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High-resolution melting curve analysis for high-throughput genotyping of NOD2/CARD15 mutations and distribution of these mutations in Slovenian inflammatory bowel diseases patients

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Abstract. Inflammatory bowel diseases (IBD) are usually classified into Crohn's disease (CD) and ulcerative colitis (UC). *NOD2/CARD15* was the first identified CD-susceptibility gene and was confirmed as the most potent disease gene in CD pathogenesis. Three *NOD2/CARD15* variants, namely two missense polymorphisms R702W (rs2066844) and G908R (rs2066845), and a frame shift polymorphism L1007fs (rs2066847), were associated with CD in Caucasian populations. High resolution melting analysis (HRMA) with saturation LCGreen dyes was previously reported as a simple, inexpensive, accurate and sensitive method for genotyping and/or scanning of rare variants. For this reasons we used qPCR-HRMA for genotyping *NOD2/CARD15* variants in 588 Slovenian IBD patients and 256 healthy controls. PCR-RFLP was used as a reference method for genotyping of clinical samples. The optimization of an HRM experiment required careful design and adjustment of main parameters, such as primer concentration, $MgCl_2$ concentration, probe design and template DNA concentration. Different HRMA approaches were tested and used to develop a reliable and low-cost SNP genotyping assays for polymorphisms in *NOD2/CARD15* gene. Direct HRMA was the fastest and cheapest HRMA approach for L1007fs and R702W polymorphisms, yet for G908R polymorphism sufficient reliability was achieved after introduction of unlabeled probe. In association analysis, we found statistically significant association of L1007fs ($p = 0.001$, OR $= 3.011$, CI95% $= 1.494$–6.071) and G908R ($p = 2.62 \times 10^{-4}$, OR $= 14.117$, CI95% $=$ 1.884–105.799) polymorphisms with CD patients. At least one of *NOD2/CARD15* polymorphisms was found in 78/354 (22.03%) in CD patients, 25/197 (12.69%) in UC patients and in 26/256 (10.15%) in healthy controls. We have successfully implemented *NOD2/CARD15* HRMA assays, which may contribute to the development of genetic profiles for risk prediction of developing CD and for differential diagnosis of CD vs. UC.

Keywords: High-resolution melting analysis, *NOD2/CARD15*, inflammatory bowel diseases

List of abbreviations

CD	Crohn's disease
DMSO	Dimethyl sulfoxide
DSA	Disease-susceptibility allele
ΔT_m	Difference in melting temperatures (e.g. between C/C and T/T)
HPLC	High-performance liquid chromatography
HRMA	High resolution melting analysis
IC	Indeterminate colitis
IBD	Inflammatory bowel disease
MAF	Minor allele frequency
OR	Odds ratio
PCR	Polymerase chain reaction
qPCR	Quantitative (real-time) PCR
RFLP	Restriction fragment length polymorphism
SNP	Single nucleotide polymorphism
Tm	Melting temperature
UC	Ulcerative colitis

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1. Introduction

Inflammatory bowel diseases (IBD) are usually classified into Crohn's disease (CD) and ulcerative colitis (UC). In a recent genome-wide association study, three *NOD2/CARD15* disease susceptibility alleles (DSAs), namely two missense polymorphisms R702W (rs2066844) and G908R (rs2066845), and a frame shift polymorphism L1007fs (rs2066847), have shown the most significant association with CD [1]. In addition to CD, *NOD2/CARD15* polymorphisms might also play important role in Blau syndrome [2] and graft-versus-host disease [3]. Therefore a high-throughput and cost-effective genotyping method is needed for diagnostic screening of *NOD2/CARD15* DSAs. There are several conventional genotyping methods available, but most of them require a post-PCR separation step which is time-consuming and also increases the risk of contamination of PCR products [4]. On the other hand, most of the close-tube methods require expensive fluorescently labeled probes [5] or primers [6]. High-resolution melting analysis (HRMA) was introduced as a closed-tube genotyping method, where the post-PCR separation step is avoided [7,8]. HRMA also offers significant savings, ease of use and increased sample throughput, compared to other screening methods [9,10]. Although HRMA enables the detection of heterozygotes and most homozygotes, in some cases an unlabeled probe has to be introduced to enhance homozygote discrimination [4]. Unlabeled probes are usually introduced when the difference in melting temperatures (Tm) between two homozygotes is small (0.00–0.25°C) [11]. HRMA was previously described as a powerful diagnostic method for polymorphism scanning in several clinically important genes [12–14]. However, no HRMA assays for *NOD2/CARD15* DSAs have been reported to this date. Therefore, our primary aim was to design and optimize *NOD2/CARD15* HRMA genotyping assays and to evaluate the genotyping efficiency of qPCR-HRMA by comparing it to the conventional PCR-RFLP genotyping method. In addition we report the distribution of *NOD2/CARD15* DSAs in Slovenian general population and in IBD patients.

2. Materials and methods

2.1. Experimental subjects and DNA extraction

We analyzed genotyping results of 588 Slovenian IBD patients including 354 CD patients, 197 UC patients and 37 patients with intermediate colitis, as well as 256 healthy unrelated blood donors as a control group. Patients were enrolled in the study as described previously [15]. In this study 49% of IBD patients were male and 51% were female. Mean age of IBD patients was 38.6 years and mean age at diagnosis was 27.17 years. Experiments were undertaken with the understanding and written consent of each individual, and the study conforms with The Code of Ethics of the World Medical Association (Declaration of Helsinki) [16]. Study was also approved by the Ethical Committee of the Republic of Slovenia. Genomic DNA of 255 IBD patients and 256 healthy controls was extracted from whole blood lymphocytes, according to manufacturer's protocol, using a combination of Ficoll-Paque PLUS (GE Healthcare Bio-Sciences, Sweden) and TRI REAGENT (Sigma-Aldrich, USA) reagents. DNA samples of 333 IBD patients were extracted from paraffin-embedded biopsy sections after tissue digestion, using standard phenol/chloroform extraction and ethanol precipitation as described previously [15].

2.2. Design of primers and unlabeled probe

Primers and unlabeled probe were designed with Primer3 software [17] and synthesized by standard phosphoramidite chemistry (Invitrogen, USA). Several factors were taken into account during design of the primers. Sequence variations were positioned at the center of amplicons. Primers used in PCR-RFLP of G908R variant produced 223 bp amplicon which was inappropriate for qPCR-HRMA since sample genotypes could not be distinguished in melting analysis. Thus, we designed primers for G908R variant that resulted in 100bp amplicon. Additionally, because nearest neighbor thermodynamic model [18] predicts no difference of melting temperatures for G:C transition in G908R variant, we expected indistinct separation of homozygous wild-type vs. homozygous mutant melting curves. Therefore we designed unlabeled probe which was blocked on the 3'-hydroxyl terminus with a three carbon (C3) alkyl group to prevent extension by *Taq* polymerase during PCR. Sequences of primers, probe and amplicon lengths for *NOD2/CARD15* DSAs are shown in Table 1.

2.3. PCR-RFLP optimization

PCR-RFLP was used to obtain reference genotypes from CEPH DNA samples and from DNA samples of 588 patients and 256 healthy controls. PCR-RFLP optimization parameters are summarized in Table 2.

Table 1
Primers and probe used for PCR amplification

SNP	Primer and probe sequence 5'– 3'	Amplicon length (bp)
L1007fs (rs2066847)	F: CTGGCTAACTCCTGCAGT	217
	R: ACTGAGGTTCGGAGAGCT	
G908R (rs2066845)	F*: GGTCCACTTTGCTGGGACCA	100
	R*: TCACCCAAGGCTTCAGCCAG	
	F: GGTCCACTTTGCTGGGACCA	223
	R: TCACCCAAGGCTTCAGCCAG	
	P: ATTCTGGCGCAACAGAGTG	
R702W (rs2066844)	F: TTCCTGGCAGGGCTGTTGTC	133
	R: AGTGGAAGTGCTTGCGGAGG	

F, forward primer;
R, reverse primer;
P, unlabeled probe;
The underlined base in the probe sequence indicates the position of the variation.
*Primers used only for PCR-HRMA.

Table 2
PCR-RFLP parameters

PCR-RFLP reaction parameter	Value		
	L1007fs	G908R	R702W
Annealing temperature [°C]	57	58	63
Primer concentration [nM]	187	250	187
Final c(MgCl2) [mM]	3	2	2
Vol. / vol. DMSO added [%]	0	0	5
Cycles	35	35	35
Restriction enzyme [units] (enzyme name)	0.5 (*Bsp* LI)	0.5(*Hha* I)	1 (*Msp* I)
Cleavage time 37°C [hours]	4	4	4
Restriction fragments [bp]	wt/wt: 217, C/C: 180 + 37	C/C: 172+51, G/G: 223	C/C: 21+54+58, T/T: 21+112

Table 3
qPCR-HRMA optimization parameters and optimal values

Optimization parameter qPCR-HRMA	Tested values	Optimal value		
		L1007fs	G908R	R702W
Annealing temperature [°C]	55–65	57	60	63
Primer concentration [nM]	125–500	187	250	187
Final c(MgCl2) [mM]	1.5; 2; 2.5; 3	3	3	2
DMSO added [vol. / vol.]	0; 5	0	0	5
Cycles	40, 45, 50, 55	45	45	45
Ramp rate [°C/s]	0.11; 0.06; 0.04; 0.03	0.04	0.04	0.04
qPCR-HRMA with unlabeled probe		rs2066845 (G908R)		
Primer asymmetry ratio	1:5, 1:10, 1:20	/	1: 5	/
Cycles	40, 45, 50, 55	/	55	/

2.4. PCR-HRMA optimization

qPCR-HRMA was performed in 96-well plates on the LightCycler 480 2.0 instrument (Roche, USA). The final reaction volume was 10 μL; 8μL of reaction master mix were added to 2 μL of template DNA. Optimal qPCR-HRMA parameters were selected from experiments with the highest concordance between HRMA and PCR-RFLP genotypes (Table 3). Final DNA concentration ranged from 10–40 ng/μL as determined by absorbance at 260 nm on NanoDrop 2000 spectrophotometer (ThermoScientific, USA). Cycling conditions were performed using the following protocol: initial denaturation at 95°C for 10 min, followed by denaturation (95°C, 15s), annealing (50–65°C, 10s) and extension (72°C, 15s). Samples with known genotypes were used as reference and were run in triplicates for each of *NOD2/CARD15* polymorphisms. Raw fluorescence data were analyzed with two algorithms given by the software provided with the LC480 instrument. In "Gene scanning" algorithm, raw fluorescence data were normalized, shifted and subtracted from a refer-

ence melting curve to obtain difference plots. Here, differences in melting curve shapes were used to determine the sample genotypes. On the other hand "Tm calling" algorithm, is based on calculation of negative of first derivative of fluorescence data, where distinctive melting curve peaks and Tms (melting temperatures) were obtained to discriminate between different genotypes.

2.5. Statistical analysis

We used the two-sided Fisher's exact test to compare *NOD2/CARD15* genotype and allele frequencies between control group and IBD patients. Statistical tests were calculated using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) statistical package. Odds ratios (OR) and corresponding 95% confidence intervals (CI95%) were calculated with SPSS 17.0 software. In all tests $p < 0.05$ was considered to indicate statistical significance. Hardy-Weinberg equilibrium for genotype frequencies was checked in the control group.

3. Results

3.1. Optimization and evaluation of qPCR-HRMA assays for NOD2/CARD15 variants

Optimized parameters for qPCR-HRMA *NOD2/CARD15* assays are summarized in Table 3.

3.1.1. L1007fs

HRM analysis in combination with "Gene scanning" algorithm of 217 bp amplicon displayed three types of melting curves, which correlated with wild-type (wt/wt), heterozygous (C/wt) and mutant homozygous (C/C) genotypes (Fig. 1a). Two rare homozygous mutants were found by HRMA in CD patients, and were subsequently confirmed by PCR-RFLP. Genotyping results obtained from qPCR-HRMA were 100% concordant with results from PCR-RFLP.

3.1.2. G908R

With HRM analysis of 100 bp amplicon in combination with "Gene scanning" algorithm we were able to discriminate between homozygotes and heterozygotes (Fig. 1b). Discrimination between homozygotes was achieved by introducing of unlabeled probe in combination with the "Tm calling" algorithm (Fig. 3). Unlabeled probe annealed to C/C (mutant) and partially to G/C amplicons and produced unique probe-amplicon melting transitions, which were used for genotype discrimination. As illustrated in Fig. 2 probe-amplicon melting transitions were observed between 63°C–68°C and amplicon-amplicon melting transitions were observed between 87°C–89°C. A rare homozygous mutant (C/C) was found in CD patients, which was subsequently confirmed by PCR-RFLP. Genotyping results obtained from qPCR-HRMA were 100% concordant with results from PCR-RFLP.

3.1.3. R702W

As illustrated in Fig. 1c qPCR-HRMA assay for R702W DSA in combination with "Gene scanning" algorithm displayed two types of melting curve shapes that correlated to wild-type (C/C) and heterozygous (C/T) genotypes. No homozygous mutants (T/T) were found in patients or controls. Genotyping results obtained from qPCR-HRMA were 100% concordant with results from PCR-RFLP.

In total, DNA samples of 588 IBD patients and 256 healthy controls were successfully genotyped for *NOD2/CARD15* DSAs by qPCR-HRMA and confirmed by PCR-RFLP.

In addition to previously mentioned SNPs, two other potential SNPs reported in the dbSNP database also map within the *NOD2/CARD15* amplicons used in our experiments. First SNP rs58586167 is located 4 bases upstream of G908R polymorphism with non-validated status at the time of this study. The second SNP rs35285618 is a low frequency (MAF = 0.013) SNP located 19 bases downstream of R702W polymorphism and was so far detected only in African Americans. To exclude potential interference with our HRMA assays, a PCR-RFLP analysis of these two SNPs was conducted in Slovenian IBD patients and in healthy controls. We found no individuals positive for any of the two polymorphisms, suggesting that these SNPs are either very rare or not present in the Slovenian population.

3.2. Association analysis on Slovenian IBD patients

We genotyped the samples of 588 Slovenian IBD patients and 256 healthy unrelated blood donors using PCR-RFLP and qPCR-HRMA methods. A group of 37 patients with indeterminate colitis (IC) was not included in statistical analysis as a separate group due to small sample size. Statistically significant association was found for L1007fs DSA in CD patients ($p = 0.001$, OR = 3.011, CI95% = 1.494–6.071), but not in UC patients ($p = 0.504$, OR = 0.885, CI95% = 0.334–2.346), compared to healthy controls (Table 4). Minor

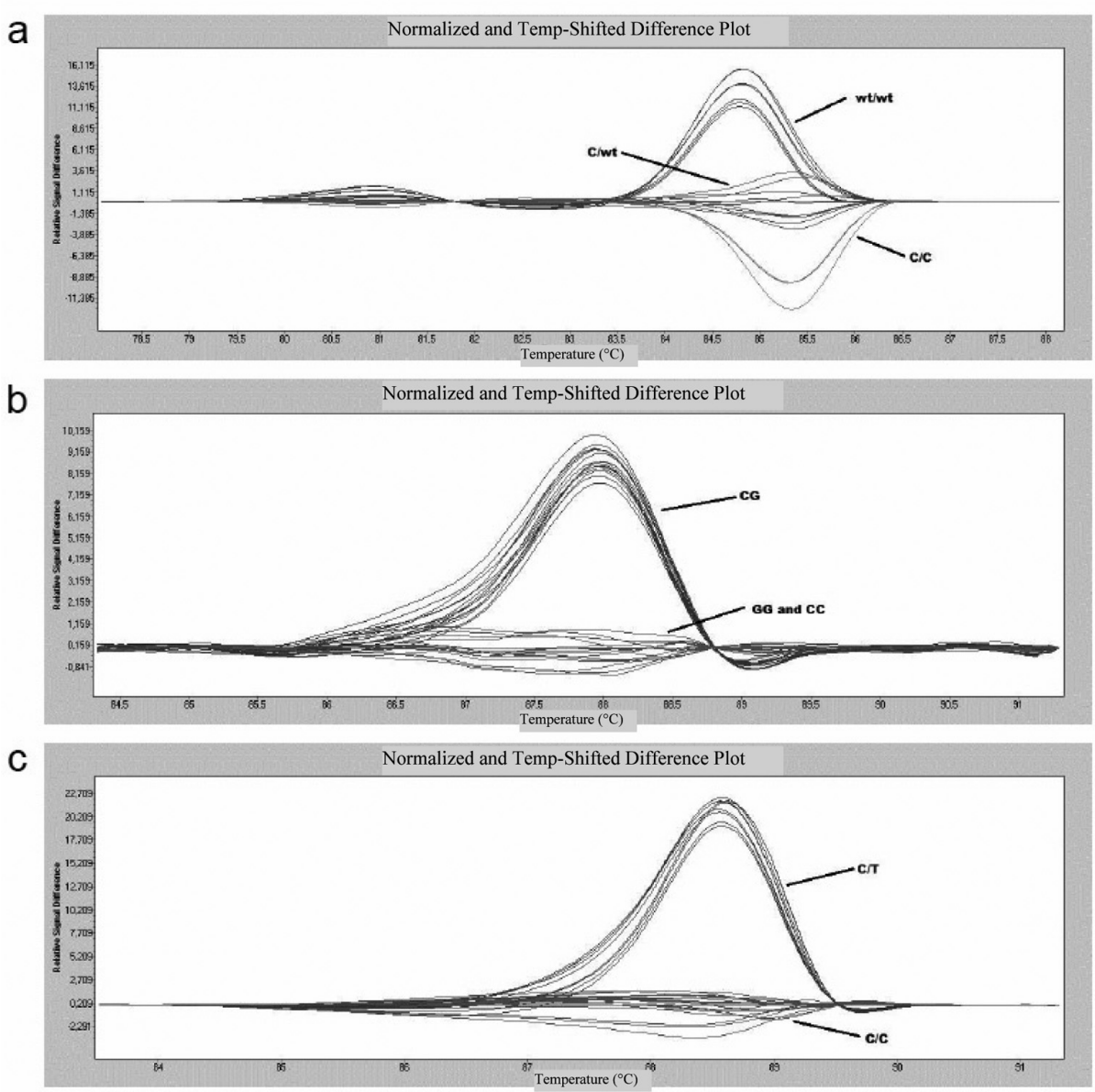

Fig. 1. Difference plots of *NOD2/CARD15* polymorphisms. Difference plots were obtained subsequent to raw fluorescence data normalization, Tm-shift and subtraction from a reference melting curve by "Gene scanning" algorithm. a) L1007fs (rs2066847). Genotypes of L1007fs polymorphism were clearly distinguished and grouped into three groups. wt/wt represents melting curves of wild-type homozygotes, C/wt melting curves of heterozygotes and C/C melting curves of mutant homozygotes. b) G908R (rs2066845). Heterozygotes were unambiguously distinguished from both groups of homozygotes due to altered melting curve shapes. Differentiation of homozygotes was difficult because of similar melting curve shapes of G/G and C/C homozygotes. c) R702W (rs2066844). Wild-type homozygotes and C/T heterozygotes were clearly distinguished. No homozygous mutants (T/T) were found in our study. C/C represents wild-type homozygotes and C/T heterozygotes.

allele frequency (MAF) of L1007fs polymorphism was 2% in control group, 5.9% in CD group and 1.8% in UC group. L1007fs MAF was also significantly higher in CD patients as compared to UC patients ($p = 0.001$, OR = 3.404, CI95% = 1.512–7.663). In case of G908R DSA, we found statistically significant association in CD patients ($p = 2.62 \times 10^{-4}$, OR = 14.117, CI95% = 1.884–105.799) and UC patients ($p = 0.013$, OR = 9.389, CI95% = 1.150–76.641) as compared to healthy controls. G908R MAF was 0.2% in control group,

Table 4
Results of association analysis of *NOD2/CARD15* DSAs in Slovenian IBD patients and healthy controls

	L1007fs			G908R			R702W		
	C	CD	UC	C	CD	UC	C	CD	UC
Homozygotes (wt)	237	311	188	252	330	184	238	323	185
Heterozygotes	10	37	7	1	17	7	15	30	11
Homozygotes (mt)	0	2	0	0	1	0	0	0	0
Total	247	350	195	253	348	191	253	353	196
Homozygotes (wt) [%]	96.0	88.86	96.41	99.6	94.82	96.34	94.1	91.50	94.39
Heterozygotes [%]	4.0	10.57	3.59	0.4	4.89	3.66	5.9	8.50	5.61
Homozygotes (mt) [%]	0	0.57	0	0	0.29	0	0	0	0
MAF	0.02	0.059	0.018	0.002	0.027	0.018	0.03	0.042	0.028
p-value		0.001	0.504		2.62×10^{-4}	0.013		0.156	0.527
OR		3.011	0.885		14.117	9.389		0.450	0.945
CI95%		1.494–6.071	0.334–2.346		1.884–105.799	1.150–76.641		0.773–2.729	0.429–2.081

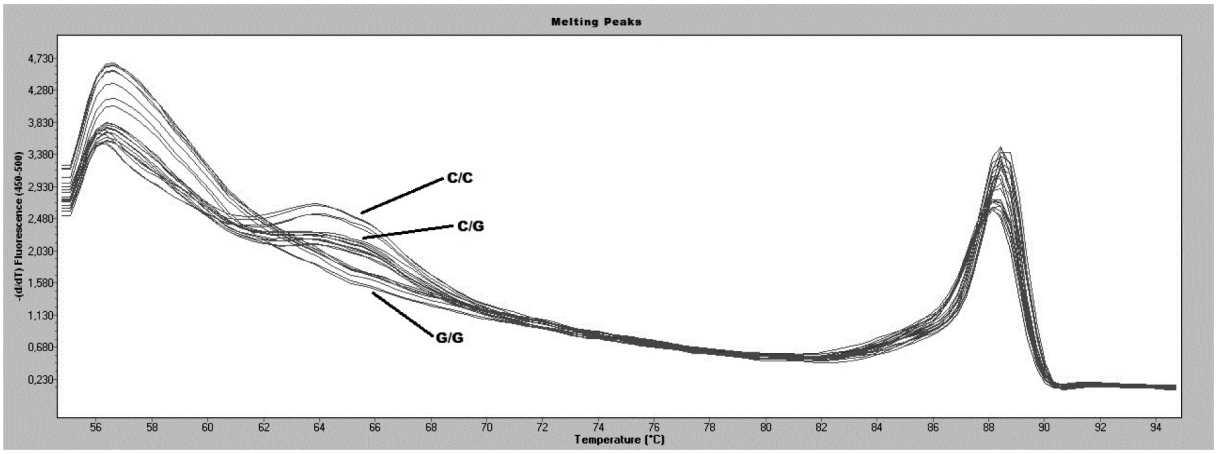

Fig. 2. Melting peaks of G908R (rs2066845) amplicon and unlabeled probe melting. Negative of first derivative of raw fluorescence data (-dF/dT) was calculated by "Tm calling" algorithm and presented on y – axis against temperature (T) on x–axis. Unlabeled probe annealed to C/C (homozygous-mutant) and partially to heterozygous G/C amplicons. This resulted in a unique probe-amplicon melting transitions (62°C–68°C), which were used for differentiation of G908R genotypes. Differentiation of genotypes by amplicon melting was not possible, since the amplicon melting peaks were confined to a narrow temperature interval (88.2°C–88.5°C).

2.7% in CD patients and 1.8% in UC patients. We found no statistically significant association of R702W DSA with CD and UC patients. Detailed results of association analysis are summarized in Table 4. The frequency of carriers of any polymorphism was significantly higher in CD patients compared to healthy controls ($p = 6.53 \times 10^{-5}$, OR = 2.5, CI95% = 1.551–4.028) and also when compared to UC patients ($p = 0.004$, OR = 1.944, CI95% = 1.192–3.171) (Table 5). At least one of *NOD2/CARD15* DSAs was found in 78/354 (22.03%) CD patients, 25/197 (12.69%) in UC patients and 26/256 (10.15%) in healthy controls. Additionally, we found 9/354 (2.54%) compound heterozygotes in CD patients and none in UC patients or in control group. In addition, mutant homozygotes for L1007fs and G908R polymorphisms were found only in group of CD patients.

Table 5
Prevalence of at least one *NOD/CARD15* DSA in Slovenian IBD patients and controls

	At least one mutation		
	C	CD	UC
Any variant	0.1015	0.2203	0.1269
p-value		6.53×10^{-5}	0.243
OR		2.500	1.286
CI95%		1.551–4.028	0.717–2.304

OR, odds ratio; CI95%, confidence interval 95%; C, controls; CD, Crohn's disease; UC, ulcerative colitis.

4. Discussion

We have developed three qPCR-HRMA genotyping assays for *NOD2/CARD15* DSAs and compared them to standard PCR-RFLP assays. As reported in previous studies [19,20], we also observed that the success of

Table 6
Minor allele frequencies of *NOD2/CARD15* polymorphisms in European IBD patients and controls

		R702W			G908R			L1007fs		
European population	Authors (year) [Reference]	CD	UC	C	CD	UC	C	CD	UC	C
Belgium	Esters et al. (2004) [25]	12.9	7.8	5.8	6.0	3.2	1.8	8.6	1.4	3.0
Croatia	Cukovic-Cavka et al. (2006) [26]	13.9	/	5.5	4.4	/	1.1	11.8	/	4.4
Czech Republic	Hosek et al. (2008) [27]	13.0	2.0	1.0	3.0	0.0	0.0	22.0	11.0	6.0
Denmark	Vind et al. (2005) [28]	0.0	/	1.5	2.6	/	1.0	16.4	/	2.1
England	Ahmad et al. (2002) [29]	12.5	/	5.2	3.3	/	1.4	9.4	/	1.6
Finland	Heliö et al. (2003) [30]	3.3	1.5	1.8	0.6	0.0	0.0	4.8	3.0	1.7
France	Heresbach et al. (2004) [31]	11.5	/	4.7	3.7	/	1.6	9.0	/	4.2
Germany	Hampe et al. (2002) [32]	10.5	/	4.8	5.2	/	0.7	14.5	/	4.1
	Buning et al. (2004) [33]	7.2	2.1	3.6	4.2	2.1	2.1	12.2	4.3	2.1
Greece	Gazouli et al. (2005) [34]	10.0	7.1	1.0	14.2	13.5	3.5	17.9	3.5	6.0
Hungary	Buning et al. (2005) [35]	7.1	3.1	2.6	3.0	1.6	1.2	10.8	2.3	2.2
	Nagy et al. (2005) [36]	10.3	/	4.7	2.7	/	1.4	8.9	/	2.4
Iceland	Thjodleifsson et al. (2003) [37]	0.0	/	/	0.0	/	/	0.0	/	/
Ireland	Bairead et al. (2003) [38]	7.0	/	4.0	3.0	/	1.0	4.0	/	1.0
Italy	Giachino et al. (2004) [39]	9.0	10.9	5.9	4.3	2.7	1.4	6.3	0.5	2.3
	Vavasori et al. (2004) [40]	1.2	/	0.8	5.2	/	2.0	11.2	/	1.2
	Annese et al. (2004) [41]	9.0	/	5.0	5.5	/	2.0	7.7	/	1.3
Netherlands	van der Linde et al. (2007) [42]	8.8	4.7	5.9	6.1	0.0	0.7	11.0	2.3	1.9
Norway	Hampe et al. (2002) [32]	4.3	/	2.8	0.9	/	1.2	2.6	/	1.2
Portugal	Ferreira et al. (2005) [43]	12.2	/	4.0	2.8	/	1.3	6.8	/	1.6
Scotland	Arnott et al. (2004) [44]	7.2	2.6	5.5	1.8	2.0	0.2	4.6	3.0	1.4
Slovakia	Bartosova et al. (2009) [45]	9.9	2.86	8.97	3.96	1.43	1.92	16.83	8.57	5.77
Slovenia	This study	5.1	3.0	3.0	2.6	1.7	0.2	5.9	2.2	2.0
Spain	Nunez et al. (2004) [46]	6.7	/	5.8	4.5	/	1.0	4.5	/	1.0
Serbia	Protic et al. (2008) [47]	20.6	1.5	14.8	5.3	0.0	0.0	15.3	7.7	0.0
Switzerland	Ruegg et al. (2004) [48]	5.7	/	/	2.8	/	/	4.7	/	/

qPCR-HRMA genotyping strongly depends on careful optimization of PCR parameters and adjustment of DNA concentration to similar values. Although there were some reports [12,21,22] on normalization (premelting, post-melting, sensitivity) and Tm-shift parameters for the LightCycler software, so far no guidelines how to approach these parameters were reported. In our case as well, we observed that the optimal normalization and Tm-shift parameters should be established arbitrarily and determined experimentally. In the course of our work, we have observed that short amplicons reduce the poor discrimination of homozygous samples as was reported previously [11]. Heterozygotes were clearly distinguished from homozygotes in all three studied *NOD2/CARD15* polymorphisms. Additionally, we were able to distinguish homozygous wild-types and mutants for R702W and L1007fs polymorphisms. According to previous reports, HRMA with unlabeled probe was used when the Tm differences were insufficient to distinguish between different homozygotes [4,23]. In case of *NOD2/CARD15* G908R polymorphism the G:C transition created insufficient differences in Tm. Unambiguous discrimination of homozygous samples was eventually achieved after the introduction of unlabeled probe. In a previous study [24], it was reported that HRMA has limited sensitivity for single nucleotide insertion-deletion variants located immediately adjacent to mononucleotide runs. In this study, L1007fs (rs2066847), an insertion of a cytosine adjacent to 3 cytosine repeat was fully detectable for heterozygous and homozygous mutant genotypes.

Previous studies show, that there is a strong evidence for regional heterogeneity within European populations in the contribution of *NOD2/CARD15* to disease susceptibility, which may reflect to differing founder populations (Table 6). Our study is the first report of the distribution of three major *NOD2/CARD15* DSAs in Slovenian IBD patients and healthy controls. We found that *NOD2/CARD15* DSAs are as frequent (22.03%) in Slovenian CD patients and healthy controls (10.15%) as in Hungary [36], Italy [49], Netherlands [50], Portugal [28], Denmark [28], Scotland [44] and Switzerland [48], and higher than in Finland [30], and lower than in Serbia [47] and Croatia [26]. Similar to majority of European studies we also report a significant association of L1007 and G908R with CD patients. The difference of MAFs between Slovenian CD patients and Slovenian healthy controls was highest for L1007fs polymorphism (3.9%) as compared to G908R polymorphism (2.4%) and R702W polymorphism (2.1%),

suggesting that L1007fs plays most important role in *NOD2/CARD15* CD – associated risk in Slovenian population, thus placing Slovenians in the European average. On the other hand, the highest difference of MAFs between CD patients and controls was reported for the R702W polymorphism in Portuguese [43] (8.2%) and French [31] (6.8%) populations. The MAF of R702W in Slovenians was slightly increased in CD patients and decreased in UC patients, though it showed no significant association with CD and UC patients vs. healthy controls as was also the case in the majority of studies conducted in European populations. Interestingly, we found the association of G908R with Slovenian UC patients which was however previously reported only in Greek [34] and Scottish [44] studies. The MAF of G908R in Slovenian UC patients is similar to that reported in several other European studies (Table 6) [35,45,51,52], but lower frequency of this polymorphism was detected in healthy controls in Slovenian population as compared to other European populations. Our findings may indicate a possible role of *NOD2/CARD15* polymorphisms in UC patients, implicating a need for further replication in a larger cohort. Four percent of CD patients were homozygote or compound heterozygote for *NOD2/CARD15* polymorphisms, yet we have not detected a single compound heterozygote or homozygote individual among healthy controls suggesting *NOD2/CARD15* compound heterozygote and homozygote status may be particularly reliable and highly specific marker for risk prediction for developing CD in Slovenian population.

In conclusion, the results of this study suggest that HRM analysis yields significant savings on analysis time and costs although costs for the development and optimization of the new HRM assays should be also taken into a consideration. HRMA has proven as a simple high-throughput technique for screening for polymorphisms of *NOD2/CARD15* gene. We report significant association of L1007fs with CD and G908R with CD and UC. We have successfully implemented *NOD2/CARD15* HRMA assays, which may contribute to the development of genetic profiles for risk prediction of developing CD and for differential diagnosis of CD vs. UC.

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