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Efficiency of two available kits for amplification of three EGFR SNPs in patients with NSCLC: 181946 G/A (rs2293347), –191 C/A (rs712830) and –216G/T (rs712829) with GC-rich regions

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ABSTRACT

Aim. To conduct a comparative analysis of two available kits that contain all necessary reagents and additives in a single reaction mixture, including 100 mM Tris-HCl, 100 mM KCL, 4 mM MgSO4, 0.2% of Tween 20, for the amplification of the three most common EGFR (epidermal growth factor receptor) gene polymorphisms in patients with non-small cell lung cancer (NSCLC): 181946 G/A (rs2293347), -191 C/A (rs712830), and -216G/T (rs712829).

Materials and methods. The protocol for genotyping 181946C>T, 191C>A and -216G/T was refined according to previously reported data. Polymerase chain reaction (PCR) products measuring 197 bp were detected using electrophoresis in a 2% agarose gel, followed by staining with ethidium bromide.

Results. The Biomaster HS Taq-PCR Color 2× and Biomaster LR HS PCR 2× reagent kits were effective for amplification of 181946 G/A polymorphism located in the intron of the *EGFR* gene. Additionally, polymorphisms -191 C/A (rs712829) and -216G/T (rs712829), located in the promoter region and containing a high GC content, were successfully amplified using the Biomaster LR HS PCR 2× kit.

Conclusion. The present study shows that the Biomaster HS Taq-PCR Color 2× and Biomaster LR HS PCR 2× reagent kits are effective for amplification of 181946 G/A polymorphism located in the intron of the *EGFR* gene. Furthermore, the EGFR SNP -191 C/A, located in the promoter region with a high GC content, was successfully amplified using the Biomaster LR HS PCR 2× reagent kit.

Keywords: additives, amplification, EGFR, NSCLC, polymorphism, PCR, reagents

Conflict of interest. The authors declare the absence of obvious or potential conflicts of interest related to the publication of this article.

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Conformity with the principles of ethics. All patients signed an informed consent to participate in the study. The study was approved by the local Ethics Committee at N.N. Blokhin National Medical Research Center of Oncology (Protocol No. 5 of 02.10.2022).

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Эффективность двух доступных наборов для амплификации трех нуклеотидных полиморфизмов, содержащих GC-богатые участки: 181946 G/A (rs2293347), –191 C/A (rs712830) и –216G/T (rs712829), у больных немелкоклеточным раком легкого

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РЕЗЮМЕ

Цель исследования – сравнительный анализ двух доступных наборов, содержащих в одной реакционной смеси все реагенты и добавки, включая 100 mM Tris-HCl, 100 mM KCL, 4 mM MgSO4, 0,2% of Tween 20, необходимых для амплификации трех наиболее часто встречающихся у больных немелкоклеточным раком легкого (НМРЛ) полиморфизмов гена рецептора эпидермального фактора роста EGFR: 181946 G/A (rs2293347), –191 C/A (rs712830) и –216G/T (rs712829).

Материалы и методы. Протокол для генотипирования 181946C>T, 191C>A и -216G/T был уточнен в соответствии с ранее представленными данными. Продукты полимеразной цепной реакции (ПЦР) размером 197 bp детектировали с помощью электрофореза в 2%-м агарозном геле с последующим окрашиванием этидиум бромидом.

Результаты. Наборы реактивов Biomaster HS Taq-PCR Color 2× и Biomaster LR HS PCR 2× были эффективны для амплификации 181946 G/A, локализованной в интроне гена *EGFR*. Кроме того, полиморфизмы –191 C/A (rs712829) и –216G/T (rs712829), расположенные в промоторном участке и содержащие чрезвычайно высокое количество GC, успешно амплифицировались с помощью набора Biomaster LR HS PCR 2×.

Заключение. В настоящем исследовании показано, что наборы реактивов Biomaster HS Taq-PCR Color 2× и Biomaster LR HS PCR 2× эффективны для амплификации 181946 G/A, локализованного в интроне гена *EGFR*. Кроме того, EGFR SNP -191 C/A, локализованный в промоторном участке с крайне высоким содержанием GC-пар, успешно амплифицировался с помощью набора реактивов Biomaster LR HS PCR 2x.

Ключевые слова: добавки, амплификация, EGFR, НМРЛ, полиморфизм, ПЦР, реагенты

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

The epidermal growth factor receptor (EGFR) belongs to the receptor tyrosine kinase family. It has a strong impact on cell growth and differentiation of both healthy and lung cancer cells and its oncogenic potential is recognized and established [1–3]. Several previously described *EGFR* single nucleotide polymorphisms (SNPs) are associated with regulation of receptor protein synthesis [1, 2, 4]. Based on the *EGFR* role in carcinogenesis, proliferation, and differentiation, it is important to study *EGFR* SNPs in non-small cell lung cancer (NSCLC) patients [3]. However, *EGFR* promoter region that we have studied is one of the extremely guanine-cytosine (GC) rich regions with up to 75.45% GC base pairs [5], so it required special amplification conditions.

Polymerase chain reaction (PCR) is a worldwide used technique for EGFR mutation detection. However, unique optimization of each PCR protocol is required, especially when GC rich regions are amplified, because they tend to form secondary structures that interrupt standard PCR amplification [6–8]. We have shown previously that a laborious PCR optimization strategy was especially necessary for SNPs amplification in the promoter region of *EGFR* [5, 9]. Until recently, there was no available complete reagent mixtures for amplification of these complex GC rich regions. In this study, for the first time, we have tested Biomaster LR HS PCR 2× (BiolabMix, Russia) and HS Taq-PCR Color 2× (BiolabMix, Russia) kits with reaction mixtures for amplification of three SNPs: 181946 G/A, –191 C/A, and –216 G/T. The tests were conducted via a polymerase chain reaction with restriction of fragment length polymorphism (PCR-RFLP) method.

MATERIALS AND METHODS

The protocol for genotyping 181946C>T, 191C>A and –216 G/T was refined according to previously reported data. PCR products measuring 197 bp were detected using electrophoresis in a 2% agarose gel, followed by staining with ethidium bromide.

Sample preparation. QIAamp DNA Blood Mini Kit (Qiagen, Germany) and DNA Kits (Invitrogen/Life Technologies, Carlsbad, CA, USA) where used for isolation of DNA sample from blood of healthy volunteers and formalin-fixed paraffin-embedded (FFPE) blocks from NSCLC tumor tissue. At least three analyses were performed in each group of respondents. An identical sample from each group is the sample used to determine the same polymorphism. The material was divided so that the test reagents were always tested on the same sample in order to avoid errors or false findings.

Concentration of both DNA samples was measured by Qubit® Fluorometer (Invitrogen/Life Technologies, Carlsbad, CA, USA). Local Ethics Committee approved of this study and the usage of tissue samples for scientific study.

Genotyping protocol. The protocol for 181946C>T genotyping was adjusted according to the previously reported data [4] with a few modifications. Namely, the temperature profile of PCR using KAPA Taq Hot Start PCR Kits was as follows: initial denaturation at 95 °C for 5 min; denaturation at 94 °C for 30s; annealing at 55 °C for 30s; extension at 72 °C for 60 s (in 45 cycling steps); and final extension at 72 °C for 7 min. The total volume of the PCR reaction mixture was 25 µl, with 0.5 µl of genomic DNA, 0.4 µM of each primer, 0.2 mM of each dNTPs, magnesium concentration was adjusted for 1.7 mM $MgCl_2$, and 1 U KAPA Taq DNA polymerase in 1× PCR buffer

A. 244 bp PCR products of were detected via 2% agarose gel electrophoresis with ethidium bromide staining.

For 191C>A and –216 G/T *EGFR* polymorphisms genotyping, we have applied the previously described protocol [1] with several modifications [5, 9]. All PCR reactions were performed via KAPA Taq Hot Start PCR Kits (Kapa biosystems, Boston, MA, USA). The temperature profile of PCR was as follows: initial denaturation at 95 °C for 5 min; denaturation at 94 °C for 30 s, annealing at 63 °C for 30 s, extension at 72 °C for 60s (in 45 cycling steps); and final extension at 72 °C for 7 min. The total volume of the PCR reaction mixture was 25 µl, with 0.4 µl genomic DNA, 0.4 µl of each primer, 0.2 mM of each dNTPs, 1.5 M betaine, and 1U KAPA Taq DNA polymerase in 1 × PCR buffer A (with 1.5 mM $MgCl_2$). 197 bp PCR products were detected via 2% agarose gel electrophoresis with ethidium bromide staining.

Biomaster HS Taq-PCR Color 2× (BiolabMix, Russia) and Biomaster LR HS PCR 2× (BiolabMix, Russia) containing 100 mM Tris-HCl, 100 mM KCL, 4 mM MgSO4, 0,2% of Tween 20 and DMSO were tested according to recommendations of the manufacturer and adjusted via the same PCR-RFLP protocols for both SNPs. PCRs were performed in total volume of 25 µl, with 0.4 or 0.5 µl genomic DNA and 0.4 µM of each primer. Temperature profile of the reaction was the same as previously described.

PCRs performed with this mixture had initial denaturation at 94 °C for 4 min, and the rest of the temperature profile of the reaction was the same and previously described.

Cfr42I restriction enzyme, (Fermentas/Thermo Fisher Scientific, Vilnius, Lithuania) was used for –191C>A RFLP digestion. Fast Digest TfiI, (PfeI) restriction enzyme (Fermentas/Thermo Fisher Scientific, Vilnius, Lithuania) was used for 181946C>T RFLP digestion. Products of the reaction were detected via a 3% agarose gel electrophoresis.

RESULTS

This study showed effectiveness of the studied kits for amplification of three most important *EGFR* SNPs in tissue samples of NSCLC patients containing GC rich region. For 181946 G/A, PCR analyses with both Biomaster kits produced 244 bp sized fragments (Fig. 1). Neither of the tested Biomaster kits affected subsequent RFLP analyses. Namely, RFLP products were as follows: 244 bp for 181946G and 171 bp for 181946 A (Fig. 2).

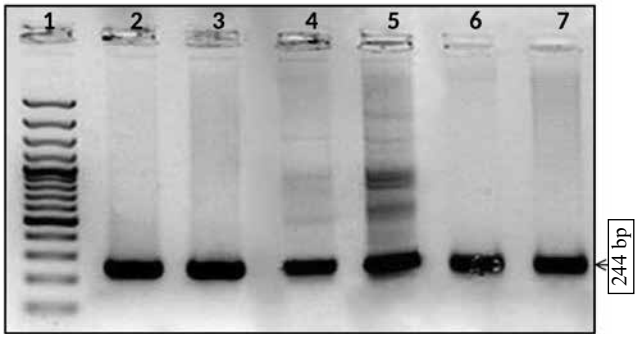

Fig. 1. PCR products of 181946G/A (D994D) (rs2293347) polymorphism amplification using Biomaster HS. Taq-PCR Color (2×) and Biomaster LR HS PCR (2×) in separate reaction mixtures. Lane 1 – markers, lane 2 – control DNA, lane 3 – NSCLC DNA, lane 4 – control DNA with Biomaster HS Taq-PCR Color (2×), lane 5 – NSCLC DNA with Biomaster HS Taq-PCR Color (2×), lane 6 – control DNA with Biomaster LR HS PCR (2×), lane 7 – NSCLC DNA with Biomaster LR HS PCR (2×)

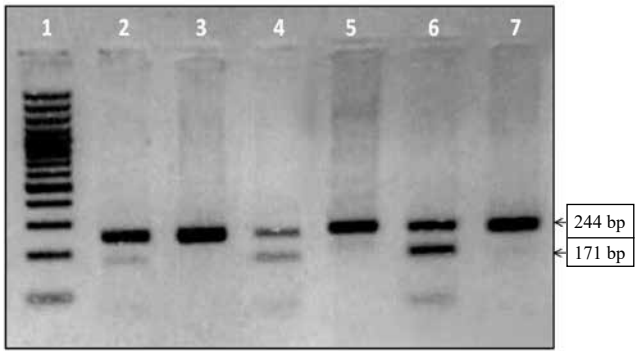

Fig. 2. PCR-RFLP products for 181946G/A (D994D) (rs2293347) polymorphism with Biomaster HS Taq-PCR. Color (2×) and Biomaster LR HS PCR (2×) in separate reaction mixtures. Lane 1 – standard, lane 2 – control DNA, clane 3 – NSCLC DNA, lane 4 – control DNA with Biomaster HS Taq-PCR Color (2×), lane 5 – NSCLC DNA with Biomaster HS Taq-PCR Color (2x), lane 6 – control DNA with Biomaster LR HS PCR (2×), lane 7 – NSCLC DNA with Biomaster LR HS PCR (2×)

Biomaster LR HS PCR 2× was tested for –191 C/A and the appropriate PCR amplification was detected (Fig. 3). Biomaster HS Taq-PCR Color 2× produced no desired 197 bp PCR products (Fig. 3). Instead, a lot of smears and extra bands above the expected band size were visible in the

agarose gel. Since there was no amplification for Biomaster HS Taq-PCR Color 2×, we have performed RFLP analysis only with Biomaster LR HS PCR 2× kit and visualized the 191 bp fragment that corresponds to wild type homozygote for both DNA samples (–-191GG) (Fig. 4). Results of PCR-RFLP products for -216G/T (rs712829) polymorphism using Biomaster LR HS PCR (2×) reaction mixture are shown in Fig. 5.

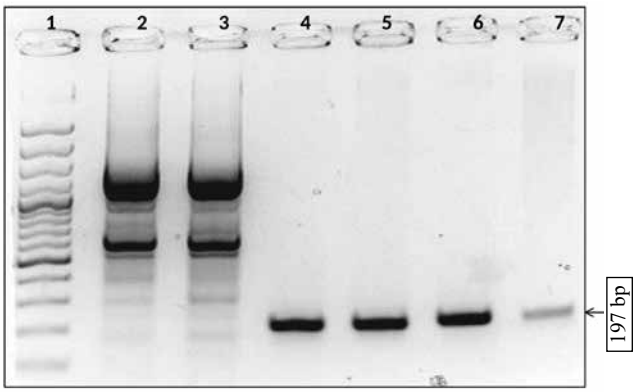

Fig. 3 PCR products for –191C/A (rs712830) and –216G/T (rs712829) polymorphisms with Biomaster HS.Taq-PCR Color (2x) and Biomaster LR HS PCR (2×) in separate reaction mixtures. Lane 1 – standard, lane 2 – control DNA with Biomaster HS Taq-PCR Color (2×), lane 3 – NSCLC DNA with Biomaster HS Taq-PCR Color (2×), lane 4 – control DNA with Biomaster LR HS PCR (2×), lane 5 – NSCLC DNA with Biomaster LR HS PCR (2×), lane 6 –control DNA,lane 7 – NSCLC DNA

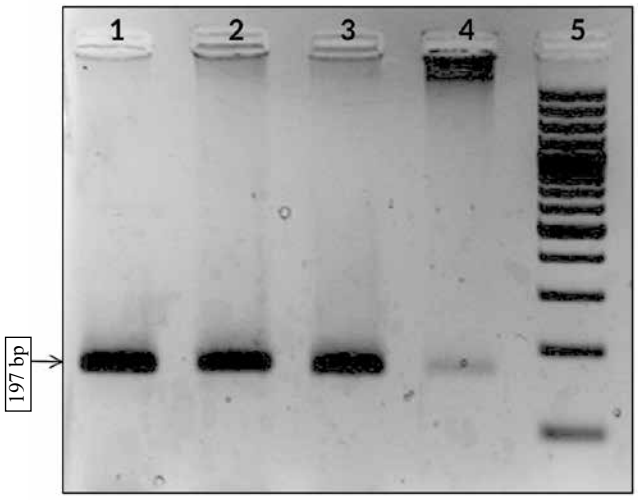

Fig. 4 PCR-RFLP products for –191C/A (rs712830) polymorphism with Biomaster LR HS PCR reaction. Lane 1 – control DNA with Biomaster LR HS PCR (2×), lane 2 – NSCLC DNA with Biomaster LR HS PCR (2×), lane 3 – control DNA, lane 4 – NSCLC DNA, lane 5 – marker

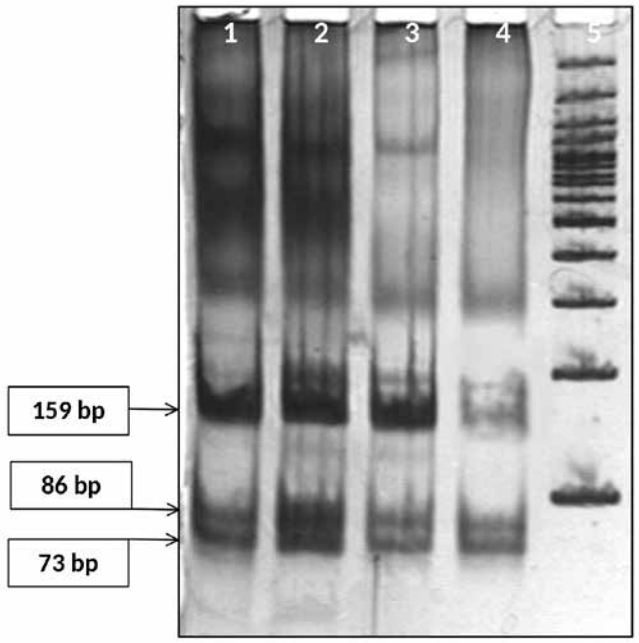

Fig. 5 PCR-RFLP products for –216G/T (rs712829) polymorphism with Biomaster LR HS PCR reaction mixture (2×). Lane 1 – control DNA with Biomaster LR HS PCR (2×), lane 2 – NSCLC DNA with Biomaster LR HS PCR (2×), lane 3 – control DNA, lane 4 – NSCLC DNA, lane 5 – marker

DISCUSSION

Conventional PCR protocol is essential for a wide range of biological experiments, including the study of EGFR, but every single PCR requires specific set of conditions. Although it might be time- and cost-consuming – from designing primers, setting up materials, reagents, reaction mixtures, thermal cycling conditions etc. to troubleshooting strategy – optimization of PCR is inevitable, especially when GC rich regions are amplified [3, 5, 10]. These regions with a high number of GC base pairs form secondary structures, show resistance to denaturation, and lead to incorrect primer attachment.

Several attempts were made to resolve these problems, including modification of primers or temperature conditions [11], and usage of different additives [7, 9]. Enhancing reagents are usually used to increase yield and specificity of the reaction in such complex DNA templates [6, 8, 11–13]. Previously we have adjusted appropriate DNA template concentration, thermal cycling conditions, and optimal $MgCl_2$ concentration for PCR amplification of *EGFR* SNPs in promoter regions with GC content up to 75.45% [3, 5] and tested the effects of different additives that enhance PCR specificity [7, 9].

In this study, we reported the benefits of Biomaster kits usage since both of them include all of the components necessary for PCR reaction including additive and all reagents in one mixture and, thus, minimize the risk of contamination. In addition, the master mix preparation time is reduced. The results of this study showed that both of the Biomaster kits produced a more successful PCR reaction when compared to PCR reactions without them. Biomaster LR HS PCR 2× has a combination of HS-Taq and Pfu DNA polymerases with stabilizers of DNA polymerases and appropriated additives that help to conduct a PCR reaction. It even contains 0.2% of Tween 20 that was shown to reduce the number of unspecific bands [14]. The manufacturer suggested adding a little more DMSO if necessary, as it was previously confirmed to be an important part of a PCR reaction. PCR buffers used for master mix usually include $MgCl_2$, while Biomaster LR HS PCR 2× buffer contains $MgSO_4$, which might increase polymerase activity [8].

This kit is suitable for long, complex regions and for GC rich fragments. We also demonstrate that the expected PCR-RFLP products for −191C>A from lung cancer sample were visualized via gel electrophoresis (Fig. 3 and 4). It was not even a long PCR fragment (244 bp). This kit, as well as Biomaster HS Taq-PCR Color 2×, worked properly for 181946 G/A (Fig. 1 and 2).

Biomaster HS Taq-PCR Color 2× is used for conventional PCR and for templates up to 5 kbp in length. It already contains dyes so there is no need to load a buffer during gel electrophoresis. Due to its high density, reaction mixture sinks in the well of the agarose gel easier. This kit is not suitable for −191C>A because it lacked to produce desired PCR products (Fig. 3).

CONCLUSION

In this study, we have shown that Biomaster HS Taq-PCR Color 2× and Biomaster LR HS PCR 2× are effective for 181946 G/A amplification located in the introne of the *EGFR* gene. In addition, *EGFR* SNP –191C/A, located in the promoter region with extremely high GC content, was successfully amplified with Biomaster LR HS PCR 2×.

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Authors' contribution

Jurisic V. – concept and design development of the article, sample preparation, collection of material, performing the biochemical section of the study; literature analysis; data analysis and interpretation; writing and designing the text of the manuscript. Obradovic J. – sample preparation, performing the biochemical section of the research. Tosic N. – performing the section of radiation studies. Pavlovic S. – performing the biochemical section of the study. Gulyaeva L.F. – collection of material. Gershtein E.S. – translation of the article; participation in the concept development of the article. Kushlinskii N.E. – scientific content editing; approval of the final version of the manuscript.

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