

Rapid and highly sensitive pharmacogenetic diagnostics using real-time PCR

Heiko Petersen (1), Sabine Wind (1), Timm Greve (2), Geena Luc (2), Eckart Schnakenberg (2)
(1) Labor Dres. Fenner, Bergstr. 14, Hamburg, Germany
(2) artus GmbH, Königstr. 4a, Hamburg

Introduction

5-fluorouracil is a cytostatic drug generally used for the therapy of tumor diseases. It is metabolized by the enzyme dihydropyrimidine dehydrogenase (DPYD; EC 1.3.1.2). So far, several mutations have been identified within the DPYD gene leading to reduced enzyme activity.

It is known that patients with no or reduced activity of the dihydropyrimidine dehydrogenase bear an elevated risk for the appearance of severe side-effects (according to WHO level 3-4). The most common mutation by far, accounting for 52% of mutant alleles, is the G-to-A transition at the first nucleotide of the splice donor site of intron 14, IVS14+1G>A leading to deletion of exon 14.

For the detection of exon 14 skipping we developed and compared two different methods based on PCR/RFLP and real-time PCR. Here, we present a reliable and rapid method for the detection of the exon 14 skipping using LightCycler™ PCR which is commercially available. This genotyping procedure may help to individually optimize medical therapies and to lower the risk of adverse drug reaction in 5'-FU therapies.

Method

For exon 14 genotyping we established a method which is based on PCR/RFLP and compared with another method applying melting point analysis of LightCycler™ PCR.

After DNA extraction we amplified a fragment using the primer SKF (5'CTAAAGGCTGACTTTCCAGACTAC; 5 μM) and SKR (5'CAGCAAAGCAACTGGCAGATT; 5 μM). A total volume of 50 μl TaqPCR Master Mix (QiaAmp) was prepared including 5 μl DNA.

The amplification procedure for PCR/RFLP was 5 min 95 °C and 0.5 min 94 °C, 0.5 min 60 °C, 0.5 min 72 °C for 35 cycles and 10 min at 72 °C. After amplification 2 μl of the PCR fragment was digested with 10 U SnaBI (New England Biolabs) at 37 °C for 2.5 hours. Digested PCR fragments were visualized by 3% Ready Agarose Gel (BioRad).

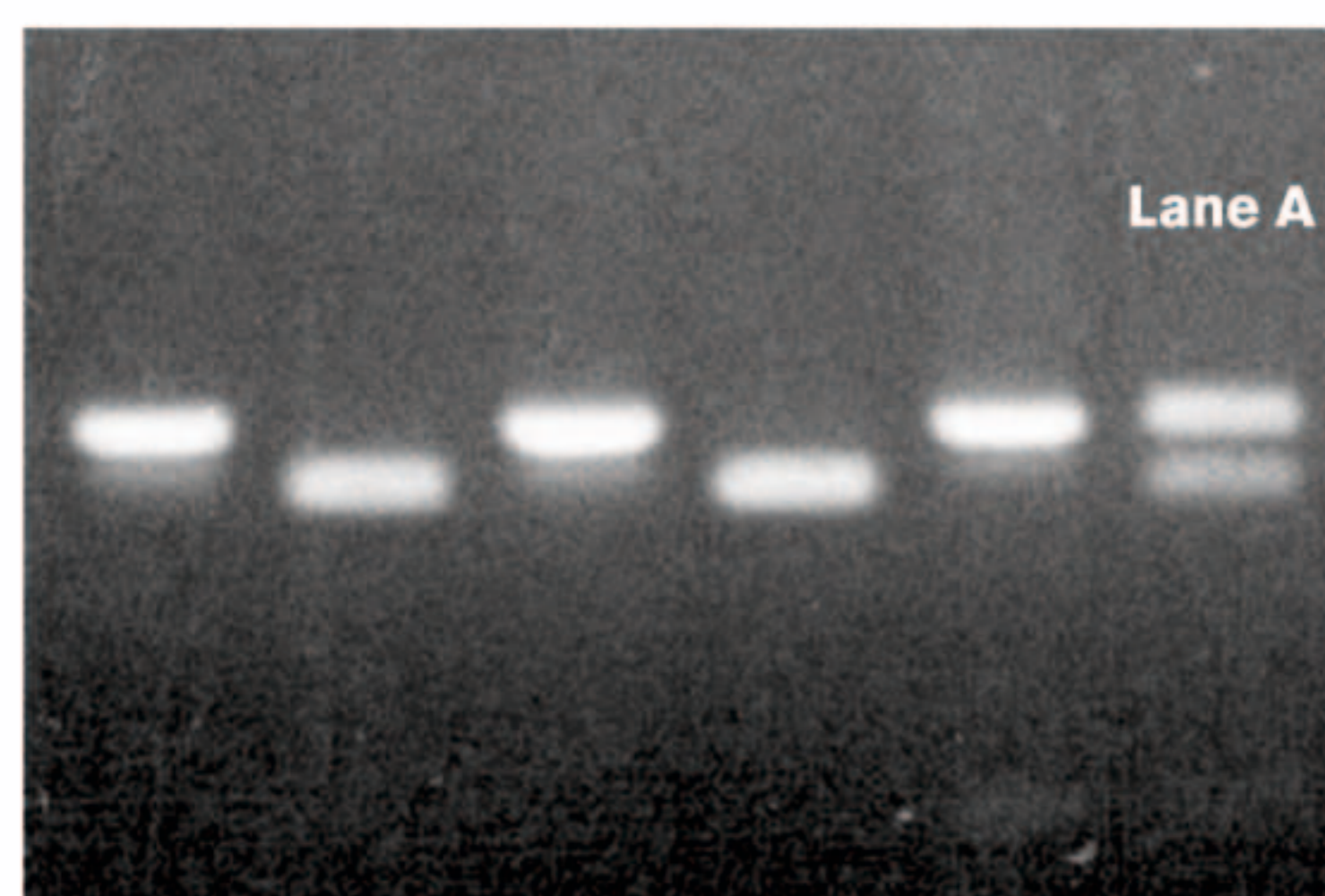


Fig. 1: RFLP pattern of exon 14 skipping within dihydropyrimidine dehydrogenase (DPYD) gene. Lane A shows a heterozygous carrier of exon 14 skipping.

For LightCycler™ PCR we used 10 min 95 °C, a touch down step of ten cycles for 5 sec 95 °C, 15 sec 65 °C and a secondary target temperature of 55 °C following by 20 sec 72 °C. The amplification step was performed using 35 cycles comprising of ten second at 95 °C, 10 sec 55 °C and 18 sec 72 °C. After amplification melting curve analysis was performed according to the instructions or the manufacturer (artus).

Results

Exon 14 skipping of the gene dihydropyrimidine dehydrogenase (DPYD) was performed by two different methods. We analysed the first nucleotide of the splice donor site of intron 14, IVS14+1G>A leading to deletion of exon 14. Using PCR/RFLP and LightCycler™ PCR we obtained the same results with both methods.

We amplified a fragment of 155 bp which is not digested if a subject is a homozygous carrier of the genetic variant. Fig. 1 shows carriers of the wild type and one heterozygous subject of exon 14 skipping using the PCR/RFLP method. Melting points of carrier of the wild type was identified at 57 °C while carriers of the genetic variant showed a melting point of 47 °C. DNA of 240 subjects were analysed.

DNA were selected randomly and not preselected for patients with adverse drug reaction. In total, we identified two subjects to be carrier of the heterozygous splice variant leading to an exon 14 skipping. None of the analysed subjects here showed a homozygous form of this genetic variant. Using LightCycler™ PCR the melting point analysis of thirty different DNA are shown in Fig. 2 including one heterozygous carrier of the exon 14 skipping.

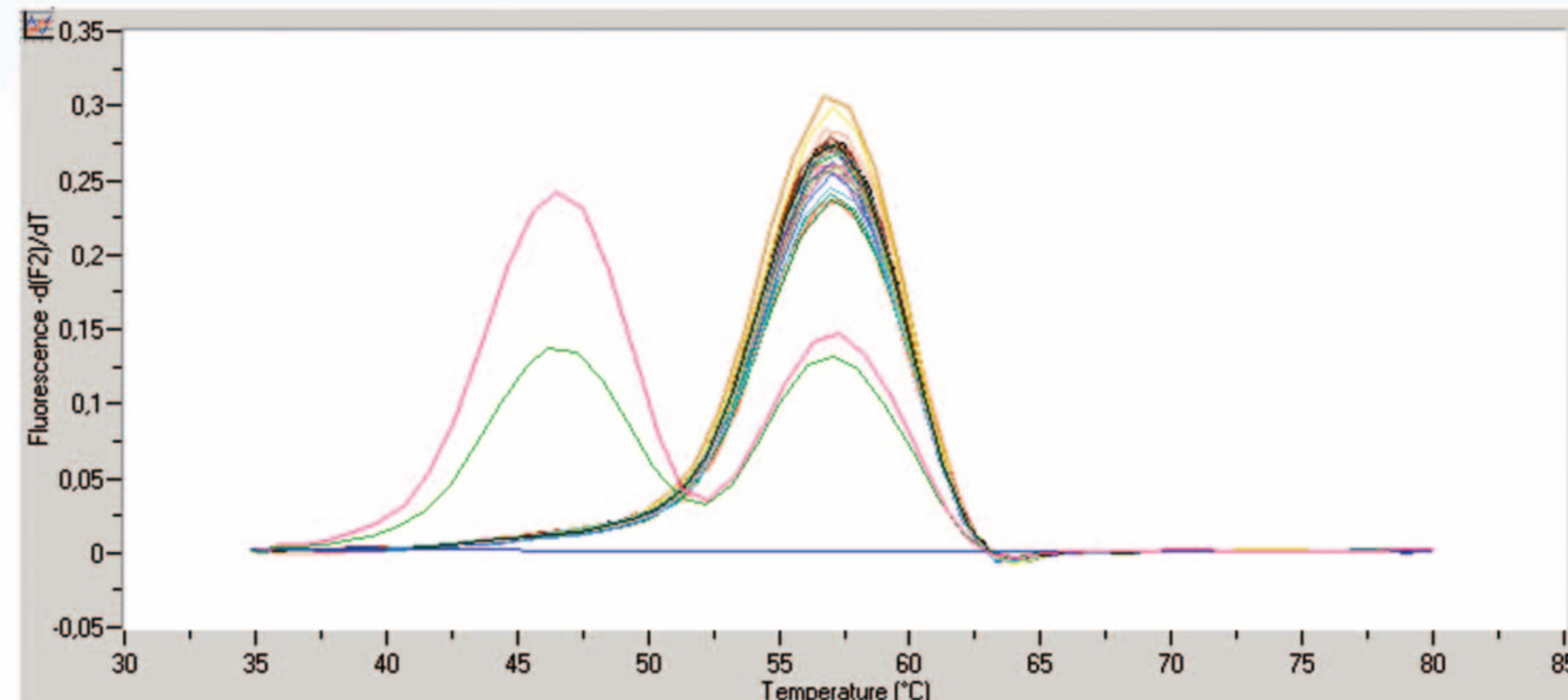


Fig. 2: Fluorogram for the detection of the nucleotide exchange IVS14+1G>A by means of the RealArt™ DPD LC PCR Reagents in the fluorimeter channel F2/BackF1.

Discussion

Besides influences such as age, sex, nutrition and comedication, genetic factors may influence enzyme activity. It is known that patients with severely reduced activity of the dihydropyrimidine dehydrogenase (DPYD) bear an elevated risk for the appearance of severe side-effects under the therapy with 5-fluorouracil (1).

Out of 240 randomly selected subjects we identified two heterozygous carriers of the splice donor site of intron 14 using two methods PCR/RFLP and LightCycler™ PCR. This is in the same range as published from other data (1-1.8% of the European population; www.aio-portal.de/nav/rund0202/r18.htm).

Until now several heterozygous patients have been identified in different clinical studies including a case report of a patient with topical 5-fluorouracil treatment (2). Using LightCycler™ PCR we present a robust and reliable method for the detection of exon 14 skipping within the dihydropyrimidine dehydrogenase (DPYD) gene.

References

- (1) Raída M, Schwabe W, Hausler P, Van Kuilenburg AB, Van Gennip AH, Behnke D, Hoffken K. Prevalence of a common point mutation in the dihydropyrimidine dehydrogenase (DPD) gene within the 5'-splice donor site on intron 14 in patients with severe 5-fluorouracil-related toxicity compared with controls. Clin Cancer Res, 7, 2832-9, 2001.
- (2) Johnson MR, Hageboutros A, Wang K, High L, Smith JB, Diasio RB. Life-threatening toxicity in a dihydropyrimidine dehydrogenase-deficient patient after treatment with topical 5-fluorouracil. Clin Cancer Res, 5, 2006-11, 1999.