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Proceedings of the fifth international RASopathies symposium: When development and cancer intersect

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Abstract

This report summarizes and highlights the *fifth International RASopathies Symposium: When Development and Cancer Intersect*, held in Orlando, Florida in July 2017. The RASopathies comprise a recognizable pattern of malformation syndromes that are caused by germ line mutations in genes that encode components of the RAS/mitogen-activated protein kinase (MAPK) pathway. Because of their common underlying pathogenetic etiology, there is significant overlap in their phenotypic features, which includes craniofacial dysmorphology, cardiac, cutaneous, musculoskeletal, gastrointestinal and ocular abnormalities, neurological and neurocognitive issues, and a predisposition to cancer. The RAS pathway is a well-known oncogenic pathway that is commonly found to be activated in somatic malignancies. As in somatic cancers, the RASopathies can be caused by various pathogenetic mechanisms that ultimately impact or alter the normal function and regulation of the MAPK pathway. As such, the RASopathies represent an excellent model of study to explore the intersection of the effects of dysregulation and its consequence in both development and oncogenesis.

Keywords

cardio-facio-cutaneous syndrome; clinical trial; Costello syndrome; Legius syndrome; neurofibromatosis type 1; Noonan syndrome; RAS/MAPK; RASopathies; signal transduction pathway; therapy

1 | INTRODUCTION

Signal transduction pathways that are drivers in oncogenesis are also critical pathways in human development and cellular homeostasis. As an example, the RAS pathway plays an essential role in the regulation of growth, differentiation, cell cycle, cell senescence, and apoptosis, all of which have been studied in the context of cancer (Stephen, Esposito, Bagni, & McCormick, 2014). The RAS pathway is also critical to normal development. The RASopathies are a group of medical genetic syndromes that are caused by germ line mutations in genes that encode components or regulators of the RAS/mitogen-activated protein kinase (MAPK) pathway (Rauen, 2013; Tidyman & Rauen, 2016). These components or regulators are the same as those that, when mutated somatically, can also lead to cancer. The RASopathies exhibit numerous overlapping phenotypic features, which may include craniofacial dysmorphology, cardiac malformations, cutaneous, musculoskeletal and ocular abnormalities, neurocognitive impairment, and an increased cancer risk. RASopathies can be caused by several pathogenetic mechanisms that ultimately impact or alter the normal function and regulation of the MAPK pathway. Therefore, the RASopathies represent an

excellent model of study to explore the intersection of the effects of dysregulation and its consequence in both development and oncogenesis.

The *Fifth International RASopathies Symposium: When Development and Cancer Intersect* convened on July 28th to July 30th, 2017 in Orlando, Florida, United States. Clinicians, basic scientists, physician-scientists, advocate leaders, trainees, students, and individuals with RASopathies and their families attended. The meeting was chaired by K.A.R. and F.M. Over 150 registrants participated. The overall goal of this 3-day symposium was to provide an open forum for attendees to share and discuss basic science and clinical issues setting forth a solid framework for future research and translational applications for therapy and best practices for individuals with a RASopathy. Educational funds were provided in part by National Institutes of Health grant number R13CA217038; the March of Dimes grant #4-FY17–900, Pharmaceutical Research and Manufacturers of America (PhRMA), International Costello Syndrome Support Group, Onconova Therapeutics Inc, EveryLife Foundation for Rare Diseases, University of Alabama at Birmingham, School of Medicine, Department of Genetics, Children's Tumor Foundation, GeneDx, Prevention Genetics, Costello Syndrome Family Network, CFC International, the Noonan Syndrome Foundation, and We Work for Health. These proceedings provide the clinical and scientific communities with an executive summary of the clinical translational research symposium.

2 | SESSION 1: WHAT DEFINES A RASOPATHY?

Session 1, chaired by M.Z., explored the question, "What defines a RASopathy?" Advocate speakers L.R. (cardio-facio-cutaneous syndrome; CFC), E.H. (Costello syndrome; CS), B.O. (neurofibromatosis type 1; NF1), and M.E. (Noonan syndrome; NS) began by discussing issues related to aging. Differences between childhood and adulthood issues, conditions that improve, and conditions that worsen or develop with age were addressed from individuals' and syndromic perspectives. Consistently, general health was reported to become more stable with age, particularly for those disorders, where serious and stressful health issues occur frequently in infancy and childhood. Affected individuals develop more self-confidence as adults and learn how to better cope with being different. For NS, an improvement of cognitive performance was mentioned. In contrast, pain, fatigue, and weakness were consistent complaints in adults affected by all the RASopathies. Various neurological and neuropsychiatric problems were reported to develop or worsen, such as depression, anxiety, memory deficits, dementia, and mobility issues. Problems in transition from pediatric to adult care and difficulties accessing appropriate care in adulthood were reported. The patient and family advocates strongly requested the researchers and medical experts to stay interested in issues that adults live with and to continue to heed advocacy support groups.

Following the advocates' discussion, F.M. provided an overview of the RAS signaling pathway, and K.A.R. presented a clinical overview of the RASopathies. The RAS/MAPK pathway is a highly conserved signal transduction pathway that controls cell proliferation. This pathway is present in flies, worms, and mammals. It connects growth factor receptors at the cell surface with transcription factors in the nucleus and is regulated by RAS proteins that act as simple binary ON/OFF switches. In resting cells, RAS proteins are kept in their

off state by proteins called GAPs (GTPase activating proteins), of which the NF1 protein neurofibromin is the most important. When growth factors bind to receptors, RAS proteins are turned ON, which activates, among other effectors, a kinase cascade initiated by RAF kinases (ARAF, BRAF, or CRAF). RAS activates RAF kinases by directly binding and recruiting them to the cell membrane. RAF kinase then activates MEK, which in turn activates ERK. Active ERK translocates into the nucleus to phosphorylate transcription factors that turn on the production of proteins that, in turn, drive cells through the cell cycle into S-phase. Although the RASopathies are a group of medical genetic syndromes caused by germ line mutations in genes that encode components or regulators of the RAS/MAPK pathway, somatic mutations of these components or regulators can also lead to cancer. In the RASopathies, the pathway is hyperactive, causing uncontrolled cell proliferation and dysregulated differentiation. Activation can occur at almost any level in the pathway. Given the common mechanisms underlying RAS/MAPK pathway activation and dysregulation, the RASopathies exhibit numerous overlapping phenotypic features, which may include craniofacial dysmorphology, cardiac malformations, cutaneous, musculoskeletal and ocular abnormalities, neurocognitive impairment, and an increased cancer risk. RASopathies can be caused by several pathogenetic mechanisms that ultimately impact or alter the normal function and regulation of the MAPK pathway. These diverse mechanisms can include functional alteration of GTPases, RASGAP proteins, RAS guanine exchange factors, kinases, scaffolding or adaptor proteins, ubiquitin ligases, phosphatases, and pathway inhibitors—again, the same mechanisms that can be altered in oncogenesis. Although the spectrum of mutations that cause RASopathies and sporadic cancers are different, the molecular basis of these diseases is similar. Furthermore, drugs developed to treat sporadic cancers may have efficacy in RASopathies and vice versa. A better understanding of the pathway in healthy cells and during development, as well as in diseases caused by dysregulation of the pathway, will lead to better therapies in the near future. In concluding the plenary discussion, it was pointed out that there is an obvious lack in knowledge about long-term outcome in RASopathies. Strategies to improve understanding were discussed, including the need for comprehensive medical studies specifically addressing the state of health in adults.

3 | SESSION 2: SYNDROMIC AND SPORADIC CANCERS OF THE RAS PATHWAY

Session 2, led by K.G., focused on the syndromes and the somatic cancers caused by mutations in components of the RAS pathway. The Keynote presentation by N.R. discussed a neurofibroma mouse model, Dhh::Cre; Nf1 fl/fl. Preclinical studies using STAT3 antisense oligonucleotides in combination with MEK inhibitors caused cell death, micro-environmental changes, and shrinking of neurofibromas in mice, suggesting that MEK inhibition may be used to treat young individuals with neurofibromata. The relevance of these preclinical studies was reflected in the presentation by B.C.W. on NF1-related plexiform neurofibromas. Long-term studies on neurofibroma growth delineated a natural growth pattern with the most rapid plexiform neurofibroma growth in young children and concern for transformation to malignant peripheral nerve sheath tumor (MPNST) in adolescent and adult patients with tumor growth more than 20% per year. A phase 1 trial

with the MEK1/2 inhibitor selumetinib for plexiform neurofibromas resulted in tumor shrinkage in most patients, and a phase 2 study is ongoing. Distinct nodular lesions (DNL) were identified by MRI as precursor lesions to MPNST; identification of DNLs may become medically actionable. C.M.L. reviewed pediatric sarcomas, specifically rhabdomyosarcomas. Although CS shows the highest incidence of embryonal rhabdomyosarcoma, other RASopathies show a small increase in the risk for these tumors. Treatment protocols will likely become more personalized in response to the genomic landscape of the individual tumor. H.C. reported on the syndromic etiology of juvenile myelomonocytic leukemia (JMML). RASopathies, including NF1 and NS, predispose to JMML. Certain PTPN11 mutations (targeting p. D61 or T73) are associated with a high-risk for myeloproliferation. Although sporadic JMML is typically aggressive, JMML in individuals with NS shows a large spectrum of clinical presentation from a very aggressive neonatal disease to a benign and transitory myeloproliferative neoplasm. The CBL missense mutation p.Y371H may be particularly prone to JMML, and in this syndrome, JMML often has a benign course with no need for hematopoietic stem cell transplantation. Thus understanding the genomic drivers of syndromic and sporadic malignancies will provide the underpinning for a precision medicine approach to these conditions. There is also a clear unmet medical need for longitudinal follow-up of patients and better ascertainment of the risk of myeloproliferative neoplasm in adulthood.

4 | SESSION 3: HUMAN DEVELOPMENT: EFFECTS ON ORGAN SYSTEMS

Session 3 was moderated by Ashley Cannon and centered on the multiple organ systems affected in the RASopathies. This session highlighted ongoing research pertaining to the dermatologic, nervous, cardiovascular, lymphatic, and gastrointestinal systems. Ashley Cannon presented a prospective natural history study of cutaneous neurofibromas (cNFs) in adults with NF1 that quantitatively showed slow yet significant increases in volume and number over an 8 year period. These data may provide insight into cNF development and benefit clinical trials targeting cNFs. She also described a new clinical trial targeting cNFs with the MEK1/2 inhibitor selumetinib. E.Y. (Lauren Weiss Laboratory) discussed the generation of neurons using iPSC (induced pluripotent stem cells) from CFC individuals and compared morphology and activity with control cells. The CFC cells exhibited decreased progenitor cell populations, indicating that neuronal cells matured more rapidly. E.M.U. described work utilizing mutant HRAS iPSC from patients with CS and transgenic mice. Data from both models indicate that astrocytes expressing mutant HRAS dysregulates cortical maturation during development. He also proposed a previously unknown function of astrocytes in regulating interneuron maturation and potential regulators, such as SNAI2 and periostin, which could be possible drug targets for treating cognitive impairment. G.Z. presented data from a comprehensive survey-based evaluation of pain in 67 patients with a RASopathy. Chronic pain was reported in 55% of the cohort (CS = 68%, CFC = 55%, NS = 45%). Laser-evoked potential studies indicate that peripheral nociceptive transmission is not the cause of pain and is, instead, an altered modulation and elaboration of the painful stimulus at a central level. These results highlight the significance of accurate pain characterization and treatment to improve quality of life for individuals with a RASopathy. B.D.G. discussed modeling cardiovascular involvement in RASopathies. Cardiomyocytes

derived from wild type or mutant BRAF iPSCs exhibited hypertrophy when cocultured with mutant BRAF-derived fibroblast-like cells, which is TGFβ-dependent. Cardiomyocytes derived from mutant HRAS iPSCs exhibited increased beat-to-beat variability, recapitulating features of atrial arrhythmias seen in CS. Cell lines and transgenic *Drosophila melanogaster* with various RASopathy mutations are being used to determine those associated with hypertrophic cardiomyopathy and to identify novel therapeutic compounds. M.I. described advanced lymphatic imaging and interventions in nine patients with NS. Dynamic contrast-enhanced MR lymphangiography and intranodal lymphangiography showed significant central lymphatic abnormalities in all patients. In a subset of patients, intranodal lipiodol injection or embolization of the thoracic duct resulted in symptom resolution. These findings emphasize lymphatic system surveillance, imaging, and interventions in patients with NS. C.S. (Maria Kontaridis Laboratory) discussed modeling gastrointestinal abnormalities using iPSCs derived from NS individuals with various SHP2 mutations. NS cultures had smaller goblet cells with decreased mucin production, whereas mutations associated with NS with multiple lentigines (NSML) exhibited goblet cell hyperplasia, enlargement, and increased mucosal secretion. Studies of SHP2 transgenic mice also showed an abnormal goblet cell phenotype. These data suggest that SHP2 is an important regulator of secretory function in the intestine.

5 | SESSION 4: DEVELOPMENTAL PERSPECTIVE: MODELING RASOPATHIES IN ANIMALS AND IN SILICO

Session 4 was moderated by S.Sha. and focused on modeling pathogenic RASopathy genetic variants in development. S.S. described effects of pathogenic genetic alterations in the MEK1 gene on the RAS/MAPK pathway using the terminal patterning system of the *Drosophila* embryo as a model. Although expression of activated MEK1 increased basal signaling in the central domain of the embryo, it leads to reduction in ERK phosphorylation and a loss-of-function effect at the termini, causing partial loss of the terminal structures. They propose that this contextual effect could be a result of negative feedback, triggered by precocious activation of the RAS pathway, leading to desensitization of the cells to subsequent endogenous signals. E.P. presented results of Perlara's drug discovery efforts using model organisms (nematodes and flies) as patient avatars for rare genetic disorders. He reported on the identification of a novel compound "PERL101" that rescued a nematode model of late-onset Niemann-Pick Type C. Their platform serves as a rapid, cost-effective strategy for primary screening prior to lead optimization in patient cells and preclinical animal models. E.C.S. developed mathematical models to analyze the multiple processes that regulate the RAS/MAPK signaling pathway in cancers and to study effects of perturbations because of disease-causing genetic variants. The model predictions revealed novel pathological processes that have since been validated biochemically. These computational modeling methods can be used for non-cancer RASopathies and allow for rapid understanding and better prediction of the downstream effect of pathologic mutations in the complex RAS/MAPK pathway genes. A.S. reported on deficits in light-off jump habituation, a high-throughput learning test, in *Drosophila* models of nearly 100 genes implicated in intellectual disabilities and autism. These gene defects converged on RAS/MAPK signaling. Furthermore, in RASopathy *Drosophila* models, deficits in habituation

learning originate from increased Ras signaling in inhibitory GABAergic neurons. Lamotrigine, an anticonvulsant and a partial agonist of the hyperpolarization activated cyclic nucleotide gated potassium channel 1 (a recently identified NF1 downstream effector), partially corrected the habituation deficits in NF1 *Drosophila* models administered in adulthood, confirming previous findings by Ype Elgersma's group performed in mice. The efficiency of the *Drosophila* habituation learning test allows for unbiased screening of drug libraries, providing novel opportunities to identify compounds that reverse cognitive phenotypes associated with RASopathies.

6 | SESSION 5: RAS PATHWAY MECHANICS

Led by M.M., Session 5 focused on the biochemical complexities of the RAS pathway. W.Y.C.H. (Jay Grove Laboratory) discussed a discrimination mechanism of Ras activation by SOS on membranes based on dynamics. Simultaneous imaging of individual SOS molecules and localized RAS activation on supported membrane microarrays maps the activation timing resulting from receptor-mediated membrane recruitment of SOS. The gamma-like shape of the activation time distribution reveals rate-limiting kinetic intermediates in the release of autoinhibition and establishes a basis for kinetic proofreading in the activation of RAS. Once activated, a single SOS molecule is highly processive, capable of activating hundreds of RAS molecules. Together, these results suggest that the timing of RAS activation on membranes can play a central role in signal transduction. J.A. spoke on quantifying the cellular effects of RAS pathway mutations with live-cell imaging. A major unanswered question is what differentiates normal from pathological RAS/ERK signaling. It is known that the dynamic patterns of ERK and AKT activity including the strength, frequency, and duration of their activation are essential for proper signaling. Using live-cell imaging, one is able to collect data on mutant-driven ERK and AKT signaling that is far more accurate and detailed than was previously available. These data will reveal the quantitative limits of RAS signal behavior, allow rational choices about which drugs to give to patients with different mutations and result in a mathematical model linking kinase activity and downstream gene expression programs. Finally, M.T. spoke on the allosteric control of RAF activation by dimerization. RAF protein kinases are maintained in an auto-inhibited state in quiescent cells, owing to an intramolecular interaction between their N-terminal regulatory region and their kinase domain. He showed that MEK binding to KSR1 selectively induces BRAF–KSR1 heterodimerization, which allows BRAF to phosphorylate MEK molecules not bound to KSR1. Together, it appears that RAS and MEK collaborate to impinge on the conformation of BRAF and KSR proteins, which in turn drives BRAF–KSR1 dimerization and BRAF transactivation.

7 | SESSION 6: POTENTIAL THERAPEUTICS

Session 6, moderated by D.A.S., examined potential therapeutics and focused on promises and challenges of RAS pathway drug development. The first presentation by B.S.A. discussed the potential role of splice switching oligonucleotides (SSOs) for exon 2 skipping in the RASopathies. The concept was initially based on an individual having CS with a specific mutation in *HRAS* (c.35_36GC > TG; p.G12V) presenting an attenuated phenotype because of exon 2 skipping. Studies showed that HRAS exon 2 has an intrinsically weak 3′

splice site that makes splicing of exon 2 dependent on exonic splicing regulatory sequences (SRS). Employing HRAS and KRAS minigenes, his group was able to show that exon 2 is weakly defined in both RAS genes. Then, they designed HRAS and KRAS SSOs targeting SRS's which successfully mediated HRAS exon 2 skipping in T24 bladder cancer cells and KRAS exon 2 skipping in MiaPaCa pancreatic cancer cells, demonstrating the potential for the translation of SSO-based therapies for specific cancers, CS, and other RASopathies. S.M.F. next presented data about a novel small molecule RAS binding domain antagonist, rigosertib, and its effects for treatment of JMML, which can be associated with NF1 and NS. Findings from studies using rigosertib with myelodysplastic syndrome (MDS), a neoplastic disease of the marrow that has been associated with a multitude of genomic abnormalities including those of RAS or RAS effector proteins, was also discussed. Current clinical trials are underway investigating rigosertib as a single intravenous agent in adult high-risk MDS and as an oral agent in combination with azacitidine with the goal of improving marrow function and overall survival. C.C.G. discussed the difficulty associated with traditional target-based drug discovery approaches compared to target-agnostic approaches. He presented on the use of high-throughput image-based screens at the level of individual cells to develop complex and subtle phenotypic signatures as the basis for a broad drug discovery platform with goals of targeting a large number of disorders. Development was underway for testing a variety of RASopathies with this approach, starting first with those that result from loss of gene function. The final presentation of the session was by P.J.S.S. who discussed data, using pancreatic cancer cell lines with oncogenic RAS mutations, supporting a model that RAS-dependent dimerization of RAF is enhanced by specific phosphorylation on tyrosine 341 (Y341) of CRAF. PhosphoY341 CRAF in RAS-mutant cancer cells can be triggered by the tyrosine kinase SRC (and related SRC family kinases) and blocked by SRC inhibitors raising the possibility that kinases mediating CRAF Y341 phosphorylation may represent a potential therapeutic target in selected RASopathies.

8 | SESSION 7: NEXT GENERATION: JUNIOR INVESTIGATOR POSTER SESSION ABSTRACTS AND CLOSING KEYNOTE

In this session, chaired by B.K., there were four presentations by abstract finalists, who each received a Junior Investigator Award in recognition of the high quality of their work. The session ended with a closing Keynote by F.M.

Nadine Hauer (Friedrich-Alexander University Erlangen-Nürnberg) systematically phenotyped and exome sequenced 565 patients with short stature. A diagnosis was obtained in approximately 30% of patients. A RASopathy was diagnosed in 2%. A mutation in *RRAS* (one frameshift and one missense) was found in two of the remaining patients, by a candidate gene approach. Functional analysis is ongoing. Tamar Green (Stanford University) discussed the outcome of a comparison study of 12 children with NS, versus age- and sex-matched controls, using a fast-spoiled gradient-recalled echo sequence on a 3T MRI scanner. Significantly, smaller anatomic brain volumes were seen in the caudate, putamen, and pallidum in NS. Corpus striatum abnormalities have been associated with ADHD, which has a high prevalence in NS, and similar brain changes have been reported in NS mouse models. The correspondence of these findings will support treatment evaluations in the mouse model.

Fabrice Jaffre (Beth Israel Deaconess Medical Center) studied the RAF1 p.S257L/+ mutation in cardiomyocytes developed from human iPSCs. There is a very strong association between the presence of this mutation and severe neonatal hypertrophic cardiomyopathy (HCM). Increased RAF1 activity was confirmed by elevated phospho-MEK1/2, but ERK expression was only modestly enhanced in mutant cells. Myofibrillar disarray was rescued by ERK1/2 inhibition, but without an effect on HCM. Simultaneous MEK and ERK inhibition rescued both. Lucy Young (UCL Cancer Institute, London) reported on the effect of PP1 and MRAS mutations on dephosphorylation of the inhibitory 14–3-3 binding site on RAF, critical in RAF activation. Dephosphorylation and activation are controlled by a heterotrimeric complex consisting of MRAS, SHOC2, and protein phosphatase PP1. Cellular studies support the model that SHOC2, MRAS, and PPP1CB mutations found in patients with NS enhance complex formation, and thus, RAF dephosphorylation and activation of the RAS pathway.

F.M. presented the closing Keynote address. RAS proteins play a major role in human cancer, causing about one million deaths per year. RAS-driven cancers are refractory to most therapeutic protocols and present a major unmet clinical need. RASopathies, including NF1, affect as many as 300,000 in the United States alone and represent a major clinical need with few effective therapeutic options. Targeting RAS proteins themselves present a technical challenge. These proteins do not possess deep pockets to which small molecules could bind, and are highly conserved and essential to normal cell function. Nevertheless, progress has been made in identifying small molecules that bind either by taking advantage of covalent binding that offsets the need for high-affinity non-covalent binding or by using biophysical techniques that detect low-affinity interactions. Of these, NMR-based fragment discovery has been the most widely used. Recently, a new technique called second harmonic generation has been used to identify novel RAS-binders. Results of this approach were presented. While targeting RAS proteins has been directly challenging, tremendous progress has been made in developing drugs that target downstream pathways, especially the MAPK pathway. Clinical success has been achieved in NF-deficient tumors, a remarkable achievement, but this approach has not yet been successful in tumors driven by mutant RAS. Other approaches to targeting RAS-driven malignancies include unbiased synthetic lethal approaches aimed at finding genes that RAS depends on, whether they are in the direct RAS pathway or not, and immunotherapy-based efforts.

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Correlegan, Prevention Genetics, and GeneDx. C.C.G. is a stock owner and Board member of Recursion Pharmaceuticals, a biopharmaceutical company engaged in discovery and development of therapies for rare disease and certain RASopathies. M.I. is a consultant and research grant recipient from Guerbet Corp. M.M. receives honoraria for serving on the following Scientific Advisory Boards: Novartis, Genentech-Roche, Merck, and Kyras Therapeutics. E.P. is a Perlara stockholder and serves on the Board of Directors.

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