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High resolution melting (HRM) analysis for the detection of ER22/23EK, *Bcl*I, and N363S polymorphisms of the glucocorticoid receptor gene

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ABSTRACT

Polymorphisms in the glucocorticoid receptor (GR) gene have been associated with altered sensitivity to glucocorticoids. We designed a high-resolution melting (HRM) assay to detect, simultaneously, the three most intriguing GR polymorphisms, selected on the bases of clinical relevance and frequencies in caucasian population as described in literature. HRM enables the detection of ER22/23EK and N363S genotypes but fails to discriminate homozygous mutant for the B*cl*I polymorphism from wild-type samples, however a simple spike experiment leads to a clear discrimination between these genotypes. The analyses were performed on a cohort of 70 healthy Caucasian subjects. The method was validated by restriction fragment length polymorphisms; HRM results were found to be in 100% concordance with those observed with the restriction enzymes. We also employed this method on a population of 40 Crohn Disease patients; the analysis demonstrated a significantly higher frequency of the *Bcl*I polymorphism in patients than in healthy volunteers.

This is, at now, the less expensive and time-and work-saving method to detect GR mutations, providing precision, fast screening and high throughput capabilities.

1. Introduction

Glucocorticoid (GCs) hormones play a key role in metabolic and immunological homeostasis. They regulate many physiological processes and, moreover, due to their immunosuppressive and antiinflammatory actions, synthetic GCs are widely employed in the treatment of a variety of immune diseases [1,2].

The glucocorticoid receptor (GR) belongs to the superfamily of nuclear receptors that are present in the cytoplasm and act as transcription factors to regulate gene expression. Following cortisol binding, a conformational change occurs that leads to dissociation of the receptor from a large complex of proteins, of which heat shock protein (HSP) 90 is the most important [3,4]. This engaged ligand-bound receptor moves to the nucleus, where it exerts its function in several ways [5]. The GR can initiate transcription through binding to GC-responsive elements of the target genes. The GR can also affect gene transcription through direct protein–protein interaction and can either activate or repress expression of target genes [6,7].

Polymorphisms – generally defined as common variations at the DNA level with a frequency of more than 1% in the normal population – that are present in the GR gene, may impair formation of the glucocorticoid-GR complex and alter transactivation and/or transrepression processes. We selected a panel of three polymorphisms within the GR gene with a putative effect on GC sensitivity on the basis of the previously described clinical relevance and frequencies in caucasian population [8–11].

One of the most interesting polymorphisms consists of two linked single-nucleotide mutations in codons 22 and 23 (exon 2 of the GR gene) [9]. The first mutation in codon 22 does not result in an amino acid substitution (GAG→GAA), as both triplets code for a glutamic acid residue (E) whereas the mutation in codon 23 (AGG→AAG) causes a change from arginine (R) to lysine (K).

The mRNA normally undergoes alternative translation initiation, resulting in a longer isoform (GR-A), starting from the first AUG codon (Met-1), and a shorter isoform, GR-B, starting from an internal, in frame AUG codon (Met-27); as ER22/23EK polymorphism is in close proximity to the Met-1 and Met-27 translation initiation start sites, this genetic variant may affect the ratio between GR-A and GR-B synthesis, finally causing a relative decrease in GC sensitivity [12–14].

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Indeed, a population-based cohort study in the elderly showed an association with higher post-DEXAMETHASONE (DEX) cortisol levels as well as diminished cortisol suppression after a 1 mg DEX suppression test, suggesting a relative GC resistance in ER22/23EK carriers [8].

This polymorphism is also associated with a better metabolic and cardiovascular health profile, and an increased survival rate [15].

Another selected variant is the single nucleotide polymorphism N363S, which is an A1218G point mutation in exon 2 causing an asparagine to serine amino acid substitution in codon 363.

This N-terminal domain of the receptor modulates transcription activation, and hyperphosphorylation of serine residues could enhance glucocorticoid-regulated gene expression [16].

The 363S allele has been indeed associated with increased sensitivity to GCs, increased insulin response to DEX, a tendency towards lower bone mineral density, and increased body mass index (BMI) [17,18].

As extensively reported by literature, the clinically most relevant polymorphism of the GR gene is the *BclI* polymorphism.

Initially described as an intronic restriction fragment length polymorphism (RFLP) of the GR gene, producing a short fragment of 2.3 kb and a large fragment of 4.5 kb [19], since then, it has been investigated in several association studies for its putative role in obesity [20,21].

This mutation was recently identified as a C/G nucleotide localized in intron 2, 647 bp far from exon/intron junction. The C allele is the most frequently occurring and thus it can be considered the wild-type allele, while the G allele is associated with hypersensitivity to glucocorticoids such as dexamethasone [10]. Moreover it is known to be associated with abdominal obesity [22], insulin resistance and increased sensitivity to GCs [23].

Table 1
Characteristics of the studied Polymorphism, primer sequences and amplicon length.

Polymorphisms	Accession no.	Primer sequences	Amplicons
N363S	rs6195	Forward: caggatcagaagcctatt Reverse: cagagtccccagagaag	111 bp
ER22/23EK	rs6190	Forward: ctccaaagaatcattaactcc Reverse: tcctcctcttagggttttata	103 bp
BclI	(Not available)	Forward: tcacagggttcttgccataa Reverse: acttgagaacttgcaggaacat	125 bp

In the present study, we developed a high-resolution melting assay to detect, simultaneously, the three most intriguing GR polymorphism described in literature and we evaluated their biological association to both Crohn Disease (CD) and response to GC therapy.

2. Materials and methods

Genomic DNA was extracted, using the "salting out" method [24], from at least 200 μL of whole blood from 70 healthy Caucasian subjects and 40 Crohn Disease patients, divided into responders and non-responders on the basis of their response to Glucocorticoid treatment.

All subjects signed a written agreement; the study was approved by Local Ethics Committees.

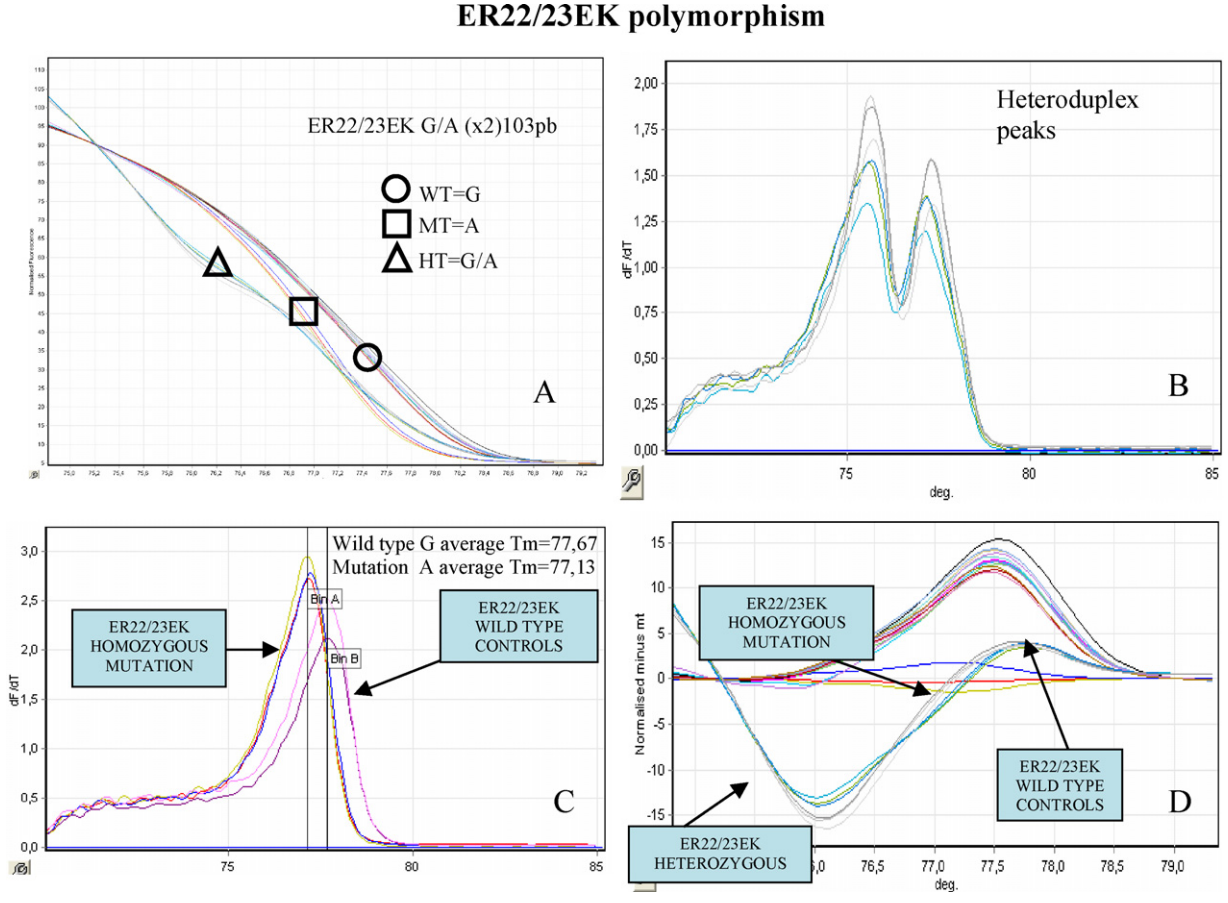

Fig. 1. Graphical visualizations for the identification of the ER22/23EK mutation. (A) Normalized melting curves of the three genotypes from genomic samples and plasmid controls. (B) Melting curves show heteroduplexes formation in the heterozygous population. (C) Temperature shift between wild-type and homozygous mutant genotypes. (D) A difference plot of the plasmid control; genomic and plasmid DNAs are compared to the median plasmid control mutant to produce the plot.

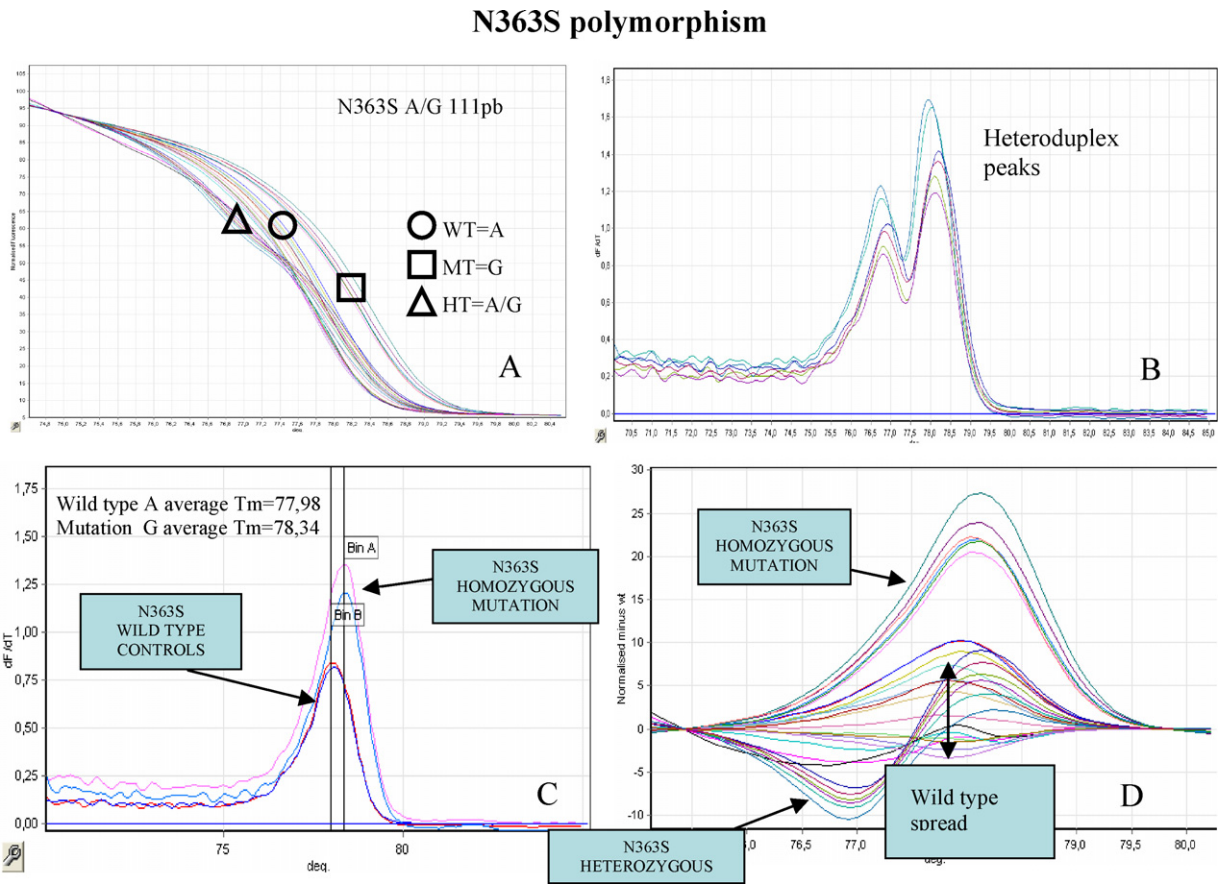

Fig. 2. Graphical visualizations for the identification of the N363S mutation. (A) Normalized melting curves of the three genotypes from genomic samples and plasmid controls. (B) Melting curves show heteroduplexes formation in the heterozygous population. (C) Temperature shift between wild-type and homozygous mutant genotypes. (D) A difference plot of the wild-type plasmid control; genomic and plasmid DNAs are compared to the median plasmid control to produce the plot.

Primer sequences (showed in Table 1) were designed to obtain the best HRM performance, avoiding, as much as possible, hairpin and primer–dimer formation and maintaining the amplicon length under 120 base pairs.

The homozygous mutant genotypes ER22/23EK and N363S are extremely rare; therefore, in order to characterize the melting curves of the amplicons, we used two distinct plasmid constructs obtained by cloning PCR products from heterozygous genotypes DNA in the pGEM®-T Easy Vector (Promega, Madison, WI) according to the manufacturer instructions. PCR reactions were performed with ER22/23EK forward and reverse and N363S forward and reverse primer pairs, respectively, and constructs containing the mutant amplicons were selected by RFLP, verified by sequencing on ABI 310 (Applied Biosystem, Foster City, USA) and 1 ng of each was utilized in subsequent PCR reactions.

Polymerase chain reaction (PCR) cycling and High Resolution Melting (HRM) analysis for the allelic discrimination were designed to allow detection of all SNPs in a single run and performed on the Rotor-Gene 6000™ (Corbett Research, Mortlake, New South Wales, Australia). All the analyses were run according to the following conditions: one step at 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 56 °C for 20 s, 72 °C for 30 s and a melt from 70 to 85 °C rising at 0.1 °C/s.

The reaction mixture consisted of 10 ng of genomic DNA, 2 mM $MgCl_2$, 500 nM of each primer, 200 μM dNTPs, 1× PCR buffer, 0.5 U of HotStartTaq polymerase (Diatheva, Fano, ITA), 1× EvaGreen intercalating dye (Biotium Inc, Hayward, CA), 5% DMSO and double distilled water to a volume of 12.5 μL.

The linear dynamic range and the intra- and inter-assay variabilities of polymorphisms' detection were determined by preparing DNA serial dilutions ranging from 1 to 200 ng/μL and performing all PCR reactions in triplicate.

Samples were purified using the Qiagen PCR purification kit (Qiagen, Valencia, CA).

All ER22/23EK, *Bcl*I and N363S templates were digested, respectively, with MnlI, BclI and Tsp509I endonuclease enzymes for comparison with genotype results obtained from HRM analysis (New England Biolabs, Beverly, MA).

When HRM analysis yielded unclear results, due to a poor quality of DNA, samples' genotypes were verified by DNA sequencing.

Differences in genotype frequencies between cases and controls and between responders and non-responders to GC therapy were tested using standard Chi-squared tests. Odds ratios and confidence limits were assessed, using standard methods, to evaluate the eventual association between SNPs and CD and between SNPs and response to GC therapy.

Statistical analyses were performed using MedCalc® (MedCalc, Mariakerke, Belgium).

Deviation from Hardy–Weinberg Equilibrium (HWE) was tested for each polymorphism using the on-line software "HWE-calculator" (http://www.oege.org/software/hardy-weinberg.shtml).

3. Results

HRM analysis enables the detection of homozygous wild-type, homozygous mutant and heterozygous mutant genotypes for the ER22/23EK and N363S mutation but not for the C/G SNP *Bcl*I (Figs. 1–3). For this polymorphism we tested three primer pairs for

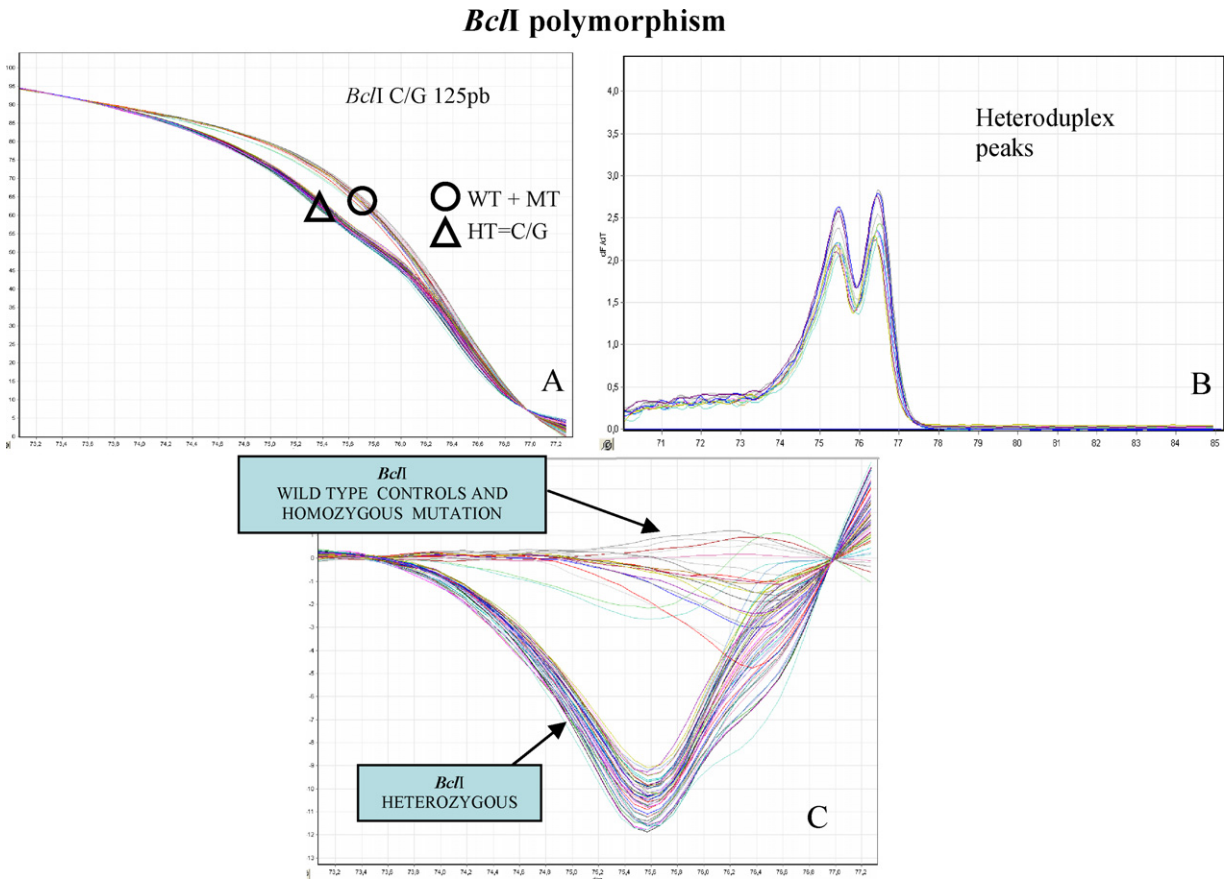

Fig. 3. Graphical visualizations for the identification of the *Bcl*I mutation. (A) Normalized melting curves of the three genotypes from genomic samples. (B) Melting curves show heteroduplexes formation in the heterozygous population. (C) A difference plot of the wild-type control; genomic DNAs are compared to the median wild-type control to produce the plot. As the melting temperature of the homozygous wild-type amplicon equals that of the homozygous mutant amplicon, no differences can be observed in fluorescence, making the discrimination between these two genotypes extremely difficult.

amplicons ranging between 70 and 120 bp with the same results: HRM analysis allowed us to detect with certainty only heterozygous samples and discriminate homozygous mutants from wild-type samples only after a spike experiment, that is, the voluntary contamination of an unknown sample with a known wild-type sample, that only when coupled to a sample homozygous for the mutation leads to the formation of heteroduplexes ensuring a clear discrimination of this genotype.

Linearity and reproducibility of the assays were assessed over a DNA concentration range of 5–100 ng/μL (data not shown).

The three genetic variants can be revealed simultaneously by HRM (Fig. 4) and, conveniently, using the same reaction mixture.

The genotype distributions in this study population did not differ from those observed in other studies [17,25].

All the samples tested by HRM were found to be in 100% (100/100) concordance with the restriction enzymes results or sequencing data.

We did not find any ER22/23EK or N363S homozygote for the mutation in our population.

Regarding the biological results, all SNPs tested were found to be in HWE.

Odds Ratios did not demonstrate any significant association of SNPs with CD or response to therapy.

Chi-square statistic revealed a significantly different ($p = 0.005$) distribution between cases and controls only for the *Bcl*I polymorphism (Table 2).

Table 2
Genotype frequencies in studied populations.

Polymorphisms	Genotypes	Controls (No. 70)	CD (No. 40)	*p*-Value	Responder (No.20)	Non-responder (No.20)	*p*-Value
ER22/23EK [G_G→A_A]	WT	68 (97%)	38 (95%)	0.718	19 (95%)	19 (95%)	0.745
	HT	2 (3%)	2 (5%)		1 (5%)	1 (5%)	
	MT	0	0		0	0	
N363S [A→G]	WT	69 (99%)	39 (97%)	0.613	20 (100%)	19 (95%)	0.07
	HT	1 (1%)	1 (3%)		0	1 (5%)	
	MT	0	0		0	0	
*Bcl*I [C→G]	WT	31 (44%)	24 (60%)	0.005	13 (65%)	11 (55%)	0.286
	HT	36 (51%)	12 (30%)		5 (25%)	7 (35%)	
	MT	3 (4%)	4 (10%)		2 (10%)	2 (10%)	

WT, wild-type; HT, heterozygous; MT, mutation; CD, Crohn Disease.

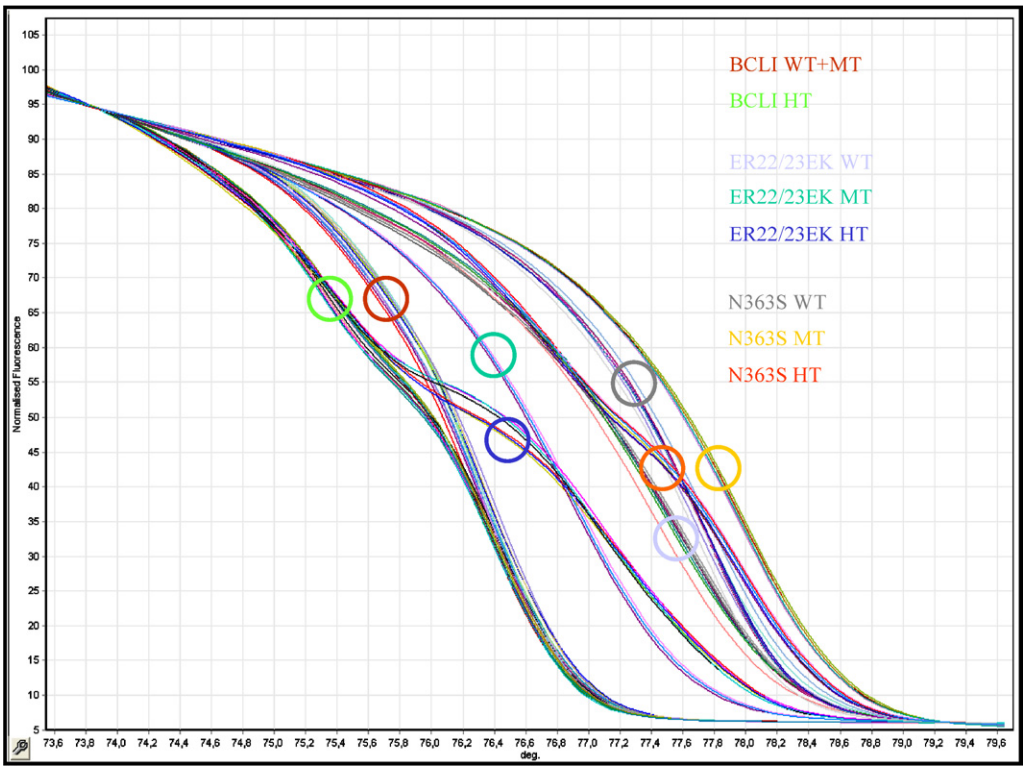

Fig. 4. Normalized melting curves of the *Bcl*I, ER22/23EK and N363S polymorphisms. A typical plot showing the melting curves of the three polymorphisms; the three genotypes for each SNPs (wild-type, heterozygous and homozygous for the mutation) are highlighted by colour rings.

4. Discussion

Polymorphisms within the GR gene are well described and already associated to various diseases; moreover, are suspected to contribute to impaired response to GC therapy. We therefore developed rapid PCR-based screening of the selected SNPs.

We employed this method on a population of 40 CD patients. The analysis demonstrated a significantly higher frequency of the *Bcl*I polymorphism in CD patients than in healthy volunteers. Although the exact meaning of such a different distribution remains unclear, similar observations were reported from other authors [26]. On the other hand, results from Odds Ratios did not highlight any association of the SNPs to CD or responsiveness to GC, probably because of the small population tested.

Despite the low genotype frequencies of almost two of the selected SNPs (ER22/23EK and N363S), the precision and the fast-screening capabilities make this method the less expensive and most rapid way to detect GR mutations, allowing appropriate therapeutic choices for patients with suspected glucocorticoid-related pathologies or for those that undergo GC therapies.

GCs are, in fact, widely employed in clinical treatment of immune diseases such as asthma and chronic intestinal inflammations, and to prevent rejection after organ transplants. It is well known that GC sensitivity and, as a consequence, the efficacy of treatment are variable among patients, as measured by a DEX suppression test [27]. Some patients develop side effects even on relatively low doses of therapeutically administered GCs, whereas others need a high dose to establish clinical effects without manifestation of side effects at all [28,29]. This suggests that each subject, when treated with GCs, needs an individually optimized dose to maintain a balance between benefits of GC treatment and its adverse effects (e.g. diabetes mellitus, peptic ulcer, hosteoporosis, skin atrophy, psychosis, glaucoma, and many others) [28].

Moreover, this method can be a useful tool to accelerate those researches based on the screening of a large amount of samples; the ability to detect simultaneously the three mutations can be in fact very helpful for epidemiological studies.

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