Next-Generation Sequencing Based Testing for Disorders of the Skeleton

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Abstract While disorders that involve the skeleton are common, most forms of genetic skeletal disorders are typically rare and not encountered routinely in clinical practice. The presentations and etiologies of genetic forms of skeletal disorders are very heterogeneous; therefore, they can be challenging to diagnose. An accurate diagnosis is very important for counseling regarding the natural history and recurrence risks as well as for appropriate management. Detailed medical and family history, physical examination, radiological evaluations, laboratory, biochemical and molecular tests are all important components in the assessment of genetic skeletal disorders. Molecular testing using next-generation sequencing (NGS) techniques can help identify the pathogenic genetic variants and thus confirm the diagnoses of specific bone disorders, even in conditions which there are overlapping clinical, radiographic and histological features. As there are limitations and advantages in using whole exome sequencing versus targeted gene panels, the decision of which test to use, should be made based on a case-by-case basis.

Keywords Skeletal Disorders • Bone Development • Molecular Diagnosis • Genetics Next-Generation Sequencing

1 Introduction

Disorders that involve the skeleton are commonly encountered in clinical practice. These disorders can result from numerous causes including age-related processes (e.g. senile osteoporosis), hormonal imbalances (e.g. postmenopausal osteoporosis

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and hyperparathyroidism-related bone loss), medications (e.g. corticosteroid-induced avascular necrosis), kidney and gastrointestinal disorders (e.g. renal osteodystrophy), and developmental anomalies of the bone (e.g. achondroplasia). Some bone disorders like osteoporosis have a high prevalence and have been estimated to affect over ten million individuals in the United States [1]. In contrast, *constitutional* errors of bone development, which typically manifest in childhood, are relatively rare conditions. These developmental disorders of bone have a collective incidence of 1 in 5000 births and can be categorized into: dysostoses (malformations of single skeletal elements), disruptions (malformations of bones due to non-skeletal causes), skeletal dysplasia (developmental disorders that involve bone and/or cartilage), and osteolyses (dissolution of preexisting bone) [2, 3]. Such disorders can present with short stature, abnormal patterning, altered size and structure of the bones, increased bone fragility, and secondary involvement of the nonskeletal tissues. Many disorders of the skeleton whether they are developmental or acquired, early or late-onset, have significant impact on the lives of affected individuals. An accurate diagnosis is important for counseling regarding the natural history and recurrence risks as well as for appropriate management. This chapter focuses on the diagnostic challenges in some genetic forms of skeletal disorders and the role of next-generation sequencing techniques in their diagnosis.

2 Genetic Forms of Skeletal Disorders

Genetic forms of skeletal disorders are heterogeneous in their presentations and etiologies. A "nomenclature" was developed in the 1970s in an attempt to classify these disorders, and these classifications have been updated and revised over the years [4–7]. The recognition of new phenotypes and the rapid advances in the molecular diagnostic techniques have led to significant increase in the number of disorders and identification of the causative genes. These have necessitated a more thorough evaluation of the nosology and classification of genetic skeletal disorders. The 2015 classification by the Nosology group of the International Skeletal Dysplasia Society identified over 430 conditions and categorized them into 42 groups based on molecular, biochemical, and/or radiographic criteria [8]. The conditions included those with primary bone involvement as well as overgrowth syndromes and lysosomal storage disorders with significant skeletal manifestations. While delving into further specifics of the classification are beyond the scope of this chapter, a review of the classification highlights the genetic and clinical heterogeneity of these disorders. Overall, there are 336 genes that have been identified to cause 436 disorders. Mutations in the same gene can give rise to phenotypically distinct disorders (e.g. metatrophic dysplasia and brachyolmia due to *TRPV4* mutations) or varying severity of the same disorder (e.g. *COL1A1* mutations in osteogenesis imperfecta types I [mild] vs. type II [perinatal lethal]), while mutations in different genes can give rise to disorders with overlapping clinical features (e.g. ciliopathies with major skeletal involvement). Mutations in genes encoding extracellular matrix proteins, transcription factors, signal transducers, enzymes, cellular

transporters, chaperone proteins, intracellular binding proteins, RNA processing molecules, and ciliary proteins can present with skeletal involvement of varying severity and patterns.

3 Diagnostic Challenges in Genetic Skeletal Disorders

An accurate diagnosis of genetic skeletal disorders requires detailed medical and family history, physical examination, radiologic evaluations, as well as laboratory, biochemical, and molecular tests. Most forms of genetic skeletal disorders are typically rare and are not encountered in routine clinical practices. Hence, their diagnosis and treatment are often performed by centers with specialized expertise. Some of the pertinent questions that may help to narrow the diagnostic considerations include: (1) Is the bone involvement primary or a part of multisystem involvement (e.g. lysosomal storage disorders, overgrowth syndromes, or inflammatory osteoarthropathy)? (2) Is the involvement localized to a few bones (dysostoses) or is it generalized (typically skeletal dysplasia)? (3) Is there a particular pattern of bone involvement (e.g. ribs and vertebral bones involvement in spondylocostal dystotoses vs. vertebral bones and the ends of the long bones in spondyloephiphyseal dysplasia)? (4) Is there a particular part of bone involved - epiphyseal or ends of the bones (e.g. multiple epiphyseal dysplasia types 1–6) vs. diaphyseal or midsection of long bones (e.g. diaphyseal dysplasia) vs. metaphyseal or the part of the bone joining epiphyses to the diaphysis (e.g. metaphyseal dysplasia, Jansen type)? (5) If the long bones are involved, is the involvement predominantly the proximal (rhizomelic), middle (mesomelic), distal (acromelic), or combinations thereof (acromesomelic)? (6) Are there specific diagnostic clues on exam or X-rays (e.g. blue sclera, tooth abnormalities in type I collagen-related osteogenesis imperfecta or interosseous membrane calcification and exuberant callous formation in osteogenesis imperfecta type V)? (7) For disorders of increased bone fragility, are they associated with decreased (e.g. osteogenesis imperfecta) or increased bone mineral density (e.g. osteopetrosis)?

Systematic assessment based on the site, severity, and nature of involvement can lead to the diagnosis in many genetic disorders of the bone without the further need for confirmatory molecular testing (e.g. achondroplasia). However, many a time, the diagnosis is not apparent and further molecular tests may be necessary.

4 Molecular Diagnosis of Genetic Skeletal Disorders

The ability to identify the pathogenic genetic variants that cause specific bone disorders can be helpful in the diagnosis given overlapping clinical, radiographic and histological features in many conditions. Until recently, molecular diagnostic testing for skeletal dysplasias was limited to sequencing a single or a few select genes by the Sanger sequencing method. This approach is effective when the possibility of the provisional diagnosis being correct is high and the number of genes to be

interrogated is few. However, in scenarios wherein there is genetic heterogeneity or the phenotype is not distinct enough to make a clinical diagnosis, interrogating numerous genes known to cause the phenotype would be a more time- and cost-effective strategy. For example, when the clinical and radiologic features are suggestive of a metaphyseal dysplasia, it would be more reasonable to investigate the seven genes that are known to cause eight conditions within this group at one time. Alternatively, when the majority of individuals with a particular disorder harbor pathogenic variants in one or few genes (e.g. *COL1A1* and *COL1A2* in osteogenesis imperfecta) and only a minority of affected individuals have mutations in one of the numerous other associated genes (e.g. *CRTAP*, *PPIB, LEPRE1, WNT1, FKBP10, SERPINF1* etc.), Sanger sequencing of the most commonly mutated genes followed by panel testing when required may be a reasonable approach.

5 Next-Generation Sequencing in Genetic Skeletal Disorders

Next-Generation Sequence (NGS) technologies have had a significant impact on the diagnosis of genetic disorders. Whole exome sequencing (WES) and targeted gene panels have been increasingly used in clinical practice. WES has the advantage of being able to sequence the entire coding portion of the genome and has been shown to have a diagnostic yield rate of 25% [9, 10]. Targeted gene panels focus on a set of genes known to cause particular phenotypes and typically have deeper coverage for the regions of interest. Currently, numerous gene panels are available for clinical diagnosis of a wide range of genetic skeletal disorders (genetic testing registry http://www.ncbi.nlm.nih.gov/gtr/ and GeneTests https://www.genetests.org). These range from large panels of over 150 genes for diagnosis of "many forms of skeletal dysplasia," to panels of over 50 genes for diagnosis of "disproportionate short stature," to assays that aim to assist in diagnoses of focused phenotypes like "osteogenesis imperfecta", "low bone mass", "osteopetrosis", high bone mass", and "Stickler syndrome", amongst others.

Table 1 A total of 34 genes responsible for disorders with high bone mass and low bone mass were utilized to create a next-generation sequencing based panel test. The total number of coding exons (CDS) and targeted bases are also shown

High Bone Mass Panel: *ANKH, CA2, CLCN7, CTSK, FAM123B, FAM20C, LEMD3, OSTM1, SOST, TCIRG1, TGFB1, TNFRSF11A, TNFRSF11B, TNFSF11, TYROBP* (15 genes) **Low Bone Mass Panel:** *ALPL, B4GALT7, COL1A1, COL1A2, COL3A1, COL5A1, COL5A2, CRTAP, FBN1, FKBP10, LEPRE1, PLOD2, PLOD3, PPIB, SERPINF1, SLC34A1, SLC39A13, SLC9A3R1, SP7* (19 genes)	
Number of CDS	602
Target size	98,962 bp (CDS ± 20 bp)
Enrichment	In solution capture library
Sequencing info	Illumina HiSeq 2000, 75 cycle, single-end

Table 2 Sequencing statistics for a total of 11 representative samples tested for disorders of high and low bone mass. All of the exons with low coverage (i.e., any base with coverage <20×) were "gap-filled" with Sanger sequencing

Sample ID	Mean coverage (bp)	Total reads per 100 bp	Minimal coverage*	# of CDS <10×	# of CDS <20×
#1	1148 ± 543	1572 ± 734	0/21×	12	14
#2	1201 ± 561	1656 ± 766	0/23×	11	13
#3	997 ± 430	1373 ± 591	0/22×	12	15
#4	969 ± 455	1328 ± 616	0/23×	14	20
#5	881 ± 402	1207 ± 545	0/24×	12	16
#6	1155 ± 568	1579 ± 765	0/21×	13	19
#7	1106 ± 546	1513 ± 737	0/23×	13	17
#8	1211 ± 586	1659 ± 792	0/20×	10	14
#9	523 ± 262	715 ± 356	0/21×	18	21
#10	945 ± 474	1290 ± 639	0/20×	13	16
#11	1072 ± 549	1465 ± 745	0/28×	13	19

* refers to the value of lowest coverage of all exons/ the coverage for 1st CDS >20×

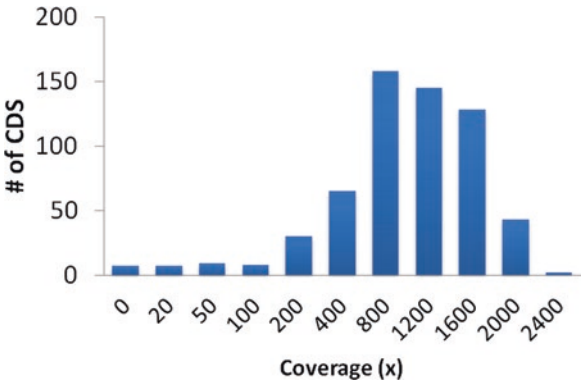

Fig. 1 Coverage depth for 602 coding exons in a representative panel used for diagnosis of disorders with high and low bone mass. Note that a vast majority of exons have deep coverage

Our previous experience on a diagnostic panel of disorders of low and high bone mass that included 34 related genes spanning 602 exons with complete coverage for coding exons from NGS and Sanger sequencing revealed 100% concordance while detecting previously identified pathogenic variants during the validation phase (Tables 1 and 2) [11]. The diagnostic utility of the panel was further underscored by the fact that a molecular diagnosis was achieved in four individuals (three with osteogenesis imperfecta and one with osteopetrosis) in whom, the genetic cause for the phenotype was not known.

As compared to exome or whole genome sequencing, panel testing offers advantages that include deeper coverage and fewer regions with insufficient coverage that could translate to decreased false negative rate (Fig. 1). In addition, regions with insufficient coverage, regions with high homologous sequences, and pseudogenes may be resolved by specifically designed PCR primers followed by

NGS. Panel-based testing is also typically more cost-efficient and is associated with fewer variants of uncertain significance (VUS) and incidental findings.

The increased use of panel-based tests has fueled the rapid growth and uptake of these diagnostic modalities in the clinic. The ability to interrogate multiple relevant genes in a single test is an attractive option for patients and physicians for whom such testing is associated with decreased costs and turn-around time, and increased diagnostic efficiency.

6 Other Diagnostic Evaluations

Biochemical tests may be useful for diagnosis in certain disorders. Some examples include urine oligosaccharides for mucopolysaccharidoses, low plasma alkaline phosphatase and elevated pyridoxal 5′-phosphate in hypophosphatasia, and abnormal sterol metabolites in chondrodysplasia punctata 2, X-linked [12, 13]. Skin biopsy and analysis of collagen secretion and amount are helpful in diagnosing osteogenesis imperfecta though this has currently been replaced by molecular diagnosis [14]. Tissue histology is typically not routinely performed but could be informative (e.g. osteogenesis imperfecta type IV). These additional modalities could be beneficial in confirmation of diagnosis when molecular testing reveals variants of uncertain significance [15].

7 Utility of an Accurate Diagnosis

(a) **For management**

The utility of making an accurate diagnosis cannot be overstated. Establishing a diagnosis provides psychological benefits and "closure" to families, enables access to the necessary support services, and guides the initiation of appropriate treatment and surveillance measures [16–18]. For example, a diagnosis of moderate-to-severe form of osteogenesis imperfecta may prompt the initiation of bisphosphonate therapy from infancy. Such therapy can be of utility in improving the bone mineral density [19–23]. Enzyme replacement therapies have been approved or being evaluated for some genetic skeletal disorders (e.g., Morquio A syndrome, hypophosphatasia) and their use is typically initiated after a definitive diagnosis [24, 25]. Many forms of genetic disorders of bone are associated with patterning defects (e.g. abnormal digits of the hand), scoliosis, or other bone malformations that may need surgical interventions. In addition, many disorders can be associated with extra-skeletal complications including neurologic (e.g., brain stem compression in achondroplasia and Morquio A syndrome), auditory (e.g., nerve entrapment in osteopetrosis and osteogenesis imperfecta), visual (e.g., optic nerve compression in osteopetrosis) and pulmonary systems (restrictive lung disease due to rib cage abnormalities). An appropriate diagnosis can thus be of significant use in initiating disease-specific surveillance measures.

(b) **For reproductive decisions**

Establishing a molecular diagnosis is important in counseling for recurrence risks and guiding reproductive decisions. Individuals with skeletal dysplasia consider the risk of transmitting the condition and the medical impact of the condition on a child as major concerns with respect to having children [26]. A not-so-infrequent-scenario is when the abnormalities of bone are detected prenatally during ultrasound examinations. Recognition of specific skeletal anomaly on ultrasound is extremely challenging and thus a definitive diagnosis is often dependent on molecular confirmation [27]. An accurate molecular diagnosis may be important for decisions regarding continuing the pregnancy or preparing to deliver the child at a tertiary care center. Panel testing could especially be of utility in such situation wherein a diagnosis may have to be reached in a short period of time. Many laboratories now offer panel-based testing for prenatal diagnosis of genetic skeletal disorders.

(c) **For evaluation of a heritable cause for fractures vs. non-accidental trauma**

Distinguishing fractures due to a genetic form of brittle bone disorder from acquired causes can have significant implications. Children with osteogenesis imperfecta can present with many fractures in various stages of healing. This is also the case in children who sustain non-accidental trauma (NAT) due to physical abuse. NAT is the leading cause of fractures in infancy and typically mandates reporting to appropriate authorities. Thus, differentiating a heritable form of bone disorder that predisposes to fracture from NAT can have medical, social, as well as legal consequences. Whereas often, the history, location and type of fractures, and other associated injuries may help in differentiating between osteogenesis imperfecta and NAT, this is not always the case. Hence, comprehensive molecular testing could be of significant utility in such scenarios.

8 Strengths and Limitations of Panel Testing in Clinic

Targeted panel tests typically provide deeper coverage than untargeted capture and sequencing of the exome or genome. The gaps in sequence due to the presence of pseudogenes or GC-rich regions are typically known and can be supplemented with Sanger sequencing of such regions to comprehensively interrogate the genes of interest. Some panels can be more affordable than whole exome sequencing. However, panel testing has limitations. The pace of discoveries in genetic disorders of bone typically makes any panel inadequate within a short span of time. Adding new genes and revalidation of such panels imposes burden of costs and time on the diagnostic laboratories. When bones are involved along with other organ manifestations, the differential diagnosis may be broad enough that whole exome sequencing could yield better results than targeted sequencing.

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