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High-resolution melting (HRM) analysis for the detection of single nucleotide polymorphisms in microRNA target sites

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ABSTRACT

Background: The function of microRNAs (miRNAs) depends on the binding of miRNAs to their target sequences in the 3'UTR of messenger RNAs (mRNAs), which enhances the degradation of mRNAs and consequently, represses their expression. Single nucleotide polymorphisms (SNPs) in the miRNA target sequences may affect or impair the binding of miRNAs. Studies have shown that SNPs in miRNA target sites (miR-TS-SNPs) have a great influence on diverse biological functions, including pharmacogenomics and disease susceptibilities in human.
Methods: High-resolution melting (HRM) analysis was applied for investigating the allele frequencies of 3 miR-TS-SNPs (PLA2G2A, IL-16, and NOD2) in acute leukemia. We also compared the genotypes of acute lymphoblastic leukemia patients at initial diagnosis and complete remission.
Results: HRM analysis revealed 3 genotypes (both homozygous and heterozygous) in the 3 miR-TS-SNPs. The allele frequencies of all 3 miR-TS-SNPs were similar in normal individuals and patients with acute myelogenous leukemia. Most patients with acute lymphoblastic leukemia had the same genotypes at initial diagnosis and complete remission.
Conclusions: Large scale scanning of case–control studies for miR-TS-SNPs may contribute to the investigation of their roles and pathogenesis mechanisms in human diseases. Our study showed that HRM analysis can be an efficient tool for studies of miR-TS-SNPs.

1. Introduction

MicroRNAs (miRNAs) are a group of non-protein-coding, endogenous, small RNAs that regulate gene expression via translational repression. By binding to the target sites in the 3' untranslated regions (UTRs) of messenger RNAs (mRNAs), miRNAs inhibit translation and induce cleavage or decay of their target mRNAs [1–4]. Initially, an miRNA gene is transcribed into a primary miRNA, which is 500–3000 bp in length, then processed by Drosha into a precursor miRNA with 70–100-bp-long precursor miRNA with a stem-loop structure. Finally, the precursor miRNA is further processed by Dicer in the cytoplasm to yield an appropriate 22 bp long, single-stranded mature miRNA (MIR). The binding of an MIR to its target mRNA is determined by Watson-Crick complementarity between the seed region of an MIR and the target site in the 3'UTR of the given mRNA [1,4–6]. Up to 30% of human protein-coding genes are predicted to be regulated by miRNAs, and abundant evidence has revealed the association of miRNAs with various human diseases, such as leukemia and lymphoma [7–10], lung cancer [11–13] and neurodegenerative disorders [14].[1] Single nucleotide polymorphisms in miRNA genes (miR-SNPs) or [2] in miRNA target sites (miR-TS-SNPs) may influence the transcription of miRNA primary transcripts, the processing of miRNAs, or miRNA-target interactions [15–18]. A bioinformatics survey of the entire human genome revealed a low intensity of miR-SNPs (around 1.3 SNPs per kb) and a high frequency of miR-TS-SNPs that disrupt target sites or create novel ones [16]. Emerging evidence suggests that miR-TS-SNPs have important biological effects. For example, polymorphism in the dihydrofolate reductase (DHFR) 3' UTR results

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[1] Single nucleotide polymorphisms in miRNA genes: miR-SNPs
[2] Single nucleotide polymorphisms in miRNA target sites: miR-TS-SNPs

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in increased expression of DHFR protein and mRNA, leading methotrexate resistance [10,19]. An epidemiological study also showed that miR-TS-SNPs in CD86 and insulin receptor genes correlate with sporadic colorectal cancer [20]. In the present study, we applied high-resolution melting (HRM) analysis to examine 3 potential miR-TS-SNPs (PLA2G2A, IL-16 and NOD2) in healthy individuals and patients with acute leukemia to understand the biological relevance of miR-TS-SNPs and the effectiveness of HRM in miR-TS-SNP detection.

2. Materials and methods

2.1. Patient samples and DNA extraction

Bone marrow samples were obtained at initial diagnosis from patients with acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL) in Kaohsiung Medical University Hospital. Bone marrow samples were also collected from ALL patients after complete remission. Peripheral blood samples from healthy individuals were obtained from Kaohsiung Medical University Hospital. Purified blood granulocytes were separated by density-gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare Bio-science AB, Uppsala, Sweden). Blood granulocyte DNAs was extracted using the Nucleospin® Blood Kit (Macherey-Nagel) according to the manufacturer's instructions. This study was approved by the Institutional Review Board (IRB) of Kaohsiung Medical University Hospital.

2.2. Candidate miR-TS-SNP selection

Methods for identifying the miR-binding sites and availability of comprehensive genomic databases of SNPs provided an opportunity to predict and investigate the biological relevance of miR-TS-SNPs [21]. Yu et al. performed a genome-wide analysis of SNPs located in miRNA-binding sites, compared their frequencies in normal tissues and cancers through the mining by EST database mining and conducted case–control studies to determine the allele frequencies in patients with cancers. They found 12 miR-TS-SNPs that displayed an different allele frequencies in human cancers [22]. Landi et al. investigated the abilities of 79 mir-TS-SNPs, which were involved in cancer-associated pathways to alter the miRNA binding. Fifteen candidate miR-TS-SNPs were identified to be potentially relevant to cancers [20]. From the polymorphisms identified by the 2 groups, we selected 3 candidate miR-TS-SNPs for our study: PLA2G2A, which is associated with human cancer development and prognosis [23,24]; IL-16, an important regulator of human T-cell differentiation and activation [25,26]; and NOD2, a key element for hematopoietic function during graft-versus-host disease [27].

2.3. Assay design and PCR conditions

Robust and reproducible HRM analysis depends on good amplicon design. PCR products smaller than 250 bp are generally recommended for HRM analysis. In this study, primers were designed using the Primer3 software and are listed in Table 1. All the primers synthesized were of standard molecular biology quality (Protech Technology Enterprise Co., Ltd, Taiwan). Each PCR product with a single band was resolved on 2% agarose gels and visualized after staining with ethidium bromide.

2.4. HRM analysis

HRM analysis comprised 3 phases: a PCR reaction, DNA melting process and gene scanning for data analysis. All the 3 programs were performed on a single instrument, the Lightcycler® 480 Real-time PCR system (Roche Diagnostics, Penzberg, Germany) and operated with 96-well closed-tube platforms by the Lightcycler® 480 Gene Scanning Software. Each PCR reaction was carried out in a mixture containing the following reagents: 10 μl of Lightcycler® 480 High-Resolution Melting Master (Roche Diagnostics) (Taq polymerase, nucleotides and the High-Resolution Melting Dye), 0.25 μM of each primer, 2.5 mM $MgCl_2$ and 30 ng DNA. The final volume of PCR reactions was 20 μl. The PCR programs started with a denaturation-activation step at 95 °C for 10 min, followed by a 45-cycle program (denaturation at 95 °C for 15 s, annealing at 58 °C for 15 s, and elongation at 72 °C for 15 s, with fluorescence measurement using a single acquisition mode).

The melting program comprised 3 steps: denaturation at 95 °C for 1 min, renaturation at 40 °C for 1 min, and finally melting with continuous fluorescence measurement from 60 °C to 90 °C (25 acquisitions per °C). After the melting process was completed, gene scanning by the Gene-scanning software began with normalization of melting curves and equalization to 100% as the initial fluorescence and to 0% as the fluorescence remnant after DNA dissociation. Then the temperature axes of the normalized curves were shifted to the point in which the entire dsDNAs were completely denatured. Finally, difference plots were calculated by subtracting the curves of the wild-type and mutant DNAs. Thus the difference plot analysis can help cluster the samples into groups.

2.5. Direct DNA sequencing

Direct DNA sequencing was performed to confirm the results of HRM analysis. The samples were purified using the PCR-M™ clean-up system (VIOGEN) and the PCR products were directly sequenced. The sequencing process was carried out in a final volume of 10 μl of the purified PCR products, 2.5 μM of one of the PCR primers and 1 μl of ABI-PRISM terminator cycle-sequencing kit v3.1 (Applied Biosystems). A 25-cycle PCR program with denaturation at 96 °C for 10 s, annealing at 50 °C for 5 s, and elongation at 60 °C for 4 min was conducted in the ABI Prism 310 Genetic Analyzer (Applied Biosystems).

2.6. Statistics

The frequencies of the different genotypes of miR-TS-SNPs in PLA2G2A, IL-16 and NOD2 were calculated. The frequencies of the different genotypes of each gene in the healthy individuals were tested using Hardy-Weinberg equilibrium equation. The frequencies of the different genotypes in healthy individuals and AML patients were analyzed using Pearson chi-square test. The genotype changes in ALL patients before and after treatment were analyzed using McNemar-Bowker test.

3. Results

3.1. HRM analysis of miR-TS-SNPs of PLA2G2A, IL-16 and NOD2

The miR-TS-SNPs of 3 genes (PLA2G2A, IL-16 and NOD2) were studied using HRM analysis in healthy individuals, patients with

Table 1
Primers for PCR amplification and HRM analysis.

Gene	SNPs	Forward primer (5'→3')	Reverse primer (5'→3')	PCR Products
PLA2G2A	rs11677 (C/T)	GCAGGAGCCCTTCTATACCC	AGGCACACAGCACTACACCA	250 bp
IL-16	rs1131445 (C/T)	CTGCTGGCCTGAATGCTTAT	AGGCAGGTTTGTGTCAGCTC	199 bp
NOD2	rs3135500 (A/G)	TAGGCCGTTCCTTCAAAGC	GTTCACGGCCATGTTGTCTA	194 bp