

Rapid Detection Method for the Four Most Common *CHEK2* Mutations Based on Melting Profile Analysis

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Abstract

Introduction *CHEK2* is a tumor suppressor gene, and the mutations affecting the functionality of the protein product increase cancer risk in various organs. The elevated risk, in a significant percentage of cases, is determined by the occurrence of one of the four most common mutations in the *CHEK2* gene, including c.470T>C (p.I157T), c.444+1G>A (IVS2+1G>A), c.1100delC, and c.1037+1538_1224+328del5395 (del5395).

Methods We have developed and validated a rapid and effective method for their detection based on high-resolution melting analysis and comparative-high-resolution melting, a novel approach enabling simultaneous detection of copy number variations. The analysis is performed in two polymerase chain reactions followed by melting analysis, without any additional reagents or handling other than that used in standard high-resolution melting.

Results Validation of the method was conducted in a group of 103 patients with diagnosed breast cancer, a group of 240 unrelated patients with familial history of cancer associated with the *CHEK2* gene mutations, and a 100-person control group. The results of the analyses for all three groups were fully consistent with the results from other methods.

Conclusion The method we have developed improves the identification of the *CHEK2* mutation carriers, reduces the cost of such analyses, as well as facilitates their implementation. Along with the increased efficiency, the method maintains accuracy and reliability comparable to other more labor-consuming techniques.

Key Points

The aim of the study was to develop and validate a rapid inexpensive method for the accurate and reliable detection of p.I157T, IVS2+1G>A, del5395, and c.1100delC in the *CHEK2* gene.

The developed methodology is based on high-resolution melting analysis and comparative-high-resolution melting, a novel approach enabling simultaneous detection of copy number variations, and allows for the detection of p.I157T, IVS2+1G>A, del5395, and c.1100delC in two polymerase chain reactions followed by melting analysis, without using any additional equipment or handling.

Obtained results indicate that along with the increased efficiency, the method maintains accuracy and reliability comparable to other more labor-consuming techniques.

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1 Introduction

CHEK2 (Checkpoint kinase 2, MIM 604373) is a tumor suppressor gene, and its functional product regulates cell division. The gene is located on the long arm of chromosome 22 [1], consists of 16 exons, and encompasses 54,092 bp. First assumptions of the malignant nature of the *CHEK2* gene, appeared in 1999, when the gene defects were associated with Li–Fraumeni syndrome 2 (MIM #609265) [2].

The *CHEK2* gene encodes a 543-amino acid checkpoint kinase playing a key role in the DNA damage repair. CHK2 protein, belongs to the ATM-CHK2 pathway activated by double-stranded DNA breaks. CHK2 is selectively phosphorylated and activated by ATM (ataxia-telangiectasia mutated) to trigger a wide range of distinct downstream responses for DNA damage [3]. One of the proteins activated by CHK2 is TP53. The serine phosphorylation at position 20 of the protein and interaction with CHK2 leads to the stabilization and cell-cycle arrest in the G1 phase [1, 4].

Changes in the structure of the *CHEK2* gene, affecting the functionality of the expression product, are associated with an increased risk of various cancers, including breast, colon, prostate, ovaries, kidney, thyroid, or lung [5–8]. The increased risk of these cancers, in a significant percentage of cases, is determined by the presence of one of the four most common mutations within the gene [9–12]. The first one is a missense mutation c.470T>C (p.I157T), while the other three: c.444+1G>A (known as IVS2+1G>A), c.1037+1538_1224+328del5395 (known as del5395), and c.1100delC cause premature termination of the gene translation. Their incidence in Poland is respectively 4.8, 0.4, 0.4, and 0.2 % [13, 14].

CHEK2 truncating mutations (c.1100delC and IVS2+1G>A, del5395) increase the risk of breast, prostate, and thyroid cancers. [15]. The risk of developing breast cancer increases approximately 2.4-fold, and the incidence of mutations in breast cancer patients is approximately 2.5 %. The risk of prostate cancer increases approximately 2.3-fold. The mutations are observed in approximately 2.5 % of all prostate cancers, and in 5 % of familial prostate cancers. The risk of prostate cancer increases approximately 5-fold, if it occurred among first- and second-degree relatives. For papillary thyroid carcinoma, the risk increases approximately 5-fold, and these mutations occur in approximately 4 % of all thyroid cancers [8, 13].

The *CHEK2* missense variant p.I157T, which is much more common in northern and eastern Europe than the three remaining alleles, increases the risk of breast cancer 1.5-fold and the risk of prostate cancer 1.6-fold. The risk of prostate cancer is increased approximately 3-fold, if it

occurred among first-degree relatives. In papillary thyroid carcinoma, mutation occurs in approximately 9 % of patients, and increases the risk approximately 2-fold [16]. This mutation also results in an approximately 2-fold increase risk of kidney and colon cancers, and is present in approximately 10 % of patients from both groups [8, 11, 13]. Furthermore, it increases the risk of low-grade malignant ovarian cancer approximately 2-fold in 10 % of ovarian cancers G1; and ovarian tumors of borderline malignancy about 2.5-fold maintaining a frequency of approximately 11.5 % [17].

The substantial role of p.I157T, IVS2+1G>A, del5395, and c.1100delC variants in increasing the risk of various cancers, raises the need for the development of their detection methods. In many cases, the increased risk of cancer may be conditioned by any of these four mutations, thus the presence of all of them should be examined. The detection of these four variants is offered by each commercial laboratory performing genetic testing. Such methods based on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), allele specific amplification-polymerase chain reaction (ASA-PCR), or the use of denaturing high performance liquid chromatography (DHPLC) have already been described [8, 14, 18, 19]. These methods allow for the detection of particular mutations in separate studies. Additionally, in some of them (e.g., DHPLC or PCR-RFLP), the PCR products require further processing, which increases the workload. Moreover, the DHPLC method needs expensive equipment in the form of a DHPLC analyzer.

In recent years, the development of techniques based on a fluorescence analysis has been observed. These include the method of high-resolution melting (HRM) [20]. The principle of the HRM technique is the observation of previously amplified DNA fragments during a denaturation process. Insight into the process is facilitated by the presence of a fluorescent dye in the reaction mixture, which intercalates only double-stranded DNA molecules, giving a strong fluorescent signal. In the course of a slow temperature increase, double-stranded fragments denature into single-stranded forms, which is reflected in a decrease of fluorescence of the sample resulting from a dissociation of the dye. The result of the measurement of the fluorescence throughout the entire process is a melting curve. In the case of mutations within the analyzed fragment, there is a change in the melting profile, which can be observed by comparing the results to the wild-type profile.

A recently described, comparative-high-resolution melting (C-HRM) method is an improvement of the standard HRM. C-HRM is also based on data from melting analysis, but in addition to the detection of small sequence changes, it allows a semi-quantitative analysis of the amplified fragments, thus the detection of copy number

variations (CNVs). The detailed description of the method had been presented previously by Borun et al. [21]. The analysis is based on the use of a multiplex polymerase chain reaction (PCR) of two fragments: a target and a reference, followed by melting analysis in the exponential growth phase of PCR. The result for wild-type samples is a characteristic pattern of a peak height corresponding to the reference and the analyzed amplicons, conditioned by the initial amount of template for both products. In the case of analysis of samples with a rearrangement of the analyzed fragment, the ratio is affected by either a deletion or a duplication of the target sequence.

The aim of the study was to develop and validate a rapid inexpensive method for the accurate and reliable detection of p.I157T, IVS2+1G>A, del5395, and c.1100delC in the *CHEK2* gene. The developed methodology is based on HRM and C-HRM analysis. It allows for the detection of these four mutations in two PCRs followed by melting analysis, without using any additional equipment or handling.

2 Materials and Methods

2.1 Assay Design

The method for detecting four common *CHEK2* gene mutations was developed based on the standard technique of

HRM and its modification C-HRM, which in addition to small mutations screening, enables the detection of CNVs. Application of HRM and C-HRM in this case is possible because of the location of investigated variants. The study of the occurrence of IVS2+1G>A and p.I157T is performed using a single PCR product analyzed by HRM because these mutations are located upstream of exon 5 and within exon 5, and the designed primers encompass both variants. In our study, we used the C-HRM to detect a large rearrangement of the *CHEK2* gene, resulting in a deletion of 5395 bp, comprising exons 10 and 11, using the primer pair amplified on a fragment of intron 10-11 undergoing deletion. At the same time in the reaction, the reference fragment is also amplified, comprising the exon 12, which simultaneously allows the detection of c.1100delC (Fig. 1). Primer sets for both HRM and C-HRM analysis are designed to the annealing temperature of 60 °C. The sequences and concentrations for each assay are shown in Table 1.

2.2 HRM and C-HRM Conditions

The products were amplified using the HRM type-it kit (Qiagen, Venlo, Netherlands) on the DNA samples at a concentration of 50 ng/μL diluted in AE buffer (Qiagen). The analysis was performed on a Rotor-Gene® Q equipment (Qiagen). PCR was carried out for the 30 cycles (with a 5-min pre-incubation at 95 °C) of 95 °C for 10 s, 60 °C

Fig. 1 Assay design and location of mutations detected by comparative-high-resolution melting

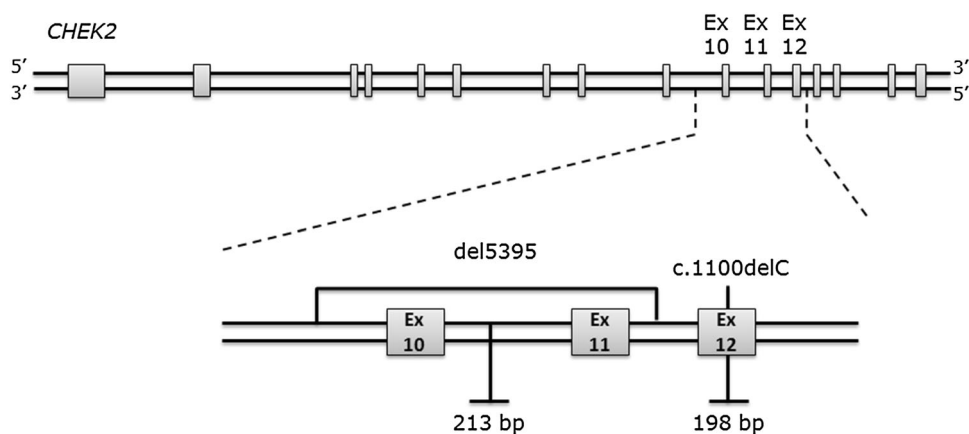


Table 1 Primer sequences and concentrations in the designed assays

Designed assays	Forward primer sequence	Final forward primer concentration (μM)	Reverse primer sequence	Final reverse primer concentration (μM)
1 IVS2+1G>A and p.I157T	CCGAACATACAGCAAGAAACA	0.69	AAAGGTTCCATTGCCACTGT	0.69
2 c.1100delC	CAATAGAAACTGATCTAGCCTACGTGT	0.56	AGAACTTCAGGCGCCAAGTA	0.56
del5395	AGGTGCAGCATCCTGTTCG	0.50	AGGATTGCTCCCAATCACTG	0.50

for 30 s, and 72 °C for 10 s, the products were then melted and PCR was continued to the 40th cycle in the same conditions followed by another melting process. The first melting analysis (used for CNV detection in C-HRM) was performed during the exponential phase of the PCR from 70 to 90 °C by raising the temperature by 0.3 °C at each step after which the second one, designed to detect small changes in the sequence, was carried out with a higher resolution raising the temperature by 0.1 °C at each step.

2.3 Assay Validation

The method was validated on a group of 103 female patients with diagnosed breast cancer (in all patients, the four most common mutations in the *BRCA1* gene had been excluded) (breast cancer [BC] group), a group of 240 unrelated patients with familial history of cancer associated with the most common *CHEK2* gene mutations (CC group), and a control group consisting of 100 unrelated healthy individuals from the Polish population (Control group). The patients were derived from the Cancer

Prevention Epidemiology Center in Poznan. Because of the rarity of the homozygous c.470C/C variant, this sample was derived from the International Hereditary Cancer Center in Szczecin and included in the tests for correct validation of the method. All patients declared informed consent and the studies were approved by the local Ethics Committee of the Poznan University of Medical Sciences (approval no. 459/10). The study groups were tested for *CHEK2* mutation status using our combined HRM and C-HRM assay and conventional methods including PCR-RFLP and ASA-PCR described by Cybulski et al. [13]. The detection of del5395 was performed using the ASA-PCR assay developed by us. Three primers (forward: GGAATCCCTGTGATTAGGA, reverse1: AGGCATCTTCCAGAAATGAG and reverse2: GCGCATGTGATTA CTCATTC) encompassing deleted exons were situated near the deletion endpoints and the presence of del5395 is visualized as an additional PCR product (Fig. 2d). All the analyses using developed assays were performed in the presence of control samples representing all possible genotypes of each variant.

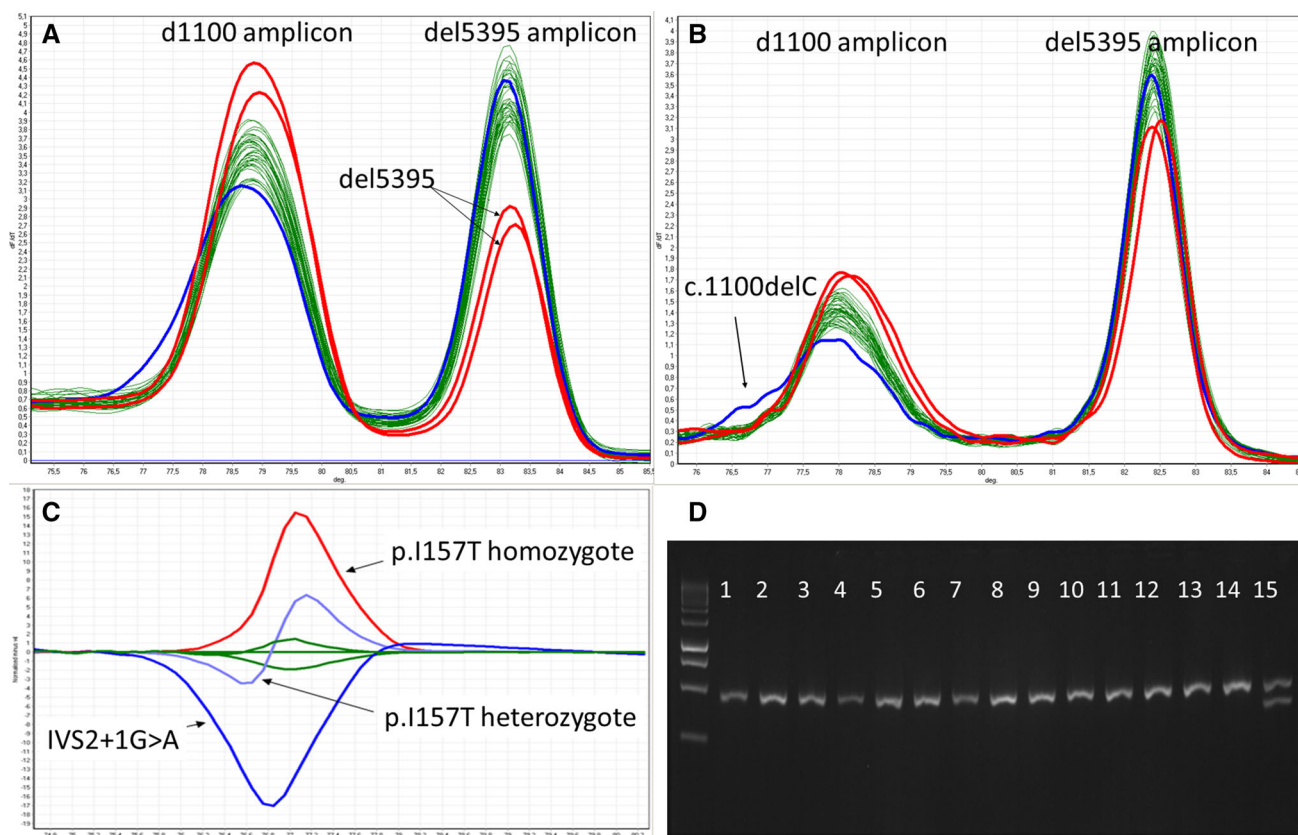


Fig. 2 Example results obtained using the developed assays. **a** Simultaneous detection of del5395 and c.1100delC using comparative-high-resolution melting assay; melting profiles after the 30th cycle showing the detection of del5395. **b** Melting profiles after the 40th cycle showing the detection of c.1100delC (red curves, samples with del5395; green curves, wild-type samples; blue curve, sample with

c.1100delC). **c** Simultaneous detection of IVS2+1G>A and p.I157T with high-resolution melting (red curve, homozygous variant p.I157T; purple curve, heterozygous variant p.I157T; green curves, wild-type samples; blue curve, sample with heterozygous variant IVS2+1G>A). **d** Detection of del5395 using the developed ASA-PCR assay (samples 1–14, wild type; sample 15, del5395 carrier)

Table 2 Analysis of results for each studied variant [(high-resolution melting and comparative-high-resolution melting results were entirely consistent with those obtained by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and allele specific amplification-polymerase chain reaction (ASA-PCR))]

	IVS2+1G>A	c.470T>C (p.I157T)	c.1100delC	del5395
BC group	2/103 (1.94 %)	4/103 (3.88 %)	1/103 (0.97 %)	1/103 (0.97 %)
CC group	5/240 (2.08 %)	13/240 (5.42 %)	0/240 (0 %)	1/240 (0.42 %)
Control group	1/100 (1 %)	4/100 (4 %)	0/100 (0 %)	0/100 (0 %)

BC breast cancer, CC cancer associated with the most common *CHEK2* gene mutations

3 Results

3.1 del5395 and c.1100delC

The normalized peak height ratio of the target to the reference amplicon should be in the range of 0.9–1.1 for wild-type samples, and 0.4–0.6 for samples with the 5.4-kbp deletion. Using the C-HRM method in the group of 103 female patients with diagnosed BC, we detected one case of del5395 and one case of c.1100delC (Fig. 2a, b). In the group of 240 individuals with familial cancer history, we detected only one case of del5395, while no mutations were detected in the control group (Table 2). Analysis of all samples was carried out twice. The results of the ASA-PCR analyses (used for detection of both del5395 and c.1100delC) for all three groups were fully consistent with the results obtained by C-HRM. A pooled analysis of detected mutations is presented in a Supplementary Figure 2.

3.2 IVS2+1G>A and c.470T>C

Two substitutions were detected using single amplicon analysis with the standard HRM technique (Fig. 2c). Missense substitution c.470T>C (p.I157T) in the BC group were detected in four (3.88 %) patients. In the group of patients from families with familial cancer history associated with *CHEK2*, the change was detected in 13 of 240 (5.42 %). In the control group, four people were carriers of the heterozygous p.I157T variant (4 %). IVS2+1G>A was detected in 2 of 103 (1.94 %) BC patients, and in 5 of 240 (2.08 %) from the CC group. In the Control group, IVS2+1G>A was observed at a frequency of 1 % (1/100). The results of PCR-RFLP for both changes were fully consistent with those obtained by HRM (Table 2). Although we did not detect any homozygous c.470T>C, we included the positive control with this genotype, and it was successfully identified by our assay (Fig. 2c). A pooled analysis of detected mutations is presented in a Supplementary Figure 1.

4 Discussion

In this study, we have developed and validated a rapid and efficient method for the detection of four common *CHEK2* gene mutations increasing the risk of cancers of various organs. This method is based on two PCRs followed by melting analysis. The first PCR enables the detection of both p.I157T, IVS2+1G>A point mutations using a single amplicon analyzed by standard HRM. The second PCR enables simultaneous detection of del5395 and c.1100delC using the C-HRM method developed by the authors.

To date, in the literature, only a few methods to detect the aforementioned mutations of the *CHEK2* gene have been described [13, 14, 22, 23]. MRC-Holland Company has developed a set (P190) for the analysis of multiplex ligation-dependent probe amplification, comprising in its composition probes for all *CHEK2* coding exons (including 10 and 11, which comprises del5395), and a specific probe for the c.1100delC detection. However, none of these techniques allow for the simultaneous detection of all the four changes of the gene. Moreover, these methods are time consuming, more expensive, and some need advanced equipment [14]. Given the fact that all the aforementioned changes are associated with conditioning the majority of cancers associated with the *CHEK2* gene (especially prostate, breast, and thyroid), it is important to test individuals at risk for the presence of all four variants.

The advantage of the developed assays is the ability to examine the presence of all four changes under the same PCR conditions. Despite the requirement of an additional melting process in the exponential growth phase of the PCR to detect del5395, it does not affect further analysis. Thus, in a simultaneous analysis, using only one device, we can conduct the detection of all the four sequence changes. We decided to examine substitutions c.470T>C and IVS2+1G>A on the basis of one amplicon comprising both changes. This is owing to the proximity of the changes that would result in the formation of intermediate products in the case of using two pairs of primers in the multiplex reaction. Both changes are easily recognized because they differently affect the melting temperature of the amplicon.

One of the limitations of the developed approach is the requirement of the use of a HRM device enabling both PCR and HRM (to perform melting during the exponential phase of PCR in C-HRM). It is also likely that the primers concentrations in the multiplex assay would have to be optimized for other HRM kits because of the kits composition differences. Moreover, C-HRM, as with other quantitative methods, requires high-quality genetic material for analysis. Degraded or contaminated DNA samples could produce false results. Finally, it must be taken into account that HRM is a screening method, and all samples with melting profiles different from the wild type should be directly sequenced to confirm genotyping results.

5 Conclusion

The developed methodology enables the improvement of the genetic diagnostics of patients, and reduces the cost of such analyses, and also facilitates their implementation. Therefore, it is possible to cover a larger group of patients, and at the same time, increase the efficiency of identification of people at risk. HRM is one of the most cost-effective screening tools for the detection of small mutations with higher efficiency and requires less time and labor, compared with other methods. Development of the C-HRM allowed the detection of large rearrangements (CNVs), without changing the specificity of the method, which further increase the efficiency of mutation detection. HRM devices are increasingly common and present in most diagnostic laboratories, which allows for a broad application of the developed methodology in everyday genetic diagnostics.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval and informed consent All patients declared informed consent and the studies were approved by the local Ethics Committee of the Poznan University of Medical Sciences (approval no. 459/10).

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