Ambry Genetics®

WHITE PAPER

Enhancing Exome Sequencing with RNA Analysis: Functional Insights and Clinical Applications

Introduction

Exome sequencing (ES) has transformed our understanding of genetic disorders by identifying variants within the protein-coding regions of the genome. Despite the potential of ES as a primary diagnostic tool, 15-22% of patients tested receive variants of uncertain significance (VUS).[1] DNA analysis alone is insufficient to reveal the functional implications of some variants at the RNA and protein levels—crucial factors in determining their pathogenicity. By supplementing DNA testing with RNA analysis, more variants can be definitively classified, increasing the diagnostic yield of ES testing while decreasing the VUS rates.

DNA testing may detect variants, but it does not provide information on their functional effects (Table 1). RNA analysis plays a pivotal role in classifying variants predicted to impact the splicing process. Approximately 15-60% of disease-causing variants are known to affect splicing, a critical process in gene expression where introns are removed, and exons are joined to form mature mRNA.[2-3] Splicing variants can result in various consequences including exon skipping, intron retention, and the use of alternative splice sites, leading to the production of aberrant proteins or altered gene expression levels (Figure 2).

>15%
of disease-causing variants are known to affect splicing[2-3]

Enhancing Variant Interpretation in Rare Disease: The Critical Role of RNA Analysis

What Does RNA Analysis Provide?

RNA analysis provides essential insights into the functional impacts of DNA variants, offering a more nuanced understanding of their potential

RNA Testing Provides Additional Insight for Multiple Variant Types
▪ Establish pathogenicity for intronic VUS
▪ Increase confidence in likely pathogenic and pathogenic classifications of intronic variants
▪ Correct misclassification of canonical variants where observed impact ≠ predicted impact
▪ Uncover clinically significant synonymous variants
▪ Establish pathogenicity for missense variants with splice impact

Table 1. The table outlines the various applications and benefits of RNA testing in genetic variant analysis. It highlights how RNA testing can provide functional evidence, thereby enhancing the accuracy and comprehensiveness of genetic variant interpretation.

contributions to human disease. By examining RNA transcripts, scientists can determine whether a variant affects splicing, thereby influencing gene function (Figure 2).[4-5] This approach has proven valuable in other specialties, such as hereditary cancer multigene panel testing, by enhancing the classification and identification of genetic variants through insights into their functional consequences.[6]

Bridging Gaps in Understanding Rare Disease

The clinical interpretation and classification of spliceogenic variants are challenging due to the immense complexity of splicing mechanisms. By integrating RNA analysis, we can detect and evaluate mis-splicing associated with identified DNA variants. RNA analysis compares the RNA transcript data to internally developed control sequences to identify abnormal transcripts that could explain the patient's phenotype (Figure 1).[5] An experienced lab will accumulate expertise in interpreting and leveraging complex RNA data for a more accurate classification. The American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) guidelines advocate for the use of RNA evidence in variant classification.[7] According to these guidelines, RNA analysis can serve as strong or moderate evidence, depending on the context, to support the classification of variants. For instance, the detection of aberrant splicing resulting in a frameshift can provide strong evidence for pathogenicity of the causative variant.

Insights from Oncology Applications

Experience from DNA-RNA testing in oncology highlights the importance of RNA analysis in variant classification.[6] This experience shows how RNA data can uncover the functional consequences of genetic variants that DNA sequencing alone might miss. For example, RNA studies have clarified VUS in oncology by characterizing aberrant splicing patterns.[6] These insights are crucial for accurately interpreting genetic variants and can be applied to the rare disease field to improve diagnostic precision.

Spliceogenic Variants Are More Prevalent than Gross Deletions and Duplications

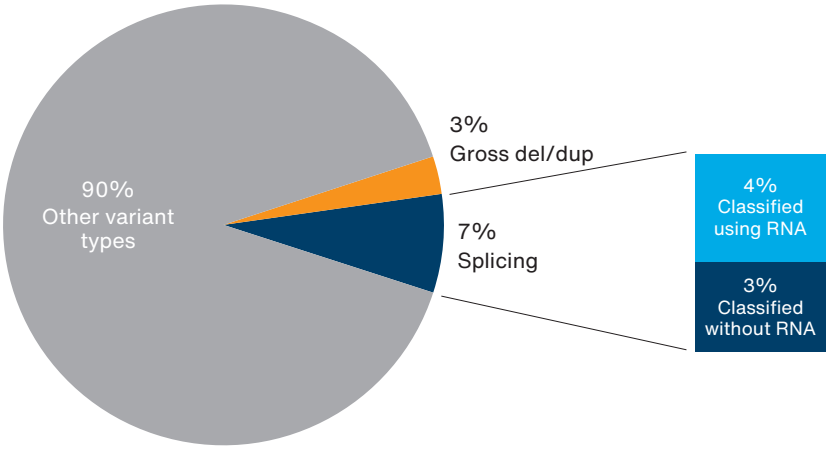

Figure 1. The prevalence of spliceogenic variants that can be classified with RNA is comparable to that of gross deletions and duplications. Labs should consider standardization of both analyses.

With experience testing 800,0000 hereditary oncology patients, Ambry Genetics has made a significant observation: RNA-dependent splicing variants (which require RNA analysis for classification as likely pathogenic or pathogenic) occur more frequently than gross deletions and duplications (Figure 1).[8] This finding is particularly noteworthy when considering that gross deletion/duplication detection is already a standard component of DNA-only genetic testing. While this data is derived from germline oncology testing, the principles and benefits of RNA analysis are generalizable across disease states. There is utility for RNA data to provide accurate variant classifications across genetic contexts. By incorporating RNA analysis as a standard practice, patients are offered comprehensive testing that increases diagnostic yield.

Splicing Variations: From Normal Processing to Aberrant Outcomes[1]

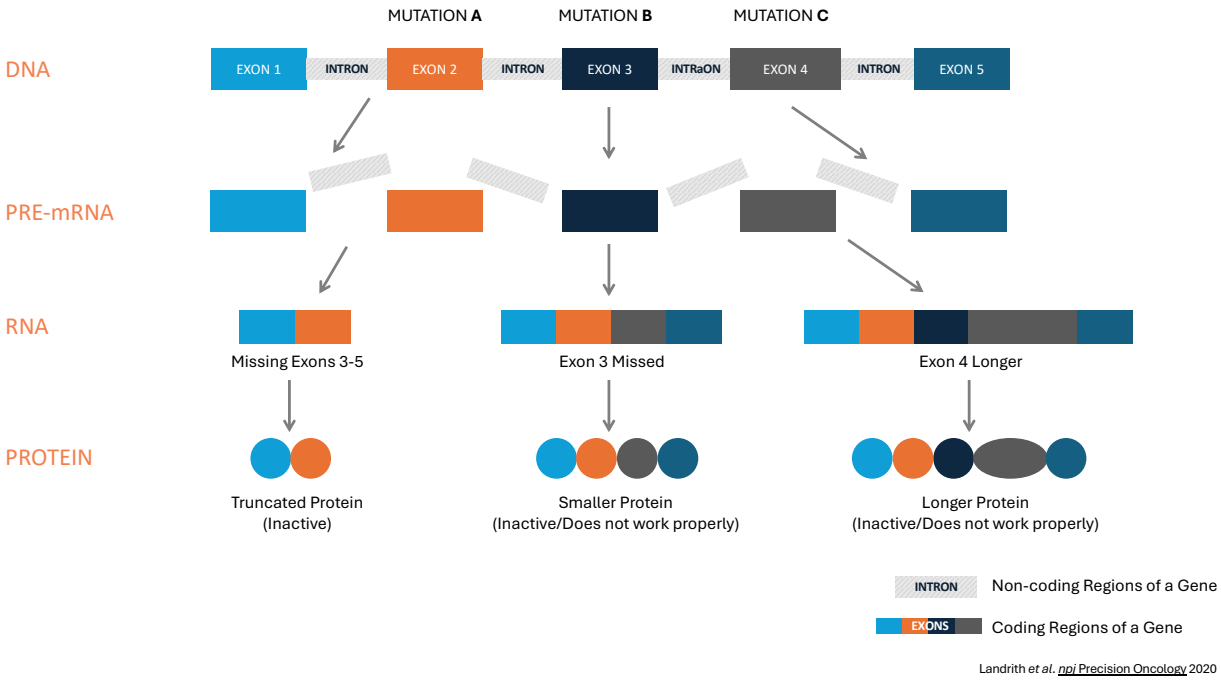

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Figure 2A. During gene transcription, both coding sequences (exons) and non-coding sequences (introns) are copied to form precursor messenger RNA (pre-mRNA). This pre-mRNA is subsequently processed by the spliceosome, a large RNA-protein complex that removes introns and joins exons to produce mature messenger RNA (mRNA). Alternative splicing can generate multiple protein isoforms from a single gene, contributing to protein diversity in eukaryotes. However, genetic variants can cause aberrant splicing, leading to abnormal proteins.

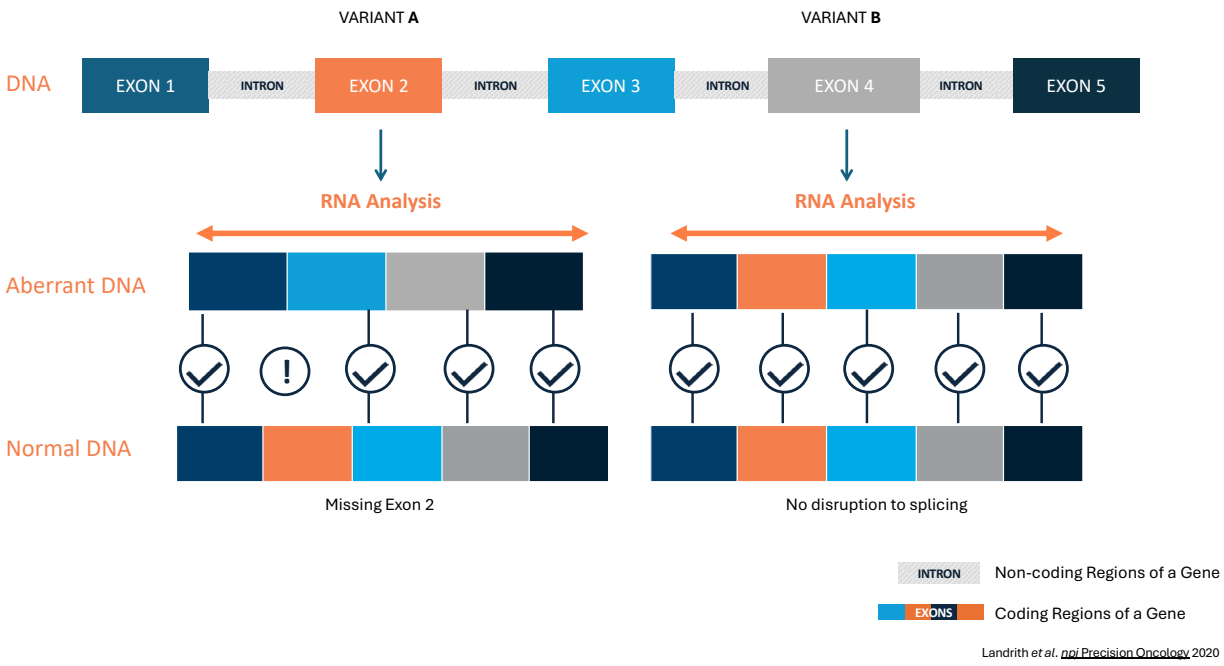

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Figure 2B. Not all splice impacts are pathogenic, so we assess the nature of the splice impact. At Ambry Genetics, RNA analysis is systematically integrated into the variant classification process. To identify mis-splicing, we compare the RNA transcript data from our patient to a reference sequence. This helps us determine whether the RNA has an aberrant or normal sequence. We look for any differences that might result in disrupted protein function, which could explain the patient's phenotype.

In Silico Prediction Tools Alone Are Insufficient for Accurate Variant Classification

While advancements in splice prediction algorithms have improved the accuracy of *in silico* prediction tools like SpliceAI, they continue to be less accurate than performing RNA sequencing.[9] By relying solely on prediction models, the sensitivity and specificity of the test decreases due to false positive and false negative results. The clinical interpretation and classification of spliceogenic variants remain complex due to the intricate nature of splicing mechanisms. Our data has shown that variants with no splicing impact predicted by *in silico* models can cause complete disruption.[10] Therefore, splice prediction models are a tool that can aid in interpretation of splicing variants, but they are not a substitute for RNA analysis.

Whole Blood Exhibits Adequate Disease-Relevant Gene Expression for Rare Disease Analysis

Gene expression varies significantly based on tissue type, which influences the availability of suitable specimens for RNA analysis. While there was initial concern that RNA analysis in rare diseases is limited by difficulty in accessing disease-relevant tissues, recent research counters this notion. Studies have demonstrated that more than half of all genes exhibit adequate disease-relevant expression levels in whole blood.[11-13] By leveraging whole blood RNA analysis, healthcare professionals can gain critical insights into the pathogenicity of variants in rare diseases, addressing primary concerns about tissue accessibility.

Unveiling ExomeReveal™: Translational Genomics in Your Clinic

ExomeReveal™ Filtering: A Superior BI Pipeline

ExomeReveal employs a sophisticated bioinformatics (BI) pipeline to ensure comprehensive interrogation of potentially diagnostic variants, a cornerstone in the diagnosis and understanding of rare genetic diseases. This BI pipeline functions by leveraging advanced computational tools and data analysis methodologies to process genetic datasets, thereby identifying and annotating variants with potential clinical implications.

Ambry's BI pipeline integrates a broad range of internal and external data points to ensure that important variants are not overlooked. It incorporates factors such as familial inheritance patterns, population frequencies, and the location of genetic alterations.[14] Notably, while HPO (Human Phenotype Ontology) terms are often used in many BI pipelines to link gene-disease relationships or patient phenotypes, ExomeReveal uses HPO data differently. Instead of relying on HPO terms to filter out variants, HPO data is used manually to prioritize candidate variants and genes that survive initial filtering. This approach helps retain more potentially relevant variants, especially in cases where the patient's phenotype is limited.[15]

Additionally, ExomeReveal includes robust measures to address variants predicted to impact splicing, guided by SpliceAI scores and our established internal thresholds. Overall, ExomeReveal supports a thorough and nuanced approach to genetic variant analysis, leading to more accurate diagnostic outcomes and enhanced patient care.

Specimen Collection: Ensuring RNA Integrity

RNA is inherently unstable and prone to degradation by RNA enzymes, necessitating special considerations when obtaining specimens for analysis. Traditionally, genetic testing has utilized Ethylene Diamine Tetraacetic Acid (EDTA) blood tubes, which are less reliable for RNA analysis. However, collection in PAXgene® DNA tubes, designed specifically for RNA analysis, inhibits RNA-degrading enzymes and protects RNA from degradation, maintaining the integrity of the sample for a longer period of time. This makes PAXgene® tubes particularly useful for clinical diagnostics and research.

Variant Selection: Targeting the Essentials

RNA studies will not inform classification of all variants. Factors that influence the utility of RNA studies for a given variant are detailed in Table 2. Retrospective review of Ambry's exome cohort suggests that as many as 3.5% of reported variants of uncertain significance (VUS) could benefit from RNA analysis.[19] Targeted RNA studies are a practical way to generate the evidence required for ES VUS resolution of carefully selected variants in the absence of transcriptome analysis. The data collected from these studies will provide valuable insights for future advancements in RNA data interpretation and validation.

Indicators of RNA Utility
Sufficient phenotype overlap with reported patient phenotype
The gene-disease relationship has at least moderate gene-disease validity (GDV)*
The variant is suspected to be spliceogenic
The gene has sufficient disease-relevant expression in the blood
The mechanism for the gene-disease relationship is loss of function

Table 2: The table outlines various factors that influence the utility of RNA analysis for a given variant.

* Gene-disease validity is the assessment of the relationship between a gene and a disease. Gene-disease relationships with a moderate GDV or higher are characterized and considered to be associated with the described phenotype.[20]

Emerging Evidence from ExomeReveal™ Early Access Program

In collaboration with over 50 partner organizations, patients were recruited for the ExomeReveal Early Access Program. The inclusion criteria were broad, including any patient with a clinical indication for exome sequencing, allowing for a comprehensive assessment of the test's clinical utility. A total of 212 Early Access cases were reported during this study period. Based on carefully determined eligibility criteria (Table 2), 13 cases were deemed eligible for RNA analysis. The analysis was performed on 14 unique variants across 13 genes, representing 6% of the cases. Gene expression in blood was sufficient for genes spanning various phenotypic categories, including neurologic and cardiac phenotypes.

6% of cases in this cohort advanced to RNA analysis

29% of upgraded variants (VUS > VLP) were predicted to be benign by *in silico* models

3% increase in diagnostic yield

Notably, RNA analysis led to an upgraded classification for seven unique variants, initially classified as VUS and reclassified as likely pathogenic (VLP) following RNA analysis (Figure 3). 29% of these upgraded variants, including *JAG1* c.886+3A>G, had low spliceAI prediction scores (<0.1), which predicted a benign impact on splicing. However, RNA analysis showed significant impacts on splicing, evidenced by a percent spliced in (PSI) greater than 40%[17], underscoring the pitfalls of relying solely on *in silico* prediction tools. The integration of targeted RNA analysis into clinical exome sequencing has proven impactful with RNA analysis impacting 14% of positive results, thus leading to definitive diagnoses. Overall, RNA analysis provided a 3% absolute increase in diagnostic yield.[18] This enhancement demonstrates the critical importance of RNA analysis in refining diagnostic accuracy and improving patient outcomes in clinical genomics.

The Impact of RNA Analysis on Variant Classification

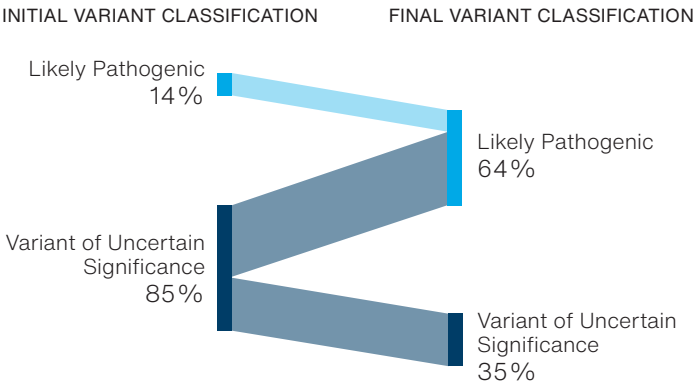

Figure 3. The results of RNA analysis provided sufficient evidence to reclassify half of variants of uncertain significance to likely pathogenic.

Classification remained unchanged for seven variants (Figure 3). RNA analysis supported the pathogenicity of two variants initially classified as likely pathogenic (LP). RNA data for one of the VUS variants failed our quality metrics and was not used. For the remaining 4 variants, we established that the variants had a significant impact on splicing, advancing our knowledge of the variants. Accurate variant classification is dependent on assessment of various lines of evidence. While RNA analysis provided additional insights into the impact of the variants, more evidence is needed to clarify their classification.

Conclusions

In conclusion, the integration of RNA analysis into exome sequencing (ES) at Ambry Genetics represents a significant leap forward in genetic diagnostics for rare diseases. While ES alone has a diagnostic yield of up to 35%, the addition of RNA analysis boosts this yield by almost 10%. This adds another 3% overall to yield in the ES cohort.[18] This advancement is particularly impactful in the realm of rare diseases, where many patients present with unique variants and disorders. By generating RNA data in real-time for each affected case, this approach eliminates the need for prolonged waiting periods typically associated with scientific publications. Consequently, it paves the way for earlier diagnoses, potentially transforming the landscape of patient care in rare genetic disorders. This integration marks a significant advancement in genetic diagnostics, opening new avenues for patient benefit.

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