Table 1: Amplification primer sequences and PCR product size. *NRIP1* is a monoexonic gene. We designed eight overlapping amplicons to cover the entire coding sequence of this gene.

PRIMER	SEQUENCE 5' → 3'	PCR product size (bp)
1F	TTCTAGTTCTGCCTCCTTAAC	554
1R	ACATTTCTGGCAGTGCATTTC	
2F	GATCAGGTACTGCCGTTGA	528
2R	CGAATCTTCCTGATGTGACT	
3F	GTGCTATGGTGTTGCATCAAG	572
3R	TGCAGGTTATAAGAACTCACTGG	
4F	CATCATCAAGCAAACTGATGGC	577
4R	AGCCCTCAGGGAGTACACAA	
5F	CTTCAATTGCTACTTGGCCAT	582
5R	GTAGTCAACCAACAGGTCCT	
6F	CTGGAAACACAGATAAACCGATAGG	584
6R	TGGCACTTCTAGAATCAAAG	
7F	AGATAGTTACCTGGCAGATG	572
7R	TCCTACTTTCCCTGAGCACT	
8F	CAGTTGCATGGATAACAGGA	645
8R	GTATTGGTTACTGGTGATG	

(NCBI) http://www.ncbi.nlm.nih.gov/. According to standard mutation nomenclature [9], we employed the most frequent allele in the first position and the rarer allele in the last position.

We employed automated DNA sequencing methods to scan the entire coding sequence of *NRIP1* gene in selected specimens. Overlapping PCRs covering the entire gene were designed and PCR products were purified and bi-directionally sequenced using the corresponding pair of primers (Table 1). Sequencing reactions were performed using the CEQ Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter, Inc) according to the manufacture's instructions. Fluorograms were analyzed on CEQ™ 8000 Genetic Analysis System following the manufacturer's instructions (Beckman Coulter, Inc).

Genotyping

To verify the DNA variants detected during the sequencing process and to perform association studies, we employed Fluorescent Resonance Energy Transfer (FRET) protocols. We designed and synthesized amplification primers and fluorescent detection probes for all the DNA variants identified within the *NRIP1* gene. The selected primer pairs and detection probes are summarized in Tables 1 and 2. Real-time PCR was performed in the LightCycler system (Roche Applied Science) using reaction conditions previously published by us [10].

The conditions to obtain optimal melting curves for FRET analysis and spectrofluorimetric genotypes were 95°C for 0 s, 63°C for 25 s, 45°C for 0 s and 80°C for 0 s (with a temperature-transfer speed of 20°C/s in each step, except the last step, in which the speed of temperature transfer was 0.1°C/s). In the last step, a continuous fluorometric register was performed fixing the gains of the system at 1, 50, and 50 on channels F1, F2, and F3 respectively. Genotype results using real time-PCR are shown in Figure 1a. To test the specificity of these assays, selected amplicons of different melting patterns were re-sequenced using an automated DNA sequencer (Beckman Coulter CEQ 2000XL, data not shown).

Statistical Analysis

To compare allele and genotype frequencies between patients, control and super-control groups, we performed conventional chi-square tests with Yates correction or Fisher exact test using Statcalc (EpiInfo 5.1, Center for Disease control, Atlanta, GA). For statistical analysis of genotype distribution, test for deviation of Hardy-Weinberg equilibrium or two-point association studies, we employed six different tests adapted from Sasieni (deviation from Hardy-Weinberg equilibrium, allele frequency differences test, heterozygous test, homozygous test, allele positivity test and Armitage's trend test) [11]. These calculations were performed in the online resource at the Institute for Human Genetics, Munich, Germany http://ihg.gsf.de. Significant thresholds for statistical studies were fixed at $p < 0.05$.

Results

Looking for somatic mutations within the *NRIP1* gene, we determined the complete coding sequence of the candidate gene in 20 selected and unrelated somatic endometriotic tissues using bi-directional automated capillary DNA sequencing. In our primary sequencing project we