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A Comparison of Cosegregation Analysis Methods for the Clinical Setting:

Cosegregation Analysis Comparison

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Abstract

Quantitative cosegregation analysis can help evaluate the pathogenicity of genetic variants. However, genetics professionals without statistical training often use simple methods, reporting only qualitative findings. We evaluate the potential utility of quantitative cosegregation in the clinical setting by comparing three methods. One thousand pedigrees each were simulated for benign and pathogenic variants in *BRCA1* and *MLH1* using United States historical demographic data to produce pedigrees similar to those seen in the clinic. These pedigrees were analyzed using two robust methods, full likelihood Bayes factors (FLB) and cosegregation likelihood ratios (CSLR), and a simpler method, counting meioses. Both FLB and CSLR outperform counting meioses when dealing with pathogenic variants, though counting meioses is not far behind. For benign variants, FLB and CSLR greatly outperform as counting meioses is unable to generate evidence for benign variants. Comparing FLB and CSLR, we find that the two methods perform similarly, indicating that quantitative results from either of these methods could be combined in multifactorial calculations. Combining quantitative information will be important as isolated use of cosegregation in single families will yield classification for less than 1% of variants. To encourage wider use of robust cosegregation analysis, we present a website (www.analyze.myvariant.org) which implements the CSLR, FLB, and Counting Meioses methods for *ATM*, *BRCA1*, *BRCA2*, *CHEK2*, *MEN1*, *MLH1*, *MSH2*, *MSH6*, and *PMS2*. We also present an R package, CoSeg, which performs the CSLR analysis on any gene with user supplied parameters. Future variant classification guidelines should allow nuanced inclusion of cosegregation evidence against pathogenicity.

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RESOURCES

CoSeg R Package:

CRAN - <https://cran.r-project.org/web/packages/CoSeg/index.html>

R-Forge - <https://r-forge.r-project.org/projects/coseg/>

Online Tools:

CSLR(Original Authors' website) - <https://www.msbi.nl/cosegregation/default.aspx>

CSLR, FLB, and Counting Meioses website - <http://www.analyze.myvariant.org/>

Keywords

Bayes Factor; Counting Meioses; Likelihood Ratio; Linkage Analysis; Variants of Uncertain Significance

INTRODUCTION

Cosegregation analysis can be an important component of evaluating the pathogenicity of newly identified genetic variants in cancer risk genes. For cancer traits, the power of cosegregation analysis depends largely on the number of genotyped individuals in the pedigree, the distance of relationship between affected individuals, and the minimum age of disease onset in affected individuals[1]. Regardless of the strength of evidence from any specific pedigree, the results of quantitative cosegregation results can be used to support either benign or pathogenic classification in multifactorial analysis[2].

While robust statistical methods exist to quantify the evidence for cosegregation in any gene and disease, they are complicated and require the use of tools that are difficult to use without a strong background in statistical genetics[3]. Because of this, genetics professionals have used informal methods to communicate qualitative impressions about cosegregation such as: “segregates with disease” or “does not segregate with disease”. In addition, many published reports of families with specific genetic variants in established disease genes do not include formal cosegregation analyses [4–8]. As such, strategies have been proposed to simplify quantitative analysis so that genetics professionals without statistical genetics training can estimate cosegregation statistics[9]. These shortcuts may or may not generate quantitative results and often consist of counting the number of meioses separating affected individuals heterozygous for a variant of uncertain significance (VUS) [9, 10]. ACMG-AMP standards[10] for variant classification allow cosegregation data to be included in variant classification, but do not provide guidance for incorporating quantitative results. For example, non-segregation with disease is considered as strong evidence against pathogenicity, yet what constitutes non-segregation for incompletely penetrant and adult onset diseases is not described.

In this paper we evaluate a recently proposed meiosis counting method along with two more robust methods. These more robust methods incorporate data about age of onset and penetrance, adjust for proband ascertainment, and utilize individuals in the pedigree who are unaffected or not heterozygous for the variant. Specifically, we evaluate the following methods for cosegregation analysis: counting meioses as recently defined by Jarvik and Browning[9], the full-likelihood method for Bayes factors (FLB) as defined by Thompson, Easton, and Goldgar[3], and co-segregation likelihood ratios (CSLR) as defined by Mohammadi et al.[11]. The FLB and CSLR methods each rely on similar underlying statistical models, but allow somewhat different assumptions of variant frequency, use different methods to define disease penetrance, and handle age of onset in different ways (See Appendix).

In order to facilitate more widespread use of quantitative cosegregation analysis we have developed an open-source, freely available, R package[12] that implements the CSLR

method for many popular genes and also has the ability to analyze custom genes. Additionally, it contains functions to simulate pedigrees, along with cancer status, based on historical demographic data and current penetrance estimates for several genes. For those unaccustomed to the R software, we also developed a webtool[13] which performs all three analyses, FLB, CSLR, and counting meioses, for variants in 9 common genes and counting.

MATERIALS AND METHODS

Pedigree Generation

To provide a basis for comparison, we simulated pedigrees with benign and pathogenic variants in *BRCA1* and *MLH1* using published United States of America (U.S.) demographics[14–16]. These demographics included age at marriage, age at death, and number of offspring surviving to adulthood for males and females during each decade since 1900. Our goal was to generate pedigrees that would be similar in size and shape to those of individuals receiving genetic testing for hereditary breast, ovarian, or colon cancer at the clinic. Briefly, we began with a seeded age that is sampled from a skewed normal distribution derived from the age distribution of individuals receiving hereditary cancer testing at the University of Washington[17], extended up three generations to create a founder with the variant in question and then expanded the pedigree down with each descendant having 0.5 probability of inheriting the variant of interest from a parent with the variant. We used published population demographic measures for average marriage age, number of offspring living to adulthood, and mortality[14–16]. Phenotypes were sampled based on age and genotype status using penetrance functions taken from published results for *BRCA1*[18] and *MLH1*[19, 20]. The population demographics and penetrance functions are all embedded in the newly available CoSeg R package[12].

We simulated 1,000 U.S. families each for pathogenic *BRCA1* variants, benign *BRCA1* variants, pathogenic *MLH1* variants, and benign *MLH1* variants (4000 simulated families total). For method comparisons we were forced to omit pedigrees with more than 50 individuals due to the computational burden of the CSLR method. This limited our comparisons to small and medium sized families; however, this family size is more consistent with the size of families that are typically recruited for VUS classification studies, so we believe our conclusions are appropriate. After omitting pedigrees with more than 50 individuals, we compared analyses on 803, 809, 799 and 821 simulated pedigrees with a pathogenic *BRCA1* variant, a benign *BRCA1* variant, a pathogenic *MLH1* variant, and a benign *MLH1* variant, respectively.

Method Implementation

We analyzed these simulated pedigrees using the counting meioses, FLB, and CSLR methods. For the cases considered in this paper, the counting meioses method by Jarvik amounts to 2^{N-1} , where N is the number of meioses separating all the individuals in the pedigree that are affected and heterozygous for the VUS. For calculating the FLB, we followed input from Thompson[3] and used the FASTLINK[21, 22] implementation of the LINKAGE program[23, 24]. For calculating the CSLR, we created a new implementation of this method for the CoSeg R package[12](See Appendix). The CoSeg package was built

using the freely available R statistical software, and is available through CRAN. We created this implementation instead of using the original author's website implementation[25] because we were able to input specific penetrance parameters which more closely matched the underlying model simulation and thus make the comparison more equal. Computational limitations are similar for both the CoSeg implementation[12] and the original web implementation[25]. We treated genotypes of family members who were deceased as unknown in the analysis. All phenotypes on the other hand, including those of deceased individuals, were considered known. The assumption of known phenotypes is reasonable since we are dealing with small, 3-generation, families meaning any deceased individual will likely have a living, adult offspring that knows parental health history. While implementing the methods we considered computational burden. Counting meioses has the smallest burden and can be implemented to analyze pedigrees with thousands of individuals. FLB using FASTLINK is able to comfortably handle single pedigrees with up to 600 individuals. CSLR is only able to comfortably handle around fifty individuals in a pedigree. The computational burden of CSLR can double for each added individual so it is unlikely that this method will be able to handle much larger pedigrees even using additional computational resources. To tackle this hurdle the authors of the CSLR method suggest a method of pruning uninformative individuals before analysis[11].

Score Comparison and Evaluation of Implications for Variant Classification

FLB and CSLR methods return results of Bayes factors and likelihood ratios, which can be used interchangeably in this context. Meiosis counting returns values similar to likelihood ratios that are discrete factors of 2. In order to facilitate clear comparisons between each method we present comparisons of likelihood ratio equivalent values on log2 scale in tables and figures throughout this paper. Assuming a uniform neutral prior probability of 50% for simulated variants, likelihood ratio can be used to evaluate post-test probability for pathogenicity using the equation: $PPa = LR/(1+LR)$.

ACMG-AMP guidelines for variant classification do not include quantitative cutoffs for variant classification, and provide limited guidance for cutoffs for qualitative cosegregation data[10]. Because of this we based our cutoffs on the international agency for research on cancer (IARC) guidelines to evaluate the strength of evidence for variant classification[2]. Our cutoffs for the probability of pathogenicity (PPa) were $PPa \leq 0.01$, $0.01 < PPa \leq 0.05$, $0.05 < PPa \leq 0.95$, $0.95 < PPa \leq 0.99$, and $0.99 < PPa$ for definitely benign, likely benign, uncertain, likely pathogenic, and definitely pathogenic, respectively.

RESULTS

Method Comparison

Histograms of the three methods and overlaid density plots of FLB and CSLR of the resultant values for simulated pathogenic and *BRCA1* variants are shown in Figure 1 and 2, respectively. Note that results are given on a Log base 2 scale (as opposed to the Lod score which is base 10) since counting meioses yields discrete values that are powers of 2. For pathogenic variants, all 3 methods generated strong evidence for pathogenicity in a similarly small number of families, but FLB and CSLR were able to generate some information for

more families than the meiosis counting method (Figure 1). FLB and CSLR methods also generated some information against pathogenicity for some pathogenic variants. For benign variants however, FLB and CSLR greatly outperformed counting meioses (Figure 2). Counting meioses was not able to generate evidence against pathogenicity for any variants, where FLB and CSLR methods were able to generate evidence against pathogenicity for many variants. This difference can be seen Figure 3, which shows a benign *BRCA1* pedigree with the results of all methods shown. Here, CSLR and FLB have likelihood ratios (LR) of 0.45 and 0.43, respectively, and the meioses counting methods results in LR=2. The FLB and CSLR methods generated weak evidence supporting pathogenicity for many benign variants. It should be noted that pedigrees were simulated to have one individual with disease and the variant, as this is an assumption of all three methods. Similar results were obtained for the simulated pedigrees with a pathogenic and benign variant in *MLH1* (See Appendix).

The FLB and CSLR methods for simulated *BRCA1* variants were highly correlated ($r^2 = 0.94$, $p < 10^{-15}$, Figure 4). Furthermore, a Bland Aldman plot of FLB and CSLR for *BRCA1* variants shows a mean difference of -0.08 and standard error of 0.41 (See Appendix), indicating that the methods produce similar output across the range of expected results. Contrastingly, the counting meioses and FLB were not correlated ($r^2 = 0.14$, $p < 10^{-15}$) with Bland Aldman mean difference of -0.81 and standard error of 1.40. Likewise, counting meioses and CSLR were not correlated ($r^2 = 0.17$, $p < 10^{-15}$) with Bland Aldman mean difference of -0.89 and standard error of 1.16. Similar results were observed for simulated *MLH1* variants (See Appendix).

Implications for VUS Classification

None of the methods were able to correctly classify more than 1% of pedigrees with a high degree of certainty using single pedigrees (Table 1). In fact, the vast majority of variants would be classified as uncertain using data from single families under the guidelines of Plon[2]. FLB and CSLR methods provided weak or no evidence for 99% of pedigrees. The weak evidence for 95% of FLB and 99% of CSLR results supported correct classification in 61% of pedigrees and incorrect classification (not supporting pathogenicity for simulated pathogenic variants or supporting pathogenicity for benign variants) in 39% of pedigrees evaluated using either FLB or CSLR methods. Counting meioses provided no evidence for 93% of the pedigrees.

DISCUSSION

We have shown that meiosis counting should be avoided if accurate quantitative methods are available. Indeed, for 93% of simulations, the counting meioses method was unable to make use of any data and provided zero support for or against pathogenicity. This is because counting meioses relies on affected carriers as the sole units of information whereas both the FLB and the CSLR can utilize all available phenotype and genotype information. Counting meioses or other qualitative strategies are especially problematic in light of ACMG-AMP standards[10], which suggest lack of evidence for cosegregation may be considered strong evidence against pathogenicity.

Although counting meioses is more comparable to the other quantitative methods for pathogenic variants, this feature may be lost in the clinic when dealing with VUS, as most are eventually classified as benign[26–28]. Furthermore, for pathogenic variants, the equivalent of almost one whole extra separating meiosis worth of information (mean difference -0.81 and -0.89 for FLB and CSLR respectively) can be gained from using one of the more robust methods. This extra information should not be overlooked when dealing with small families.

The counting meioses method cannot quantitatively classify benign variants. This is illustrated in Figure 3 where both of the robust methods are able to correctly yield evidence that the variant is benign whereas counting meioses is likely to provide evidence supporting pathogenicity. This discrepancy may be amplified for larger families. In the extreme case, finding two distant relatives with the same disease and genetic variant will yield a significant result using counting meioses regardless of how many other relatives with the variant do not have the disease in the family, since the presence of these unaffected individuals is not accounted for in the method. Thus, we illustrate that the counting meioses method should be used with caution for variants with incomplete penetrance, as noted by authors of the most recent paper advancing this method[9].

On the whole, it appears that the CSLR and FLB methods produce similar results (Figure 4). A closer inspection, reveals that FLB narrowly edges out the CSLR (Table 1) with a slightly greater success rate. This may be due to our simulated pedigrees being more closely aligned to the assumptions of the FLB model. Specifically, the penetrance function we used for simulated pedigrees may be better captured by the bins approximation of the FLB method than the Normal distribution approximation of the CSLR method (See Appendix). It is difficult to say which penetrance function is closer to the truth. Since differences may be dependent entirely on implementation of penetrance models, we cannot conclude which of the two methods is optimal. It unfortunately appears that, in the clinical setting, classification of variants of uncertain significance is usually not possible with any of the methods tested even in the best case scenario where all living family members are genotyped. There is simply not enough information in the average U.S. family to classify variants in *BRCA1* or *MLH1*, using cosegregation analysis alone. Although bigger families would provide more evidence[1], these are not commonly seen in the clinical setting.

In spite of this, quantitative cosegregation analysis can be useful in variant classification because it can be easily combined with other quantitative information. For example, one can combine results across multiple families sharing the same variant. We have shown that FLB and CSLR methods can be considered equivalent, with a mean difference of -0.08 suggesting that they can be used interchangeably for most cases. Because they are nearly equivalent, quantitative likelihood ratios from either method might be multiplied (or log likelihoods added) to yield combined likelihood ratios if penetrance used for both methods are equivalent. Combining quantitative counting meioses results is also possible if a variant is seen in more than one independent family, but the authors proposing the counting meioses method caution against it, and our analysis indicates this caution is appropriate. Quantitative cosegregation results may also be combined with other types of data, such as in silico data,

splice data, or tumor data as has been established in several published multifactorial analyses[29–32].

Both the FLB and CSLR method were initially defined as general statistical methods that were presented without specific instructions for implementation, though Mohammadi and colleagues already created a web tool that implements CSLR for *BRCA1* and *BRCA2*[3, 11, 25]. In keeping with the goals of this paper to evaluate cosegregation methods and make cosegregation tools more readily available, we present an R package: CoSeg. This package expands the CSLR method for analyzing variants in other genes including *ATM*, *CHEK2*, *MEN1*, *MLH1*, *MSH2*, *MSH6*, and *PMS2*. Users can also input custom penetrance parameters for their desired gene. The CoSeg R package also contains functions to count meioses and simulate pedigrees using historical population demographics (See Appendix). For those unfamiliar with the R software we have developed a website[13] that implements all three methods for the same genes as CoSeg. We hope these tools will allow genetics researchers and clinical genetics professionals to more easily apply robust cosegregation analysis and share results with others in public databases such as ClinVar as sharing of quantitative cosegregation results would aid in classifying VUS.

In conclusion, we compared 3 methods for cosegregation analysis (counting meioses, FLB, and CSLR) on simulated families likely to be seen in a clinical setting. Either of the more robust methods (FLB or CSLR) provide much more information than counting meioses and should be used if available. Even under ideal conditions cosegregation analysis alone likely cannot classify a VUS, and will be most useful when combined across families or with other variant specific data. To aid geneticists in the use of these better methods, we present the CoSeg R package[12] which contains the code that we used for this study. We also present the www.analyze.myvariant.org website, which is a web interface that implements each of the methods described in this manuscript for *BRCA1*, *BRCA2*, *MLH1*, *MLH2*, *MSH6*, *PMS2*, and several other cancer risk genes using pedigrees coded in a simplified linkage format in excel or tab delimited text files. We hope that these results and the availability of these tools improve rare variant classification in cancer risk genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A histogram of analysis results of the pedigrees for the different methods. For convenience, a density plot is also shown though it is omitted for counting meioses since this yields discrete values. Pedigrees were simulated with a pathogenic BRCA1 variant.

Figure 2.

A histogram of analysis results of the pedigrees for the different methods. For convenience, a density plot is also shown though it is omitted for counting meioses since this yields discrete values. Pedigrees were simulated with a benign BRCA1 variant.

Figure 3.

An example of a simulated benign pedigree with likelihood ratios for the three methods.

Note that all living individuals were assumed to be genotyped for the analysis though this need not be the case. For reference, the log2 values are -1.15 , -1.22 , and 1 for CSLR, FLB, and Meioses Counting respectively.

Figure 4.

A scatter plot($r^2 = 0.94$, $p < 10^{-15}$) of the likelihood ratio against the Bayes factor colored by whether the pedigrees were simulated with a pathogenic or benign BRCA1 variant. A diagonal line is added for reference.

Number of families with given probabilities for correct classification for different classes of BRCA1 or MLH1 variants. These cutoffs were adapted from Plon[2]. See appendix for results separated into benign and pathogenic variants.

Table 1

	Incorrectly Classified	Moderate evidence - incorrect classification	Weak evidence - incorrect classification	No evidence	Weak evidence - correct classification	Moderate evidence - correct classification	Correctly Classified
Method	P 0.01	0.01 < P 0.05	0.05 < P < 0.5	P=0.5	0.5 < P < 0.95	0.95 P < 0.99	0.99 P
FLB	3	10	1210	128	1848	27	6
CSLR	3	4	1277	0	1933	14	1
Meioses Counts	0	0	11	2995	224	2	0