Center for Genomic Interpretation

ELEVATEGENETICS CLARITY

Vetting data used by laboratories to classify genetic/genomic variants.

For Research Use Only. Not for clinical use.

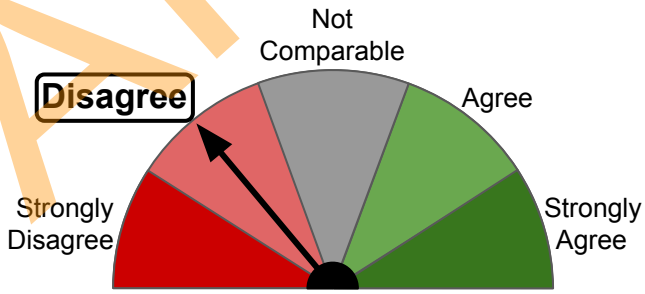
CGI's high stringency research-use-only classification of

BRCA2 c.425+3A>G germline

is

VARIANT OF UNCERTAIN SIGNIFICANCE (VUS)

CGI's summary of the available evidence is in Appendices A-C.



CGI AGREEMENT METER WITH ORIGINAL REPORT

The original report received by CGI was from [Laboratory], which classified this germline variant as "Likely Pathogenic" (see Appendix D). CGI's research-use-only, high-stringency classification **DISAGREES with the original classification from** [Laboratory].

CGI recommends that this research-use-only **ELEVATE**GENETICS CLARITY™ report be shared with the laboratory that generated the original report for their re-consideration and possible generation of an amended report.

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FAQs:

1. Is any genetic variant classification guaranteed accurate?

- No. As stated within the ACMG guidelines[1], "Variant analysis is at present imperfect and the variant category reported does not imply 100% certainty." While CGI prides itself on excellent research-use-only variant classifications, the science remains imperfect. CGI is dedicated to continually improving the science of clinical variant interpretation.

3. Why does CGI's research-use-only variant classification sometimes disagree with the classification from the originating laboratory?

- Sometimes CGI may not have access to all of the information that the originating laboratory has. CGI will re-evaluate its conclusion on this report if the originating laboratory provides additional relevant information within 10 days as part of this patient's care pathway.
- However, most often disagreement occurs because laboratories and companies frequently use different variant classification protocols to assess the same publicly available evidence. CGI's assessment protocol is strict. CGI's variant classification method is designed to minimize false positives.

4. Does CGI evaluate all the publicly available evidence for the variant?

- CGI endeavors to evaluate all the publicly available evidence for your variant. CGI uses a strict variant classification protocol to triage out evidence that CGI determines is of weak or no clinical value for variant classification. CGI's **ELEVATE**GENETICS CLARITY™ report summarizes all of the evidence CGI used to arrive at its research-use-only classification.

5. Will CGI talk to the originating laboratory/company that issued the original report?

- Yes, at stakeholder request and direction.

REFERENCE

1. Richards S. et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genetics in Medicine* 2015;17:405-424.

Description of Appendices

Appendix	Description
A	**Variant Classification Evidence Summary**
	A1 CGI's Variant Classification (for research use only)
	A2 CGI's Highest-Value Variant Data Summary
B	**Gene-Disease Association Evidence**
	B1 ClinGen's Information on Gene-Disease Association
	B2 CGI's Gene-Disease Citations Measure
C	**Variant Specific Data Work Up**
	C1 Allele Frequency Data from Population Databases
	C2 Functional Features Analysis
	C3 *in silico* pre-mRNA Splicing Prediction
	C4 Literature & Locus Specific Database Evidence Assessment
D	**Originating Reports**
	D1 Originating Report Received by CGI
E	**CGI Agreement Meter Explanation**
	E1 Matrix for "CGI Agreement Meter With Original Report"

Appendix A - Variant Classification Evidence Summary

A1. CGI's Variant Classification (for research use only)

NM_000059.4(BRCA2):c.425+3A>G	
Gene-Disease Association (Appendix B)	*BRCA2* gene, and not necessarily this variant, receives a "Definitive" category from ClinGen for its association with Hereditary Breast and Ovarian Cancer in an autosomal dominant inheritance pattern, and also for Fanconi Anemia in an autosomal recessive inheritance pattern. The gene is also relevant to other diseases including Prostate Cancer. Only a small fraction of the gene's variants will be disease causing.
Unusual points to note	This variant may either fully or partially disrupt a splice site donor in *BRCA2*. However, disruption of this donor site has recently been demonstrated to NOT significantly contribute to increased risk for Hereditary Breast and Ovarian Cancer. Compensatory mRNA transcripts that are presumed functional appear to be the most likely rescue mechanism.
CGI research use only Classification of the Variant (Appendix A-A2)	**Variant of Uncertain Significance (VUS)** Absent strong or moderate evidence. Some conflicting evidence.

A2. CGI's Highest Value Variant Data Summary:

	Biochemistry Findings		**Person, Family or Population Findings**	
	Disease Causality	**Disease Non-Causality**	**Disease Association**	**Disease Non-Association**
Strong Evidence	None	None	None	None
Moderate Evidence	None	None	None	None
Conflicting Evidence	According to [Laboratory], this specific variant may either fully or partially disrupt a splice site donor in *BRCA2*. However, disruption of this donor site has recently been demonstrated to NOT significantly contribute to increased risk for Hereditary Breast and Ovarian Cancer. Compensatory mRNA transcripts that are presumed functional appear to be the most likely rescue mechanism.			
Variant Specific Caveats	None		None	

Appendix B - Gene-Disease Association Evidence

CGI's interpretation of a specific genetic variant requires that the gene's association with at least one disease be well defined. Independent of CGI, the ClinGen Gene Curation Workgroup is continually advancing gene level clinical validity methods. When available, CGI defers to the current ClinGen definition of Gene-Disease evidence strength. CGI accepts ClinGen "Definitive" and "Strong" associations.

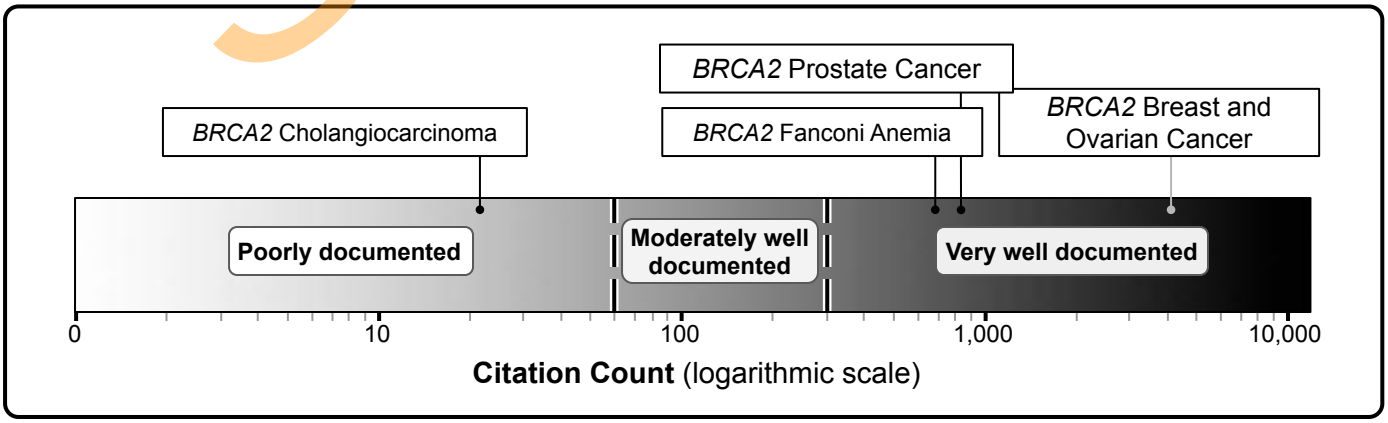
When a qualifying ClinGen gene-disease association is not yet available, CGI estimates the strength of gene-disease association knowledge with CGI's Gene-Disease Citation Measure which has been calibrated against the ClinGen Gene-Disease curation list, and ACMG's secondary findings gene list. CGI searches for citations of the gene with diseases listed in the OMIM resource maintained by Johns Hopkins University. CGI accepts "Very well documented" and "Moderately well documented" from its calibrated citation measure estimate illustrated below.

For gene-disease associations with insufficient evidence, and thus not accepted by CGI, CGI is unable to make a determination as to its research-use-only variant classification. In these situations, unless otherwise specified, CGI defaults its interpretation of the variant to "Variant of Uncertain Significance", as the entire gene is uncertain with regards to human disease risk.

B1. ClinGen's Information on Gene-Disease Association (not the variant)

Gene: *BRCA2*	**ClinGen Gene Curation Workgroup Gene-Disease Evidence**					
Disease	Not Yet Available	Contradictory	Limited or None	Moderate	Strong	Definitive
Breast-Ovarian cancer, familial, susceptibility to, 2						✓ Definitive Autosomal Dominant 13 Sep 2017
Fanconi anemia complementation group D1						✓ Definitive Autosomal Recessive 21 Dec 2018

B2. CGI's Gene-Disease Citations Measure (not the variant)



Appendix C - Variant Specific Data Work Up

C1. Allele Frequency Data from gnomAD Population Database

Date accessed	19 Dec 2020	
Variant Evaluated	**NM_000059.4(BRCA2):c.425+3A>G**	
	Genome Aggregation Database (gnomAD v2.1.1)	**Genome Aggregation Database (gnomAD v3.1)**
Genomic Coordinate(s)	13:32899324 (GRCh37)	13:32325187 (GRCh38)
Populations & Sample Sizes Populations are derived from a variety of disease and unselected patient cohorts. gnomAD: For absent variants, only the overall study configuration is provided. For variants present, the representation at the specified genomic coordinate(s) is provided.	57,787 XX Exomes, 6,967 XX Genomes, 67,961 XY Exomes & 8,741 XY Genomes combined: African/African American: 8,128 Exomes + 4,359 Genomes. Amish: 0 Exomes + 0 Genomes. Latino/Admixed American: 17,296 Exomes + 424 Genomes. Ashkenazi Jewish: 5,040 Exomes + 145 Genomes. East Asian: 9,197 Exomes + 780 Genomes. Finnish: 10,824 Exomes + 1,738 Genomes. Middle Eastern: 0 Exomes + 0 Genomes. Non-Finnish European: 56,885 Exomes + 7,718 Genomes. South Asian: 15,308 Exomes + 0 Genomes. Other: 3,070 Exomes + 544 Genomes. Total: 125,748 Exomes + 15,708 Genomes = 141,456 Total persons	38,947 XX & 37,209 XY Genomes combined: African/African American: 20,744 Genomes. Amish: 456 Genomes. Latino/Admixed American: 7,647 Genomes. Ashkenazi Jewish: 1,736 Genomes. East Asian: 2,604 Genomes. Finnish: 5,316 Genomes. Middle Eastern: 158 Genomes. Non-Finnish European: 34,029 Genomes. South Asian: 2,419 Genomes. Other: 1,047 Genomes. Total: 76,156 Genomes
Depth of Coverage at the Coordinate(s)	Sufficient: Per-base mean depth of coverage: ~30x Exome (~54% of individuals with coverage over 30x), ~44x Genome (~70% of individuals with coverage over 30x)	Sufficient: Per-base mean depth of coverage: 30x Genome (~67% of individuals with coverage over 30x)
Variant frequency in population samples represented	**Absent** (While pathogenic variants are often rare, most rare variants are benign).	

C2. Functional Features Analysis

Variant:	**NM_000059.4(BRCA2):c.425+3A>G** Potential alternate name(s): IVS4+3A>G, IVS3+3A>G
Genomic location:	Chr13:32899324 (on Assembly GRCh37) Chr13:32325187 (on Assembly GRCh38)
Known mechanism of disease:	Null variants would be considered disease causal.
RNA Transcripts:	More than one transcript is described by GENCODE v 32 and RefSeq
Functional location of the variant across all RNA transcripts (GENCODE v 32):	All transcripts maintain the same functional position within this gene: Intronic and proximal to a splice donor. This specific intron of interest is NOT the final intron.
Functional impact:	This variant is intronic and has the potential to disrupt RNA splicing in a way that could lead to RNA nonsense mediated decay resulting in loss of function of the gene. However, in silico RNA splice predictions predict only a MODERATE disruption in splicing of the native donor site (see Appendix C-C3). "Internal evidence" cited in [Laboratory B]'s ClinVar evidence claims complete disruption of the splice site has not been independently evaluated by the researcher producing this **ELEVATE**GENETICS CLARITY™ report. **Most importantly however, a likely rescue mechanism is present, see Appendix C-C4. Variants at, or very close to the c.425 splice donor site of *BRCA2* that COMPLETELY disrupt that splice donor site are very unlikely to be a major cause of Hereditary Breast or Ovarian Cancer. The hypothesis is that there are "rescue" transcripts that are formed that are presumed functional, and that delete exons 4-5 inclusive. Therefore, even if *BRCA2* c.425+3A>G completely disrupts the native splice site, likely alternative transcripts are triggered which compensate for the loss of the native splice site.**

C3. *in silico* pre-mRNA Splicing Prediction

Variant evaluated in context of reference sequence: NM_000059.4(BRCA2):c.425+3A>G

Does the variant qualify for pre-mRNA splicing analysis? YES
The gene has a known Loss of Function mechanism for disease, and the variant is proximal to an RNA donor site.

in silico NNSplice predictions:

NNSplice **reference** coding sequence input (5′ to 3′, UPPER CASE = Native exon):
AGCAGATGATGTTTCCTGTCCACTTCTAAATTCTTGTCTTAGTGAAAGgtatgatgaagctattatattaaaatatttaaatgaaacattttcctacata

NNSplice **reference** seq prediction #1. (Underlined=predicted exon. No underline=predicted intron):
GTGAAAGgtatgatg
Considered the native donor site. Predicted score = 0.94 (predicted very strong)

NNSplice **variant** coding sequence input (5′ to 3′, UPPER CASE = Native exon):
AGCAGATGATGTTTCCTGTCCACTTCTAAATTCTTGTCTTAGTGAAAGgtgtgatgaagctattatattaaaatatttaaatgaaacattttcctacata

NNSplice **variant** seq prediction #1. (Underlined=predicted exon. No underline=predicted intron):
GTGAAAGgtgtgatg
Considered the native donor site. Predicted score = 0.68 (predicted moderate)

in silico MaxEntScan predictions:

MaxEntScan **reference** coding sequence input (5′ to 3′, UPPER CASE = Native exon):
AAGgtatga

MaxEntScan **reference** seq prediction #1. (Underlined=predicted exon. No underline=predicted intron):
AAGgtatga
Considered native donor site. Predicted score = 9.11 (predicted strong)

MaxEntScan **variant** coding sequence input (5′ to 3′, UPPER CASE = Native exon):
AAGgtgtga

MaxEntScan **variant** seq prediction #1. (Underlined=predicted exon. No underline=predicted intron):
AAGgtgtga
Considered native donor site. Predicted score = 4.59 (predicted moderate)

in silico pre-mRNA splicing prediction summary:

MODERATE disruption in splicing of native donor site. However, CGI considers this in silico prediction only informative information, rather than Strong or Moderate lines of evidence for pathogenicity.

The variant was evaluated in the context of the native RNA splice donor site.
NNsplice predicts and MaxEntScan predict decreased utilization of the native splice site, but not abolishment.

C4. Literature and Locus Specific Database Evidence Assessment

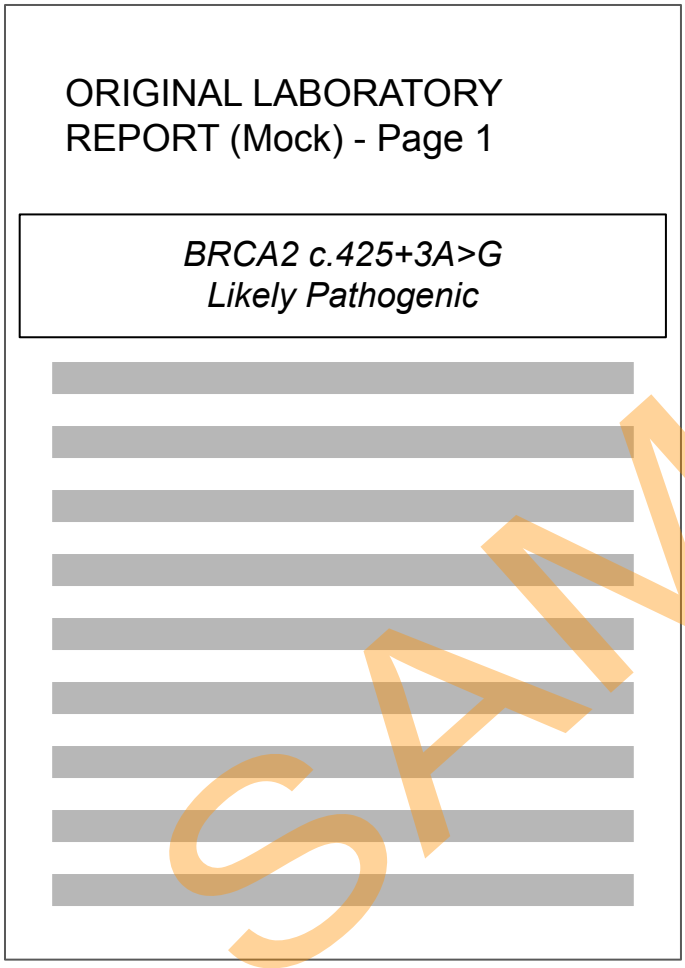
Variant evaluated:	**NM_000059.4(BRCA2):c.425+3A>G Alternate name(s): IVS4+3A>G**	
Reference	**Data Description**	**Evidence Evaluation**
PMID: 21638052 Brandão RD et al. Breast Cancer Res. Treat., 2011 Oct;129:971- 82. -[Laboratory B] cites this publication in ClinVar evidence.	Demonstrates that a nucleotide change in the final base of exon 4, c.425G>T, completely abolishes the native splice site.	**INFORMATIVE - Yet does not trigger a classification code** This publication is NOT directly about the variant of interest, c.425+3A>G. Rather, it is informative about the native splice donor site for which position c.425+3 would influence. However, the publication does not explore potential rescue mechanisms for when the native site is disrupted. Those potential rescue mechanisms have since been explored in PMID 35050751, Nix P et al. JCO Precis Oncol. 2020 Nov;4:730-735. PMID 35050751 will be addressed in the next row in this table.
PMID: 35050751 Nix P et al. JCO Precis Oncol. 2020 Nov;4:730-735.	Demonstrates that a nucleotide change in the final base of exon 4, c.425G>T, completely abolishes the native splice site. However, the study also demonstrates reading frame rescue (with the deletion of exons 4 and 5) and human phenotype rescue.	**INFORMATIVE - Yet does not trigger a classification code** This publication is NOT directly about the variant of interest, c.425+3A>G. Rather, it is informative about the native splice donor site for which position c.425+3 would influence. Of greatest importance, this publication demonstrates that when the splice donor site at BRCA2 c.425 is disrupted, some full length and in frame BRCA2 transcript is generated which is missing exons 4 and 5. This is the hypothesized explanation for why the clinically validated history weighting algorithm tool shows a “Benign” significance for this variant. Thus all variants disrupting this splice site probably lead to the same “benign” impact, but such classifications cannot be made without further evidence. Thus c.425+3A>G should not presently be downgraded to benign or likely benign, but should remain VUS given this very strong counter evidence against a pathogenic classification.
ClinVar Database, Variant ID 495460, accessed 20 May 2022	[Laboratory A] VUS (Mar 28, 2016) [Laboratory B] Likely Path. (Oct 11, 2019) [Laboratory C] VUS (Feb 4, 2020) [Laboratory D] VUS (Submitted Sep 02, 2021) [Laboratory E] VUS (Submitted Sep 30, 2021)	**“Internal Data” DISQUALIFIED** Labs A, C, D and E provide no additional relevant data not described in references listed above. Note that [Laboratory B] provides the following in their evidence text: “RNA studies have demonstrated that this [c.425+3A>G] alteration results in abnormal splicing in the set of samples tested ([Laboratory] internal data). Another close match alteration at this donor site, BRCA2 c.425G>T, causes the same defect- complete skipping of coding exon 3 (also known as exon 4 in the literature) (Brandão RD et al. Breast Cancer Res. Treat., 2011 Oct;129:971- 82).” However, the language “abnormal splicing” is nonspecific and could mean complete disruption of the native splice site, or partial disruption. In the next sentence in the evidence quote, complete disruption of the splice site is implied. For *BRCA2*, complete disruption is the most stringent requirement for triggering ACMG PVS1 (null) “Very Strong” evidence if there are no rescue mechanisms, which the PMID: 35050751 data suggests there is. Thus using a PVS2 line of evidence puts the classification at higher risk for being a false positive classification, and we recommend not using PVS2 evidence here given the counterevidence.
Evidence obtained from Literature or Locus Specific Databases that can be input for CGI’s variant classification:		**NONE**

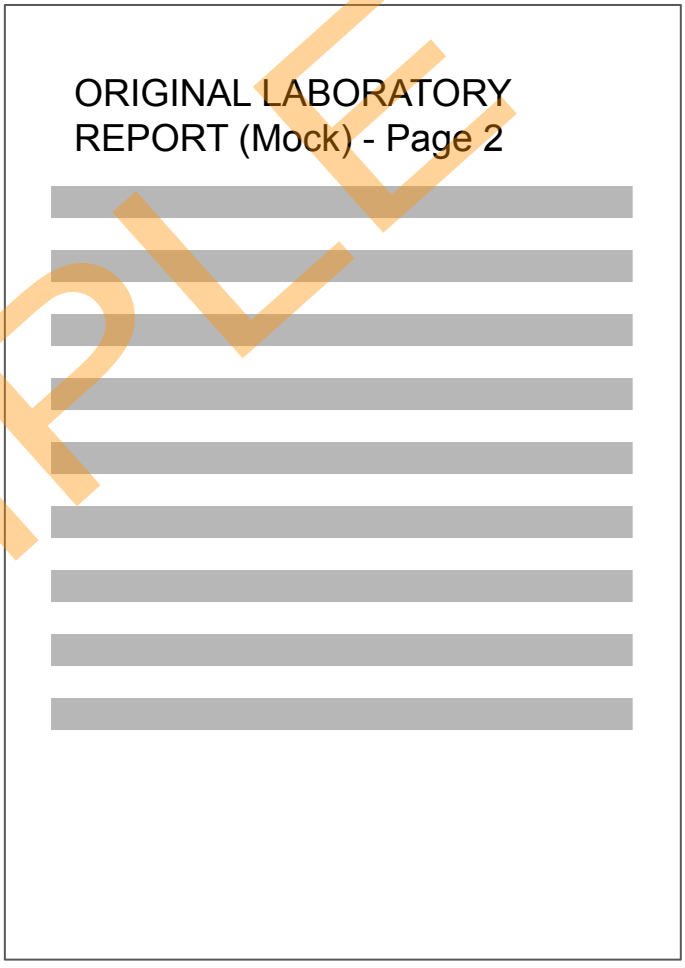
Evidence is “DISQUALIFIED” when it is redundant, or of no, low, or insignificant quality or quantity to be applicable in a clinical setting. Most “Disqualified” evidence represents data in the earliest, and most error and biased prone stages of basic research.

Appendix D - Originating Report

D1. Relevant Pages from Originating Report Received by CGI:

[LABORATORY REPORT FOR PATIENT]





Appendix E - CGI Agreement Meter Explanation

E1. Matrix for "CGI Agreement Meter With Original Report"

		Variant Classification from the originating report				
		Pathogenic	Likely Pathogenic	VUS	Likely Benign	Benign
CGI's research-use -only variant classification	Pathogenic	Strongly Agree	Agree	Strongly Disagree	Strongly Disagree	Strongly Disagree
	Likely Pathogenic	Agree	Strongly Agree	Disagree	Strongly Disagree	Strongly Disagree
	VUS	Strongly Disagree	Disagree	Strongly Agree	Disagree	Strongly Disagree
	Likely Benign	Strongly Disagree	Strongly Disagree	Disagree	Strongly Agree	Agree
	Benign	Strongly Disagree	Strongly Disagree	Strongly Disagree	Agree	Strongly Agree