*Technical paper*

# High Resolution Melting Curve Analysis for High-Throughput SNP Genotyping in IL23R Gene and Association of IL23R with Slovenian Inflammatory Bowel Diseases Patients

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## Abstract

Single nucleotide polymorphism (SNP) analysis is important tool in the studies of genetic factors associated with complex diseases and with genetically influenced response to drug therapy (pharmacogenetics). Recently, a new generation of generic dsDNA binding dyes (LCGreen$^{TM}$) contributed to the development of fast and low-cost method for SNP detection and/or genotyping based on high resolution melting (HRM) analysis. The aim of our study was to develop HRM assay for *IL23R* gene (rs7517847) and to perform association study in Slovenian inflammatory bowel diseases (IBD) patients. We genotyped 345 Slovenian healthy controls and 295 IBD patients including 159 with Crohn's disease (CD) and 136 with ulcerative colitis (UC) for rs7517847 polymorphism in *IL23R* gene using standard RFLP and optimized HRM methods.

In this study, we showed, that HRM is a simple, fast and reliable method for genotyping of clinical samples where homozygotes (GG and TT) were determined by »Tm calling method« and difference between homozygotes and heterozygotes was determined by different melting curve shape using »gene scanning method«. With combination of results from »Tm calling« and »gene scanning« methods, we achieved 98,6% concordance between PCR-RFLP and PCR-HRM results, based on the analysis of 640 samples. We found statistically significant association of *IL23R* polymorphism with Slovenian Crohn's disease patients when comparing genotype and allele frequencies between CD patients and controls. Allele frequency of minor allele G was 0,46 in controls and was reduced to 0,33 in CD patients ($p < 0,001$, OR = 0,588). The frequency of T/T genotype carriers was higher in CD patients (50,3%) than in controls (26,7%, $p = 0,002$, OR = 2,558). We found weak association between *IL23R* polymorphism and Slovenian UC patients. Carriers of T/T genotype have higher risk for UC ($p = 0,035$, OR = 1,599). These results suggest *IL23R* plays important role in CD and UC development in Slovenian patients.

**Keywords**: SNP genotyping, high resolution melting, DNA dyes, inflammatory bowel diseases, LC Green Plus

## 1. Introduction

Single nucleotide polymorphisms (SNPs) are a powerful tool in genetic association studies, where they are commonly used as markers in research of complex diseases. One example of a typical complex disease, where environmental factors and several genes play role, are human inflammatory bowel diseases (IBD), usually classified into Crohn's disease (CD) and ulcerative colitis (UC). Although the precise cause of IBD is not known yet, a number of association studies and genome-wide association studies[1–6] confirmed several genes involved in a risk and the pathogenesis of the disease, including *ATG16L1, IL23R, NOD2* and *PTGER*. *IL23R* is one of the most potent IBD genes, as reported from a recent genome-wide

study (odds ratio = 2, 50),[7] and codes for the interleukin 23 receptor, which is present on the surface of several types of immune cells, including T – cells, natural killer cells, monocytes, and dendritic cells.[8] Several SNPs in or near the gene region have been found to influence the risk of developing CD. This association has been found primarily in Caucasian populations, where intronic variant rs7517847 was reported to be the most significant.[9, 10, 11] Several other reports have also confirmed the association of *IL23R* variants with other auto-inflammatory conditions, such as psoriasis[12] and ankylosing spondylitis.[13] In order to confirm genotype – phenotype correlation in the above mentioned diseases, genotyping of individuals for *IL23R* SNPs is essential.

There are many methods for the SNP genotyping; however, the most of these techniques require an additional separation step that makes them less favorable for high-throughput genotyping. Examples of such methods are single-strand conformation polymorphism analysis,[14] denaturing gradient gel electrophoresis,[15] restriction endonuclease analysis and denaturing HPLC.[16] In contrast; closed-tube systems enable automation, greatly decrease the risk of laboratory contamination of PCR products and significantly reduce analysis time. Melting curve data acquisition and analysis, as an example of closed-tube system, could be performed in less than 10 min after PCR. Conventional closed-tube genotyping techniques, however, require fluorescently labeled probes, which are costly and capable of detecting only a single allele.[17] On the contrary, high-resolution melting (HRM) does not require expensive fluorescent labels and unlabeled probes.[18] The power of the DNA melting analysis depends on the instrument resolution,[19] double stranded DNA (dsDNA) dye[20] and purity of the PCR product. In HRM analysis, heterozygotes are particularly easily differentiated from homozygotes, because the heteroduplexes formed before the melting step has a characteristic melting profile. Homozygotes are more difficulty differentiated because their melting curves are usually very similar, often with the small differences in their melting temperatures (Tm).[21] Approximately 84% of all human SNPs result in an A:T to G:C interchange with a Tm difference of approx. 1 °C in short amplicons (< 100 bp). In the remaining 16%, the base pair is inverted or neutral (e.g. A:T to T:A or G:C to C:G) and the Tm difference is smaller. In approx. 4% of human SNPs, nearest-neighbor symmetry calculations predict no difference in Tm[22]. In such a case, mixing with known genotype is necessary for complete genotyping. According to amplification of a heterozygote, SNPs are classified into four classes, which result from grouping of six different binary combinations of bases by homoduplex and heteroduplex products. The *IL23R* variant (rs7517847) belongs to 2nd SNP class, where the predicted Tm difference between homozygotes ranges from 0,5–1,4 °C.[21] The aim of this study was to conduct a case-control association study of *IL23R* variant on a cohort of 295 Slovenian IBD patients and 345 healthy controls and to explore if the variant influences the risk for developing IBD. We used HRM to develop reliable and low-cost SNP genotyping assay that did not require additional labeled probes or primers. This was accomplished by designing PCR assays for one short (87 bp) and for one medium-sized (259 bp) amplicon that harbored *IL23R* SNP (rs7517847). Additionally, two assays were designed to test the influence of amplicon size on HRM genotyping resolution efficiency.

# 2. Experimental

## 2. 1. Materials and Methods

### 2. 1. 1. Samples and DNA Extraction

In this study, we have enrolled 295 Slovenian patients with IBD, including 159 with CD and 136 with UC, as described previously.[23] In brief, 49% of patients in this study were male and 51% female. The mean age of patients was 38 years (38,6 +/– 14 years) and mean age of diagnosis was 27 years (27,17 +/–12,16 years). Study was approved by the Ethical Committee of the Republic of Slovenia (approval No. 57/03/20 of March 21, 2000). Informed consent was obtained from all patients.

The DNA from, IBD patients was isolated from paraffin-embedded biopsy sections after tissue digestion using standard phenol/chloroform extraction and ethanol precipitation. The DNA from 345 unaffected and unrelated blood donors was extracted from the whole blood lymphocytes according to manufacturer's protocol using a combination of Ficoll-Paque PLUS (GE Healthcare Bio-Sciences, Uppsala, Sweden) and TRI REAGENT (Sigma-Aldrich, Saint Louis, Missouri, USA) reagents. The final DNA concentration ranged from 20 – 50 ng/μL as determined by absorbance at 260 nm.

We used CEPH DNA (http://www.cephb.fr/en/cephdb) as reference in HRM optimization process. Genotypes for CEPH samples were obtained from HapMap project (http://www.hapmap.org/cgi-perl/gbrowse/hapmap26_B36).

### 2. 1. 2. Primer Design

Primers were designed using Primer3 software (MIT Center for Genomic Research, Cambridge, MA, USA, http://fokker.wi.mit.edu/primer3/input.htm) and synthesized by standard phosphoramidite chemistry (Invitrogen, Carlsbad, USA). Primer sequences were analyzed to minimize the likelihood that undesired products would co-amplify and interfere with the target sequence melting curves. Sequence variations were positioned at the center of amplicons. Table 1 shows PCR primer sequences and amplicon lengths.

We designed two different pairs of primers to acquire two *IL23R* amplicons with different lengths. Primer

**Table 1.** Primers used for real-time PCR amplification and SNP genotyping.

| Gene | Sense primer 5' – > 3' | Antisense primer 5' – > 3' | Amplicon length (bp) |
|---|---|---|---|
| IL23R | CCATCTCACTGTCTCCTCTC | GGCTCCAGTTTCTAGCCTAC | 87 |
| IL23R | TCTGCCAATTCCCTAAAC | AAGTAGGTGTGGATTGCC | 259 |

pair that resulted in 87 bp long amplicon was used in HRM. As the amplicon length increases, the difference in Tm between genotypes becomes smaller.[24] Primer pair that resulted in 259 bp long amplicon was used in RFLP. The 259 bp long amplicon was cleaved with restriction enzyme to produce fragments with different lengths needed for discrimination between genotypes. Both sets of primers were used to test the influence of amplicon size on HRM genotyping efficiency resolution.

### 2. 1. 3 PCR-RFLP

PCR amplification and restriction fragment length polymorphism (RFLP) were used to obtain reference genotypes from CEPH reference DNA samples and from DNA samples of patients and controls. PCR was performed in 0,2 mL PCR strip tubes on T1 Thermocycler (Biometra, Germany), using 10-µL final reaction volumes with 2 µL of template DNA (20–50 ng/µL). The reaction mixture contained 1 x PCR Buffer, 2 mM $MgCl_2$, 0,2 mM of each dNTP, 0,5 µM of each primer, 0,5 U of *Taq* Polymerase (Fermentas, Lithuania) and PCR grade water. Cycling conditions were performed using the following protocol: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation (95 °C, 30 s), annealing (55 °C, 30 s), extension (72 °C, 30 s) and final extension (72 °C, 5 min). After amplification the resulting PCR products were mixed with restriction enzyme *Hpy* F3I (Fermentas, Lithuania) and cleaved at 37 °C for 16 hours. Resulting fragments were separated by length on agarose gel electrophoresis.

### 2. 1. 4 PCR-HRM

PCR and HRM reactions were performed on a LightCycler 480 2.0 instrument (Roche Diagnostics, Indianapolis, IN, USA), using 10-µL reaction volumes with 2 µL of DNA, 1 x PCR Buffer, 3 mM $MgCl_2$, 0,2 mM of each dNTP, 0,5 µM of each HRM primer, 1 x LC Green Plus (Idaho Technology, USA) and 0,5 U of *Taq* Polymerase (Fermentas, Lithuania). Cycling conditions were performed using the following protocol: initial denaturation at 95 °C for 1 min, followed by 40 cycles of denaturation (95 °C, 1s), annealing (58 °C, 1s) and extension (72 °C, 10s). After amplification, the samples were heated to 95 °C for 1 min and rapidly cooled to 40 °C for 1 min at rate of 1 °C/s, to induce heteroduplex formation before melting. Melting curve data were obtained by continuous fluorescence acquisition from 55 to 90 °C with a thermal transition rate of 0,1 °C/s. Genotyping was based on negative first derivate melting curves and comparison of unknowns to genotyped controls. Data were analyzed using software provided with the LC480 instrument, where two methods were used to determine sample genotypes. »Gene scanning« method is based on normalization, temperature shift of fluorescence data and calculation of difference plot to discriminate between different genotypes. »Tm calling« method, on the other hand, is based on calculation of negative of the first derivative of the fluorescence data, where melting curve peaks are obtained to discriminate between different genotypes.

## 3. Results and Discussion

### 3. 1. High Resolution Melting Analysis of *IL23R*

High-resolution dye LCGreen Plus used in our study allowed the identification of both heterozygous and homozygous samples, thus making scanning and genotyping of PCR products possible. Although both, 87 bp and 259 bp amplicons were tested for HRM analysis, only genotype data from the shorter amplicon was in full coherence with the results obtained by RFLP analysis. Altogether 640 samples were genotyped by PCR-RFLP and PCR-HRM methods. Melting curve analysis of 87 bp amplicon by »gene scanning« method is shown in Figures 1A, B, C, D.

The original HRM data (Figure 1A) were normalized by defining linear baselines before and after melting transitions, which were designated values of 100% and 0%, respectively. Within each sample, the fluorescence of each acquisition was calculated as a percentage of fluorescence between the top and bottom baselines at each acquisition temperature, as depicted on Figure 1B.

Different genotypes were easily distinguished after normalization (Figure 1B). Normalized melting curves were adjusted to eliminate slight temperature and salt variation between samples by shifting each curve along the temperature axis for 5% of normalized fluorescence, as shown in Figure 1C.

Figure 1C shows, that heterozygotes (G/T) have different melting curve shapes and were unambiguously distinguished from homozygotes G/G and T/T. Note that homozygotes G/G and T/T have similar curve shape, whereas heterozygotes G/T were in between with broader melting transition. To further discriminate between genotypes, difference plots were obtained by subtracting the temperature-overlaid, normalized curves from one of the wild-type (G/G) curves, as shown in Figure 1D.

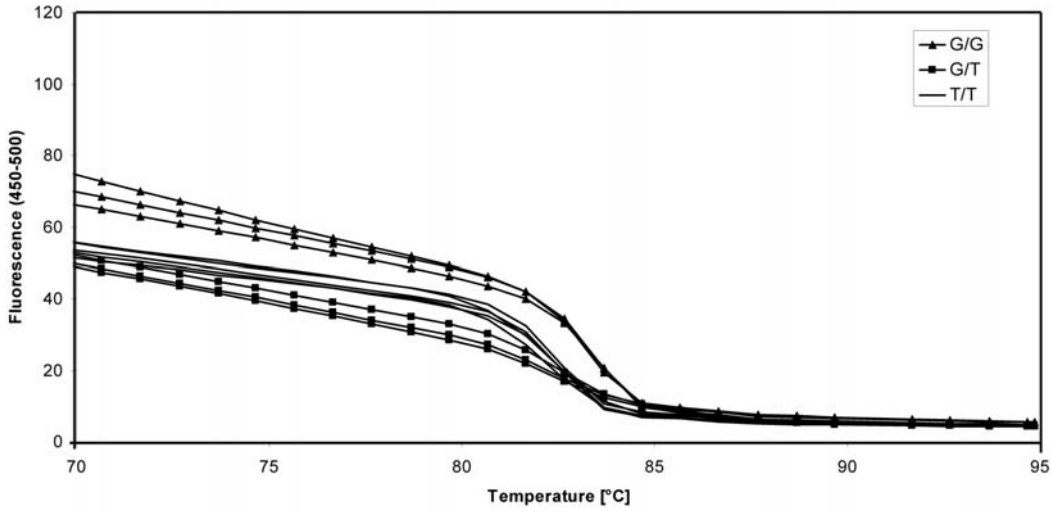



**Figure 1A.** Original HRM data. Melting curves include three different individuals for each of three genotypes and each individual run in triplicate.

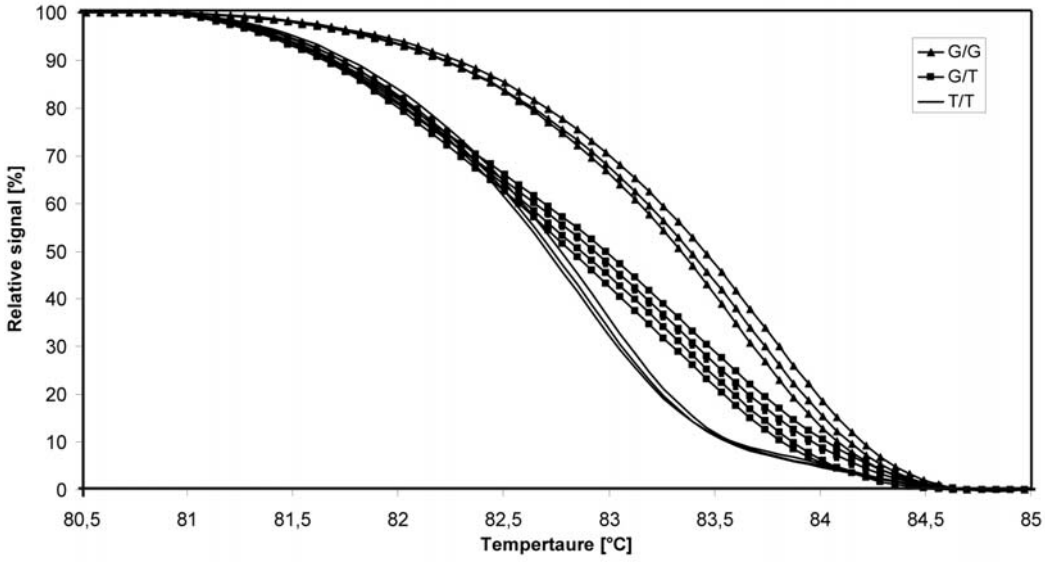



**Figure 1B.** Normalized HRM data.

The average peak of relative signal difference in Figure 1D for T/T homozygotes was at 83,19 °C, and 82,44 °C for G/T heterozygotes. Note that, because of the shift in the temperature of the curves in Figures 1C and D, the temperature axis no longer reflects absolute temperatures, but rather reflects temperature differences relative to superimposed segments of the curves. A total of 627 of 640 tested samples (98%) were determined correctly by HRM »gene scanning« method when compared to RFLP results. Four homozygotes G/G (0,6%), were incorrectly grouped as T/T homozygotes and were subsequently correctly determined by »Tm calling« method. The other nine samples (1,4 %) were negative and subsequent measurements on spectrophotometer showed poor A260/280 (0,56–1,14) and A260/230 (0,44–0,98) ratios, suggesting low DNA quality and the presence of small molecule contaminants in the DNA preparation.

We also performed the alternative »Tm calling« method to additionally differentiate between genotypes as shown in Figure 1E

Figure 1E shows that homozygotes G/G and T/T melted at different temperatures and could be easily distinguished from each other. The data obtained from »Tm calling« method are summarized in Table 2.

The calculation of theoretical Tm is based on nearest-neighbor thermodynamic model, but practical Tm is the one measured by the instrument and could differ from theoretical Tm due to fluctuations in reaction conditions (e.g. salt concentration), that influence melting and are not taken into account in the model calculation.

The efficiency of Tm calling method for 12 and 92 samples was calculated by comparing genotyping results with results from RFLP. The efficiency of ho-

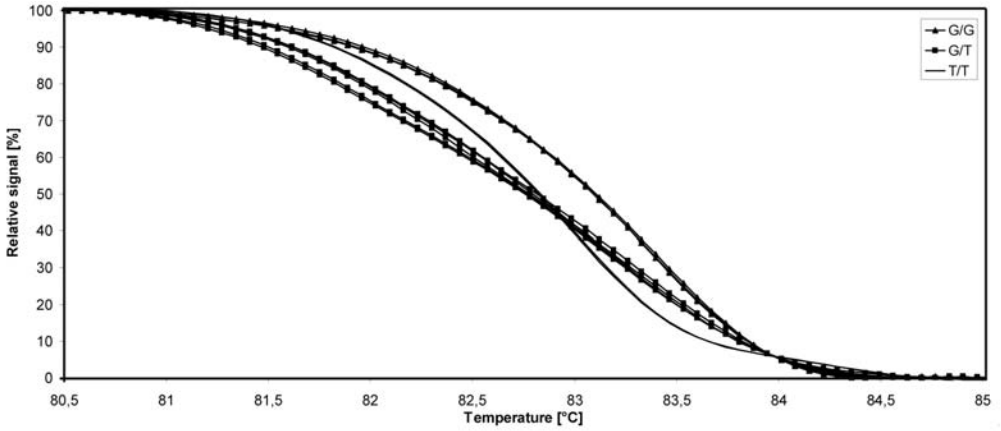



**Figure 1C.** Normalized and temperature shifted HRM data.

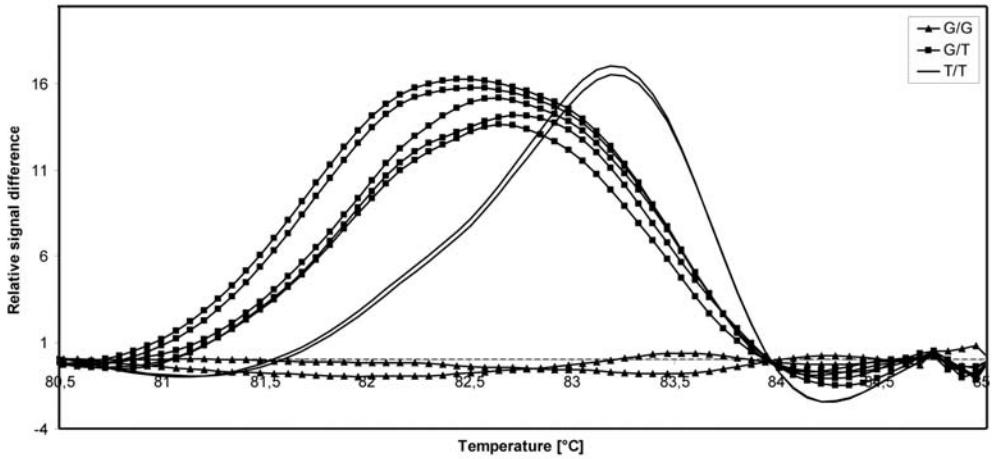



**Figure 1D.** Normalized and temperature shifted HRM difference plot.

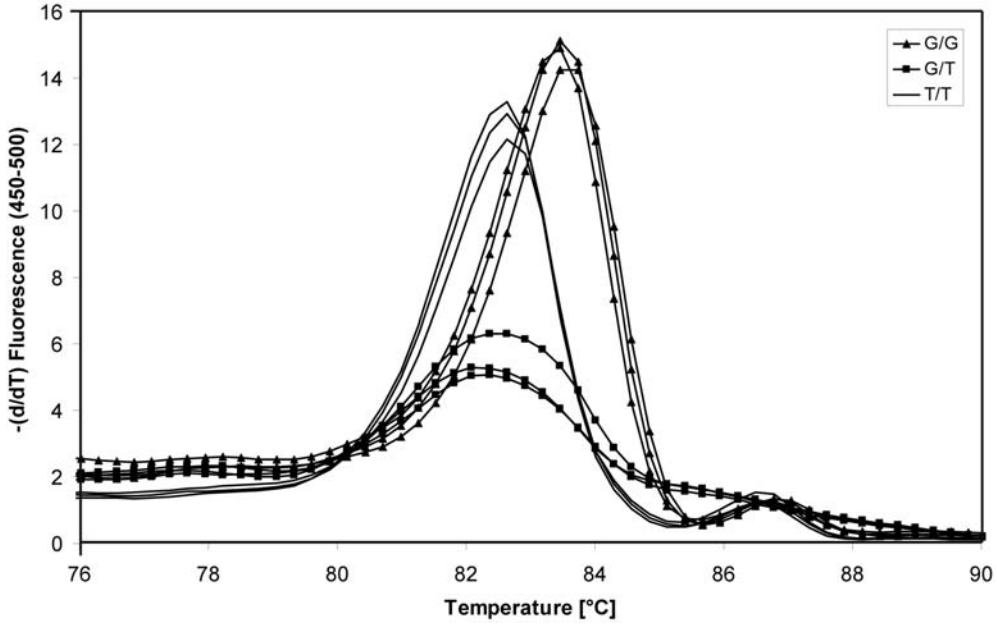



**Figure 1E.** Derivative melting curves of amplicon (87bp) melting for genotyping of the IL23R gene.

**Table 2.** Tm calling data.

| No. Samples | $T_m$ [°C] (theoretical) | $\Delta T_m$ [°C] (theoretical) | $T_m$ [°C] (practical) | $DT_m$ [°C] (practical) | STD | Homozygotes differentiation | Heterozygotes differentiation |
|---|---|---|---|---|---|---|---|
| 12 | G: 72,9<br>T: 72,4 | 0,5 | G:82,90<br>T: 82,37 | 0,53 | G:0,40<br>T: 0,18 | 100% | 100% |
| 92 | G: 72,9<br>T: 72,4 | 0,5 | G:83,07<br>T: 82,44 | 0,63 | G:0,44<br>T: 0,33 | 95% | 79% |

*STD = standard deviation. * $T_m$ = melting temperature.
*$\Delta T_m$ = difference in melting temperatures (e.g. between G/G and T/T genotypes).

mozygote determination by Tm calling method was 100% in our initial analysis of 12 samples, however when the sample size increased to 92 samples the efficiency decreased to 95%. The efficiency of heterozygote determination was 100% in 12 samples analyzed, yet it decreased to 79% in 92 samples analyzed. Although »Tm calling« did not prove as a very robust method, it was successfully used to complement »gene scanning« method.

With combination of results from »Tm calling« and »gene scanning« methods, we achieved 98,6% concordance between PCR-RFLP and PCR-HRM results based on the analysis of 640 samples. Using combination of both, »gene scanning« and »Tm calling« methods we were able to unambiguously distinguish different genotypes, therefore no mixing of DNA samples with known genotypes (spike-in principle) was needed to enhance the differences between the melting curves.

### 3. 2. RFLP Analysis

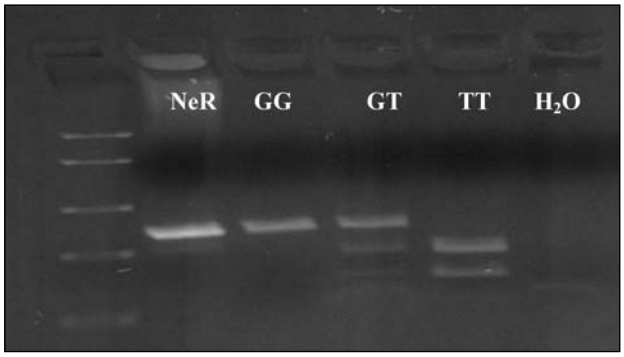
The restriction analysis of *IL23R* gene revealed 259 bases long fragment for G/G genotype, 168 and 91 bases long fragments for T/T genotype and 259, 168 and 91 bases long fragments for G/T genotype as shown on Figure 2. RFLP was used as a reference method to evaluate the accuracy of high resolution amplicon melting. 345 Slovenian healthy controls and a total of 295 IBD patients were genotyped by RFLP method.

## 3. 3. Association Study Analysis

We genotyped 345 Slovenian healthy controls and a total of 295 IBD patients (159 with CD and 136 UC), for intronic rs7517847 polymorphism in *IL23R* gene. We found statistically significant association of *IL23R* polymorphism with Crohn's disease patients when comparing genotype and allele frequencies between CD patients and controls (Tables 3 and 4).

The allele frequency of minor allele G was 0,46 in controls and was reduced to 0,33 in CD patients ($p < 0,001$, OR = 0,588). The frequency of protective allele G was also decreased in case of UC compared to controls, but only weak association was found ($p = 0,13$, OR = 1,252), suggesting a significantly less important protective effect of the minor allele for UC compared with CD.

As shown in Table 4, the frequency of T/T genotype carriers was significantly higher in CD patients (50,3%) than in controls (26,7%, $p = 0,002$, OR = 2,558). The frequency of T/T genotype carriers was also significantly higher in UC patients ($p = 0,035$, OR = 1,599) compared to healthy controls, suggesting a major effect on susceptibility to CD and a more modest effect on UC. Our results are in concordance with two other association studies, in Caucasians in Great Britain and in Ashkenazi Jews in the USA, which also confirmed the association of *IL23R* variant with IBD.[10,11] Compared to Slovenian healthy controls, the allele frequencies of minor allele G were similar in both healthy populations (SLO: 0,45; GB: 0,45; USA, 0,44;). Interestingly, the allele frequency of the minor allele G was also very similar in both populations of IBD



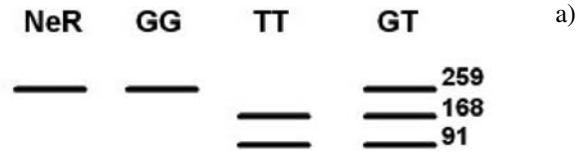




**Figure 2.** Restriction analysis of IL23R gene (rs7517847). a) Restriction scheme for IL23R gene, where NeR represents unrestricted sample, G/G, G/T and T/T genotype standards and $H_2O$ a blank sample.

**Table 3.** Case–Control Allele Frequencies of *IL23R* (rs7517847).

| Patients | Allele frequencies – controls (n = 345) | Allele frequencies – patients | p-value* | Confidence interval 95% | OR* |
|---|---|---|---|---|---|
| IBD (n = 295) | G:0,46; T:0,54 | G:0,38; T:0,62 | **0,006** | 0,310–0,826 | 1,382 |
| CD (n = 159) | G:0,46; T:0,54 | G:0,33; T:0,67 | **< 0,001** | 0,466–0,776 | 0,588 |
| UC (n = 136) | G:0,46; T:0,54 | G:0,40; T:0,60 | 0,13 | 0,942–1,664 | 1,252 |

* P-values were obtained with Fisher exact test in SPSS v. 14.00 software.
* OR = odds ratio
* n = number of individuals included in study

**Table 4.** Case–Control Genotype Frequencies of *IL23R* (rs7517847).

| Patients | Genotype frequencies – controls (n = 345) | Genotype frequencies – patients | Patients vs controls (TT vs GT&GG)** |
|---|---|---|---|
| IBD (n = 295) | GG:18,6%<br>GT:54,8%<br>TT:26,7% | GG:14,6%<br>GT:47,0%<br>TT:38,4% | **p = 0,002**<br>OR = 1,717<br>1,224–2,409 (95% CI) |
| CD (n = 159) | GG:18,6%<br>GT:54,8%<br>TT:26,7% | GG:8,2%<br>GT:41,5%<br>TT:50,3% | **p = 0,002**<br>OR = 2,558<br>1,346–4,797 (95% CI) |
| UC (n = 136) | GG:18,6%<br>GT:54,8%<br>TT:26,7% | GG:17,6%<br>GT:45,6%<br>TT:36,8% | **p = 0,035**<br>OR = 1,599<br>1,048–2,439 (95% CI) |

*P-values were obtained with Fisher exact test in SPSS v. 14.00 software.
*OR = odds ratio
*n = number of individuals included in study
**only the recessive model for T allele (TT vs GT&GG) was used in the association between IL23R rs7517847 SNP and IBD according to previous study[11]

patients when compared to Slovenian IBD patients. (SLO: 0,38; B: 0,34; USA, 0,33).

## 4. Conclusions

We have developed HRM genotyping assay for *IL23R* variant rs7517847 for genotyping of clinical samples and proved 98,6 % efficiency of developed HRM assay, when using RFLP as a reference genotyping method. With HRM analysis, differences between homozygotes and heterozygotes were easily distinguished by different Tms and melting curve shapes, therefore no mixing of DNA samples with known genotype with amplicons of unknown genotypes was needed to enhance the differences between melting curves. We found statistically significant association of *IL23R* polymorphism with CD patients when comparing genotype and allele frequencies and with UC patients when comparing genotype frequencies to healthy controls. These results suggest that *IL23R* gene has a major effect on susceptibility to CD and a more modest effect on UC in Slovenian population.

**List of abbreviations**

| | |
|---|---|
| CD | Crohn's disease |
| dsDNA | double stranded DNA |
| $\Delta T_m$, | Difference in melting temperatures (e.g. between G/G and T/T) |
| GB, | Great Britain |
| HPLC, | High-pressure liquid chromatography |
| HRM, | High resolution melting |
| IBD, | Inflammatory bowel disease |
| PCR, | Polymerase chain reaction |
| RFLP, | Restriction fragment length polymorphism |
| SLO, | Slovenia |
| SNP, | Single nucleotide polymorphism |
| Tm, | Melting temperature |
| UC, | Ulcerative colitis |
| USA, | United States of America |

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## Povzetek

Analiza polimorfizmov posameznega nukleotida (ang. SNP za single nucleotide polymorphism) in mutacij je ključnega pomena pri ugotavljanju genetskih dejavnikov tveganja za nastanka kompleksnih bolezni in genetsko pogojenega odziva posameznikov na zdravila (farmakogenetika). Razvoj nove generacije fluorescentnih označevalcev (LCGreen), ki se z večjo afiniteto vežejo na dvojno vijačnico DNK, je omogočil razvoj nove metode za hitro in cenovno ugodno analizo polimorfizmov SNP in mutacij na osnovi analize talilne krivulje visoke ločljivosti (ang. HRM za High-resolution melting). Namen naše študije je bil razvoj metode HRM za gensko tipizacijo polimorfizma rs7517847 v genu *IL23R* in izvedba asociacijske študije za omenjeni polimorfizem pri slovenskih bolnikih s kronično vnetno črevesno boleznijo (KVČB). V asociacijski študiji smo z uporabo standardne metode PCR-RFLP in optimizirane metode PCR-HRM določili genotipe za polimorfizem gena *IL23R* (rs7517847) za 345 zdravih posameznikov in za 295 bolnikov s KVČB, med katerimi je bilo 159 bolnikov s Crohnovo boleznijo (CB) in 136 bolnikov z ulceroznim kolitisom (UK). Ugotovili smo, da je HRM enostavna, hitra in zanesljiva metoda za določanje genotipov v kliničnih vzorcih. Razlike med skupinama homozigotov (GG in TT) smo ugotavljali z algoritmom »Tm calling«, heterozigote in homozigote pa smo razlikovali na osnovi različnih oblik talilnih krivulj z algoritmom »gene scanning«. S kombinacijo obeh algoritmov za analizo HRM podatkov smo določili genotipe za 640 vzorcev in ugotovili 98,6% skladnost z genotipi, ki so bili določeni z referenčno RFLP metoda na istih vzorcih. V asociacijski študiji smo s primerjavo alelnih in genotipskih frekvenc med zdravimi posamezniki in bolniki s CB odkrili statistično značilno povezavo med polimorfizmom gena *IL23R* in skupino bolnikov s CB. Pri zdravih posameznikih je bila alelna frekvenca alela G 0,46, pri bolnikih s CB pa 0,33 ($p < 0,001$, OR = 0,588). Frekvenca posameznikov z genotipom T/T je bila pri bolnikih s CB (50,3%) višja kot pri skupini zdravih posameznikov (26,7%, $p = 0,002$, OR = 2,558). Odkrili smo tudi šibko povezavo med polimorfizmom gena *IL23R* in bolniki z UC, kjer so imeli nosilci genotipa T/T višje tveganje za UC ($p = 0,035$, OR = 1,599). Ti rezultati kažejo, da igra gen *IL23R* pomembno vlogo v patogenezi pri slovenskih bolnikih s CB in UC.