normal *BRaf* promoter/enhances and subject to normal patterns of differential mRNA splicing and usage of initiator AUG codons. Even tissue-restricted expression of $BRaf^{V600E}$ in the developing mouse embryo is a lethal event, suggesting that sustained activation of BRAF → MEK → ERK MAP kinase signaling is incompatible with survival. This may explain the lack of mutations encoding $BRAF^{V600E}$ in humans with CFC syndrome. However, expression of oncogenic $BRaf^{V600E}$ in cells of the adult lung, thyroid, pancreas or in melanocytes leads to rapid development of benign tumors that generally fail to progress to malignancy unless combined with silencing of tumor suppressor genes such as *Trp53*, *Pten* or *Ink4a/Arf*. The implications of these observations for pharmacological targeting of $BRAF^{V600E}$-driven cancers will be discussed.

Modeling Genetic Syndromes of the Ras/MAPK Pathway in Mice

Carmen Guerra. We have generated mouse models for CS, CFC and NS by knocking in the corresponding mutations found in human patients within murine embryonic stem cells. For CS, we have inserted the oncogenic G12V miscoding mutation in the endogenous H-*Ras* locus. H-$Ras^{+/G12V}$ and H-$Ras^{G12V/G12V}$ mice are viable and display many of the defects observed in CS patients including facial anomalies, cardiomyopathies and low tumor incidence. In addition, these mice revealed phenotypes that resembled the hyperemotivity, hypersensibility and cognitive impairments observed in children with CS. Perhaps more importantly, H-Ras^{G12V} mice develop an age-dependent systemic hypertension induced by alterations in the renin-angiotensin II system, a condition that has been observed but not well characterized in CS patients. As expected, this hypertensive condition can be prevented by captopril treatment. We are currently using this CS mouse model to evaluate by genetic means whether inhibition of Farnesyl-Transferase, an enzyme essential for the oncogenic activity of H-Ras oncogenes may provide therapeutic benefit to CS patients. To this end, we have crossed the H-$Ras^{G12V/G12V}$ strain with $FTase^{lox/lox}$ mice previously developed in our laboratory. We hope to be able to present preliminary results at the meeting. For CFC, we have used a mouse strain that expresses low levels of a B-Raf^{V600E} oncogenic allele, thus displaying hyperactive B-Raf protein kinase activity at levels similar to those induced by *bona fide* B-Raf mutations present in CFC patients. These mice are viable and display developmental abnormalities highly reminiscent of those observed in CFC patients. They include reduced life span, facial anomalies, smaller size, cardiomegaly, increased respiratory frequency and seizures. In addition, we have observed other defects in these mice so far not reported in CFC patients, mainly the appearance of cataracts and pheocromocytoma tumors. These mice were engineered so that the B-Raf^{V600E} allele could be conditionally inactivated by expression of the FLPase recombinase. We are currently deleting this hyperactive allele at various times during late embryonic and postnatal development to determine the potential reversibility of the various defects observed in this strain. B-$Raf^{+/LSLV600Efrt}$ mice should also be useful to test the potential therapeutic effect of available B-Raf and Mek inhibitors. Finally, we have just developed the K-Ras^{V14I} knock-in mouse strain as a model for NS. Results from the initial characterization of these mice in hemi- and homozygocity will be presented at the meeting. Full Author List: Carmen Guerra, Jelena Urosevic, Alberto J. Schuhmacher, Vincent Sauzeau, Marta Cañamero, Xosé R. Bustelo. and Mariano Barbacid.

Mice With Endogenous Expression of HrasG12V Have Developmental Defects Consistent With Costello Syndrome and Develop Neoplasms Associated With Copy Number Imbalances of the Oncogene

James A. Fagin. The *HRAS* gene is of particular interest from the standpoint of development, because de novo germline mutations of *HRAS* cause CS, a congenital disorder caused by germline mutations of genes encoding effectors in the Ras/MAPK signaling pathway. Most individuals affected with CS have *HRAS* mutations encoding mutant proteins with comparatively weak transforming activity, primarily HRASG12S. The G12V mutant of HRAS has the lowest GTPase activity among various amino acid substitutions at codon 12 and the highest transformation potential, and is the most common HRAS mutation of this codon in cancer. Accordingly, there has only been one child reported with a germline HRASG12V mutation, which was associated with a particularly severe form of CS. We developed mice with germline endogenous expression of oncogenic HrasG12V to study effects on development and on mechanisms of tumor initiation. They had high perinatal mortality, abnormal cranial dimensions, malocclusion, defective dental ameloblasts and nasal septal deviation, consistent with some of the features of human CS. Older animals (40–58 weeks) developed mild myocardial fibrosis, but no other significant histological abnormalities in the heart, valves or arterial wall. These mice developed skin and forestomach papillomas and angiosarcomas, which were associated with HrasG12V allelic imbalance and augmented Hras signaling. Some tumors had lost the wild-type *Hras* allele, whereas the majority had excess copies of mutant *Hras*. The importance of *Hras* gene dosage on tumorigenesis was confirmed in mouse embryonic fibroblasts expressing either endogenous or supraphysiological levels of the oncoprotein, in which only the latter cooperated successfully with either cMyc or T antigen to induce cell transformation. Endogenous expression of HrasG12V was also associated with a higher mutation rate in vivo. Hence, tumor initiation by HrasG12V likely requires augmentation of signal output, which in papillomas and angiosarcomas is achieved via an imbalance of the ratio of mutant-to-wild-type *Hras* alleles, which may in turn be favored by a higher mutation frequency in cells expressing the oncoprotein.

Animal Models to Study the Effects of Ras/MAPK Signals on Skin and Hair Development Provide a Novel Platform for Studying Biomarkers and Treatments for Costello, Cardio-Facio-Cutaneous and Noonan Syndromes

Benjamin D. Yu. The skin phenotypes of CS and CFC suggest that the skin may be an ideal platform to discover and validate biomarkers for the Ras/MAPK pathway. The availability of mouse models with human CS/CFC skin phenotypes and the relative ease

of in which tissue can be obtained from patients further support the idea that the skin could be used not only to screen for disease but also to determine treatment response. Here we demonstrate that Ras over-activation has specific effects on hair patterning, which can be appreciated through microscopic and molecular studies of isolated hair shafts. Using this model, we have identified the cells and developmental pathways that are influenced by Ras. Importantly, by understanding the effect of Ras signaling on hair development, safe trials could be performed to determine whether cutaneous phenotypes can be locally rescued by topical application of small molecules. Full Authors List: Benjamin D. Yu, Anandaroop Mukhopadhyay, Suguna Krishnaswami and Heather Reilly-Rhoten.

Animal Models of Human Neuro-Cardio-Facial Cutaneous (Ras/MAPK) syndromes

Benjamin G. Neel. The SH2 domain-containing protein-tyrosine phosphatase SHP2, encoded by *PTPN11* in humans (*Ptpn11* in mice), plays a key role in RAS/ERK activation in response to most, if not all, receptor tyrosine kinase (RTK), cytokine receptor and integrin signaling pathways, and may also have cell-type and signal-dependent functions in other downstream signaling cascades. Previous studies have suggested that all signaling functions of SHP2 depend on its catalytic (PTP) activity. Germ line gain-of-function (GOF) *PTPN11* mutations cause 50% of the cases of the autosomal dominant disorder NS, whereas more highly activated, somatic alleles cause juvenile myelomonocytic leukemia (JMML) and other hematological malignancies. Surprisingly, LEOPARD syndrome (LS), another autosomal dominant developmental disorder with strong phenotypic similarities to NS, is caused by catalytically impaired, *PTPN11* mutants that can act as dominant negative mutants in transfection assays. Other genes mutated in NS include *SOS1*, *KRAS*, and *RAF1* (*CRAF*). Remarkably, some NS-associated RAF1 mutants show increased in vitro kinase activity, whereas others are associated with either normal or reduced kinase activity. Unlike PTPN11-associated NS, in which hypertrophic cardiomyopathy (HCM) is rare, human NS caused by RAF1 mutants with increased kinase activity (but not with the other type of RAF1 mutants) shows a strong correlation with HCM. We have generated and extensively analyzed mouse models for *PTPN11*-associated NS, mutant *PTPN11*-evoked JMML, and a zebrafish model for Shp2 LOF and its disease-associated mutants. We have also generated mouse models for increased (L613V) and decreased (D486N) RAF1 activity. These models reveal novel PTP-independent functions for Shp2 and Raf1, and provide a conceptual basis for the distinct and shared pathological features of these disorders. Full Author List: Benjamin G. Neel, Gordon Chan, Rodney A. Stewart, Xue Wu, Jeremy Simpson, Demetrios Kalaitizidis, Toshiyuki Araki, Wentian Yang, Jeffery L. Kutok, Peter Backx and A. Thomas Look.

Animal Models for Noonan Syndrome and LEOPARD Syndrome

Bruce D. Gelb. The Ras/mitogen-activated protein kinase (MAPK) signal transduction pathway altered in patients with NS and related disorders is evolutionarily conserved in the animal kingdom. In the fruit fly, numerous aspects of development, such as the seven photoreceptors of the eye, rely on Ras/MAPK signaling, and mutant alleles exist for all relevant genes. Using genetic tools available for *Drosophila* research, we have generated several transgenic fruit fly models that permit the expression of mutant Ras pathway proteins associated with NS and related disorders in a temporally and spatially controlled manner. For this presentation, our findings with respect to the developmental perturbations caused by these disease-associated transgenic alleles will be shown as well as insights garnered about gene–gene interactions. Particular emphasis will be placed on the perturbations in memory formation engendered by gain-of-function alleles modeling the *PTPN11* form of NS. As certain aspects of memory formation are also conserved in the animal kingdom, these findings provide testable hypotheses concerning neurocognitive development in mouse models.

Genetic Networks and Control of Cancer Susceptibility

Allan Balmain. Self-renewing epithelial tissues such as the skin or intestine undergo frequent tissue damage due to infection by exogenous organisms or exposure to environmental chemicals or radiation. Skin cancer is the commonest form of human cancer, and most other tumors also arise in epithelial tissues. Quantitative genetics has identified polymorphisms within gene regulatory factors or their target genes which cause phenotypic variation and influence disease susceptibility. Human studies have also demonstrated strong associations between polymorphic variation and regulation of gene expression. Applying expression Quantitative Trait Locus (eQTL) approaches to mouse strains with differing susceptibility to diseases, such as obesity, has identified signaling hubs that may be important targets for drug development. We used network construction methods to analyze the genetic architecture of gene expression in normal mouse skin in a cross between tumor-susceptible *Mus musculus* and tumor-resistant *Mus spretus*. We demonstrate that gene expression motifs representing different constituent cell types within the skin such as hair follicle cells, hematopoietic cells, and melanocytes are under separate genetic control. Motifs associated with inflammation, epidermal barrier function, cell cycle control and proliferation are differentially regulated in mice susceptible or resistant to tumor development. The intestinal stem cell marker *Lgr5* is identified as a candidate master regulator of hair follicle gene expression, and the Vitamin D receptor (*Vdr*) links epidermal barrier function, inflammation, and tumor susceptibility. These gene expression networks undergo substantial rewiring during development of benign and malignant skin tumors. Novel network approaches offer substantially greater power than classical methods for identification of genetic factors that contribute to cancer susceptibility.

Clinical Proteomic Approaches to Identify Biomarkers for Ras/MAPK Genetic Disorders

Richard R. Drake. The term clinical proteomics describes the analysis and characterization of the proteins and peptides present

in a particular fluid, cell or tissue-type. In contrast to the genetic information in these systems which are relatively defined and stable, the protein and peptide constituents (i.e., proteomes) are cell and tissue specific, and dynamically change over time in response to different physiological situations. While there are many analytical approaches to characterize proteins and peptides, the past decade has seen a dramatic increase in the use of mass spectrometry based methods, particularly in efforts to define protein changes reflective of disease states (i.e., biomarkers). Mass spectrometers are used because of their absolute sensitivity and specificity to detect mass values of target molecules, as well as an inherent capability to detect multiple analytes in complex samples. In relation to clinical applications, current mass spectrometry-based proteomic technologies possess an equal wealth of possibilities for their use, but a corresponding amount of limitations that have hampered their overall utility. A brief overview of the basis for these promises and challenges will be presented in relation to the instrumentation, sample collection, storage and processing techniques. For the analysis of clinical samples related to Ras/MAPK genetic disorders, collection of urine and saliva represent the least invasive options for use in proteomic-based studies. Previous studies have established that physiological changes in the genitourinary tract and the kidney are reflected by changes in the proteome present in urine. Urinary proteins have also been shown to remain relatively stable after collection, and large quantities can be collected in a non-invasive manner. Initial results from the proteomic profiling of individual urine samples from subjects with CS and CFC will be presented. Issues related to their collection, processing and preparation for mass spectrometry analysis will also be discussed.

Genetics and Stage Specificity of Drug Response in AML

Garry Nolan. Previously, we demonstrated the presence of co-existing blast subpopulations with distinct signaling profiles in adult AML, using phospho-specific flow cytometry. We identified an adverse signaling profile of high inducible phospho-Stat5 and phospho-Stat3 at diagnosis, which was >95% predictive of future relapse in our small cohort (n = 30). Subsequent studies from our group and industrial collaborators with independent patient cohorts has confirmed these results. Activated Stat5 and Stat3 promote survival and proliferation, which may explain why a subclone with enhanced signaling through these pathways would escape drug treatment. Recently, we extended our studies to pediatric AML, and observed dramatic signaling heterogeneity between patients. Our pilot study included one set of paired diagnosis/relapse samples. The GM-CSF-inducible pStat3hi/pStat5hi subset present in this patient at diagnosis doubled in size at relapse, suggesting clonal selection had occurred. To confirm this, we sorted each putative subclone from the diagnosis and relapse samples based on their phospho-flow signaling profiles, and performed copy number analysis by array CGH. The pattern of copy number alterations (CNAs) in each subclone was different, but the presence of several conserved CNAs allowed us to retrace the natural history of each subclone as will be detailed in the presentation. This approach is very powerful because it eliminates much of the noise introduced by patient-to-patient variability: Only the CNAs that differ between subclones in a patient could explain the differences in signaling behavior. A larger cohort of clinical samples will be needed to identify recurring CNAs with distinct functional consequences. Taken together, it is clear from our studies that the cytokine activated Stat 3/5 double positive subpopulation is strongly correlated with a negative outcome in patients treated with Ara-C and daunorubicin. I will demonstrate this and stochastic modeling of other phenotypic markers that suggest a differentiation stage specific resistance to therapy that can predict outcome and survival in AML, and possibly other, myeloid diseases.

Raf Pathway Inhibitors. Selectivity Matters

Gideon Bollag. The discovery of the Ras oncogene led to considerable interest in the anti-cancer activity of Ras pathway inhibitors. The identification of Raf kinase as an important Ras effector subsequently led to drug discovery efforts to identify potent inhibitors of Raf kinase. One such discovery project led to the identification of sorafenib, a potent inhibitor of C-Raf kinase activity in biochemical assays. Despite the successful development of sorafenib as a therapeutic option for patients with metastatic kidney and liver cancers, an understanding of the mechanistic role of C-Raf inhibition remains enigmatic even 10 years since its initial discovery. In the intervening years, identification of the *BRAF* oncogene led to renewed efforts to discover Raf pathway inhibitors. Structure-guided optimization yielded PLX4032, a potent and highly selective inhibitor of oncogenic B-Raf kinase activity. In preclinical cellular and in vivo studies, PLX4032 demonstrates potent anti-cancer activity against tumors bearing oncogenic *BRAF* mutations, while showing no effect on tumors lacking *BRAF* mutations. In non-clinical toxicology studies, PLX4032 exerts no adverse effects even at the highest doses administered, confirming the high degree of selectivity of this compound. Given the favorable pharmacology, PLX4032 has advanced to human clinical trials in cancer patients. Early clinical data have now revealed that PLX4032 has anti-cancer activity in patients with metastatic melanoma. Indeed, significant tumor regressions have been confirmed in patients whose tumors harbor the *BRAF* oncogene; no regressions have been observed in patients lacking *BRAF*-mutated tumors. Thus, Raf inhibition affords an attractive therapeutic option for patients with diseases that rely on the Raf pathway.

Advances in the Development of MEK Inhibitors for Pharmacological Intervention of the Ras/MAP Kinase Pathway

Judith S. Sebolt-Leopold. A number of drugs are being developed to block different components of the Ras–Raf–MEK–mitogen-activated protein kinase (MAPK) kinase cascade. The MAPK kinase kinase MEK has received increasing interest in recent years based on its amenability to the development of exquisitely selective inhibitors that effectively block MAPK signaling. CI-1040 (PD184352) was the first MEK inhibitor to enter the clinic and has served as a prototype for subsequent pharmaceutical improvements. Subsequently, during the past 10 years a multitude of highly potent and bioavailable

MEK inhibitors have emerged as clinical candidates. While MEK inhibitor trials have primarily targeted oncology patients, pharmaceutical companies are also exploring the feasibility of developing these agents for inflammatory indications, such as rheumatoid arthritis. As a MEK inhibitor has not yet won regulatory approval, MEK remains an unprecedented kinase target, and as such carries with it unanswered questions regarding its ultimate safety profile. However, toxicities observed in early clinical trials with MEK inhibitors are proving to be manageable. As expected for agents of this mechanistic class, common toxicities include diarrhea and skin rash. Importantly, there are also encouraging clinical signs of antitumor activity in these trials as evidenced by objective responses in melanoma patients. Because of the high incidence of B-*raf* mutations in the melanoma patient population, many companies are placing a heavy emphasis on this tumor indication for early clinical testing. However, the high incidence of *ras* and *raf* mutations in many other tumor indications, including those of lung, colorectal, pancreatic, and thyroid origin, make these reasonable candidate populations for further study as well. Since it relatively straightforward to prospectively test for *ras* and *raf* mutational status, MEK inhibitors are therefore especially suited to a personalized medicine approach. An overview of the MEK inhibitor field will be provided and will address the current challenges facing their development. Looking forward, there is no longer a need for further improvements in enzyme potency. The playing field now shifts to clinical trial design and how best to use them. Optimization of clinical activity in tumors with extensive genetic heterogeneity is one of the many current challenges that now needs to be addressed. MEK inhibitors will likely find broader application as other targeted agents become available for rational combination regimens. In particular, activation by Ras of the phosphatidylinositol 3-kinase (PI3K)/AKT survival signaling pathway will compromise therapeutic gain from solely blocking the MAPK pathway in many tumors. Agents targeting key steps in this pathway, including PI3K, AKT, and mammalian target of rapamycin (mTOR), are logical candidates for MEK inhibitor combination trials.

Therapeutic Potential of Farnesyltransferase Inhibitors

Jackson B. Gibbs. The enzyme called farnesyltransferase catalyzes a lipid modification on many proteins in the cell. The ras oncogene proteins (called Harvey, Kirsten, and N-ras) are among the substrates of farnesyltransferase. In the case of Ras, placing the lipid on its tail (the "CAAX" box) is essential for its function since Ras proteins are active only when attached to a membrane compartment. In 1990, when farnesyltransferase was discovered, it was thought that inhibition of this enzyme could serve as the Achilles heel to inhibit Ras function. The importance of Ras as a tumor promoting gene gone awry in many human cancers had been known for some time, but efforts to inhibit its function proved difficult. An enzyme like farnesyltransferase afforded a target that could be targeted by medicinal chemistry efforts, and several pharmaceutical companies raced to develop such inhibitors. In preclinical models, the inhibitors exhibited excellent anti-tumor activity, particularly against tumors having a mutated Harvey-Ras gene which is very sensitive to inhibition of farnesyltransferase. The inhibitors also were active against tumors having mutated Kirsten or N-Ras, but the activity was weaker. The goal, of course, was to test these potential drugs in clinical trials in humans with cancer. But, the inhibitors also offered valuable pharmacological probes to explore the biology of farnesylation. In these studies, we learned that another enzyme (geranylgeranyltransferase) can add a different lipid to Kirsten and N-Ras proteins when farnesyltransferase is inhibited and allow these forms of Ras to function (a highly significant observation given that mutations in Kirsten and N-Ras are far more frequent than mutations in Harvey-Ras). Investigators also discovered other proteins in the cell whose function also depended on farnesyltransferase, and that inhibiting the lipid modification on these proteins contributed to the anti-tumor activity of FTIs. So, now instead of thinking about farnesyltransferase as the Achilles heel to inhibit Ras function, it was instead considered to be a general housekeeping enzyme that was necessary for cell proliferation. Several clinical trials evaluated the activity of FTIs against solid tumors and leukemias. One of the inhibitors was also tested in children having defects in the NF1 gene. Overall, the results of these trials were disappointing with little evidence for anti-tumor activity. None of the FTIs tested in humans generated sufficient positive clinical data that could enable registration as new drug. Nevertheless, there have been some learnings. Although FTIs have activity against normal cells and indeed in preclinical studies demonstrate side effects such as myelosuppression, the compounds in general have been well tolerated in humans. While FTIs have not proven useful to treat cancer, these compounds may have utility treating genetic disorders driven by mutations in the Harvey-ras gene or disorders having defects in other farnesylated proteins that underlie the respective disease.

Molecular and Cellular Mechanisms Underlying the Learning Disabilities Associated With Disruptions of Ras/MAPK Signaling: From the Lab to the Clinic

Alcino J. Silva. Ras-dependent activation of MAPK has a key role in synaptic plasticity and learning and memory, and disruptions in this signaling pathway underlie a number of genetic disorders that affect cognitive function. For example, mutations in the *NF1* gene, encoding Neurofibromin, a p21Ras GTPase Activating Protein (GAP), cause learning disabilities and attention deficits. Our studies have shown that the learning and memory deficits of a mouse model of NF1 ($nf1^{+/-}$) are caused by excessive Ras/MAPK signaling leading to hyperphosphorylation of synapsin I, and subsequent enhanced GABA release, which in turn result in impairments in the induction of long-term potentiation (LTP), a cellular mechanism of learning and memory. Consistent with increased GABA-mediated inhibition, we found evidence for hypoactivation of key brain regions in fMRI studies of NF1 patients. Recently, we discovered that statins, at concentrations ineffective in controls, can reverse the enhanced p21Ras activity in the brain of $nf1^{+/-}$ mice, rescue their LTP deficits, and reverse their spatial learning and attention impairments. Strikingly, recently completed pilot clinical trials (collaboration with the Elgersma laboratory in Rotterdam) uncovered suggestive evidence that statins may also be able to reverse

cognitive deficits in children with NF1. Additionally, our laboratory is studying the molecular, cellular, systems and behavioral mechanisms of other genetic conditions that involve Ras/MAPK signaling and learning disabilities. We will provide an update of these other studies.

Cardio-Facio-Cutaneous Syndrome Alleles Are Active During Zebrafish Development and Are Sensitive to Small Molecule Inhibitors

E. Elizabeth Patton. The Ras/MAPK pathway is critical for human development, and plays a central role in the formation and progression of most cancers. Children born with germ-line mutations in *BRAF*, *MEK1*, or *MEK2* develop CFC syndrome, an autosomal dominant syndrome characterized by a distinctive facial appearance, heart defects, skin and hair abnormalities, and mental retardation. CFC syndrome mutations in *BRAF* promote both kinase-activating and kinase-impaired variants. CFC syndrome has a progressive phenotype, and the availability of clinically active inhibitors of the MAPK pathway prompts the important question as to whether such inhibitors might be therapeutically effective in treatment of CFC syndrome. To study the developmental effects of CFC mutant alleles in vivo we have expressed a panel of 28 BRAF and MEK alleles in zebrafish embryos to assess the function of human disease alleles and available chemical inhibitors of this pathway. We find that both kinase-activating and kinase-impaired CFC mutant alleles promote the equivalent developmental outcome when expressed during early development. *BRAF* CFC mutations promote an additive effect during development, consistent with both the kinase-active and kinase-impaired *BRAF* CFC mutations acting as gain-of-function mutations during development. We find that treatment of CFC-zebrafish embryos with inhibitors of the FGF-MAPK pathway can restore normal early development. Importantly, we find a developmental window in which treatment with a MEK inhibitor can restore the normal early development of the embryo, without the additional, unwanted developmental effects of the drug. CFC syndrome has a progressive phenotype, and as many of the phenotypic effects develop postnatally, patients may be helped by systemic therapies after birth. Our work shows the zebrafish system as a tractable tool for medical and research geneticists to explore allele activity and therapeutic potential, and establishes a foundation to propel forward the clinical discussion and scientific strategy for assessing the suitability of using currently available cancer drugs to treat the progressive phenotypes of CFC in children. Full Author List: Corina Anastasaki, Anne L. Estep, Richard Marais, Katherine A. Rauen, and E. Elizabeth Patton.

A Clinical Trial Strategy for Children With Hutchinson-Gilford Progeria Syndrome

Mark W. Kieran. Hutchinson-Gilford progeria syndrome (HGPS or Progeria) is a rare autosomal-dominant genetic syndrome characterized by certain features of premature aging. While the clinical phenotype is almost pathognomonic for this disease, it is the accelerated cardiovascular and cerebrovascular disease that results in death, typically in the second decade. Through the creation of an international organization (the Progeria Research Foundation), support for the identification of the gene responsible for the disease was achieved (lamin A). When mutated, the modified lamin A results in an abnormal transcript called progerin. While a detailed molecular understanding of how progerin causes the specific traits associated with this disease remain poorly defined, alterations in the processing of lamin A appear to be an important component in causing disease progression. In particular, the presence of a truncated lamin A molecule linked to a farnesyl group, whose normal function is to transiently sequester lamin A to the cell membrane, results in persistence of this protein at the nuclear membrane. Critical in vitro and preclinical studies have supported the role of this abnormal farnesylated protein in HGPS and have demonstrated that drugs that target farnesylation, and possibly geranylation, can reverse some aspects of cellular atypia, as well as certain aspects of the disease in animal models. With these advances in our understanding of the disease, and at least three classes of drugs that can specifically target critical pathways implicated in HGPS, we have developed a clinical trial strategy in an attempt to ameliorate the severity of the disease. To achieve this goal, an ongoing detailed analysis of the natural history and phenotype of patients with HGPS is required and was provided by the Progeria Research Foundation. This talk will detail the development of these trials, selection of the outcome measures, and choice of the drugs being used. This information may provide a pathway by which other gene mutational syndromes can seek to develop similar comprehensive treatment strategies that are likely to result in more effective therapies for their patients.

Development of Treatments for NF1

Bruce R. Korf. NF1 is a complex multisystem disorder with a wide range of variability of expression and unpredictable rate of progression. Major sources of morbidity include effects of both benign and malignant tumors, learning disabilities, skeletal dysplasias, vascular stenoses, and other problems. Management to date has been limited to counseling, anticipatory guidance, and treatment of specific complications. Benign tumors are typically treated surgically and malignancies with chemotherapy. There is, however, no current approach to slowing or reversing the overall progression of the disorder. Elucidation of the role of the *NF1* gene product, neurofibromin, in the Ras signaling pathway has opened the door to development of new approaches to therapy using drugs that target this pathway. The possibility of conducting clinical trials, though, raises major challenges in terms of definition of outcomes and recruiting sufficient numbers of participants to conduct a conclusive study. Natural history data have been collected on some phenotypes, including plexiform neurofibromas, learning disabilities, and skeletal dysplasias. There are also tools available for assessment of quality of life. Genetic testing is routinely available; though testing provides very limited ability to stratify the disorder due to only a small number of genotype–phenotype correlations established to date. There have been several ad hoc clinical trials conducted in NF1 patients, and for the past several years the Department of Defense Neurofibromatosis Research Program has funded the NF Consortium to conduct clinical trials. The NF Consortium has one currently open trial targeting plexiform neuro-

fibromas with sirolimus (rapamycin). Endpoints are time to progression and reduction of tumor volume. A second placebo-controlled trial of Lovastatin for cognitive disorders in children with NF1 will launch early summer 2009. A third trial for low grade glioma is currently under final review. The Consortium approach offers the advantages of utilizing a set of clinical data collection sites, which improves the ability to rapidly recruit participants, and also facilitates management of the regulatory and data coordination functions. Additional trials aimed at tumor and non-tumor manifestations of neurofibromatosis are being explored.

Fragile X: A Time for Targeted Treatments

Randi J. Hagerman. Fragile X syndrome (FXS) is caused by a full mutation, an expansion of the CGG sequence at the 5' end of the *FMR1* gene. The full mutation leads to methylation of the gene and a subsequent deficit of transcription and therefore a deficit of FMRP, the protein produced by *FMR1*. The allele frequency of the full mutation is approximately 1 in 2,500 in the general population. FMRP is an RNA binding protein that regulates the translation of many messages important for synaptic plasticity. Typically FMRP is an inhibitor of translation so when FMRP is deficient there is up regulation of a variety of proteins. There is enhancement of the downstream components of the metabotropic glutamate receptor 5 pathway (mGluR5) leading to enhanced long term depression (LTD) and also enhanced levels of matrix metalloproteinase 9 (MMP9) levels in patients with FXS. Enhanced LTD leads to long, thin and immature synaptic connections in FXS. These findings have led to the use of targeted treatments in FXS including the use of mGluR5 antagonists and minocycline, which lowers the level of MMP9. The use of mGluR5 antagonists in the mouse model of FXS has improved synaptic structure and plasticity, improved behavior, eliminated seizures, and improved cognition. Preliminary studies of a single dose of fenobam, an mGluR5 antagonist, in adult patients with FXS suggested improved behavior and CNS function as measured by prepulse inhibition (PPI). Further studies of mGluR5 antagonists are underway in patients with FXS. The use of minocycline for 1 month in the fragile X knockout mouse after birth has lead to maturation of synaptic structure, improved behavior and cognitive benefit. A survey of minocycline treatment in patients with FXS suggests improvements in language, attention and academic abilities. Therefore a controlled trial of minocycline treatment in children and adults with FXS is underway. Further molecular studies in lymphocytes of patients with FXS has demonstrated up regulation of the mTOR pathway and down regulation of PTEN consistent with the large head and autism phenotype of individuals with fragile X syndrome. FXS is the most common single gene cause of autism and these molecular findings suggest significant overlap with other neurodevelopmental disorders that have autism spectrum as part of their phenotype, such as tuberous sclerosis. FMRP also regulates a number of other genes associated with autism including the neuroligins and the neurorexins. Further study of the commonalities across disorders may lead to the development of new targeted treatments for FXS. At the upper end of the premutation (55–200 CGG repeats) of *FMR1* there are lowered levels of FMRP which can lead to features of FXS in patients. In addition there is up-regulation of *FMR1*- mRNA levels in the premutation range which leads to RNA toxicity and both aging problems and neurodevelopmental problems in some individuals with the premutation. We commonly see autism spectrum in boys with the premutation in addition to ADHD. RNA toxicity leads to dysregulation of a number of proteins including lamin A/C, heat shock proteins and alpha B crystallin causing enhanced neuronal cell death especially with stress.

Ras/MAPK Pathway From Beside to Bench and Back: Towards Clinical Trials

Roger J. Packer. Although the conditions which result in molecular alterations in the Ras/MAPK signaling pathway, at first glance, seem phenotypically heterogeneic, there are significant overlaps as regards neurodevelopment, cardiovascular, and other issues. This overlapping symptomatology allows the possibility of developing specific therapeutic protocols targeting defects in the Ras/MAPK signaling pathway to alter the course of illness and, even conceivably, reverse some of the manifestations. In NF1, the most common genetic condition with an activation of the pathway, clinical trials are already open evaluating biologic agents interfering with Ras signaling or activated downstream targets for varied conditions, including progressive plexiform neurofibromas, low-grade gliomas, and neurocognitive abnormalities. It is unclear whether one biologic agent will be sufficient, due to compensatory pathways, and there is significant interest in coupling these drugs for better efficacy. Major issues that still need to be explored are the long-term toxicity of many of these drugs on the development of the nervous system, their potential mutagenesis due to activation of feedback loops, and their impact on bone growth. It has already been shown that an mTor inhibitor, rapamycin, can effectively slow the growth of low-grade gliomas in tuberous sclerosis, another condition where the Ras/MAPK pathway is activated. Because of the relative rarity of some of the conditions, such as CFC, CS, and LEOPARD syndrome, it will be necessary for institutions to work collaboratively on these clinical protocols, as it is unlikely that one institution will accrue enough patients to efficiently perform such trials. In addition, there needs to be an agreement in what the outcome parameters are to be and what the eligibility criteria should be across studies; if this is not done, the studies will not be interpretable across different centers and progress will be slowed. One mechanism which has been found to be particularly effective in childhood cancer, especially in the management of rarer forms of childhood cancer, has been the development of cooperative groups with a well-formed infrastructure to rapidly perform clinical trials. Recently, the neurofibromatosis community has embraced this approach, and through the Department of Defense a neurofibromatosis clinical trials consortium has been opened and is accruing patients on multiple trials for children with NF1. A similar consortium is under discussion for patients with NF2. The cooperative group mechanism theoretically allows for the more rapid development of clinical trials and their completion. The infrastructure does not have to be reinvented for each clinical trial and multiple manifestations of disease can be addressed. Smaller institutions, with innovative investigators, can be extremely important in such consortiums, as their ability to share in progress in such rare diseases is not limited by the small number of eligible patients at

their institution. In addition, studies can be planned sequentially, allowing for more rapid progress. Results can be assessed centrally, making outcomes more reproducible and possibly valid. A well-organized infrastructure allowing the rapid accrual of patients, results in clinical trials becoming more attractive to funding agencies and industry. The consortium mechanism is not without its potential pitfalls and can be slowed by in-fighting and unnecessary bureaucracy. However, if done appropriately, a clinical trials consortium for these Ras/MAPK pathway abnormalities holds the promise of dramatically improving the rate and quality of translational investigations.

Aligning Interests Between the Public and Corporate Sectors to Advance Rare Disease Research

Teri Melese. The scale of the challenge to bring transformative therapeutics to patients with common or rare diseases, and the escalating costs of doing so, increasingly requires cross-disciplinary collaboration. At the same time there is a growing imperative for companies to access innovation outside of their own four walls. In order to identify breakthrough strategies for new therapies that will not result in costly failures late in development, there is a need to fill several knowledge gaps to understand more about the molecular underpinnings of disease and the individual response of patients to treatment. As a result, there is a tremendous opportunity to address industry-wide healthcare initiatives together. Rare diseases are therapeutically "neglected" because the costs of drug development cannot be recouped by drug sales due to small patient numbers and the inability in some cases for specific populations to pay for medicines. Of the more than 6,000 rare diseases, fewer than 200 have any therapy. Public/Private Teams are able to facilitate advances in rare disease research by bringing together key complementary resources. Academic Medical Centers and Foundations bring the experts in the biology and genetics of the diseases, experts in patient care, and the biological samples that are the basis for molecular studies. Biotechnology and pharmaceutical companies are an important source of compounds and biologics that will ultimately target key components of pathways involved in rare disease. However, companies face numerous challenges when asked to split indications for a specific compound. As an example there are concerns about price structures; especially when pricing for the two indications is different. There is the potential to run into side effects in a particular population that might not be relevant in the disease setting for which the drug is primarily intended, and might affect the label and thus curtail commercial prospects for a compound. In addition, in the case of the constellation of syndromes being discussed at the symposium, there are difficulties involved in adapting to a pediatric cohort. However, on the positive side, there is an advantage in obtaining accelerated development in a population with no proven therapies. A consideration is that companies might take on the clinical development in a public private partnership for a marketed drug, or a drug in clinical development and, either donate compounds for a very rare condition, or price at the level for the marketed indication. Some rare disease research is currently being advanced in such consortiums or networks; examples of which are progeria and fragile X. Perhaps for an abandoned molecule, companies might consider assigning all rights to the university to clear the path for commercialization. A Federal initiative to advance therapeutic development was formed in May of 2009 called TRND, Therapeutics for Rare and Neglected Disease and will be overseen by the NIH Office of Rare Diseases Research (ORDR) and involve the National Human Genome Research Center and the National Chemical Genomics Center (NCGC). The plan is for this program to work together with academic medical centers, universities and the corporate sector to advance rare disease research and treatment.

POSTER ABSTRACTS

In addition to the platform presentations summarized above, fourteen research groups submitted abstracts and presented their work at the poster session.

Mouse Models of Costello Syndrome

John P. Flaherty, Leslie Haynes, Leah Rae Donahue, and David E. Bergstrom

The Jackson Laboratory. Bar Harbor, ME, USA

CS is a rare, complex, developmental disorder characterized by a constellation of features including failure to thrive, characteristic facies, delay in intellectual development, hypertrophic cardiomyopathy, arrhythmia, and predisposition to both benign and malignant tumors. Past studies identifying gain-of-function mutations in the *HRAS* gene as the causative basis of human CS and strong conservation with the mouse ortholog, *Hras1*, have made the development of a mouse model of CS a reality. To confirm these results and extend animal modeling studies to include various allelic forms of the disease, we are developing five additional mouse models of CS in the CJ7 line of mouse 129S1/Sv-*Oca2*$^{+}$ *Tyr*$^{+}$ *Kitl*$^{Sl\text{-}J}$/J (JAX JR #000090) embryonic stem (ES) cells. Using a recombineering-based "knock-in" approach, we have completed construction of five vectors, each containing a *lox*P-flanked, neomycin resistance (*Neo*^{r}) selection cassette transcribed in the opposite direction of *Hras1* in intron 1; and site-directed mutations to encode each of the following five pathogenic alleles—G12A encoded by the *Hras1* amino acid 12 codon; GCA G12A encoded by the *Hras1* amino acid 12 codon, GCC; G12S encoded by the *Hras1* amino acid 12 codon, AGC; G12V encoded by the *Hras1* amino acid 12 codon, GTA; G12V encoded by the *Hras1* amino acid 12 codon, GTT. ES cells are currently being electroporated with each of the five vectors. Confirmation of proper targeting events, microinjection, development/breeding of chimerae and identification of ES-derived ("germline") mice carrying the CS alleles will follow. Each strain will be deposited into The Jackson Laboratory Repository allowing broad access to the research community. Designed suppression of the CS alleles by the oppositely transcribed *Neo*^{r} transcript will be relieved by crossing to inner cell mass- or germline-specific "deleter" *Cre* lines such as *Meox2-Cre* or *Zp3-Cre*, respectively. Together, these five models hold the potential for uncovering allelic and codon preference influences on development and neoplasia in CS, identifying CS modifying genes, and dissecting tissue-specific facets of CS using spatially controlled *Cre* lines. This work was supported by a generous gift from Dr. Christopher Earl, BIO Ventures for Global Health.

Genomic Duplication of *PTPN11* is an Uncommon Cause of Noonan Syndrome

John M. Graham, Jr.[1], Nancy Kramer[1], Bassem A. Bejjani[2], Christian T. Thiel[3], Claudio Carta[4], Giovanni Neri[5], Marco Tartaglia[4], and Martin Zenker[3]

[1]Cedars-Sinai Medical Center at UCLA, Los Angeles, CA; [2]Signature Genomic Laboratories, Spokane, WA; [3]University Hospital Erlangen, University of Erlangen-Nuremberg, Germany; [4]Istituto Superiore di Sanità, Rome, Italy; [5]Università Cattolica del Sacro Cuore, Rome, Italy

NS is a genetically heterogeneous disorder caused most commonly by activating mutations in *PTPN11*. We report a patient with hypotonia, developmental delay and clinical features suggestive of NS. High-resolution chromosome analysis was normal, and sequence analysis of *PTPN11*, *SOS1*, *KRAS*, *BRAF*, *RAF1*, *MEK*, and *MEK2* was also normal. Array CGH revealed a single copy gain of nine BAC clones at 12q24.11q24.21 (8.98 Mb in size), which encompassed *the PTPN11* locus at 12q24.13 and was confirmed by FISH analysis. A similar case was reported and speculated that such duplications might account for 15–30% of NS cases with no detectable mutation in NS genes. We screened more than 250 NS cases without mutation in known NS disease-causing genes by quantitative PCR, and none of these cases produced results in the duplicated range. We also explored the possibility that de novo changes affecting the untranslated region (UTR) of the *PTPN11* transcript might represent an alternative event involved in SHP2 enhanced expression. DHPLC analysis and direct sequencing of the entire 3′UTR in 36 NS patients without mutation in known genes did not reveal any disease-associated variant. These findings indicate that duplications of *PTPN11* represent an uncommon cause of NS, and functionally relevant variations within the 3′UTR of the gene do not appear to play a major role in NS. However, recurrent observations of NS in individuals with duplications involving the *PTPN11* locus suggest that increased dosage of SHP2 may have dysregulating effects on intracellular signaling.

Modeling Genetic Syndromes on the Ras/MAPK Pathway in Mice

Carmen Guerra[1], Jelena Urosevic[1], Alberto J. Schuhmacher[1], Vincent Sauzeau[2], Marta Cañamero[1], Xosé R. Bustelo[3], and Mariano Barbacid[1]

[1]Centro Nacional de Investigaciones Oncológicas, Madrid, Spain; [2]Inserm 915-Institut du thorax. Faculté des Sciences de Nantes, Nantes, France; [3]CSIC-University of Salamanca, Salamanca, Spain

Understanding the causative role of the Ras/MAPK pathway in Neuro-cardio-facio-cutaneous (NCFC) syndromes, requires the development of genetically modified mouse models carrying the same mutations that have been recently identified in these patients. Mouse models will help to acquire a basic knowledge of the role of the specific mutations in genes of the Ras/MAPK pathway in the developmental defects of these patients. More importantly, these mouse models will be used to assay therapeutic strategies that hopefully will help to treat and or palliate some of the symptoms of these patients. We have recently published the generation of an H-RasG12V knock-in mouse model which are viable and besides their low incidence of tumors, displays some of the features of CS patients: facial anomalies, increased emotivity and sensitivity, and more importantly cardiomyopathies, the most relevant alterations of these patients. These mice will help to characterize the developmental defects of CS patients and to evaluate therapeutic strategies. Indeed, we have described in these mice a frequent alteration in CS patients that was not well described before: an age-dependent renin-angiotensin II dependent systemic hypertension, which can be prevented by captopril treatment. We are currently evaluating in this CS mouse model, by genetic approaches, the therapeutic value of FTIs in CS patients. We have also developed two new knock-in mouse models for two other germline mutations in the Ras/MAPK pathway: B-RafV600E and K-RasV14I. B-RafV600E knock-in mice, which displays reduced expression of the B-RafV600E knock-in allele (10% of the total B-Raf expression), results in typical alterations of CFC patients: reduced life span, facial anomalies, shorter stature, cardiomegaly, increased respiratory frequency and seizures. We have found other alterations not described yet in CFC patients, such as pheocromocytoma and cataracts, which are currently under characterization. Finally, we have just developed a K-RasV14I knock-in mouse model, mutation that has been found in some NS patients. Results from the initial characterization of this mouse model will be presented in this meeting.

Molecular Analysis and Long-Term follow-Up study in Noonan Syndrome and Related Disorders

Tomoko Kobayashi, Yoko Aoki, Tetsuya Niihori, Shoko Komatsuzaki, Shigeo Kure, and Yoichi Matsubara

Tohoku University School of Medicine, Sendai, Japan

NS, CS, and CFC are autosomal dominant congenital anomaly syndromes characterized by a distinctive facial appearance, heart defects, mental retardation and tumor predisposition. Germline mutations in *PTPN11*, *HRAS*, *KRAS*, *BRAF*, *MEK1/2*, *SOS1*, and *RAF-1* have been identified in NS and related disorders. We suggested that disorders with mutations of molecules in the Ras/MAPK cascade may be comprehensively termed "the Ras/MAPK syndromes." To re-classify these syndromes according to molecular diagnosis and re-evaluate clinical manifestations, a website including the details on the MAPK syndromes and mutation data (http://www.medgen.med.tohoku.ac.jp/RasMapksyndromes.html) was launched. According to our mutation analysis of the eight genes, mutations were identified in 151 of 250 patients (60%) with NS and related disorders. Evaluation of detailed clinical manifestations revealed a genotype–phenotype correlation among mutation-positive patients. Pathogenic mutations in as yet unidentified genes remain to be elucidated in the rest of patients. In order to assess the precise prevalence, natural history, prognosis and the rate of tumor development in Japanese patients, we designed a long-term follow-up protocol of patients with CS and CFC.

Cardiac Abnormalities in Costello Syndrome: Clinical and Molecular Analysis

Angela E. Lin[1], Steven D. Colan[2], Mark E. Alexander[2], Robert Hamilton[3], Bronwyn Kerr[4], Leslie Smoot[3], A. Micheil Innes[5], Jeanne Baffa[6], Katia Sol-Church[7], Elizabeth Hopkins[8], Deborah Stabley[7], and Karen W. Gripp[8]

[1]MassGeneral Hospital for Children, Boston, MA; [2]Children's Hospital of Boston, MA; [3]Hospital for Sick Children, Toronto, Canada; [4]CMMUH, NHS Trust, Manchester, England; [5]Alberta

Children's Hospital, Calgary, Canada; [6]Deparmentt of Cardiology; [7]Department Biomedical Research, and [8]A.I duPont Hospital for Children, Wilmington, DE

Introduction: Cardiac abnormalities in CS are important diagnostic and management features, and overlap with other Ras/MAPK pathway syndromes. We report on clinical and molecular studies from an ongoing study of CS, with attention to classification, severity of cardiac abnormality, progression and outcome, and genotype–phenotype correlation. **Methods**: We studied 61 patients with CS; 58 patients enrolled from July 2003 to June 2007 in an IRB approved study with informed consent, plus 3 patients who were part of a study of the prenatal features of CS. We also studied 83 *HRAS*+ literature patients, and compared selected features to those of other Ras/MAPK pathway syndromes. **Results**: In ~80% of the patients, the self-reported parental data form was supplemented with medical records and/or published data. GENERAL: CS was diagnosed by age 1 year in 16 patients (26%); 22 patients (36%) had been followed between 5 and 18 years since their diagnosis. Though most patients (48, 79%) were 5–18 years, 25% (12) were older than 18 years. Most patients were white (49, 75%) with a slight excess of females (34, 54%). Cancer occurred in 10 patients (16%), 7 of whom had hypertrophic cardiomyopathy (HCM). GENOTYPE: All patients had an *HRAS* mutation, most commonly G12S (51, 84%), followed by G12A, G12C (3 each), G12C (2) and T58I, A146V (1 each). G12S was as common among patients with a single cardiac defect (21, 41%) as those with more complex hearts of two or more defects (18, 35%). CARDIAC PHENOTYPES: Patients were classified based on the presence of no (11, 18%), one (19, 31%), two (14, 23%), three (14, 28%) or four (2, 5%) cardiac abnormalities. I. Congenital heart defects (CHD) were present in 28 (45%) broadly defined to include valve anomalies, especially pulmonic stenosis (PS) (12 patients), aortic/mitral/tricuspid and polyvalvar anomalies (8 patients). PS rarely progressed. Mitral valve obstruction was usually related to HCM rather than a primary valve dysplasia. II. HCM was present in at least 36 patients (59%), typically isolated subaortic septal hypertrophy, with /without obstruction (21, 34%). Other types were global left hypertrophy (4 patients), diffuse, mild interventricular hypertrophy (5 patients), unspecified or variable (5 patients). HCM was chronically severe or progressed in 14 patients, stabilized in 12 patients, and resolved in 8 patients. One pt had an operative biopsy showing myocardial disarray. Eight patients underwent myectomy. III. Arrhythmia in CS consisted of atrial tachycardia (27, 44%) especially chaotic atrial rhythm/multifocal atrial tachycardia, ectopic atrial tachycardia (15, 25%). Late onset atrial fibrillation in a 16-year-old is a new finding. In 8 patients (13%) the arrhythmia was chronically severe or worsened. Two patients had ablations. IV. Mild-moderate aortic root dilation was documented in 3 (5%); there was no aortic dissection. Systemic hypertension requiring treatment was reported in 3 (5%), pulmonary hypertension occurred in 1 infant. OUTCOME: There were 4 (7%) deaths, 3 in newborns associated with fetal and postnatal atrial tachycardia; the cause of the sudden death in a 5-year-old boy with severe HCM was probably cardiac. **Conclusions**: This longitudinal analysis of a large ongoing cohort of *HRAS*-proven CS patients confirms the high frequency of cardiac abnormalities, and refines previous observations. PS is rarely progressive; mitral valve disease is usually related to HCM. Atrial tachycardia has a high frequency in CS, and is an important cause of morbidity and mortality in infancy. The resolution of HCM, and the fluctuating pattern of involvement suggests HCM differs from non-syndromic HCM, although the detection of myocardial disarray is a similarity to sarcomeric disease. In anticipation for possible therapeutic trials, the delineation and classification of cardiac abnormalities, especially HCM, may play an important role as a metric.

Perturbation in ERK1/2 Signaling Result in Developmental Deficits Associated With NCFC Syndromes

Joanna Pucilowska[a], Jason Newbern[b], Ivy Samuels[a], J. Colleen Karlo[a], Sulagna Saitta[c], William Snider[b], and Gary Landreth[a]

[a]Case Western Reserve University School of Medicine, Cleveland, OH; [b]University of North Carolina, Chapel Hill, NC; [c]The Children's Hospital of Philadelphia, University of Pennsylvania School of Medicine, Philadelphia, PA

Neuro-cardio-facial cutaneous (NCFC) syndromes are genetically related disorders arising from mutation of elements within a common genetic pathway necessary for the activation of the ERK MAP kinases. These syndromes are typified by cardiac and craniofacial defects arising from perturbations of neural crest development. Importantly, these syndromes are also characterized by a high incidence of cognitive deficits and psychiatric disease. Saitta and others have identified individuals with a 1 Mb microdeletion on chromosome 22 which encompasses the *MAPK1* gene encoding ERK2. These individuals exhibit cardiac and craniofacial abnormalities accompanied by microcephaly and neurodevelopmental deficits. Similarly, deletion or duplication of 16p11.2, encompassing the *MAPK3* gene encoding ERK1, is associated with autism, congenital heart defects and facial anomalies. We have developed murine models of these syndromes in which the ERK genes have been inactivated. Conditional inactivation of ERK2 in the developing neural crest (using Wnt1-cre) resulted in major craniofacial defects including cleft palate, mandibular hypoplasia and shortened maxilla. These phenotypes were exacerbated upon the additional loss of one or both ERK1 alleles. Phenotypic severity was correlated with impaired pharyngeal arch morphogenesis, consistent with perturbation of neural crest development. Knockout of upstream elements in the ERK signaling cascade, MEK1/2 or B/C-raf, within the neural crest, resulted in similar phenotypes. In comparison, little is known about the central nervous system effects of perturbation of ERK signaling pathway. Conditional inactivation of *Erk2* within the developing telencephalic progenitors alters the dynamics of neural progenitor proliferation. *Erk2* knockout mice exhibit thinner cortex due to generation of fewer neurons that is accompanied by an increase in the number of astrocytes in the mature brain. These mice exhibit profound deficits in learning and memory as well as anosmia. Mice in which both Erk isoforms are inactivated in developing telencephalon at E13.5 through the action of GFAP-cre, exhibit a phenotype that is more severe then observed upon inactivation of Erk2 alone. *Erk1/2* inactivation results in a dramatic decrease in neural progenitor proliferation at E14.5 and the near complete loss of basal intermediate neural progenitors that are normally present in the subventricular zone. These changes correlate with a decrease in Map2+ neurons and an increase in uncommitted Nestin+ progenitors in the developing cortex. Importantly, at E16.5 we observe an even greater exacerbation of proliferation

and differentiation defects during neurogenesis than observed in mice lacking only Erk2. The perturbations of neural progenitor cell cycle dynamics were associated with the suppression of cyclin D1 and elevated p27 levels, both of which are downstream effectors of Erk1/2. Furthermore, double S-phase labeling suggests a significant lengthening of the G1 phase of the cell cycle of cortical progenitor cells in mutant animals compared to wild type mice. In conclusion, the developing human brain is exquisitely sensitive to gain as well as loss of function alterations in proteins of the ERK kinase pathway. Our data documents a critical role of Erk1/2 in regulating neural progenitor cells dynamics during cortical development that may also underlie the neurodevelopmental defects observed in NCFC syndromes.

Comprehensive Mutation Analysis in Noonan Syndrome and Functional Characterization of a Novel *KRAS* Mutation

Md. Abdur Razzaque[1], Yuta Komoike[1], Tsutomu Nishizawa[1], Michiko Furutani[1,3], Toru Higashinakagawa[2], and Rumiko Matsuoka[1,3]

[1]International Research and Educational Institute for Integrated Medical Sciences; [3]Tokyo Women's Medical University, Tokyo, Japan; [2]Waseda University, Tokyo, Japan

NS is a developmental genetic disorder which is characterized by short stature, unusual facial characteristics, mental retardation, developmental delay and a wide spectrum of congenital heart defects, most commonly pulmonic stenosis (PS) and hypertrophic cardiomyopathy (HCM). The genes that cause the NS are involved in the Ras–MAPK signaling pathways. We have studied a cohort of 66 clinically confirmed NS patients of Japanese origin. We screened for mutations in the *PTPN11, KRAS, HRAS, ERAS, NRAS, SOS1, Grb1, Grb2, ARAF, BRAF, RAF1, MEK1*, and *MEK2*, and identified mutations 28 in the *PTPN11*, 6 in the *SOS1*, 1 in the *KRAS*, 1 in the *BRAF* and 10 in the *RAF1*. We previously demonstrated a loss-of-function *BRAF* mutation and gain-of-function *RAF1* mutations are a cause of NS. In this study, we characterized a novel mutation in the *Kras* with an amino acid substitution Asparagine to Serine at codon 116 (N116S). To explore the functional consequences of the mutation we determined the ability of Kras mutant identified in NS to activate its downstream effectors by measuring the endogenous phosphorylation status of Erk by Western blotting in cells transfected with constructs constitutively expressing the wild type (WT) Kras or NS associated mutant Kras or oncogenic mutant Kras (G12D). NS-associated *Kras* mutation resulted in enhanced Erk activation. To examine whether the mutation we found in NS has any oncogenic activity or not, we examined the growth in DMEM containing 1% fetal calf serum of NIH3T3 fibroblast cells expressing the WT Kras or NS associated mutant Kras or oncogenic mutant Kras. NS associated Kras mutation had no significant effect on cell proliferation in compared with the oncogenic Kras mutation. In addition, Morpholino knocked-down zebrafish embryos of kras caused heart and craniofacial malformations, and expression of mutated kras resulted in maldevelopment of the heart. Taken together, our findings implicate Kras-N116S mutation exhibits gain -of-function in vitro and expression of this mutant causes maldevelopment of the heart in zebrafish, suggesting a causative agent of NS.

Functional Consequences of a Glutamic Acid Duplication in the Switch 1 Region (p.E37dup) of HRAS Identified in a Boy With Some Features of Costello Syndrome

Georg Rosenberger[1], Lothar Gremer[2], Torsten Merbitz-Zahradnik[2], Susanne Morlot[3], Kerstin Kutsche[1], and Mohammad Reza Ahmadian[2]

[1]University Medical Center Hamburg-Eppendorf, Hamburg, Germany; [2]Heinrich Heine University Medical Center, Düsseldorf, Germany; [3]Medical Centre WagnerStibbe, Hannover, Germany

HRAS plays a central role in the transduction of signals elicited by receptor activation to diverse downstream pathways, thereby controlling many aspects of cellular physiology. Somatic mutations of *HRAS* changing glycine at position 12 or 13 have been identified in various human tumors and lead to constitutive HRAS activation by impairing its GTP hydrolysis activity. The same *HRAS* missense mutations, with p.G12S being the most common, have been detected as germline mutations in patients with CS. CS is a developmental disorder characterized by short stature, mental retardation and heart and skin abnomalities. We identified a de novo heterozygous 3-bp duplication in *HRAS* leading to a duplication of glutamic acid 37 (p.E37dup) in a male patient with some clinical features reminiscent of CS. Here, we compare the consequences of the p.E37dup mutation on HRAS function with HRAS wild-type and $HRAS^{G12V}$. By quantitative fluorescence methods we demonstrated that p.E37dup marginally influenced kinetics of guanine nucleotide binding and Sos1-catalyzed nucleotide exchange. Recombinant $HRAS^{E37dup}$ showed decreased intrinsic GTP hydrolysis activity by threefold, whereas RasGAP neurofibromin-stimulated GTP hydrolysis was completely abolished. Remarkably, interaction of $HRAS^{E37dup}$ with neurofibromin and various effectors was found to be drastically diminished. Accordingly, cell-based co-precipitation of the active GTP-bound form of HRAS with p.E37dup by any effector protein tested (RAF1, PI3K, PLCε, and RALGDS) was markedly impaired supporting the notion that the switch 1 region of $HRAS^{E37dup}$ underwent drastic structural changes. Our data suggest that $HRAS^{E37dup}$ is predominantly present in the active, GTP-bound form in the cell, but most likely is not able to properly transduce signals due to diminished effector binding. However, in COS-7 cells expressing $HRAS^{E37dup}$ we detected increased MEK, ERK, and AKT phosphorylation after growth factor stimulation. Thus, although binding of $HRAS^{E37dup}$ to Ras binding domains of various effectors is impaired, $HRAS^{E37dup}$-expressing cells seem to escape from down-regulating RAS-dependent signaling pathways by yet unknown compensatory mechanisms. The molecular consequences of p.E37dup, particularly increased MAPK and AKT signaling, may account for the clinical manifestation similar to CS in the affected boy.

Ocular Manifestations of Ras/MAPK Dysregulation: A Comparison of CFC, Costello, Noonan Syndromes, and Neurofibromatosis 1

Suma P. Shankar[1], Teri L. Young[2], and Katherine A. Rauen[1]

[1]University of California San Francisco, San Francisco, CA; [2]Duke University, Durham, NC

CFC, CS, NS, and NF1 are developmental disorders that belong to the recently described group of syndromes called RASopathies.

These are caused by germline mutations in genes encoding key components of Ras/mitogen activated protein kinase (MAPK) signaling transduction pathway. Several in vitro and animal model studies have shown that signaling through the MAPK is required for normal ocular development. We evaluated ocular manifestations in CFC and CS patients using ophthalmic medical records and parental reports. We compare these findings to previously published eye findings in NS and NF1. CFC is a congenital disorder caused by mutations in genes of the Ras/MAPK pathway—*BRAF*, *MEK1*, and *MEK2*. CFC is characterized by cardiac defects, distinctive craniofacial appearance and cutaneous abnormalities. We analyzed retrospective patient records from 51 CFC patients with confirmed molecular diagnosis. Visual problems were reported in 49/51 (96%) patients. All patients had cognitive developmental delay. Other commonly reported manifestations were strabismus 37/51 (72%)—exotropia 28/37, esotropia 4/37; refractive errors 30/51 (58%)—myopia 12/30, hyperopia 18/30; nystagmus 21/51 (41%); ptosis 7/51 (13%) and optic nerve hypoplasia in 18/51 (35%). Other features were problems with depth perception, abnormal head posture and amblyopia. CS is caused by missense mutations in the gene *HRAS*. CS is characterized by failure to thrive, neurocognitive delay, distinctive facial features, cardiac involvement, musculoskeletal and cutaneous features. Significant ocular features included strabismus 16/34 (48%); refractive errors 19/34 (55%) with myopia (14/19) occurring significantly more than hyperopia (2/19), type of refractive error unknown (3/19); nystagmus 12/34 (35%) and optic nerve anomalies in 4/34 (0.12%). NS is caused by mutations in genes *PTPN11*, *KRAS*, *SOS1*, and *RAF1*. NS is characterized by a variable degree neurocognitive delay, short stature, distinctive craniofacial features, congenital cardiac anomalies, and bleeding disorders. Previously reported ocular manifestations include hypertelorism, strabismus, anterior segment changes, retinitis pigmentosa, ERG abnormalities and optic nerve abnormalities. NF1 is caused by germline mutations in the *NF1* gene. It is characterized by the presence of cafe-au-lait maculae, intertriginous freckling, neurofibromas and plexiform neurofibromas, and osseous dysplasia. Iris Lisch nodules and optic pathway glioma are the most common ocular features. Others include glaucoma, conjunctival hamartomas, retinal astrocytic hamartomas, and choroidal hamartomas. The high occurrence of ocular features in the syndromes of the Ras/MAPK pathway suggests that it plays an important role in human eye and visual pathway development. Early interventions are likely to benefit patients and warrant routine ophthalmologic evaluation. Further characterization of the ocular manifestations in larger number of mutation positive patients with Ras/MAPK syndromes is likely to help in the development of potential treatment strategies.

Dermatologic Findings in 55 Mutation Positive Individuals With Cardio-Facio-Cutaneous Syndrome

Dawn H. Siegel[1], Jill McKenzie[2], and Katherine A. Rauen[3]

[1]*Oregon Health & Science University, Portland;* [2]*University of Washington Medical Center, Seattle, WA;* [3]*University of California San Francisco, San Francisco, CA*

The dermatologic findings were analyzed in a cohort of 55 individuals with identified CFC causing mutations. Dermatologic surveys were designed by the authors and distributed to the subjects through CFC International or directly by the authors (K.A.R. and D.H.S.) between July of 2006 and October 2008. A second follow up survey was collected between December 2007 and October 2008. Cutaneous features were present in all mutation positive CFC subjects with no clear genotype–phenotype correlation elucidated in this study. Common features reported included sparse eyebrows, sparse hair at the temples, and poor hair growth. Numerous acquired melanocytic nevi and an increased incidence of infantile hemangiomas were additional findings. The analyzed data were collected via surveys filled out in most cases by the parents of the subjects rather than by a physician. The dermatologic features of CFC syndrome are distinctive and help to distinguish it from the other Ras/MAPK pathway syndromes.

An Innovative Approach to Specialty Genetics: The UCSF NF/Ras Pathway Genetics Clinic

Michelle N. Strecker and Katherine A. Rauen

University of California San Francisco, San Francisco, CA

Providing comprehensive care for individuals with rare syndromes is often challenging; particularly given that specialty health care providers cannot be expected to be well-versed on a plethora of rare disorders. Individuals with rare syndromes also frequently experience difficulty transitioning from pediatric to adult care due to their multi-specialty needs. We have developed a new clinical model to serve individuals with syndromes caused by germline mutations in genes that encode various components of the Ras/mitogen activated protein kinase (MAPK) pathway. These syndromes include: NF1, NS, CS, CFC, LEOPARD syndrome, Legius syndrome, capillary malformation-AV malformation syndrome and gingival fibromatosis. Although individually, these genetic syndromes are rare, when taken together, these syndromes may represent one of the most common classes of Mendelian genetic disorders. The common underlying pathogenetic mechanism of the Ras/MAPK disorders leads to significant overlap in phenotypic features as well as the resulting specialty care needs. It is this overlap that inspired the idea of a pathway-based (instead of a disease-based) genetics clinic. Building off of the Children's Tumor Foundation's neurofibromatosis satellite clinic model, we developed a comprehensive network of over 50 specialists from pediatric and adult care, as well as practitioners in reproductive endocrinology, obstetrics and gynecology and perinatology. Through this pathway-based approach to care, we are able to provide comprehensive management of complex cases and multidisciplinary referrals to our carefully selected network of specialists; all of whom are located at a single tertiary care center. Our ability to provide care throughout the life cycle allows us to facilitate smooth transitions from pediatric to adult multidisciplinary care. In the past 5 months alone (January 2009 to May 2009), the clinic served 36 individuals with complex health needs: three with optic pathway gliomas; two with malignant peripheral nerve sheath tumors; two with longstanding chronic pain; and both a child and an adult with NF1-associated hypertension. We made 14 referrals for cardiac evaluations, 5 for endocrinology, 2 for gastroenterology, 18 for neurology and nine for ophthalmology. Preliminary patient feedback has been very positive. In order to help insure the continued success of the clinic in meeting patient needs, we have established an external patient advocacy advisory board and an internal scientific

advisory board to provide feedback and guidance as the clinic continuously evolves. As we move toward becoming a center for translational medicine, we will serve as a hub for ongoing research and clinical trials. We anticipate that this novel clinic will serve as a model for other institutions.

Prenatal Phenotype and Neonatal Presentation of Cardio-Facio-Cutaneous Syndrome

Susan H. Tran and Katherine A. Rauen

University of California, San Francisco, San Francisco, CA

Objective: To delineate the prenatal phenotype and neonatal characteristics of CFC. **Study Design**: From January 2007 to September 2008, a survey was conducted via CFC International. Data was collected on CFC mutation type, maternal characteristics, pregnancy, delivery, and neonatal outcomes, with medical record review performed when available. Summary statistics were calculated using Microsoft Excel. **Results**: We identified 38 individuals with diagnostic mutations in BRAF (n = 30), MEK1 (n = 6), or MEK2 (n = 2) genes. The median age of participants was 8.5 years. Hyperemesis gravidarum, gestational hypertension, preeclampsia, and gestational diabetes occurred in 13%, 5%, 8%, and 8% of pregnancies, respectively. Prenatal testing included: 0/3 abnormal first trimester nuchal translucency ultrasounds; 0/14 abnormal amnio or CVS karyotype studies; and abnormal second trimester maternal serum triple marker screening in 28% of respondents. Decreased fetal movement was reported in 29%. Various second and third trimester ultrasound abnormalities were reported (Table). Delivery occurred via spontaneous vaginal, operative vaginal, or cesarean delivery in 35%, 19%, and 46%, respectively, and at a median gestational age of 35.9 weeks with median birth weight of 3,233 g. Neonatal complications included feeding tube requirement (57%), irregular heart beat (24%), and hypoglycemia (11%). **Conclusion**: CFC is a multiple congenital anomaly disorder characterized by craniofacial anomaly, ectodermal abnormalities, congenital heart defects, and developmental and growth delay. In mutation-positive individuals, the prenatal phenotype includes polyhydramnios, renal and cardiac abnormalities, macrocephaly, and macrosomia on second and third trimester ultrasound. Elevated rates of operative delivery and neonatal complications were also noted.

Rates of ultrasound findings in CFC syndrome

Ultrasound finding	n (%)
Polyhydramnios	25/38 (66%)
Renal abnormality	8/36 (22%)
Other abnormalities	7/34 (21%)
Macrocephaly	7/35 (20%)
Macrosomia	6/36 (17%)
Heart abnormality	6/35 (17%)
Brain	3/35 (9%)
Neck	3/34 (9%)
Bones	2/36 (6%)
Face	0/35 (0%)

Perturbation in ERK1/2 Signaling Results in Cardiac and Glandular Defects Associated With NCFC Syndromes

Michiko Watanabe[a], Natalie Cherosky[a], Jason Newbern[b], Ivy Samuels[a], Yong-Qiu Doughman[a], Jamie Wikenheiser[a], Madhusudhana Gargesha[a], Ganga Karunamuni[a], J. Colleen Karlo[a], Sulagna Saitta[c], William Snider[b], and Gary Landreth[a]

[a]Case Western Reserve University School of Medicine, Cleveland, OH; [b]University of North Carolina, Chapel Hill, NC; [c]The Children's Hospital of Philadelphia, University of Pennsylvania School of Medicine, Philadelphia, PA

Neuro-cranio-facial cutaneous (NCFC) syndromes arise from mutations in genes that impact the ERK/MAP kinase pathway. Phenotypes associated with a different class of syndromes (DiGeorge and related syndromes), also appear to arise from the disturbance of this pathway. Individuals with the latter syndromes have cardiac and craniofacial defects associated with perturbations of neural crest development. Saitta and colleagues have identified individuals with a 1 Mb microdeletion on chromosome 22 which encompasses the MAPK1 gene encoding ERK2. These individuals exhibit cardiac and craniofacial abnormalities. Deletion or duplication of 16p11.2, encompassing the MAPK3 gene encoding ERK1, is also associated with congenital cardiac and craniofacial defects. This spectrum of defects in individuals lacking ERK pathway genes was replicated in mice that were lacking ERK genes only in neural crest cells. Neural crest cell-specific deletion of the genes for upstream elements of the ERK cascade (B-RAF and C-RAF, MEK1 and MEK2) as well as deletion of a critical downstream effector, the transcription factor serum response factor (SRF), resulted in defects similar to those that resulted from the deletion of ERKs. From these and other findings, we favor the hypothesis that neural crest cells are particularly sensitive to perturbations involving either over-stimulation or under-stimulation of the ERK pathway, and that these perturbations can result in the craniofacial and cardiac defects observed in several classes of human syndromes. Neural crest cells are known to have profound effects on the development of the heart. They influence physiology of the tubular heart, regulate the differentiation of the secondary heart field, are involved in the septation of the outflow tract of the heart, provide cells for the innervation of the heart, and influence the maturation of the conduction system of the heart. Our analyses of the hearts of embryonic mice with ERK pathway components perturbed in neural crest cells (NCC) suggest that several roles for the NCC are disturbed in these mice. We identified conotruncal defects such as persistent truncus arteriosus (PTA) and double-outlet right ventricle (DORV) that have been identified in individuals with DiGeorge and related syndromes. In addition we observed ventricular wall abnormalities. Further analysis of the steps in neural crest cell development of these transgenic embryonic mice will be pursued to identify the mechanisms that lead to these and other cardiac defects. Analysis of the expression pattern of the activated form of ERK (pERK) revealed the surprising finding that it is present at discrete sites at defined windows of developmental time. By focusing on cardiac development, we discovered that pERK is expressed in the cytoplasm and nuclei of NCC from the time they are within the neural tube, when they reach the pharyngeal arches and form the bulk of the mesenchyme surrounding the secondary heart field, and when they enter the endocardial matrix of the outflow tract of the heart proper. The elements that activate pERK within NCCs at these stages in their development are the focus of our studies. Other tissues relevant to heart development that express pERK are the epicardial cells that give rise to the vessels

of the heart. With regard to epicardial expression of pERK, our recent findings indicate an intriguing link between ERK signaling in epicardial cell lines and the development of lymphatics. Investigation of this connection may elucidate why individuals with NS and other related syndromes may suffer lymphedema.

A Restricted Spectrum of *NRAS* Mutations Causes Noonan Syndrome

Martin Zenker[1], Ion C. Cirstea[2], Kerstin Kutsche[3], Amy E. Roberts[4], Denise Horn[5], Bruce D. Gelb[6], Mohammad R. Ahmadian[2], and Marco Tartaglia[7]

[1]University of Erlangen-Nuremberg, Erlangen, Germany; [2]Heinrich-Heine University Medical Center, Düsseldorf, Germany; [3]Universitätsklinikum Hamburg-Eppendorf, Hamburg, Germany; [4]Children's Hospital Boston, Boston, USA; [5]University Medicine of Berlin, Germany; [6]Mount Sinai School of Medicine, New York, USA; [7]Istituto Superiore di Sanità, Rome, Italy

NS, CFC and the related disorders of the neuro-cardio-facial-cutaneous spectrum are known to be caused by constitutional dysregulation of the Ras/MAPK signaling pathway. Based on the evidence that NS and CFC are genetically heterogeneous with mutations in known disease genes accounting for only 70–80% of cases, a systematic scanning of the entire coding sequence of the *NRAS* gene as another obvious candidate gene was performed on a cohort of 533 individuals with phenotype fitting or suggestive of these disorders and tested negative for mutations in previously identified disease genes. We identified two previously unreported nucleotide changes (c.149C>T and c.179G>A) predicting the amino acid substitutions T50I and G60E, each shared by two unrelated individuals. Three cases were sporadic, and in the remaining family transmitting the trait, the missense change co-segregated with disease. All mutation carriers displayed typical features of NS. Both the identified *NRAS* mutations affected amino acid residues that show strong evolutionary conservation among RAS orthologues and paralogs. Transient expression of both the NS-associated NRAS mutants resulted in enhanced ERK phosphorylation in a stimulus-dependent manner. In $NRAS^{G60E}$, these effects were related to accumulation in the active form and GAP resistance, whereas $NRAS^{T50I}$ was not found to be enriched in the GTP-loaded state, suggesting a different mechanism of functional dysregulation for this mutant. We conclude that NS can be caused by altered signaling through NRAS in a way that resembles KRAS dysregulated function in NS and CFC. Similarly to what observed for the germline *KRAS* mutations, NS-causing *NRAS* lesions do not affect residues recurrently mutated in human cancers, and appear to be less activating in vitro compared to the cancer-associated defects. Based on the large size of the patient cohorts included in the study, germline *NRAS* mutations are estimated to occur with a prevalence of less than 1% in patients with NS.

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Non-Hodgkin lymphoma in a patient with cardio-facio-cutaneous syndrome

Akira Ohtake, MD, PhD[1], Yoko Aoki, MD, PhD[2], Yuka Saito, MD[2], Tetsuya Niihori, MD, PhD[2], Atsushi Shibuya, MD, PhD[1], Shigeo Kure MD, PhD[2], Yoichi Matsubara MD[2]

[1]Department of Pediatrics, Saitama Medical University, Moroyama, Saitama, Japan.
[2]Department of Medical Genetics, Tohoku University School of Medicine, Sendai, Japan.

Correspondence to:
Yoko Aoki, MD, PhD,
Department of Medical Genetics, Tohoku University School of Medicine, 1-1 Seiryo-machi, Sendai 980-8574, Japan.
Telephone: +81-22-717-8139; Fax: +81-22-717-8142
E-mail: aokiy@mail.tains.tohoku.ac.jp

Key words: cardio-facio-cutaneous syndrome, KRAS, BRAF, RAS/MAPK, leukemia

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Summary: Cardio-facio-cutaneous (CFC) syndrome is a multiple congenital anomaly/mental retardation syndrome characterized by a distinctive facial appearance, ectodermal abnormalities and heart defects. Clinically, it overlaps with both Noonan syndrome and Costello syndrome. Mutations in *KRAS*, *BRAF* and *MAP2K1/2* (MEK1/2) have been identified in patients with CFC syndrome. *BRAF* mutations are involved in more than 80% of CFC syndrome patients, and we have previously reported that two CFC patients with *BRAF* mutations developed acute lymphoblastic leukemia. Here we report a boy with CFC syndrome who developed non-Hodgkin lymphoma. At two months of age, he developed pneumonia with pleurisy and was diagnosed as having non-Hodgkin lymphoma (precursor T-cell lymphoblastic lymphoma) by cytopathologic examination of pleural fluid. He was suspected of having Noonan syndrome because of his facial appearance, webbed neck and cubitus valgus. Precursor T-cell lymphoblastic lymphoma was treated by TCCSG NHL 94-04 protocol. At 9 years of age, he was clinically re-evaluated and diagnosed as having CFC syndrome because of his distinctive facial appearance, multiple nevi and moderate mental retardation. Sequencing analysis revealed a germline p.A246P (c.736G>C) mutation in *BRAF* previously reported in CFC syndrome. Molecular diagnosis and careful observation should be considered in children with CFC syndrome.

Key words: RAF, RAS-MAPK, non-Hodgkin lymphoma, cardio-facio-cutaneous syndrome, Noonan syndrome

Cardio-facio-cutaneous (CFC) syndrome is a multiple congenital anomaly/mental retardation syndrome characterized by heart defects, facial dysmorphism, ectodermal abnormalities and mental retardation.[1, 2] Typical facial characteristics include a high forehead with bitemporal constriction, hypoplastic supraorbital ridges, downslanting palpebral fissures, a depressed nasal bridge and posteriorly angulated ears with prominent helices. Affected individuals present with heart defects, including pulmonic stenosis (PS), atrial septal defects and hypertrophic cardiomyopathy. Ectodermal abnormalities including sparse, friable hair, multiple nevi and hyperkeratotic skin lesions are noted. CFC syndrome has many clinical features in common with those of Noonan syndrome and Costello syndrome. Of these three syndromes, Noonan syndrome is most frequent, its incidence being estimated to be 1 in 1000 to 1 in 2500 live births. Compared with the other two syndromes, Noonan syndrome has lower frequencies of mental retardation (24-35%), heart defects (50-67%) and skin abnormalities (2-27%),[3] while CFC patients show a high frequency of growth failure (78.9%), mental retardation (100%), heart defects (84.2%) and skin abnormalities (68.4%).[2] Costello syndrome is characterized by mental retardation, high birth weight, neonatal feeding problems, curly hair, redundant skin, nasal papillomata and tumor predisposition. Wrinkled palms and soles, hyperpigmentation and joint hyperextension, which have been commonly reported in Costello syndrome but not in CFC syndrome, have been observed in 30 to 40% of the mutation-positive CFC patients.[4] Thus, differential diagnosis of these syndromes is difficult because of their overlapping clinical manifestations.

Mutations in molecules in the RAS/MAPK pathway have been identified in these disorders: HRAS in Costello syndrome,[5] *PTPN11*, *KRAS*, *SOS1*, *RAF1* and *NRAS* in Noonan syndrome[6-12] and *KRAS*, *BRAF* and *MAP2K1/2* in individuals with CFC syndrome.[2, 13] Therefore, these syndromes are termed NCFC syndrome or RAS/MAPK syndromes.[14, 15] Approximately 10% patients with Costello syndrome have been shown to have malignant tumors, including neuroblastoma, rhabdomyosarcoma and bladder carcinoma. For Costello syndrome, tumor screening protocols have been proposed.[16] In contrast, little attention has been paid to development of tumors in patients with CFC syndrome.

We herein report a Japanese patient with CFC syndrome who developed non-Hodgkin lymphoma at 2 months of age. Although he had been suspected to having Noonan syndrome, re-evaluation of clinical manifestations and identification of a *BRAF* mutation led to a rediagnosis of CFC syndrome. Our observations, together with a literature review of previous patients, suggest the importance of careful observation for malignancy in CFC syndrome.

CASE REPORT

The propositus was a 12-year-old Japanese boy. He was the first son of unrelated healthy parents. At birth, his father was 27 years old and his mother was 27 years old. Delivery at 40 weeks was done by obstetrical vacuum extraction and birth weight of the patient was 3,488 g (+ 0.8 SD), length was 49.6 cm (+ 0.1 SD), and occipitofrontal circumference (OFC) was 35 cm (+1.0 SD). At two months of age, coughing, rhinorrhea and feeding difficulty were observed. He was admitted our hospital because his chest roentgenogram showed right lung pneumonia with pleurisy. The echocardiography was normal and a computed tomography (CT) revealed right atelectasis and right pleural effusion. There were no mediastinal or axillary lymphadenopathies. Laboratory finding

were as follows: hemoglobin 10.4 g/dl, white blood cells 9.1 X 10^9/L and platelets 404 X 10^9/L. Levels of serum lactate dehydrogenase (LDH) and C-reactive protein were normal. Pleural effusion aspirate had a milky-white appearance, a triglyceride content of 2529 mg/dl and an LDH level of 1656 IU/dl. Cytological examination of pleural fluid showed highly cellular specimens. The majority of these cells were lymphoblasts. These cells showed T-cell phenotype: positive for CD2, CD3, CD5 and CD7 by immunophenotyping study using flow cytometry. No other infiltration was identified by CT/MRI imagings, bone marrow aspiration and Garium scintigram. He was then diagnosed as having T-cell Lymphoblastic lymphoma by cytopathologic examination of pleural fluid (Stage III). At admission, the patient was diagnosed as having Noonan syndrome because of the following anomalies: hyperterolism, downslanting palpebral fissures, low nasal bridge, low set and posterior rotated ears, micrognathia, short and webbed neck, cubitus vulgus, funnel chest and wide nipples. His chromosomal analyses of both peripheral lymphocytes and lymphoma cells showed a normal karyotype of 46, XY.

Induction therapy, which consisted of vincristine, predonisolone, tetrahydropyranyl adriamycin, cyclophosphamide and E. coli asparaginase, was performed for three months. The patient had a good clinical response to the chemotherapy and the chemotherapy was terminated before the entire protocol was finished. No relapse was observed afterwards.

At the age of nine years, he had a distinctive facial appearance, including hypertelorism, underdeveloped supraorbital ridges, sparse and highly arched eyebrows, bilateral ptosis, depressed nasal bridge, concave nasal ridge, broad nasal base, anteverted nares, long philtrum, everted lower lip, and low set and posterior rotated ears (Fig.1). Other features included a webbed/short neck, multiple nevi in his face, cubitus valgus, pectus excavatum, widely spaced nipples and short stature of -3.1 SD. Results of growth hormone provocation tests with arginine and insulin were both normal. Echocardiography and ECG revealed no cardiac abnormalities. His IQ has not been accurately examined, but he attends to a class for the handicapped. At 9 years of age, he was suspected to having CFC syndrome because of his facial appearance, mental retardation and multiple nevi, although no heart defects have been noted.

Mutation analysis

Genomic DNA from blood leukocytes from the patient was isolated by a standard protocol. Fifteen exons with flanked introns in which mutations have been identified in CFC patients as well as *HRAS* exon1, in which mutations are clustered in more than 90% of patients with Costello syndrome, were examined amplified by polymerase chain reaction (PCR). These were exons 1, 2 and 5 in *KRAS*, exons 6 and 11-16 in *BRAF*, exons 2 and 3 in *MAP2K1* and exons 2,3 and 7 in *MAP2K2*.[2,4] The PCR products were gel-purified and sequenced on an ABI PRISM 310 automated DNA sequencer (Applied Biosystems). This study was approved by the Ethics Committee of Tohoku University School of Medicine. We obtained informed consent for the sample and specific consent for a photograph. Sequencing analysis showed a G to C change at nucleotide 736, resulting in an A246P mutation of *BRAF* in the heterozygous form.

DISCUSSION

The patient with CFC syndrome herein reported developed right lung pneumonia with pleurisy at 2 months of age. He was firstly diagnosed as having Noonan syndrome. The cytopathologic examination of lymphoblasts in pleural fluid showed T-cell phenotype

and the patient was diagnosed as having precursor T-cell lymphoblastic lymphoma. At 9 years of age, a *BRAF* A246P mutation was identified in the patient. We concluded that the patient had CFC syndrome because of his distinctive facial features, including underdeveloped supraorbital ridges, sparse and highly arched eyebrows, ptosis, growth failure, moderate mental retardation and multiple nevi in skin, although no heart defects were observed.

Mutations in *BRAF* identified in CFC patients partially overlap those identified in cancers. V600E mutation, which is most frequently identified in cancers, has never been detected in CFC patients and activation of downstream ERK is weaker in germline mutations than V600E.[15] The association with malignancy is rarer in CFC syndrome than in Costello syndrome with germline *HRAS* mutations, 10% of which develop malignant tumors including rhabdomyosarcoma, neuroblastoma and bladder carcinoma. Four patients with CFC syndrome have been found to develop malignant tumors (Table 1). We have previously reported two patients with *BRAF* mutations who developed ALL.[2, 17] A CFC patient with a MEK1 mutation has been reported to have develop hepatoblastoma after cardiac transplantation.[18] To date, 104 CFC patients with *BRAF* mutations and 42 with CFC patients with MEK1/2 mutations have been reported.[15] The frequency of the association with malignant tumors cannot be neglected. Careful observation should be considered in children with CFC syndrome and related disorders.

Precursor T-cell lymphoblastic lymphoma accounts for approximately 33% of pediatric non-Hodgkin lymphoma and most commonly involves the mediastinum and lymph nodes.[19] The precise incidence of NHL in children is not known and development of NHL in early infantile period is rare.[20] It is possible that our patient developed NHL at two months of age due to having the germline mutation in the proto-oncogene *BRAF*.

The contribution of somatic mutations in *BRAF* to hematologic malignancy has been controversial. Lee et al. identified *BRAF* mutations in four cases with diffuse large B cell lymphoma (two cases with G468A, one with G468R and one with D593G) [21] and four cases with leukemia (G468A) (Table 2) .[22] Dustafsso et al. investigated exons 11 and 15 of *BRAF* in 29 cases with pre-B ALL (25 cases), T-cell ALL (3 cases) and undifferentiated ALL (1 case) and identified *BRAF* mutations in six cases (21%).[23] Christiansen et al. identified three *BRAF* mutations in 3/51 therapy-related AML, but not in therapy-related MDS.[24] In contrast, Davidsson et al. did not identify any *BRAF* mutations in 92 B-cell precursor ALL and 17 T-cell ALL.[25] In other studies, no *BRAF* mutations were identified in 86 cases with childhood ALL,[26] in 104 cases with AML[27] and in 65 cases with JMML.[28] Recently, it has been postulated that two general types of gene mutation cooperate leukemogenesis.[29] Class I mutations are mutations with receptor tyrosine kinases or genes downstream in the RAS/RAF/MEK/ERK pathway which result in a proliferative or survival advantage. Class II mutations impair hematologic differentiation. BRAF is a member of RAF serine/threonine family and transmits the RAS signal to downstream MEK/ERK. Somatic mutations in *BRAF* have been identified in approximately 7% of tumors, including malignant melanoma, pancreatic tumors and lung cancer.[2] *BRAF* mutations are involved in Class I mutation and possibly contribute to leukemogenesis.

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