DOCTORAL THESIS

The Clinical Description, Molecular Etiology, and Pathophysiological studies in Cutaneous Skeletal Hypophosphatemia Syndrome: a Mosaic Disorder of Activating RAS Mutations

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Syndrome: a Mosaic Disorder of Activating RAS Mutations

5. DISCUSSION

In the present work, the clinical features of CSHS have been characterized through detailed study of a well-defined cohort, supported by findings from an exhaustive literature review. In addition, a series of experimental *in vitro* and *in vivo* models have been developed in an attempt to replicate certain aspects of CSHS to better understand the underlyling pathophysiology of the disease. Key features of the analysis include a broad spectrum of skeletal findings, the novel observation of age-related clinical improvement in skeletal disease, evidence that phosphate and active vitamin D treatment, but not nevi removal, are effective at healing rickets and improving hypophosphatemic symptoms, and the lack of reports of malignant transformation of skeletal lesions. It must be emphasized that *RAS*-specific sequencing has not been performed in most reported cases compatible with CSHS, thus at present we cannot attribute the genesis of this syndrome and its manifestations solely to hyperactive RAS. Nevertheless, given that *RAS* mutations have been identified in the majority of epidermal and melanocytic nevi in its isolated and syndromic forms [57-60], and in other CSHS cases subject to specific sequencing [62], we reckon that RAS mutations represent CSHS's main molecular etiology.
To the best of our knowledge, the research here displayed represents at this day the most thorough effort to conceive this syndrome from a clinical and translational standpoint and to establish the role of RAS in FGF23 production.

The following subsections are structured to discuss the results of the work in relation to the thesis objectives.

5.1 Comprehensive analysis of CSHS clinical spectrum and natural history (Objective I)

The detailed phenotyping of the CSHS cohort and the analysis derived from an exhaustive literature review has allowed us to describe several clinical and pathophysiological features of CSHS, with a special focus on the skeletal component of the disorder.
As expected in patients with hypophosphatemic rickets and skeletal dysplasia, fractures and deformities affected the majority of CSHS subjects. Similar to other mosaic disorders, such as MAS [4], the distribution and location of the dysplastic skeletal lesions varied, affected all skeletal compartments, and was not limited to the side of the body with the nevus. The spine was the skeletal site least affected by dysplasia. However vertebral lesions are often difficult to discern on plain radiographs, which was often the only study performed, and the lower prevalence of spine involvement could be attributed to this. Scoliosis, on the other hand, was extremely common. The etiology of scoliosis in CSHS is not clear. It could be related to osteomalacia, but it should be noted that scoliosis has also been reported in association with other ENS in the absence of hypophosphatemia [186]. Hemibody asymmetry, impaired mineralization and possible underlying dysplastic vertebrae could account for the high prevalence of scoliosis in CSHS.

While skeletal dysplasia is a defining feature of CSHS, it was not mentioned in a third of the published cases. In these reports, it was impossible to determine if skeletal dysplasia were truly absent or if it were simply not appreciated or reported. The overall incidence of skeletal dysplasia was likely underestimated, given that in some cases it was clear from the images in the paper [53, 167, 170, 173] or confirmed personally by the authors [50] that dysplastic lesions were present, but either not reported or reported simply as rachitic changes. In addition, abnormalities in phosphate were not reported in 7/51 subjects from the published reports, although 4 of these subjects had a past medical history of numerous insufficiency fractures [161, 189, 190, 193] suggesting that hypophosphatemia might have been present at some point, but was not recognized. However, it is also possible that these cases in which hypophosphatemia was not detected were either erroneously classified as CSHS, or on a less severe end of the clinical spectrum.

Unlike other genetic forms of FGF23-mediated hypophosphatemia, in which rickets develops in infancy (e.g., X-linked hypophosphatemic rickets), the onset of rachitic changes in CSHS is delayed. The range of the age of onset of hypophosphatemic manifestations in our cohort and in the literature review was broad (mean: 4 years, median 2.7 years, SD 3.6, range 1-14 years), emphasizing the importance of routine phosphate assessment in pediatric patients with ENS. In addition to the delayed clinical onset, an age-related remission of mineral abnormalities was also appreciated in some patients. These important findings are similar to what is seen in patients with FD. In FD, which like CSHS is a disease of skeletal stem cells caused by somatic mosaic expression of gain-of-function mutations in GTPases ($G_s\alpha$) [199], delayed onset is attributed to the time it takes for patients to develop a sufficient mass of dysplastic tissue, while the loss of phosphate wasting is attributed to age-dependent apoptosis of mutation-bearing skeletal stem cells [144]. It is intriguing to speculate that a similar mechanism, age-dependent apoptosis and/or oncogene-induced senescence [200], may be responsible for the age-related waning

of symptoms in CSHS. Prospective long-term follow-up of our cohort and other CSHS patients will help determine the likelihood of skeletal disease resolution in these patients.

Since the availability of FGF23 assays, elevations in FGF23 have been consistently reported in CSHS. All cases in which complete biochemical panels were assessed, displayed a profile compatible with FGF23-mediated hypophosphatemia. Therefore, it is overwhelming likely that excess FGF23 is the cause of hypophosphatemia in CSHS, although secondary hyperparathyroidism due to vitamin D insufficiency, as observed in some cases, is probably contributes to lower serum phosphate and impair mineralization.

The best, currently approved, medical therapy for the hypophosphatemia of CSHS is the combination of oral phosphate and active vitamin D (e.g. calcitriol). Typically, 20-40 mg/kg of elemental phosphorus is given split among 3-5 daily doses. Active vitamin D is necessary given that FGF23 suppresses the action of 1-α-hydroxylase, which is necessary for the conversion inactive 25-hydroxy vitamin D to active 1,25-dihydroxy vitamin D. Not surprisingly, and similar to what was attempted in the early days of X-linked hypophosphatemia treatment, most subjects treated with non-active analogs of vitamin D did not experience clinical recovery. An optimized regimen with good patient adherence has the potential to reverse symptoms associated with rickets [201]. However, non-adherence is common due to the frequent dosing requirements (≥ thrice daily) and gastrointestinal side effects of oral phosphate. Emerging therapies that may prove effective in the treatment of CSHS, and other disorder feauturing FGF23-mediated hypophosphatemia, include cinacalcet, which diminishes the phosphaturic effects of FGF23 [202], and an anti-FGF23 monoclonal antibody, which blocks the action of FGF23 [203]. In fact, there is currently an ongoinig trial of aniti-FGF23 antibody in ENS-related hypophosphatemia (cNCI trial # NCT02722798). In addition, FGFR1 inhibitors, such as BGJ398, which act on the RAS pathway, and which are associated with an increase in serum phosphate, may be a potential treatment for CSHS [204].

Neoplastic growth was frequent in our cohort and published reports, which is not surprising given that *RAS* mutations are known to lead to unchecked cell proliferation and tumor development [205]. Although the vast majority of the neoplasms that have been identified in CSHS (table 4.9) have not undergone genetic sequencing, we assume that they are probably directly associated to CSHS's pathophysiology since the same *RAS* mutations that cause CSHS are frequently detected in those neoplasms in their sporadic forms [206]. In regards to CSHS' nevi malignant potential, CMN are the lesions that have the highest risk for cancerous transformation, albeit the risk for melanoma development appears to be very variable depending on the study (<1-5%) [207]. Currently is unclear whether preventive surgical excision reduces the risk of melanoma. It is not uncommon for NS to transform into secondary benign tumors over time such astrichoblastoma or syringocystadenoma papilliferum, although the risk for malignant transformation, most frequently in the form of a basal cell carcinoma, is very low (<1%) and more common in patients 40 years or older [208]. The risk for malignant transformation of KEN is extremely rare [206]. Therefore, it is not currently recomended to remove EN for cancer prevention.

Despite the abundance of dysplastic and mutated skeletal tissue in CSHS, there were no reports of osteosarcoma or other skeletal malignancies. This could be related to the scarcity of *RAS* mutations found in bone cancer [209]. The lack of published cases of bone cancer in CSHS is comforting, but due to the small number of subjects reported it is advisable to err on the side of caution and evaluate the bone lesions periodically.

The extra-cutaneous/extra-osseous abnormalities detected in our cohort and in the CSHS literature are similar to those encountered in other subtypes of nevoid syndromes, i.e. disorders characterized by the association of epidermal and/or melanocytic nevi with mosaic abnormalities in other tissues. We speculate that the postnatal phenotype, which ultimately defines a nevoid syndrome subtype e.g. Schimmelpenning syndrome [11], CSHS, or neurocutaneous melanosis [210] among others-, depends on the tissues derived from the multipotent stem cell that was initially mutated.

No apparent association with sex or ethnic background was detected with CSHS's incidence. This may be explained by the fact that postzygotic mutations are considered random events and not the result of Mendelian inheritance [3],

The most common cutaneous findings in our cohort and in the literature were EN. However, given that CSHS is very rare and that EN occurs in approximately 1/1000 births [8], it is likely that the relative prevalence and association of CSHS with PPK is much higher, given that PPK is extremely rare (only ~30-40 reports in the literature [15]), yet it was found in 16/51 of the published cases and in CSHS106. The skin lesions that characterize PPK, both epidermal and melanocytic nevi, originate from the ectoderm but have different embryonic origins (epidermis and neural crest, respectively). Yet, both types of lesions bear the same mutation implying mutagenesis in an early common progenitor prior to ectodermal differentiation [58]. It is likely that this mutated ectodermal progenitor derived from an even more primitive mutated pluripotent cell, explaining the high incidence of abnormalities in tissues derived from non-ectodermal layers (such as the skeleton) in PPK. In contrast, EN originate from the epidermis, so they can be caused by mutations either in very early progenitors, which could result in large cutaneous lesions and multi-systemic syndromes, a.k.a. ENS, or by mutations in more differentiated cells that result in small and solitary birthmarks, which constitute the most common presentation for an EN. The low frequency of giant CMN could be attributed to its extremely low incidence (1<20,000 newborns) [13].

5.2 Insights on the physiopathology of FGF23 overproduction in CSHS (Objective II)

The absence of additional mutations in the exome data from our index cases observed in our prior study [61], suggest that an activating *RAS* mutation is sufficient to drive FGF23-mediated hypophosphatemic rickets. Yet, the tissue responsible for secreting FGF23 in excess in CSHS remains undetermined. In spite of the lack of a conclusive answer, several observations indicate that FGF23 is probably not produced by the skin lesions. First, the supernatant obtained from ground nevi in CSHS101 and CSHS105 did not demonstrate measurable levels of FGF23 when measured via ELISA (Figure 4.7). The results were validated as the positive controls for this experiment were ground PMTs from a patient with TIO, which showed very high levels of FGF23. In addition, FGF23 inmunohistochemical tests performed on the nevoid skin of 5 CSHS patients were negative [61]. Further, other investigators have failed to identify *FGF23* via qPCR and immunohistochemistry in the nevi [17, 104]. Nevi also appear unlikely to be the source of FGF23 as most syndromic and non-syndromic cases of EN and CMN, despite deriving from the same *RAS* mutations identified in CSHS, are not associated with hypophosphatemia. Moreover, it is highly improbable that all the different types of skin cells (e.g. keratinocytes, sebocytes, and melanocytes) affected in the spectrum of CSHS, are capable of secreting FGF23. Finally, data from studies on MAS, in which FGF23 has been detected in the mutant FD cells, is consistent with the hypothesis that dysplastic CSHS bone cells have the potential of secreting FGF23 [150].

Contrary to our hypothesis, the skin has been postulated as the pathological source of FGF23 in CSHS. This was initially proposed by Aschinberg et al. who reported on a boy with EN and severe rickets in whom removal of skin lesions was stated to result in normalization of blood phosphate [56]. The excised tissue was infused into a dog and was reported to result in increased renal phosphate excretion. However, a careful analysis of the data revealed that serum phosphate did not decrease in the dog, which is inconsistent with the hypothesis that the excised tissue led to dysregulated phosphate homeostasis. Similarly, Ivker et al. described a girl with severe hypophosphatemia, despite being appropriately treated with oral medication, in which nevi removal was eventually associated with an increase in serum phosphate. However, in that report it was not clear if oral treatment supplementation had been discontinued, and/or if compliance with treatment prior to surgery had been adequate [168]. Outcomes in other subjects in whom nevi removal was reported to be associated with an increase in serum phosphate were also confounded by concomitant treatment of hypophosphatemia with oral medications. Importantly, with careful observation in subjects CSHS101 and CSHS102, phosphate status was unchanged after nevi removal. Therefore, it is unlikely that in CSHS the source of excess FGF23 is overproduction by the skin. Taking into account that normal bone is the physiological source of FGF23 [209], and that skeletal dysplasia is identified in most subjects with CSHS, it is more likely that dysplastic bone is the source of pathologically secreted FGF23.

We conducted several *in vitro* and *in vivo* experiments to investigate whether bone and/or bone cells baring activating *RAS* mutations expressed/produced higher levels of FGF23. However, none yielded positive results. In *in vitro* experiment I, cellular damage became visible after 10 days of adenoviral transduction. Lack of *FGF23* expression was expected (Figure 4.12) as we

assumed that this would negatively impact on hBMSC differentiation and potential hormone production. Paradoxically, what lead us in the first place to developing an adenoviral system was the relative lack of hazardous effects adenovirus are known to exert on cells compared to other techniques of gene transfer. In fact, prior to this experiment, we had employed plasmid transfection and retroviral transduction with the same *NRAS* constructs on BMSC (data not presented), but early apoptosis (~3 days) prompted us to utilize a less hazardous system.

To address this technical problem, mutated hBMSC from a CSHS patient were employed as it appeared as an ideal solution to overcome the harmful effects derived from artificial gene transfer. Nevertheless, *FGF23* gene expression remained negative in these cells as well (Fig. 4.15). The fact these experiments were unyielding is not completely surprising since currently, there are no published reports that have shown BMCSs producing or even expressing *FGF23*. IDG-SW3 cells, on the other hand, were known to express *Fgf23*, at least at the transcriptional level [151]. Despite this promising feature, adenoviral transduction with the *NRAS* constructs did not increase *Fgf23* expression at any time point (Figure 4.13). The possibility that dysplastic bone cells may secrete paracrine factors that would induce *Fgf23* expression by healthy osteocytes was also investigated by treating IDG-SW3 cells with media in which mutant CSHS hBMCs had been cultured. Again, this approach did not yield positive results (Figure 4.14).

We think that failure to retrieve positive data with these models relies on the difficulty of replicating CSHS physiopathology *in vitro*. Considering that the skeletal dysplasia in CSHS probably originates from cells derived from mutated skeletal stem cells, BMSCs appear, at first glance, as the most attractive option to study CSHS physiopathology *in vitro* instead of IDG-SW3 cells, which are a model for differentiated bone cells. Unfortunately, the ability of BMSCs to undergo osteogenic differentiation *in vitro* is limited with the current protocols.

Our lack of success with these experiments prompted us develop a set of transgenic mouse strains in an effort to replicate the mineral abnormalities observed in CSHS by selectively expressing *Ras* mutations in bone tissue. Unfortunately, to date our mouse models did not show higher levels of F*gf23* expression in the bone or decrease in serum phosphate. These murine experiments are discussed in depth in section 5.3.

In addition to the uncertainties regarding the source of FGF23, the molecular events that lead to an increase of FGF23 production secondary to RAS hyperactivity in CSHS are also unclear. Data derived from several studies point to FGFR1 signaling in bone cells as a reasonable link between RAS and FGF23 (FGFR association with the RAS pathway is discussed in section 5.3). Activating *FGFR1* mutations have been identified in osteoglophonic dysplasia, a rare skeletal dysplasia that has been sporadically associated with FGF23-mediated hypophosphatemia [89]. In line with these findings, the introduction of a conditional deletion of *Fgfr1* in osteocytes in a mouse model of Fgf23 overproduction, has shown to decrease Fgf23 protein levels. [122]. In the same study, and consistent with our hypothesis, the administration of MAPK inhibitors to a osteoblast-like cell line reduced *Fgf23* expression [122]. Moreover, it has been demonstrated that local bone factors that bind to FGFR1, such as FGF2, stimulate *FGF23* expression [123], whereas pharmacological inhibition of FGFR1 has shown to block it [204]. It is implied from the aforementioned data that FGFR1signaling is involved in FGF23's physiological regulation. The

identification of a fusion gene composed of fibronectin (FN1) and *FGFR1* in number of PMTs, provided additional convincing evidence for the role of FGFR1 signaling in the regulation of FGF23, in these cases in the setting of a pathological event [212].

The hypoxia-inducible pathway also links RAS and FGF23. Hypoxia is known to stimulate Ras activity, which in turn induces HIF1α expression [213]. HIF1α increases in several hypoxic conditions including cancer and ferropenia. As discussed in the Introduction, ferropenia induces FGF23 expression both in subjects with ADHR, a disease caused by mutations in FGF23's proteolytic site, and healthy individuals. However, only high intact FGF23 levels are detected in ADHR subjects in this setting due to aberrant protein degradation [117]. *In vitro* studies on ADHR have demonstrated that pharmacological induction of HIF1α leads to increased FGF23 expression and that this increase can be blunted with MAPK inhibitors [118], consistent with a role for the Ras pathway in the regulation of FGF23.

Additional data implicating hyperactive RAS with pathological FGF23 secretion was suggested by a case-report that described a woman with a colon adenocarcinoma harboring an activating *KRAS* mutation who also had oncogenic osteomalacia; inmunohistochemical tests were performed on the tumor and demonstrated the presence of FGF23 [214].

Given that CSHS appears to arise from *RAS* mutations, it is surprising that neither frank hypophosphatemia nor rickets are commonly reported in germline RASopathies. Nonetheless, a recent study comparing bone mineral density (BMD) in Costello syndrome, which is caused by germline *RAS* mutations, and healthy controls, revealed that blood phosphate in affected patients was lower in the group of Costello patients compared to a healthy control group [215]. Increased urinary phosphate excretion was detected in some subjects, but FGF23 levels were not assessed, so the status of phosphate homeostasis in patients with Costello syndrome remain unclear at this point. On a similar note, hypophosphatemia has been reported sporadically in subjects with neurofibromatosis type 1 (NF1), [216-218]. NF1 is caused by mutations in the neurofibromin 1 gene which encodes a protein that downregulates RAS by enhancing RAS' GTPase activity [219]. In contrast to the mild renal phosphate wasting found in Costello patients, the abovementioned NF1 cases exhibited severe hypophosphatemia and osteomalacic manifestations, and elevated FGF23 was found in those in whom it was assessed. Interestingly, these symptoms commenced suddenly during adulthood as occurs most times in TIO secondary to PMTs. Possibly related to the pathophysiology of hypophosphatemia in NF1 are 2 reports on schwanomas, tumors occasionally found in the setting of NF1, that behaved clinically as PMTs [220, 221].

5.3 The effects of hyperactive RAS in dysplastic bone formation (Objective III)

RAS signaling has a well-established role in all stages of skeletal development, therefore it is not surprising that *RAS* mutations in mesenchymal stem cells lead to alterations in skeletal development. In regards to RAS signaling in skeletal development, FGFRs play a prominent role. FGFRs are membrane bound tyrosine kinases that upon ligand binding can stimulate several pathways critical for cell proliferation and differentiation, the Ras-dependent MAPK pathway being the most frequently involved [222]. Relative to their role in skeletal formation, FGFR1 and FGFR2 have demonstrated to be key osteoblastogenesis and limb development, whereas growth plate formation and differentiation depends mainly on FGFR3 and to a lesser extent to *FGFR1* expression [223]. The importance of FGFR signaling during skeletogenesis is highlighted by the fact that most chondrodysplastic and cranyosinostosis syndromes, in addition to other skeletal dysplasias, are secondary to activating *FGFR* mutations (e.g. *FGFR3* mutations in achondroplasia, *FGFR2* mutations in Crouzon syndrome) [224, 225].

Germline RASopthies provide additional and very obvious evidence of Ras hyperactivity in abnormal skeletal development. Orthopedic manifestations that are frequently detected in germline RASopathies include scoliosis, kyphosis, pectum carinatum, and hand deformities among others, some of which can also be found in CSHS subjects [226]. Nonetheless, dysplastic skeletal lesions similar to those observed in CSHS have not been reported in germline RASopathies. Perhaps certain distinctive features of CSHS's pathophysiology such as the timing of the mutation during the postzygotic stage, the specifc mutations identified in CSHS, and the interaction of mutant with WT cells that occur in mosaicism, could potentially explain the phenotypical differences between CSHS and germline RASopathies.

The first window into the potential pathophysiological effects of these hyperactive *RAS* mutations in bone came from the direct histological examination of bone samples from patients with CSHS. In the case of subject CSHS105, bone was taken from a radiographically dysplastic skeletal region, but microscopically, the tissue did not appear to have any abnormality other than osteomalacia (Figure 4.6). This could represent a sampling error, but consistent with this observation were the findings from a patient with CSHS in which a bone biopsy was also performed in a dysplastic-appearing area and no histological abnormalities were noted beyond osteomalacia [52]. In contrast, small islands of dysplastic-appearing fibrous cells were observed in histologic sections from the rib of CSHS104 (obtained at autopsy) (Figure 4.5) in which the presence of the *NRAS* mutation was also verified. Nonetheless, these fibrotic islands were small, thus they may not represent the mixed lytic/sclerotic lesions seen on imaging. Further study of additional bone samples from patients with CSHS is necessary to better characterize the histological features of the CSHS skeletal dysplasia. Osteomalacia, on the other hand, has been confirmed in the aforementioned bone samples as well as in the literature. Given that dysplastic-appearing bones seem to be more prone to fracture, it may be possible that the most prominent effect of these *RAS* mutations on the skeleton, in addition to a hypothetical effect on FGF23 production, is to inhibit local mineralization, instead of inducing structural abnormalities

in the organic component of the bone as seen in conditions such as FD. Direct comparison of bone from a non-dysplastic area vs a dysplastic bone lesion within the same individual could be very informative for this matter.

In an effort to further characterize CSHS bone histology, CSHS hBMSCs (which had a mix population of WT and mutant cells) were transplanted to immunocompromised mice. Subcutaneous ossicle formation is a powerful system for accessing the intrinsic bone-forming (or dysplasia-forming) capacity of skeletal progenitor cells that is accomplished in part by by removing cells from the complex *in vivo* milieu [144, 152]. Unfortunately, inadequate bone formation was observed with both the CSHS and WT hBMCSs ossicles, so conclusions regarding CSHS bone histology with this assay could not be drawn. While succesful subcutaneous ossicle formation can be informative, this technique is notorious for its poor reproducibility. Several factors that are difficult to control for may alter the ability to form ossicles e.g. the specific lot of FBS used for cell culture, the degree of immunodeficiency in the mouse strain (in some inmunocrompromised strains, immunodeficiency tends to drift over time), defective cellular cryopreservation, cellular death during hydroxyapatite incubation, and the source of hydroxyapatite particles are some examples. The problem we experienced was not unique to CSHS. Other well-established hBMCS transplant assays, e.g. FD [144], have also experienced difficulties. Currently, new carriers are being investigated in our laboratory to improve this technique that lead to superior bone formation than with hydroxyapatite. In addition, new strains of immunocompromised mice are now being studied which are also delivering better and more consistent results. At present we are waiting to corroborate the benefits of these modifications on better established models before repeating the studies with our CSHS cells, which are in short supply.

Perhaps the ultimate model for studying a disease is the generation of transgenic animal models. The mutated gene can be introduced globally, in a specific tissue, and/or expression controlled temporally via inducible systems. Towards this end, mouse strains were developed to study the effects of *Ras* mutations in the bone. While transgene expression was clearly seen in skeletal tissue (Figure. 4.18), phenotypic abnormalities were not detected in the *in vivo* inducible models. In the case of the non-inducible model, none of the pups born had a positive genotype.

There are several potential reasons that could explain the lack of an observable pathological skeletal phenotype in these mouse models. First, with the tamoxifen-inducible Cre-Lox systems, transgenic recombination only takes places in cells that are expressing the selected cre driver in the specific moment that tamoxifen is administrated (and in their daughter cells). Therefore, it is possible that even after using high doses of tamoxifen for 5 days, the *Kras G12D* transgene was not activated in enough bone cells to induce a skeletal dysplasia. One could argue that recombination appeared very abundant in the luciferase assay, but the luciferase reporter is known to deliver a hypersensitive signal. This means that luciferase is a good reporter to obtain a "yes/no" answer, but does not accurately quantify the amount of recombination. So while there was clearly reporter transgene expression in the appropriate tissue, it is still possible that the number of cells that actually underwent Cre recombination was insufficient to generate a skeletal phenotype.

Considering that CSHS probably arises from a primitive pluripotent stem cell, another possibility for the lack of results in the case of mouse model 1, is that the collagen 3.6 driver targeted cells (i.e. immature osteoblasts) that are probably too differentiated to generate a CSHS phenotype. In line with this thought, Remoli et al. generated a *knock-in* mouse model aiming to replicate FD by activating the mutant Gsα construct in early osteoblasts through a constitutively activated Col 2.3 driver (which is very similar to the Col 3.6 driver) [227]. The results showed a mouse with a skeletal phenotype, but not that of FD, supporting the hypothesis that targeting differentiated cells to express the mutant transgene is not appropriate for disorders that are originated in more primitive cells.

To address this potential technical issue, we modified our experimental design by replacing the tamoxifen-inducible collagen 3.6 Cre driver for the tamoxifen-inducible Prx1-Cre driver. As previously mentioned, *Prx1* is expressed in the limb bud and craniofacial mesenchyme during embryonal development [158], and in a small subset of periosteal cells in postnatal life that are thought to have osteochondral potential [159]. This promoter has been used in skeletal developmental models [158] and in fracture models created to assess the origins of skeletal progenitors during soft callus formation [159].
In our model, transgene induction was performed initially in post-weaned mice (21 days of postnatal life), but this approach did not lead to any visible radiographic or histological abnormality. In thinking that the key resided in activating the Kras transgene during embryogenesis, pregnant dams from mouse model 1 and 2, were treated with tamoxifen (data not presented), but this culminated systematically in abortion due to tamoxifen's toxicity.

Consequently, we further modified our approach and developed a non-inducible model with a Prx1 constitutive Cre driver. However, as previously discussed, none of the pups that were born from these breeding pairs harbored both the Prx1Cre and LSL-KrasG12D transgenes, implying that widespread expression of mutant *Kras* G12D in tissues expressing *Prx1* is lethal *in utero*. This raises the point that certain mutations are so potent that even at the tissue level they need to exist in the mosaic state in order to be viable.

An additional possible explanation for our lack of positive data is that *KRAS* G12D might not be an analogous mutation, at least at the bone level, to *HRAS* G13R and *NRAS* Q61R (the mutations that have been identified in CSHS), and therefore does not yield a CSHS skeletal phenotype. Currently, it is accepted that the 3 main RAS proteins, HRAS, NRAS and KRAS, have both overlapping and non-redundant actions [65]. An example of RAS proteins demonstrating overlapping actions is seen in our cohort: both subjects CSHS101 and CSHS105 have EN, rickets and a skeletal dysplasia, but subject CSHS101 harbors an *NRAS* Q61R mutation whereas CSHS105 harbors a *HRAS* G13R mutation. In contrast, subject CSHS104 harbored a *NRAS* Q61R mutation but had a GCMN instead of an EN indicating that the same *RAS* mutation can also generate a different phenotype depending on the developmental program of the cell that was initially mutated. Non-redundancy is evident in the prevalence of specific *RAS* mutations in certain cancers and benign tumors e.g. *KRAS* mutations are the most frequent in pancreatic tumors, whereas *NRAS* and *HRAS* mutations are especially prevalent in melanoma and bladder cancer, respectively [65]. Further, studies have shown that *Hras* and *Nras* deficiency

does not impair embryonic viability in mice [228], whereas *Kras* is necessary for embryonic development pointing to a unique role for this protein [229].

Taking into account our experimental results, we are planning to generate a fourth *in vivo* model. In this new mouse model a *Hras* G12V transgene designed with tetracycline response element will be targeted to the early limb bud mesenchyme cells, and a subset of the craniofacial mesenchyme, by the a constitutively activated Prx1Cre and a Lox-Stop-Lox-reverse tetracycline-controlled transcriptional activator transgene.The latter gene will be responsible of activating the *Hras* mutant transgene, but will only be expressed in Prx1Cre positive cells and will only be activated after doxycycline administration. We believe that this model has several advantages compared to the previous ones:

a. The analogy of the *Hras* G12V mutation to that of the mutations identified in CSHS patients appears greater than that of *Kras* G12D. This transgene has also been identified in subjects with epidermal nevus [155].
b. Doxycycline can be administered safely during pregnancy. It lacks tamoxifen's abortive effects.
c. Doxycline's dose can be regulated. This is advantageous since perhaps a full standard dose given during pregnancy may induce complete transgene activation which may lead to *in utero* lethality as seen in mouse model 3. We also anticipate that by regulating doxycycline's dose, the creation of a mosaic phenotype will be facilitated.
d. This model also offers the possibility to study the effects of the *Hras* mutation postnatally.

To date, owing largely to technical issues, we haven't been able to model the specific effects of *RAS* mutations in the bone, and therefore we have not been able to delinieate the specific mechanisms by which RAS hyperactivity induces dysplastic and unmineralized bone tissue and investigate its potential role in FGF23 overproduction. For these reasons, we are persisting in making technical modifications in the will of generating an *in vivo* model that will eventually allow us to clarify mechnistally the role of the activating *RAS* mutations in the genereation of the phenotype observed in CSHS. In addition, we expect that the ongoing collection of prospective data on CSHS patients by our group and others will enhance our understanding of this rare, fascinating, and we think ultimately biologically informative syndrome.

6. STUDY LIMITATIONS AND STRENGTHS

1. **Small sample size:** The number of patients in our cohort is very small. Hence, the results of our clinical analyses need to be validated with additional patients before generalizing our inferences to other CSHS patients. Nonetheless, our cohort is still the largest reported and most thoroughly phenotyped to date so it does provide valuable clinical information for physicians and patients.

2. **Limited acces to CSHS102 records:** This patient was not personally examined by us and we only had limited access to her clinical records thus, the clinical characterization of this patient is less complete compared to that of the other subjects of our cohort.

3. **Lack of long-term follow-up in our CSHS cohort:** This limits our understanding of the natural history of the syndrome.

4. **The retrospective nature of the literature review:** This limitiation is coupled to the possibility of misclassifying some of the reported subjects as having CSHS. On the other hand, the literature review presented is the most exhaustive and thorough reported review on the topic which has allowed us to further characterize the syndrome and to support important findings from our cohort such as and age-related improvement of symptoms and lack of malignant transformation of the bone.

5. ***RAS*-specific sequencing has not been performed in most reported CSHS individuals:** Thus, at present we cannot attribute the genesis of this syndrome and its manifestations solely to hyperactive RAS.

6. **Successful CSHS *in vitro/in vivo* models have not been generated:** This has hampered our quest for answers regarding the CSHS's physiopathologic mechanisms. Nonetheless, reporting negative data prevents other scientists from conducting uninformative experiments. In addition, certain aspects of the experiments have been informative, e.g. the fact that BMSCs do not express FGF23 thus should not be used as model FGF23-producing system or that the widespread expression of a potent Kras mutation in developmental skeletal tissues is lethal in mice.

7. CONCLUSIONS

7.1 General Conclusion

CSHS is a complex, multisystem mosaic syndrome secondary to activating *RAS* mutations in the GTPase domain that affects the skin, the bone, phosphate homeostasis, and non-cutaneous/non-ossesous tissues. The study of patients with the disease, and the analysis of tissues harboring such mutations obtained from patients or from experimental models, provide a biological framework in which to study the effects of hyperactive RAS in different organs and the role of RAS signaling in FGF23 regulation.

7.2 Specific Conclusions

1. Prevalence of tumorigenesis appears increased in CSHS. However, to date, there is no evidence that there is an increased risk for malignant transformation of the dysplastic bone lesions.

2. Hypophosphatemia does not appear to be congenital in CSHS and tends to present after the first year of life.

3. FGF23 has not been detected in nevi from CSHS patients. Currently there is no convincing evidence that excising the nevus will improve phosphate status.

4. It is possible that CSHS-related hypophosphatemia resolves over time.

5. The incidence of CSHS is proportionally much higher in PPK than in other nevi subtypes, but it is more frequently associated with EN, owing to the relatively higher incidence of EN vs. PPK in general population.

6. Adenoviral gene transfer can be hazardous to bone marrow stromal cells.

7. Activating *RAS* mutations do not induce an increase in FGF23 at the translation and/or transcriptional level in human bone marrow stromal cells or IDG-SW3 cells.

8. Ossicle formation assays performed through the subcutaneous implantation of bone marrow stromal cells with hydroxyapatite particles in immunocompromised mice is a techinique that has poor reproducibility.

9. Postnatal induction with tamoxifen of Kras G12D in cells expressing the 3.6 kb segment of collagen 1α1 and *Prx1* does not deliver a skeletal phenotype or induce systemic mineral abnormalities.

10. The widespread expression of *Kras G12D* in Prx1-expressing embryonal cells is embryonically lethal in mice.

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9. ANEX I

Original publication generated from the work of this thesis:

1. Ovejero D, Lim YH, Boyce AM, Gafni RI, McCarthy E, Nguyen TA, Eichenfield LF, DeKlotz CM, Guthrie LC, Tosi LL, Thornton PS, Choate KA, Collins MT. **Cutaneous skeletal hypophosphatemia syndrome: clinical spectrum, natural history, and treatment.** Osteoporos Int. 2016 Dec;27(12):3615-3626

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ORIGINAL ARTICLE

Cutaneous skeletal hypophosphatemia syndrome: clinical spectrum, natural history, and treatment

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Abstract

Summary Cutaneous skeletal hypophosphatemia syndrome (CSHS), caused by somatic *RAS* mutations, features excess fibroblast growth factor-23 (FGF23) and skeletal dysplasia. Records from 56 individuals were reviewed and demonstrated fractures, scoliosis, and non-congenital hypophosphatemia that in some cases were resolved. Phosphate and calcitriol, but not skin lesion removal, were effective at controlling hypophosphatemia. No skeletal malignancies were found.

Purpose CSHS is a disorder defined by the association of epidermal and/or melanocytic nevi, a mosaic skeletal dysplasia, and an FGF23-mediated hypophosphatemia. To date, somatic *RAS* mutations have been identified in all patients whose affected tissue has undergone DNA sequencing. However, the clinical spectrum and treatment are poorly defined in CSHS. The purpose of this study is to determine the spectrum of the phenotype, natural history of the disease, and response to treatment of hypophosphatemia.

Methods Five CSHS subjects underwent prospective data collection at clinical research centers. A review of the literature identified 45 reports that included a total of 51 additional patients, in whom the findings were compatible with CSHS. Data on nevi subtypes, bone histology, mineral and skeletal disorders, abnormalities in other tissues, and response to treatment of hypophosphatemia were analyzed.

Results Fractures, limb deformities, and scoliosis affected most CSHS subjects. Hypophosphatemia was not present at birth. Histology revealed severe osteomalacia but no other abnormalities. Skeletal dysplasia was reported in all anatomical compartments, though less frequently in the spine; there was no clear correlation between the location of nevi and the skeletal lesions. Phosphate and calcitriol supplementation was the most effective

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therapy for rickets. Convincing data that nevi removal improved blood phosphate levels was lacking. An age-dependent improvement in mineral abnormalities was observed. A spectrum of extra-osseous/extra-cutaneous manifestations that included both benign and malignant neoplasms was present in many subjects, though osteosarcoma remains unreported.
Conclusion An understanding of the spectrum, natural history, and efficacy of treatment of hypophosphatemia in CSHS may improve the care of these patients.

Keywords Epidermal nevus syndrome · FGF23 · Hypophosphatemic rickets · RASopathies · Skeletal dysplasia

Introduction

Cutaneous skeletal hypophosphatemia syndrome (CSHS) refers to the association of epidermal and/or melanocytic nevi, mosaic skeletal dysplasia, and fibroblast growth factor 23 (FGF23)-mediated hypophosphatemia [1]. We reported the finding of somatic activating *RAS* mutations in nevoid skin and dysplastic bone in six CSHS subjects establishing a role of RAS hyperactivity in the syndrome's pathogenesis [1, 2]. However, the clinical spectrum and natural history of this disease remain poorly described. This is due, in part, to its extreme rarity and diagnostic uncertainties in published reports, which have been variably reported as components of epidermal nevus syndromes (ENS). Optimal clinical management in CSHS has likewise not been established. Believing that skin lesions were the source of FGF23 excess, removal of nevi has been advocated as a potential treatment for the hypophosphatemia [3], but the efficacy of this approach has not been systematically investigated and remains unclear given uncertainty as to the tissue source of FGF23 in CSHS.

The purpose of this study is to describe the phenotype, clinical course, and response to treatment for mineral abnormalities in a series of CSHS subjects. Findings from this series were supported by an extensive review and analysis of all published reports likely to represent CSHS. Together, these data provide the first report on the spectrum, course, and treatment of the disease.

Subjects and methods

Subjects

Four subjects with CSHS were evaluated at the NIH Clinical Center and participated in an NIH IRB-approved protocol. Subjects are identified as CSHS101, 104–106. Clinical records and radiographs of an additional subject evaluated in another clinical center, CSHS102, were also reviewed. All subjects gave informed consent/assent. Selected clinical features of these subjects have been previously reported [1, 2]. All subjects underwent clinical phenotyping, including history and physical exam, biochemical evaluation, and imaging.

Genetic and histological evaluation of CSHS bone

A bone biopsy of radiographically appearing dysplastic bone was performed for clinical purposes from the anterior iliac crest of subject CSHS105 at the age of 17. The sample was divided in three similar pieces: one piece was fixed in 70 % ethanol and subsequently embedded undecalcified in methylmethacrylate. The specimen was sectioned, and the slides stained with Goldner's trichrome to assess mineralization status. Analysis of microstructural and kinetic indices was performed using OsteoMeasure (Osteometrics, Atlanta, GA, USA), and outcomes were expressed according to the ASBMR's standardized nomenclature [4]. Another section of the biopsy was decalcified and subsequently embedded in paraffin, sectioned, and stained with hematoxylin-eosin. The third piece was used to assess for the presence of the *HRAS* G13R, which is the mutation found in the patient's nevi. Bone marrow stromal cells (BMSCs) were isolated from the specimen and single colonies derived as previously reported [5]. Genomic DNA was isolated from colonies of clonal cells using the DNeasy Tissue Kit according to the manufacturer's instructions (Qiagen). DNA was amplified in a GeneAmp PCR System 9700 (Applied Biosystems) with primers, *HRAS* forward: AGGTGGGGCAGGAGACCCTGTAG and *HRAS* reverse: AGCCCTATCCTGGCTGTGTCCTG. Sanger sequencing was performed on a 3730 DNA Analyzer (Applied Biosystems) on the PCR product with *HRAS* primer (TGGGCTCGCCCGCAGCAGCTGCTGGCACCTGG). Results were processed with the Chromas Lite 2.0 software.

Literature search strategy

A Medline search was performed in an effort to identify previously reported individuals with CSHS. The following term combinations were used without restrictions: "nevus rickets," "nevus hypophosphatemia," "nevus fibrous dysplasia," and "phakomatosis pigmentokeratotica." "Fibrous dysplasia" was used in the search, as there were some reports in which this term was used to describe individuals that in retrospect clearly had CSHS.

Report selection

Case reports of subjects with epidermal nevi (EN), congenital melanocytic nevi (CMN), or phakomatosis pigmentokeratotica (PPK) (coexistence of EN and speckled lentiginous nevi) were selected when rickets and/or focal dysplastic skeletal lesions

were part of the subject's medical history, and family history was negative for inherited skeletal disorders.

Results

CSHS cohort

Demographic information and clinical findings from the current cohort are summarized in Table 1. Biochemical data are displayed in Online Resource 1.

Cutaneous nevi

Dermatological findings in our series included EN (CSHS101, 102, 105), PPK (CSHS106), and giant CMN (CSHS104), the last defined as a CMN reaching ≥20 cm in adulthood [6]. Representative images are displayed in Fig. 1a–d.

Features of mineral abnormalities and skeletal dysplasia

Hypophosphatemia and mosaic bone lesions coexisted in all of the subjects from our series. Hypophosphatemia was not present at birth and had a variable age of presentation ranging from 16 months in CSHS104 to 12 years in CSHS105. The most common initial symptoms attributed to hypophosphatemia were bone pain, limb length discrepancy, bone deformities, and impaired mobility.

Blood and urine biochemistries were consistent with FGF23-mediated hypophosphatemia in all the subjects from our series. These included elevated FGF23, renal phosphate wasting (as defined by a tubular reabsorption of phosphate <85 %), elevated alkaline phosphatase, and normocalcemia in the absence of evidence for intrinsic tubular dysfunction or kidney disease (Online Resource 1). PTH values were sometimes elevated in the setting of vitamin D deficiency. 1,25-dihydroxyvitamin D values were variable.

Radiographs demonstrated a broad skeletal phenotype that included dysplastic foci of mixed lytic and sclerotic bone, cortical irregularities, and classic rachitic features (Fig. 2). Marked osteoid accumulation was observed in the plastic-embedded bone specimen from the iliac crest in subject 105, although hematoxylin-eosin (H&E) staining of the same specimen did not demonstrate evidence other abnormal findings (Fig. 3). DNA isolated from BMSCs corroborated the presence of the *HRAS* G13R mutation in approximately 30 % of the colonies (Online Resource 2), suggesting that even at the tissue level the lesions are mosaic. 2D histomorphometry revealed severe osteomalacia in this area (mean, SD): osteoid volume/bone volume 59 % (1.48, 0.93), total osteoid surface 69.4 % (12.1, 4.64), bone volume/total volume 27 % (23.2, 4.37), trabecular thickness 125 μm (133, 22), trabecular separation 338 μm (570, 99), trabecular number 2.16 trabeculae/mm (1.75, 0.23), cortical thickness 783 μm (1202, 314), eroded surface 2.44 % (4.09, 2.33), and mineralizing surface 19 % (9.7, 4.9).

The location and laterality of dysplastic skeletal lesions were compared with coexistent skin lesions. CSHS101 and 102 had unilateral nevi with ipsilateral bone lesions; CSHS105 had unilateral nevi with bilateral dysplasia; and CSHS104 and 106 had bilateral nevi and bilateral dysplasia. Both the appendicular and axial skeleton were affected in all subjects from our cohort. The pelvis and skull were the most commonly involved areas of the axial skeleton. Vertebral involvement was identified only in CSHS106.

Response to therapy

Oral phosphate and calcitriol were prescribed to all subjects in an effort to treat hypophosphatemia and related symptoms. When subjects adhered to the prescribed doses, all experienced significant symptomatic and/or biochemical improvements, e.g., healed rickets in CSHS101, decreased pain and weakness in CSHS105, initiation of ambulation in CSHS104, and improved biochemical parameters in CSHS106. In CSHS102, mineral abnormalities appeared less responsive to treatment during childhood but improved dramatically during adolescence.

Nevi removal has been postulated as a potential therapy for hypophosphatemia. However, no effects on mineral abnormalities were detected in either CSHS101 or 102 after undergoing extensive CO_2 laser ablation and surgical excision, respectively.

Skeletal disease course

Resolution of biochemical abnormalities, alleviation of hypophosphatemia-related symptoms, and increase in BMD occurred in CSHS105 at age 17 (Fig. 4). Similarly, CSHS102 experienced a decrease in fracture rate with improved response to medication at age 12. The findings in these two subjects were suggestive of age-related regression of skeletal/mineral homeostasis abnormalities in CSHS, a finding which we sought to corroborate in the published literature.

Extra-skeletal/extra-cutaneous manifestations

Findings, including benign tumors, were found in non-osseous/non-cutaneous tissues in all the subjects from the cohort. These are listed in Table 1, and representative images are displayed in Fig. 5.

Literature review

The search terms employed retrieved 39 reports that described 47 subjects with findings compatible with CSHS [1, 3, 7–42].

Table 1 Demographic information and clinical findings from the CSHS cohort

Subject	Sex	Ethnicity	Nevi type	Mutation [1], Lim et al. JAAD	Age of rachitic onset (years)	Pathologic fractures	Scoliosis	Other skeletal deformities	Laterality of skin vs. bone lesions	Response to oral phosphate and calcitriol	Spontaneous improvement of mineral alterations	Extra-osseous/ extra-cutaneous manifestations
CSHS101	F	Caucasian	EN	*NRAS* Q61R	2	Yes	No	Windswept deformity in lower extremities	Unilateral nevi; ipsilateral skeletal dysplasia	Healed rickets	No (f/u until age 9)	Brainstem lipoma; thyroid nodule; splenic hemangiomas
CSHS102	F	Caucasian	EN	*HRAS* G13R	1.5	Yes	Yes	Coxa vara; lower extremities bowing and deformities secondary to fractures	Unilateral nevi; ipsilateral skeletal dysplasia	Limited effect during childhood; improved labs and ↓ fracture in adolescence	Yes. Decrease of fracture rate, age 12 (f/u until age 14)	Subaortic valve stenosis
CSHS104	F	African American	CMN	*NRAS* Q61R	1.3	Yes	No	Upper and lower extremity bowing	Bilateral nevi; bilateral skeletal dysplasia	Initiation of ambulation; Improved labs	No (deceased at age 4 due to pericardial effusion)	CNS melanosis; ocular dermoid Pericardial effusion
CSHS105	M	Caucasian	EN	*HRAS* G13R	12	Yes	Yes	Pronounced leg length discrepancy	Unilateral nevi; bilateral skeletal dysplasia	↓ pain ↓ weakness	Yes. Resolution of mineral abnormalities at age 17 (f/u until age 19)	Colpocephaly body asymmetry
CSHS106	F	Hispanic	PPK	*HRAS* G13R	5	Yes	Yes	Bowing of lower extremities; leg length discrepancy	Bilateral nevi; bilateral skeletal dysplasia	Improved labs	No (f/u until age 14)	Body asymmetry

F female, *M* male, *EN* epidermal nevus, *CMN* congenital melanocytic nevus, *PPK* phakomatosis pigmentokeratotica, *f/u* follow-up, *CNS* central nervous system

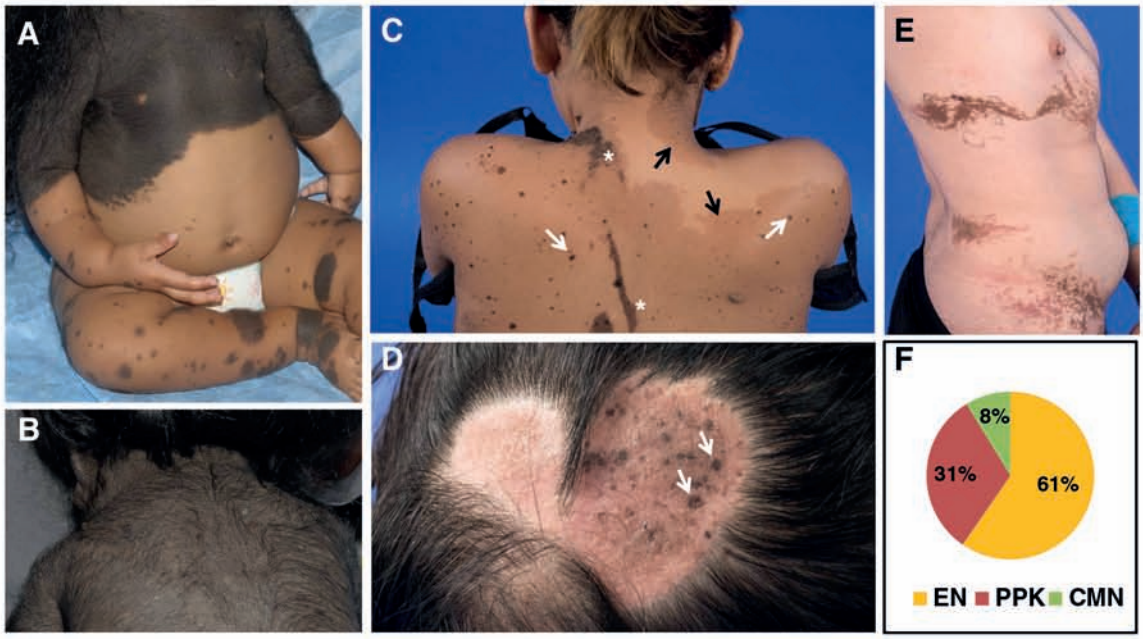

Fig. 1 Cutaneous nevi in CSHS. **a** Giant congenital melanocytic nevus (CMN) (CSHS104) composed of nevomelanocytes (neural crest origin). **b** Hair follicles may be enlarged and infiltrated by these cells, giving the observed hairy appearance. **c** Phakomatosis pigmentokeratotica (PPK) (CSHS106) characterized by speckled lentiginous nevi (*white arrows*), a subtype of melanocytic nevi (neural crest origin), and nevus sebaceous (NS) (*asterisk*), a subtype of epidermal nevus (epidermal origin). Superimposed café-au-lait macules are frequently present in these patients (*black arrow*). **d** NS (CSHS106) seen as a round waxy alopecic scalp plaque containing small speckled lentiginous nevi (*arrows*). **e** Epidermal nevus (EN) (CSHS 104) characterized by hyperplasia of elements of epidermal origin; e.g., sebaceous glands or keratinocytes. **f** Distribution of nevi types in 51 CSHS patients identified in published reports. EN in 31/51 (61 %) [1, 8, 10–12, 18–30, 32, 35, 36, 39–43, 46, 47], PPK in 16/51 (31 %), [3, 9, 11, 13, 14, 16, 17, 31, 33, 37–39, 44, 45, 48, 49], and giant CMN in 4/51 subjects (8 %) [7, 12, 15, 34] (color figure online)

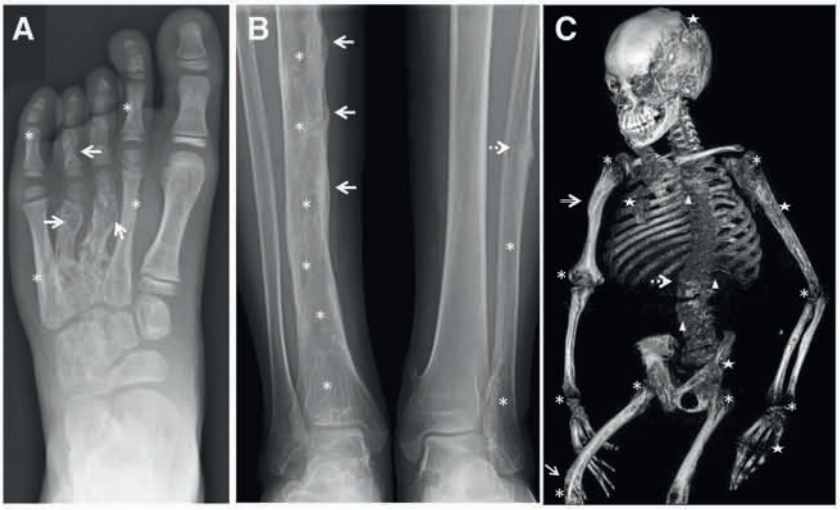

Fig. 2 Representative radiographic skeletal features in CSHS. **a** *Left* foot radiograph (CSHS101) showing dysplastic lesions in phalanges and metacarpals of digits 2–5. In contrast, the bones of the first ray are spared from dysplasia, consistent with the mosaic nature of the syndrome. Dysplastic bone lesions are characterized by areas of mixed lysis/sclerosis. In this radiograph, the lytic component is more prominent in digits 3 and 4 (*arrows*), while the sclerotic component is more noticeable in digits 2 and 5 (*asterisks*). **b** Radiograph of mid and lower shafts of tibiae and fibulae (CSHS105). The *right* tibia shows cortical pseudofractures with periosteal reaction along the mid shaft (*arrows*) in addition to irregular lucent changes indicative of dysplasia (*asterisks*). A healing stress fracture is also observed in the mid left fibula (*dashed arrow*), with lucent changes in its mid and distal shaft (*asterisks*). The left tibia appears normal and unaffected, suggesting that dysplastic bones are more prone to fracture under the same systemic hypophosphatemic milieu than non-dysplastic bones. **c** Skeletal 3D CT reconstruction (CSHS106) showing severe rickets (*asterisks*) and lower limb bowing (*arrow*) secondary to osteomalacia. Patchy areas with lytic lesions are seen throughout the skeleton (*stars*) in addition to areas of sclerosis (*compound arrow*). This was the only patient in the cohort in which vertebral dysplasia was identified (*triangles*). Scoliosis, a frequent manifestation in CSHS, was severe in this patient (*spotted arrow*)

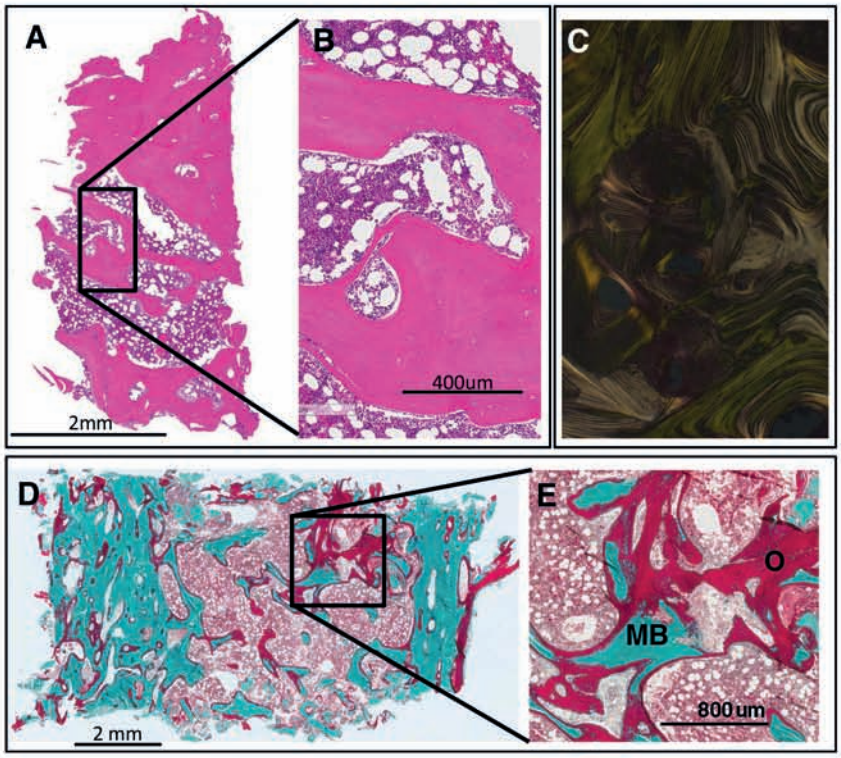

Fig. 3 Bone histopathology. An iliac crest biopsy was performed in an area that appeared dysplastic on imaging in subject CSHS105. The same *HRAS* G13R mutation that had been identified in the skin was identified in bone marrow stromal cells extracted from this sample. **a**, **b**. H&E of the iliac crest does not exhibit any noticeable histopathological abnormality. **c**. Polarized light shows normal lamellar distribution of the collagen fibrils of the sample shown in **a**, **b**. **d**, **e**. Goldner's trichrome stain of an undecalcified sample of the biopsy showing areas with excessive accumulation of osteoid indicative of severe osteomalacia. O (*red*) = osteoid; MB (*green*) = mineralized bone (color figure online)

Seven additional reports were identified from references of the reports retrieved [43–49]. This resulted in a total of 45 reports with 51 subjects, which were included in the literature review. Sixty-one percent of the subjects were male. Ethnic backgrounds were diverse (Online Resource 3).

Cutaneous nevi

A similar spectrum of cutaneous findings to that of our CSHS cohort was identified in the literature review; EN affecting 57 %, PPK 32 %, and giant CMN 8 % of the patients (Fig. 1e).

Features of mineral abnormalities and skeletal dysplasia

Coexistence of hypophosphatemia and focal bone lesions was observed in 26/51 subjects described in the literature [1, 7–9, 11, 12, 14, 16, 17, 19, 22, 24, 25, 27–29, 32, 35–37, 41, 42, 45, 49]. In 18/51 reports, hypophosphatemia and/or rickets was reported without mention of skeletal dysplasia, and radiographs were either not provided or did not demonstrate dysplasia [3, 11–13, 15, 18, 20, 21, 23, 26, 31, 33, 34, 38, 39, 48]. Seven of fifty-one reports described dysplastic lesions on radiographs, but serum phosphate was either not reported [40, 43, 44, 46, 47] or was within the normal age-related normal range at the time of the report [10, 30].

Published reports, in which biochemistries were provided, showed similar results to that of our cohort, except for the aforementioned patients in whom phosphate was within the normal range (Online Resource 1). FGF23 levels were measured and elevated in all six subjects from the literature in whom it was assessed [1, 7, 8, 17, 18, 32].

The mean age of onset of hypophosphatemia in the published reports was 4 years (median 2.7 years, SD 3.6, range 1–14 years). The most common initial symptoms were similar to our cohort and included bone pain, limb length discrepancy, bone deformities, and impaired mobility. Four subjects [3, 19, 23, 27] had no evidence of rickets or hypophosphatemia in early childhood but developed these conditions later in childhood, indicating that systemic mineral abnormalities are not congenital.

Fractures and deformities are common in CSHS. Fractures were reported in 29/51 subjects (57 %) in the literature [1, 3, 8–14, 17–20, 22, 24–27, 29, 35, 36, 41–44, 47–49] and were frequently in areas of dysplastic bone. Limb deformities, predominantly bowing, were reported in 36/51 (70 %) [1, 3, 7, 9, 11–13, 15–17, 19–22, 24, 25, 27, 29–34, 37–39, 41–43, 47–49]. Scoliosis was described in 21/51 (41 %) [1, 3, 7, 9, 12–14, 16, 19, 20, 22, 24, 27, 30, 31, 34, 39–41, 44, 47, 48].

Literature reports of dysplastic bone lesions were similar to those in our cohort and were often described as "radiolucent," "sclerotic," "cyst-like," "lytic," and/or "fibrous dysplasia-like."

Bone biopsy specimens were examined in four patients. Osteomalacia was identified in 3/4, without any evidence of dysplasia [11, 14, 17]. "Hemangiomas" were described in the fourth biopsy, and it does not appear that the presence of osteomalacia was assessed [36].

In respect to the location and laterality of dysplastic bone and skin lesions, in patients with unilateral skin lesions ($n = 15$), skeletal lesions were ipsilateral in 10/15 (67 %)

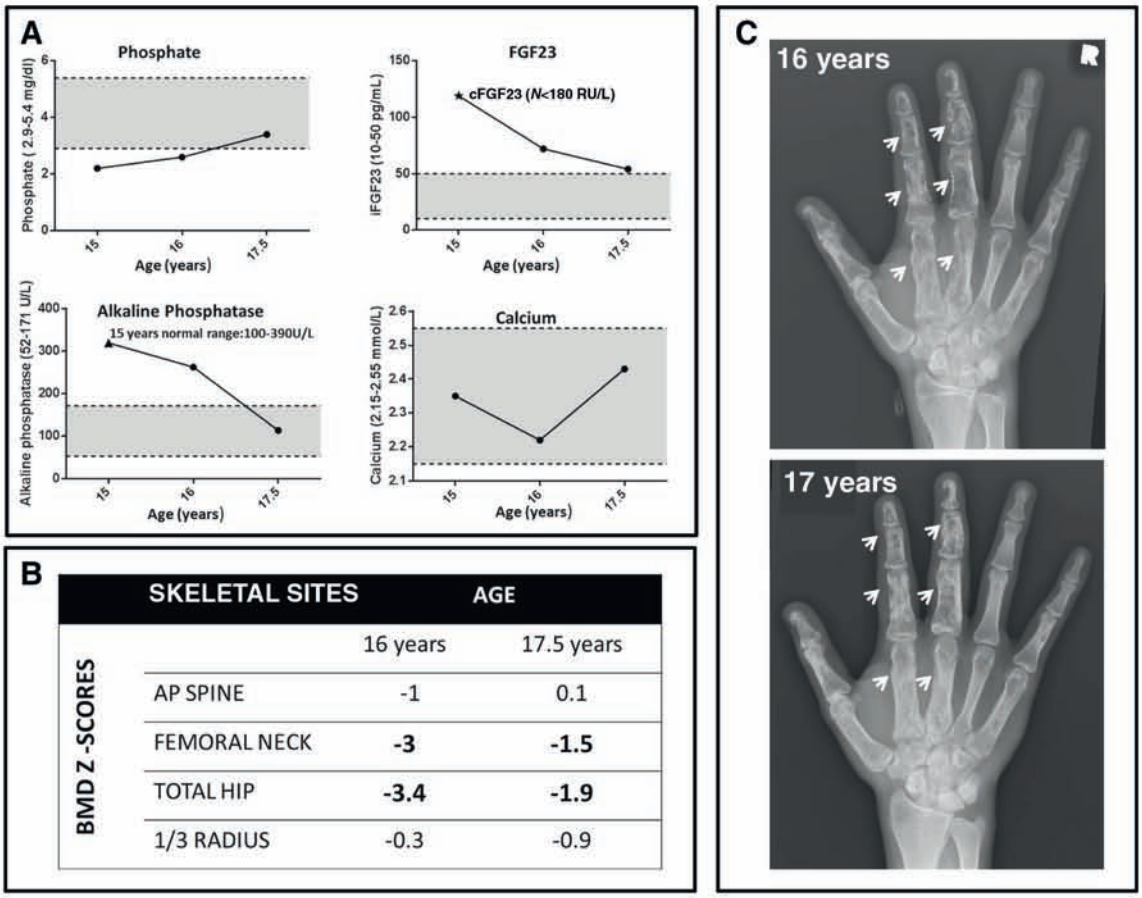

Fig. 4 Changes in mineral metabolism and radiographic findings in CSHS105 following treatment with phosphate and calcitriol. **a**. Fasting biochemistries, measured when off medication for at least 48 h, had normalized by age 17.5 years. Initially FGF23 was measured using a "C-terminal" assay that detects both C-terminal fragments and intact molecule ((star) Immutopics, San Clemente, CA); subsequently, FGF23 was measured using an assay that measures only intact FGF23 assay (Kainos, Japan). *Shaded areas* denote normal ranges for a 17-year-old. **b**. Bone mineral density (BMD) Z-scores by DXA increased from baseline at age 16 to age 17.5. **c**. Dysplastic skeletal lesions appeared more radio dense at age 17 vs. age 16 (*arrows*), consistent with an increase in mineral deposition in the dysplastic bone

[10, 11, 14, 16, 17, 27, 35, 36, 40, 45] and bilateral in 5/15 (33 %) [1, 8, 29, 42, 44, 49]. Bilateral nevi and bilateral skeletal lesions were reported in five patients [9, 24, 32, 46, 47], while bilateral skin lesions with unilateral skeletal dysplasia were described in two patients [30, 37].

Skeletal dysplasia was reported in all skeletal compartments. Both the appendicular and axial skeletons were affected in 16 patients from the literature [1, 8–10, 14, 17, 28–30, 36, 42, 44–47, 49, 50]. Dysplasia limited to the appendicular skeleton was described in nine patients [11, 12, 19, 24, 27, 32, 35, 37, 40], while one report described dysplastic lesions exclusively in the axial skeleton [16]. The pelvis and skull were the most commonly involved areas of the axial skeleton, whereas vertebral involvement was identified only in two patients from the literature [8, 44].

Hypophosphatemia/rickets and response to treatment

Treatment approaches and efficacy differed among the reported patients from the literature. Of the 26 patients treated with a combination of phosphate supplements and calcitriol (or other active vitamin D analogs), 19/26 (73 %) experienced significant clinical improvement, e.g., resumed ambulation, decreased pain, and healing of radiographic rickets [11, 12, 14, 15, 19, 21, 25, 27, 29, 32, 35, 37, 38, 48]. In 4/26 (15 %) [17, 18, 20, 22], the initial response was not optimal but improved over time. In 2/26 (8 %), efficacy appeared limited [12, 13]. Of the 11 patients treated with non-active vitamin D (ergo- or cholecalciferol) [3, 7, 9, 11, 16, 24, 28, 31, 34, 38], symptomatic improvement was reported in only 2/11 (18 %) [16, 28]. However, some of these patients underwent nevi removal in addition to phosphate and active vitamin D replacement. In an effort to avoid the potential confounding effect of nevi removal on the efficacy of oral medication, response to medication was also analyzed in patients who did not undergo nevi removal. In these patients, including CSHS104–106, oral medication had an independent positive effect on symptoms and blood phosphate levels [11, 12, 14, 15, 21, 22, 27–29, 32, 35, 37, 39].

Twenty of fifty-one patients from the literature underwent surgical excision, dermabrasion, or CO_2 laser ablation of the

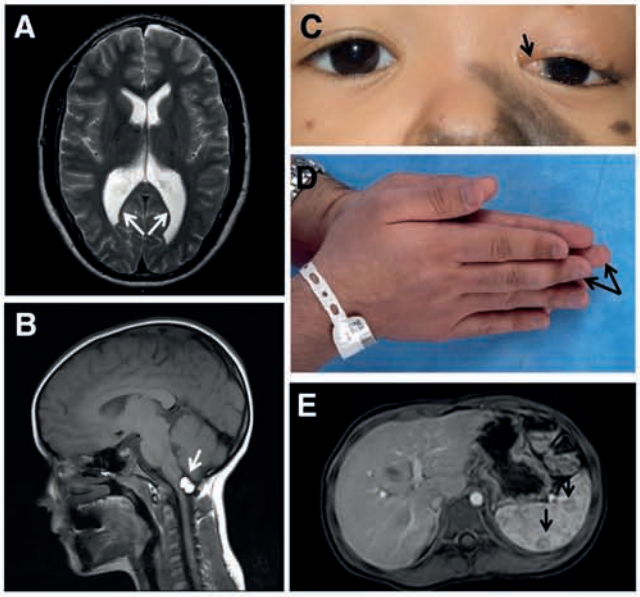

Fig. 5 Representative extra-osseous/extra-cutaneous manifestations in CSHS. **a**. Enlarged posterior horns of the lateral ventricles in CSHS105 (*arrows*). **b**. Intracranial lipoma in CSHS101 (*arrow*). **c**. Limbal dermoid in CSHS104 (*arrow*). **d**. Hemibody asymmetry in CSHS105 (*arrows*). **e**. Splenic hemangiomas in CSHS101 (*arrows*)

nevi in an effort to correct the hypophosphatemia. Serum phosphate and hypophosphatemic symptoms were unchanged in 7/20 (35 %) [12, 14, 22, 27, 29, 36] and improved in 12/20 (60 %) [16–20, 26, 31, 38, 41, 48]. However, attribution of improvement in blood phosphate levels to skin lesion removal was confounded by the fact that phosphate and calcitriol/vitamin D were either not discontinued or even initiated at the time of nevi removal. In two patients, a direct association between nevi removal and a subsequent increase in serum phosphate was suggested [3, 20].

Skeletal disease course

Evidence of clinical improvement in mineral metabolism (e.g., drug dose reduction, fracture cessation, resolution of hypophosphatemia) was reported in ten patients from the literature, most of whom were over 17 years old (Online Resource 4). However, hypophosphatemia and/or osteomalacic manifestations persisted in five young adults (≤23 years) [11, 14, 17, 36, 44] and one older adult [9]. Of note, skeletal dysplasia remained apparent on imaging despite clinical improvement.

Extra-osseous/extra-cutaneous manifestations

A detailed list of extra-skeletal/extra-cutaneous abnormalities is included in Online Resource 5. Neurological abnormalities were reported in 23/51 (45 %) of the patients from the literature. Intellectual disability, developmental delay, ventricular enlargement, and EEG abnormalities were the most frequent findings. Ophthalmological disorders, predominantly colobomas, and ocular dermoids were identified in 13/51 patients (26 %). Body asymmetry was described (or observed in the images provided) in 11/51 patients (21 %). Angiomatous malformations and/or cardiac problems were also reported in 4/51 patients from the literature (8 %). Precocious puberty occurred in 4/51 (8 %) of the patients. Benign neoplasms were frequent, whereas malignant transformation was less common, albeit reported (5 malignant vs. 16 benign tumors) (Online Resource 6). Interestingly, osteosarcoma has not been reported to date in association with CSHS.

Discussion

The clinical features and natural history of CSHS were detailed through combining the findings from a cohort of subjects that underwent a thorough analysis with the findings from patients identified in an exhaustive literature review. Key features of the analysis include a broad spectrum of skeletal findings, the novel observation of age-related clinical improvement in skeletal disease, evidence that phosphate and active vitamin D treatment, but not nevi removal, are effective at healing rickets and improving hypophosphatemic symptoms, and the lack of reports of malignant transformation of skeletal lesions. Given that *RAS* mutations have been identified in the vast majority of epidermal and melanocytic nevi in which it has been assessed [51–54], as well as in recently identified reports of CSHS [1, 8], the findings presented here provide clinical context for RAS pathway activation in the pathogenesis of CSHS. However, since *RAS*-specific sequencing was not performed in the patients from the literature, some caution must be exercised in attributing all of the manifestations cited solely to RAS pathway activation.

As expected in patients with hypophosphatemic rickets and skeletal dysplasia, fractures and deformities affected the majority of CSHS subjects. Similar to other mosaic disorders, such as McCune-Albright syndrome [55], distribution and location of the dysplastic skeletal lesions varied, affected all skeletal compartments, and were not limited to the side of the body with the nevus. The spine was the skeletal site least affected by dysplasia. However, vertebral lesions are often difficult to discern on plain radiographs and the lower prevalence of spine involvement could be attributed to this. Scoliosis, on the other hand, was extremely common. The etiology of scoliosis in CSHS is not clear. It could be related to osteomalacia, but it should be noted that scoliosis has also been reported in association with other ENS in the absence of hypophosphatemia [39]. Hemibody asymmetry, impaired mineralization, and possible underlying dysplastic vertebrae could account for the high prevalence of scoliosis in CSHS.

In spite of detecting a high number of mutation-bearing colonies in cultured bone marrow stromal cells from the iliac crest biopsy performed on CSHS105, the H&E-stained histologic sections did not reveal any specific dysplastic findings. This could represent sampling error, but consistent with this observation were the findings from a patient with CSHS in which a bone biopsy was performed in a dysplastic-appearing area, and no histological abnormalities were noted beyond osteomalacia [17]. In contrast, in our prior report, small islands of dysplastic-appearing fibrous cells were observed in histologic sections from the rib of CSHS104 (obtained at autopsy), in which the presence of the *RAS* mutation was also verified [1]. Nonetheless, these fibrotic islands were small, thus they may not represent the mixed lytic/sclerotic lesions seen on imaging. Further study of additional bone samples from patients with CSHS is necessary to better characterize the cellular and tissue features of the CSHS skeletal dysplasia. Osteomalacia, on the other hand, has been confirmed in both bone samples from our cohort (CSHS104, 105), as well as in the literature [11, 14, 17]. Given that dysplastic appearing bones seem to be more prone to fracture, it may be possible that the most prominent effect of these *RAS* mutations on the skeleton, in addition to a hypothetical effect on FGF23 production, is to inhibit mineralization of mutation-bearing bone-cells. Comparison of bone from a non-dysplastic area vs. a dysplastic bone lesion within the same individual could be very informative for this matter.

While skeletal dysplasia is a defining feature of CSHS, it was not mentioned in a third of the published reports that we suspect or represent cases of CSHS. In some of the reports, in which there was no mention of skeletal dysplasia, it was clear from either the images in the paper [19, 22, 25, 41] or those confirmed by the authors [7] that dysplastic skeletal lesions were present. It is also likely that in the cases in which rachitic changes were reported, there was also an accompanying skeletal dysplasia. Similarly, abnormalities in phosphate were not reported in 7/51 subjects, although four of these subjects had a past medical history of numerous insufficiency fractures [10, 43, 44, 47], suggesting that hypophosphatemia had been present. Therefore, the overall estimation of the presence of skeletal disease likely represents an underestimation.

Unlike other genetic forms of FGF23-mediated hypophosphatemia, in which rickets develops in infancy (e.g., X-linked hypophosphatemic rickets), the onset of rachitic changes in CSHS is delayed. The range of the age of onset of hypophosphatemic changes in our cohort and in the literature review was broad (4 years (median 2.7 years, SD 3.6, range 1–14 years), emphasizing the importance of routine phosphate assessment in pediatric patients with ENS.

Since the availability of FGF23 assays, elevations in FGF23 have consistently been reported in CSHS. Complete biochemical panels from reported individuals, in which FGF23 was not assessed, also displayed a profile compatible with FGF23-mediated hypophosphatemia. Therefore, excess FGF23 is the most probable cause of hypophosphatemia in CSHS, although secondary hyperparathyroidism due to vitamin D insufficiency, as observed in some cases, cannot be eliminated as the cause, or at least a contributing factor to lower serum phosphate and impaired mineralization. We speculate that hyperactive RAS signaling is responsible for FGF23 overproduction, but the precise mechanisms, by which this would lead to an increase in FGF23, and its tissue source, remain unclear. The skin, as the pathological source of FGF23, was initially proposed by Aschinberg et al. who reported a boy with EN and severe rickets, in whom removal of fibroangiomas (not EN) normalized blood phosphate [3]. The excised tissue was infused into a dog and was reported to result in increased renal phosphate excretion. However, a careful analysis of data revealed that serum phosphate did not decrease in the dog, making it unlikely that the excised tissue led to dysregulated phosphate homeostasis. Similarly, Ivker et al. described a girl with severe hypophosphatemia, despite being appropriately treated with oral medication, in which nevi removal was eventually associated with an increase in serum phosphate; however, in that report, it was not clear if oral treatment supplementation had been discontinued and/or if compliance with treatment prior to surgery had been adequate [20]. Outcomes in other subjects, in whom nevi removal was reported to be associated with an increase in serum phosphate, were also confounded by concomitant treatment of hypophosphatemia with oral medications. Importantly, with careful observation in subjects CSHS101 and CSHS102, phosphate status was unchanged after nevi removal. Therefore, it is unlikely that in CSHS, the source of excess FGF23 is overproduction by the skin. Supporting this conclusion is the fact that the vast majority of syndromic and non-syndromic cases of EN and CMN are not associated with hypophosphatemia. Furthermore, it is improbable that skin cells of very different origins (e.g., keratinocytes, melanocytes, sebocytes), which are differentially affected in patients with CSHS, are capable of secreting FGF23. In addition, when directly investigated, FGF23 was not found to be expressed in nevi [1, 26, 32]. Taking into account that normal bone is the physiological source of FGF23 [56] and that skeletal dysplasia is identified in most subjects with CSHS; it is most likely that dysplastic bone is the source of pathologically secreted FGF23.

The best, currently approved, medical therapy for the hypophosphatemia of CSHS is the combination of oral phosphate and active vitamin D (e.g., calcitriol). Active vitamin D is necessary given that FGF23 suppresses the action of 1-α-hydroxylase, which is necessary for the conversion of inactive 25-hydroxy vitamin D to active 1,25-dihydroxyvitamin D. Emerging therapies that may prove effective in the treatment of CSHS include cinacalcet, which diminishes the phosphaturic effects of FGF23 [57], and an anti-FGF23 monoclonal antibody, which blocks the action of FGF23 [58]. In addition, the selective FGF receptor inhibitor, BGJ398, which has action in the RAS pathway and which is

associated with an increase in serum phosphate, may be a potential treatment for CSHS [59].

Delayed onset and age-related remission of mineral abnormalities in CSHS is an important observation of our study and similar to what is seen in patients with fibrous dysplasia of bone. In fibrous dysplasia, which like CSHS is a disease of skeletal stem cells caused by somatic mosaic expression of gain-of-function mutations in GTPases ($G_s\alpha$) [60], delayed onset is attributed to the time it takes for patients to develop a sufficient mass of dysplastic tissue and the loss of phosphate wasting attributed to age-dependent apoptosis of mutation-bearing skeletal stem cells [61]. It is intriguing to speculate that a similar mechanism, age-dependent apoptosis, and/or oncogene-induced senescence [62] may be responsible for the age-related waning of symptoms in CSHS. Prospective long-term follow-up of our cohort and other CSHS patients will help determine the likelihood of skeletal disease resolution in these patients.

The most common cutaneous findings in our cohort and in the literature were EN. However, given that CSHS is quite rare and that EN occurs in approximately 1/1000 births [63], it is likely that the association of CSHS with PPK is much higher, given that PPK is very rare (only ~30–40 reports in the literature [64]), yet it was found in 16/51 of the published cases and in CSHS106. The skin lesions that characterize PPK have different embryonic origins (epidermis and neural crest) but bear the same mutation [52]. This implies mutagenesis in an earlier cellular progenitor, in contradistinction to EN, which is exclusively of epidermal origin. This may explain the higher incidence of abnormalities in tissues derived from non-ectodermal layers (such as the skeleton) in PPK vs. most cases of EN.

Neoplastic growth was frequent in our cohort and published reports, which is not unexpected given that *RAS* mutations are known to lead to unchecked cell proliferation and tumor development [65]. Surprisingly, despite abundance of dysplastic and mutated skeletal tissue in CSHS, there were no reports of osteosarcoma or other skeletal malignancies an observation that parallels the scarcity of *RAS* mutations in sporadic bone cancer (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/).

Our study has two major limitations: (1) long-term follow-up of our CSHS cohort is lacking, as well as (2) the retrospective nature of the literature review and the possibility of misclassifying some of the reported subjects as having CSHS. Despite this, our report offers insight into clinical features and natural history of the syndrome. This provides guidance for clinicians who evaluate and treat patients with CSHS and suggests a developmental, physiologic framework within which to understand and study CSHS going forward.

Summary

CSHS is a rare mosaic and sporadic disorder. Somatic *RAS* mutations have been identified in the affected tissues from all patients that have undergone sequencing. The incidence of CSHS is proportionally much higher in PPK than in other nevi subtypes, but it is more frequently associated with EN, owing to the relatively higher incidence of EN vs. PPK in general population. Concomitant rickets and skeletal dysplasia are reported in most CSHS subjects. Skeletal dysplasia may affect all skeletal sites and its distribution is not dependent on nevi location. Although skeletal dysplasia is less frequent in the spine, scoliosis is very common and likely multifactorial. Histological analysis of bone with dysplastic appearance on imaging did not reveal any particular features beyond osteomalacia. FGF23-mediated hypophosphatemia and attendant symptoms are typically detected during childhood and not present shortly after birth. Calcitriol (or analogs) and phosphate supplementation may be the best treatment currently available, but more directed therapies are in development. Convincing evidence supporting nevi removal as an effective treatment for hypophosphatemia is lacking, but fortunately, mineral abnormalities may remit spontaneously with age. Similarly to other ENS, neurological and ophthalmological abnormalities are the most common extra-osseous/extra-cutaneous abnormalities, followed by body asymmetry. Risk of developing tumors appears increased in CSHS, but malignant transformation of the bone has not been reported.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

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10. ANEX II

Publications associated to the work of this thesis:

1. Lim YH, Ovejero D, Sugarman JS, Deklotz CM, Maruri A, Eichenfield LF, Kelley PK, Jüppner H, Gottschalk M, Tifft CJ, Gafni RI, Boyce AM, Cowen EW, Bhattacharyya N, Guthrie LC, Gahl WA, Golas G, Loring EC, Overton JD, Mane SM, Lifton RP, Levy ML, Collins MT, Choate KA. **Multilineage somatic activating mutations in HRAS and NRAS cause mosaic cutaneous and skeletal lesions, elevated FGF23 and hypophosphatemia.** Hum Mol Genet. 2014 Jan 15;23(2):397-407.

2. Lim YH, Ovejero D, Derrick KM; Yale Center for Mendelian Genomics., Collins MT, Choate KA. **Cutaneous skeletal hypophosphatemia syndrome (CSHS) is a multilineage somatic mosaic RASopathy**. J Am Acad Dermatol. 2016 Aug;75(2):420-7.

3. 3. Ovejero D, Collins MT. **RAS in FGF23: another piece in the puzzle.** J Clin Endocrinol Metab. 2014 Jan;99(1):63-6.

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Multilineage somatic activating mutations in *HRAS* and *NRAS* cause mosaic cutaneous and skeletal lesions, elevated FGF23 and hypophosphatemia

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Pathologically elevated serum levels of fibroblast growth factor-23 (FGF23), a bone-derived hormone that regulates phosphorus homeostasis, result in renal phosphate wasting and lead to rickets or osteomalacia. Rarely, elevated serum FGF23 levels are found in association with mosaic cutaneous disorders that affect large proportions of the skin and appear in patterns corresponding to the migration of ectodermal progenitors. The cause and source of elevated serum FGF23 is unknown. In those conditions, such as epidermal and large congenital melanocytic nevi, skin lesions are variably associated with other abnormalities in the eye, brain and vasculature. The wide distribution of involved tissues and the appearance of multiple segmental skin and bone lesions suggest that these conditions result from early embryonic somatic mutations. We report five such cases with elevated serum FGF23 and bone lesions, four with large epidermal nevi and one with a giant congenital melanocytic nevus. Exome sequencing of blood and affected skin tissue identified somatic activating

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mutations of *HRAS* or *NRAS* in each case without recurrent secondary mutation, and we further found that the same mutation is present in dysplastic bone. Our finding of somatic activating *RAS* mutation in bone, the endogenous source of FGF23, provides the first evidence that elevated serum FGF23 levels, hypophosphatemia and osteomalacia are associated with pathologic Ras activation and may provide insight in the heretofore limited understanding of the regulation of FGF23.

INTRODUCTION

Skin and skeletal lesions appearing in localized patterns are generally mosaic disorders that result from embryonic somatic mutations (1–3). The distribution and pattern of lesions are determined by the type of progenitor cell affected and the timing of mutation during embryogenesis (1,2). Epidermal nevi and congenital melanocytic nevi (EN and CMN) appear in a wide range of sizes from small subcentimeter lesions to extensive segmental lesions affecting a large fraction of the skin surface area (4,5). While both are typically limited to the skin, in rare syndromic forms including epidermal nevus syndrome (ENS), they can be accompanied by abnormalities in other organ systems, including the eyes, brain and bone (5–8). The additional systemic abnormalities almost exclusively occur in the setting of extensive skin surface area involvement, consistent with early embryonic somatic mutation in a multipotent progenitor (6–8). Since somatic mutations can occur at any point post-fertilization, timing determines the relative potential of the affected cell (9,10). Mutations prior to germ layer specification, for example, could result in progeny capable of ectodermal, mesodermal or endodermal fates as is postulated to occur in ENS (11). Later embryonic mutations affect cells with more limited potential, and thus tend to be smaller and involve a single germ layer as in the skin limited form of EN and NS (11,12).

EN, nevus comedonicus and giant CMN have been found in association with precocious puberty, cognitive dysfunction, arterial stenosis and skeletal malformations (7,13–15). Depending on the combination of cutaneous lesions and associated abnormalities, various nomenclatures such as Schimmelpenning–Feuerstein–Mims syndrome (16,17), Becker's nevus syndrome (18) and ENS have been applied, but these disorders have significant clinical overlap, suggesting a common pathogenesis.

Hypophosphatemic rickets rarely occurs in EN (27 reported cases) and CMN (1 case) (14,19–39). All reported affected individuals had skin lesions over 10–60% of the body surface area with involvement of other organ systems variably reported. In addition, each exhibited skeletal dysplasia, and in all cases in which it was assessed, low levels of blood phosphate, phosphaturia and elevated blood fibroblast growth factor-23 (FGF23) (26,32,35). FGF23 is a bone-derived hormone that regulates phosphorus homeostasis and vitamin D metabolism at the level of the renal proximal tubule cell by reducing expression of sodium-phosphate cotransporters types IIa and IIc (NPT2a, NPT2c), inhibiting 1-alpha hydroxylase activity, and enhancing 24-hydroxylase activity (40,41). Thus both renal phosphate reabsorption and synthesis of 1,25 $(OH)_2$ vitamin D3, which mediates the gastrointestinal absorption of phosphate, are impaired by elevated FGF23, collectively contributing to the development of rickets and osteomalacia (40,42). In the reported cases of hypophosphatemic rickets with skin lesions, skin involvement was widespread and abnormalities in other organ systems were frequently identified (14,27,28,30,33,36). Given the broad spectrum of cutaneous and systemic findings, we have designated this disorder cutaneous-skeletal hypophosphatemia syndrome (CSHS). The generally large size of these skin lesions and presence of systemic findings are consistent with a shared origin from an early somatic mutation.

Isolated and syndromic keratinocytic and sebaceous nevi, as well as melanocytic nevi have been shown to result from somatic activating mutations in codons 12, 13 and 61 of *RAS* gene family members, which cause cell proliferation via the MAP kinase pathway (43–46). Skin lesions from one case of Schimmelpenning syndrome with hypophosphatemic rickets were also found to have somatic activating mutations in *HRAS* (47). The Mendelian forms of hypophosphatemic rickets typically present in childhood and can be transmitted in an autosomal dominant, recessive or X-linked fashion (48–51). Almost all feature either elevated FGF23 or inappropriately normal FGF23 levels in the setting of hypophosphatemia. Autosomal dominant hypophosphatemic rickets results from missense mutations in *FGF23* that affect the C-terminal RTHR cleavage site, blocking degradation and leading to increased circulating FGF23 levels (51). X-linked hypophosphatemia is caused by inactivating mutations in the endopeptidase-encoding *PHEX* gene (49), while autosomal recessive hypophosphatemic rickets 1 and 2 (ARHR1, ARHR2) result from mutations in *DMP1* and *ENPP1*, respectively (48,50). Reports of activating mutations in FGF receptor 1 (*FGFR1*), as well as a single *de novo* translocation causing increased circulating levels of cleaved α-Klotho, a cofactor for FGF23 binding to FGFR1 in the renal tubules, have also been found to increase levels of FGF23 resulting in hypophosphatemic rickets (52,53).

The etiology of hypophosphatemia in the setting of skin lesions has remained unknown. Some reports have described improvement of phosphate homeostasis upon destruction of skin lesions via excision or ablative techniques, suggesting the skin to be a source of a phosphatonin (19,28,54). However, qRT–PCR using RNA extracted from affected skin did not demonstrate FGF23 expression in the skin, suggesting that the skin-derived phosphatonin may be an unknown substance or that the excess FGF23 is produced elsewhere (32). Others have postulated that the focal bone lesions may be the source of FGF23 in ENS (25). We have employed exome and Sanger sequencing of blood and tissue samples from a cohort of five patients, to determine the genetic mechanism of the pathophysiology of CSHS.

RESULTS

To identify the genetic basis of hypophosphatemia, elevated FGF23 levels, osteomalacia and skin lesions in CSHS, we investigated five such cases, four with EN affecting multiple

Table 1. Elevated FGF23 and hypophosphatemia in the CSHS patient cohort

Patient	Cutaneous findings	Age	Sex	FGF23 (RU/ml)	Phosphate (mg/dl)	Calcium (mg/dl)	ALKP (U/l)	PTH (pg/ml)
CSHS101	EN	5	F	276	2.0	9.1	007000	45.5
CSHS102	EN	12	F	279	2.3	9.7	003660	47.4
CSHS103	EN	15	F	527	1.5	9.3	006510	90.0[b]
CSHS104	Giant CMN	4	F	795	1.5	8.5	01081	88.5[b]
CSHS105	EN	16	M	104.5[a]	2.2	8.7	003460	47.9

Cutaneous findings, age at time of reported laboratory values, sex and values confirming the diagnosis of CSHS are presented. All patients show elevated FGF23, hypophosphatemia, normocalcemia, elevated alkaline phosphatase (ALKP) and normal 25(OH)D (calcidiol). 1,25(OH)2D (calcitriol) levels are not shown since they were confounded by the concomitant treatment with oral calcitriol. Tubular reabsorption of phosphate (TRP%) values ranged from 73 to 85.7%, consistent with renal phosphate wasting.

Reference normal values: FGF23, average for <18 years: 25–140 RU/ml; phosphate: 3.0–4.5 mg/dl; calcium: 9.0–10.5 mg/dl; alkaline phosphatase (ALKP): 30–120 U/l; parathyroid hormone (PTH), 10–60 pg/ml (115–117).

[a]CSHS105 had the intact FGF-23 measured, which is recorded in pg/ml, rather than the intact + C-terminal FGF-23, measured in RU/ml. The normal range of intact FGF-23 is 8–54 pg/ml.

[b]CSHS103 and 104 presented with low vitamin D when values were measured. Given their normal calcium levels, the elevated PTH represents secondary hyperparathyroidism, the normal physiological response to low vitamin D.

developmental segments of the skin and a single case with a giant CMN (Table 1, Fig. 1). All cases exhibited skeletal findings that included foci of dysplastic bone and fractures; most had rickets, and available lesional bone tissue demonstrated osteomalacia (Fig. 2). Histologic examination of dysplastic bone revealed fibroblast-like spindle-shaped cells surrounded by a relatively dense collagen matrix, an appearance consistent with cells in the osteogenic lineage (Fig. 3). In addition, pathologic lesions were observed in other tissues (Supplementary Material, Fig. S1). Affected individual CSHS101 had a brainstem lipoma, a thyroid nodule and splenic hemangiomas; CSHS102 manifested subaortic valve stenosis; CSHS103 had an eccrine poroma; CSHS104 displayed a left intraventricular choroidal mass and a non-pigmented mass in the medial canthus of the right eye; CSHS105 had brain abnormalities including colpocephaly and periventricular white matter paucity. These findings were consistent with somatic mosaicism for mutations contributing to multisystem disease.

Exome sequencing was performed on DNA samples from affected skin and peripheral blood leukocytes of individuals CSHS101, 102, 103 and 105, and on DNA extracted from archival affected skin from CSHS104 (Supplementary Material, Table S1). Genetic variants were annotated and compared with identify somatic mutations present solely within affected tissue and not in germline (blood) DNA. In the four samples with matched blood and skin lesion DNA, we found a single heterozygous somatic mutation, in each case a known activating mutation in either *HRAS* or *NRAS* (55–63). In addition, we found a known activating RAS mutation in the lesion that did not have matched blood DNA; in this case, the mutation was absent in DNA prepared from normal bone marrow, demonstrating its somatic origin. The mutations included c.182A>G, p.Q61R in *NRAS*; c.182A>G, p.Q61R in *HRAS*; c.37G>C, p.G13R in *HRAS* (Table 2). All these mutations were confirmed by direct Sanger sequencing (Supplementary Material, Fig. S2). No germline mutations were found in other genes implicated in congenital disorders of phosphate metabolism (*FGF23*, *FGFR1*, *FGF2*, *FGF7*, *DMP1*, *DMP4*, *ENPP1*, *KL*, *MEPE*, *PHEX*, *SLC34A1*, *SLC34A3*, *CLCN5*, *VDR*, *OPN*, *SFRP4*, *GALNT3* and *GALNT8* genes) (64–98). Furthermore, germline samples were searched for genes harboring novel protein-altering variants in more than one subject, and none were found (see Materials and Methods). Finally, plots of differences in B-allele frequency between tumor and blood did not deviate from 0.5 across the genome, excluding large segments with loss of heterozygosity in tissue (Supplementary Material, Fig. S3). Collectively, these findings suggest that somatic *RAS* mutation alone is sufficient to cause CSHS.

To further examine the etiology of elevated FGF23 and hypophosphatemia in CSHS patients, we examined dysplastic bone from cases CSHS102 and CSHS104 using Sanger sequencing, and found the same *HRAS* G13R and *NRAS* Q61R mutations present in the patients' EN and CMN, respectively. The mutations were absent in normal bone (Fig. 4). While nevus tissue of individual CSHS 104 was strongly positive via immunohistochemical staining for MelanA, a transmembrane protein expressed by melanocytes, the dysplastic bone tissue was MelanA negative, confirming that it arose independently (Fig. 5).

Given prior reports suggesting the skin lesions could be a source of elevated serum FGF23 in CSHS, we next examined affected skin tissue from four cases in our cohort (101,102,103,104) for FGF23 expression using immunohistochemical staining. Immunostaining was performed using normal skin as a negative control and a tumor-induced osteomalacia (TIO) sample as a positive control. In addition, a sporadic nevus sebaceus sample arising from a somatic *HRAS* G13R mutation was examined (45). None of the skin samples demonstrated expression of FGF23, while the TIO specimen demonstrated strongly positive staining (Supplementary Material, Fig. S4).

DISCUSSION

We have described a cohort of five cases of hypophosphatemia, skeletal dysplasia with osteomalacia and cutaneous lesions in developmental mosaic patterns, accompanied by various associated clinical findings. We define CSHS as a multisystem skin-bone disorder in which all patients have elevated circulating levels of FGF23. Further, we present the first exome sequencing analysis of CSHS in which we identified somatic activating *RAS* mutations in skin lesions of all five cases and in affected dysplastic bone of two subjects.

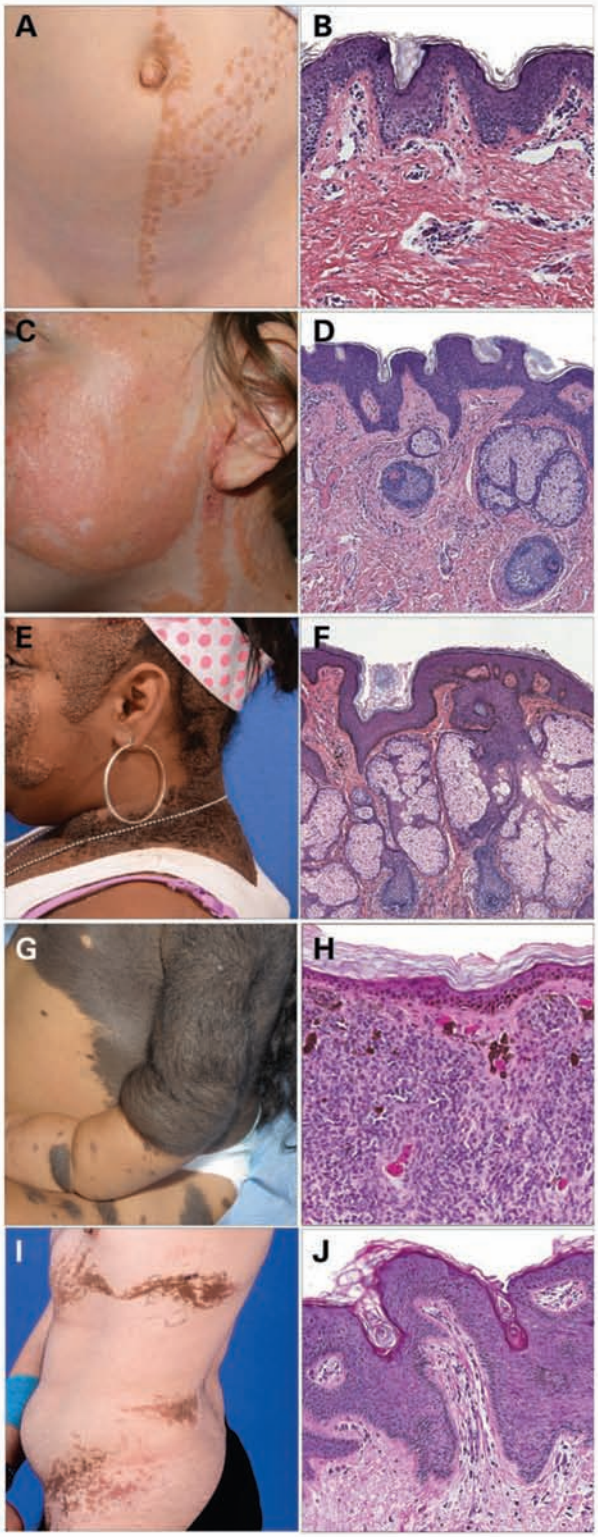

Figure 1. Clinical and histological features of CSHS. All photomicrographs are at ×10 magnification. (**A** and **B**) Affected individual CSHS101 is a 7-year-old Caucasian female who presented at birth with linear epidermal nevi restricted to the left side of her body distributed from the neck to the calf. Histopathology shows thickening of the epidermis (acanthosis) and papillomatosis. (**C** and **D**) CSHS102 is a 12-year-old Caucasian female with nevus sebaceus on the left side of her head and neck. Histology of lesional skin from the cheek shows sebaceous hyperplasia, thickening of the stratum corneum (hyperkeratosis) and papillomatosis. (**E** and **F**) CSHS103 is a 15-year-old black female with widespread keratinocytic epidermal nevi on the torso and sebaceous nevi on the scalp and cheek, with brown verrucous papules and plaques covering the scalp, face, torso and extremities, as well as linear white plaques on the scalp and torso. Histological examination shows marked sebaceous hyperplasia, hyperkeratosis and papillomatosis. (**G** and **H**) CSHS104 is a black female who presented with a giant melanocytic nevus covering the entire posterior torso at birth, extending across the flanks to the anterior chest. Round, raised, hairy and pigmented plaques determined to be satellitosis of the congenital nevi were located on her extremities. There was a 0.5 × 12 cm linear tan epidermal nevus on the left forearm (not shown). Histology of tissue from her back tissue shows melanocytes infiltrating the full thickness of the dermis, melanin deposition and hyperkeratosis. She died at 4 years of age from a large pericardial effusion that occurred during sedation for an MRI. (**I** and **J**) CSHS105 is a 16-year-old Caucasian male born with whorls of raised pink to tan plaques across the central right chest and nipple, as well as lesions extending around the flank towards the right back, all consistent with keratinocytic epidermal nevi. Histology of lesions shows hyperkeratosis, acanthosis and papillomatosis.

A similar pattern of mosaicism is found in McCune–Albright syndrome (MAS), a condition in which affected individuals demonstrate elevated FGF23, focal dysplasia of the bone, and cutaneous café-au-lait lesions (99,100). Somatic activating mutations in *GNAS*, which encodes the signaling protein $G_s\alpha$, cause MAS and have been found in the fibrous dysplasia lesions, affected skin and other affected tissues. In MAS, *in vitro* expression assays of mutant $G_s\alpha$ in osteogenic cells suggest a role for $G_s\alpha$ activation in FGF23 production and/or processing (101–105). Furthermore, just as the degree of clinical findings in MAS are dependent on the developmental timing and location of the postzygotic *GNAS* mutation during embryogenesis, the timing and location of the postzygotic *RAS* mutation in CSHS may determine the type, extent and distribution of mutated progeny cells in the body, and thus the spectrum of clinical phenotypes. Our data suggest that other rare clinical findings in patients with congenital nevi may also be attributable to somatic *RAS* mutations.

Our discovery of recurrent activating *RAS* mutations in tissues derived from different germ layers provides evidence that embryonic mutations in a common progenitor of skin and bone lead to CSHS, and suggests that the extracutaneous findings in CSHS and other ENS s result from the pleiotropic effects of *RAS* mutations acting in different tissues. Similar to postzygotic *RAS* mutations in a multipotent progenitor giving rise to both sebaceous and melanocytic nevi in phacomatosis pigmentokeratotica, *RAS* mutations found in CSHS are likely acquired during early embryogenesis in a cell destined to differentiate into both the skeletal and cutaneous systems (106). The absence of additional intersecting mutations in the exome data from our index cases and the presence of an activating *RAS* mutation in bone suggest that an activating *RAS* mutation is sufficient to drive hypophosphatemic rickets via increased production of FGF23. Support for the role of Ras in FGF23 regulation includes recent findings in a pre-osteoblast cell line and primary cultures of bone marrow stromal cells, which show that Ras is activated in response to hyperphosphatemia, an established stimulus for FGF23 secretion *in vivo* (107–109). Further support for the role of *RAS* mutations in FGF23 regulation is the recent finding of an activating somatic *KRAS* mutation (G12V) in a hepatic metastasis of an adenocarcinoma that was accompanied by elevated FGF23 levels and showed FGF23 expression on

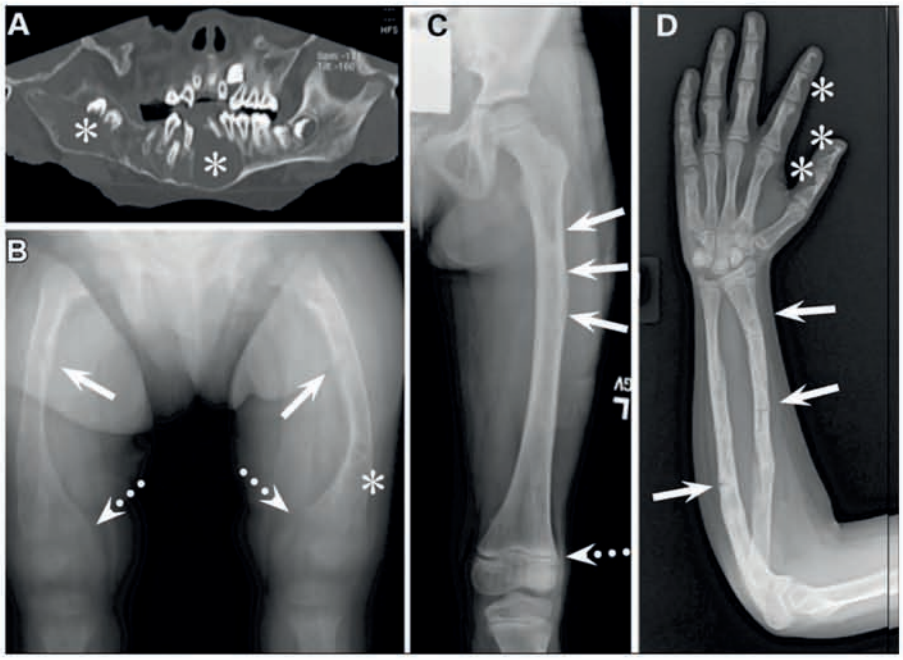

Figure 2. Radiographic features of CSHS. (**A**) A CT scan of the jaw of patient CSHS103 shows large areas of dysplastic bone (asterisks) that appear as lytic lesions. The skeletal disease in the mandible displays an aggressive expansion that displaces and resorbs the tooth roots. (**B**) A radiograph of the femurs of patient CSHS104 demonstrates healing fractures due to osteomalacic bone (solid arrows), evidence of rickets (ill-defined, frayed and widened growth plates, dotted arrows), and dysplastic bone with mixed lytic and sclerotic changes (asterisk). (**C**) A radiograph of the femur of patient CSHS101 at age 7 reveals a stretch of dysplastic bone with a primarily sclerotic appearance at this age (solid arrows). At a younger age, the same lesion was more lytic in nature (not shown). As a result of medical therapy, the growth plate is normal, with no evidence of rickets (dotted arrows). (**D**) A radiograph of the arm of patient CSHS102 shows multiple unhealed fractures (arrows). Dysplastic lesions with lytic changes are seen in some of the phalanges (asterisks).

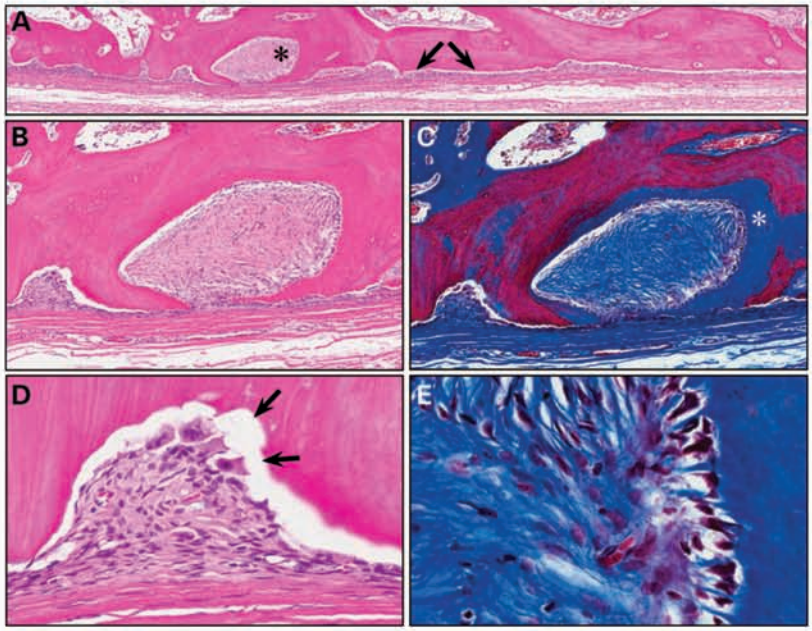

Figure 3. Dysplastic skeletal lesions from the rib of CSHS104. (**A**) Low power (×2) view from the pleural of the side of the rib shows a thin layer of cells that underlie the periostium along the length of the section of rib depicted (arrows). Asterisk denotes focus of dysplastic cells. (**B**) Higher power (×4) H&E of the area of dysplastic tissue marked by the asterisk in section (A) is shown. The lesion consists of a collection of fibroblast-like spindle-shaped cells in a relatively dense collagen matrix that appears to arise from the layer of dysplastic cells demonstrated in (A). (**C**) A Masson's trichrome stain of the same region seen in (B) displays both blue-staining collagen in the matrix surrounding the dysplastic fibroblast-like cells and an extensive area of osteoid surrounding the collection of dysplastic cells (asterisk). (**D**) A higher power (×10) view of the smaller area dysplastic cells seen in (B and C) show a collection of cells similarly arising from the layer of dysplastic cells seen in (A) as well as active osteoclast resorption of adjacent lamellar bone (arrows), suggesting that the dysplastic cells of CSHS can induce osteoclastogenesis. (**E**) A high power (×40) view of the region seen in (C) demonstrates collagen bundles connecting the osteoid with the pericellular matrix. The collagen bundles are parallel to the mineralizing surface (Sharpey's fibers) and interdigitate between the atypical but osteoblast-like cells that appear to have produced the adjacent osteoid.

Table 2. Somatic mutations in HRAS and NRAS identified by exome sequencing

Sample	Gene	Base change	Protein change	No. of reads in tissue		No. of reads in blood		*P*-value
				Ref.	Non-ref.	Ref.	Non-ref.	
CSHS101	*NRAS*	A>G	Q61R	097	033	129	0	1.3×10^{-11}
CSHS102	*HRAS*	G>C	G13R	188	093	159	0	9.0×10^{-22}
CSHS103	*HRAS*	A>G	Q61R	119	054	034	0	1.2×10^{-5}
CSHS104	*NRAS*	A>G	Q61R	235	152	NA	NA	NA
CSHS105	*HRAS*	G>C	G13R	029	015	069	0	1.3×10^{-6}

The *P*-values denoting the significance of the differences in reference (ref.) and non-references (non-ref.) reads in tissue versus blood were calculated using a one-tailed Fisher's exact test. In all cases, the respective *RAS* mutation carried the lowest *P*-value among all SNVs, and was the only variant with a quality score of ≥ 50 and a *P*-value of $<1.0 \times 10^{-3}$. After correction for multiple testing, a value of 1.7×10^{-6} is considered the threshold for genome-wide significance. Given the biopsy technique, which samples both normal and affected tissue, admixture accounts for reduced mutant allele fraction in some samples.

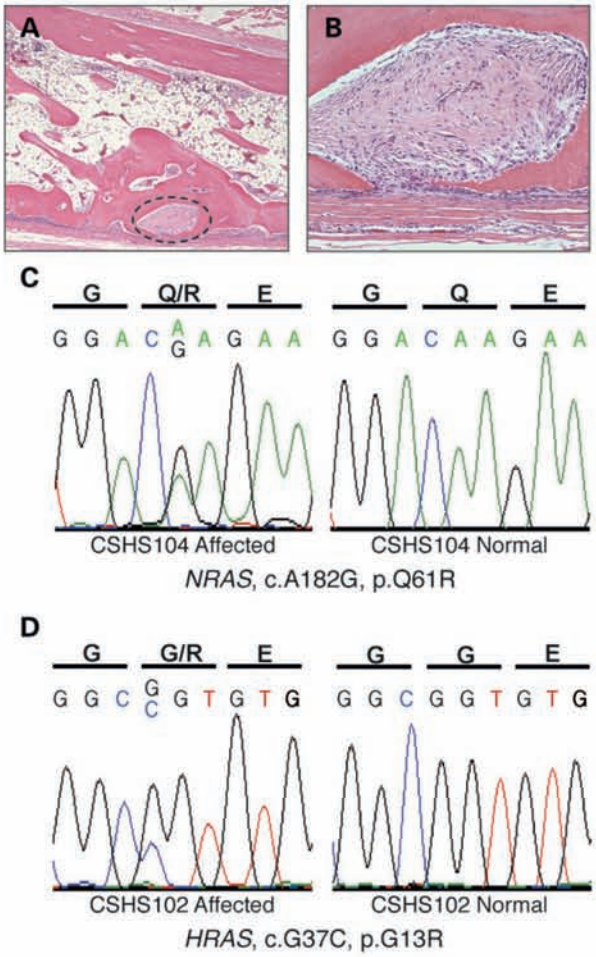

Figure 4. *NRAS* Q61R is present in dysplastic, but not normal, bone in CSHS102 and 104. (**A**) The region within the dotted line on a ×2 view of an affected rib from patient CSHS104 shows a representative region of dysplasia. DNA was extracted from cores of tissue excised from this region. (**B**) A ×20 view of the same region shown in (A) demonstrates an area of fibrous tissue composed of spindle-shaped cells set in a dense collagen matrix and in intimate association with the adjacent lamellar bone. (**C**) Sanger sequencing of DNA from affected bone tissue from patient CSHS104 shows the same heterozygous *NRAS* Q61R mutation that was found in the patient's cutaneous lesion (left panel). Sequence of DNA extracted from the patient CSHS104's unaffected rib is wild type (right panel). (**D**) Sanger sequencing of DNA from a bone sample appearing grossly sclerotic from patient CSHS102 shows the heterozygous *HRAS* G13R mutation that was found in the patient's nevus (left panel). DNA from a region near the cortical bone is wild type (right panel).

immunohistochemistry (110). This suggests similarity between CSHS and oncogenic osteomalacia (a.k.a.TIO), a disorder in which primarily mesenchymal tumors secrete sufficient amounts of FGF23 to cause hypophosphatemia (41). These reports implicate Ras proteins in the regulation of FGF23, and our findings in CSHS provide the first evidence, suggesting that activating *RAS* mutations in bone cause elevated FGF23, hypophosphatemia, focal skeletal dysplasia and osteomalacia.

MATERIALS AND METHODS

Human subjects

All subjects provided written consent to the study protocol, which was approved by the Yale Human Investigation Committee. Each subject provided punch biopsies of affected skin as well as a blood sample. For one deceased case, archival tissue was obtained. One biopsy was used for direct DNA preparation in parallel with blood, and the other embedded for subsequent frozen sectioning.

DNA isolation

Genomic DNA from EN was obtained from fresh skin biopsies or 1 mm cores of formalin-fixed paraffin-embedded (FFPE) specimens, using DNeasy Micro (Qiagen, Valencia, CA, USA) with added deparaffinization performed for FFPE tissue. Fresh bone samples were powderized in liquid nitrogen and digest overnight in 10% (w/v) extraction buffer (EDTA 0.5 M, 1% *n*-lauroyl sarcosinate) and 10% (w/v) proteinase K (Qiagen, Valencia, CA, USA), and DNA was extracted using a standard phenol–chloroform protocols. FFPE samples were deparaffinized before proceeding as done for fresh bone. Blood DNA was also isolated via a standard phenol–chloroform protocol.

Whole-exome sequencing

Bar-coded libraries of sheared DNA isolated from tissue and blood were prepared, and whole-exome capture was performed (EZ Exome 2.0, Roche, Nutley, NJ, USA) by the Yale Center for Genome Analysis. Illumina HiSeq 2000 and 2500 instruments were used for sequencing blood samples pooled 6 per lane and tissue samples pooled 4 per lane with 75 bp paired end reads. Resulting reads were aligned to hg19 human reference using Efficient Large-scale Alignment of Nucleotide Databases

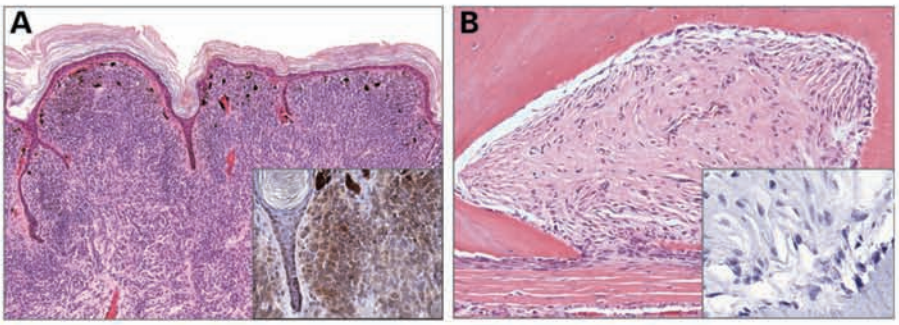

Figure 5. Affected skin, but not affected bone, shows staining for MelanA. Skin (**A**) and bone (**B**) specimens from patient CSHS104 confirmed to carry the *NRAS* Q61R mutation were sectioned and immunostained with melanocyte marker MelanA, to confirm the affected bone to be osteogenic and independent. Nuclei of abundant dermal melanocytes within a congenital nevus stain strongly brown for MelanA (A: ×10, ×20 insert), while a cellular region of dysplastic bone shows no staining (B: ×10, ×20 insert). A hematoxylin counterstain highlights nuclei in both panels.

software (ELAND, Illumina). Aligned reads were subsequently processed via a Perl script, which trimmed sequence to the targeted intervals and removed PCR duplicates. Single nucleotide variants (SNVs), deletions and insertions were identified using SAMtools software (111), and all variants were annotated for functional impact using a Perl script (112). We filtered our data to examine tissue variants with $\geq$2 non-reference reads in tissue and $\leq$4 non-reference reads in blood. The resulting data was filtered in Excel to exclude variants in 1000 Genomes (release 05/2011) and the National Heart, Lung and Blood Institute exome database (release ESP6500) from further analysis. Tissue variants in each sample were then filtered to examine coding mutations (missense, nonsense, splice site SNVs and insertions and deletions) with SAMtools quality scores $\geq$50 and coverage $\geq$8 and which were not present in 2577 control exomes. We then employed a one-tailed Fisher's exact test to compare mutant and wild-type read numbers in tissue and blood (genome-wide threshold for significance $\sim 1.7 \times 10^{-6}$, after Bonferroni correction for multiple testing of $\sim$30 000 genes). Aligned reads were examined with the Broad Institute Integrative Genomics Viewer (IGV) (113) to exclude variants resulting from alignment error.

Analysis of exome data for LOH

Exome CNV was employed to identify LOH segments of $\geq$1 Mbp with a *P*-value of $\leq$0.015 (114). LOH data were plotted by dividing the number of B-allele (non-reference) reads by the total number of reads independently for both tissue and blood at each SNV position. The difference in B-allele frequency values between tissue and blood was plotted against the genomic location.

Sanger sequencing

Verification of *RAS* mutations identified by exome sequencing was performed via standard PCR using Kapa 2G Fast polymerase (Kapa Biosystems, Woburn, MA, USA) and Sanger sequencing. The primers used are as follows:

HRAS Exon 2F: AGGTGGGGCAGGAGACCCTGTAG
HRAS Exon 2R: AGCCCTATCCTGGCTGTGTCCTG
HRAS Exon 3F: AGAGGCTGGCTGTGTGAACTCCC
HRAS Exon 3R: ACATGCGCAGAGAGGACAGGAGG
NRAS Exon 3F: GGGACAAACCAGATAGGCAGAAATGG
NRAS Exon 3R: TGGTAACCTCATTTCCCCATAAAGATTC

Immunohistochemistry

In order to exclude that the focus of dysplastic bone was the result of melanocytic metastasis and to confirm its osteogenic origin, the bone lesion was stained for melanocytic marker MelanA. Five-micrometer sections from FFPE tissue were deparaffinized using a xylene–ethanol gradient, rehydrated and rinsed in phosphate-buffered saline (PBS). Heat-induced antigen retrieval was performed by immersion in a modified pH 6.0 citrate buffer at 95°C for 30 min (Dako, Carpenteria, CA, USA). Slides were cooled, rinsed in PBS and endogenous peroxidase activity blocked by incubation in 3% H_2O_2 for 10 min, followed by washing, blocking in 10% normal goat serum and incubation with a 1:50 dilution of anti-MelanA primary antibody (IR633, Dako, Carpenteria, CA, USA). Slides were washed in PBS and incubated with an anti-mouse HRP secondary antibody for 1 h prior to substrate detection with 3,3′-diaminobenzidine tetrahydrochloride employed as a chromagen (LSAB+, Dako, Carpenteria, CA, USA). Hematoxylin with 1% ammonium hydroxide posttreatment was employed as a counterstain.

Investigation of the skin as potential source of FGF23 was done via staining affected skin tissue with FGF23 antibody (Immunotopics, San Clemente, CA, USA). Six-micrometer sections of frozen or FFPE lesional skin tissue were either fixed using a 1:1 mixture of acetone and methanol and rinsed in PBS or deparaffinized using a xylene-ethanol gradient, rehydrated and rinsed in PBS, respectively. For FFPE sections, heat-induced antigen retrieval was performed as previously described. Slides were cooled, rinsed and blocked in 10% normal donkey serum and 1% bovine serum albumin, prior to incubation for 2 h at room temperature with a 1:100 dilution of anti-FGF23 primary antibody (IR633, Dako, Carpenteria, CA, USA). Slides were washed in PBS and incubated with an anti-goat Cy3 fluorescent secondary antibody for 1 h at room temperature, and washed again. Slides were incubated with 1× DAPI diluted in PBS for 3 min and mounted using Mowiol.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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Cutaneous skeletal hypophosphatemia syndrome (CSHS) is a multilineage somatic mosaic *RAS*opathy

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Background: We recently demonstrated multilineage somatic mosaicism in cutaneous skeletal hypophosphatemia syndrome (CSHS), which features epidermal or melanocytic nevi, elevated fibroblast growth factor (FGF)-23, and hypophosphatemia, finding identical *RAS* mutations in affected skin and bone.

Objective: We sought to: (1) provide an updated overview of CSHS; (2) review its pathobiology; (3) present a new patient with CSHS; and (4) discuss treatment modalities.

Methods: We searched PubMed for "nevus AND rickets," and "nevus AND hypophosphatemia," identifying cases of nevi with hypophosphatemic rickets or elevated serum FGF-23. For our additional patient with CSHS, we performed histopathologic and radiographic surveys of skin and skeletal lesions, respectively. Sequencing was performed for *HRAS*, *KRAS*, and *NRAS* to determine causative mutations.

Results: Our new case harbored somatic activating *HRAS* p.G13 R mutation in affected tissue, consistent with previous findings. Although the mechanism of FGF-23 dysregulation is unknown in CSHS, interaction between FGF and MAPK pathways may provide insight into pathobiology. Anti-FGF-23 antibody KRN-23 may be useful in managing CSHS.

Limitations: Multilineage *RAS* mutation in CSHS was recently identified; further studies on mechanism are unavailable.

Conclusion: Patients with nevi in association with skeletal disease should be evaluated for serum phosphate and FGF-23. Further studies investigating the role of *RAS* in FGF-23 regulation are needed. (J Am Acad Dermatol 2016;75:420-7.)

Key words: congenital melanocytic nevus; cutaneous skeletal hypophosphatemia syndrome; epidermal nevus; fibroblast growth factor-23; mosaicism; nevus syndrome; rickets.

GENETIC MOSAICISM

Mosaic organisms harbor 2 or more genetically distinct cell types. The generation of a mosaic requires a nonlethal somatic mutation in 1 cell of a developing embryo; this mutant cell divides and gives rise to mutant daughters that populate 1

Abbreviations used:

CSHS: cutaneous skeletal hypophosphatemia syndrome
FGF: fibroblast growth factor
FGFR: fibroblast growth factor receptor

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or more parts of the organism.[1] Germline mosaicism occurs when a mutation affects germ cell progenitors, allowing the mutation to be inherited by subsequent generations, whereas pure somatic mosaicism spares germ cells and is thus noninheritable. Genetic mosaicism of the skin can often be appreciated as lesions appearing along the lines of Blaschko, which follow the dorsal-ventral migration pattern of mutant ectodermal progenitors. Other patterns have been observed in somatic mosaicism of the skin, including phylloid patterns and large coatlike patches crossing the midline.[1]

CAPSULE SUMMARY

- Cutaneous skeletal hypophosphatemia syndrome results from multilineage activating *RAS* mutations in skin and bone.
- The presence of *RAS* mutation in bone suggests that this drives abnormal fibroblast growth factor-23 regulation.
- Excision or laser ablation of nevi is not recommended in patients with hypophosphatemia.

NEVUS SYNDROMES: A SPECTRUM OF GENETIC MOSAICISM

Congenital melanocytic nevi and epidermal nevi that include both keratinocytic and sebaceous subtypes are examples of somatic mosaicism arising via postzygotic activating *RAS* mutations.[2-4] Laser capture microdissection and whole exome sequencing found causative *RAS* mutations in epidermal keratinocytes and sebocytes of the lesions, whereas the underlying dermis, blood leukocytes, and adjacent, unaffected skin were wild type. In phacomatosis pigmentokeratotica, *RAS* mutations are found in both keratinocytes and melanocytes, giving rise to both organoid nevi and speckled lentiginous nevi.[5]

Although most cases of epidermal or melanocytic nevi are nonsyndromic, some occur with abnormalities in other organs, including the eye, brain, muscle, and vasculature.[6-10] Nevi with systemic findings (nevus syndromes) highlight the spectrum of potential end organ effects of *RAS* mosaicism, which depend on mutation timing during development. Schimmelpenning-Feuerstein-Mims syndrome, which features sebaceous nevi variably associated with neurologic abnormalities such as intellectual disability and epileptic seizures, along with ocular and skeletal deformities, is likely a result of an early mutation affecting a multipotent progenitor.[2,11] Nearly all cases of syndromic nevi, especially those with abnormalities in nonectoderm-derived tissues, demonstrate extensive skin surface involvement, consistent with early embryonic somatic mutation.[12]

CUTANEOUS SKELETAL HYPOPHOSPHATEMIA SYNDROME

Cutaneous skeletal hypophosphatemia syndrome (CSHS) features epidermal or melanocytic nevi and hypophosphatemic rickets with elevated levels of a serum phosphatonin, fibroblast growth factor (FGF)-23.[13] Patients often require phosphate and calcitriol supplementation to maintain mineral homeostasis.

In 1977, Aschinberg et al[14] reported the first case of CSHS in a 5-year-old boy with linear verrucous nevi and severe rickets. Serum phosphate and tubular resorption of phosphate were low, indicating renal phosphate wasting (2.0 mg/dL, normal: 3.0-4.5 mg/dL), whereas serum alkaline phosphatase was high. Serum parathormone and calcium levels were within normal limits. At that time, FGF-23 had not been identified. Interestingly, surgical excision of fibroangiomas from the face and left lower limb resulted in reduction of musculoskeletal pain and normalization of phosphate levels within 4 weeks. The authors postulated a secretory mechanism originating from the skin for pathobiology. They tested this hypothesis by homogenizing excised lesions and injecting them into a dog, and within 1 hour postprocedure found increased renal wasting of phosphate secondary to decreased reabsorption, although without changes in serum phosphate.[14] The authors did not find similar amelioration of phosphate excretion after excision of an epidermal nevus in the same patient, although subsequent reports did. Ivker et al[15] reported a female infant with CSHS who, despite medical therapy, exhibited a low serum phosphate of 0.87 to 0.97 mmol/L (normal <1 year of age: 1.56-2.29 mmol/L), along with an extensive linear epidermal nevus involving various parts of the body. At 21 months of age, areas of the nevus were excised, with histopathologic confirmation of verrucous epidermal nevus. Shortly after the operation, serum phosphate values transiently climbed to 1.51 mmol/L, but later dropped, prompting subsequent nevus excisions at 27 months and stabilization of serum phosphate at 1.29 to 1.61 mmol/L.[15,16] It was unclear whether oral medication was continued during this period. Lastly, in a 2003 report by Saraswat et al[17] of a 22-year-old man with phacomatosis pigmentokeratotica and hypophosphatemic rickets, normalization of serum phosphate and reduction in phosphaturia was observed after carbon-dioxide laser ablation of the skin lesions. It is unknown whether this normalization was

Table I. Patients with cutaneous skeletal hypophosphatemia syndrome demonstrate elevated fibroblast growth factor-23 and hypophosphatemia in the setting of multilineage somatic *RAS* mutation

Patient no.[13]	Skin	Age, y/sex	*RAS* mutation	Serum FGF-23, RU/mL	Phosphate, mg/dL	Additional findings
CSHS101	KEN	5/F	*NRAS* Q61R	276	2.0	Brainstem lipoma; thyroid nodule; splenic hemangiomas
CSHS102	KEN/NS	12/F	*HRAS* G13R*	279	2.3	Subaortic valve stenosis
CSHS103	KEN/NS	15/F	*HRAS* Q61R	527	1.5	Eccrine poroma
CSHS104	GCMN	4/F	*NRAS* Q61R*	795	1.5	Intraventricular choroidal mass; mass in medial canthus of right eye
CSHS105	KEN	16/M	*HRAS* G13R	104.5†	2.2	Colpocephaly; periventricular white matter paucity
CSHS106	PK	12/F	*HRAS* G13R	98†	1.2	None

Reference normal values: FGF-23, average for age <18 y: 25-140 RU/mL; phosphate, 3.0-4.5 mg/dL.
CSHS, Cutaneous skeletal hypophosphatemia syndrome; *F*, female; *FGF*, fibroblast growth factor; *GCMN*, giant congenital melanocytic nevi; *KEN*, keratinocytic epidermal nevi; *M*, male; *NS*, nevus sebaceus; *PK*, phacomatosis pigmentokeratotica.
**RAS* mutation was identified in both skin and dysplastic bone.
†Intact FGF-23 is measured in pg/mL, the normal range of which is 8-54 pg/mL. Intact + C-terminal FGF-23 is measured in RU/mL.

sustained as there was no follow-up reported, and medications or supplements were not withheld during the procedure. Collectively, these reports suggest a parallel between the pathobiology of CSHS and tumor-induced osteomalacia, in which a phosphaturic mesenchymal tumor secretes FGF-23 ectopically. Indeed, some have referred to nevus syndromes as a subtype of tumor-induced osteomalacia, in which resection of the phosphaturic tumor quickly normalizes mineral panels and alleviates symptoms of osteomalacia.[18] Because FGF-23 assays were not performed for the aforementioned patients with nevus syndrome, it remains unknown whether the rickets resulted from elevated FGF-23 or an alternative rachitogenic substance.

We recently determined the genetic basis of CSHS, which falls within the nevus syndrome spectrum. Our investigation included 5 patients with CSHS (CSHS 101-105) (Table I) with keratinocytic, sebaceous, or giant congenital melanocytic nevi occurring in association with hypophosphatemic rickets.[13] All epidermal nevi appeared in a Blaschkoid pattern, whereas the giant congenital melanocytic nevi had a coatlike pattern. Lesional histopathology was characteristic: epidermal nevi demonstrated acanthosis, papillomatosis, hyperkeratosis, and sebaceous hyperplasia (within nevus sebaceus), whereas the giant congenital melanocytic nevi showed infiltration of melanocytes throughout the full thickness of the dermis along with hyperkeratosis. Radiographic survey revealed coexistence of rachitic features with large regions of dysplastic bone. Besides the skin and skeleton, all patients exhibited additional pathologic lesions in other tissues (Table I). We studied the effects of epidermal nevus ablation and found that neither surgical excision nor laser ablation correlated with improvement in mineral status or patient-reported quality of life. Immunolocalization and quantitative PCR (qPCR) studies did not detect FGF-23 expression within nevi from any of the 5 patients, consistent with prior reports.[19]

We used exome sequencing to discover activating *RAS* mutations within the epidermis of the skin lesions, and found identical *RAS* mutations within dysplastic bone (Table I). There was no evidence of loss of heterozygosity or secondary mutation that might otherwise account for pathogenesis. The presence of an identical *RAS* mutation in tissues of both ectodermal (skin) and mesodermal (bone) derivation suggested that the mutation occurred before gastrulation leading to multilineage mosaicism.

Since our initial report, we studied a 12-year-old girl with phacomatosis pigmentokeratotica, severe rickets, and skeletal dysplasia (CSHS 106) (Table I). The Yale School of Medicine Internal Review Board reviewed and approved of this study. The work described has been carried out in accordance with the Declaration of Helsinki principles, and informed consent was obtained from all patients. Skin findings included compound-type congenital melanocytic nevi on the right upper aspect of the back and front of the left thigh, linear epidermal nevus on the left aside of abdomen and side of forearms, and nevus sebaceus with an area of alopecia on the right scalp and right temporal region (Fig 1, *A* and *B*). There was a linear verrucous nevus stretching from the right ear to the neck (Fig 1, *C*). Histopathologic examination of a skin biopsy specimen from the abdominal keratinocytic nevus demonstrated marked acanthosis and papillomatosis (Fig 1, *D*). Radiographic studies revealed diffuse skeletal hypomineralization, with

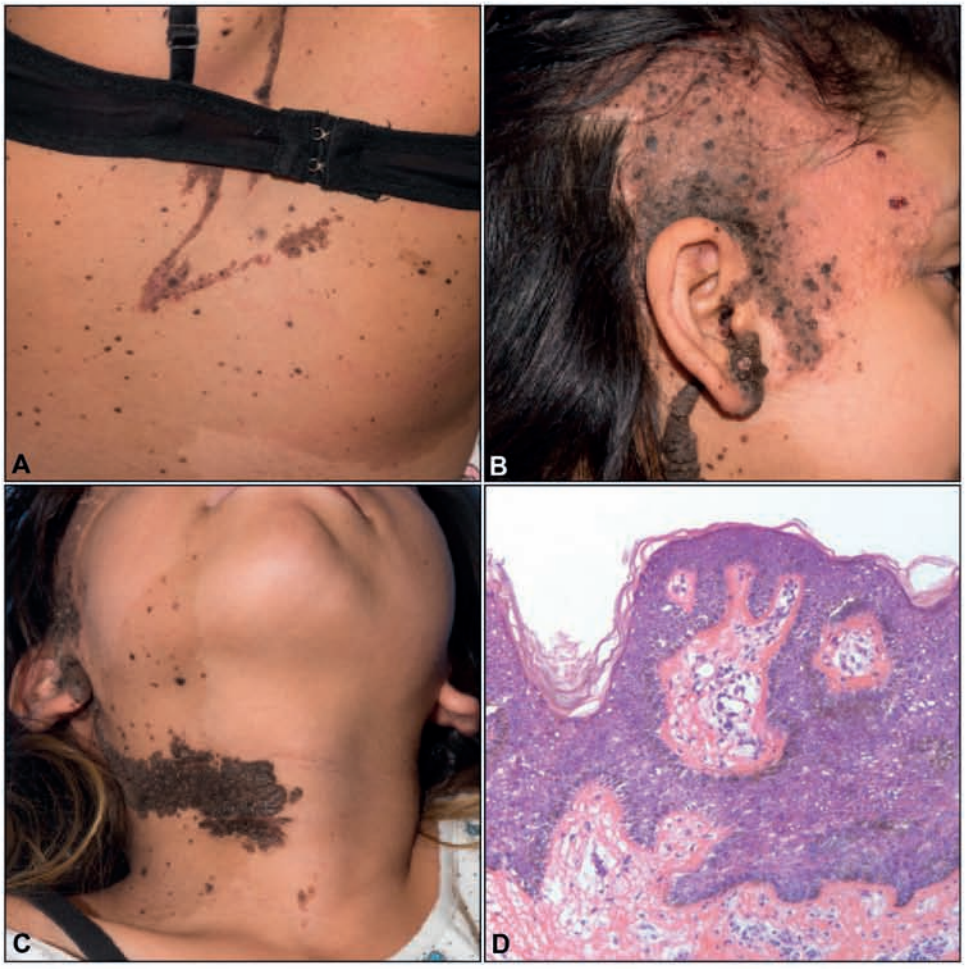

Fig 1. Cutaneous skeletal hypophosphatemia syndrome. Clinical and histopathologic features. **A**, Congenital melanocytic nevi and keratinocytic epidermal nevi in Blaschkoid pattern on the back. **B**, The right temporal region shows alopecia and papules of verrucous nevi. **C**, Verrucous and keratinocytic epidermal nevus limited to the right side extends from the ear to the midline of the neck. **D**, Histopathology from a 3-mm punch biopsy specimen of abdominal keratinocytic epidermal nevus shows acanthosis and papillomatosis. (Original magnification: ×10.)

lytic foci in the left hand and foot (Fig 2). Vertebral bodies were shortened with evidence of dysplasia, some with hemibody asymmetry, all of which likely contributed to significant scoliosis. Skeletal maturation was delayed and she was wheelchair-dependent. No pathology was found in other organ systems. Characteristic changes in laboratory values included significantly low phosphate and elevated FGF-23 levels (Table I). Targeted Sanger sequencing of all exons of Harvey rat sarcoma viral oncogene homolog (*HRAS*), Neuroblastoma RAS viral oncogene homolog (*NRAS*), V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) identified somatic mutation in *HRAS* c.37G>C, G13R in the epidermis (Fig 3); based on our previous cohort, we presume that foci of dysplastic bone harbor this *HRAS* mutation. The mutation was further verified via restriction fragment length polymorphism (RFLP) digestion, as the mutation creates a de novo *Eag*I site (data not shown).

FGF-23 IN CSHS

FGF-23 is a 30-kd phosphatonin normally secreted from osteocytes, which regulates both phosphate and vitamin-D homeostasis by modulating the expression of renal phosphate transporters and calcitriol-metabolizing enzymes, respectively.[20-24] Transgenic mice that overexpress FGF-23 demonstrate reduced levels of sodium phosphate cotransporter 2a (NaPi-2a) and 2c (NaPi-2c) in the proximal tubules, increasing urinary excretion of phosphate, whereas ablation of FGF-23 leads to hyperphosphatemia.[23,24] Also suppressed by FGF-23 is 1-alpha hydroxylase, necessary for the conversion of

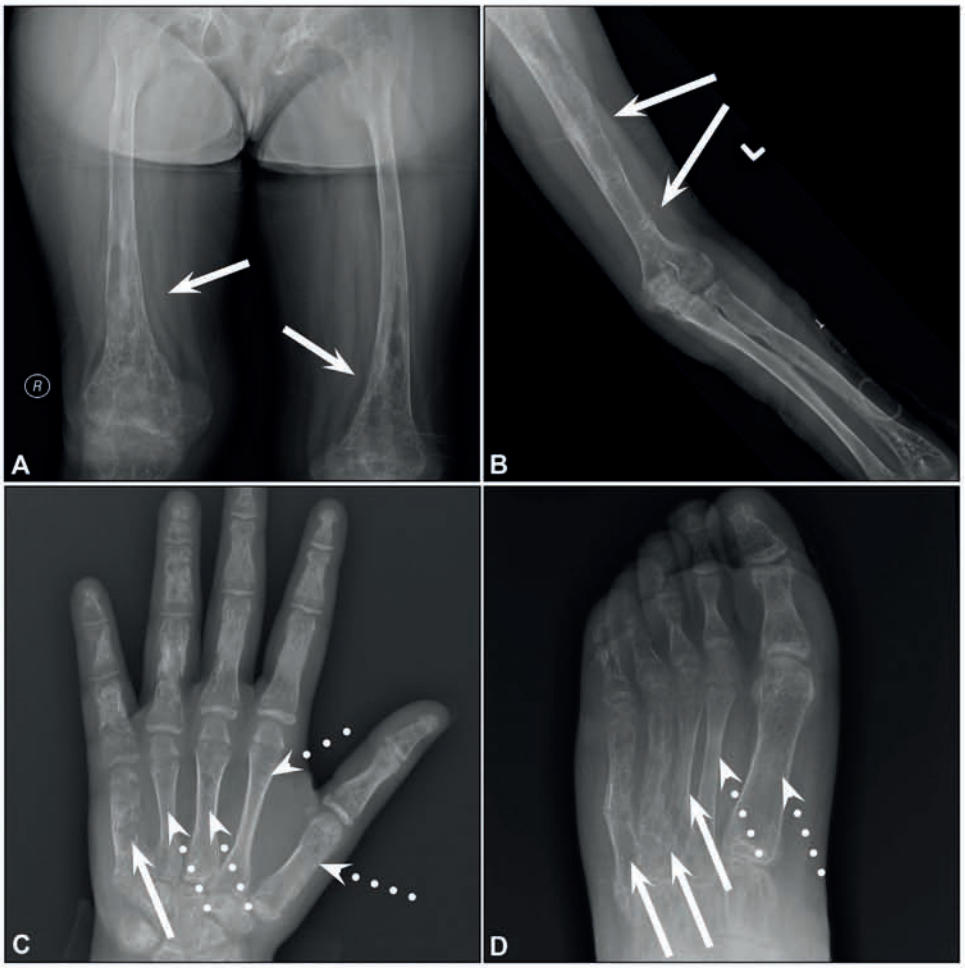

Fig 2. Cutaneous skeletal hypophosphatemia syndrome (CSHS). Skeletal survey. **A**, Radiograph of the femurs demonstrates stretches of dysplastic bone with mixed sclerotic and lytic changes (*arrows*). **B**, Multiple transverse defects in the shaft of the humerus representing pseudofractures (Looser zones or Milkman lines), which is a sign of osteomalacic bone (*arrows*) in the setting of dysplastic bone. Consistent with the mosaic nature of CSHS, some rays are dysplastic (**C**, fifth ray of the hand [*solid arrow*]; **D**, third, fourth, and fifth rays of the foot [*solid arrow*], and others are unaffected [*dotted arrows*]).

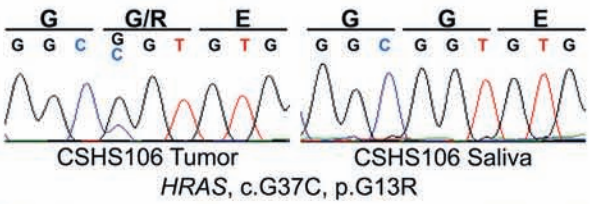

Fig 3. Somatic *RAS* mutation in patient 106 with cutaneous skeletal hypophosphatemia syndrome. Sanger sequencing of DNA isolated from the patient's epidermal nevus demonstrates *HRAS* p.G13R mutation that is absent in the saliva.

calcifediol (25-hydroxyvitamin D) to bioactive calcitriol (1,25-dihydroxyvitamin D_3), whereas 24-hydroxlase, a catabolic enzyme of calcitriol, is increased.[20,21] In the setting of pathologic overexpression, the combination of reduced phosphate and bioavailable vitamin D—which also facilitates absorption of phosphate in the gut—promotes rachitic symptoms including frequent fractures and immobility. Likewise, familial tumoral calcinosis, which results from loss of function mutations in FGF-23, is characterized by hyperphosphatemia caused by increased reabsorption of phosphate in the kidney.[25]

For CSHS, given the lack of FGF-23 expression in skin and the presence of activating somatic *RAS* mutations in the bone, we must consider a direct mechanism by which Ras signaling can influence FGF-23 regulation. The *RAS* family of guanosine triphosphate-ases (GTPases)—*HRAS*, *KRAS*, and *NRAS*—is known for its role in carcinogenesis, as activating missense mutations in codons 12 (glycine), 13 (glycine), and 61 (glutamine) are

found in a large number of cancers.[26] Normally, Ras is activated when bound to guanosine-5′-triphosphate (GTP), and inactivated upon hydrolysis of bound GTP to guanosine diphosphate (GDP). *RAS* mutations identified in neoplasms and nevi favor the binding of GTP and/or prevent GTP hydrolysis, rendering the Ras protein and its downstream signals constitutively active.[27] Although no direct association between *RAS* and *FGF-23* has been observed, there is clear evidence for a role of Ras in human skeletal development, and an established FGF receptor (*FGFR*)-*RAS*-mitogen activated protein kinase (MAPK) signaling pathway that regulates expression of genes important for cell growth and survival.[28-31] FGFR1, for example, activates Ras in an FGF2-dependent manner to induce catabolism in articular chondrocytes, whereas FGFR3 was found to activate the Ras axis for cells to acquire resistance to vemurafenib in v-Raf murine sarcoma viral oncogene homolog B V600E (BRAF V600E) mutant melanoma.[32,33] Osteoglophonic dysplasia resulting from activating FGFR1 mutation features elevated FGF-23 and hypophosphatemia, whereas antibody-mediated activation of FGFR1 in mice was found to increase serum FGF-23 and induced hypophosphatemia.[34] NVP-BGJ398, a pan-specific FGFR inhibitor, further ameliorated hypophosphatemia and improved calcitriol biosynthesis in the Hyp mouse model.[35] Moreover, a recent study of 14 phosphaturic mesenchymal tumors demonstrated a fibronectin-FGFR1 fusion in 9 of them (9/14, 60%), implicating tumor-induced osteomalacia to be a consequence of FGFR1 overexpression.[36] It is thus possible that constitutively active *RAS* mutations, which drive downstream signaling of FGFR1, could also induce overproduction of FGF-23.

Alternatively, a *RAS*-mediated mechanism may be indirect, because regulation of FGF-23 synthesis is complex with numerous endocrine, paracrine, and autocrine inputs from the bone, gut, kidneys, and parathyroid glands.[37-40] Given that CSHS is likely to arise from a mesoderm-ectoderm progenitor, other cells derived from these germ layers that are important for FGF-23 regulation may harbor *RAS* mutations. It is possible that a mosaic background provides an interface between mutant and wild-type cells and may be required for FGF-23 secretion, as it is yet unknown whether FGF-23 is secreted by mutant or adjacent wild-type cells. Evidence in favor of this hypothesis include germline *RAS*opathies such as Noonan or Costello syndromes, which feature constitutional activating *RAS* mutations without elevated FGF-23 or hypophosphatemic rickets, although patients with Costello syndrome occasionally demonstrate hypomineralized enamel.[41,42] Furthermore, *RAS* mutant cell phenotypes are modified by intercellular communication with neighboring cells.[26,43]

MANAGEMENT OF CSHS

Given our current understanding of the pathogenesis of CSHS, excision or ablation of nevi as treatment for hypophosphatemia is not advised. Although some case reports suggest a therapeutic response, the results are confounded by concomitant oral medication or lack of follow-up. Patients with CSHS/nevus syndrome may undergo potentially painful removal procedures with no improvement.[13,19] Not all patients in whom phosphate levels normalized were subject to nevi removal and there is evidence that phosphaturia improves over time.[44] We propose that early initiation of therapy consisting of oral phosphate and calcitriol can improve rachitic symptoms, and help heal dysplastic bone by enhancing mineralization. A multidisciplinary approach involving dermatology, endocrinology, pediatrics, orthopedic surgery, and physical rehabilitation is critical to mitigate complications and to maximize clinical outcomes. As CSHS is a *RAS*opathy, agents targeting the MAPK pathway are theoretically possible for severe cases.[45,46] Notably, KRN-23, a human anti-FGF-23 antibody that is administered subcutaneously once per month, has shown promising results in X-linked hypophosphatemia, another disease of FGF-23 excess. KRN-23 may similarly prove useful in CSHS, especially for patients with poor compliance with or response to oral medication.[47,48]

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RAS in FGF23: Another Piece in the Puzzle

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The first piece of the puzzle that is the evolving story of fibroblast growth factor-23 (FGF23) physiology was identified in 1959 by Andrea Prader (1). He postulated the existence of a phosphate-regulating hormone as the cause of acquired rickets in a child who was cured by excision of a bone-derived tumor. More pieces were added in 1976, 1989, and 1992, respectively, by the discovery of the Hyp mouse as a model of X-linked hypophosphatemic rickets (XLH) and elegant parabiosis and renal transplant studies in hyp mice that clearly demonstrated that a circulating factor was responsible for the phosphate wasting in Hyp mice (2–4). A key piece was identified in 2000 when the autosomal dominant hypophosphatemic rickets (ADHR) consortium discovered that mutations in *FGF23* were responsible for ADHR (5). In rapid succession, FGF23 was found to be elevated in patients with XLH, tumor-induced osteomalacia, and fibrous dysplasia (FD) of bone (6, 7). Another important piece was added when it was discovered that bone-derived cells were the source of FGF23, not only in the dysplastic bone cells of FD but also in normal bone (7).

Insight into the molecular mechanism of FGF23 action was demonstrated in several models in 2004 when it was shown that FGF23 regulated serum phosphate and $1,25(OH)_2$ vitamin D_3 levels by direct action on renal sodium phosphate cotransporters and vitamin D 1-α hydroxylase (8–10). Important pieces of the puzzle of FGF23 signal transduction were put in place in 2006. It was shown that whereas the action of FGF23 could be potentially transduced by one of several FGF receptors (especially FGFR1[IIIc]), it was the coreceptor Klotho that was required for full signal transduction as well as imparting tissue specificity to the action of FGF23 (11, 12).

Some pieces of the transcriptional, translational, and post-translational regulation of the FGF23 puzzle are known, but gaps remain. For example, there are data to suggest that phosphate and $1,25(OH)_2$ vitamin D_3 may feedback and regulate FGF23 levels. However, the data are not compelling enough to account for all or even most of FGF23 regulation (reviewed in Ref. 13). An important missing piece relates to what is observed in renal failure, the disease with the highest levels of FGF23. Here, blood levels of FGF23 begin to rise even before serum phosphate levels, and the degree of elevation of blood phosphate is insufficient to account for the degree of elevation of FGF23 (14). In addition, the importance of post-translational modification in regulating FGF23 levels is demonstrated by loss of function of the Golgi-associated enzyme galactosyltransferase N-acetylgalactosaminyltransferase 3 (ppGalNacT3). Mutations in *GALNT3*, which codes for ppGalNacT3 result in the disease of functional FGF23 deficiency, familial tumoral calcinosis (15). Yet direct evidence for glycosylation of intact FGF23 in human samples is lacking. Support for post-translational regulation as a modifiable process was demonstrated in FD (16). An increase in intracellular cAMP levels by the activating mutations in the GTP-binding regulatory protein $G_s\alpha$ that cause FD resulted in coordinated alterations in both ppGalNacT3 and furin enzyme activity. Furin is the enzyme that may be responsible for the inactivating cleavage of intact FGF23 that takes place at its subtilisin-like proprotein convertase sequence, ^{176}RHTR^{179}. The result is an increase in the ratio of the hormonally inactive C-terminal FGF23 to hormonally active intact FGF23. A perhaps more dramatic example of a role for post-translational processing in FGF23 regulation was seen in the differences

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Abbreviations: ADHR, autosomal dominant hypophosphatemic rickets; CSHS, cutaneous-skeletal hypophosphatemia syndrome; DMP1, dentin matrix protein-1; EN, epidermal nevus; ENPP1, ectonucleotide pyrophosphatase/phosphodiesterase 1; ENS, EN syndrome; FD, fibrous dysplasia; FGF23, fibroblast growth factor-23; GALNT3, galactosyltransferase N-acetylgalactosaminyltransferase 3; PHEX, phosphate regulating gene with homologies to endopeptidases on the X chromosome; XLH, X-linked hypophosphatemic rickets.

between the C-terminal and intact FGF23 levels in iron-deficient control subjects and iron-deficient subjects with ADHR (17). Iron deficiency resulted in increased intact FGF23 levels and hypophosphatemia in ADHR subjects, but normal intact FGF23 levels, euphosphatemia, and elevated C-terminal FGF23 in iron-deficient control subjects. These data were interpreted to suggest that iron deficiency caused an increase in FGF23 transcription and translation in both ADHR subjects and controls. Control subjects were able to increase FGF23 processing (as manifested by increased C-terminal levels) and maintain euphosphatemia, but ADHR subjects, due to the mutations in FGF23, were unable to process FGF23 properly and became hypophosphatemic. In vitro studies indicated that hypoxia inducible factor-1α, which is downstream of the PI3K/AKT/mTOR and MAPK/ERK pathways, may be a key pathway linking iron deficiency to increased FGF23 expression (18), but these results have not been confirmed by in vivo or human studies.

More pieces to the puzzle that have been identified but whose places have yet to be assigned are the mechanism(s) by which the extracellular protein, dentin matrix protein-1 (DMP1), and the transmembrane enzymes—phosphate regulating gene with homologies to endopeptidases on the X chromosome (PHEX), and ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1)—regulate FGF23 (reviewed in Ref. 13). Mutations in PHEX are the molecular underpinning of XLH, and those in DMP1 and ENPP1 cause forms of autosomal recessive hypophosphatemic rickets. Although there is speculation as to how these mutations cause FGF23-mediated hypophosphatemia, the mechanisms are far from clear or settled. Other missing pieces are the absence of a phosphate-sensing mechanism to account for phosphate regulation of FGF23, and the absence of a vitamin D-responsive element on the human FGF23 promoter to account for $1,25(OH)_2$ vitamin D_3 regulation of FGF23.

The paper in this edition of the *JCEM* by Avitan-Hersh et al (19) points to another, perhaps key piece of the puzzle of FGF23 biology. The authors present a case of a patient with a mosaic disorder consisting of epidermal nevus (EN), a thymoma, and FGF23-mediated hypophosphatemia in association with a skeletal dysplasia. The authors found a G13R-activating mutation in *HRAS* in the available affected tissues (thymoma and EN) that was not present in lymphocytes. The association of EN with extracutaneous tissues, such as eyes and brain, occasionally bone, and rarely hypophosphatemia, is known as EN syndrome (ENS). The fact that the cutaneous lesions follow the lines of Blaschko and that extracutaneous lesions are often unilateral or segmental suggests that the disease is mosaic in nature. Ras mutations (*NRAS*, *KRAS*, or *HRAS*) have recently been identified as being responsible for most cases of EN and ENS (20). The newest piece of the puzzle, highlighted by this report, is the identification of a Ras mutation in association with FGF23-mediated hypophosphatemia and a skeletal dysplasia in ENS. Similar to Avitan-Hersh et al (19), Lim et al (21) recently published five cases of congenital mosaic skin diseases (EN and congenital melanocytic nevi) associated with segmental bone lesions, hypophosphatemia, and excess FGF23. This constellation of findings has been called cutaneous-skeletal hypophosphatemia syndrome (CSHS). Exome sequencing of lesional skin and unaffected tissue and Sanger sequencing of available bone were compared and identified mutations in *HRAS* or *NRAS* in all five cases without recurrent secondary mutations in unaffected tissues. Both Avitan-Hersh et al (19) and Lim et al (21) demonstrated in CSHS causative mutations in tissues derived from different germ layers: eg, ectoderm (skin), mesoderm (skeletal), and endoderm (thymic epithelium). Analogous to what is thought to be the case in McCune-Albright syndrome, the mutation likely occurred very early in embryogenesis, before gastrulation, leading to the survival of mutated clones in tissues of diverse germinal origin.

There is debate as to the cause of the hypophosphatemia in ENS. In the few cases of ENS with hypophosphatemia in which it has been investigated, serum FGF23 was elevated. Reports investigating FGF23 expression in skin lesions and/or a decrease in serum FGF23 after laser or surgical ablation of skin lesions are conflicting (reviewed in Ref. 21). Skeletal tissue from patients with CSHS has not yet been adequately analyzed for the presence or absence of FGF23. Although it remains to be proven, we contend that the source of FGF23 in CSHS is likely dysplastic bone. In support of this are the facts that the mutation was present in affected bone, normal bone is the physiological source of FGF23, and perhaps most importantly, in the approximately 27 cases of ENS with hypophosphatemia reported since 1969, all were associated with a skeletal dysplasia (reviewed in Ref. 21). Clearly though, this is an area of the puzzle where pieces remain missing.

Perhaps the most intriguing question raised by Avitan-Hersh et al (19) and Lim et al (21) is: What is the relationship between activation of the Ras pathway and FGF23? Ras proteins are small GTPases that switch to active and inactive conformations when bound to GTP and GDP, respectively (discussion of Ras is reviewed in Ref. 22). Unlike $G_s\alpha$, which has efficient intrinsic GTP binding and GTPase activity, Ras proteins require guanine exchange factors such as SOS1 and SOS2 to activate the "on" state and require activation of GTPase activity by GTPase activator proteins, such as neurofibromin and

p120GAP. Ras proteins are key signaling molecules involved in many cellular processes including cell growth, differentiation, and survival. They respond to a number of extracellular ligands and activate several signaling pathways, including the MAPK/ERK and/or PI3K/Akt/mTOR pathways. In addition, Ras-activated pathways have the potential to cross talk with multiple other pathways at multiple points. The most relevant members of the Ras subfamily are HRAS, KRAS, and NRAS. The respective genes are found on different chromosomes, but all three are highly homologous. In fact, the first 85 amino acids are identical. This 100% homologous region includes the all-important GTPase activity domain, to which amino acids 12, 13, and 61 are critical. Amino acid substitutions in these codons, which can lead to constitutively active Ras, are found in approximately 30% of all human cancers and most patients with EN. Although no clear genotype/phenotype correlation exists, nor are there clear roles for one Ras vs another in normal cellular function, certain Ras mutations are found with greater frequency in certain cancers, eg, *KRAS* in pancreatic cancer and *NRAS* in melanoma. The presumption is that these mutations are lethal in germline transmission and survive only by mosaicism. In contrast, in some cases of Noonan syndrome or cardiofaciocutaneous syndrome, germline activating *Ras* mutations can be found at amino acids other than 12, 13, or 61. These mutations are thought to have relatively lower kinase activity, at least in vitro. Because these very active *Ras* mutations in patients with EN, ENS, and CSHS are associated with cancers, it is prudent to maintain a high level of suspicion for malignant transformation in affected tissues and follow patients closely. In fact, malignant transformation of skin lesions in EN or ENS has been reported, but to date malignant transformation in skeletal tissues has not.

Suspecting now that Ras activation may be important in FGF23 physiology, and given that Ras is able to interact/cross talk with many signaling pathways, Ras may be the missing piece of the FGF23 puzzle that will allow for the ordering of the many scattered pieces. It may be possible to tie hypoxia inducible factor-1α activation, important in iron deficiency, with $G_s\alpha$ activation in FD, and even allow for a better understanding of such disparate and unexplained observations as the roles of PHEX, DMP1, ENPP1, and others. A requirement of the model, however, will be that it also explains dysplasia (FD, CSHS) and neoplasia (tumor-induced osteomalacia) and accounts for the coordinated regulation of transcription, translation, post-translational processing, and secretion of biologically active FGF23. Putting the pieces in place should prove interesting and fun!

Acknowledgments

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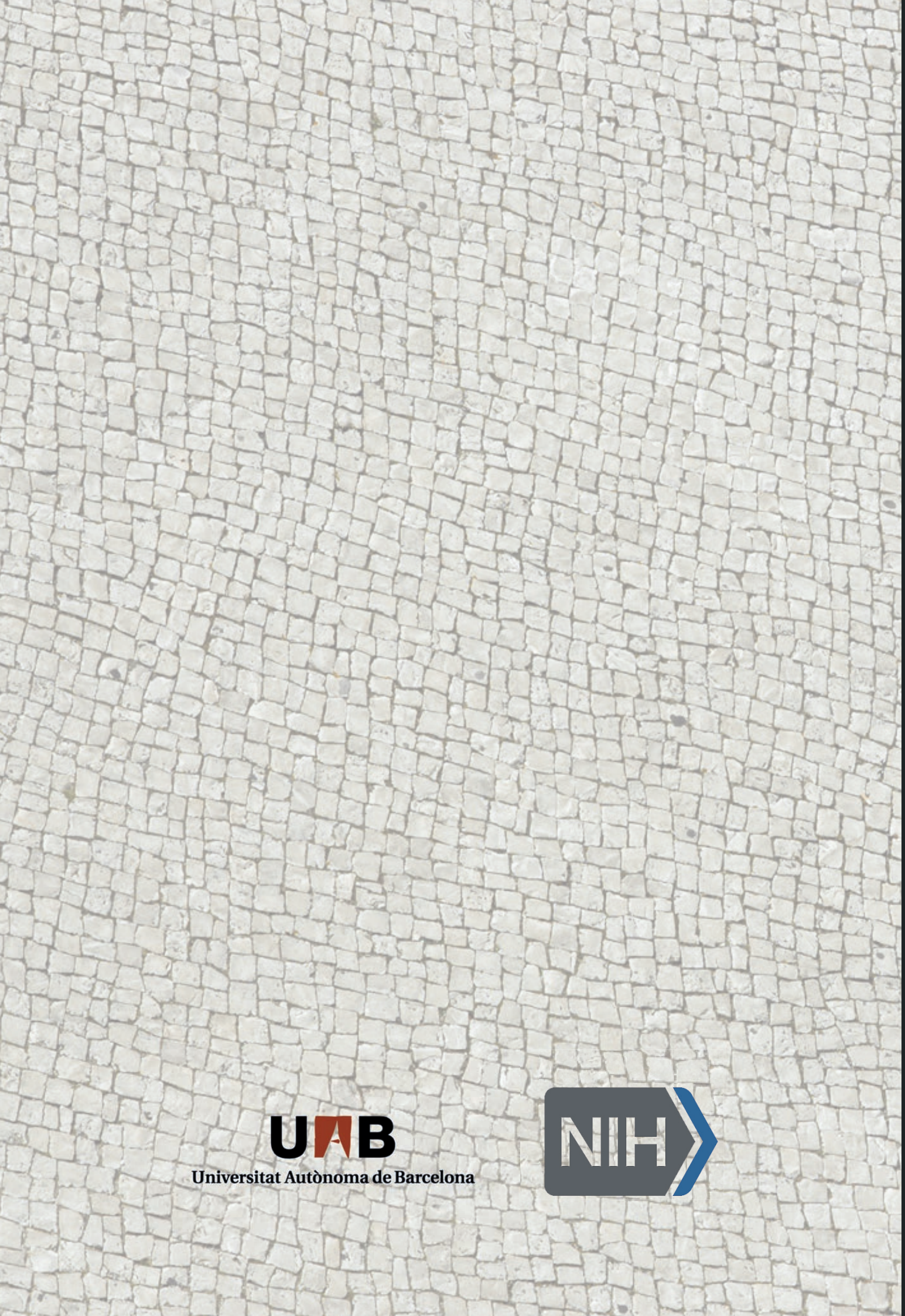
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