Homozygosity Mapping using Whole-Exome Sequencing: A Valuable Approach for Pathogenic Variant Identification in Genetic Diseases

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Abstract: In the human genome, there are homozygous regions presenting as sizeable stretches, or 'runs' of homozygosity (ROH). The length of these ROH is dependent on the degree of shared parental ancestry, being longer in individuals descending from consanguineous marriages or those from isolated populations. Homozygosity mapping is a powerful tool in clinical genetics. It relies on the assumption that, due to identity-by-descent, individuals affected by a recessive disease are likely to have homozygous markers surrounding the disease locus. Consequently, the analysis of ROH shared by affected individuals in the same kindred often helps to identify the disease-causing gene. However, scanning the entire genome for blocks of homozygosity, especially in sporadic cases, is not a straight-forward task. Whole-exome sequencing (WES) has been shown to be an effective approach for finding pathogenic variants, particularly in highly heterogeneous genetic diseases. Nevertheless, the huge amount of data, especially variants of unknown clinical significance, and the presence of false-positives due to sequencing artifacts, makes WES analysis complex. This paper briefly reviews the different algorithms and bioinformatics tools available for ROH identification. We emphasize the importance of performing ROH analysis using WES data as an effective way to improve diagnostic yield.

1 INTRODUCTION

Mendelian diseases are caused by pathogenic variants in genes that follow the biological inheritance laws originally proposed by Gregor Mendel. The disease-causing gene may be in an autosome or in a sex-chromosome, and may be dominant or recessive. Individually, these diseases are considered to be rare, but collectively they occur at a high rate, with an estimated 7.9 million children being born annually with a serious birth defect of genetic origin (Christianson et al., 2006).

Sanger sequencing has been the gold standard in molecular diagnostics of Mendelian diseases and is still the first choice to confirm a suspected diagnosis, enabling accurate genetic counselling. However, for diseases with genetic heterogeneity, such as hereditary myopathies or primary ciliary dyskinesia, gene-by-gene Sanger sequencing is not the most cost-effective or efficient approach (Oliveira et al., 2015; Pereira et al., 2015). Technological advances over the past decade has led to the development of high-throughput sequencing platforms, revolutionizing the sequencing capabilities and boosting the use of this so called "next-generation sequencing" (NGS) both in research and in clinical diagnostic settings. With this technology, the human genome can be completely sequenced, allowing the simultaneous analysis of multiple genes. The application of NGS in Mendelian diseases focuses firstly on exonic regions of DNA, as the majority of disease-causing mutations are found in exons or in the flanking intronic regions (Ng et al., 2010).

Marriage between close biological relatives increases the probability of the offspring inheriting two deleterious copies of a recessive gene. Thus, children from consanguineous couples have a higher incidence of autosomal recessive disorders (Bittles, 2001). In addition, as alleles are parts of haplotypes, not only will the affected descendant have two identical copies of the ancestral allele, but the

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surrounding DNA segment (haplotype) will also be homozygous. Thus, the child will be homozygous for that segment, the so-called runs of homozygosity (ROH) (McQuillan et al., 2008). These homozygous segments that are identical by descent (IBD) are generally longer in consanguinity cases. Nevertheless, even in the absence of known recent inbreeding, ROH can be detected in geographically isolated populations and historical bottlenecks (Pemberton et al., 2012). The ROH length is dependent on the degree of shared parental ancestry and its age. Recent inbreeding events/parental consanguinity tend to have longer ROH (measuring tens of Mb) since there are fewer recombination events interrupting the segments that are IBD. Conversely, older ROH are generally much shorter because the homozygous stretches have been split down by repeated meioses over the generations, with the exception of genomic regions where the recombinetion rates are lower (McQuillan et al., 2008).

2 HOMOZYGOSITY MAPPING

Homozygosity mapping (also known as autozygosity mapping), consists in the identification of homozygous regions in the genome. This is a powerful strategy to associate new genes to diseases (Alkuraya 2010; Goodship et al., 2000). As mentioned, affected individuals are likely to have two IBD alleles at markers located in the vicinity of the disease locus and thus will be homozygous for these markers. This method relies on the search for ROH that are shared by affected individuals in the same family. However, scanning the genome for blocks of homozygosity, although simple and efficient, requires sophisticated techniques such as the use of numerous microsatellite markers or high-density single nucleotide polymorphism (SNP) genotyping. For most autozygosity mapping projects, multipoint linkage analyses under a recessive disease model have been performed, with software such as GENEHUNTER (Kruglyak et al., 1996), SIMWALK2 (Sobel et al., 2002), MERLIN (Abecasis et al., 2002) or ALLEGRO (Gudbjartsson et al., 2000).

In general, haplotypes are inspected manually for homozygous regions that are shared by all affected individuals, and can be inferred to be IBD if genotypes from the parents or other close relatives are available. But, in practical terms, conventional parametric methods of multipoint linkage analysis for large datasets in complex consanguineous families are often difficult, because of the time and computational power required, since homozygous blocks among affected individuals tend to be large (mean 4.4 Mb) and contain dozens or hundreds of genes. Moreover, genotyping errors of SNP array platforms and poorly represented chromosomal regions also limit the potential of this technology. If ROH are incorrectly mapped by the introduction of erroneous heterozygous genotypes, the analysis of the causative gene will be compromised.

3 NEXT-GENERATION SEQUENCING

NGS, and in particular whole-exome sequencing (WES), prompted great advances in the study of genetic diseases and gene discovery (Boycott et al. 2013; Ng et al., 2010). However, this technology has still some limitations (Sirmaci et al., 2012). Firstly, some deleterious variations may be in non-coding regions, which cannot be detected by WES. Genetic and phenotypic heterogeneity in affected individuals makes exome sequencing difficult to interpret. Sequencing errors related to poor capture efficiency, mechanical and analytical errors, as well as misalignment of repetitive regions, lead to erroneous results. These hamper the analysis and impose the need to validate candidate causative variants by Sanger sequencing. Moreover, WES analysis imposes the need of applying filtering strategies. This step is critical as it will limit data analysis and consequently influence the results. For instance, almost half of the variants may be excluded for being synonymous. Despite the fact that these are usually not considered deleterious, numerous synonymous mutations have been implicated in human diseases (Sauna & Kimchi-Sarfaty, 2011). In addition, during WES analysis a frequency filter is usually set to exclude variants with minor allele frequency above a certain threshold (above 1% in most cases). This threshold should be set in accordance with the expected prevalence of the disease. Even so, considering that in recessive disorders carriers do not show any signs of the disease, the frequency of damaging alleles in populational variant databases can still be higher than the established threshold. This would lead to the erroneous exclusion of this variant during filtering.

Table 1: List of bioinformatic tools developed for ROH detection and their main features.

Software	OS	UI	Algorithm	Main features / experimental design	Input data files	ROH size range (Mb)	Other features	Ref.
Homozygosity-Mapper	Unix/Linux (web server [a])	GUI	Detection of homozygous blocks of selectable length	Perform autozygosity mapping from SNP arrays and NGS data	VCF files and SNP geno-types	> 1.5	• Independent of parameters like family structure/allele frequencies. • Robust against genotyping errors. • Integrated with GeneDistiller (candidate gene search engine).	Seelow et al., 2009
PLINK	Unix/Linux, Mac OS, Win.	CLI & GUI	Sliding-window	WGAS analysis tool set. Estimation and use of IBD in the context of population-based studies	BED, PED, and FAM files	[0.5 - 1.5] > 1.5	• Makes a variety of standard association tests. • Maps disease loci that contain multiple rare variants in a population-based linkage analysis. • Integrated with Haploview.	Purcell et al., 2007
GERMLINE	Unix/Linux	CLI	Sliding-window	Designed for genome-wide discovery of IBD segments shared within large populations (SNP arrays)	PED, MAP and Hap-map files	> 1.5	• Overcomes the computational barrier of pairwise analysis and can scale the analysis linearly with the sample size.	Gusev et al., 2008
HomSI	Unix/Linux, Mac OS, Win.	GUI	Sliding-window	Identify ROH in consangui-neous families from NGS data	VCF files	> 1.5	• Takes into account the distribution of the variants within genomic coordinates. • Reported to be consistent with data derived from SNP microarrays.	Gormez et al., 2014
H^3M^2	Unix/Linux	CLI	Heterogeneous hidden Markov model	Analyse medium and short ROH obtained from WES data	BAM files	< 0.5 [0.5-1.5] > 1.5	• Reported to be more accurate than GERMLINE and PLINK, especially in the detection of short and medium ROHs.	Magi et al., 2014
Agile-Genotyper and Agile-VariantMapper	Win.	GUI	User-controllable visualization of homozygous regions	ROH analysis WES data and SNP genotyping file data	SAM; tab-delimited text files	> 1.5	• AgileVariantMapper uses the genotypes of all positions found to be polymorphic. • AgileGenotyper deduces genotypes from positions previously found to be polymorphic in the 1000 Genomes Project data set.	Carr et al., 2013

Footnote: [a]-http://www.homozygositymapper.org; CLI- command-line interface; GUI- graphical user interface; NGS-Next-generation sequencing; OS- operative system; Ref.- references; ROH- runs of homozygosity; SNP- single nucleotide polymorphism; UI- user interface; VCF- variant call format; WES- whole-exome sequencing; WGAS- whole-genome association studies; Win.- Windows.

3.1 Homozygosity Mapping using WES Data

Recent studies have clearly demonstrated the power and the effectiveness of applying homozygosity mapping to WES data, in an attempt to identify causative genes for Mendelian disorders (Gillespie et al., 2014; Shamseldin et al., 2015). This approach has the advantage of unraveling the causal variant irrespective of the gene involved. The homozygosity map allows narrowing down of the target data sets; examination at the base-pair level then enables identification of candidate causative variants.

This approach would start by mapping WES reads against a reference genome (human hg19 in our examples). Data derived from whole-genome and RNA sequencing can also be used. This step is usually performed through a bioinformatic pipeline that generates SAM/ BAM (Sequence /Binary Alignment Map) and, at the end of the process, a variant call format (VCF) file. These files are then used as input for homozygosity mapping analysis. The position and zygosity of the obtained sequence variants can be used to retrieve/infer ROH regions. As all bioinformatic approaches, there is an error rate associated that is difficult to estimate, since it is highly dependent on the WES metrics and the sequencing platform used.

Table 1 lists some of the tools available to perform ROH. For instance, the web-based tool HomozygosityMapper (Seelow & Schuelke 2012), allows users to interactively analyze NGS data for homozygosity mapping. Furthermore, PLINK (Purcell et al., 2007) and GERMLINE (Gusev et al., 2008), originally developed for the analysis of SNP array data, are tools based on sliding-window algorithms. In a sliding window analysis, the statistics are calculated for a small frame of the data. The window incrementally advances across the region of interest and, at each new position, the reported statistics are calculated. In this way, chromosomes are scanned by moving a window of a fixed size along their entire length and variation in genetic markers across the region of interest can be measured. This type of analysis reveals how variation patterns change across a surveyed genomic segment (Srinuandee & Satirapod 2015). EXome-HOMozygosity is an example in which a sliding-window algorithm (PLINK) is applied for WES-based ROH detection (Pippucci et al., 2011).

However, the sliding-windows approaches cannot be used easily with short/medium ROH sizes. In order to solve this issue, Magi et al proposed a new algorithm, H^3M^2, that is capable of detecting smaller ROH (Magi et al., 2014). AgileGenotyper (Carr et al., 2013) and HomSI (Gormez et al., 2014) are other tools that can be used for the graphical visualization of ROHs.

3.2 Case Studies

The following examples are shown to elucidate the relevance and limitations of WES-based ROH. Two patients listed in Table 1 have recessive conditions caused by homozygous pathogenic variants identified by WES. There was no indication of parental consanguinity, and patient P2 had a sibling affected by the same condition.

In both cases, retrospective analysis was based on genome-wide homozygosity mapping performed using HomozygosityMapper algorithm.

Data obtained from patient P1 revealed the presence of two long stretches of homozygous SNPs in chromosomes 1 and 17 (with 19 and 17 Mb, respectively) (Figure 1, top panel). The affected gene (*CCDC103*) is located in one of these regions, specifically in chromosome 17. In this example the proposed approach would be suitable to generate a considerably shorter list of candidate *loci*, and consequently reduce the number of variants to be evaluated in terms of their pathogenicity. In the second example the same strategy was applied in a patient with a rare neuromuscular disease.

Table 2: Cases selected to illustrate the use of homozygosity mapping using WES data in patients with rare genetic diseases.

Patient	Phenotype	Genotype	Reference
P1, male adult	Infertility due to total sperm immotility. Clinical features compatible with Kartagener syndrome. No known parental consanguinity.	Homozygous pathogenic variant in *CCDC103* gene: Chr17(h19):g.42978470G>C	Pereira et al., 2015.
P2, female neonate	Severe neuromuscular disease, congenital hypotonia, respiratory distress and bone fractures. No known parental consanguinity.	Homozygous pathogenic variant in *ASCC1* gene: Chr10(hg19):g.73970545dupC	Oliveira et al., (submitted).

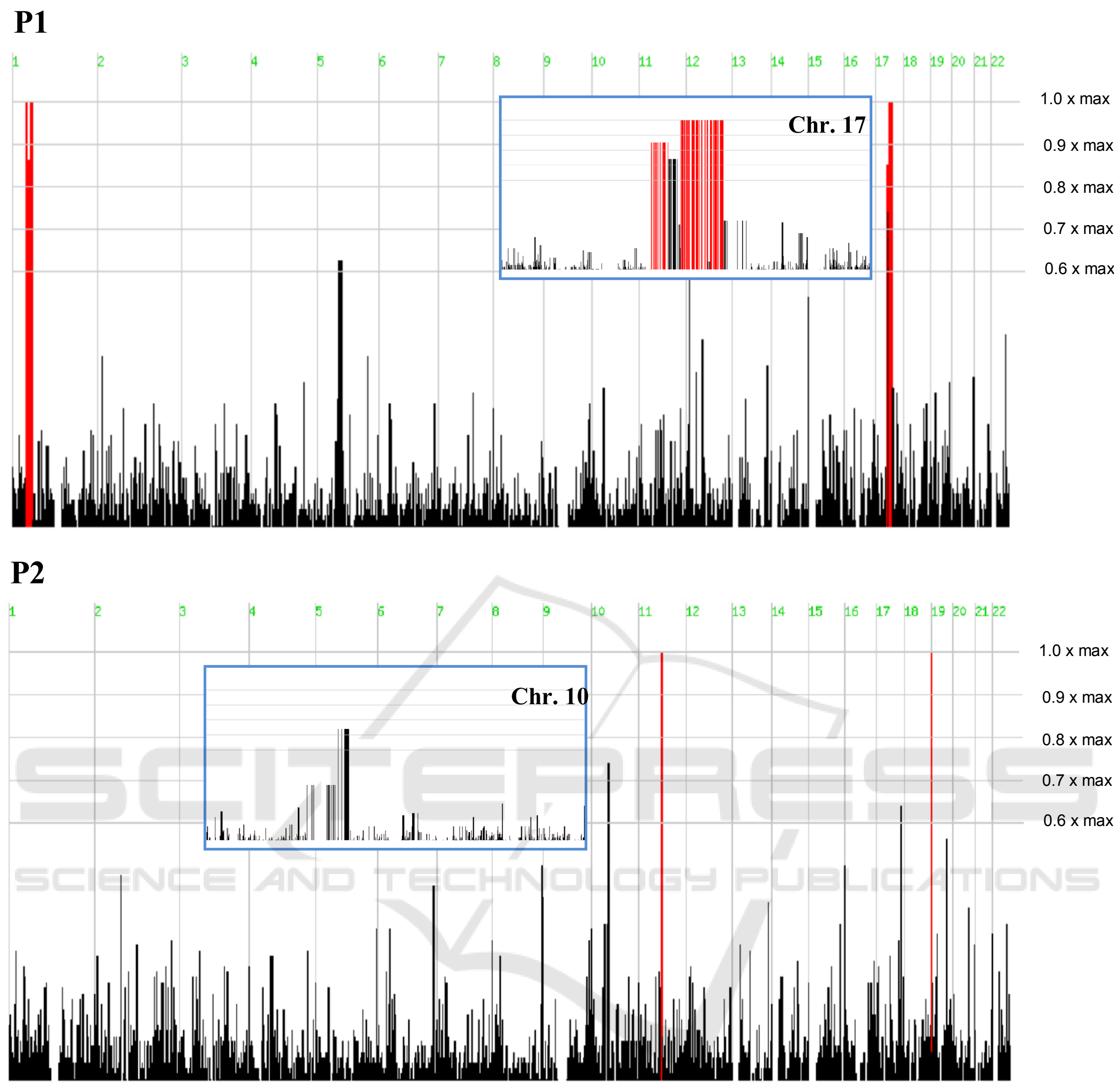

Figure 1: Genome-wide homozygosity mapping using WES and the HomozygosityMapper software. Results are shown for patients P1 and P2. Longer ROH are shown in red color. Inserted blue boxes show the genomic regions in more detail where the disease-related gene is located.

Results for patient P2 revealed two smaller ROH (1.8 and 0.2 Mb) in chromosomes 11 and 19 respectively (Figure 1, bottom panel). However, the *ASCC1* gene, carrying the disease-causing variant, is located in chromosome 10. In this last example, and considering the algorithm used, the analysis based on the assumption of a homozygous variant located in a ROH would clearly fail. The causal gene would not be included in the list of candidate genes, most likely due to the size of the actual ROH where the genetic defect is located. In the first example, the long ROH can be attributed to consanguinity (although it was not formally confirmed), while in the second case, a distant common ancestor could explain the presence of a rare pathogenic variant in a smaller ROH tract.

4 CONCLUSIONS

The aim of this position paper is to revisit homozygosity mapping as an important tool for clinical genetics in cases where a recessive disease is suspected. We have reviewed the proposed algorithms and available bioinformatic tools designed for ROH detection based on WES data. Selection of appropriate algorithms should mainly consider specific features of the case under study, such as the genetic context, the ROH size and the number of relatives affected by the same condition.

Monogenic disorders have been studied by classical approaches aiming to unravel several disease-causing genes. The presence of numerous genes in a candidate genomic region was a limiting factor, considering the costs and the time required, to screen mutations by Sanger sequencing. Another limitation with the "traditional" approaches is more evident when they are used to study trios (only the parents and their affected child) or larger families with only one or two affected members. The genotype data extracted in these familial contexts generally remain statistically insufficient for classical analytical approaches (Jorde, 2000).

As compared with conventional homozygosity mapping that uses known SNPs, WES has the added advantage of allowing the identification of the actual disease-causing variant. Therefore, instead of using two different procedures, one for identifying candidate loci and the other to identify the genetic defect itself at the nucleotide level, both can be performed in a single step by WES. Nonetheless, there are still limitations and further bioinformatic developments are required. Considering the examples presented, there are sensitivity issues especially if the genetic defect is in a small ROH. Finally, we consider that it would be useful to develop a bioinformatic tool that combines variant filtering and homozygosity mapping (Appendix), which currently need to be performed separately.

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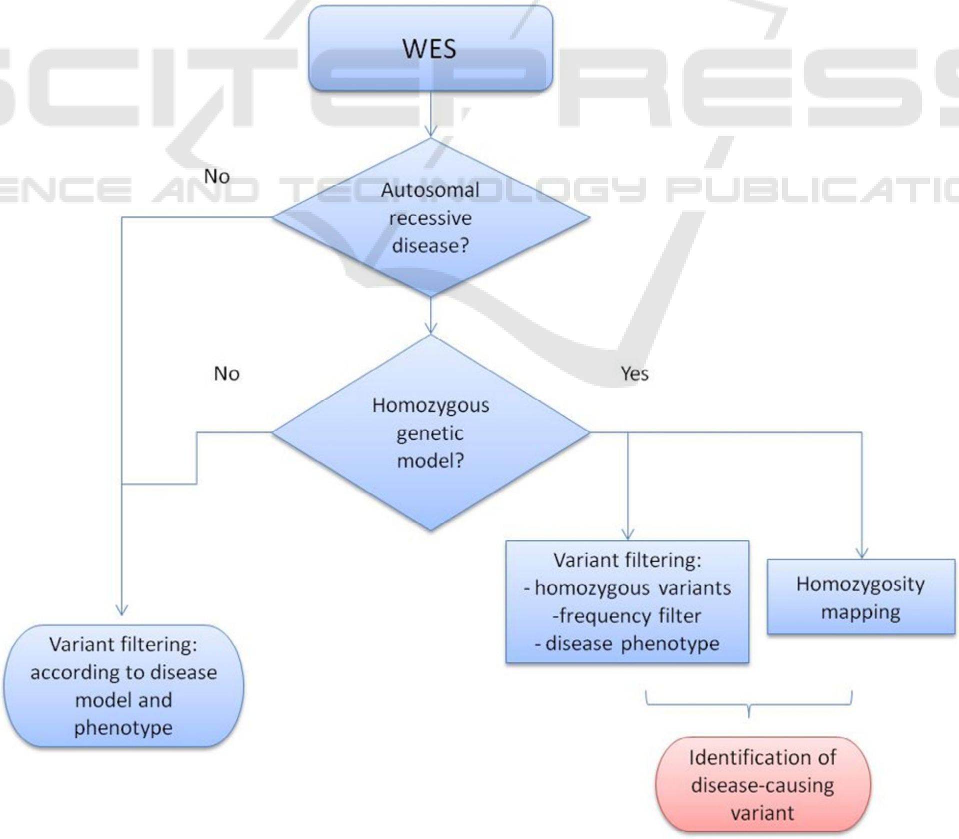
APPENDIX


WES
No
Autosomal recessive disease?
No
Yes
Homozygous genetic model?
Variant filtering: according to disease model and phenotype
Variant filtering:
- homozygous variants
-frequency filter
- disease phenotype
Homozygosity mapping
Identification of disease-causing variant

Appendix: Integration of homozygosity mapping in the analysis workflow of whole-exome sequencing (WES).