RESEARCH NOTE

Detection of new single nucleotide polymorphisms by means of real time PCR

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Real time polymerase chain reaction (RT-PCR) is a new technique in molecular genetics which allows quantification of polymorphic DNA regions and genotyping of single nucleotide polymorphisms (SNPs) in one run. A by-product of real time PCR is the opportunity to identify new SNPs in the proximity of gene loci of interest. In this paper methodological details are presented and exemplified by a newly detected SNP in codon 155 of the catechol-O-methyltransferase (COMT) gene in a patient with severe alcoholism.

Some years ago genotyping of single nucleotide polymorphisms was conducted in two steps. First a DNA region of approximately 200 basepairs around the SNP was amplified millions to billions of times by means of a PCR before the PCR product had to be sequenced or digested by an enzyme with ensuing gel electrophoresis in order to genotype the polymorphic regions. The invention of RT-PCR extremely facilitates the analysis, because quantification of target DNA sequences and genotyping can be done in one single PCR run. Besides the higher speed of RT-PCR machines – notably less than one hour for one PCR run – the risk for contamination is minimised because the PCR product remains in the machine for genotyping. Moreover, the amplification process can be monitored online so that unsuccessful PCR runs can be terminated betimes.

Online monitoring of the amplification process as well as genotyping by melting curve analysis is possible by the use of hybridisation probes. These hybridisation probes are sequence-specific oligonucleotide probes, labelled by fluorescence dyes. For the detection of a SNP two hybridisation probes are required, one that binds to the DNA strand in a way that the polymorphic region is covered (sensor hybridisation probe) and a second probe (anchor probe) that binds to a site in close proximity (only 1–5 bases between the probes) to the sensor probe. The sensor probe is labelled by fluorescein and the anchor probe by LC Red 640. During amplification the hybridisation probes anneal to the amplified DNA segment. By means of an LED the RT-PCR machine excites the fluorescein of the sensor probe which then emits green light. The energy of the green light in turn excites the LC Red 640 dye which then emits red fluorescent light. The energy transfer is referred to as fluorescence resonance energy transfer (FRET). The optical unit of the RT-PCR machine is able to measure the intensity of the red light at 640 nm. The more new target DNA sequences are built the stronger is the FRET signal, because more probes can hybridise to the DNA. Therefore, the FRET signal is a direct measure of DNA copies in a PCR run. In the elongation phase of the PCR run the temperature is raised causing the displacement of the hybridisation probes from the DNA.

After amplification, point mutations can be detected by a melting-curve analysis. Hereby the temperature is slowly raised from 40° to 75°C and the critical temperature, T_m, at which the hybridisation probes are melted off the DNA strand, is an indicator for the presence or absence of a mutation. If the sensor probe is designed in a way that it perfectly fits the DNA strand, then it melts off at a higher temperature than in case there is a mismatch of one base (the single nucleotide mutation) causing the probe to melt off earlier at a lower temperature.

The result of the melting curve analysis can yield three characteristic curves: a curve with a single early peak (homozygous wildtype), a curve with a single late peak (homozygous mutant) and a curve with two peaks (heterozygous genotype). It has to be mentioned that the

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sizes of the amplitudes are irrelevant for the determination of genotypes because they are only an indicator for the amount of DNA and the concentration of hybridisation probes in the reaction tubes.

The precision of the RT-PCR is extremely high always resulting in nearly exactly the same melting points for the three possible genotypes. Just this high precision makes it possible to detect new SNPs on the target DNA. The hybridisation probes are designed in a way that they can detect a certain SNP on the target DNA. But if there is an additional SNP on the target DNA the melting curves will deviate from the standard curves resulting in a lower Tm than expected. A rule of thumb is that when the Tm differs more than 1.5°C from the standard curve than this is a strong hint for the presence of a new mutation in the DNA region covered by the hybridisation probes. Since RT-PCRs are conducted to investigate gene loci of high interest (mainly SNPs on coding regions or promoter regions) the detection of a new SNP in close proximity of those are very promising.

In the following we will illustrate the principle of detecting new SNPs by RT-PCR by an example of a new SNP on codon 155 of the COMT gene.

The hypothesis of a final common pathway of reward (Spanagel and Weiss 1999) has markedly influenced the search for candidate genes relevant for addiction. Therefore, especially polymorphisms of the dopaminergic (DA) system coding for receptors, transporters or enzymes involved in DA metabolism have predominantly been investigated (Noble 2000; McKinney *et al*. 2000). One of those DA associated candidate genes is the catechol-o-methyltransferase (COMT) gene. COMT is an enzyme which has a crucial role in the metabolism of catecholamines by inactivating them in the synaptic cleft. The COMT gene is located at the q11 band of human chromosome 22, and until now more than 800 SNPs have been identified on it but only 23 of these SNPs are located in coding regions. The most prominent SNP on the COMT gene is a G → A transition in codon 158 of the COMT gene resulting in 3 to 4-fold difference in COMT enzyme activity (Lachman *et al*. 1996) by coding for the synthesis of the amino acid methionine instead of valine (VAL158MET SNP). According to the literature homozygosity for the high activity allele (VAL/VAL genotype) and for the low activity allele (MET/MET genotype) is found in approximately 25% of Caucasians, each. Heterozygotes (VAL/MET genotype) have intermediate levels of COMT activity (Lachman *et al*. 1996; Syvänen *et al*. 1997).

Results with respect to addiction and the COMT VAL158MET polymorphism are heterogeneous: For example David *et al*. did not find any association with smoking behaviour (David *et al*. 2002). However, associations between the COMT-polymorphism and alcoholism have already been reported (Kauhanen *et al*. 2000; Wang *et al*. 2001) or denied (Ishiguro *et al*. 2000; Hallikainen *et al*. 2000). With the aim to replicate the findings reporting a positive association between the VAL158MET SNP and alcoholism and to test for interactions between the COMT gene and other DA relevant candidate genes we started to genotype alcoholics with a history of severe alcohol abuse who were presently in therapy in a psychiatric hospital. Genotyping of the COMT polymorphisms was performed by real time PCR using fluorescence melting curve detection analysis by means of the Light Cycler System (Roche Diagnostics, Mannheim, Germany). The primers and hybridisation probes used (TIB MOLBIOL, Berlin, Germany) and the PCR protocols were as follows: For COMT: forward primer: 5′-GGGCCTACTGTGGCTACTCA-3′; reverse primer: 5′-GGCCCTTTTTCCAGGTCTG-3′; anchor hybridisation probe: 5′-LCRed640-TGTGCATGCCTGACCCGTTGTCA-phosphate-3′; sensor hybridisation probe: 5′-ATTTCGC-TGGCATGAAGGACAAG-fluorescein-3′ (the sensor hybridisation probe was designed to be complementary to the MET allele). The localization of the hybridisation probes and the primers are illustrated in figure 1. The PCR run comprised 55 cycles of denaturation (95°C, 0 s, ramp rate 20°C $^{s-1}$), annealing (57°C, 10 s, ramp rate 20°C $^{s-1}$) and extension (72°C, 10 s, ramp rate 20°C $^{s-1}$) which followed an incubation period of 10 min to activate the FastStart Taq DNA Polymerase of the reaction mix (Light Cycler FastStart DNA Master Hybridisation Probes, Roche Diagnostics, Mannheim, Germany). After amplification a melting curve was generated by holding the reaction time at 40°C for 2 min and then heating

Figure 1. Schematic diagram of the location of the primers and probes in relation to the mutation. Forward primer (light green): position 1822–1841; Reverse primer (light green): position 1983–1999; Mutation (dark green): position 1947; Sensor hybridisation probe: position 1936–1958; Anchor hybridisation probe: position 1960–1982.

slowly to 95°C with a ramp rate of 0.2°C s^{-1}. The fluorescence signal was plotted against temperature to yield the respective melting points (T_m) of the two alleles. T_m for the VAL allele was 59.00°C and 64.50°C for the MET allele (see figure 2). It has to be mentioned that we designed the sensor probe in such a way that it perfectly matched the mutated MET-allele and not the wildtype, resulting in a higher T_m for the MET-allele than for the VAL-allele.

Melting curve analyses revealed a new atypical melting curve for one of the alcoholic patients with a Tm of 62.70°C, located between melting points of the VAL and the MET allele. This yields strong evidence for a mutation located near codon 158 – obviously located in a region to which the hybridisation probes bind. In order to verify this result and to more precisely determine the location of the mutation we sequenced the PCR product after purification (see figure 3). Sequencing results showed a silent SNP, a C → T transition in the third position of codon 155 in one allele. Moreover, this patient was homozygous for the MET allele in codon 158. Due to an additional mismatch the SNP in codon 155 results in a weaker binding of the sensor hybridisation probe during real time PCR. Therefore the T_m of this patient with the genotype MET/MET lies between the Tm's of the VAL and the MET alleles.

In more than 800 analyses in our laboratory on healthy subjects and alcoholics the new SNP in codon 155 could not be detected before, indicating the low prevalence of this mutation. Although, the C155T polymorphism is a silent mutation it may be of scientific and clinical interest because numerous examples have been reported in the literature for linkages between silent mutations and psychopathological behaviour, especially if a silent mutation is located within a splice site. However, since the variation is located in an open reading frame, it is unlikely that it influences a splicing event.

In the case of a silent mutation a second possibility has to be considered which can cause functional implications. Due to differences in the availability of a specific tRNA a reduction of translation rate is caused. In the case of the C155T SNP this is suggested by the reduced absolute and relative codon usage frequency of the TTT codon as compared to the TTC wildtype codon, both coding for phenylalanine (absolute usage per thousand codons: 15.36 vs 20.72; relative frequency of each codon among

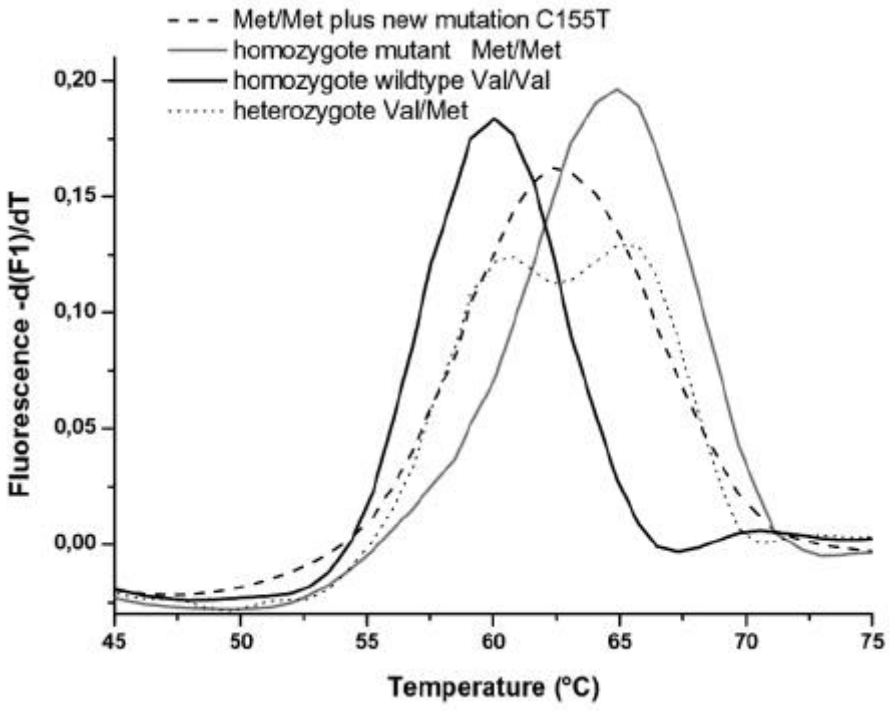

Figure 2. Results of the melting curve analyses of the COMT polymorphism. Melting temperature (T_m) is calculated by taking the first negative derivate (–dF/dT) of the melting curve. A single late peak indicates a mutation on both alleles (MET/MET genotype). A single early peak indicates the homozygote wildtype (VAL/VAL genotype). Two peaks indicate a heterozygote sample (VAL/MET genotype). The curve with the single peak between the homozygote VAL/VAL and MET/MET genotypes indicates the new additional mutation in codon 155. The heights of the amplitudes (fluorescence) of the curves are irrelevant. Of importance is only the temperature of the peak of the curves (T_m).

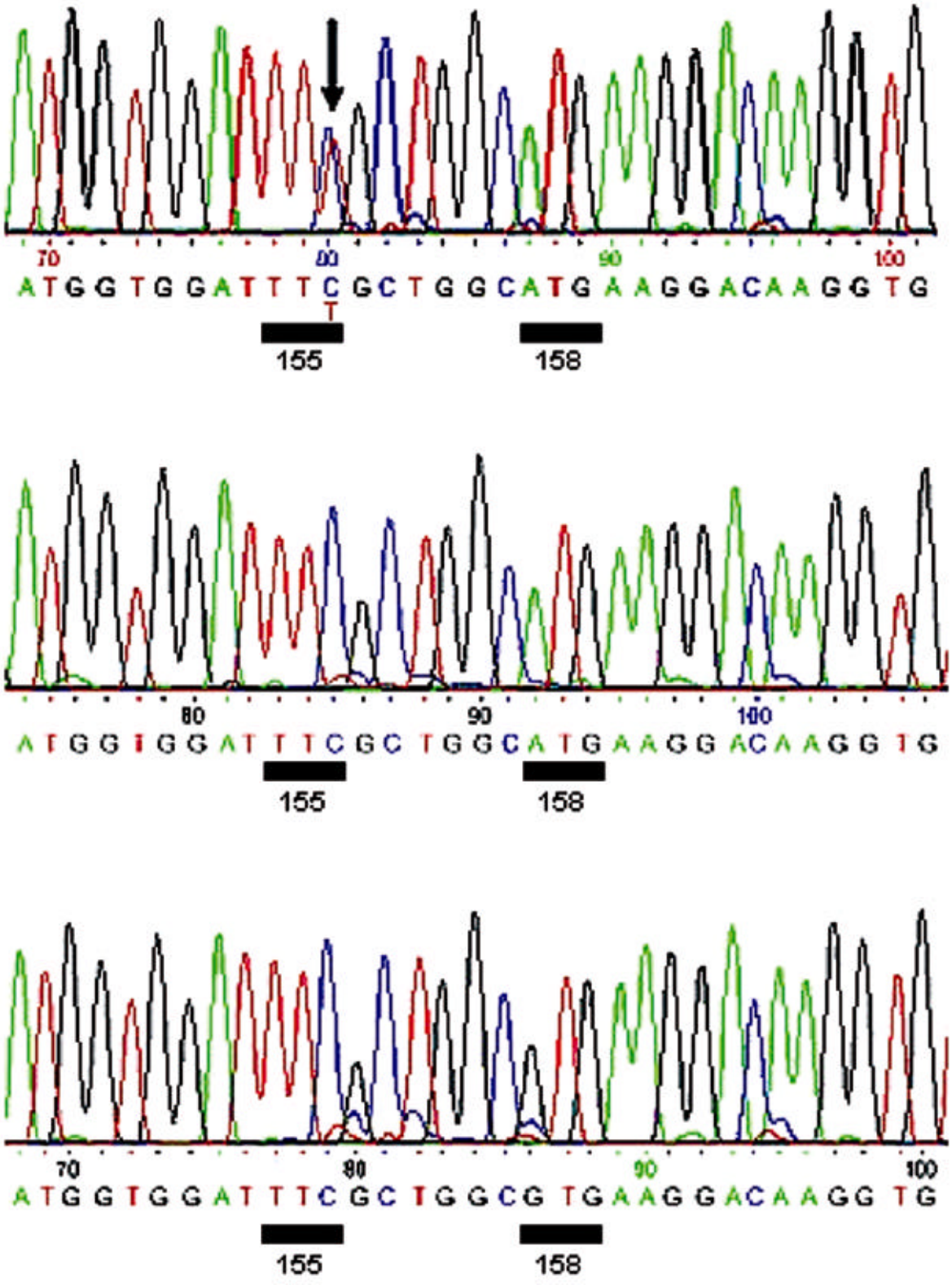

Figure 3. Sequencing results. Upper panel: homozygote mutant (MET/MET) in codon 158 and new SNP (C→T transition) in codon 155 (see arrow). Patient is heterozygote for this SNP. Medium panel: homozygote mutant (MET/MET) in codon 158; Lower panel: homozygote wildtype (VAL/VAL) in codon 158.

synonymous codons: 0.43 vs 0.57; Guigo *et al*. 1999). However, this difference in codon usage for phenylalnine in the mammalian translation system is far from impressive supporting the hypothesis that the new SNP in codon 155 does not seems to be of clinical relevance for alcoholism.

Results demonstrate that the method of melting curve analysis does not only allow to genotype with a perfect reliability but it also increases the probability to detect new SNPs. The detection of an abnormal melting curve, i.e. an earlier or later peak, yields strong evidence for a new mutation. Since only highly interesting gene regions are investigated by real time PCR, this method increases the chance to detect functional SNPs or SNPs that are in linkage disequilibrium with functional gene regions. The fantastic thing about this method is that the researcher does not need to pay attention to large sequencing results but that the new mutation is directly visible by the mere inspection of the melting curves.

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