When splicing is not all or none: GT>GC 5′ splice-site variants as a model for intermediate effects and challenges in variant classification

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Abstract

Variants with intermediate functional effects—those that neither eliminate nor fully maintain normal gene function—represent an underrecognized source of genetic complexity and constitute a grey zone that challenges variant classification. Here, we address this issue using GT>GC (+2T>C) 5′ splice-site variants as a tractable model, as approximately 15% of such substitutions retain variable amounts of wild-type (WT) transcript. Using residual WT transcript as a quantitative readout, we first show that disease-associated GT>GC variants known to generate substantial WT transcript (for example, *SPINK1* c.194+2T>C, *HBB* c.315+2T>C, and *BRCA2* c.8331+2T>C) consistently complicate classification efforts. We then carried out a locus-wide assessment of all 26 GT>GC substitutions in

CFTR, applying SpliceAI delta donor-loss scores as an initial screen and cross-checking classifications in expert-curated database. This evaluation indicated that three variants with low or intermediate SpliceAI scores are likely to retain WT transcript. Minigene analyses of four selected *CFTR* variants, together with additional examination of a *BAP1* GT>GC variant with conflicting clinical interpretations, illustrate the methodological challenges in predicting and experimentally quantifying residual WT transcript. These results emphasize that (i) *in silico* predictors cannot reliably discriminate between complete and partial splice disruption and (ii) experimental systems differ in their ability to detect subtle splicing outcomes. Overall, our integrative analysis shows that GT>GC variants generating appreciable WT transcript exemplify a broader class of intermediate-effect alleles that complicate both computational prediction and experimental assessment, underscoring the need for classification frameworks that incorporate quantitative functional data and better capture the continuum of variant effects.

Keywords

ACMG/AMP variant classification guidelines; ClinVar; Incomplete penetrance; Intermediate functional effects; Loss-of-function (LoF) variants; Missing heritability; Residual wild-type transcript; SpliceAI; Variant effect spectrum; Variants of uncertain significance (VUS)

Introduction

Accurate classification of genetic variants remains a central challenge in human genetics.[1,2] The increasing use of a genotype-first or genomic-first approach,[3,4] enabled by exome and genome sequencing, has greatly magnified this challenge. In many cases, the mode of inheritance becomes apparent only after genetic analysis, and disorders presumed to follow a simple Mendelian pattern may instead have a complex, multifactorial, or oligogenic basis. This complexity underscores the limitations of the widely used American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) framework.[5] Its dichotomization of variants as either “pathogenic” or “benign,” although useful for clinical consistency, cannot account for the full

54 spectrum of variant effects that contribute to human genetic disease.[3,6-9] It should be noted that the
55 current ACMG/AMP category "variant of uncertain significance (VUS)"[5] is not a biological entity but
56 a temporary designation expected to resolve as either pathogenic or benign once sufficient evidence
57 becomes available.

58 For clarity, we focus here on variants that act through a loss-of-function (LoF) mechanism. In
59 contrast to variants at the two extremes—those causing complete or near-complete loss of function of
60 the affected allele, typically responsible for classic Mendelian disease phenotypes, and those retaining
61 complete or near-complete normal function—variants with intermediate effects are challenging to
62 interpret because they lie outside the scope of binary pathogenicity models. This challenge is
63 illustrated by the multiple partial LoF variants identified in *SPINK1* (MIM: 167790).[8] Despite their
64 clear biological reality and clinical relevance, such variants remain underexplored and are not
65 adequately addressed by any widely established classification frameworks, which offer limited
66 guidance for interpreting quantitative or intermediate functional effects and thereby risk systematic
67 over- or under-classification.

68 In this study, we explore GT>GC (+2T>C) 5′ splice-site variants as a tractable model to
69 investigate how intermediate functional effects challenge classification. Although substitutions at
70 canonical GT donor or AG acceptor sites typically abolish normal splicing,[10,11] a subset of GT>GC
71 substitutions defy this expectation. Specifically, a meta-analysis of 45 disease-associated variants,
72 together with full-length gene splicing assay (FLGSA) data from 103 substitutions, showed that 15–
73 18% retain residual wild-type (WT) transcript, in some cases at levels approaching 80% of normal.[12]
74 This variability can be clinically significant. For instance, in cystic fibrosis, as little as 5% normal
75 cystic fibrosis transmembrane conductance regulator (CFTR) activity may be sufficient to prevent
76 pulmonary disease,[13,14] whereas in the hemophilias, factor VIII or IX levels above 5% may lead to
77 only mild bleeding diatheses.[15,16] Conversely, in disorders typically caused by complete LoF alleles,
78 GT>GC variants may not be invariably pathogenic if substantial WT transcript is preserved, as we
79 previously proposed.[12]

80 Using residual ("leaky") WT transcript levels as a quantitative measure, this study illustrates the
81 difficulties inherent in classifying GT>GC variants that generate substantial yet incomplete amounts of

82 WT transcript. Our analysis highlights the challenges posed by intermediate functional effects and
83 reinforces the need for classification frameworks capable of capturing the full continuum of variant
84 effects.[6,8]
85

86 # Methods

87 **Identification and selection of GT>GC variants**

88 Our 2019 study[12] served as the starting point. From the FLGSA-analyzed GT>GC variants reported
89 therein, we included all variants that both retained WT transcript and were registered in ClinVar[17]
90 (https://www.ncbi.nlm.nih.gov/clinvar/; accessed September 5, 2025) for analysis. To identify
91 additional disease-associated GT>GC variants with evidence of residual WT transcript generation
92 published since 2019, we conducted a Google-based literature search using the keyword "+2T>C,"
93 complemented by cascade reference tracking and cross-referencing within the peer-reviewed literature
94 (up to September 5, 2025). As in our previous work,[12] we focused on studies in which RNA analysis
95 was performed on material derived from affected individuals, and simple heterozygotes were excluded
96 unless residual WT transcript generation from the variant allele was explicitly demonstrated by allele-
97 specific reverse transcription-PCR (RT-PCR) in the original study. At the single-gene level, all 26
98 possible *CFTR* (MIM: 602421) GT>GC variants were subjected to initial evaluation. Finally, a *BAP1*
99 (MIM: 603089) variant, c.783+2T>C (GenBank: NM_004656.4, NG_031859.1), identified during the
100 literature search as having conflicting classifications,[18,19] was included for additional minigene splicing
101 analysis.
102

103 ***In silico* splicing predictions**

104 *In silico* predictions were obtained using SpliceAI[20] via the SpliceAI Lookup tool
105 (https://spliceailookup.broadinstitute.org/; accessed September 5, 2025) with the following settings:
106 genome version, hg38; GENCODE, basic; maximum distance, 10,000 bp; and REF & ALT scores
107 displayed. The resulting delta donor loss (ΔDL) scores, derived from comparisons between WT and
108 variant sequences, were used for analysis.

109 **Variant classification or description in public databases**

110 Selected variants were queried in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/)[17] to determine their
111 current classification status. The Clinical and Functional TRanslation of CFTR database (CFTR2;
112 http://www.cftr2.org/)[21] and the CFTR-France database (https://cftr.chu-montpellier.fr/)[22] were
113 consulted to evaluate *CFTR* variant classifications. Variant information from the Cystic Fibrosis
114 Mutation Database (CFTR1; http://www.genet.sickkids.on.ca/) was used to assist in the classification
115 of two *CFTR* variants that were not registered in either CFTR2 or CFTR-France. Our evaluation of the
116 variant classification or description data in these databases was last updated on October 28, 2025.

117

118 **Minigene splicing analysis of selected *CFTR* GT>GC variants**

119 ***Variant selection***

120 Of the 26 possible *CFTR* GT>GC variants (GenBank: NM_000492.4, NG_016465.4), we selected all
121 three with low SpliceAI DL scores (≤0.56)—c.2490+2T>C, c.3717+2T>C and c.4242+2T>C—
122 together with one variant (c.3873+2T>C) that had a high SpliceAI score (0.98) but was previously
123 reported to generate WT transcript,[23] for minigene splicing analysis.

124

125 ***Construction of pET01 and pSPL3 minigene expression vectors***

126 For each selected +2T>C variant, the corresponding WT genomic sequences cloned into the pET01
127 and pSPL3 exon trapping vectors[24] were always identical. Each *CFTR* WT insert comprised 213–217
128 bp from the 3′ end of intron N−1, the entire exon N, and 213–216 bp from the 5′ end of intron N (N =
129 variant-affected intron). Cloning of WT constructs and introduction of the selected +2T>C variants,
130 followed by Sanger confirmation, were performed by GENEWIZ Biotech Co. (Suzhou, China).

131

132 ***Cell culture, transfection, RT-PCR, and sequencing***

133 Experiments were carried out essentially as previously described.[24] Human embryonic kidney 293T
134 (HEK293T) cells were maintained in DMEM (Gibco) supplemented with 10% fetal calf serum
135 (Procell). Cells (3.5×10^5/well) were seeded in 6-well plates 24 h before transfection. Per well, 2.5 µg
136 WT or variant plasmid were transfected using HieffTrans Universal Transfection Reagent (Yeasen).

137 Forty-eight hours later, total RNA was extracted with the FastPure Cell/Tissue Total RNA Isolation
138 Kit V2 (+gDNA wiper) (Vazyme). RT was carried out using the HiScript III 1st Strand cDNA
139 Synthesis Kit (Vazyme), incorporating 2 μL 5×gDNA wiper Mix, 2 μL 10×RT Mix, 2 μL HiScript III
140 Enzyme Mix, 1 μL Oligo (dT)20VN, and 1 μg total RNA. RT-PCR was performed in a 25-μL reaction
141 mixture containing 12.5 μL 2×Taq Master Mix (Vazyme), 1 μL cDNA, and 0.4 μM each primer. The
142 primer pairs were as follows: pET01 vector—forward 5′-GAGGGATCCGCTTCCTGGCCC-3′,
143 reverse 5′-CTCCCGGGCCACCTCCAGTGCC-3′; pSPL3 vector—forward 5′-
144 TCTGAGTCACCTGGACAACC-3′, reverse 5′-ATCTCAGTGGTATTTGTGAGC-3′. The PCR
145 program comprised an initial denaturation step at 94°C for 5 min, followed by 35 cycles of
146 denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, and a final
147 extension step at 72°C for 7 min. RT-PCR products were separated on agarose gels, excised, and
148 purified (Omega Bio-Tek Gel Extraction Kit). Sequencing primers were the same as those used for
149 RT-PCR, and reactions were performed with the BigDye Terminator v3.1 Cycle Sequencing Kit
150 (Applied Biosystems).
151
152 **Full-length and minigene splicing analysis of *BAP1* c.783+2T>C**
153 The full-length genomic *BAP1* WT insert, spanning from the translation initiation codon to the
154 termination codon (GenBank: NM_004656.4, NG_031859.1), was amplified from genomic DNA of a
155 normal control using long-range PCR. The resulting fragment was cloned into the pcDNA3.1/V5-His-
156 TOPO vector. These procedures, as well as subsequent transfection and RT–PCR, were performed
157 essentially as previously described.[12]
158 Minigene splicing analysis followed the same procedures as for *CFTR* variants, except that two
159 WT constructs (and their corresponding variant constructs) were generated. The *BAP1* Exon 9 insert
160 comprised 337 bp from intron 8, exon 9, and 302 bp from intron 9, whereas the *BAP1* Exon 8–10
161 insert comprised 197 bp from intron 7, exon 8, intron 8, exon 9, intron 9, exon 10, and 243 bp from
162 intron 10.

Results

Analytical approach

Building on our 2019 findings that some GT>GC variants retain WT transcript,[12] we analyzed three successive variant categories (Figure 1). First, we re-examined variants previously characterized by FLGSA. Second, we considered disease-associated GT>GC variants reported since 2019 that generate WT transcript. Third, we evaluated all possible GT>GC variants in *CFTR* and functionally tested four selected variants. Across these categories, we assessed residual WT transcript levels, genetic and clinical findings, SpliceAI ΔDL scores, and database classifications, where available. In addition, we conducted minigene splicing analysis of the *BAP1* c.783+2T>C variant, which has conflicting classifications.[18,19] Integrating these findings, we delineated the classification challenges posed by variants with intermediate functional effects—hereafter referred to conceptually as grey-zone variants.

Comparing disease-associated and FLGSA-analyzed GT>GC variants

In our previous study,[12] the proportion of GT>GC variants retaining WT transcript was similar between disease-associated and FLGSA-analyzed variants: 15.6% (7 of 45) versus 18.4% (19 of 103). The magnitude of residual WT transcript levels, however, differed markedly. The seven disease-associated variants yielded only low levels, from trace amounts up to 15% normal, whereas of the ten variants generating exclusively WT transcript (as judged by gel electrophoresis), eight retained levels ranging from 13.7% to 84% of normal, with only two falling below 5%.

The consistently low WT transcript levels observed for the seven disease-associated variants most likely reflect clinical ascertainment bias, as noted previously.[12] Nonetheless, of the four variants retaining 10–15% WT transcript, three—*CAV3* (MIM: 601253) c.114+2T>C (GenBank: NM_033337.3, NG_008797.2),[25] *CD40LG* (MIM: 300386) c.346+2T>C (GenBank: NM_000074.3, NG_007280.1),[26] and *DMD* (MIM: 300377) c.8027+2T>C (GenBank: NM_004006.3, NG_012232.1)[27]—were associated with milder clinical phenotypes; the remaining variant, *SPINK1* c.194+2T>C (GenBank: NM_001379610.1, NG_008356.2), is discussed below.

189 The corresponding WT constructs for the 103 FLGSA-analyzed substitutions were invariably
190 preselected to yield a single or quasi-single RT–PCR band of the expected WT transcript size,
191 consistent with their respective reference mRNA sequences.[12] We also analyzed *HBB* (MIM: 141900)
192 c.315+2T>C (GenBank: NM_000518.5, NG_059281.1),[28] despite its WT construct producing two
193 strong bands—one corresponding to the WT transcript and the other representing an aberrantly spliced
194 transcript. This variant also generated WT transcript in FLGSA,[12] bringing the total number of WT
195 transcript–generating GT>GC variants identified by FLGSA to 20.
196
197 **Detailed analysis of three FLGSA-analyzed GT>GC variants**
198 Of the 20 GT>GC variants previously shown by FLGSA[12] to generate residual WT transcript, only
199 three were found to be registered in ClinVar[17] (https://www.ncbi.nlm.nih.gov/clinvar/; assessed
200 September 5, 2025). These three variants, described below, illustrate how the presence of residual WT
201 transcript can challenge variant classification.
202
203 ***SPINK1 c.194+2T>C***
204 Among GT>GC variants, *SPINK1* c.194+2T>C (GenBank: NM_001379610.1, NG_008356.2) is
205 distinctive in that transcript expression has been assessed both *in vivo* (using RNA from an affected
206 homozygote)[29] and *in vitro* using FLGSA.[12] Both approaches yielded concordant results, with
207 approximately 10% of normal WT transcript levels from the variant allele.[30] SpliceAI predicts a ΔDL
208 score of 0.33, consistent with a substantial yet incomplete reduction in normal splicing.
209 Our in-depth analysis of *SPINK1*-related chronic pancreatitis[8] indicated that this ~10% residual
210 WT transcript places c.194+2T>C in the grey zone, conferring a well-defined genetic effect that is too
211 weak for "pathogenic" under current ACMG/AMP criteria[5] but too strong for the proposed "risk
212 allele" category.[7] We therefore suggested classifying it as "predisposing," reflecting its partial LoF
213 nature and intermediate genetic impact.[8] ClinVar lists this variant as pathogenic/likely pathogenic
214 (VCV000132142.62; accessed October 28, 2025), but convergent evidence from functional assays, *in*
215 *silico* prediction, and clinical and genetic data supports its role as proof of concept highlighting the
216 need for expanded classification frameworks.[8]

217 ***HBB c.315+2T>C***

218 This variant was identified during routine thalassemia screening in a 34-year-old individual who
219 exhibited hypochromia and microcytosis consistent with a thalassemia trait rather than β-thalassemia
220 major. The individual was compound heterozygous for *HBB* c.315+2T>C and *HBB* c.79G>A
221 (p.Glu27Lys) (GenBank: NM_000518.5, NG_059281.1).[28] The original authors reported that *HBB*
222 c.315+2T>C had a limited effect on splicing efficiency, stating that it "results in expression that is
223 approximately two-thirds of the normal allele."[28] However, this estimate was based on hemoglobin
224 quantitation and was likely confounded by the *trans*-inherited *HBB* c.79G>A (p.Glu27Lys) variant.
225 The latter likely retains substantial normal function, as most individuals homozygous for *HBB*
226 c.79G>A (p.Glu27Lys) exhibit only mild hypochromic microcytic anemia.[31]

227 Our FLGSA analysis confirmed that *HBB* c.315+2T>C allows appreciable production of WT
228 transcript.[12] Here, using ImageJ (https://imagej.net/ij/), we compared the intensity of the WT transcript
229 band derived from the variant allele with that from the WT allele in the original gel.[12] The variant
230 allele was estimated to retain approximately 30% of WT transcript output, providing a more direct and
231 reliable measure of its residual transcript level. Notably, the orthologous rabbit variant also generated
232 WT transcripts,[32,33] supporting conservation of the partial splicing effect across species. The SpliceAI
233 ΔDL score for *HBB* c.315+2T>C was 0.72.

234 *HBB* alleles in β-thalassemia have traditionally been classified as β^0 (complete loss of β-globin
235 synthesis), β^+ (moderate-to-severe reduction), or β^{++} ("silent," minimal reduction).[34] *HBB* c.315+2T>C
236 was originally described as a mild β^+ allele,[28] consistent with our FLGSA finding of significant but not
237 complete loss of WT transcript.[12] Nevertheless, it is currently annotated in ClinVar as "pathogenic"
238 (VCV000869355.2; accessed October 28, 2025)—a designation that may warrant re-evaluation in
239 view of its substantial residual WT transcript generation and the mild hematological phenotype
240 observed in the 34-year-old compound heterozygote.

241

242 ***MGP c.94+2T>C***

243 *MGP* (MIM: 154870) c.94+2T>C (GenBank: NM_000900.5, NG_023331.2) was shown by FLGSA[12]
244 to retain approximately 80% of WT transcript, whereas SpliceAI predicted a strong splice-disrupting

245 effect (ΔDL score, 0.97). Despite this discrepancy, ClinVar lists *MGP* c.94+2T>C as "likely
246 pathogenic" (VCV001987659.2; accessed October 28, 2025), based on a single clinical testing
247 submission (SCV003031993.2) with unknown affected status and no published case reports. This
248 classification appears to rest on four implicit assumptions:
249 (1) the variant alters a canonical donor splice site;
250 (2) such variants generally disrupt splicing;
251 (3) disruption of canonical sites typically abolishes protein function; and
252 (4) LoF variants in *MGP* are known to be pathogenic.
253 As this example shows, ClinVar classifications may at times rely on inferred reasoning rather than
254 direct experimental evidence.
255
256 **Disease-associated GT>GC variants reported since 2019**
257 We next examined disease-associated GT>GC variants reported since 2019, focusing on those with
258 WT transcript generation documented in samples derived from affected individuals. Among the five
259 variants identified, *SBDS* (MIM: 607444) c.258+2T>C (GenBank: NM_016038.4, NG_007277.1)[35]
260 and *SRP68* (MIM: 604858) c.184+2T>C (GenBank: NM_014230.4, NC_000017.11),[36] produced only
261 trace (~2%) or low amounts of WT transcript, with SpliceAI ΔDL scores of 0.93 and 0.37,
262 respectively. *TNXB* (MIM: 600985) c.12469+2T>C (GenBank: NM_001365276.2, NG_008337.2)
263 retained a moderate level of WT transcript by allele-specific RT–PCR,[37] consistent with its very low
264 ΔDL score of 0.01, and has been associated with moderate Ehlers–Danlos–like manifestations in
265 congenital adrenal hyperplasia.[37] The remaining two variants—*BRCA2* (MIM: 600185) c.8331+2T>C
266 (GenBank: NM_000059.4, NG_012772.3) and *DOCK8* (MIM: 611432) c.3234+2T>C (GenBank:
267 NM_203447.4, NG_017007.1)—are discussed below.
268
269 ***BRCA2 c.8331+2T>C***
270 *BRCA2* c.8331+2T>C (GenBank: NM_000059.4, NG_012772.3) is associated with a high SpliceAI
271 ΔDL score (0.93) and is listed in ClinVar as "pathogenic/likely pathogenic" (VCV000267692.71;
272 accessed October 28, 2025). However, allele-specific RT–PCR in a heterozygous carrier demonstrated

273 approximately 60% of WT transcript from the variant allele.[38] A validated *BRCA1/2* history-weighting
274 algorithm, which compares cancer histories with expectations for pathogenic versus benign variants,[39]
275 yielded a benign call.[38] Moreover, *BRCA2* appears relatively tolerant of reduced transcript dosage, and
276 one study suggested that variants producing more than 5% WT transcript should, in the absence of
277 other supporting evidence, be considered VUS.[40] While the original report reclassified c.8331+2T>C
278 from "pathogenic" to a "VUS,"[38] the combined evidence appears to point toward a benign
279 interpretation under current ACMG/AMP principles.
280
281 ***DOCK8* c.3234+2T>C**
282 Biallelic LoF variants in *DOCK8* cause autosomal recessive hyper-IgE syndrome-2 (MIM: 243700).
283 *DOCK8* c.3234+2T>C (GenBank: NM_203447.4, NG_017007.1) was reported in the homozygous
284 state in an infant with severe viral infections.[41] T cells from this individual showed a predominance of
285 WT transcript, normal DOCK8 protein accumulation, intact cellular function, and no DOCK8
286 deficiency phenotype. The clinical presentation was instead explained by a homozygous *IFNAR1*
287 (MIM: 107450) nonsense variant (c.1156G>T, p.Glu386Ter; GenBank: NM_000629.3).[41] Despite this
288 evidence for near-complete WT transcript generation, lack of disease relevance, and a modest
289 SpliceAI ΔDL score (0.49), three of the four ClinVar submissions for *DOCK8* c.3234+2T>C
290 (VCV000573664.15; accessed October 28, 2025) classified it as "likely pathogenic," while one
291 classified it as "benign."
292 *BRCA2* c.8331+2T>C and *DOCK8* c.3234+2T>C exemplify how variants predicted—or
293 assumed—to be fully disruptive can nevertheless yield intermediate or even near-complete levels of
294 WT transcript, underscoring the need for direct transcript-level evidence for accurate classification.
295 These two variants are summarized in Table 1 alongside the three FLGSA-analyzed examples
296 (*SPINK1* c.194+2T>C, *HBB* c.315+2T>C, and *MGP* c.94+2T>C).
297
298 **Locus-wide analysis of *CFTR* GT>GC variants**
299 Having examined GT>GC variants that retain WT transcript generation across multiple genes, we next
300 asked how frequent and systematic this phenomenon might be within a single locus. We selected

301 *CFTR* (GenBank: NM_000492.4, NG_016465.4), in which biallelic LoF variants cause cystic fibrosis
302 (CF; MIM: 219700),[42,43] as a model system because it harbors 26 GT donor splice sites (Table S1), is
303 among the most extensively studied disease-related genes, and benefits from expert-curated databases
304 CFTR2[21] and CFTR-France[22] that provide authoritative variant annotations.

305 Building upon our previous[12,30] and current findings, we observed that although some GT>GC
306 variants retaining WT transcript are associated with high SpliceAI ΔDL scores, most have low scores.
307 We therefore used SpliceAI ΔDL scores as the starting point for this analysis, complemented by cross-
308 examination of variant classifications in CFTR2 (http://www.cftr2.org/; accessed October 28, 2025)
309 and CFTR-France (https://cftr.chu-montpellier.fr/; accessed October 28, 2025) (Figure 2A). Notably,
310 all 16 *CFTR* GT>GC variants that we grouped as "pathogenic" had SpliceAI ΔDL scores greater than
311 0.80. These included 13 designated as "CF-causing" in CFTR2, one absent from CFTR2 but listed as
312 "likely pathogenic" in CFTR-France (c.3468+2T>C), and two absent from both CFTR2 and CFTR-
313 France but classified by us as "pathogenic" based on clinical and genetic data in CFTR1
314 (http://www.genet.sickkids.on.ca/; accessed October 28, 2025) (c.2908+2T>C and c.2988+2T>C).
315 Eight *CFTR* GT>GC variants were not registered in these three databases, of which seven also scored
316 above 0.80 and one had a score of 0.56 (c.3717+2T>C). The remaining two *CFTR* GT>GC variants—
317 one listed as a VUS in CFTR-France (c.2490+2T>C) and one reported in CFTR1 in two individuals
318 with suspected CF (c.4242+2T>C)—had SpliceAI ΔDL scores below 0.56 (Figure 2A,B). Table S1
319 provides full details of all 26 *CFTR* GT>GC variants, including the rationale for our classifying two as
320 "pathogenic."

321 Assuming that the three variants with relatively low SpliceAI ΔDL scores (c.2490+2T>C, 0.35;
322 c.3717+2T>C, 0.56; and c.4242+2T>C, 0.15) generate WT transcript, at least 11.5% of the 26 *CFTR*
323 GT>GC variants would retain WT transcript. This proportion is broadly consistent with the 15–18%
324 frequency observed across genes in our previous study.[12]

325 Among these three *CFTR* variants with relatively low SpliceAI ΔDL scores, only c.4242+2T>C
326 had previously undergone functional analysis. Using an expression minigene (EMG) system
327 comprising the full *CFTR* coding sequence and intronic regions tailored to the variant under study,
328 generation of residual WT transcript from the c.4242+2T>C allele was confirmed by Sanger

329 sequencing, and mature CFTR protein was detected in transfected cells by immunoblotting.[23] The only
330 other *CFTR* GT>GC variant reported to yield WT transcripts is c.3873+2T>C, although this was
331 mentioned only briefly and without accompanying experimental data.[23] Notably, c.3873+2T>C is
332 classified as "CF-causing" in both CFTR2 and CFTR-France, consistent with its high SpliceAI ΔDL
333 score (0.98) (Figure 2B).

334 We tested the three variants with low SpliceAI ΔDL scores, together with c.3873+2T>C, in
335 minigene assays using both pET01 and pSPL3 vectors (Figure 3; see also Figure S1). Findings for
336 each variant are detailed below.

337

338 ***c.2490+2T>C (intron 14)***

339 WT constructs in both minigene vectors generated multiple RT–PCR products, including the WT
340 transcript. Importantly, WT transcript from the variant allele was detected in the pET01 vector (Figure
341 3A), consistent with its modest SpliceAI ΔDL score (0.35). Although this variant is not classified by
342 CFTR2, it is listed in CFTR-France as a VUS, having been reported in two asymptomatic
343 homozygotes. In CFTR1, it was described in the heterozygous state in the partner of an individual
344 affected by congenital bilateral absence of the vas deferens (Figure 2B). Furthermore, ClinVar lists
345 this variant under "conflicting classifications of pathogenicity" (VCV000370078.23; accessed October
346 28, 2025), based on six clinical-testing submissions ("pathogenic" × 3, "likely pathogenic" × 2, and
347 "uncertain significance" × 1). Only one submission specified a known affected status—homozygosity
348 for c.2490+2T>C, labeled "pathogenic," in an individual with fetal cystic hygroma
349 (SCV001737053.1). Another submission, designated "likely pathogenic" (SCV001363801.5),
350 explicitly noted that "no occurrence of c.2490+2T>C in individuals affected with cystic fibrosis and
351 no experimental evidence demonstrating its impact on protein function have been reported." Taken
352 together, the clinical, genetic, computational, and experimental evidence indicate that c.2490+2T>C is
353 unlikely to represent a pathogenic *CFTR* variant in the context of classic CF.

354 ***c.3717+2T>C (intron 22)***

355 No WT transcript from the variant allele was detected in either minigene vector (Figure 3B), despite
356 its intermediate SpliceAI ΔDL score (0.56). This variant is not listed in CFTR2, CFTR-France,
357 CFTR1, or ClinVar (Figure 2B).

358

359 ***c.3873+2T>C (intron 23)***

360 No WT transcript from the variant allele was observed in either vector (Figure 3C); this finding is
361 consistent with its high SpliceAI ΔDL score (0.98), its designation as "CF-causing" in both CFTR2
362 and CFTR-France, and its "pathogenic" classification in ClinVar (VCV000053828.18; accessed
363 October 28, 2025) (Figure 2B). Moreover, consistent with its description in CFTR1, this variant is
364 among the *CFTR* alleles recurrently identified in individuals with clinically diagnosed CF in
365 Norway.[44] Taken together, c.3873+2T>C represents a pathogenic *CFTR* variant causing classic CF.
366 The level of WT transcript generated from the c.3873+2T>C allele, as previously reported,[23] is
367 presumably minimal.

368

369 ***c.4242+2T>C (intron 26)***

370 No WT transcript from the variant allele was detected in either minigene vector (Figure 3D), in
371 contrast to the earlier EMG report of residual WT transcript and protein production[23] and to its very
372 low SpliceAI ΔDL score (0.15). Notably, this variant is not listed in CFTR2 or CFTR-France but
373 appears in CFTR1 as present in two individuals with suspected cystic fibrosis (Figure 2B). ClinVar
374 lists c.4242+2T>C as "pathogenic/likely pathogenic" (VCV000035884.8; accessed October 28, 2025),
375 based on three submissions ("pathogenic" × 1 and "likely pathogenic" × 2), all from clinical genetic
376 testing laboratories. The collection method was described as "clinical testing" for two submissions
377 (SCV002630543.2 and SCV004675881.2), both with unknown affected status, and as "curation" for
378 the remaining submission (SCV000052191.3). Taken together, this variant may best be classified as a
379 VUS at present.

380 In summary, locus-wide analysis of *CFTR* confirms that a subset of GT>GC variants retain WT
381 transcript. Functional testing of selected *CFTR* GT>GC variants highlights both inter-assay variability
382 and the inherent limitations of minigene systems in fully recapitulating native splicing.[24]
383

384 **Challenges of splicing assays: the case of *BAP1* c.783+2T>C**

385 As illustrated by *CFTR* variants, *in vitro* splicing assays may fail to yield clear or consistent results,
386 complicating variant classification. This challenge is further exemplified by *BAP1* c.783+2T>C
387 (GenBank: NM_004656.4, NG_031859.1). Because heterozygous germline LoF variants in *BAP1*
388 underlie tumor predisposition syndrome 1 (TPDS1; MIM: 614327) and *BAP1* c.783+2T>C affects the
389 canonical intron 9 donor splice site, this variant was initially classified in ClinVar as "likely
390 pathogenic," as noted by Goldberg et al.[18] These authors reclassified it as a VUS based on the
391 following considerations:

392 (1) the variant was identified in seven families (six Jewish Iraqi and one non-Jewish) with
393 nonclassical *BAP1*-related TPDS1 tumor types;
394 (2) the variant exhibited low penetrance;
395 (3) the variant had a relatively high allele frequency in Jewish Iraqi individuals (3.6%);
396 (4) the variant had a low SpliceAI score (0.08); and
397 (5) no aberrantly spliced transcripts were detected by RT-PCR using RNA from blood cells of
398 two probands.

399 A subsequent study[19] identified a methodological limitation: the forward primer used by Goldberg
400 et al.[18] was located within exon 9, rendering it incapable of detecting exon 9 skipping. Using a primer
401 in exon 8, Sculco et al.[19] analyzed *BAP1* c.783+2T>C heterozygotes and observed an alternative
402 splicing pattern consistent with exon 9 skipping. These authors proposed reinstating a "likely
403 pathogenic" classification while acknowledging that the effect of *BAP1* c.783+2T>C on generating
404 aberrant transcripts might be incomplete.

405 To further clarify this issue, we performed additional splicing assays (Figure 4; Figure S2). We
406 first attempted FLGSA, as the *BAP1* genomic sequence from start to stop codon (<8 kb) falls within
407 the upper size limit of the insert cloned into the pcDNA3.1/V5-His-TOPO vector routinely used in this

408 assay.[12] However, the WT construct, when transfected into HEK293T cells, failed to yield clear RT-
409 PCR bands (Figure 4A). Notably, in our previous study, only 33 of 119 (28%) WT full-length
410 constructs produced a single or quasi-single band of the expected size after transfection.[12] We
411 therefore turned to minigene assays using pET01 and pSPL3 vectors. WT constructs spanning
412 genomic sequence from exon 8 to exon 10 (including exon 8, intron 8, exon 9, intron 9, and exon 10)
413 with short flanking sequences from introns 7 and 10 produced multiple RT-PCR bands, none
414 corresponding to the WT transcript defined by NM_004656.4; the corresponding variant constructs
415 also failed to generate a normally spliced transcript, although the banding patterns differed from those
416 of the WT (Figure 4B). In contrast, shorter constructs containing exon 9 with short flanking intronic
417 sequences generated the expected WT band from the WT allele, whereas only transcripts lacking exon
418 9 were produced from the variant allele (Figure 4C; Figure S2).
419 ClinVar currently lists c.783+2T>C under "conflicting classifications of pathogenicity"
420 (VCV000422670.29; accessed October 28, 2025), with five submissions of "uncertain significance"
421 and one of "likely benign." In light of these inconclusive findings, allele-specific transcript analyses in
422 heterozygous individuals—if possible in the presence of a *cis*-linked coding variant outside exon 9—
423 would be most informative. Alternatively, targeted knock-in cell models could provide additional
424 insight into the splicing effect of *BAP1* c.783+2T>C.
425

426 ## Discussion

427 Our analysis yielded several insights into the impact of GT>GC 5′ splice-site variants. Consistent with
428 findings from FLGSA,[12] we show that disease-associated GT>GC variants can generate a wide range
429 of residual WT transcript levels, from barely detectable to nearly normal. Moreover, our findings
430 confirm our earlier postulate that "GT>GC variants in human disease genes may not invariably be
431 pathogenic,"[12] as exemplified by the *DOCK8* c.3234+2T>C variant.
432 Second, GT>GC variants that generate substantial yet incomplete WT transcript levels often fall
433 within a functional grey zone, complicating their classification (Figure 5). Such indeterminacy
434 underscores the limitations of binary frameworks and has important clinical implications. A clear

435 example is *SPINK1* c.194+2T>C, which retains approximately 10 % of normal WT transcript. In the
436 simple heterozygous state, its effect is too weak to be classified as "pathogenic" yet too strong to be
437 regarded as a mere "risk" allele. However, in homozygous, compound heterozygous, or *trans*-
438 heterozygous states—or in the presence of environmental modifiers—the same variant confers a
439 substantially greater pathogenic effect.[8] This exemplifies how variants with intermediate functional
440 effects contribute to the genetic complexity of human disease. To address this challenge, we have
441 proposed expanding the ACMG/AMP variant classification framework by incorporating additional
442 categories, including a "predisposing" category.[6,8]

443 Third, our findings underscore the limited resolution of current *in silico* tools, such as SpliceAI,[20]
444 for predicting whether GT>GC variants generate WT transcript and, if so, to what extent. Whereas
445 most WT transcript–retaining variants exhibit low SpliceAI ΔDL scores, some have high values
446 (>0.80), and transcript levels show no consistent correlation with predicted scores. This limitation
447 partly reflects the intrinsic ability of GC to function as a natural donor site in approximately 1% of U2-
448 type introns.[45] More broadly, it highlights the difficulty of predicting splicing outcomes that are not
449 all-or-none, leaving many variants within a functional grey zone that current algorithms cannot readily
450 model or quantify.

451 Fourth, our results highlight the methodological challenges inherent in experimentally
452 characterizing splice-altering variants. RNA from disease-relevant tissues provides the most reliable
453 readout, yet such material is rarely available, and surrogate sources such as blood cells or
454 lymphoblastoid lines are informative only if the gene of interest is expressed. In the absence of RNA
455 from affected individuals, cell-based assays—FLGSA,[12] EMG,[23] midigene,[46] or minigene[47] systems—
456 are often used, but each has intrinsic limitations. For example, not only is FLGSA impractical for large
457 genes, but also not all successfully constructed full-length WT constructs yield the expected WT
458 transcript. The most frequently used minigene assays are performed in a highly artificial background
459 that lacks the natural genomic context required for proper splicing regulation.[48,49] Such loss of context
460 is particularly problematic for variants with intermediate effects, for which classification depends not
461 only on detecting aberrant transcripts but also on quantifying residual WT transcript from the variant
462 allele. Even when RNA from affected individuals is available, interpretation in heterozygotes can be

463 confounded by WT transcript derived from the unaffected allele. For this reason, in both our
464 previous[12] and current studies, analyses of disease-associated GT>GC variants were focused primarily
465 on homozygotes and hemizygotes. RNA-seq, although increasingly applied in clinical contexts,[50-52]
466 faces similar challenges of tissue accessibility and allele-specific quantification. Collectively, these
467 methodological obstacles underscore the need for refined experimental systems, such as those based
468 on transactivation or transdifferentiation.[53]

469 GT>GC variants that retain residual WT transcript reflect the degeneracy and context dependence
470 of splice-site recognition. As such, findings from this study can likely be extrapolated to many other
471 splice-altering variants—excluding those at the invariant +1, −1, and −2 positions—because *cis*-acting
472 motifs are degenerate, and deep intronic variants can generate novel splice sites that compete with
473 physiological ones. Although a comprehensive survey is beyond the scope of this study, several
474 observations support this view. In SpliceVarDB, approximately 50% of variants could not be assigned
475 unambiguously to "splice-altering" or "non–splice-altering" categories but instead fell into a "low-
476 frequency splice-altering" group, corresponding to weak or indeterminate evidence of
477 spliceogenicity,[54] suggesting that grey-zone variants are common. Among 12 FLGSA-analyzed
478 *SPINK1* coding variants showing aberrant splicing, nine (including presumed missense and
479 synonymous variants) also generated residual WT transcript.[55] Likewise, deep intronic variants such as
480 *CFTR* c.1584+18672A>G (GenBank: NM_000492.4, NG_016465.4)[56] and *CLRN1* c.254−643G>T
481 (GenBank: NM_174878.3, NG_009168.1)[57] have been reported to yield both aberrant and WT
482 transcripts. Collectively, these examples indicate that residual WT transcript generation is a recurrent
483 and underappreciated phenomenon across genes and variant types, producing outcomes along a
484 continuum rather than at binary extremes (Figure 5). Because residual WT transcript cannot always be
485 readily assessed in simple or compound heterozygotes, and splice-altering variants account for an
486 estimated 10–30% of all disease-associated alleles,[11,54,58] we infer that variants generating residual WT
487 transcript may represent a significant source of variable penetrance and expressivity.

488 Nonetheless, the boundaries of the functional grey zone (Figure 5) likely vary across genes,
489 depending on functional tolerance thresholds and modes of inheritance, such that an identical level of
490 residual WT transcript may be clinically inconsequential in one gene–disease context yet functionally

491 significant in another. Although developed here in the context of LoF variants for conceptual
492 simplicity, the grey-zone model may also extend to gain-of-function or dominant-negative variants,[59]
493 in which disease-associated alleles within a given gene are likewise unlikely to exert uniform effects.
494 Notably, this conceptual framework was established primarily for genes in which complete or near-
495 complete LoF alleles cause classical Mendelian diseases. As we have previously opined,[8] some
496 disease-associated genes may not necessarily harbor clearly "pathogenic" variants.

497 Taken together, our study establishes GT>GC variants as a tractable model for addressing the
498 classification challenges posed by splice-altering variants with intermediate functional consequences.
499 By using residual WT transcript as a measurable parameter—and integrating clinical and genetic data,
500 RNA analyses of material derived from affected individuals, full-length and minigene assays, and
501 computational predictions—we show that residual WT transcript generation is a recurrent and
502 biologically relevant phenomenon that frequently complicates classification. Gradual functional
503 effects are also observed for other variant types, as exemplified by missense variants in *BRCA1* (MIM:
504 113705)[60] and *SOD1* (MIM: 147450),[61] both analyzed using multiplexed assays of variant effect
505 (MAVE). These observations underscore the need to move beyond binary pathogenic/benign
506 frameworks toward models that accommodate a continuum of effects,[6,8] as efforts focused solely on
507 refining ACMG/AMP criteria within a binary framework (as reflected in recent studies[62-65]) are
508 unlikely to resolve the fundamental problem amplified by the field's transition from a "phenotype-
509 first" to a "genotype-first" approach.

510 To address this challenge, new conceptual, computational, and experimental strategies are
511 emerging. As highlighted by Whitcomb,[66] our recently proposed "pathogenic–predisposing–risk–
512 benign" framework[8] represents an important step toward resolving the variant classification challenge.
513 The integration of machine-learning models trained on large-scale population-based datasets[9] with
514 functional data generated from MAVE[67,68] holds great promise for defining the full spectrum of variant
515 effects revealed through analyses of increasingly large cohorts.[69-71] Ultimately, grey-zone variants may
516 constitute a substantial portion of those currently classified as VUS or excluded for exceeding the
517 allele-frequency thresholds set for high-penetrance disease variants. Such variants could also account
518 for part of the "missing heritability" observed in both rare and complex diseases.[72-74] Recognizing the

widespread presence of grey-zone variants and their contribution to genetic complexity will be essential for realizing the full potential of precision medicine.[75]

Our study has several limitations. First, the number of GT>GC 5′ splice site variants known to generate substantial amounts of WT transcript remains limited. Second, the level of WT transcript generated from certain GT>GC variants, as determined by FLGSA or RT–PCR using material derived from affected individuals, may differ from that occurring under true *in vivo* conditions within the pathologically relevant tissue. Third, a potential alternative impact of GT>GC variants—namely, a reduction in overall splicing efficiency without producing aberrant transcripts—could not be adequately assessed in this analysis. Likewise, GT>GC variants that generate both WT and aberrant transcripts, but in which the aberrant transcripts are degraded by nonsense-mediated decay (NMD),[76] could not be evaluated. These two scenarios could lead to underestimation of the true extent of partial LoF GT>GC variants. Fourth, while we highlighted the classification challenges posed by the variants discussed in detail, we did not systematically attempt to specify how each variant should be classified. This is primarily because, as we have previously noted, "defining precise thresholds between different variant categories remains challenging and must be tailored to each gene and disease context,"[8] and because the available functional analytic data are not conclusive or fully reliable in some cases, and the associated phenotypes were not always well defined. Despite these limitations, our integrative analysis of GT>GC variants provides a coherent framework for understanding how residual WT transcript generation shapes the functional continuum of splice-altering variants and informs their broader biological and clinical interpretation.

In conclusion, viewing GT>GC variants through the lens of residual WT transcript generation offers valuable insight into how partial LoF splice-altering variants—and, more broadly, variants with intermediate functional effects—can contribute to genetic complexity, complicate both *in silico* prediction and experimental analysis, and challenge variant classification (Figure 5).

Data and code availability

All data supporting this study are provided within the article and its supplementary materials. This study did not generate any new datasets or code.

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Author contributions

Conceptualization, J.M.C.; study design, J.H.L., H.W., W.B.Z. and J.M.C.; methodology, J.H.L., H.W., W.B.Z. and J.M.C.; experiments, J.H.L., H.W., X.Y.T. and W.B.Z.; literature search and variant collation, E.M. and J.M.C.; writing—original draft preparation, J.M.C.; writing—review and editing, J.H.L., H.W., X.Y.T., E.M., D.N.C., C.F., Z.L., W.B.Z. and J.M.C.; project administration, W.B.Z. and J.M.C.; funding acquisition, J.H.L., W.B.Z. and J.M.C. All authors have read and approved the final version of the manuscript and agree to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Declaration of interests

The authors declare no competing interests.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT 5 in order to improve readability and language. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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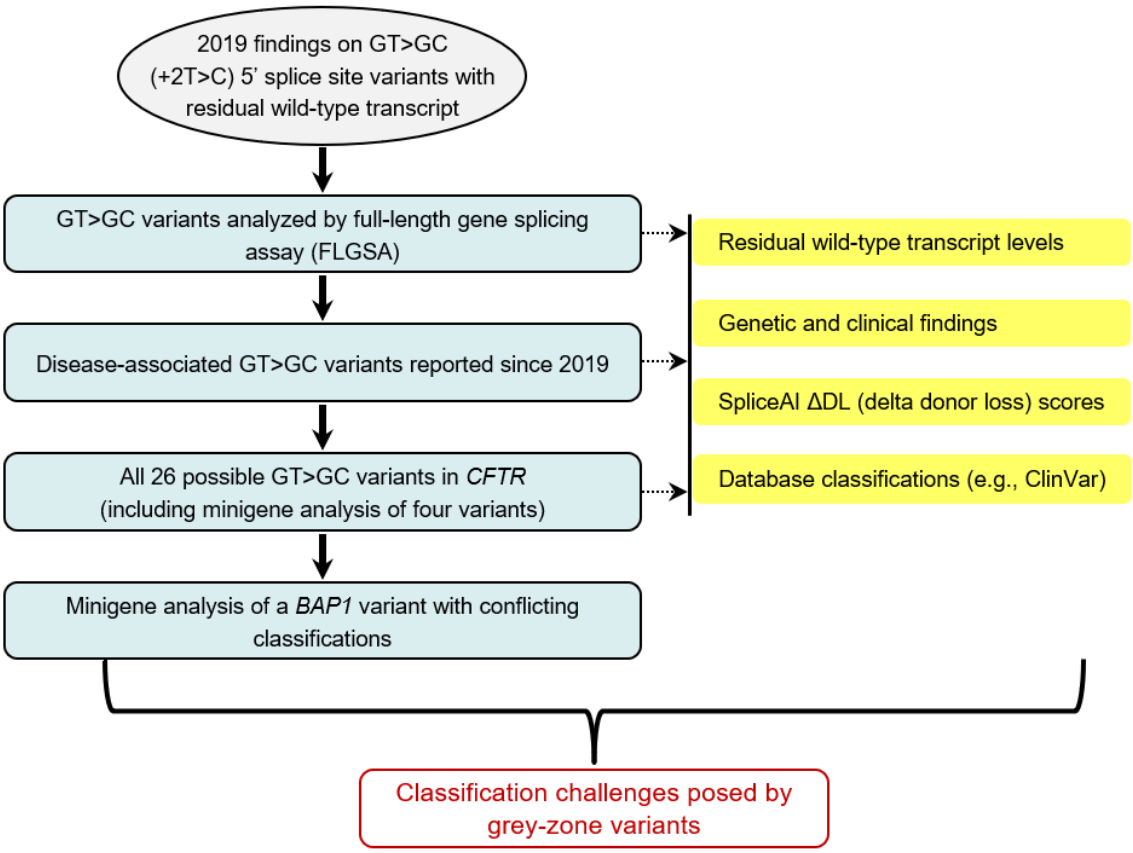
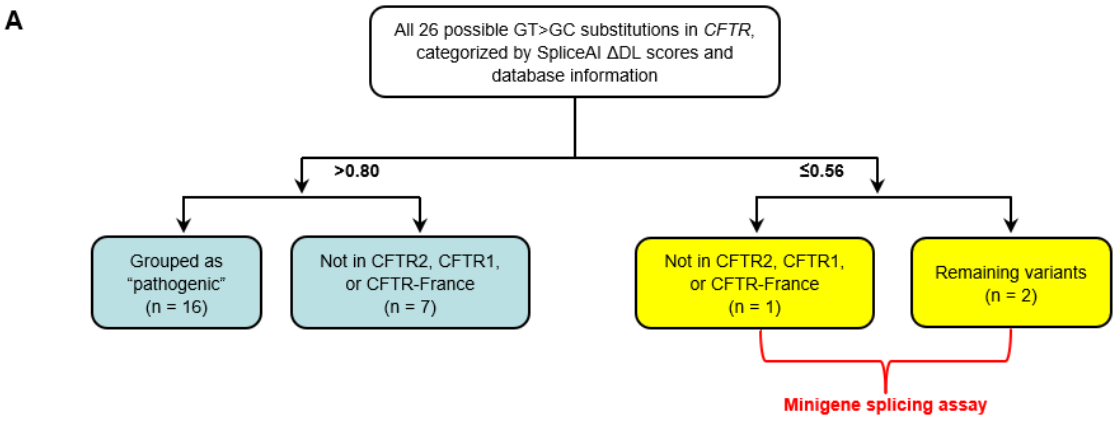


Figure 1. Scheme of the analytical approach

GT>GC variants were analyzed in three successive categories, together with minigene analysis of a *BAP1* variant with conflicting classifications. Within each category, variants were examined for four features (yellow shading). The red box highlights the central issue addressed in this study—the classification challenges posed by grey-zone variants that retain substantial yet incomplete wild-type transcript generation.



B

CFTR variants selected for functional analysis using minigene splicing assays

Variant	SpliceAI ΔDL score	Database classification and reporting				Residual wild-type transcript	
		CFTR2	CFTR-France	CFTR1	ClinVar	Previous study	This study
c.2490+2T>C	0.35	–	VUS (reported in two asymptomatic homozygotes)	Identified in heterozygous state in the partner of a CBAVD patient	CCP	–	Yes (pET01); No (pSPL3)
c.3717+2T>C	0.56	–	–	–	–	–	No in both assays
c.3873+2T>C	0.98	CF-causing	CF-causing	Commonly reported in Norwegian CF patients	P	Yes	No in both assays
c.4242+2T>C	0.15	–	–	Reported in two individuals with suspected CF diagnosis	P/LP	Yes	No in both assays

Figure 2. Analysis of GT>GC substitutions in *CFTR*

(A) Flowchart summarizing the 26 possible *CFTR* GT>GC substitutions categorized by SpliceAI ΔDL scores and database information (see Table S1 for details). Variants with low scores (≤0.56) were selected for minigene splicing assays.

(B) Four variants analyzed by minigene splicing assays. In "Database classification and reporting," note that, unlike CFTR2 and CFTR-France, CFTR1 does not classify registered variants. In "Residual wild-type transcript," "Yes" and "No" indicate whether the variant allele generated wild-type transcript. Results from this study are shown separately for the two assay systems (pET01 and pSPL3; see Figure 3), with "No in both assays" indicating absence of wild-type transcript from the variant allele in both systems. "Previous study" refers to Joynt et al.[23]

Abbreviations: CBAVD, congenital bilateral absence of the vas deferens; CF, cystic fibrosis; CCP, conflicting classifications of pathogenicity; ΔDL, delta donor loss; P, pathogenic; P/LP, pathogenic/likely pathogenic; VUS, variant of uncertain significance.

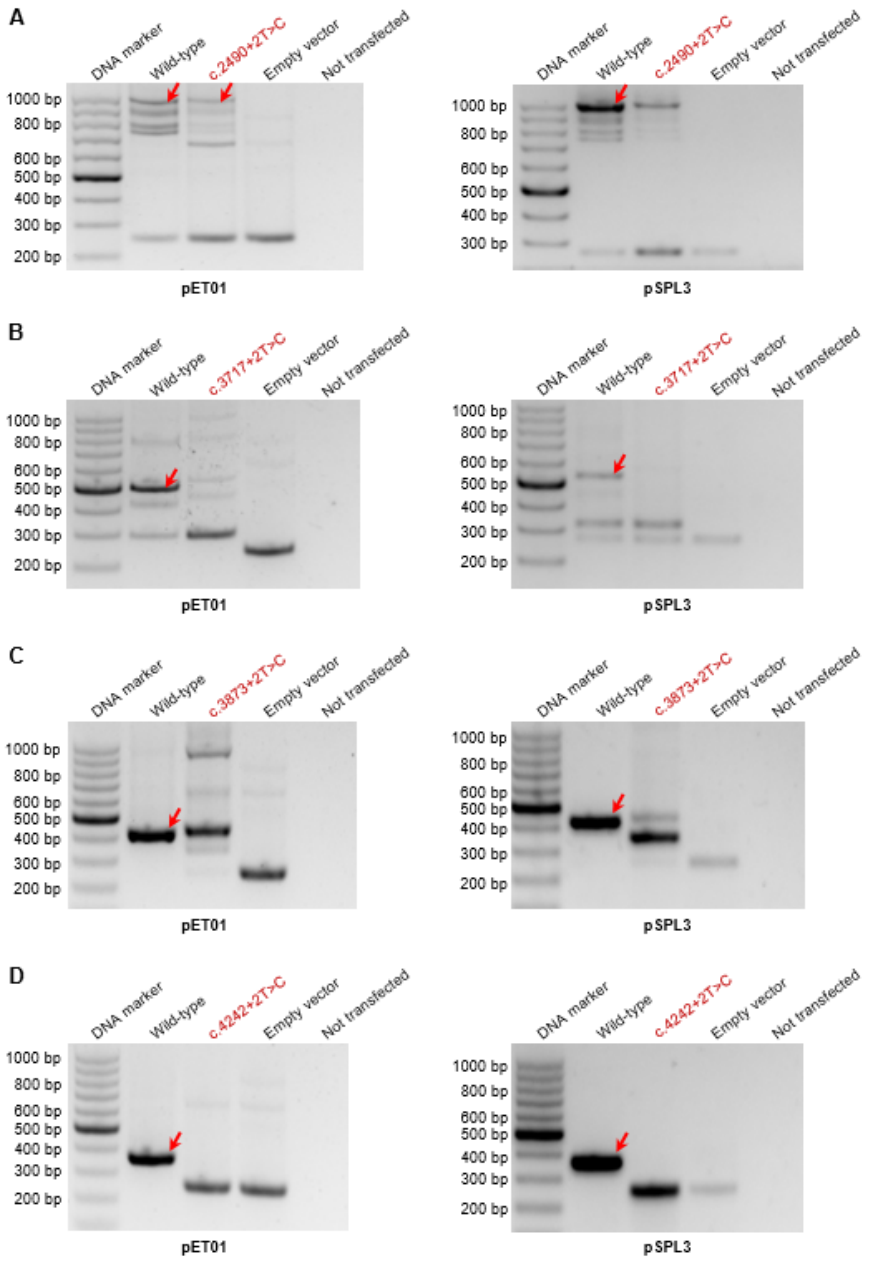


Figure 3. Minigene splicing analysis of four selected *CFTR* GT>GC substitutions using pET01 and pSPL3 vectors

Each variant was analyzed with its preceding exon and short flanking intronic sequences cloned into the respective reporter vectors. Arrows indicate bands corresponding to the normally spliced transcript, in accordance with the *CFTR* mRNA reference sequence NM_000492.4. Additional bands are annotated in Figure S1.

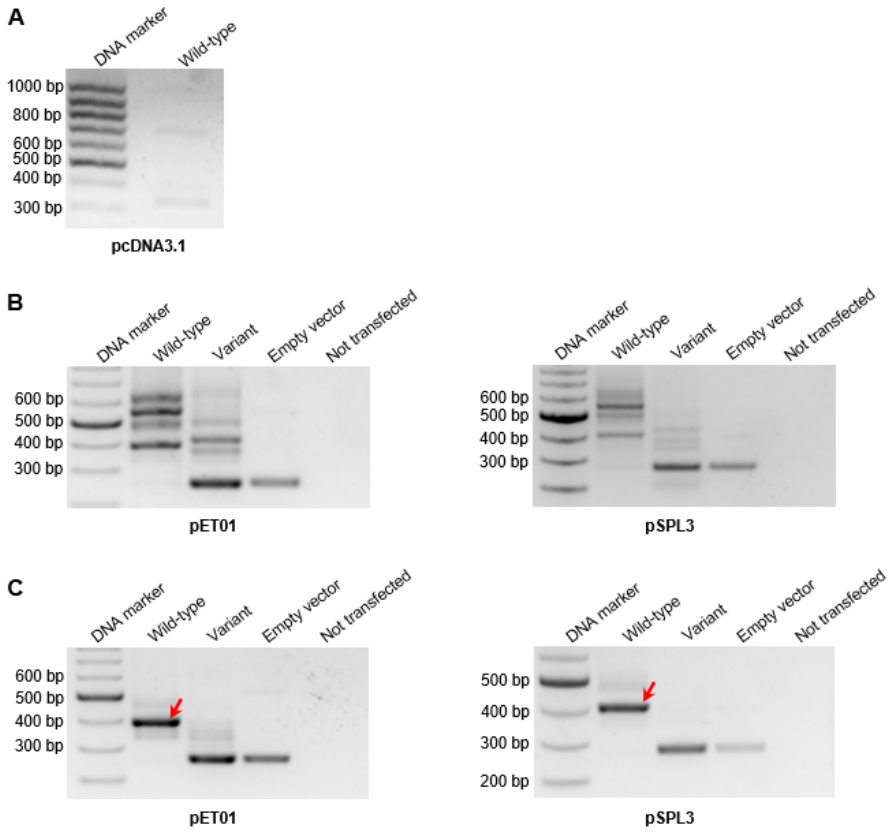


Figure 4. Splicing analysis of the *BAP1* c.783+2T>C variant in different assay contexts

(A) Full-length gene splicing assay (FLGSA). The wild-type (WT) construct failed to generate clear reverse transcription–PCR (RT–PCR) bands.

(B) Minigene analysis of exons 8–10 (including exon 8, intron 8, exon 9, intron 9, and exon 10) with short flanking sequences from introns 7 and 10 cloned into the pET01 and pSPL3 vectors. No normally spliced transcripts were detected, in accordance with the *BAP1* mRNA reference sequence NM_004656.4, even from the WT constructs. For band annotations, see Figure S2.

(C) Minigene analysis of exon 9 with short flanking intronic sequences cloned into the pET01 and pSPL3 vectors. Arrows indicate bands corresponding to the normally spliced transcript, in accordance with the *BAP1* mRNA reference sequence NM_004656.4. Additional bands are annotated in Figure S2. *BAP1* genomic reference sequence: NG_031859.1.

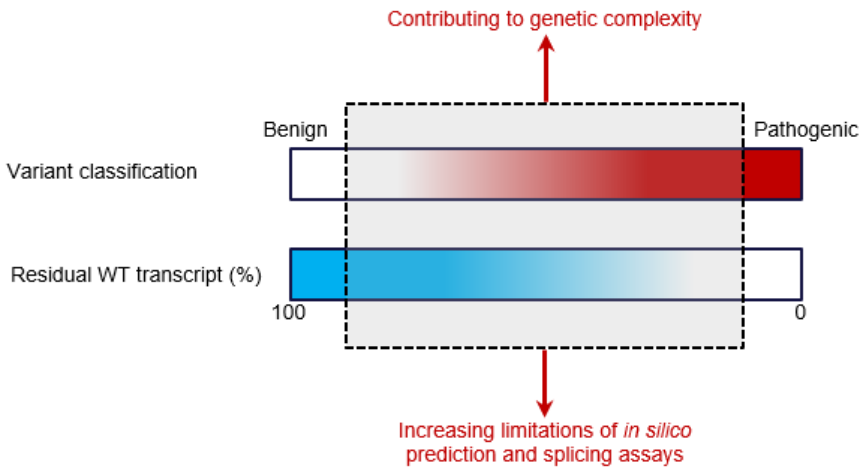


Figure 5. Conceptual model of the functional grey zone in variant classification

Residual wild-type (WT) transcript levels and variant classification lie along continua rather than binary states. Variants with intermediate WT transcript levels often fall within a functional grey zone, where *in silico* prediction and experimental analysis become increasingly difficult. Such grey-zone variants may act through variant–variant (both intra- and intergenic) or variant–environment interactions, thereby contributing to the genetic complexity of human disease. Although illustrated here with splice-altering variants, this conceptual model also applies to other variant types and disease mechanisms shaped by graded levels of functional impact.

897 **Table 1. Five GT>GC variants with residual WT transcript that present classification challenges**

Variant[a]	Reference mRNA and genomic sequences	hg38 coordinate	Residual WT transcript[b]	Assessment method	SpliceAI ΔDL score	Clinical/genetic findings	ClinVar classification
SPINK1 c.194+2T>C	NM_001379610.1, NG_008356.2	chr5-147828020-A-G	10%	RT–PCR of material from an affected homozygote;[29] FLGSA[12]	0.33	Well-defined genetic effect, too weak for pathogenic classification[8]	Pathogenic/likely pathogenic
HBB c.315+2T>C	NM_000518.5, NG_059281.1	chr11-5226575-A-G	30%	FLGSA[12]	0.72	Originally described as a mild β^+-thalassemia allele[28]	Pathogenic
MGP c.94+2T>C	NM_000900.5, NG_023331.2	chr12-14884211-A-G	80%	FLGSA[12]	0.97	Not reported in affected individuals	Likely pathogenic
BRCA2 c.8331+2T>C	NM_000059.4, NG_012772.3	chr13-32363535-T-C	60%	Allele-specific RT–PCR of affected individual–derived material[38]	0.93	*BRCA2* history-weighting algorithm[39] indicated a benign profile;[38] *BRCA2* exhibits tolerance to reduced transcript dosage[40]	Pathogenic/likely pathogenic
DOCK8 c.3234+2T>C	NM_203447.4, NG_017007.1	chr9-399261-T-C	Predominant WT	RT–PCR and protein analyses of T cells from an affected homozygote[41]	0.49	Disease explained by an unlinked homozygous *IFNAR1* nonsense variant[41]	Conflicting classifications of pathogenicity

898 Abbreviations: ΔDL, delta donor loss; FLGSA, full-length gene splicing assay; RT-PCR, reverse transcription-PCR; WT, wild-type.

899 [a]Variants are arranged in order of mention in the main text.

900 [b]Percentage of WT transcript generated from the variant allele relative to that from the WT allele.