Single gene disorders come into focus—again

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For more than 10 years, genome research has focused on finding genetic risk factors for common disorders, based on the "common disease—common variant (CDCV)" hypothesis—the intuitive but unproven assumption that for most of the common disorders like dementia, diabetes, coronary heart disease, autism, and hypertension, there are common genetic risk factors. Since 2007, after many years of growing frustration with the disappointing results of genome-wide association studies (GWAS), associated markers were identified for a wide variety of complex disorders; this was hailed as a decisive breakthrough in this field. However, these associations were only found after massively increasing cohort sizes and marker densities, meaning that the vast majority of the associated risk factors have small effects and that they are of no diagnostic and prognostic relevance. Moreover, many markers were found to be located in noncoding sequences, and thus, very few pro-

In the early 1990s, when the second 5-year plan for the Human Genome Project—which requested more money than any previous research project in biology—was written, common disorders were presented as the future target of genome research. This was a clever move to ensure continued public support for this endeavor, which had been justified previously by the prospect that it would lead to the diagnosis, prevention, and therapy of severe, but mostly rare, Mendelian disorders. Today, more than 15 years later, after billions of dollars have been spent on genome-wide association studies (GWAS), very few major genetic risk factors for common diseases have been identified, and the enthusiasm for large GWAS is dwindling. At the same time, there is renewed interest for studying single gene disorders, which are now considered by some as a better clue to the understanding of common diseases. While this is probably true, Mendelian disorders are also important in their own right, since they must be far more common than generally thought. As discussed here, various efficient strategies exist for the elucidation of single gene defects, and their systematic application in combination with novel genome partitioning and massive parallel sequencing techniques, will have far-reaching implications for health care.

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vided novel insights into the underlying pathogenetic mechanisms. Ironically, therefore, very shortly after this "breakthrough," there is growing support for the notion that for most common disorders, the CDCV hypothesis must be wrong.[1,2]

This is certainly true for mental retardation (MR)—the biggest unsolved problem of clinical genetics and the largest socioeconomic burden of health care—where most severe forms are due to defined chromosomal abnormalities or single gene defects, instead of resulting from multifactorial inheritance, ie, the interaction of many different gene variants and environmental factors. However, there is increasing evidence that single gene defects also play a significant, previously underestimated, role in other complex disorders. This has led to growing uneasiness about the validity of the idea that GWAS is the preferred approach for identifying sequence variants in the human genome that predispose to, or cause, disease. Moreover, it has raised serious doubts about the strategy, first proposed in the early 1990s and uncritically adopted by leading genome centers worldwide, to focus exclusively on complex disorders.

After the introduction of massive parallel next-generation sequencing techniques, there are now indications for a paradigm shift in this field, with a renewed focus on single gene disorders. At a recent meeting,[3] two groups reported on their efforts to unravel the molecular basis of Mendelian disorders by sequencing all exons in the genomes of patients and their unaffected parents. Moreover, leading genome researchers expressed their belief that instead of GWAS, whole genome sequencing-based, large-scale elucidation of single gene disorders will be the strategy of choice for shedding more light on the molecular architecture of common disorders.

In the late 1980s, before common disorders were proclaimed as the central target of genome research, along with overly optimistic assumptions about the medical implications of this research, the revolutionary and costly project to elucidate the structure of the human genome had been justified by the prospect that it would lead to unambiguous diagnosis, prevention, and, eventually, therapies for severe Mendelian disorders. Now, almost 20 years after the official commencement of the Human Genome Project, and 6 years after its completion, it appears that genome research is coming around full circle by once again focusing on single gene defects.

Single gene defects are important for health care

Single gene defects have significance in their own right. In contrast to many complex disorders such as type 2 diabetes and obesity, which are lifestyle-related, become manifest only later in life, or are relatively mild, single gene disorders are mostly severe, early-onset conditions, necessitating lifelong care and support. Moreover, single gene disorders are far more numerous than generally assumed, and as a group, they are certainly not rare.

According to OMIM, the comprehensive catalogue of human traits that are inherited in a Mendelian fashion (http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim), only slightly more than 2500 human genes have been linked to disease, and there are approximately 3500 Mendelian diseases for which the molecular cause is not yet known. It is likely, however, that this is a wide underestimate, and that the number of genes which are indispensable for normal embryonic and postnatal development, homeostasis, and aging is much higher.

In mice with induced defects of single genes (ie, "knockout mice"), conspicuous (disease) phenotypes or embryonic lethality are the rule rather than the exception, as discussed elsewhere.[2] In humans, the proportion of gene defects that are associated with recognizable disorders must be even higher, because relatively subtle (eg, behavioral) abnormalities are readily detectable in man, even without specific clinical examination. Milder mutations in the same genes known to cause embryonic lethality when affected by loss-of-function mutations may be compatible with life but also cause disease.

Functional considerations and empirical data from model organisms suggest that most disease-associated gene defects are inherited as recessive traits. At least in Western societies, this means that most patients will be isolated cases, due to small family sizes and the fact that in these populations, parental consanguinity is rare. In sporadic cases without specific, previously described combinations of clinical symptoms, single gene defects are unlikely to be considered as the underlying cause. In particular, this holds for patients with complex disorders and presumed multifactorial inheritance. Thus, as discussed for MR, it is likely that many Mendelian disorders have not been identified yet because in the well-studied Western populations, they do not segregate in families. Irrespective of family sizes and parental con-

sanguinity, this also holds for all severe autosomal dominant disorders conferring a significant reproductive disadvantage (eg, severe mental handicaps). Most of these patients will carry new mutations and therefore will be isolated cases as well.

For most common diseases, the possibility that there is a sizable "contamination" by monogenic forms has not been excluded, and the proportion of cases that are due to single gene defects is hitherto unknown. As indicated above, this does not hold for MR, however. Prompted by the early observation that males are more often affected than females,[4] and by the description of several large families where MR segregated in an X-linked fashion (see ref 5, for example), the hypothesis that single gene defects on the X chromosome play a major role in MR was put forward in the early 1970s.[6,7]

Since the 1990s, genetic research into the molecular causes of MR has focused on X-chromosomal genes,[8] and at the time of writing (September 2009), mutations in 90 X-chromosomal genes have been implicated in Mendelian forms of MR, demonstrating that this condition is extremely heterogeneous. Surprisingly, screening of several hundred families with X-linked MR (XLMR) has revealed that these 90 genes account for at most 50% of all mutations[9]; see also ref 10. This means that there must be many more genes on the X chromosome which are indispensable for the normal function of the human brain. The X chromosome carries about 4% of all human genes, and even though there is evidence suggesting that on the X, the density of MR genes is higher than on autosomes,[11] extrapolation of these data suggests that defects in several thousand human genes may give rise to cognitive dysfunction. However, the systematic search for these autosomal MR genes has only just begun, as discussed below.

There is increasing evidence that single gene defects also have important roles in the etiology of other complex disorders. For example, several homozygous deletions were recently described in autistic offspring of healthy consanguineous parents,[12] strongly suggesting that autosomal recessive gene defects are important causes of autism, too. In view of the growing molecular evidence that MR, autism, and schizophrenia are etiologically related,[2,13] it is likely that many cases of schizophrenia are also due to a variety of single gene defects. There is reason to believe that the same holds true for many other complex diseases that are generally considered multifactorial.[14]

Systematic elucidation of single gene disorders

There are various efficient strategies for elucidating the molecular defects underlying Mendelian disorders, as discussed in detail elsewhere.[2] Most of them consist of two steps, the chromosomal and regional mapping of the relevant defect and the search for mutations in positional and functional candidate genes.

Disease-associated balanced chromosome rearrangements

Systematic breakpoint mapping and cloning in patients with disease-associated balanced chromosome rearrangements (DBCRs) has been employed by several groups to identify genes that are truncated or inactivated by the rearrangement *(Figure 1a)*. Most de novo balanced chromosome rearrangements can be identified by conventional karyotyping, and, with an incidence of 1 in 2000, they are not rare. About 6% of these are associated with MR or other clinical abnormalities, which means that in the European Union, with its 495 million inhabitants, there must be almost 15 000 patients with de novo DBCRs, and even more familial cases. So far, only a small percentage of these patients have been identified, which argues for systematic karyotyping in all patients where a genetic cause of the disorder cannot be ruled out. Unfortunately, however, the ongoing substitution of conventional karyotype analyses with array CGH techniques (see below) means that balanced chromosome rearrangements will no longer be detected upon routine cytogenetic examination.

Mapping of chromosomal breakpoints has been facilitated by the availability of an ordered set of large overlapping genomic clones that serve as probes for fluorescent in situ hybridization (FISH). Still, determining the precise sequence of the breakpoint region remained quite time-consuming. Recently, Chen et al[15] have overcome this problem by preparative sorting of derivative chromosomes followed by next-generation sequencing in three mentally retarded patients with DBCRs, which enabled the identification of three novel candidate genes for MR. In follow-up studies, they showed that it is even possible (by paired-end sequencing) to identify breakpoint-spanning DNA fragments in total genomic DNA, ie, without prior sorting of chromosomes.[16]

Screening for microdeletions and duplications

Small deletions, barely detectable by high-resolution karyotyping, illuminated the way to pinpointing the Duchenne muscular dystrophy gene[17]; later on, microdeletions were instrumental in the identification of many other disease genes. Through the recent introduction of array-based comparative genomic hybridization (array CGH), screening of the entire human genome for submicroscopic copy number variants (CNVs) has become possible, thereby providing a very powerful new strategy for finding the molecular defects underlying Mendelian disorders *(Figure 1b)*. Employing tiling path BAC arrays or, more recently, high-density oligonucleotide arrays, apparently causative de novo microdeletions or duplications can be found in more than 10% of mentally retarded patients,[18] which means that these small variations are about as common as chromosome rearrangements that can be seen under the microscope. Recurrent CNVs that are flanked by low-copy repeats account for about half of the cases (B. de Vries, Nijmegen, personal communication, 2009), and for many of these new "genomic disorders," [19] both deletions and duplications have been observed.

Apart from CNVs causing disease, eg, by disturbing the stoichiometry of protein complexes or by unmasking recessive gene defects,[20] the vast majority of CNVs occur in healthy individuals, and most of them are functionally neutral polymorphisms. Using tiling oligonucleotide microarrays to detect CNVs greater than 450 basepairs, Conrad et al[21] have identified, on average, more than 1000 validated CNVs when comparing genomes of two unrelated individuals.

However, not all CNVs can be assigned unambiguously to one of these two groups. There is a third category of CNVs which are neither functionally neutral nor strictly pathogenic; they are significantly more common in patients with specific disorders than in healthy individuals. One of the first CNVs of this kind observed, a recurrent, sometimes familial 1 to 2 Mb deletion/duplication on chromosome 16p13, was detected in a cohort of 300 patients with autism spectrum disorder and/or MR.[22] Follow-up studies[23] have shown that this CNV, and another on chromosome 15q11.2, are among the most common and important risk factors for MR and autism known to date, both raising the risk for these diseases about 5-fold. Moreover, according to a recent report, the dup16p13.1 is also a significant risk factor for schizophrenia.[13] This CNV encompasses the *NDE1* gene, which interacts with *DISC1*, a known schizophrenia susceptibility gene, and has also been implicated in Asperger syndrome, as discussed elsewhere.[2] Thus, there is no sharp demarcation line separating functionally neutral polymorphisms and clinically relevant CNVs, and distinguishing them is not a trivial task (see below).

Linkage mapping

X-linked disorders are easily recognizable because of their characteristic pattern of inheritance. This is why they are over-represented in OMIM, and why the underlying molecular defect has been elucidated in many

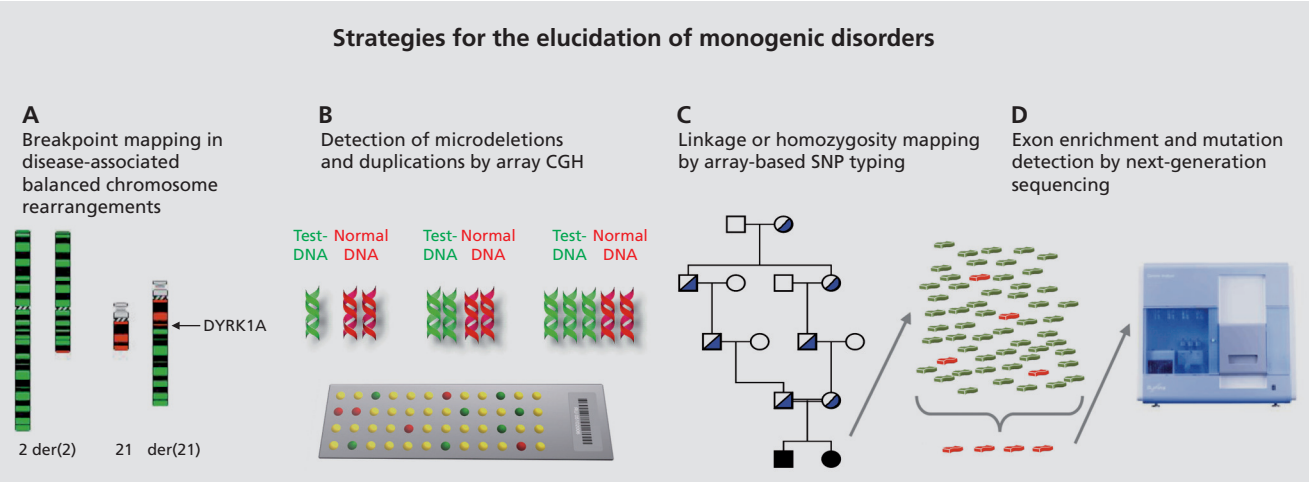

Figure 1. Strategies for the elucidation of monogenic disorders. CGH, comparative genome hybridization; SNP, single-nucleotide polymorphism

instances, as already discussed for X-linked MR. Autosomal dominant disorders also run in families, if they are not lethal in early life, or are so severe that affected individuals do not reproduce. For this reason, they are also easily identifiable, which explains why so many of them are known. In contrast, autosomal recessive disorders are likely to be under-represented, because in Western populations, most patients are isolated cases; the monogenic nature of these disorders is thus not recognized, as discussed above.

Homozygosity mapping in large, consanguineous families is the strategy of choice for mapping recessive disorders *(Figure 1c)*. Such families are common in predominantly Islamic countries of the "consanguinity belt"[24] that extends from Morocco into India. Significantly elevated miscarriage rates and a two-to-threefold higher prevalence of MR and congenital malformations in these countries are generally ascribed to malnutrition and poor standards of hygiene. However, there is evidence that these disorders are also more common in Muslim families living abroad, such as Turkish families in Germany and families from Pakistan in the UK, which suggests that recessive gene defects are another important cause.

Specific forms of autosomal recessive MR (ARMR) that are due to primary microcephaly have been investigated by homozygosity mapping in consanguineous families from Pakistan and India, which led to the identification of 7 loci and 5 microcephaly genes.[25-27] Similarly, large-scale homozygosity mapping in consanguineous Iranian families has revealed numerous novel loci and several new genes for nonsyndromic ARMR, which is thought to be more common than syndromic forms.[28-31] These studies showed that nonsyndromic ARMR is extremely heterogeneous, thereby refuting earlier speculations that, analogous to the fragile X syndrome in X-linked MR and to connexin 26 mutations in nonsyndromic deafness (eg, see ref 32), there might be frequent forms of this disorder. There is recent evidence, however, that ARMR is not quite as heterogeneous as previously suggested. Systematic homozygosity mapping and mutation screening in 250 Iranian families has identified numerous new loci for ARMR and several allelic mutations in the relevant genes (Kuss, Kahrizi, Tzschach, Najmabadi, Ropers et al, unpublished). Analogous studies have also greatly expanded our knowledge of recessive defects in other diseases such as deafness, and there is now evidence that recessive forms also exist in autism and other frequent disorders that are considered to be multifactorial.

Identification of functional candidate genes

Many of the clinically relevant deletions detected by array CGH are larger than 1 to 2 Mb, and most linkage intervals are even larger, often comprising several hundred genes. This renders mutation screening of all genes in these intervals very time-consuming and costly. Numerous software packages have been developed, including PosMed, Endeavour, and Polyphen (see ref 2) that can be employed to identify and prioritize functional candidate genes corresponding to the relevant disease phenotype. The utility of these programs depends on the specificity of the phenotype; not unexpectedly, their performance is still relatively poor for nonsyndromic MR, but much better for easily recognizable syndromes. Undoubtedly, it will improve once more is known about regulatory pathways and the interaction partners of genes and proteins.

As mutation detection techniques are rapidly evolving, sometimes either functional or positional information may suffice for finding specific gene defects. For example, fine-tuning of synaptic transmission is essential for proper brain function, and there are about 1200 proteins that are expressed predominantly in the synapse. Even with conventional Sanger sequencing techniques, screening of all synapse proteins to isolate gene defects responsible for brain dysfunction is no longer an impossible task,[33] and novel technologies are around the corner, which will further facilitate large-scale mutation screening (see below).

Why not search for the mutation directly?

In a recent attempt to identify nearly all genes involved in X-linked MR in one sweep, an international consortium has employed Sanger sequencing to screen 208 families with X-linked MR for mutations in more than 700 fully annotated X-chromosomal genes.[10] This heroic effort has revealed recurrent truncating mutations in 9 novel XLMR genes, and, notably, also almost 1000 missense changes. Some of these are allelic and probably functionally relevant, eg, there are several such mutations in the IQSEC2 gene, which codes for a guanine nucleotide exchange factor.[34] Recent follow-up studies revealed apparently pathogenic CNVs in >10% of the

families,[35] but for more than half of the families studied, the causative molecular defect is still unknown.

This pioneering study has highlighted the possibilities, but also some of the problems, that researchers will face when trying to identify a single pathogenic mutation in an entire genome full of mostly neutral sequence variants. As shown by two independent studies,[36,37] the coding portion of individual genomes contains approximately 10 000 nonsynonymous nucleotide changes, even after excluding those that are known as single-nucleotide polymorphisms (SNPs). These figures should dampen the enthusiasm of those proposing to elucidate unknown monogenic disorders by whole-genome sequencing of single patients and their healthy parents, using exon enrichment and next-generation sequencing techniques *(Figure 1d)*,[3] even though, admittedly, some of the underlying defects may be detectable in this way, depending on the nature of the relevant mutation.

There are now various efficient methods for the enrichment of exons or defined genomic intervals, including custom-made oligonucleotide arrays, commercial enrichment kits based on hybridization in solution, or advanced PCR-based techniques (for details, see the recent review by Tucker et al[38]). Preparative chromosome sorting and next-generation sequencing[39] is another attractive alternative for facilitating mutation detection when the chromosomal location of the defect is known. An advantage of this approach is that it will allow us to detect mutations everywhere on the relevant chromosome, including introns and intergenic sequences. Moreover, sequencing of sorted chromosomes yields a more even coverage than other enrichment strategies that involve PCR amplification (Chen, Wrogemann, Hu, Haas, Ropers et al, unpublished).

Each of these methods has its limitations, however, and the same holds for next-generation sequencing techniques with their usually small read length, which is a problem for (re)sequencing of repeat-rich genome segments. Still, in combination, genome partitioning methods and next-generation sequencing techniques are a great asset for the detection of mutations in defined genomic intervals, which has been one of the stumbling blocks for the large-scale elucidation of single gene disorders.

Conclusions and outlook

With the implementation of these novel methods, the stage is set for the systematic identification of single gene defects, which is overdue and will have far-reaching implications for health care. Recessive disorders likely represent the bulk of the disorders that are hitherto unknown, but they are easily overlooked in industrialized countries because most of the patients will be isolated cases, particularly those without clearly distinguishable phenotypes. Their identification and recruitment is much easier in countries where large families and parental consanguinity are common, but due to more urgent problems, like the scarcity of clean drinking water, malnutrition, or high perinatal and infant mortality, the diagnosis, prevention, and therapy of single gene defects is not high on their agenda, even though these disorders are even more common in these countries than they are in outbred Western populations. This argues for collaborations between emerging and industrialized countries, as exemplified by the long-standing collaboration between our group and an effective Iranian partner, which was instrumental in the elucidating the gene defects responsible for several recessive forms of MR, thereby paving the way for the diagnosis, prevention and—eventually—therapy of these disorders.

So far, recessive disorders are considered too rare to justify carrier screening, but this is likely to change as soon as there is a reliable and inexpensive test for all recessive disorders. According to leading manufacturers, "third-generation" sequencing technologies that enable sequencing of the entire human genome for less than $5000 US will be on the market by the end of 2010 or early in 2011, which indicates that carrier tests for all known recessive disorders will be available sooner rather than later. Indeed, the (US) National Center for Genome Resources has recently teamed up with the Beyond Batten Disease Foundation to develop such a test for approximately 448 single gene defects using available next-generation sequencing technology. With such a carrier test at hand, premarital screening can be offered to rule out the possibility that both spouses are heterozygous for defects in the same gene, and prevention programs can be set up, similar to the successful prevention of Tay-Sachs disease in Ashkenazim, which was initiated in the 1970s.[40]

Whole genome sequencing (WGS) is not only the method of choice for the large-scale elucidation of Mendelian disorders, but it is also a superior alternative for risk factor screening in complex diseases, because it is not fraught with the inherent limitations of GWAS.[2,41] There is no doubt that there exist genetic factors which

predispose individuals to disease without sufficing for disease manifestation, as discussed for CNVs that are risk factors for MR, autism, and schizophrenia. Another telling example is a deletion on chromosome 1q that seems to be a necessary but not sufficient prerequisite for thrombocytopenia/absent radius syndrome.[42] CNVs predisposing for disease can only be identified efficiently by large case-control studies; attempts to find them by investigating the normal variation, ie, by excluding all CNVs present in healthy individuals, are bound to fail because risk factors for common disorders will be found in the healthy controls, too. From the health care point of view it is unfortunate, therefore, that large sums were invested to generate inventories of normal CNVs, instead of focusing on disease-relevant CNVs right from the start—and the same criticism applies to the even more costly "1000 genome project," which uses GWS to study the normal genome variation in 1000 healthy individuals.

It is a commonly held view that mild forms of MR are multifactorial, while severe forms are largely due to catastrophic genetic defects, including chromosomal aberrations and mutations of single genes. Lehrke[6,7] assumed that MR genes and genes determining the IQ were identical, and others speculated that risk factors for mild MR might be allelic variants of these genes,[43,44] exerting a moderate effect on the IQ. As the number of MR genes is increasing, and in view of the novel methods for high-throughput mutation detection, everything seems to be in place for putting these ideas to the test. ❑

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Los trastornos por un gen único vuelven a estar en el foco de la investigación

A comienzos de los años 1990, cuando se escribió la segunda fase de cinco años del Proyecto del Genoma Humano –el cual ha requerido más dinero que cualquier otro proyecto de investigación en biología- los trastornos comunes fueron presentados como los blancos futuros de la investigación del genoma. Esta fue una medida ingeniosa para asegurar un financiamiento público continuo para este esfuerzo, el cual se había justificado previamente por las perspectivas que conducirían al diagnóstico, la prevención y el tratamiento de los trastornos Mendelianos, que si bien son graves ocurren con escasa frecuencia. Hoy día, después de más de quince años y luego de haber gastado billones de dólares en los estudios de asociación del genoma completo (EAGC), se han identificado muy pocos factores de riesgo genético importantes para las enfermedades comunes, y el entusiasmo por grandes EAGC está disminuyendo. Al mismo tiempo, hay un renovado interés en el estudio de trastornos por un gen único, los cuales son considerados ahora por algunos investigadores como una mejor pista para la comprensión de las enfermedades comunes. Aunque esto es probablemente cierto, los trastornos Mendelianos también son importantes por derecho propio, ya que ellos deben ser mucho más comunes de lo que generalmente se piensa. Como se discute aquí, existen varias estrategias eficientes para aclarar los defectos de un gen único y su aplicación sistemática en combinación con nuevas técnicas de división del genoma y de secuenciación paralela masiva, tendrán efectos de gran alcance para los cuidados en salud.

Les maladies monogéniques attirent à nouveau l'attention

*Au début des années 90, lorsque le second plan quinquennal du projet du génome humain (*Human Genome Project*) (plus dispendieux que tout autre projet de recherche précédent en biologie) a été écrit, les maladies courantes furent présentées comme la future cible de la recherche sur le génome. C'était une manœuvre intelligente destinée à s'assurer d'un soutien publique prolongé pour cette tentative, préalablement justifié par la perspective du diagnostic, de la prévention et du traitement des maladies mendéliennes sévères mais rares pour la plupart. Aujourd'hui, après plus de 15 ans et des milliards de dollars dépensés pour des études d'association sur le génome entier (GWAS pour* Genome-Wide Association Studies*), très peu de facteurs majeurs de risque génétique pour les maladies courantes ont été identifiés et l'enthousiasme pour les grandes études d'association faiblit. Au même moment, il existe un regain d'intérêt pour l'étude des maladies monogéniques, considérées maintenant par certains comme une meilleure piste pour la compréhension des maladies courantes. C'est probablement le cas, d'autant que les maladies mendéliennes sont elles-mêmes importantes puisque beaucoup plus fréquentes qu'on ne le pense généralement. Dans cet article, nous examinons les différentes stratégies efficaces pour comprendre les anomalies monogéniques et leur application systématique en association aux nouvelles techniques de partition du génome et de séquençage parallèle massif. Ces stratégies auront des implications considérables pour la Santé.*

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