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Systematic Minigene-Based Splicing Analysis and Tentative Clinical Classification of 52 *CHEK2* Splice-Site Variants

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BACKGROUND: Disrupted pre-mRNA splicing is a frequent deleterious mechanism in hereditary cancer. We aimed to functionally analyze candidate spliceogenic variants of the breast cancer susceptibility gene *CHEK2* by splicing reporter minigenes.

METHODS: A total of 128 *CHEK2* splice-site variants identified in the Breast Cancer After Diagnostic Gene Sequencing (BRIDGES) project (https://cordis.europa.eu/project/id/634935) were analyzed with MaxEntScan and subsetted to 52 variants predicted to impact splicing. Three *CHEK2* minigenes, which span all 15 exons, were constructed and validated. The 52 selected variants were then genetically engineered into the minigenes and assayed in MCF-7 (human breast adenocarcinoma) cells.

RESULTS: Of 52 variants, 46 (88.5%) impaired splicing. Some of them led to complex splicing patterns with up to 11 different transcripts. Thirty-four variants induced splicing anomalies without any trace or negligible amounts of the full-length transcript. A total of 89 different transcripts were annotated, which derived from different events: single- or multi-exon skipping, alternative site-usage, mutually exclusive exon inclusion, intron retention or combinations of the abovementioned events. Fifty-nine transcripts were predicted to introduce premature termination codons, 7 kept the original open-reading frame, 5 removed the translation start codon, 6 affected the 5′UTR (Untranslated Region), and 2 included missense variations. Analysis of variant c.684-2A > G revealed the activation of a non-canonical TG-acceptor site and exon 6 sequences critical for its recognition.

CONCLUSIONS: Incorporation of minigene read-outs into an ACMG/AMP (American College of Medical Genetics and Genomics/Association for Molecular Pathology)-based classification scheme allowed us to classify 32 *CHEK2* variants (27 pathogenic/likely pathogenic and 5 likely benign). However, 20 variants (38%) remained of uncertain significance, reflecting in part the complex splicing patterns of this gene.

Introduction

The *CHEK2* (checkpoint kinase 2) gene [MIM#604373] is composed of 15 exons and encodes the nuclear serine/threonine-kinase CHK2. CHK2 exerts multiple functions in DNA damage response and is a key guardian of genome integrity. In response to double-strand DNA breaks (DSBs), ATM Serine/Threonine Kinase (ATM) phosphorylates and activates CHK2 and in turn phosphorylates several downstream proteins essential for DSB repair by homologous recombination (1, 2).

Protein truncating variants (PTVs) in *CHEK2* and 7 other genes (*BRCA1* [MIM#113705], *BRCA2* [MIM#600185], *ATM* [MIM#607585], *PALB2* [MIM#610355], *BARD1* [MIM#601593], *RAD51C* [MIM#602774], and *RAD51D* [MIM#602954]) are unequivocally associated with breast cancer (BC) risk (3–5), with

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CHEK2 accounting for nearly 25% of all the PTVs identified in these 8 genes in large case-control Breast Cancer After Diagnostic Gene Sequencing (BRIDGES) and CARRIERS studies (second only to *BRCA2*) (3, 4). *CHEK2* PTVs are associated with moderate BC risk (2.5; absolute risk of about 25% by 80 years of age), and have been associated as well with increased risk to other types of cancer, including prostate and colorectal (6).

The National Comprehensive Cancer Guidelines (https://www.nccn.org/guidelines/guidelines-detail?category=2&id=1503, accessed March 27, 2023) recommend annual mammography at age 40 and breast magnetic resonance rmaging (MRI) at age 30 to 35 in *CHEK2* pathogenic variant carriers, a strategy that has been estimated to reduce BC mortality by 58% (7).

Nonsense, frameshift and splice-site ±1,2 variants are usually classified as pathogenic or likely pathogenic, on the grounds that they are expected to lead to the absence of transcription or nonsense-mediated decay of the resulting transcript, according to the guidelines of the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) (8). About 54% of all *CHEK2* variants reported at the ClinVar database (http://www.ncbi.nlm.nih.gov/clinvar/, accessed February 3, 2023) are of uncertain or conflicting clinical significance. Variants of uncertain significance (VUSs) limit the power of genetic tests to guide medical recommendations of carrier and non-carrier relatives.

Variants can disrupt essential gene expression steps, and hence impact disease risk, through mechanisms such as transcription initiation or splicing, among others (9–11). The splicing reactions are mediated by the spliceosome and specific consensus sequences at the exon–intron boundaries (5′ and 3′ splice sites, 5′SS and 3′ SS, respectively). Any mutation at these elements can trigger splicing anomalies that may be associated with a genetic disorder (12). Interestingly, a relevant proportion of pathogenic variants at BC genes impact pre-mRNA splicing (13, 14).

Splicing analyses are often performed by RT (Reverse transcription)-PCR in RNA from variant carriers. However, such samples are typically not available and, at any rate, expression from the accompanying wild-type (wt) allele is a confounding factor (12, 15). Alternatively, hybrid minigenes are a straightforward strategy for the initial characterization of spliceogenic variants of disease genes, including the main BC susceptibility genes where comprehensive variant analyses have been performed (16–19).

Our goal was to analyze *CHEK2* candidate variants identified in the BRIDGES project. We utilized in silico analysis of 128 *CHEK2* variants, 52 of which were selected for subsequent splicing assays using 3 minigenes that cover the 15 *CHEK2* exons. Finally, we classified the variants following ACMG/AMP-based guidelines.

Materials and Methods

Ethical approval for this study was obtained from the Ethics Committee of the Spanish National Research Council-CSIC (28/05/2018).

ANNOTATION OF VARIANTS AND TRANSCRIPTS

CHEK2 variants were obtained from the BRIDGES consortium sequencing data, derived from >60 000 BC cases and >50 000 controls. Variant data and alternative transcripts were annotated according to the Human Genome Variation Society (HGVS) guidelines on basis of the *CHEK2* GenBank NM_007194.4 (MANE selected transcript). Neither BRIDGES nor the present study interrogated genetic variants located in a nonconstitutive exon of 129 nucleotides (nt) located in between reference exons 2 and 3 (NM_001005735). Splicing events were described with a short descriptor combining the following symbols: Δ (skipping of exonic sequences), ▼ (inclusion of intronic sequences), E (exon), p (acceptor site shift), and q (donor site shift) (18). When necessary, the number of deleted or inserted nucleotides is indicated. Thus, Δ(E1q13) indicates the use of a cryptic alternative donor site 13-nt upstream of exon 1, producing a 13-nt deletion.

BIOINFORMATICS ANALYSIS

A total of 128 variants (see online Supplemental Table 1) located at the intron–exon boundaries (±10 intronic nt and the first 2 and last 2 exonic nt) were bioinformatically analyzed to identify candidate splicing variants (3), utilizing MaxEntScan (MES) (http://hollywood.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html) (20). In addition, the genetic alterations were analyzed with SpliceAI (genome version: hg38, score type: raw, max distance: 10 000) to predict splicing outcomes (https://spliceailookup.broadinstitute.org/) (21). Likely spliceogenic variants were selected if they fulfilled at least one of the following criteria: (*a*) disruption of splice site (canonical ±1,2 positions), no more than one variant per splice site, unless 2 or more variants display different predictions (e.g., exon skipping vs creation of a de novo site); or (*b*) ≥20% decrease of MES score (>40% for polypyrimidine tract single-nucleotide substitutions as they commonly cause partial or no splicing effects); or (*c*) regardless of MES scores, deletion in the polypyrimidine tract (e.g., c.593-11_593-7del) or variant at other conserved positions such as a +6 T change.

The presence of splicing enhancers/silencers in exon 6 was estimated by Hexplorer (https://www2.hhu.de/

rna/html/hexplorer_score.php) where peaks (positive values) and valleys (negative values) denote enhancer-rich and silencer-rich sequences, respectively. SpliceAid (http://www.introni.it/splicing.html) was employed to predict putative binding motifs of splicing factors in exon 6.

MINIGENE CONSTRUCTION AND MUTAGENESIS

The construction of 3 *CHEK2* minigenes (mgChk2_ex1-7, mgChk2_ex6-10, and mgChk2_ex11-15) in the pSAD (Splicing And Disease) vector (Patent_P201231427) and site-directed mutagenesis of *CHEK2* variants are described in the online Data Supplement (Supplemental Figs. 1 and 2, Fig. 1, Supplemental Tables 2 and 3). All constructs were confirmed by sequencing (Macrogen). The whole protocol is outlined in online Supplemental Fig. 3.

TRANSFECTION OF EUKARYOTIC CELLS

Approximately 2×10^5 MCF-7 (human breast adenocarcinoma cell line) and HeLa cells were grown to 90% confluency in 0.5 mL of medium (Minimum Essential Medium -MEM-, 10% fetal bovine serum, 1% nonessential amino acids, 2 mM glutamine, and 1% penicillin/streptomycin) in 4-well plates (Nunc). Cells were transiently transfected with 1 μg of minigene using 2 μL of Lipofectamine-LTX (Life Technologies). To inhibit nonsense-mediated decay (NMD), cells were treated with cycloheximide 300 μg/mL (Sigma-Aldrich) for 4 hours just before RNA extraction. RNA was purified with the Genematrix Universal RNA Purification Kit (EURx) with on-column DNAse I treatment.

RT-PCR

Retrotranscription was carried out with 400 ng of RNA and the RevertAid First Strand cDNA Synthesis Kit (Life Technologies), using the vector-specific primer 5′-TGAGGAGTGAATTGGTCGAA-3′ and the manufacturer's conditions. Then, 40 ng of cDNA were amplified with primers 5′-TCACCTGGACAACCTCAAAG-3′ and RTpSAD-RV (Patent_P201231427) using Platinum-Taq polymerase (Life Technologies), under the following thermocycling conditions: 94°C, 2 min, 35 cycles × [94°C/30 s, 60°C/30 s, and 72°C/(1 min/kb)], and 72°C, 5 min. The expected sizes of the minigene full-length (mgFL1, 2, and 3) transcripts are: 1052-nt (mgChk2_ex1-7), 593-nt (mgChk2_ex6-10), and 676-nt (mgChk2_ex11-15).

To estimate the relative proportions of each transcript, semiquantitative fluorescent RT-PCRs were undertaken in triplicate with primers RTPSPL3-FW and RTpSAD-RV labelled with 6-FAM (6-Carboxyfluorescein; blue peaks) and Platinum Taq polymerase (Life Technologies) under standard conditions, except that 26 cycles were run (22). FAM-labelled products were run with LIZ1200 (mgChk2_ex1-7, mgChk2_ex11-15) or LIZ600 Size Standards (mgChk2_ex6-10) at the Macrogen facility and analyzed with Peak Scanner_V1.0. Only peak heights ≥200 RFU (relative fluorescence units) were considered, except in case of low-quality electropherograms where cutoffs were decreased.

ACMG/AMP-BASED TENTATIVE CLASSIFICATION OF CHEK2 GENETIC VARIANTS

We classified the tested variants according to ACMG/AMP guidelines transformed into a Bayesian classification framework (23). In this approach, evidence strengths are expressed as a point scale (supporting = ±1 point, moderate = ±2 points, strong = ±4 points, very strong = ±8 points) with negative values corresponding to benign evidence and positive values corresponding to pathogenic evidence. Variant classification is performed by combining all collected evidence. Categories are specified as follows: pathogenic (P), ≥+10 points; likely pathogenic (LP), +6 to +9 points; VUS, 0 to +5 points; likely benign (LB), −1 to −6 points; and benign (B) ≤−7 points. Furthermore, we have introduced into the system the following caveats: (*a*) we have not combined experimental and predictive splicing evidence, and (*b*) we have classified as VUS variants that reach > +5 points, if all collected points stem from a single evidence.

Following ClinGen Hereditary Breast, Ovarian and Pancreatic Cancer Variant Curation Expert Panel (HBOP-VCEP) recommendations (www.clinicalgenome.org/affiliation/50039, accessed February 3, 2023), we incorporated *CHEK2* minigene read-outs into the classification system as PVS1_O/BP7_O codes of variable strength depending on the actual experimental outcome (the HBOP-VCEP recommends using PS3/BS3 codes to protein level functional data only). As most tested variants produce 2 or more transcripts, we proceeded as follows: (*a*) we assigned a specific PVS1_O (or BP7_O) code (variable strength) to each individual transcript and (*b*) we assigned an overall PVS1_O (or BP7_O) code to the variant only if pathogenic supporting transcripts (or benign supporting transcripts) reach 90% of the overall expression. To assist in the classification process, we developed a *CHEK2* adaptation of the generic PVS1 decision tree proposed by the ClinGen sequence variant interpretation working group (24). Other than PVS1_O/BP7_O, only PS4, PM2_S, PM5_S, and BS1 evidence contributed to the final classification of the 52 *CHEK2* variants (see Supplemental Material).

Results

BIOINFORMATICS ANALYSIS

We identified 128 unique *CHEK2* variants at the intron–exon boundaries in the BRIDGES data set.

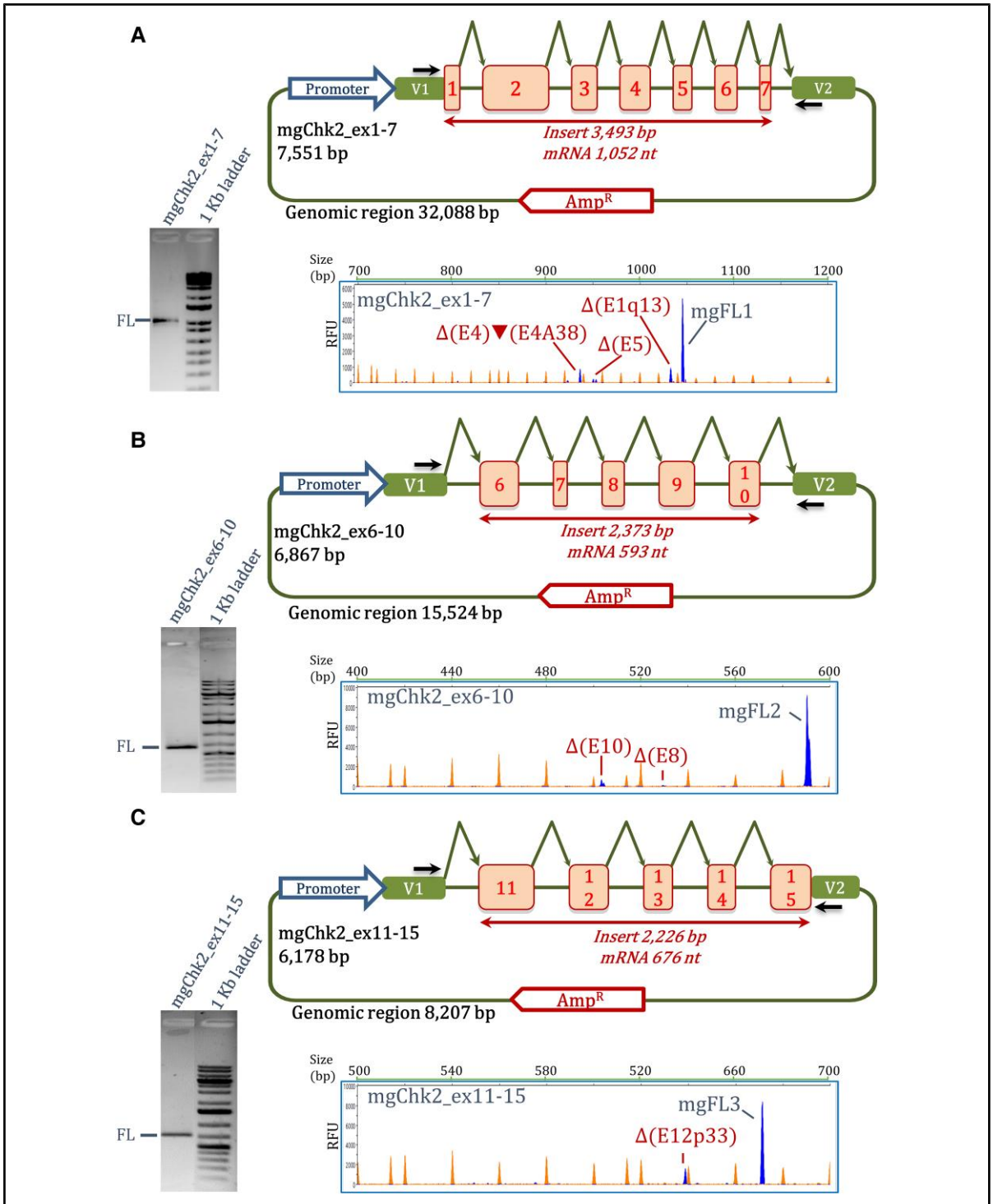

Fig. 1. Structure and functional validation of the *CHEK2* minigenes. Graphic representation of the *CHEK2* minigenes: (A), mgChk2_ex1–7; (B), mgChk2_ex6–10; (C), mgChk2_ex11–15. Exons are boxed; black arrows denote specific vector RT-PCR primers. FAM (6-Carboxyfluorescein)-RT-PCR products were analyzed by agarose (left) and fluorescent fragment electrophoreses (right; RFU, relative fluorescence units; Δ, exon skipping; ▼, intron inclusion). Color figure available online at clinchem.org.

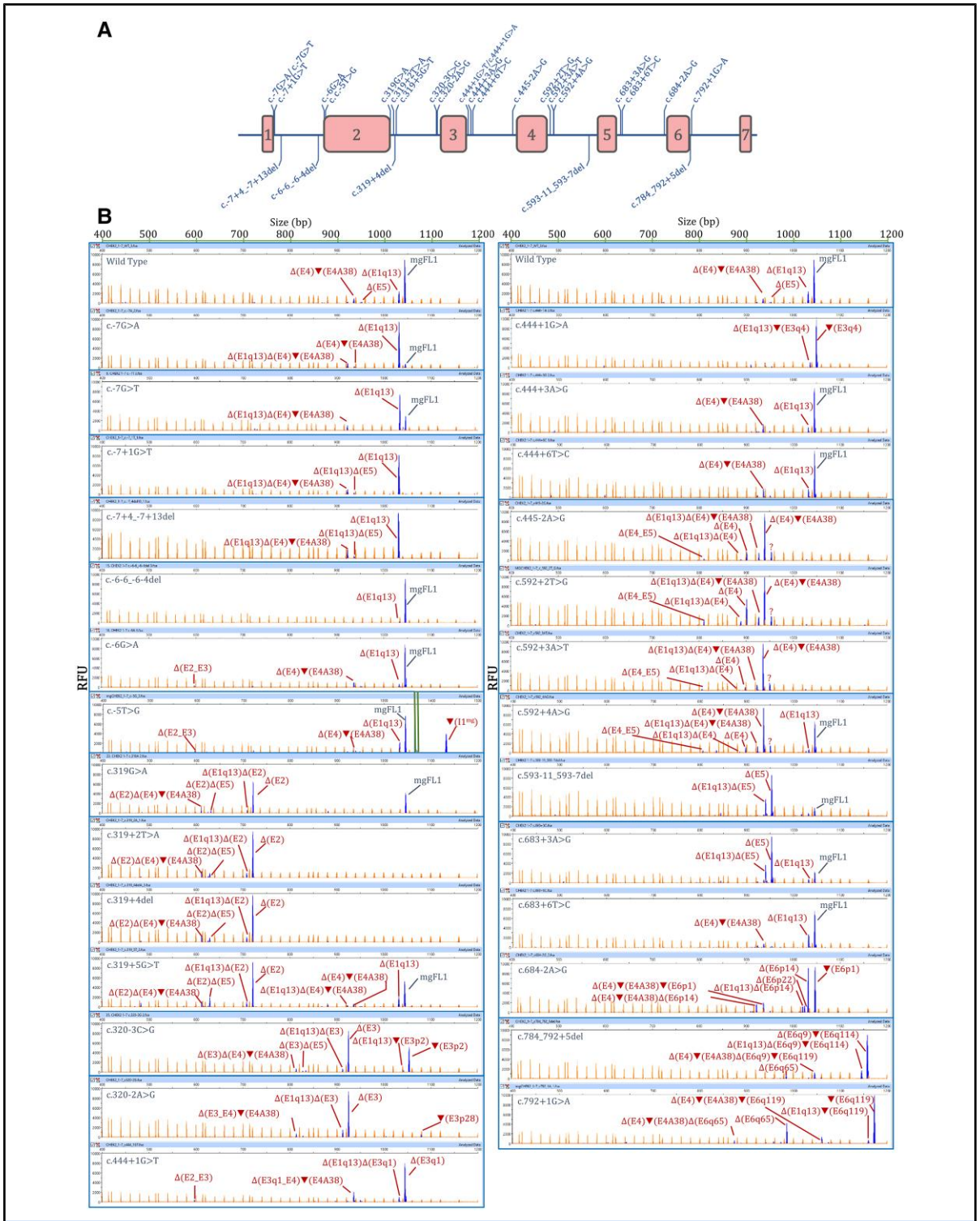

Fig. 2. Splicing functional assays of selected *CHEK2* variants in mgChk2_ex1–7 minigene. (A), Map of variants; (B), Fluorescent fragment analysis of transcripts generated by the wild type and mutant minigenes. Electropherogram of c.-5T > G is the combination of 2 images (separated by 2 vertical lines); (C), Bar graphs of the relative proportions of the different types of transcript. Color figure available online at clinchem.org.

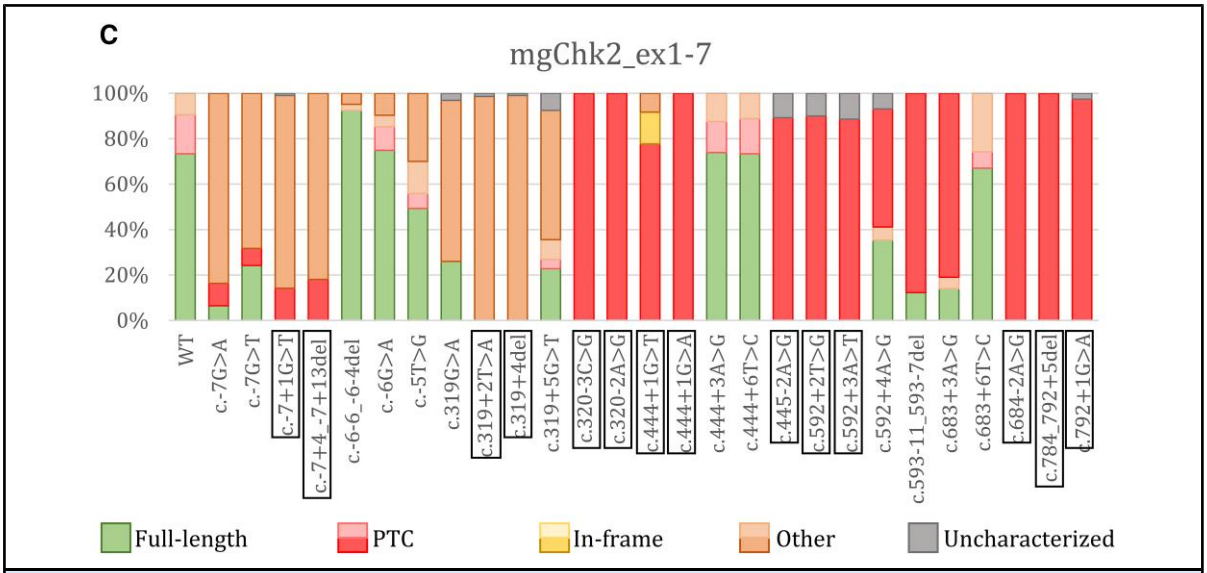

Fig. 2. Continued

Bioinformatics analysis reduced this list to 52 variants to assess in the functional assays (Supplemental Table 1). Seventeen and 35 variants were predicted to impact the 3′SS and the 5′SS, respectively. Nine of these variants (c.320-3C > G, c.444 + 1G > T, c.684-2A > G, c.793-2A > G, c.793-1G > A, c.847-14_847-2delinsGG, c.1096-6T > G, c.1260-8A > G, and c.1543-9_1546del) were predicted to concomitantly create a de novo splice site.

SPLICING ASSAYS

Fluorescent fragment electrophoresis of the 3 wt minigenes showed the expected mgFL-transcripts in MCF-7 and Hela cells. They also induced several alternative transcripts (Δ(E1q13), Δ(E4)▼(E4A38) and Δ(E5) (mgChk2_ex1-7), Δ(E8) and Δ(E10) (mgChk2_ex6-10) and Δ(E12p33) (mgChk2_ex11-15)) (Fig. 1) that had been previously reported as physiological alternative events (25, 26).

The 52 selected variants were introduced into the corresponding minigene: 27 in mgChk2_ex1-7, 14 in mgChk2_ex6-10 and 11 in mgChk2_ex11-15 (Figs. 2A-3A and C) and analyzed in MCF-7 cells. Results showed that 46 variants (88.5%) impaired splicing (at least 10% reduction of the mgFL-transcript), 34 of which resulted in severe splicing anomalies with no trace or negligible amounts (<5%) of the mgFL-transcripts (Table 1; Figs. 2B, 3B and D). Twenty of these variants affected the ±1,2 positions, while the remaining 26 variants affected other conserved positions of the splice sites, including the polypyrimidine tract, the second, antepenultimate, and last exon nucleotides, and intron positions −3, +3, +4, +5, and +6, illustrating the high spliceogenicity of any nucleotide of the splice-site consensus sequences.

Four variants of each minigene were also tested in HeLa cells where they showed similar splicing patterns (online Supplemental Fig. 4). Nevertheless, fragment analysis of variant c.320-3C > G revealed slightly different proportions of the most prevalent transcripts (Supplemental Fig. 4B) between both cell lines.

TRANSCRIPT ANALYSIS

Fluorescent fragment analysis detected, with high sensitivity, at least 89 transcripts (including the mgFL1, 2, and 3 transcripts and those carrying an exonic variant), 82 of which could be characterized by sequence and/or fragment analysis, while 7 were minor uncharacterized isoforms (Supplemental Table 4, Table 1). A highly complex pattern of transcripts was revealed, with some variants, such as c.319 + 5G > T, producing up to 11 different minigene RNA-isoforms, although, as far as we know, this result has not been confirmed in patient RNA yet. Twenty-three variants caused the use of alternative or new splice sites or upregulated the inclusion of a nonconstitutive exon of 38-nt (exon 4A; NM_001349956). Remarkably, 59 transcripts introduced premature termination codons (PTCs), including 1 mgFL-transcript containing the nonsense variant c.1459C > T [p.(Gln487*)], 55 of which are predicted to be degraded by the NMD mechanism.

Of the 23 remaining RNA-isoforms, Δ(E1q13) and ▼($I1^{mg}$) altered the 5′ untranslated region (5′UTR), 5 (with Δ(E2)) had lost the natural translation initiation codon, 7 kept the open reading frame, and 9 were full-

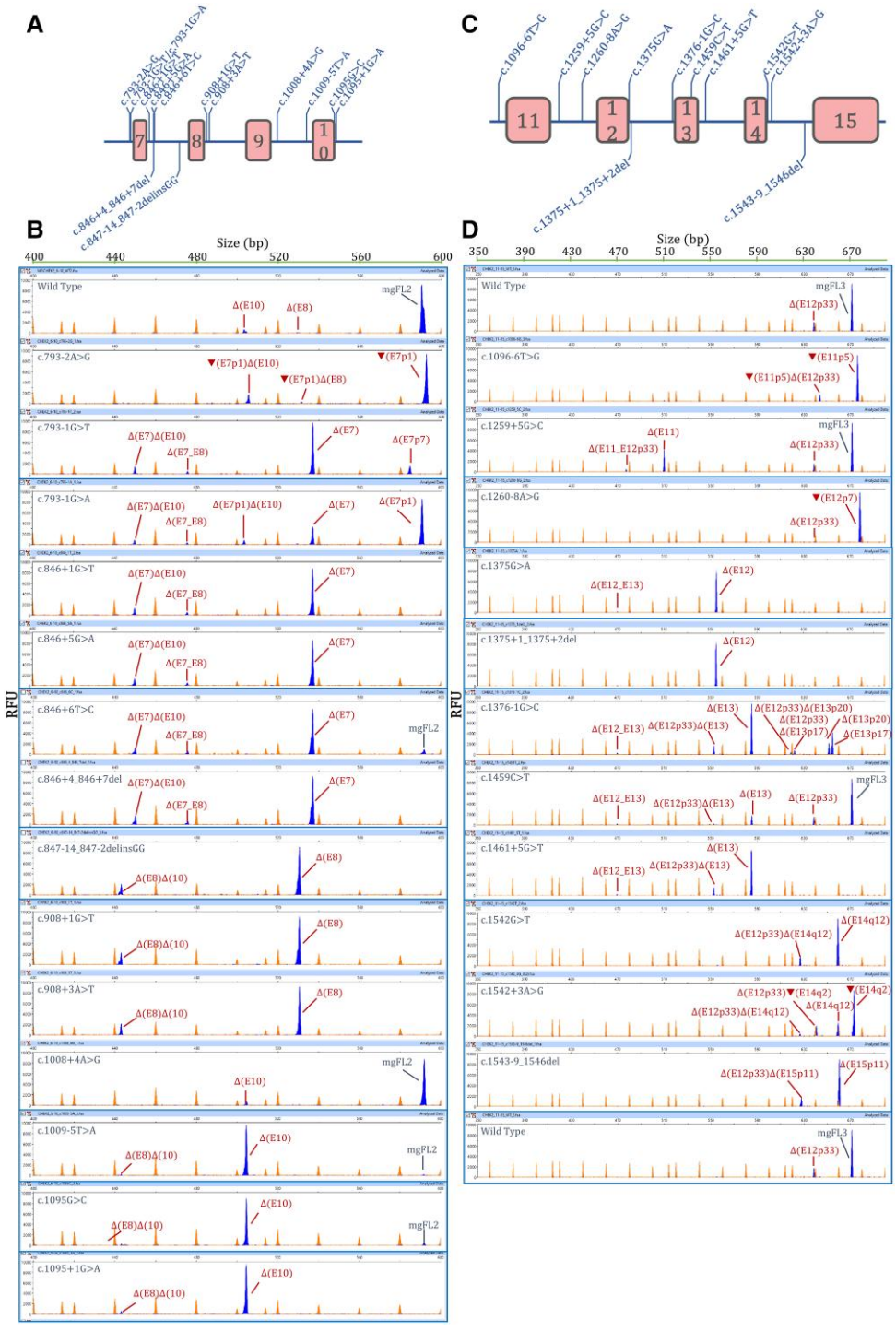

Fig. 3. Splicing functional assays of selected *CHEK2* variants in mgChk2_ex6–10 and ex11-15 minigenes. (A), Variants of mgChk2_ex6–10; (B), Fluorescent fragment analysis of wt and mutant mgChk2_ex6–10 minigenes; (C), Variants of mgChk2_ex11-15; (D), Fluorescent fragment analysis of wt and mutant mgChk2_ex11–15 minigenes; (E), Bar graphs of the relative proportions of the different types of transcripts. Color figure available online at clinchem.org.

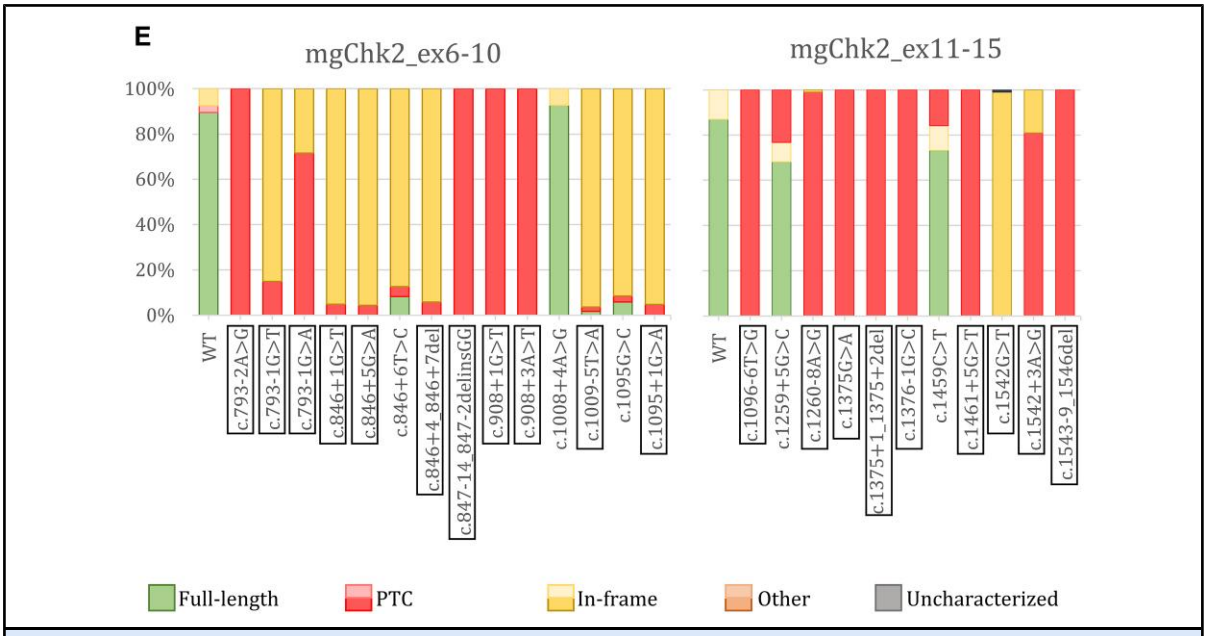

Fig. 3. Continued

length transcripts, including the wt products and those with exon variants (r.-7g > a, r.-7g > u, r.-6g > a, r.-5u > g, r.319g > a, and r.1095g > c).

ATYPICAL TG ACCEPTOR SITE

Remarkably, variant c.684-2A > G produced 2 transcripts (49.6% of the overall minigene expression) with the ▼(E6p1) event (insertion of the last nt of intron 5) that is explained by the use of a noncanonical TG acceptor site (wt, TAG/c.684-2G, TGG). TG sites are very rare accounting for about 0.02% of human 3′SS (27). To address the mechanism underlying the activation of this TG acceptor, we proceeded to map critical regions for its recognition (Fig. 4A to C). Hence, 2 overlapping 37-nt microdeletions of exon 6 (c.685_721del and c.707_743del) were engineered into the c.684-2A > G-minigene. Deletion c.685_721del (closest to the noncanonical TG) prevented its use, while c.707_743del notably reduced the use of this acceptor. So, both intervals contain sequences that promote TG recognition. Three other overlapping 14-nt microdeletions (c.685_698del, c.697_710del, and c.709_722del), which span the highest effect interval c.685_721del, were introduced and checked. Again, the closest microdeletion c.685_698del abrogated TG usage. In addition, the region c.709_722del also appears to be relevant, as its deletion reduced more than 4-fold the relative proportion of ▼(E6p1) transcripts (11.1% of the overall expression, Fig. 4B). Both microdeletions (c.685_698del, c.709_722del) remove exonic splicing enhancer-rich sequences according to Hexplorer (Fig. 4C). Consequently, it is plausible that both intervals contain essential sequences for TG acceptor identification by the splicing machinery. Notably, the c.697_710del microdeletion induced the use of other acceptors not detected in the c.684-2G variant, specifically a noncanonical GG (0.014% of human 3′SS) (27) and AG acceptors, 12 and 34 nucleotides downstream of the canonical 3′SS, respectively. Conversely, deletions c.686_721del and c.686_698del in the wt minigene did not affect the use of the canonical AG acceptor of exon 6 (Fig. 4B, below).

ACMG/AMP-BASED INTERPRETATION OF VARIANTS

After splicing analysis, we classified 2 of the 52 variants as pathogenic, 25 as likely pathogenic, and 5 as likely benign (online Supplemental Table 5). The other 20 variants remained as VUSs (Table 2). Most *CHEK2* variants investigated (44 out of 52) had been reported previously at ClinVar. Focusing our analysis on the subgroup of 22 variants with multiple submitters and no conflicts (14 LP/P and 8 VUS), our classification scheme reclassifies up to 8 variants (36%). Three VUSs were reclassified to P/LP (c.846 + 5G > A, c.1375G > A, and c.1461 + 5G > T), two VUSs to LB (c.444 + 6T > C, and c.1008 + 4A > G), and 3 P/LP variants to VUS (c.793-1G > A, c.793-1G > T, and c.1095 + 1G > A). Online Supplemental Table 6 provides details on this comparative analysis.

Table 1. Splicing outcomes of *CHEK2* splice-site variants.

Variant (HGVS)[a]	Bioinformatics summary[b]	Full-length	Transcripts[c,d]
mgChk2_ex1-7 WT (6 assays)		73.3% ± 0.8%	PTC: Δ**(E4)** ▼ **(E4A38)**: 13.2% ± 2.3% / Δ**(E5)**: 4.0% ± 0.3% OTHER: Δ**(E1q13)**: 9.5% ± 2.6%
c.-7G > A (Ex1)	[↓]5′SS (10.5 → 7.4) [=]5′SS (8.1) 13-nt upstream	6.5% ± 0.4%	PTC: Δ**(E1q13)**Δ**(E4)** ▼ **(E4A38)**: 7.6% ± 0.5% / Δ**(E1q13)**Δ**(E5)**: 2.2% ± 0.1% OTHER: Δ**(E1q13)**: 75.7% ± 2.0% / ▼ **(I1^{mg})**: 8.0% ± 2.7%
c.-7G > T (Ex1)	[↓]5′SS (10.5 → 7.4) [=]5′SS (8.1) 13-nt upstream	24.2% ± 0.3%	PTC: Δ**(E1q13)**Δ**(E4)** ▼ **(E4A38)**: 7.5% ± 0.4% OTHER: Δ**(E1q13)**: 60.2% ± 0.5% / ▼ **(I1^{mg})**: 8.1% ± 0.5%
c.-7 + 1G > T (ivs1)	[-]5′SS (10.5 → 2.0) [=]5′SS (8.1) 13-nt upstream	—	PTC: Δ**(E1q13)**Δ**(E4)** ▼ **(E4A38)**: 11.2% ± 1.6% / Δ**(E1q13)**Δ**(E5)**: 3.0% ± 0.4% OTHER: Δ**(E1q13)**: 81.2% ± 2.0% / ▼ **(I1^{mg})**: 3.6% ± 1.3% / **721-nt**: 1.0% ± 0.3%
c.-7 + 4_-7 + 13del (ivs1)	[-]5′SS (10.5 → 3.1) [=]5′SS (8.1) 13-nt upstream	—	PTC: Δ**(E1q13)**Δ**(E4)** ▼ **(E4A38)**: 14.2% ± 3.2% / Δ**(E1q13)**Δ**(E5)**: 3.8% ± 0.9% OTHER: Δ**(E1q13)**: 80.9% ± 5.0% / ▼ **(I1^{mg})**: 1.1% ± 0.9%
c.-6-6_-6-4del (ivs1)	[-]3′SS (1.7 → −3.7)	92.5% ± 0.3%	OTHER: Δ**(E1q13)**: 2.6% ± 0.2% / ▼ **(I1^{mg})**: 4.9% ± 0.5%
c.-6G > A (Ex2)	[-]3′SS (1.7 → 0.3)	74.9% ± 1.6%	PTC: Δ**(E4)** ▼ **(E4A38)**: 10.4% ± 1.4% OTHER: ▼ **(I1^{mg})**: 7.1% ± 0.6% / Δ**(E1q13)**: 5.0% ± 0.3% / Δ**(E2_E3)**: 2.6% ± 0.2%
c.-5T > G (Ex2)	[-]3′SS (1.7 → −3.2)	49.3% ± 0.6%	PTC: Δ**(E4)** ▼ **(E4A38)**: 6.6% ± 0.3% OTHER: ▼ **(I1^{mg})**: 24.4% ± 1.7% / Δ**(E1q13)**: 14.1% ± 0.2% / Δ**(E2_E3)**: 5.6% ± 1.0%
c.319G > A (Ex2) p.(Glu107Lys)	[-]3′SS (8.9 → 3.8)	26.0% ± 8.5%	OTHER: Δ**(E2)**: 53.0% ± 5.9% / Δ**(E2)**Δ**(E4)** ▼ **(E4A38)**: 9.2% ± 1.2% / Δ**(E1q13)**Δ**(E2)**: 4.7% ± 0.6% / Δ**(E2)**Δ**(E5)**: 4.0% ± 0.9% / **881-nt**: 3.1% ± 0.0%
c.319 + 2T > A (ivs2)	[-]5′SS (8.9 → 0.7)	—	OTHER: Δ**(E2)**: 73.9% ± 1.3% / Δ**(E2)**Δ**(E4)** ▼ **(E4A38)**: 9.3% ± 0.9% / Δ**(E2)**Δ**(E5)**: 9.3% ± 0.2% / Δ**(E1q13)**Δ**(E2)**: 6.1% ± 0.9% / **482-nt**: 1.4% ± 0.2%
c.319 + 4del (ivs2)	[-]5′SS (8.9 → −0.6)	—	OTHER: Δ**(E2)**: 74.2% ± 3.7% / Δ**(E2)**Δ**(E4)** ▼ **(E4A38)**: 9.8% ± 1.1% / Δ**(E2)**Δ**(E5)**: 7.6% ± 1.7% / Δ**(E1q13)**Δ**(E2)**: 7.4% ± 0.1% / **482-nt**: 1.0% ± 0.8%
c.319 + 5G > T (ivs2)	[↓]5′SS (8.9 → 4.2)	22.8% ± 0.5%	PTC: Δ**(E4)** ▼ **(E4A38)**: 2.7% ± 0.1% / Δ**(E1q13)**Δ**(E4)** ▼ **(E4A38)**: 1.4% ± 0.0% OTHER: Δ**(E2)**: 37.4% ± 2.0% / Δ**(E1q13)**: 8.8% ±

Continued

Table 1. (continued)

Variant (HGVS)[a]	Bioinformatics summary[b]	Full-length	Transcripts[c,d]
			0.4% / Δ**(E2)**Δ**(E5)**: 9.8% ± 0.9% / Δ**(E2)**Δ**(E4)** ▼ **(E4A38)**: 5.0% ± 0.6% / Δ**(E1q13)**Δ**(E2)**: 4.6% ± 0.4% / **482-nt**: 3.4% ± 0.4% / **1446-nt**: 2.2% ± 0.1% / **881-nt**: 1.9% ± 0.0%
c.320-3C > G (ivs2)	[-]3′SS (7.7 → 1.7) [+]3′SS (3.9) 2-nt upstream	—	PTC: Δ**(E3)**: 55.2% ± 0.6% / ▼ **(E3p2)**: 32.0% ± 0.6% / Δ**(E3_E4)** ▼ **(E4A38)**: 4.4% ± 0.1% / Δ**(E1q13)**Δ**(E3)**: 4.1% ± 0.0% / Δ**(E1q13)** ▼ **(E3p2)**: 2.3% ± 0.2% / Δ**(E3)**Δ**(E5)**: 2.0% ± 0.1%
c.320-2A > G (ivs2)	[-]3′SS (7.7 → −0.3)	—	PTC: Δ**(E3)**: 70.0% ± 6.5% / Δ**(E1q13)**Δ**(E3)**: 20.6% ± 7.4% / Δ**(E3_E4)** ▼ **(E4A38)**: 4.9% ± 0.6% / Δ**(E3p28)**: 4.5% ± 0.4%
c.444 + 1G > T (ivs3)	[-]5′SS (8.1 → −0.4) [+]5′SS (6.3)1-nt upstream	—	PTC: Δ**(E3q1)**: 66.9% ± 1.9% / Δ**(E1q13)**Δ**(E3q1)**: 10.8% ± 2.6% IN-FRAME: Δ**(E3q1_E4)** ▼ **(E4A38)**: 14.0% ± 4.3% OTHER: Δ**(E2_E3)**: 8.3% ± 3.5%
c.444 + 1G > A (ivs3)	[-]5′SS (8.1 → −0.1) [=]5′SS (7.6) 4-nt downstream	—	PTC: ▼ **(E3q4)**: 87.2% ± 3.3% / Δ**(E1q13)** ▼ **(E3q4)**: 12.8% ± 3.3%
c.444 + 3A > G (ivs3)	[↓]5′SS (8.1 → 5.4) [↓]5′SS (5.7) 4-nt downstream	73.9% ± 3.7%	PTC: Δ**(E4)** ▼ **(E4A38)**: 13.7% ± 0.6% OTHER: Δ**(E1q13)**: 12.4% ± 4.3%
c.444 + 6T > C (ivs3)	[↓]5′SS (8.1 → 7.3)	73.3% ± 1.6%	PTC: Δ**(E4)** ▼ **(E4A38)**: 15.5% ± 1.7% OTHER: Δ**(E1q13)**: 11.2% ± 1.7%
c.445-2A > G (ivs3)	[-]3′SS (11.3 → 3.3)	—	PTC: Δ**(E4)** ▼ **(E4A38)**: 55.3% ± 6.8% / Δ**(E1q13)**Δ**(E4)** ▼ **(E4A38)**: 18.0% ± 7.5% / Δ**(E4)**: 9.8% ± 1.2% / Δ**(E1q13)**Δ**(E4)**: 3.0% ± 1.2% / Δ**(E4_E5)**: 3.1% ± 0.6% OTHER: **952-nt**: 10.8% ± 1.5%
c.592 + 2T > G (ivs4)	[-]5′SS (8.5 → 0.8)	—	PTC: Δ**(E4)** ▼ **(E4A38)**: 46.5% ± 1.2% / Δ**(E4)**: 22.7% ± 2.6% / Δ**(E1q13)**Δ**(E4)** ▼ **(E4A38)**: 9.3% ± 0.6% / Δ**(E4_E5)**: 6.7% ± 1.8% / Δ**(E1q13)**Δ**(E4)**: 4.8% ± 0.7% OTHER: **952-nt**: 10.0% ± 0.5%
c.592 + 3A > T (ivs4)	[-]5′SS (8.5 → 1.8)	—	PTC: Δ**(E4)** ▼ **(E4A38)**: 53.6% ± 2.5% / Δ**(E4)**: 17.7% ± 1.0% / Δ**(E1q13)**Δ**(E4)** ▼ **(E4A38)**: 7.8% ± 1.1% / Δ**(E4_E5)**: 7.6% ± 1.0% / Δ**(E1q13)**Δ**(E4)**: 1.9% ± 1.7% OTHER: **952-nt**: 11.4% ± 0.2%

Continued

Table 1. (continued)

Variant (HGVS)[a]	Bioinformatics summary[b]	Full-length	Transcripts[c,d]
c.592 + 4A > G (ivs4)	[↓]5′SS (8.5 → 5.7)	35.3% ± 0.2%	PTC: Δ**(E4)** ▼ **(E4A38)**: 30.1% ± 0.3% / Δ**(E4)**: 10.2% ± 0.2% / Δ**(E4_E5)**: 5.6% ± 0.1% / Δ**(E1q13)**Δ**(E4)** ▼ **(E4A38)**: 4.8% ± 0.1% / Δ**(E1q13)**Δ**(E4)**: 1.3% ± 0.0% OTHER: **952-nt**: 6.9% ± 0.1% / Δ**(E1q13)**: 5.8% ± 0.1%
c.593-11_593-7del (ivs4)	[↓]3′SS (9.2 → 7.4)	12.3% ± 0.9%	PTC: Δ**(E5)**: 62.0% ± 1.6% / Δ**(E1q13)**Δ**(E5)**: 25.7% ± 1.2%
c.683 + 3A > G (ivs5)	[↓]5′SS (8.9 → 6.4)	13.9% ± 0.8%	PTC: Δ**(E5)**: 57.3% ± 3.2% / Δ**(E1q13)**Δ**(E5)**: 23.7% ± 2.2% OTHER: Δ**(E1q13)**: 5.1% ± 0.6%
c.683 + 6T > C (ivs5)	[↓]5′SS (8.9 → 8.1)	67.0% ± 0.5%	PTC: Δ**(E4)** ▼ **(E4A38)**: 7.3% ± 0.5% OTHER: Δ**(E1q13)**: 25.7% ± 0.3%
c.684-2A > G (ivs5)	[-]3′SS (10.2 → 2.3) [+]3′SS-TG (3.4) 1-nt upstream	—	PTC: ▼ **(E6p1)**: 40.4% ± 0.8% / Δ**(E6p14)**: 33.9% ± 0.3% / Δ**(E4)** ▼ **(E4A38)** ▼ **(E6p1)**: 9.2% ± 0.2% / Δ**(E4)** ▼ **(E4A38)**Δ**(E6p14)**: 6.5% ± 0.1% / Δ**(E6p22)**: 5.2% ± 0.3% / Δ**(E1q13)**Δ**(E6p14)**: 4.8% ± 0.7%
c.784_792 + 5del (Ex6/ivs6)	[-]5′SS (9.1 → −40.6)	—	PTC: Δ**(E6q9)** ▼ **(E6q114)**: 76.1% ± 3.7% / Δ**(E1q13)**Δ**(E6q9)** ▼ **(E6q114)**: 10.5% ± 1.8% / Δ**(E4)** ▼ **(E4A38)**Δ**(E6q9)** ▼ **(E6q114)**: 9.3% ± 0.4% / Δ**(E6q65)**: 4.1% ± 1.4%
c.792 + 1G > A (ivs6)	[-]5′SS (9.1 → 0.9)	—	PTC: ▼ **(E6q119)**: 58.7% ± 1.2% / Δ**(E6q65)**: 24.4% ± 1.0% / Δ**(E4)** ▼ **(E4A38)** ▼ **(E6q119)**: 7.7% ± 0.5% / Δ**(E1q13)** ▼ **(E6q119)**: 3.4% ± 0.9% / Δ**(E4)** ▼ **(E4A38)**Δ**(E6q65)**: 3.2% ± 0.1% OTHER: **957-nt**: 2.6% ± 0.6%
mgChk2_ex6-10 WT (6 assays)		89.5% ± 1.4%	PTC: Δ**(E8)**: 3.0% ± 0.5% IN-FRAME: Δ**(E10)**: 7.5% ± 1.5%
c.793-2A > G (ivs6)	[-]3′SS (6.2 → −1.8) [+]3′SS (5.6) 1-nt upstream	—	PTC: ▼ **(E7p1)**: 83.0% ± 0.4% / ▼ **(E7p1)**Δ**(E10)**: 14.2% ± 0.2% / ▼ **(E7p1)**Δ**(E8)**: 2.8% ± 0.1%
c.793-1G > T (ivs6)	[-]3′SS (6.2 → −2.4)	—	PTC: Δ**(E7p7)**: 11.3% ± 0.4% / Δ**(E7_E8)**: 3.7% ± 0.1% IN-FRAME: Δ**(E7)**: 74.6% ± 0.5% / Δ**(E7)**Δ**(E10)**: 10.4% ± 0.8%
c.793-1G > A (ivs6)	[-]3′SS (6.2 → −2.6) [+]3′SS (4) 1-nt downstream	—	PTC: Δ**(E7p1)**: 63.7% ± 1.3% / Δ**(E7p1)**Δ**(E10)**: 5.5% ± 0.4% / Δ**(E7_E8)**: 2.4% ± 0.1% IN-FRAME: Δ**(E7)**: 23.3% ± 0.3% / Δ**(E7)**Δ**(E10)**: 5.1% ± 0.5%

Continued

Table 1. (continued)

Variant (HGVS)[a]	Bioinformatics summary[b]	Full-length	Transcripts[c,d]
c.846 + 1G > T (ivs7)	[-]5′SS (8.3 → −0.2)	—	PTC: Δ**(E7_E8)**: 5.0% ± 0.2% IN-FRAME: Δ**(E7)**: 84.2% ± 0.2% / Δ**(E7)**Δ**(E10)**: 10.8% ± 0.2%
c.846 + 5G > A (ivs7)	[-]5′SS (8.3 → 2.1)	—	PTC: Δ**(E7_E8)**: 4.4% ± 0.5% IN-FRAME: Δ**(E7)**: 84.3% ± 1.8% / Δ**(E7)**Δ**(E10)**: 11.3% ± 1.6%
c.846 + 6T > C (ivs7)	[↓]5′SS (8.3 → 5.6)	8.2% ± 0.5%	PTC: Δ**(E7_E8)**: 4.4% ± 0.5% IN-FRAME: Δ**(E7)**: 77.5% ± 1.8% / Δ**(E7)**Δ**(E10)**: 9.9% ± 1.1%
c.846 + 4_846 + 7del (ivs7)	[-]5′SS (8.3 → −4.4)	—	PTC: Δ**(E7_E8)**: 5.8% ± 0.0% IN-FRAME: Δ**(E7)**: 80.3% ± 0.2% / Δ**(E7)**Δ**(E10)**: 13.9% ± 0.2%
c.847-14_847-2delinsGG (ivs7)	[-]3′SS (9.4 → −8.3)	—	PTC: Δ**(E8)**: 81.4% ± 2.8% / Δ**(E8)**Δ**(E10)**: 18.6% ± 2.8%
c.908 + 1G > T (ivs8)	[-]5′SS (8.5 → 0.0)	—	PTC: Δ**(E8)**: 81.4% ± 0.5% / Δ**(E8)**Δ**(E10)**: 18.6% ± 0.5%
c.908 + 3A > T (ivs8)	[-]5′SS (8.5 → −3.4)	—	PTC: Δ**(E8)**: 83.3% ± 1.3% / Δ**(E8)**Δ**(E10)**: 16.7% ± 1.3%
c.1008 + 4A > G (ivs9)	[↓]5′SS (8.6 → 6.7)	92.8% ± 0.3%	IN-FRAME: Δ**(E10)**: 7.2% ± 0.3%
c.1009-5T > A (ivs9)	[-]3′SS (6.9 → 3.8)	1.8% ± 0.1%	PTC: Δ**(E8)**Δ**(E10)**: 1.9% ± 0.2% IN-FRAME: Δ**(E10)**: 96.3% ± 0.3%
c.1095G > C (Ex10) p.(Lys365Asn)	[-]5′SS (10.6 → 7.7)	5.8% ± 0.4%	PTC: Δ**(E8)**Δ**(E10)**: 2.7% ± 0.2% IN-FRAME: Δ**(E10)**: 91.5% ± 0.3%
c.1095 + 1G > A (ivs10)	[-]5′SS (10.6 → 2.4)	—	PTC: Δ**(E8)**Δ**(E10)**: 4.8% ± 0.5% IN-FRAME: Δ**(E10)**: 95.2% ± 0.5%
mgChk2_ex11-15 WT (6 assays)		85.9% ± 1.6%	IN-FRAME: Δ**(E12p33)**: 14.1% ± 1.6%
c.1096-6T > G (ivs10)	[-]3′SS (9.2 → 2.0) [+]3′SS (3.2) 5-nt upstream	—	PTC: ▼ **(E11p5)**: 89.8% ± 1.1% / ▼ **(E11p5)** Δ**(E12p33)**: 10.2% ± 1.1%
c.1259 + 5G > C (ivs11)	[↓]5′SS (9.5 → 4.6)	68.3% ± 2.3%	PTC: Δ**(E11)**: 21.1% ± 1.4% / Δ**(E11_E12p33)**: 2.1% ± 0.3% IN-FRAME: Δ**(E12p33)**: 8.5% ± 0.6%
c.1260-8A > G (ivs11)	[-]3′SS (2.0 → −3.8) [+]3′SS (9.7) 7-nt upstream [=]3′SS (3.8) 33-nt downstream	—	PTC: ▼ **(E12p7)**: 99.0% ± 0.1% IN-FRAME: Δ**(E12p33)**: 1.0% ± 0.1%
c.1375G > A (Ex12) p.(Ala459Thr)	[-]5′SS (9.1 → 3.9)	—	PTC: Δ**(E12)**: 98.9% ± 0.3% / Δ**(E12_E13)**: 1.1% ± 0.3%
c.1375 + 1_1375 + 2del (ivs12)	[-]5′SS (9.1 → −1.8)	—	PTC: Δ**(E12)**: 100.0% ± 0.0%

Continued

Table 1. (continued)

Variant (HGVS)[a]	Bioinformatics summary[b]	Full-length	Transcripts[c,d]
c.1376-1G > C (ivs12)	[-]3′SS (6.0 → −2.1)	—	PTC: Δ**(E13)**: 51.4% ± 0.6% / Δ**(E13p17)**: 22.2% ± 0.4% / Δ**(E13p20)**: 11.2% ± 0.2% / Δ**(E12p33)Δ(E13)**: 7.9% ± 0.2% / Δ**(E12p33)Δ(E13p17)**: 3.9% ± 0.1% / Δ**(E12p33)Δ(E13p20)**: 1.9% ± 0.0% / Δ**(E12_E13)**: 1.5% ± 0.0%
c.1459C > T (Ex13) p.(Gln487Ter)	[↓]5′SS (8.6 → 5.6)	73.4% ± 0.5%	PTC: Δ**(E13)**: 12.5% ± 0.3% / Δ**(E12p33)Δ(E13)**: 1.8% ± 0.1% / Δ**(E12_E13)**: 1.5% ± 0.0% IN-FRAME: Δ**(E12p33)**: 10.8% ± 0.1%
c.1461 + 5G > T (ivs13)	[-]5′SS (8.6 → 2.2)	—	PTC: Δ**(E13)**: 85.8% ± 0.1% / Δ**(E12p33)Δ(E13)**: 12.8% ± 0.1% / Δ**(E12_E13)**: 1.4% ± 0.0%
c.1542G > T (Ex14) p.(Gln514His)	[-]5′SS (6.5 → −4.3)	—	IN-FRAME: Δ**(E14q12)**: 83.9% ± 0.3% / Δ**(E12p33)Δ(E14q12)**:15.1% ± 0.1% OTHER: **632-nt**: 1.0% ± 0.2%
c.1542 + 3A > G (ivs14)	[-]5′SS (6.5 → 1.2)	—	PTC: ▼ **(E14q2)**: 70.4% ± 0.5% / Δ**(E12p33)▼(E14q2)**: 10.7% ± 0.3% IN-FRAME: Δ**(E14q12)**: 16.9% ± 0.4% / Δ**(E12p33)Δ(E14q12)**:2.0% ± 0.6%
c.1543-9_1546del (ivs14/Ex15)	[-]3′SS (8.5 → −8.8) [+]3′SS (6.4) 11-nt downstream	—	PTC: Δ**(E15p11)**: 84.9% ± 1.8% / Δ**(E12p33)Δ(E15p11)**: 15.1% ± 1.8%

[a]HGVS, Human Genome Variation Society. Variants without any trace (or ≤5%) of the minigene full-length transcript are underlined
[b][-] Site disruption; [+] new site; [↓] the strength of the SS is reduced; [=] the strength of the SS is not altered.
[c]Transcripts are shown in bold and are annotated as follows: Δ (skipping of exonic sequences), ▼ (inclusion of intronic sequences), E (exon) and when necessary, p (acceptor shift) and q (donor shift) + nt inserted or deleted.
[d]OTHER: transcripts that eliminate the 5′UTR (Untranslated Region) region and the start codon, as well as uncharacterized transcripts.

Discussion

Splicing disruption is one of the most common deleterious alterations in BC genes (14, 22, 28), and should be investigated as a primary ethiopathogenic mechanism of variants. Actually, a large proportion of spliceogenic variants (81%; 139/171) were revealed in previous studies of *ATM*, *PALB2*, *RAD51C*, and *RAD51D* variants identified in BRIDGES (16–19). Here, we have tested 52 *CHEK2* candidate variants in 3 minigenes that cover all 15 exons of the NM_007194.4 transcript. According to Simple-ClinVar (https://simple-clinvar.broadinstitute.org/, accessed November 30, 2022), variants with impact on splicing represent approximately 15% all *CHEK2* pathogenic variants (77 out of 512 variants). However, this figure may represent an underestimation, in particular for changes other than ±1,2.

Splicing reporter minigenes represent a simple and high-capacity tool for functional analysis of putative spliceogenic variants as we and others have shown (13, 22, 29). Major advantages of this strategy are: (*a*) study of a single allele that allows the detection of even small quantities of any variant-induced transcript by fluorescent fragment electrophoresis, including the full-length one, which may be critical for its clinical interpretation; (*b*) use of any cell type relevant for the disease, whereas patient RT-PCR typically uses blood RNA; (*c*) in our hands, high reproducibility of splicing patterns, as we have shown in previous reports, which, in our opinion, may be due to maintenance of the genomic context in the minigene construct (each target exon flanked by its natural counterparts). The lack of genomic context may also represent a limitation for minigene analysis of 5′SS-exon 10 and 3′SS-exon 11 variants (c.1095G > C, c.1095 + 1G > A, and c.1096-6T > G). They were tested in 2 different minigenes without their natural neighboring exons, so splicing outcomes might slightly differ from patient RNA. However, SpliceAI

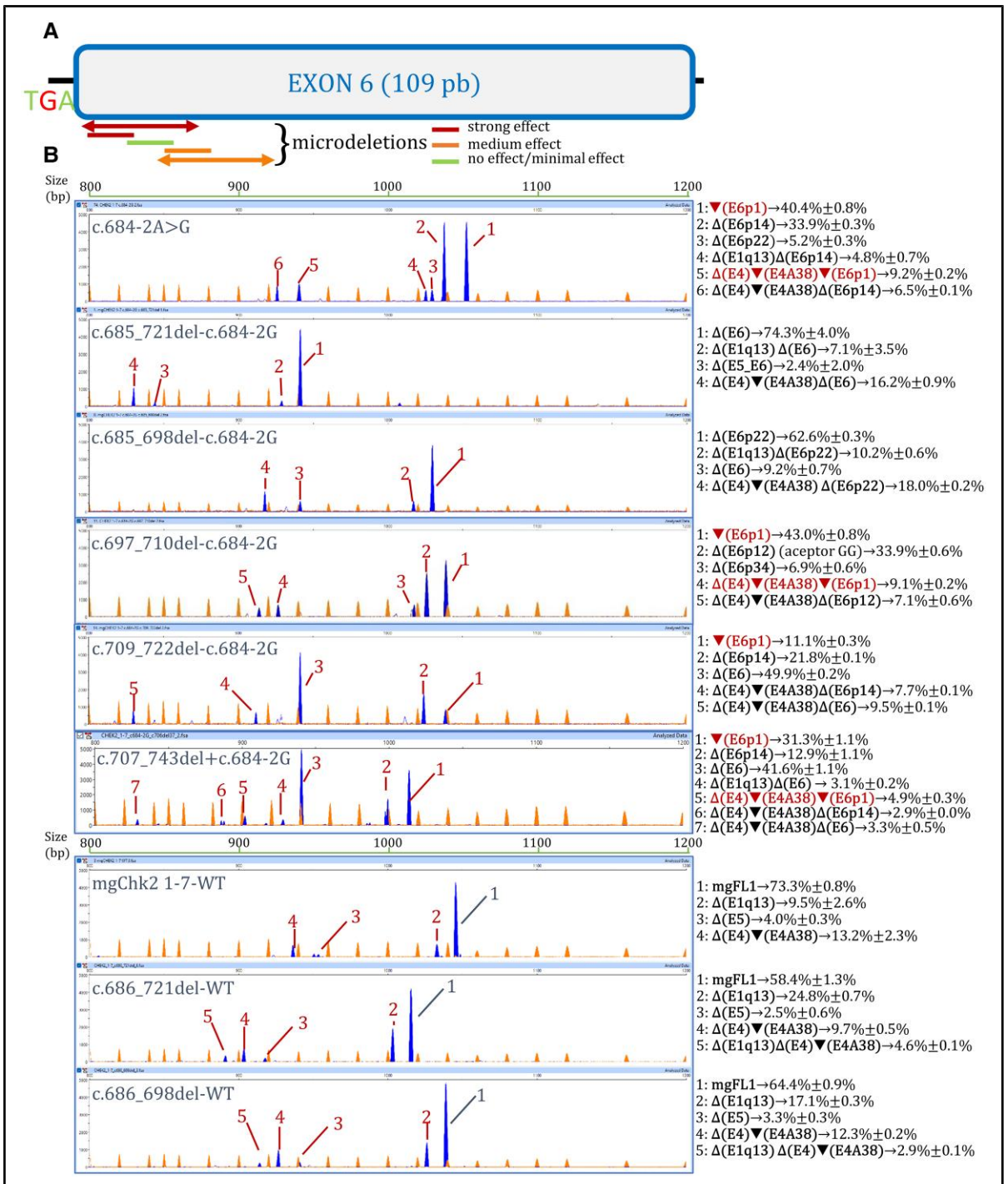

Fig. 4. Impact of exon 6 microdeletions on the TG-3′SS recognition. (A), Exon 6 microdeletions; (B), Microdeletion analysis of c.684-2A > G (above) and wt mgChk2_ex1-7 minigenes (below); (C), Hexplorer and SpliceAid analyses of exon 6. The percentage of ▼ (E6p1) transcripts of each microdeletion is indicated; (D), Comparison of the c.684-2A > G TG acceptor and the consensus sequence of 130 human TG acceptors (27). Color figure available online at clinchem.org.

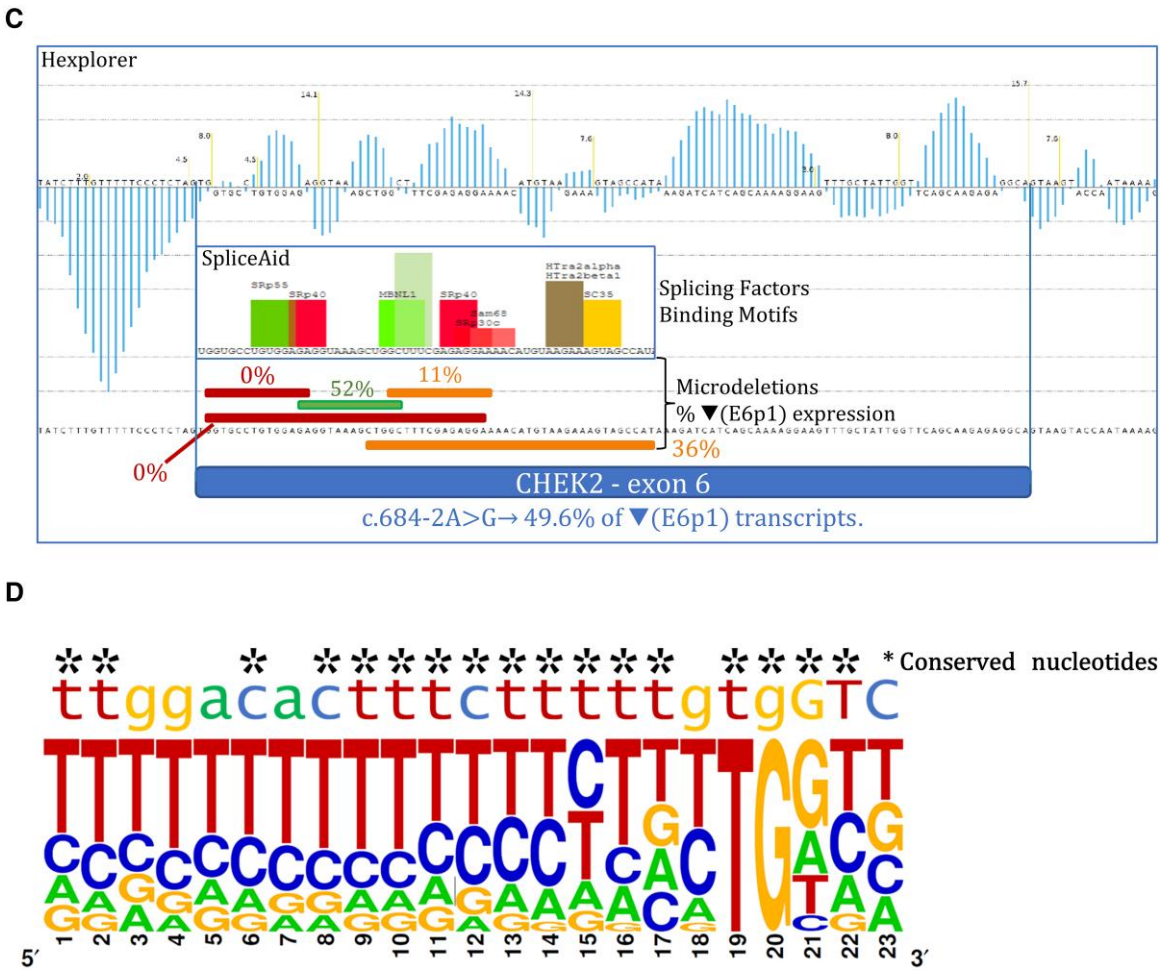

Fig. 4. Continued

predictions of these variants agree with splicing outcomes (online Supplemental Table 7), supporting the reproducibility of minigene results. Likewise, 6 *CHEK2* variants replicated the splicing profiles previously detected in patient RNA assays: c.444 + 1G > T (Δ(E3q1)) (30), c.444 + 1G > A (▼(E3q4)) (31), c.793-1G > A (Δ(E7p1)) (32), c.846 + 1G > T (Δ(E7)) (33), c.846 + 4_846 + 7del (Δ(E7)) (34), and c.1260-8A > G (▼(E12p7)) (34). Nevertheless, patient RNA of c.592 + 3A > T showed different results that might be due to technical causes (35, 36).

A high proportion of all tested variants (88.5%) disrupted splicing, corroborating the accuracy of our selection method (Supplemental Table 1; Table 1). In fact, 4 nonspliceogenic variants (c.444 + 3A > G, c.444 + 6T > C, c.683 + 6T > C, and c.1008 + 4A > G) poorly changed the MES score of canonical splice sites (Supplemental Table 1) and in 2 of them (c.444 + 3A > G and c.1008 + 4A > G), each substitution added the second most prevalent nucleotide at these positions. Twenty-six spliceogenic variants affected nucleotides other than ±1,2 positions, underlining the potential deleterious capacity of any change at the conserved nucleotides of the splice sites (Table 1; Figs. 2-3).

Fluorescent fragment electrophoresis of minigene RT-PCR reactions displayed high resolution and sensitivity that unveiled up to 89 different transcripts, 82 of which could be characterized (Supplemental Table 4). Interestingly, in breast tumor samples, *CHEK2* undergoes extensive alternative splicing with about 90 different mRNA isoforms (37). Notably, some variants displayed extraordinarily complex profiles with as many as 11 different transcripts (c.319 + 5G >

Table 2. Point-based ACMG/AMP classification of 52 *CHEK2* variants incorporating mg*CHEK2* read-outs as PVS1_O (or BP7_O) evidence of variable strength.

c.HGVS[a]	p.HGVS[a]	ACMG/AMP[b]		PVS1_O/BP7_O[c]	PS4[d]	PM2[e]	PM5[f]	BS1[g]	ClinVar[h]
c.-7G > A		VUS	+1			+1			not reported
c.-7G > T		VUS	+1			+1			not reported
c.-7 + 1G > T		VUS	+1			+1			not reported
c.-7 + 4_-7 + 13del		VUS	+1			+1			VUS (1)
c.-6-6_-6-4del		LB	−3	−4		+1			VUS (1)
c.-6G > A		LB	−4					−4	VUS (3); LB(1); B(1)
c.-5T > G		VUS	0						not reported
c.319G > A	p.(Glu107Lys)	VUS	0						VUS (2)
c.319 + 2T > A		P	+12	+8	+4				P(3); LP(8)
c.319 + 4delA		LP	+9	+8		+1			not reported
c.319 + 5G > T		VUS	+1			+1			VUS (2)
c.320-3C > G		LP	+9	+8		+1			VUS (1)
c.320-2A > G		LP	+9	+8		+1			LP (1)
c.444 + 1G > T		LP	+9	+8		+1			P(3); LP(2)
c.444 + 1G > A		P	+12	+8	+4				P(27); LP(2); VUS(1)
c.444 + 3A > G		LB	−4	−4					VUS(4); LB(2)
c.444 + 6T > C		LB	−3	−4		+1			VUS (3)
c.445-2A > G		LP	+9	+8		+1			LP (2)
c.592 + 2T > G		LP	+9	+8		+1			not reported
c.592 + 3A > T		VUS	+8	+8					LP(8); VUS(5)
c.592 + 4A > G		VUS	+1			+1			VUS (8)
c.593-11_593-7del		VUS	0						VUS(2); LB(5)
c.683 + 3A > G		VUS	+1			+1			not reported
c.683 + 6T > C		VUS	+1			+1			VUS (1)
c.684-2A > G		LP	+9	+8		+1			P(1);LP(3)
c.784_792 + 5del		LP	+9	+8		+1			not reported
c.792 + 1G > A		LP	+9	+8		+1			LP (3)
c.793-2A > G		LP	+9	+8		+1			LP (2)
c.793-1G > A		VUS	+8	+8					P(4);LP(4)
c.793-1G > T		VUS	+8	+8					LP (2)
846 + 1G > T		LP	+9	+8		+1			P(1);LP(1)
c.846 + 5G > A		LP	+9	+8		+1			VUS (3)
c.846 + 6T > C		LP	+9	+8		+1			VUS (1)
c.846 + 4_846 + 7del		LP	+9	+8		+1			P(2); LP(9); VUS(1)
c.847-14_847-2delinsGG		LP	+9	+8		+1			LP(2); VUS(1)
c.908 + 1G > T		LP	+9	+8		+1			P(2);LP(4)
c.908 + 3A > T		LP	+9	+8		+1			LP(1); VUS(1)
c.1008 + 4A > G		LB	−3	−4		+1			VUS (3)

Continued

Table 2. (continued)

c.HGVS[a]	p.HGVS[a]	ACMG/AMP[b]		PVS1_O/BP7_O[c]	PS4[d]	PM2[e]	PM5[f]	BS1[g]	ClinVar[h]
c.1009-5T > A		VUS	+5	+4		+1			VUS (1)
c.1095G > C	p.(Lys365Asn)	VUS	+5	+4		+1			VUS (1)
c.1095 + 1G > A		VUS	+5	+4		+1			LP (4)
c.1096-6T > G		LP	+9	+8		+1			VUS (2)
c.1259 + 5G > C		VUS	+1			+1			VUS (1)
c.1260-8A > G		LP	+9	+8		+1			P(1); LP(1); VUS(3)
c.1375G > A	p.(Ala459Thr)	LP	+9	+8		+1			VUS (2)
c.1375 + 1_1375 + 2del		LP	+9	+8		+1			LP(2)
c.1376-1G > C		LP	+9	+8		+1			LP(2)
c.1459C > T	p.(Gln487Ter)	LP	+9	+8			+1		P(3)
c.1461 + 5G > T		LP	+9	+8		+1			VUS (3)
c.1542G > T	p.(Gln514His)	VUS	+2	+1		+1			VUS (5)
c.1542 + 3A > G		VUS	+2	+1		+1			LP(1); VUS (3)
c.1543-9_1546del		LP	+9	+8		+1			P(1)

The table shows only ACMG/AMP evidence that has contributed to the final classification. A more detailed analysis is shown in Supplemental Table 5.1.
[a]HGVS (Human Genome Variation Society) nomenclature using NM_007194.4 as a reference.
[b]P, pathogenic; LP, likely pathogenic; VUS, variant of uncertain significance; LB, likely benign.
[c]PVS1_O/BP7_O code strength (see Supplemental Material) derived from mg*CHEK2* read-outs. See Supplemental Table 5.2 for further details.
[d]PS4 (see Supplemental Material) based on BRIDGES case-control data. See Supplemental Table 5.3 for further details.
[e]We have applied the rarity pathogenic evidence at supporting strength, as recently recommended by the ClinGen SVI (see Supplemental Material).
[f]We have applied PM5 at supporting strength to premature termination codons (see Supplemental Material).
[g]We have applied BS1 to *CHEK2* variants with a MAF > 0.05% in gnomAD (see Supplemental Material for further details).
[h]For comparative purposes, we summarize ClinVar status of the 52 variants under investigation (last accessed 03/03/2023). The number of ClinVar records is indicated between parentheses. ClinVar detailed information is provided in Supplemental Table 5.3.

T), severely hampering their interpretation. Moreover, the *CHEK2*-5′ exons 1 to 6 showed the highest variability as 52 transcripts were identified, which, indeed, accumulate the most frequent naturally occurring alternative splicing events such as Δ(E1q13), Δ(E4), ▼(E4A38), or Δ(E5) (24). This finding might reflect the intrinsic complex nature of the splicing regulation of *CHEK2*. Transcripts were generated by a variety of splicing events, such as single- or multi-exon skipping, alternative site usage of cryptic or de novo 3′ SS or 5′ SS, mutually exclusive exons (up to 9 transcripts combine skipping of exon 4 with exon 4A38 inclusion), intron retention (▼($I1^{mg}$)), or a combination of the above phenomena.

Remarkably, 8 isoforms (Δ(E1q13), Δ(E4), ▼(E4A38), Δ(E5), Δ(E4_E5), Δ(E8), Δ(E10), and Δ(E12p33)) had been previously identified as *CHEK2* physiologically occurring isoforms (26). Therefore, this lends further support to the notion that naturally occurring alternative events observed in control samples anticipate the outcome of some spliceogenic variants (i.e., some variants impact splicing by upregulating the expression of transcripts already detectable in non-carriers) (38). For instance, Δ(E1q13) and Δ(E4) ▼(E4A38) were the main induced transcripts in 4 spliceogenic variants each (Table 1).

We also noted the use of a rare de novo functional TG 3′SS (variant c.684-2A > G) that is present in some human exons (nearly 0.02%) (27). The unexpected c.684-2A > G finding is explained, at least in part, by the surrounding nucleotides that closely matches the TG consensus sequence (Fig. 4D), where 17 positions match the first or second most common nucleotide. Recognition critically depends on interval c.685_698del (complete abrogation of TG recognition when deleted), but deletions c.709_722del and c.707_743del also have a significant effect. It is likely that TG usage is mediated by splicing enhancers or silencers or even by the secondary RNA structure that might hide or expose essential binding motifs for splicing factors. Indeed, as per SpliceAid (http://www.introni.it/splicing, accessed November 23, 2022), deletions

c.685_698del, c.709_722del, and c.707_743del remove putative exon 6 enhancers such as SRp55 (Serine And Arginine Rich Splicing Factor 6), SRp40 (Serine And Arginine Rich Splicing Factor 5), SC35 (Serine And Arginine Rich Splicing Factor 2), or Tra2β (Transformer 2 Beta Homolog), among others (Fig. 4C). At any rate, the contribution of any of these SR proteins for TG recognition has to be experimentally confirmed.

Clinical interpretation of variants through a point-based ACMG/AMP clinical classification was particularly challenging because:

(i) *CHEK2* expert panel specifications of the ACMG/AMP guidelines are not yet available.
(ii) Most mgChk2 read-outs were very complex, with several transcripts detected (up to 8 different transcripts in c.592 + 4A > G mgChk2 read-out), some of them not fully characterized.
(iii) Various spliceogenic variants were leaky (expressing variable amounts of full-length transcripts).
(iv) wt minigenes (in particular mgChk2_ex1-7) produce a non-negligible level of alternative transcripts.
(v) Very little evidence other than minigene read-outs were available.
(vi) Up to 7 variants under investigation target the 5′ UTR, a region outside the scope of current ACMG/AMP guidelines.

Accordingly, our classification process resulted in a relatively high proportion of VUSs (38%). By comparing our mgChk2-based classification with ClinVar reports, we made some interesting observations regarding GT-AG and non-GT-AG intronic variants:

(a) Our data set includes 16 GT-AG variants previously reported in ClinVar as P/LP by all submitters (c.847-14_847-2delinsGG is reported as VUS by 1 of 3 submitters). Yet, we classified 3 of these variants as VUSs (c.793-1G > A and c.793-1G > T due to the lack of evidence other than the splicing assay; c.1095 + 1G > A due to splicing alterations that are not unequivocally damaging).
(b) Our data set includes 21 non-GT/AG intronic variants previously reported in ClinVar. While ClinVar reports all these as VUSs (or conflicting), we classify 8 as LP, 4 as LB, and only 9 as VUS, decreasing uncertainty.

To avoid overestimating the prior probability of pathogenicity for ±1,2 variants, we recommend developing gene-specific PVS1 decision trees in which ±1,2 variants have PVS1 codes of variable strength depending on the exact nature of the predicted splicing outcome. To this aim, we recommend using SpliceAI, a neural network that predicts splicing from a pre-mRNA sequence (21), and has been extensively validated (39). Online Supplemental Table 7 shows a comparative analysis of SpliceAI predictions and mgChk2 read-outs for 51 *CHEK2* variants (c.847-14_847-2delinsGG has been excluded, as SpliceAI does not support currently complex InDels). Overall, the analysis suggests that Δ scores <0.2 may have predictive value, and that Δ scores <0.8 (acceptor or donor loss) predict leaky variants. That said, our study also shows that RNA assays are essential to verify the effect of candidate variants on splicing.

In conclusion, we have tested 52 *CHEK2* variants in 3 minigenes containing all 15 exons of this gene. A high proportion of them (88.5%) induced splicing anomalies. We ended up classifying 5 variants as LB and 27 as P/LP. Burden analyses using case-control or family data will be needed to refine the classification method, and to provide accurate risk estimations that inform the clinical management of BC patients. In the meantime, the minigene methodology with the pSAD vector is a versatile and suitable simple approach that has allowed us to check hundreds of putative spliceogenic variants in 6 other BC genes as well as other disease genes such as *TRPM4* (MIM#606936), among others (40).

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: BRIDGES, Breast Cancer After Diagnostic Gene Sequencing; MCF-7, Human Breast Adenocarcinoma Cell Line; HeLa, Human Cervical Carcinoma Cell Line; ACMG/AMP, American College of Medical Genetics and Genomics/Association for Molecular Pathology; BC, breast cancer; VUS, variants of uncertain significance; 5′SS, 5′ splice sites; 3′SS, 3′ splice sites; RT-PCR, reverse transcription-PCR; wt, wild type; nt, nucleotide; MES, MaxEntScan; mgFL, minigene full-length transcript; P, pathogenic; LP, likely pathogenic; LB, likely benign; PVS, Pathogenic Very Strong; BP, Benign Supporting.

Human Genes: *ATM*, ATM serine/threonine kinase; *BARD1*, BRCA1 associated RING domain 1; *BRCA1*, BRCA1 DNA repair associated; *BRCA2*, BRCA2 DNA repair associated; *CHEK2*, Checkpoint kinase 2; *PALB2*, partner and localizer of BRCA2; *RAD51C*, RAD51 paralog C; *RAD51D*, RAD51 paralog D; *TRPM4*, Transient receptor potential melastatin member 4.

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