Diagnostic Exome Sequencing (DES) Coupled with Rules-based Candidate Gene Analysis Identifies a Causative *RRAS* Lesion in a Patient with a Novel RASopathy*

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BACKGROUND

- The proband is a 7 year-old male who presented to clinic with neurodevelopmental symptoms, as well as other clinical features.
- Some of the patient's symptoms were indicative of a hematological disorder- malignancy.
- Previous testing was non-informative.
- Family-trio DES is an established test that provides a molecular diagnosis in complex cases that are often refractory to diagnosis. Despite this analytical power, many laboratories and/ or institutions employing DES only analyze the approximately 25% of protein coding genes that are strongly associated with clinical disease; they leave the remainder of genes unanalyzed.
- **Rules-based candidate gene analysis** allows for virtually all of the human exome to be evaluated for its contribution to a patient's phenotype.
- Family-trio DES with rules-based candidate gene analysis was undertaken to determine the patient's molecular etiology.

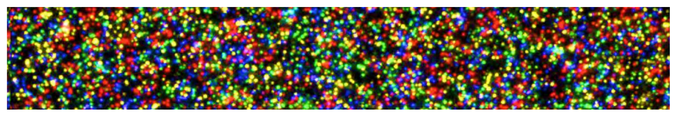
CLINICAL FEATURES

- **7 year old boy**
- **Delayed milestones (speech delay; some problems with reading and spelling)**
- **Chronic fatigue**
- **Astigmatism**
- **Scoliosis with pelvic tilt**
- **Hypermobile (Beighton 7/9)**
- **Small hands, short fingers**
- **Splenomegaly**
- **Decreased immunoglobulins**
- **Thrombocytopenia**
- **Periodic nose bleeds**

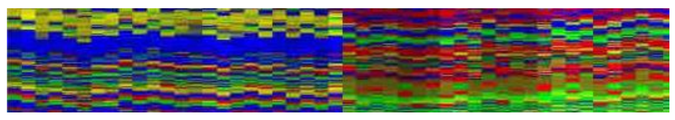
- **Slightly dysmorphic – hypertelloric appearing, small nose, crowded teeth, full lower lip with pits**
- **Craniosynostosis (premature fusion of the skull, which results in an abnormal head shape)**
- **Small ventricular septal defect (VSD)**
- **Constipation**
- **Large penis for age**
- **Hydrocele (painless buildup of watery fluid around testicles)**

METHODS

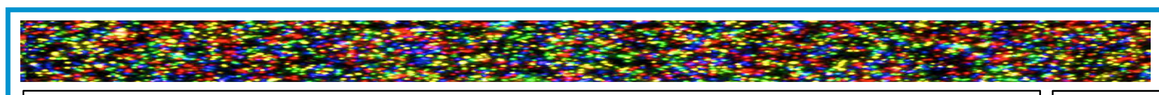
- **Patients:** Genomic deoxyribonucleic acid was isolated from whole blood extracted from the proband and relatives who were referred to Ambry Genetics (Aliso Viejo, CA) for DES.
- Informed consent was obtained from all family members involved in the testing process.



- Exome library preparation, sequencing, bioinformatic processing, and data analysis were performed as previously described (Farwell, 2015).
- Candidate alterations identified as potentially causative were confirmed using automated fluorescence dideoxy Sanger sequencing. Co-segregation analysis was performed on each available family member.



RESULTS



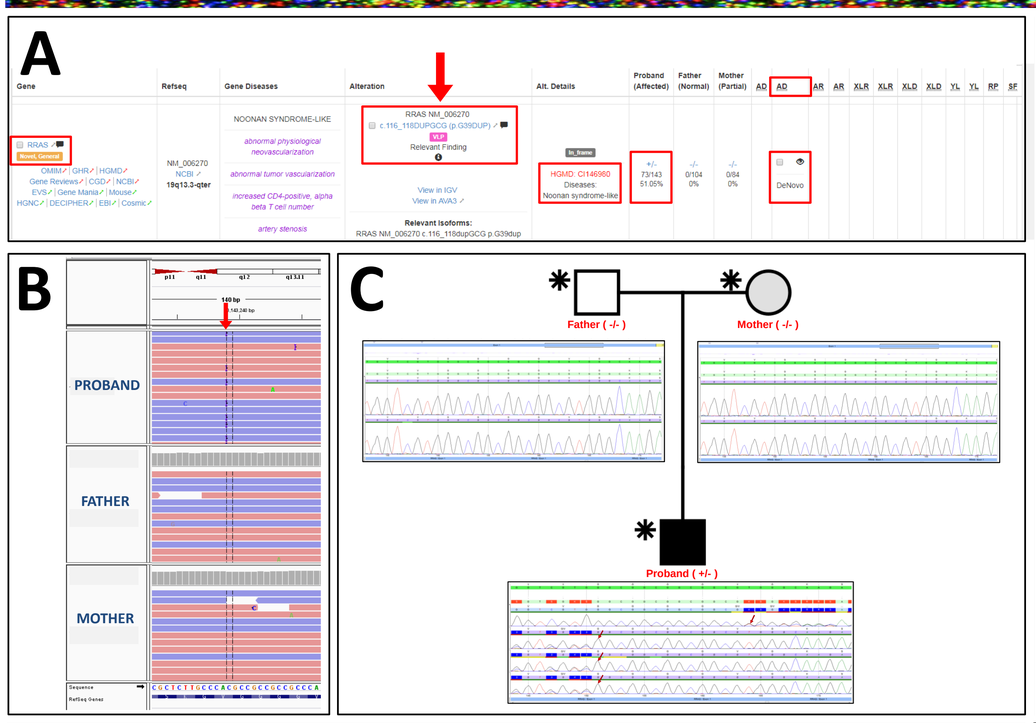


Figure 1. DES coupled with bioinformatic analysis identifies an apparently *de novo* ***RRAS*** alteration, **G39DUP** (c.116_118dupGCG), in the proband. **A)** DES and bioinformatic filtering results. Red arrow and boxes indicate the gene, alteration, inheritance, and zygosity/ number of reads. **B)** Alignment of NGS reads, as visualized with the Broad Institute's Integrated Genome Viewer (IGV). Red arrow indicates the location of the insertion, which is found in approx. half of the proband's reads but is absent in the parents' reads. **C)** Family pedigree with Sanger sequencing traces confirm the *de novo* nature of *RRAS* G39dup. Red arrows indicate the location of the duplication in the proband, * indicates exome sequencing was performed, open shape indicates unaffected family member, filled shape indicates affected family member.

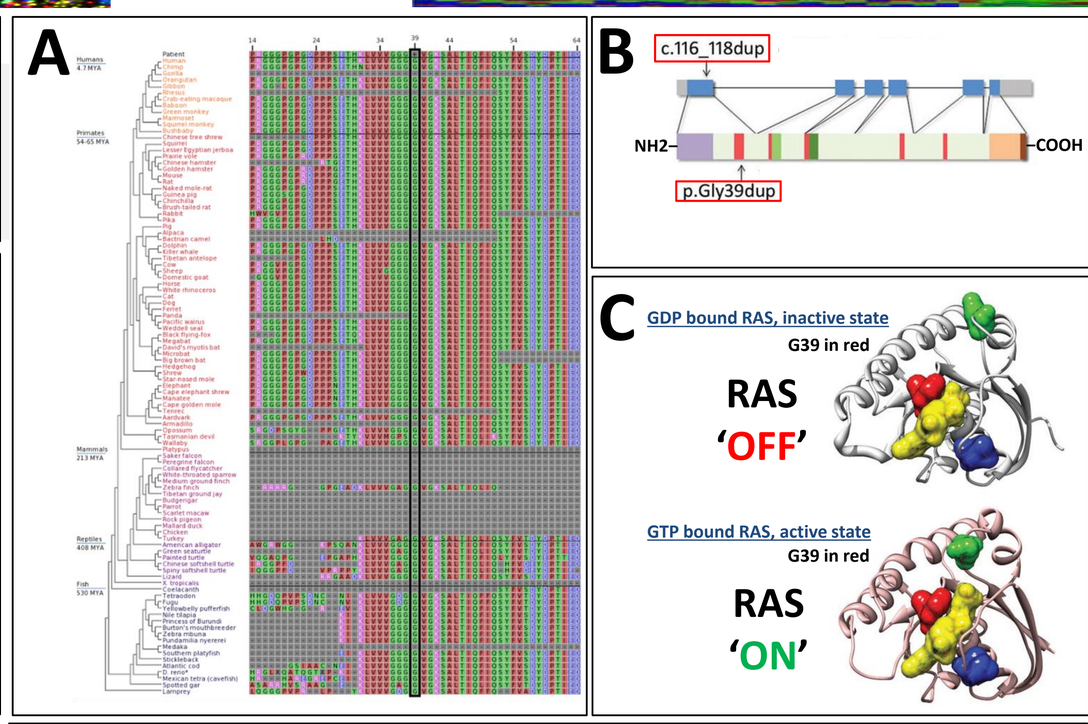


Figure 2. G39dup is situated in a highly conserved, functional region of RRAS. **A)** G39 and flanking residues are conserved across evolution. **B)** Schematic of the *RRAS* gene (top) juxtaposed with the linear protein product (bottom). Arrows indicate that c.116_118dup (G39dup) is located in *RRAS* exon 1, which encodes the first of five functional motifs (G1 to G5, red) that participate in GTP/ GDP binding and GTPase activity. Also depicted on the linear protein are the unique N-terminal region (violet), switch I (light green), switch II (dark green), hypervariable region (light brown), and the C-terminal CAAX motif (dark brown). **C)** Visualization of G39 within the 3D structure of RAS. Red surface indicates G39 and V40. Residue V55 is depicted in blue while Q87 is shown in green; GDP and GTP are coloured in yellow. Elements from panels B and C have been adapted from Flex, 2014.

RRAS G39dup IS A VARIANT, LIKELY PATHOGENIC (VLP)

- **G39dup**, which is **conserved** across evolution, impacts one or more conserved RAS **functional domains** (Fig. 2).
- **G39dup** is a ***de novo*** occurrence within the pedigree (Fig. 1).
- With a **PROVEAN** score of -8.994, G39dup is predicted to be **Deleterious** (Choi, 2015).
- *RRAS* G38 and **G39** are major mutational **hotspots** in human **cancer** (COSMIC database).
- **Analogous insertions** in **RAS family** members have been reported in cases of **Juvenile Myelomonocytic Leukemia** (**JMML**) and other malignancies (Bollag, 1996; Murugan, 2009; Reimann, 2006; Sartori, 2009).
- Alterations at **analogous residues** G12 and G13 account for the majority of **germline** *HRAS* mutations that cause **Costello syndrome** (Aoki, 2005).
- Flex, 2014 identified *RRAS* G39dup as the causative **germline lesion** in a patient presenting symptoms overlapping those of the proband. Clinical features included symptoms suggestive of **Noonan-like syndrome** and the emergence of **Acute Myeloid Leukemia** (**AML**). The authors also identified *RRAS* G39dup as a **somatic** alteration in a different patient with aggressive **JMML** that progressed rapidly to **AML**.
- Using both ***in vitro*** and ***in vivo*** functional assays, Flex 2014 demonstrated that G39dup exerts its deleterious effect(s) through a **Gain-of-Function** mechanism. G39dup increases GDP dissociation, impairs GTPase activity, and enhances signaling through the RAS pathway.

RULES-BASED CANDIDATE-GENE CRITERIA*

*Farwell Hagman, 2017

Clinical report classification	Category	Code	Criterion	Exceptions/caveats/notes
Candidate	At least VLP according to Ambry Variant Classification[17] *AND*:			For inherited compound heterozygous alterations, both must be at least VLP
	A (1 needed)[a]	C-A1	Gene located within the well-defined critical gene region of an established microdeletion/duplication syndrome with highly consistent features supportive of proposed gene–disease relationship	With consistent zygosity/model of inheritance
		C-A2	Proposed candidate gene–disease relationship categorized as at least "limited" (Clinical Validity Classification) and patient's phenotype is highly consistent with that of reported patients	Ambry's Clinical Validity Classification Scheme (data in preparation) is inspired by the ClinGen Gene Curation classification (in preparation)
	B (2 needed)[a]	C-B1	Protein colocalizes or physically interacts with the products of genes implicated in the proposed gene–disease relationship	No point if conflicting evidence; predicted damaging alterations in expression-specific tissues have a high predictive predictability[60]
		C-B2	In vivo model organism with consistent genotype produces phenotype strongly supportive of the proposed gene–disease relationship	No point if conflicting evidence; must consider specificity (e.g., short stature in mice not specific; observed in ~30% of knockouts)[59]
		C-B3	Expression profile is strongly supportive of the proposed gene–disease relationship (e.g., expression is restricted to diseased tissues)	No point if conflicting evidence; predicted damaging alterations in expression-specific tissues have a high predictive predictability[60]
		C-B4	Gene disruption experiment produces a phenotype supportive of the proposed gene–disease relationship *and* the phenotype can be rescued by addition of a wild-type gene product	No point if conflicting evidence
		C-B5	Other strong data to support relevance of the gene with the proposed gene–disease relationship	
	C (4 needed)[a]	C-C1	Gene function and/or expression profile is consistent with the phenotype (e.g., expression is not restricted to the diseased tissue)	No point if conflicting evidence
		C-C2	In vivo model organism (any genotype) produces a phenotype supportive of the proposed gene–disease relationship	No point if conflicting evidence; only applies if C-B2 is not met
		C-C3	Gene disruption experiment produces a phenotype supportive of the proposed gene–disease relationship	No point if conflicting evidence; only applies if C-B4 is not met
		C-C4	Gene is located within a microdeletion/duplication syndrome described in multiple patients with consistent features with the evaluated phenotype	No point if conflicting evidence; AD, XL only; reported microdeletions/duplications must be known to be of high penetrance; only applies if C-A1 is not met
		C-C5	Gene product is in the same family, colocalizes, or physically interacts with the products of genes implicated in diseases with overlapping features	No point if conflicting evidence; only applies if C-B1 is not met
		C-C6	Other data to support the relevance of the gene with the evaluated phenotype	
		C-1	1 of B and 3 of C	
Suspected candidate		S-1	Meets Candidate Criteria but the alteration(s) is classified as variant of uncertain significance according to Ambry Variant Classification[17]	
		S-2	Meets Candidate Criteria but phenotypic overlap is uncertain	
		S-3	At least VLP and 1 of B and 2 of C	For inherited compound heterozygous alterations, at least one must be at least VLP
		S-4	At least VLP and 3 of C	

RRAS G39dup IS A CANDIDATE FOR THE ETIOLOGY UNDERLYING THE PROBAND'S DISEASE

- *RRAS* **G39dup** (c.116_118dup) is at minimum a **VLP**.
- C-B5) OTHER STRONG DATA: A previous patient reported by Flex, 2014 harbored the same heterozygous *de novo* germline *RRAS* alteration as our proband; that is, p.G39dup. This female patient presented symptoms overlapping those of the proband, including **motor delay**, a **thick lower lip** and **hematological** abnormalities in the form of **splenomegaly** and **abnormal blood cell counts**). In addition, she developed AML, which was suspected to have been secondary to JMML. Flex, 2014 also identified a somatic *RRAS* G39dup alteration in an unrelated girl with JMML; she also displayed splenomegaly and abnormal blood cell counts.
- C-C1) GENE FUNCTION: Situated on chromosome 19q13.33, the ***Related Ras Viral Oncogene Homolog*** (***RRAS***) gene encodes a 218 amino acid (AA) member of the RAS family of small GTPases (OMIM #165090). RAS proteins act as molecular 'switches' that transduce cell-surface signals to the MAPK and PI3K/ AKT signaling cascades. In developing neurons, *RRAS* serves to control axon specification (Oinuma, 2007).
- C-C3) *IN VITRO* MODULATION OF GENE EXPRESSION AND/ OR PROTEIN ACTIVITY: Both increased and decreased *RRAS* expression lead to abnormal neuronal development *in vitro*; that is, ectopic expression of *RRAS* induces the formation of multiple axons, whereas RNAi-mediated knockdown of endogenous *RRAS* blocks axon formation (Oinuma, 2007).
- C-C4) PATHWAY/ FAMILY: Genetic alterations resulting in the constitutional dysregulation of RAS/ MAPK signaling underlie a group of disorders collectively referred to as RASopathies. RASopathies, which include diseases like Noonan Syndrome, are characterized by cardiac and growth defects, facial dysmorphism, variable cognitive deficits, and predisposition to certain malignancies (Flex, 2014). Moreover, many of the clinical features displayed by our proband are typical of those associated with RASopathies/ Noonan Syndrome. Such features include **developmental delay**; **VSD**; **bleeding propensity**; **hydrocele**; **scoliosis**, **joint hypermobility** and **hip** dysplasia; **fatigue**, and mild dysmorphia in the form of **hypertelorism** and full/ thick lips or **full/ thick lower lip**; (Croonen, 2016; Flex, 2014; Hasegawa, 2009; OMIM #163950; Pierpont, 2014, Rauen, 2013; Sznajer, 2007; Vengunta, 2011). Addissie, 2015 identified a causative *de novo* alteration, p.P34Q (c.101C>A), in the *KRAS* gene of a two month-old girl with NS. In addition to presenting features typically associated with the disease, the girl also exhibited **craniosynostosis**. Upon literature review, the authors found that approximately 10% of published cases with germline *KRAS* alterations displayed craniosynostosis. The authors indicate that germline KRAS alterations are causally involved in craniosynostosis, and moreover, that the RAS-MAPK pathway is an important mediator of bone growth in cranial sutures. They suggest craniosynostosis is a significant finding in NS.

TAKE-HOME POINTS

#1 Rules-based candidate gene analysis allows for virtually all of the genes in the human exome to be evaluated for their contribution to a patient's phenotype.

#2 By employing DES with rules-based candidate gene analysis, we determined ***RRAS* p.G39dup** to be the causative lesion in our proband. Not only does this finding recapitulate the work of Flex *et al.*, it expands the phenotypic spectrum associated with *RRAS* alterations.

#3 We propose that *RRAS* be added to the list of genes that are commonly sequenced in individuals suspected of having a RASopathy.

#4 We recommend that patients with *RRAS* alterations be monitored carefully for the emergence of hematological malignancy.

NOTE. Since the DES report was issued, the proband's malignancy has been confirmed to be **Monosomy 7 Myelodysplastic Syndrome**. He is undergoing appropriate treatment.

REFERENCES

- Addisie YA, *et al.* (2015) *Am J Med Genet A.* **167A**:2657-2663.
- Aoki Y, *et al.* (2005) *Nat Genet.* **37**:1038-1040.
- Bollag G, *et al.* (1996) *J Biol Chem.* **271**:32491-32494.
- Choi Y & Chan AP. (2015) *Bioinformatics* **31**: 2745-2747. (http://provean.jcvi.org)
- Croonen EA, *et al.* (2016) *Am J Med Genet A.* **170**:2349-2356.
- Farwell KD, *et al.* (2015) *Genet Med.* **17**:578-586.
- Farwell Hagman KD, *et al.* (2017) *Genet Med.* **19**:224-235.
- Flex E, *et al.* (2014) *Hum Mol Genet.* **23**:4315-4327.
- Hasegawa K, *et al.* (2009) *Clin Pediatr Endocrinol.* **18**:87-93.
- Murugan AK, *et al.* (2009) *Oral Oncol.* **45**:e161-166.
- Oinuma I, *et al.* (2007) *J Biol Chem.* **282**:303-318.
- OMIM (Online Inheritance in Man) [Internet]: Copyright® 2014 Johns Hopkins University. Available from www.omim.org
- Rauen KA. (2013) *Annu Rev Genomics Hum Genet.* **14**:355-369.
- Pierpont ME, *et al.* (2014) *Pediatrics.* **134**:e1149-1162.
- Reimann C, *et al.* (2006) *Leukemia.* **20**:1637-1638
- Sartori, *et al.* (2009) *Am J Clin Pathol.* **131**:478-489.
- Sznajer Y, *et al.* (2007) *Pediatrics.* **119**:e1325-1331.
- Vegunta S, *et al.* (2015) *Am J Med Genet A.* **167A**:2998-3005.

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