The Molecular Revolution in Cutaneous Biology: Era of Molecular Diagnostics for Inherited Skin Diseases

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The discovery of pathogenic mutations in inherited skin diseases represents one of the major landmarks of late 20th century molecular genetics. Mutation data can provide accurate diagnoses, improve genetic counseling, help define disease mechanisms, establish disease models, and provide a basis for translational research and testing of novel therapeutics. The process of detecting disease mutations, however, has not always been straightforward. Traditional approaches using genetic linkage or candidate gene analysis have often been limited, costly, and slow to yield new insights, but the advent of next-generation sequencing (NGS) technologies has altered the landscape of current gene discovery and mutation detection approaches.

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During the 1990s, disease gene discovery usually emanated from either genetic linkage studies (e.g., for dominant diseases or recessive diseases in consanguineous pedigrees) or from candidate gene approaches (excellent for recessive diseases associated with a reduction/absence of the target protein). For example, genetic linkage studies in epidermolysis bullosa simplex and Darier disease paved the way for subsequent identification of keratin (*KRT5*, *KRT14*) and calcium pump (*ATP2A2*) genes, respectively (Ryynänen et al., 1991; Wakem et al., 1996). Having identified a likely gene, most mutation detection methods then focused on comprehensive Sanger sequencing, or, for larger genes, an additional step of mutation screening was necessary. These approaches included denaturing gradient gel electrophoresis (Myers et al., 1987), chemical cleavage of mismatch (Cotton et al., 1988), single-stranded conformation polymorphism (Orita et al., 1989), heteroduplex analysis (White et al., 1992), conformation sensitive gel electrophoresis (Ganguly et al., 1993), and the protein truncation test (Roest et al., 1993). Choice of approach was influenced by the sensitivity of the method, the size of the gene, and its number of exons, and some comparative studies, such as for *COL7A1* mutation screening in dystrophic epidermolysis bullosa, have been reported (Whittock et al., 1999). The initial screening would then be followed by targeted Sanger sequencing, although all approaches have been gradually superseded by the increasing availability and reduced costs of NGS technologies.

For clinical application, NGS mainly centers on two techniques: whole-exome sequencing (WES) and whole-genome sequencing (WGS). Technically, WES incorporates two powerful recent developments in genetic sequencing: first, the isolation of target regions from mechanically fragmented DNA by hybridization (capture), and second, the sequencing capability to read lengths of DNA ranging from approximately 100 to several hundred base pairs. After the reads are recorded, with each nucleotide being sequenced up to 100 times, sequence alignment with a reference sequence is performed. One key challenge of large-scale data, however, lies in the number of DNA variants identified. For example, DNA from an outbred European subjected to WES would be expected to disclose approximately 25,000 variants, and therefore fast and efficient filtering tools are needed to reliably and efficiently extract the desired information from the vast dataset.

A major area of interest has been the use of WES (or WGS) for gene discovery or diagnosis. As a disease gene discovery tool, the value of WES is very clear. From perhaps the earliest report of a homozygous missense mutation in *SLC26A3* (Choi et al., 2009) in a patient with Bartter syndrome, a renal salt-wasting disease, more than 20 novel and previously uncharacterized genodermatoses, germline and mosaic, have been identified through WES, and many further discoveries are anticipated. Selected recent examples include the discovery of somatic mutations in *HRAS* and *KRAS* in nevus sebaceus (Levinsohn et al., 2013) and a new autosomal recessive epithelial inflammatory disease resulting from germline mutations in *EGFR* (Campbell et al., 2014; Ganetzky et al., 2015). For diagnostic applications, however, there are some limitations to WES, mainly centered on the management and analysis of the large-scale data that are generated. The difficulties of data management are expanded 50-fold for WGS) which may generate over 2–3 million variants (Moore et al., 2011). Nevertheless, WGS can detect mutations in noncoding regions, copy number variation, and complex chromosomal rearrangements, variations that WES is limited in its ability to detect (Schaffer, 2012). Studies have also compared diagnostic laboratory use of WES, WGS, and targeted enrichment of genes (gene panels) and have

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Abbreviations: NGS, next-generation sequencing; WES, whole-exome sequencing; WGS, whole-genome sequencing

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concluded that WES currently offers the optimal mutation detection methods for disorders that may be genetically heterogeneous and for which additional currently unknown gene pathology may contribute (Sun et al., 2015), although gene panels may represent the most practical option for well-characterized disorders in which the spectrum of molecular pathology is close to being fully known. Indeed, next-generation gene panel sequencing has already been shown to offer both accurate and rapid diagnostics (less than 72 hours to complete) for patients with suspected epidermolysis bullosa (Tenedini et al., 2015).

For families, one of the major benefits of molecular diagnostics has been the development of new options for prenatal testing, with expansion of personal choice when faced with the prospects of risk of recurrence of a genetic skin disease. The first prenatal diagnostic tests for genodermatoses (epidermolytic ichthyosis and junctional epidermolysis bullosa) were performed by fetal skin biopsy in 1980 (Golbus et al., 1980; Rodeck et al., 1980). These biopsies were performed during the second trimester with the aid of a fetoscope to visualize the fetus, although later biopsies were performed under ultrasonographic guidance. Initially, light microscopy and transmission electron microscopy were the main diagnostic tools, although during the mid-1980s, complementary immunohistochemical tests were added to improve diagnostic accuracy (Fassihi et al., 2006a). During the mid-1990s, DNA-based tests were introduced for genodermatoses. By comparison with fetal skin biopsy samples, sampling fetal DNA was performed much earlier (end of first trimester). Typically, the procedure involves sampling chorionic villi, components of the placenta that contain the same genetic material as the fetus. The sampling can be done either transcervically or transabdominally under ultrasonographic guidance, depending on the position of the placenta. DNA-based prenatal testing has been applied to a much broader range of genodermatoses. Indeed, testing for over 30 different inherited skin disorders has been reported. A fundamental prerequisite to performing DNA-based prenatal tests, however, is the need to establish informative molecular data in families with a previously affected child. Ideally, DNA samples should be obtained from the parents, the affected individual, and any other siblings to search for pathogenic mutations. This comprehensive screening of familial DNA can be helpful in ruling out occurrences of de novo mutations, nonpaternity, uniparental disomy, and germline mosaicism.

Preimplantation genetic diagnosis, first developed in 1990, is an alternative to conventional DNA-based prenatal testing (Handyside et al., 1990). The method involves testing for specific genetic abnormalities using a single cell from 8- to 10-cell–stage embryos cultured in vitro. With regard to inherited skin diseases, there has been only one reported case of successful preimplantation genetic diagnosis leading to birth of a healthy baby in a family at reproductive risk of ectodermal dysplasia–skin fragility syndrome (Fassihi et al., 2006b), a form of epidermolysis bullosa simplex resulting from mutations in the desmosomal protein plakophilin 1. The subsequent low uptake of preimplantation genetic diagnosis for genodermatoses partly relates to technical difficulties in the design and optimization of family-specific protocols. Such work is often time-consuming, expensive, associated with some health risks (e.g., ovarian hyperstimulation syndrome), and beset with some technical challenges. Notably, a single cell only contains approximately 6 pg of DNA, which makes assays vulnerable to contamination by extraneous DNA and failure of allele amplification. To counter these limitations, generic multiplex PCR assays of appropriate linked microsatellite markers for specific gene loci have been established; this approach is known as preimplantation genetic haplotyping (Renwick et al., 2006). The feasibility of preimplantation genetic haplotyping involves linkage analysis with genotyping of the couple requesting preimplantation genetic diagnosis, and potentially other family members, to construct high- and low-risk haplotypes. The methodology relies on amplification of the whole genome from a single cell to give microgram quantities of template DNA (e.g., using multiple displacement amplification), which then allows testing of multiple loci using standard DNA-based PCR protocols. Robust, licensed preimplantation genetic haplotyping protocols have been developed and applied for *LAMB3*, the gene most commonly implicated in generalized severe forms of junctional epidermolysis bullosa (Fassihi et al., 2010).

Prenatal testing by invasive procedures, including chorionic villus sampling or amniocentesis, is associated with a risk of spontaneous abortion in a small number of pregnancies (~0.5–1%). As such, noninvasive methods of prenatal testing, using fetal-derived genetic material in the maternal blood, have been pursued. These approaches were boosted in 1997 by the discovery of cell-free fetal DNA in the maternal blood (Lo et al., 1998). Free fetal DNA is mostly derived from the placenta and constitutes approximately 5% of cell-free DNA in the maternal plasma and mainly consists of short fragments (<200 base pairs). This DNA is detectable as early as day 18 after embryo transfer (Guibert et al., 2003), and its concentration increases as pregnancy progresses. Unlike rare nucleated fetal cells that can be found in the maternal circulation, there is no long-term persistence of free fetal DNA. Indeed, free fetal DNA is cleared rapidly with a mean half-life of 16 minutes (Lo et al., 1999).

Subsequently, NGS of free fetal DNA has been applied for prenatal testing, although costs have limited its clinical application. Moreover, direct sequencing of fetal DNA is limited to screening of paternal mutant alleles, given the presence of background maternal DNA. Advances in detecting epigenetic differences between maternal blood DNA and cell-free fetal DNA have been observed, suggesting that methylation assays could be very effective for screening genomes for differentially methylated CpGs that might then be used as fetal specific markers (Bunce et al., 2012; Chu et al., 2009; Ou et al., 2014). Two principal methylation assay methods are being developed: methyl-DNA immunoprecipitation or use of methyl-sensitive restriction enzymes followed by PCR approaches—the latter has shown differentially methylated CpG sites on chromosomes 13, 18, and 21 with a potential for aneuploidy testing and regions harboring submicroscopic deletion or duplication syndromes. For now, however, these new methods of discriminating and defining fetal DNA markers have yet to affect prenatal diagnostics for inherited skin diseases.

The arrival of high-throughput single-nucleotide polymorphism genotyping and, more recently, NGS technology