:
2018-02-01

Citation:

Persistent Link:
http://hdl.handle.net/11343/294067