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Fragment analysis represents a suitable approach for the detection of hotspot c.7541_7542delCT NOTCH1 mutation in chronic lymphocytic leukemia

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Mgr. Barbara Vonková

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Annotation

Chronic lymphocytic leukemia is the most common type of leukemia in adults in western countries. Despite the continual improvement of diagnostic methods and treatment, it still remains incurable. One of the main characteristic features of CLL is its clinical and biological variability. In present, the analysis of prognostic genetic markers is the most useful tool for understanding biology and clinical course of the disease and consequently the individual type of treatment. The presence of a NOTCH1 mutation is connected with shortened survival and resistance to conventional chemo-immunotherapy. In this thesis, we examined CLL patients with c.7541_7542delCT NOTCH1 mutation and the most sensitive and specific method was identified. We compared nowadays most commonly used methods: allele-specific PCR, fragment analysis and Sanger sequencing.

Declaration [in Czech]

Prohlašuji, že svoji rigorózní práci jsem vypracoval/a samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své rigorózní práce, a to v nezkrácené podobě elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

České Budějovice dne 20.4.2018 Mgr. Barbara Vonková

Přiznání spoluautorství

Svým podpisem potvrzuji plnohodnotné spoluautorství Mgr. Barbary Vonkové na článku: Fragment analysis represents a suitable approach for the detection of hotspot c.7541_7542delCT NOTCH1 mutation in chronic lymphocytic leukemia, publikovaného v Leukemia Research (IF=2,501) dne 6.9.2017. Mgr. Barbara Vonková se v rámci této publikace podílela na zpracování biologického materiálu, analýze získaných dat a také finálních úpravách výsledného manuskriptu.

Jmenovitě bylo součástí práce slečny Vonkové zpracování biologického materiálu; konkrétně separace B lymfocytů z periférní krve a následná izolace DNA pacientů s diagnózou chronické lymfocytární leukemie. Dále se spolupodílela se na optimalizaci zejména metody PCR, kde bylo zapotřebí nastavit podmínky nejvíce vhodné k průběhu pokusů. Dále její práce zahrnovala provedení alelově specifické PCR a fragmentační analýzy u části souboru vybraných pacientů.

Mezi další činnost žadatelky také patřila výpomoc při ověřování výsledků pomocí sekvenování nové generace na přístroji MiSeq (Illumina) včetně přípravy sekvenační knihovny a vyhodnocování alelově specifické PCR pomocí agarové elektroforézy. Mezi nezanedbatelné příspěvky patří také spolupráce na vyhodnocování získaných výsledků.

Eva Vávrová

Mgr. Barbara Kantorová, Ph.D.

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Research paper

Fragment analysis represents a suitable approach for the detection of hotspot c.7541_7542delCT *NOTCH1* mutation in chronic lymphocytic leukemia

Eva Vavrova[a], Barbara Kantorova[a,b], Barbara Vonkova[a], Jitka Kabathova[b], Hana Skuhrova-Francova[b], Eva Diviskova[b], Ondrej Letocha[b], Jana Kotaskova[a,b], Yvona Brychtova[b], Michael Doubek[a,b], Jiri Mayer[a,b], Sarka Pospisilova[a,b,*]

[a] Central European Institute of Technology (CEITEC) and Faculty of Medicine, Masaryk University, Kamenice 5, CZ-625 00 Brno, Czech Republic
[b] Department of Internal Medicine – Hematology and Oncology, University Hospital Brno, Cernopolni 9, CZ-613 00 Brno, Czech Republic

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ABSTRACT

The hotspot c.7541_7542delCT *NOTCH1* mutation has been proven to have a negative clinical impact in chronic lymphocytic leukemia (CLL). However, an optimal method for its detection has not yet been specified. The aim of our study was to examine the presence of the *NOTCH1* mutation in CLL using three commonly used molecular methods. Sanger sequencing, fragment analysis and allele-specific PCR were compared in the detection of the c.7541_7542delCT *NOTCH1* mutation in 201 CLL patients. In 7 patients with inconclusive mutational analysis results, the presence of the *NOTCH1* mutation was also confirmed using ultra-deep next generation sequencing. The *NOTCH1* mutation was detected in 15% (30/201) of examined patients. Only fragment analysis was able to identify all 30 *NOTCH1*-mutated patients. Sanger sequencing and allele-specific PCR showed a lower detection efficiency, determining 93% (28/30) and 80% (24/30) of the present *NOTCH1* mutations, respectively. Considering these three most commonly used methodologies for c.7541_7542delCT *NOTCH1* mutation screening in CLL, we defined fragment analysis as the most suitable approach for detecting the hotspot *NOTCH1* mutation.

1. Introduction

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia in western countries. This disease is greatly heterogeneous and has a highly unpredictable clinical course [1–5]. To improve the prognosis and prediction of treatment efficacy in CLL, genomic analyses become crucial. In addition to IGHV mutational status, cytogenetic abnormalities and *TP53* mutations, mutations in the NOTCH1 transcription factor have been proven to be clinically relevant in CLL [3]. The presence of *NOTCH1* mutations, with a predominant hotspot c.7541_7542delCT mutation detected in ~10-20% of CLL patients [2,4,6–8], is closely associated with shortened survival, resistance to conventional chemo-immunotherapy and an increased risk of CLL transformation toward high grade malignant lymphomas [1–3,6–8]. Despite the proven clinical significance of the hotspot *NOTCH1* mutation in CLL, an optimal method for its reliable detection has not yet been specified. In this study, we therefore compared the most commonly used methods of *NOTCH1* mutation screening in CLL [1,2,4,6], namely Sanger sequencing, fragment analysis and allele-specific polymerase chain reaction (PCR), to define the most suitable approach.

2. Materials and methods

The presence of the c.7541_7542delCT *NOTCH1* mutation was analyzed in 201 untreated CLL patients monitored at the Department of Internal Medicine – Hematology and Oncology at the University Hospital Brno, Czech Republic, according to the National Cancer Institute-sponsored Working Group guidelines [9]. Patients with unmutated IGHV genes were preferentially included in this cohort due to their expected increased *NOTCH1* mutation frequency [4,6,7]. Detailed patient's characteristics are shown in Table 1. Peripheral blood samples from the CLL patients examined were collected between the years 2004 and 2016 with written informed consent, according to the Declaration of Helsinki and internal Ethics Committee regulations. DNA was isolated from peripheral mononuclear cells or separated CD19+ lymphocytes (RosetteSep™ kits, StemCell Technologies Inc.; Vancouver, BC, Canada).

* Corresponding author at: Central European Institute of Technology (CEITEC) and Faculty of Medicine, Masaryk University, Kamenice 5, CZ-625 00 Brno, Czech Republic.
E-mail address: sarka.pospisilova@ceitec.muni.cz (S. Pospisilova).

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Table 1
Characterization of the examined CLL cohort; I-FISH, interphase fluorescence *in situ* hybridization.

Characteristics of CLL patients		Number of patients
Median age at diagnosis (n = 201)	64 years	
Sex (n = 201)	Male	124 (62%)
	Female	77 (38%)
Disease stage (n = 196)	Rai 0	74 (38%)
	Rai I–II	91 (46%)
	Rai III–IV	31 (16%)
IGHV gene mutation status (n = 196) 98% cut-off	Mutated	64 (33%)
	Unmutated	132 (67%)
Hierarchical cytogenetics (I-FISH; n = 190)	None aberration	43 (23%)
	del(13q) sole	63 (33%)
	+12	19 (10%)
	del(11q)	51 (27%)
	del(17p)	14 (7%)

The occurrence of the c.7541_7542delCT *NOTCH1* mutation was analyzed using three methods in parallel − Sanger sequencing, fragment analysis and allele-specific PCR on the same sample in the cohort of all 201 CLL patients. For Sanger sequencing, the part of the *NOTCH1* gene covering the mutated region was amplified with exon-specific primers using Optimase® Polymerase (Transgenomic Inc.; Omaha, NE, USA) as previously published [10]. For fragment analysis, Q5® Hot Start High-Fidelity DNA Polymerase (New England Biolabs Ltd.; Ontario, Canada) was used, following published conditions [6] with some modifications. Sequencing and fragment products were analyzed on an ABI PRISM® 3130xl Genetic Analyzer (Thermo Fisher Scientific; Waltham, MA, USA). The protocol for allele-specific PCR was adapted according to published data [10] and the received products were visualized using the QIAxcel® System (QIAGEN; Hilden, Germany) or agarose gel electrophoresis. The respective examples of hotspot *NOTCH1* mutation detection are shown in Fig. 1. In 7 patients with inconclusive mutational analysis results, the presence of the c.7541_7542delCT *NOTCH1* mutation was also confirmed using ultra-deep next generation sequencing (NGS) on the Miseq platform (Illumina Inc.; San Diego, CA, USA), as previously published [7,11]. The average coverage for the sequence position containing the hotspot *NOTCH1* mutation was 7573 reads (range 3753–18433). Detailed conditions for each method used are described in the Supplementary material (Supplemental methods). Only samples with the c.7541_7542delCT *NOTCH1* mutation being conclusively detected using (1) at least two different conventional methods or (2) one conventional method together with ultra-deep NGS, were considered as positive (for details see Table 2).

3. Results

By merging the data from the three compared methods − Sanger sequencing, fragment analysis and allele-specific PCR, we detected the c.7541_7542delCT *NOTCH1* mutation in 15% (30/201) of examined patients (detailed description in Table 2). In 10/30 of these patients, the buccal swab material was available and the somatic origin of the *NOTCH1* mutations detected was confirmed by Sanger sequencing in all cases. Comparing the efficacy of the used approaches, only fragment analysis was able to identify all 30 *NOTCH1*-mutated patients (variant allele frequency 3.88-100%; Table 2). Sanger sequencing and allele-specific PCR showed a lower detection efficiency, determining 93% (28/30) and 80% (24/30) of the c.7541_7542delCT *NOTCH1* mutations present, respectively (Table 2). The c.7541_7542delCT *NOTCH1* mutations unrecognized by both Sanger sequencing and allele-specific PCR occurred in the cases with a low percentage of mutated DNA in the examined samples (under 5%), and their presence was verified with ultra-deep NGS (patients no. 1184, 1586; Table 2).

Moreover, using fragment analysis, we registered a background signal corresponding to the c.7541_7542delCT *NOTCH1* mutation in 5/171 of the *NOTCH1*-wild type patients. Remarkably, in all of these 5 patients, ultra-deep NGS confirmed the presence of low-abundance c.7541_7542delCT *NOTCH1* mutations (variant allele frequency 1.90-4.36%). However, as the conclusive evidence for the presence of such minor *NOTCH1* mutations could not be evaluated in this study due to the missing biological material, we did not consider these samples in the analysis.

To further evaluate the detection efficacy of the methods tested, we proportionally mixed DNA from the *NOTCH1*-wild-type control and from patient no. 561, who carried the c.7541_7542delCT *NOTCH1* mutation in 100% of DNA, as determined using Sanger sequencing (Table 2). The percentage ratio of the mutated to wild-type DNA was as follows: 0%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%. Examining these samples using fragment analysis and Sanger sequencing, we were able to detect 5% of mutated DNA, while allele-specific PCR did not recognize the hotspot *NOTCH1* mutations occurring in less than 10% of DNA (for details see Fig. 2). The established detection efficiency of the particular methods resulting from the dilution experiment correlated with the *NOTCH1* mutation screening performed in this study (Table 2). The observed increased Sanger sequencing detection limit compared to the usually declared 10% of mutated DNA [7,12] was caused by the easily distinguishable frameshift character of the monitored c.7541_7542delCT *NOTCH1* mutation.

4. Discussion

Although a negative prognostic impact of the c.7541_7542delCT *NOTCH1* mutation has been repeatedly proven in CLL [1–3,6,7], determining a gold standard approach for analysing this mutation is problematic as (1) many different methods on dissimilar patient cohorts are used to analyse the *NOTCH1* gene in CLL [4,7,13], (2) there are no long-term studies evaluating the effect of patient treatment on the *NOTCH1* mutation load in CLL, (3) a clinically relevant mutation threshold has not yet been exactly defined [12]. In addition, despite a growing number of novel genomic approaches, the analysis of *NOTCH1* hotspot mutations using standard molecular biological methods is still common in clinical laboratories. For these reasons we compared three of the most commonly used conventional methods for the *NOTCH1* mutation detection in CLL to each other.

In our study, each of the assessed classical methodologies − Sanger sequencing, fragment analysis and allele-specific PCR − was able to detect all c.7541_7542delCT *NOTCH1* mutations occurring in more than 10% of DNA. This detection efficiency should be sufficient in the majority of cases; in addition, the clinical impact of minor *NOTCH1* mutations (< 10% of DNA) is questionable [12,14] and requires further evaluation. On the other hand, even low-abundance *NOTCH1*-mutated subclones might show chemo-refractoriness and they may be selectively prioritized sporadically during the CLL course [5,8,15,14]. Early detection of *NOTCH1* mutations in untreated CLL using sensitive screening methods is therefore highly recommended.

Comparing conventional detection methods, we proved that only fragment analysis was able to recognize all c.7541_7542delCT *NOTCH1* mutations present (with a range of mutated DNA from 3.88-100%). Nevertheless, in contrast to sequencing, fragment analysis does not allow the identification of point *NOTCH1* mutations. This limitation is not so relevant in the case of CLL, as the c.7541_7542delCT *NOTCH1* mutation represents the majority of registered *NOTCH1* mutations

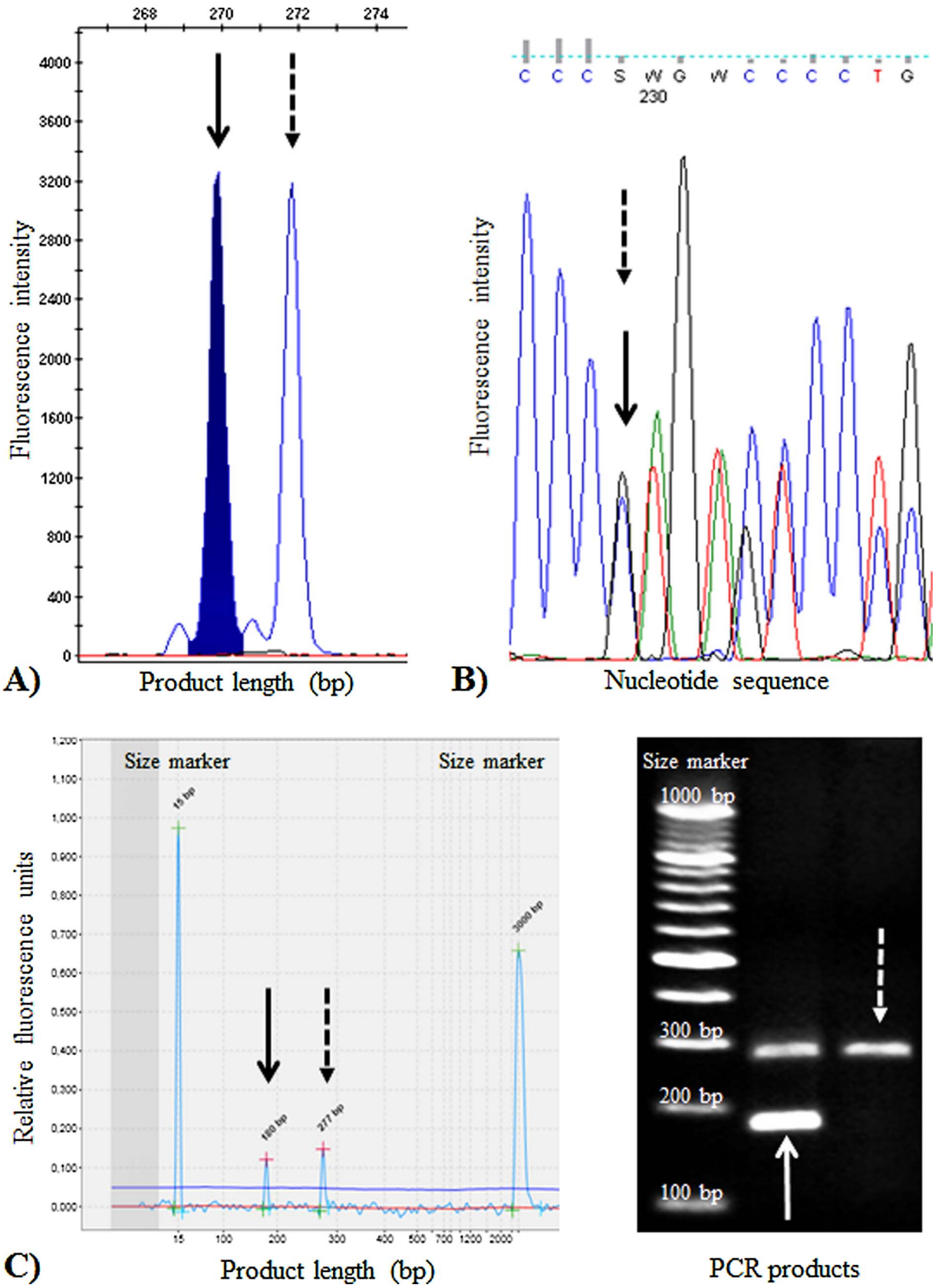

Fig. 1. Detection of c.7541_7542delCT *NOTCH1* mutation in CLL using A) fragment analysis, B) Sanger sequencing, C) allele-specific PCR visualized by QIAxcel® System and agarose gel electrophoresis; the solid black arrows indicate the presence of c.7541_7542delCT *NOTCH1* mutation; the dashed black arrows correspond to the *NOTCH1*-wild-type PCR product.

Table 2
Characterization of 30 *NOTCH1*-mutated CLL patients; AS PCR, allele-specific PCR; FA, fragment analysis; I-FISH, interphase fluorescence *in situ* hybridization; NA, not analyzed; NGS, ultra-deep next generation sequencing; NGS%, percentage of the *NOTCH1*-mutated variant reads determined based on ultra-deep NGS; SEQ, Sanger sequencing; SEQ%, proportion of c.7541_7542delCT *NOTCH1* mutation estimated based on Sanger sequencing using Mutation Surveyor DNA Variant Analysis Software (SoftGenetics®;State College, Pennsylvania, USA); *, DNA used for the method sensitivity evaluation; #, c.7541_7542delCT *NOTCH1* mutation visible in background.

Patient	I-FISH	IGHV genes	FA	SEQ	SEQ%	AS PCR	NGS%
361	+12	Unmutated	Positive	Positive	20	Positive	NA
395	None aberration	Unmutated	Positive	Positive	25	Positive	NA
427	del(13q), +12	Unmutated	Positive	Positive	50	Positive	NA
453	del(13q)	Unmutated	Positive	Positive	5	**Negative**	NA
550	None aberration	Unmutated	Positive	Positive	8	Positive	NA
561*	del(17p)	Unmutated	Positive	Positive	100	Positive	NA
595	del(13q)	Unmutated	Positive	Positive	45	Positive	NA
814	None aberration	Unmutated	Positive	Positive	7	Positive	NA
902	del(13q), del(17p)	Unmutated	Positive	Positive	70	Positive	NA
975	del(13q)	Unmutated	Positive	Positive	7	Positive	NA
1035	+12	Unmutated	Positive	Positive	5	**Negative**	NA
1080	del(11q)	Unmutated	Positive	Positive	50	Positive	NA
1087	del(13q)	NA	Positive	Positive	50	Positive	NA
1101	del(11q)	Unmutated	Positive	Positive	50	Positive	NA
1184	del(13q), del(11q)	Unmutated	Positive	**Negative**#	–	**Negative**	3.88
1187	+12	Unmutated	Positive	Positive	7	**Negative**	NA
1193	+12	Unmutated	Positive	Positive	60	Positive	NA
1200	del(13q)	Unmutated	Positive	Positive	50	Positive	NA
1228	del(13q)	Unmutated	Positive	Positive	8	Positive	NA
1266	None aberration	Unmutated	Positive	Positive	50	Positive	NA
1274	None aberration	Unmutated	Positive	Positive	50	Positive	NA
1284	del(13q), +12, del(11q)	Unmutated	Positive	Positive	50	Positive	NA
1326	del(13q)	Mutated	Positive	Positive	30	Positive	NA
1338	None aberration	Unmutated	Positive	Positive	5	**Negative**	NA
1364	NA	Mutated	Positive	Positive	25	Positive	NA
1412	+12	Unmutated	Positive	Positive	50	Positive	NA
1457	del(11q)	Unmutated	Positive	Positive	50	Positive	NA
1518	del(13q)	Unmutated	Positive	Positive	50	Positive	NA
1583	del(13q)	Unmutated	Positive	Positive	50	Positive	NA
1586	+12	Unmutated	Positive	**Negative**#	–	**Negative**	4.05

[4,7]. Although allele-specific PCR might also be beneficial in *NOTCH1* screening due to cost and time efficiency [2,13], its utilization in samples with a low mutation burden is rather restricted [13]. To overlap the limitations of the conventional methodologies used, NGS has been proposed to detect the c.7541_7542delCT *NOTCH1* mutation in CLL [16]. In our study, ultra-deep NGS confirmed the presence of all assessed *NOTCH1* mutations including the minor background mutations, which were visible in fragment analysis only and were not recognized with Sanger sequencing or allele-specific PCR (variant allele frequency 1.90-4.36%). Although we can not decisively evaluate the occurrence of such minor *NOTCH1* mutations in the examined patients due to an absence of follow-up samples, the presence of low-abundance *NOTCH1*-mutated subclones have been repeatedly reported in CLL using NGS [7,12,16,14]. However, utilizing this approach for *NOTCH1* mutation detection in CLL is controversial, as a lot of sequencing errors given by technical issues have been documented [17].

5. Conclusion

To conclude, our study determined fragment analysis as an optimal approach for the routine detection of c.7541_7542delCT *NOTCH1* mutations in CLL. As even minor *NOTCH1* mutations (present below 10% of DNA) can be identified using this method, the results of the mutation analysis should be interpreted carefully in the context of the therapy applied and with regard to the presence of other clinically relevant defects.

Authorship and disclosures

SP was the principal investigator with primary responsibility for the paper; BK designed the study; EV, BK, BV, JK, HS-F, ED and OL performed experiments for the study; JK processed clinical data; YB, MD and JM collected patient's samples; SP coordinated the research; EV and BK wrote the manuscript.

Conflict of interests

The authors declare no conflict of interests.

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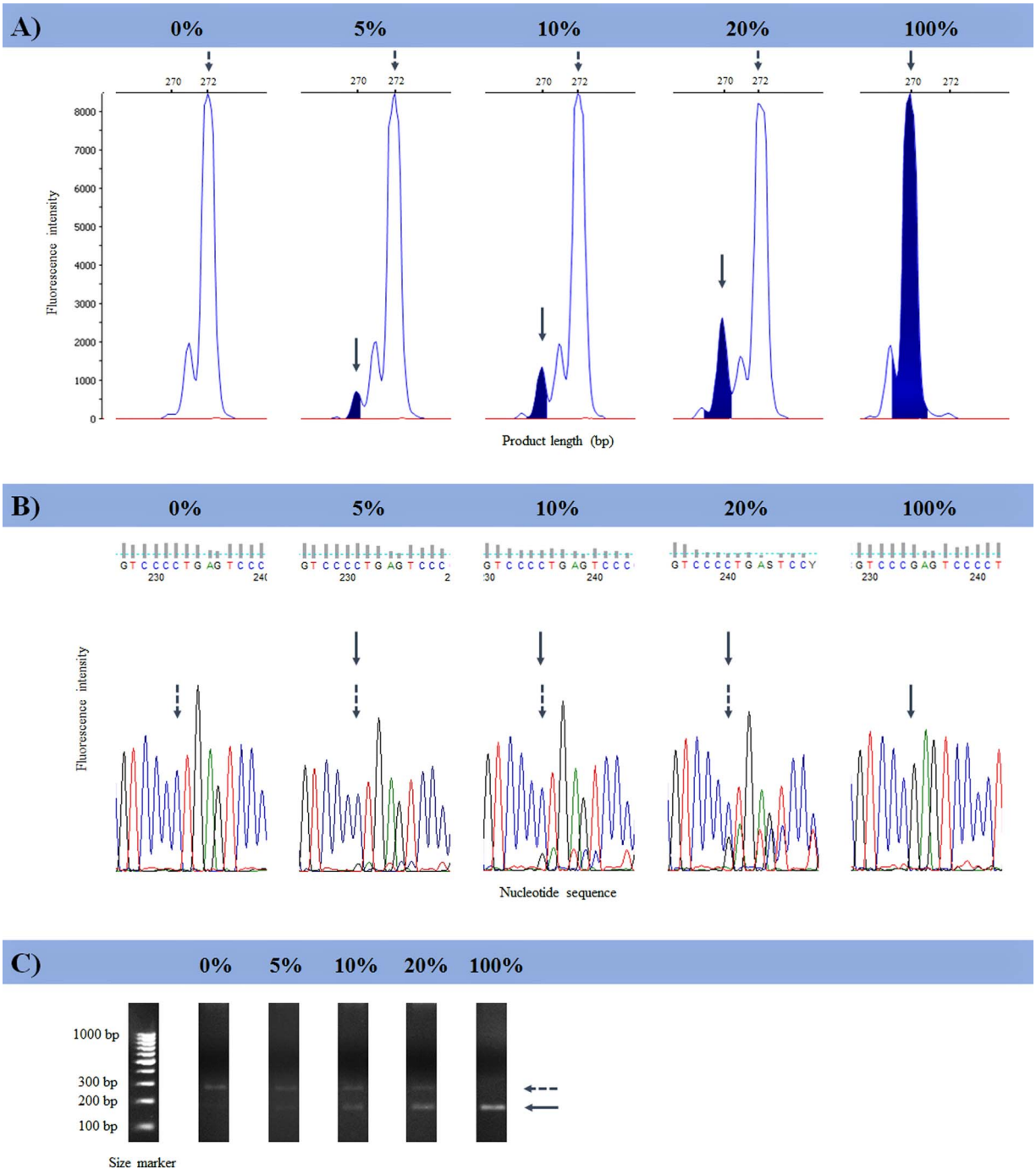

Fig. 2. Analysis of c.7541_7542delCT *NOTCH1* mutation in samples including 0%, 5%, 10%, 20% and 100% of the mutated DNA (patient no. 561) using A) fragment analysis, B) Sanger sequencing, C) allele-specific PCR visualized by agarose gel electrophoresis; the solid black arrows indicate the presence of c.7541_7542delCT *NOTCH1* mutation; the dashed black arrows correspond to the *NOTCH1*-wild-type PCR product.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.leukres.2017.08.001.

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