

Short reports

A PCR-SSP method for detecting the His63Asp mutation in the HFE gene associated with hereditary haemochromatosis

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Abstract

Hereditary haemochromatosis is an autosomal recessive disease in which there is defective regulation of iron absorption, causing gradual accumulation of excessive amounts of iron in certain organs. Recently, a candidate gene for hereditary haemochromatosis has been identified, located on the short arm of chromosome 6, telomeric to the major histocompatibility complex (MHC) and showing sequence homology to the human leucocyte antigen (HLA) class I genes. Two mutations have been found in this gene that are potential markers for haemochromatosis. The first, a cysteine to tyrosine substitution (Cys282Tyr) is strongly associated with the disease, whereas the second mutation, a histidine to aspartic acid substitution (His63Asp) shows a less obvious relation. To examine the importance of this second mutation in hereditary haemochromatosis it is important to study the links between this genotype and abnormalities of iron metabolism. A polymerase chain reaction method using sequence specific primers is described which might be useful for identifying those individuals carrying the mutation that encodes the His63Asp substitution, who might be at risk from a milder form of haemochromatosis.

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Keywords: haemochromatosis; polymerase chain reaction using sequence specific primers; HFE gene; His63Asp substitution

The haemochromatosis associated substitutions Cys282Tyr and His63Asp described by Feder *et al*,¹ caused by point mutations 845G→A and 187C→G, respectively, are located in a gene that has been assigned the name HFE by the Genome Database Nomenclature Committee, and which is situated more than 3 Mb telomeric from the major histocompatibility complex (MHC). Although a functional role in iron metabolism for a molecule with human leucocyte antigen (HLA) class I characteristics has not been demonstrated conclusively, an association between haemochromatosis and the two mutations in the HFE gene has been shown by several groups.¹⁻⁴

The incidence of homozygosity for the Cys282Tyr substitution (845A/A) in white haemochromatosis patients is 85-100%, and various methods for identifying this allele and its significance in diagnosis have been reported.¹⁻⁷ The demographic distribution of the Cys282Tyr substitution corresponds closely to the incidence of hereditary haemochromatosis, predominating in the Celtic populations of Northern Europe, a further demonstration of the extremely close association between this mutation and the disease.⁸

In contrast, the effect of the His63Asp substitution is less obvious. It has been shown that the mutant 845A allele is in complete linkage disequilibrium with the normal 187C allele (no chromosome with the Cys282Tyr substitution carries the His63Asp substitution as well).⁴ Among a group of patients with haemochromatosis studied by Beutler, ~12% of the haplotypes did not have the Cys282Tyr substitution, but of these almost 40% carried the His63Asp substitution (compared with 17% in normal controls). More significantly, in patients heterozygous for Cys282Tyr, 86% (predictably all male) carried the His63Asp substitution (they were compound heterozygotes).⁹ From gene frequency data it has been estimated that the penetrance of the compound heterozygous condition is roughly 1.0-1.5%, prompting the suggestion that the combination of the 845A and 187G alleles might produce a much milder form of haemochromatosis than the 845A/A homozygous situation, and that it would still be of benefit to identify individuals with the compound heterozygous genotype to monitor their serum iron and ferritin concentrations periodically.⁴

The polymerase chain reaction method using sequence specific primers (PCR-SSP) described here allows simple and rapid typing for the His63Asp substitution, and it could be used as part of a comprehensive genetic screening strategy for hereditary haemochromatosis.

Methods

DNA was extracted from 2 ml anticoagulated blood by a simple salting out method¹⁰ and the DNA concentration was adjusted to 50 ng/μl.

PCR amplification was carried out in a reaction volume of 10 μl consisting of 20 mM ammonium sulphate, 75 mM Tris-HCl

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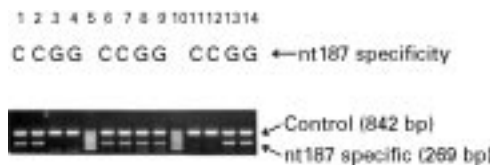


Figure 1 Gel electrophoresis from three individuals. All reactions were carried out in duplicate. Lanes 1–4, individual homozygous for the normal allele (187C/C; His63Asp^{-/-}); lanes 5–8, individual heterozygous for the mutant allele (187C/G; His63Asp^{-/+}); lanes 9–12, individual homozygous for the mutant allele (187G/G; His63Asp^{+/+}); lanes 13 and 14, molecular size markers (100 bp ladder).

(pH 9.0), 0.01% Tween, 1.5 mM MgCl₂, 200 μM dNTPs, 1.0 μM specific primers (Oswel, Southampton, UK), 0.25 μM control primers, 0.5 U DNA polymerase (Advanced Biotechnologies, Epsom, UK), and 50 ng genomic DNA. One duplicate reaction set was specific for the normal sequence (187C) and a second reaction set was specific for the mutated sequence (187G); both reaction sets used a common antisense primer (5'-CACAAAGACCTCAGACTTCCA-3') from intron 2 of the HFE sequence with a sense primer specific for either 187C (5'-GCTGTTTCGTGTTCTATGATC-3') or 187G (5'-GCTGTTTCGTGTTCTATGATG-3'), resulting in a PCR product of 269 base pairs (bp). Control primers that amplified an 842 bp fragment of the human growth hormone gene¹¹ were included in all reactions. Amplification conditions involved an initial denaturation step for one minute at 96°C, followed by five cycles consisting of denaturation for 25 seconds at 96°C, annealing for 45 seconds at 70°C, and extension for 30 seconds at 72°C. This was followed by a further 21 cycles where the annealing temperature was lowered to 65°C, and four cycles where annealing was for one minute at 55°C and extension was for two minutes at 72°C. A final extension step for five minutes at 72°C completed the amplification.

Results and discussion

The duplicate reaction sets typing for either 187C (normal) or 187G (mutant) alleles were analysed by electrophoresis in a 1.5% agarose gel; PCR products were visualised by staining with ethidium bromide and photographed under ultraviolet light. Results from three representative individuals are shown in fig 1. They show that the PCR–SSP method is highly specific for the haemochromatosis associated mutation 187C→G in the HFE gene and that the method could be used to identify individuals with the 845A/187G compound heterozygous genotype who might be susceptible to a milder form of hereditary haemochromatosis.

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A simple combined microdissection and aspiration device for the rapid procurement of single cells from clinical peripheral blood smears

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Abstract

Molecular analysis of cells from cytology specimens can help to establish a diagnosis in ambiguous cases. However, mutations in heterogeneous samples might not be detected because of the diluting effect of DNA from normal background cells. Even if a mutation were detected, it could not be traced back to a specific cell type.

Molecular analysis of single cells circumvents this problem. Both mechanical and laser assisted methods have been described for the selective procurement of cells from histology slides; however, they have the drawback of either being technically demanding or expensive. Furthermore, it is unclear whether they can be applied to cytology specimens. Finally, few

of these techniques are able to procure single cells. Therefore, we developed a simplified combined microdissection and aspiration device for the rapid procurement of single cells from clinical cytology specimens. The principle of this device, called the cytopicker, is the combination of the microdissection tool, a steel cannula, with the aspiration tool, a glass capillary connected to a vacuum, into one device. Steel cannulae are optimal for microdissection of cells from the hard matrix of cytology specimens but aspirate poorly. On the other hand, glass capillaries are suboptimal for dissecting but aspirate very well. Combining both tools into one by inserting the capillary into the cannula allows optimal dissection using the cannula (with the glass capillary withdrawn and thus protected), followed by optimal aspiration using the capillary (after being advanced through the cannula). All movements of the device are controlled by just one micromanipulator, making the cytopicker inexpensive to manufacture. The cytopicker can rapidly and simply procure single cells, such as lymphoblasts, from cytology specimens, such as peripheral blood smears. DNA from these cells can be amplified by PCR. However, precautions have to be taken to avoid contamination. Once improved further, the cytopicker might facilitate molecular analysis in the routine cytology laboratory.

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Keywords: microdissection; single cell; cytology; molecular analysis

Molecular analysis of cells derived from routine clinical samples such as histology and cytology slides is attracting increasing attention. Molecular analysis can supplement morphological assessment and aid in the diagnosis of ambiguous morphological findings. A prerequisite for molecular analysis is the procurement of morphologically defined cells from the slides, which often necessitates the retrieval of single cells. Otherwise, the DNA from contaminating normal background cells can impede molecular analysis of the cells of interest or, alternatively, if a molecular alteration is found it cannot be correlated to a specific cell.

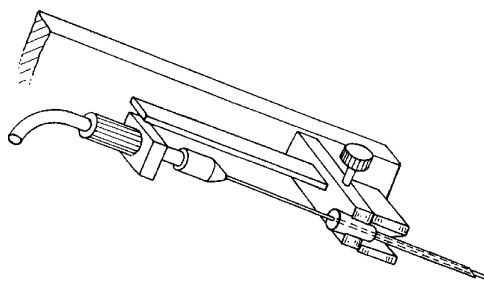


Figure 1 Design of the cytopicker. A glass capillary connected to a vacuum source is inserted into a steel cannula and can be advanced and retracted inside the stationary cannula. The cytopicker is mounted on the arm of a micromanipulator.

To circumvent these potential problems, mechanical and laser assisted microdissection and procurement methods have been described for formalin fixed, paraffin wax embedded histological sections.^{1–6} We and others have described mechanical and laser methods for cell retrieval from cytology specimens.^{7,8} These methods reliably provide cells for DNA extraction, but they either require a high degree of manual dexterity or specialised expensive equipment, thus impeding their application in the routine diagnostic laboratory. For many of these techniques, the practicability for cytological samples, which differ in their consistency from histological samples, has not been shown. In addition, few of these techniques allow the retrieval of single cells, a feature especially needed for cytology samples with scant cells.

Therefore, we developed an inexpensive device for the simple and rapid retrieval of single cells from clinical cytology specimens. We show that this device, called the cytopicker, effectively retrieves single lymphoblasts from peripheral blood smears of patients with acute lymphoblastic leukaemia (ALL).

Materials and methods

DESIGN OF THE CYTOPICKER

Figure 1 is a schematic diagram of the cytopicker. A glass capillary with an inner diameter of about 30 μm is connected via plastic tubing to a vacuum source, such as a Hamilton syringe or a piston pump. The capillary is inserted into a fine steel cannula (Microbalance

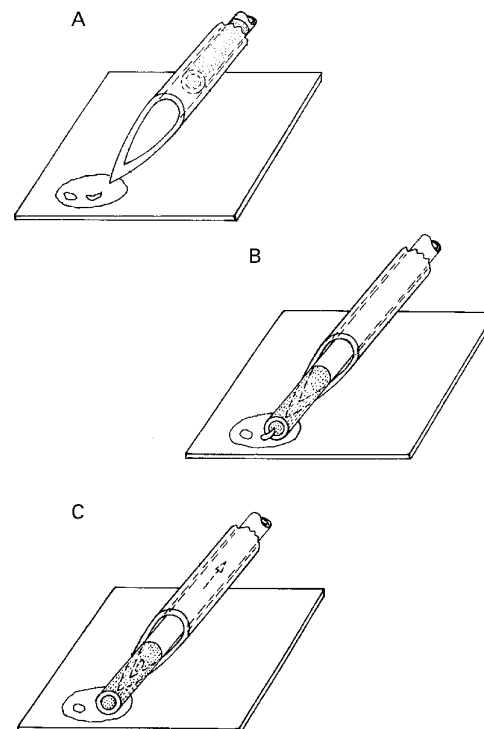


Figure 2 Application of the cytopicker. (A) A drop of oil is applied to the slide. The cell of interest is microdissected by the steel cannula with the glass capillary withdrawn. (B) After the cell of interest has been mobilised, the glass capillary is advanced and placed over the cell. (C) Suction is applied to the capillary and the cell is aspirated into the most distal part of the capillary.

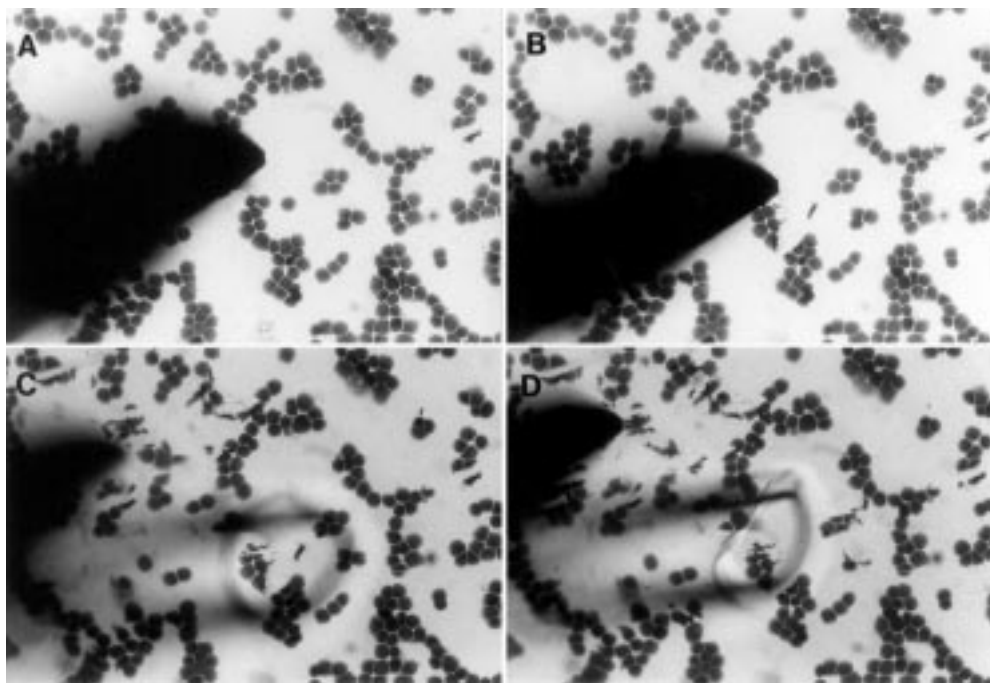


Figure 3 Microdissection and aspiration of a single lymphoblast from the peripheral blood smear of a patient with acute lymphoblastic leukaemia. (A) The lymphoblast of interest is identified and the steel cannula is placed adjacent to it. The glass capillary cannot be seen because it is retracted into the steel cannula. (B) With the tip of the cannula the lymphoblast has been microdissected and is visible as a sausage-like structure. Some surrounding cells have been damaged without having been detached. (C) The cannula is pulled back and the glass capillary is advanced and placed over the mobilised blast. (D) After suction has been applied to the capillary, the blast is aspirated into the capillary (the blast cannot be seen inside the capillary because it is hidden by the cannula).

3, Becton Dickinson; Heidelberg, Germany). The cannula (with the capillary inside) is fixed rigidly to the arm of a micromanipulator. The cytopicker is moved relative to the stationary specimen by using the micromanipulator controls. Alternatively, the cytopicker, after being lowered into the area of interest by the micromanipulator, remains stationary and the stage is moved using the microscope drive controls. The capillary can be moved back and forth within the cannula by a control on the arm of the micromanipulator.

PATIENTS AND SAMPLES

Diagnostic peripheral blood smears from three patients with ALL were used. The diagnosis of ALL was made according to standard morphological, immunological, and cytogenetic criteria.⁹ The blood smears were prepared using a standard technique, air dried, fixed, and stained with modified May-Gruenwald's eosin-methylene blue solution, followed by Giemsa stain. The glass slides were not coverslipped.

MICRODISSECTION AND ASPIRATION

Microdissection was performed using an inverted microscope (Leitz DM IL; Leitz, Leica, Germany) with the cytopicker mounted on the micromanipulator (Bachofer, Reutlingen, Germany). To decrease manipulation time, the micromanipulator was modified so that its arm automatically snapped the cytopicker into the light path. Leukaemic blasts conforming to standard morphological criteria were identified by light microscopy using $\times 1600$ magnification and oil immersion. With the capillary withdrawn, the cannula was brought within the proximity of the leukaemic blast of interest (figs 2A and 3A). The blast was mobilised with the tip of the cannula by operating the micromanipulator or the microscope stage (figs 2A and 3B). The cannula was pulled back slightly and the capillary pushed forward until its opening covered the mobilised blast (figs 2B and 3C). The blast was aspirated into the capillary by applying suction (figs 2C and 3D) and transferred into a reaction tube containing 10 μ l lysis buffer (100 mM Tris-HCl, pH 8.0, 0.1 M EDTA, pH 8.0, 1 mg/ml proteinase K,

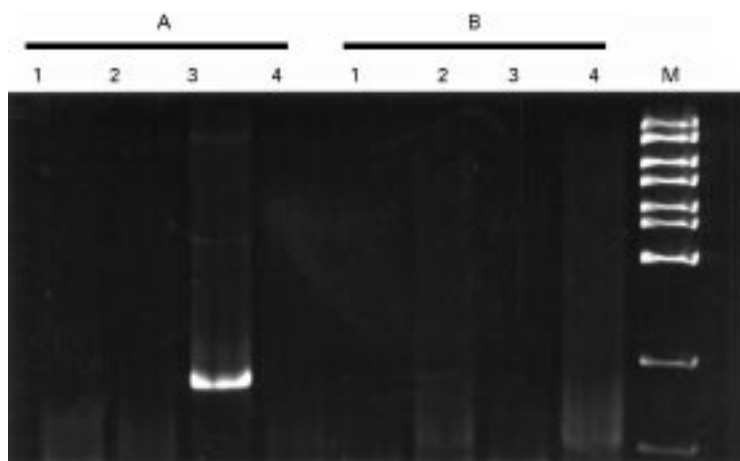


Figure 4 PCR amplification of single cells procured by the cytopicker. From each peripheral blood smear of two patients (A and B) three single cells (lanes 1–3) and oil without visible particles (lane 4) were procured with the cytopicker, lysed, and subjected to nested PCR to amplify part of the CD95 gene. PCR products were separated on an 8% polyacrylamide gel. M, 100 base pair marker.

and 1% Tween 20 (both from Sigma, Deisenhofen, Germany)) as described above. After swinging the cytopicker back into focus, the capillary was checked for complete expulsion of the blast. To control for contamination by debris in the oil covering the microdissection site, oil without visible particles was aspirated.

PCR

The single cell was lysed at 37°C overnight and the proteinase K inactivated at 95°C for 10 minutes. The lysate was used as the template for a nested PCR amplification of CD95 (APO-1/Fas), as described previously.⁷ PCR products were visualised using an 8% polyacrylamide gel stained with ethidium bromide (fig 4).

Results and discussion

Using the cytopicker, single cells of interest could be precisely microdissected away from surrounding cells using the steel cannula. Cells were mobilised en bloc without fragmentation. The mobilised cells were aspirated into the most distal part of the glass capillary. The extremely small volume of oil aspirated under constant control allowed the aspiration of the mobilised cell without visible contamination. Expulsion was always complete. The procedure to procure one single cell could be performed within 10 minutes by a trained operator; however, this is still too long for most routine laboratories. The time could be decreased by changes in the design of the cannula and capillary (see below) and by using pre-made single use cytopickers. The cytopicker did not require much practice or manual dexterity because inexperienced users were able to perform the procedure proficiently after a short time.

One has to keep in mind the limited application of the cytopicker as tested so far because the "matrix" in the background of other cytological preparations such as cervical smears, sputum samples, or fine needle aspirates from solid organs is different from that of peripheral blood smears. Also, blood films are unfixed, unlike some other cytology specimens.

We have shown previously that the whole genome of single cells microdissected and retrieved from routine cytology slides can be amplified and specific genes of interest can be analysed by means of PCR using small aliquots of the whole genome amplification.⁷ There was no contamination from surrounding cells. Whole genome amplification produces a sufficient number of DNA copies from a single cell to analyse all exons of an average sized gene for mutations, to perform multiple loci genetic analysis, or to verify molecular findings by repeat analysis. Thus, molecular analysis of

cells from cytology slides becomes practical because the time needed for retrieval of the cells is the limiting factor of this technique. However, we did not use the cytopicker in the study mentioned above but used a microdissection technique with steel cannulae only. Thus, although single cells were procured quite rapidly, the technique was difficult to master and therefore not suitable for the routine cytology laboratory.

The cytopicker is a potential solution to the problem of sample capture after microdissection, the most time consuming and often futile step of procuring single cells. It retrieves single cells in an easy and rapid way, which, as shown for patient A, can be amplified successfully by PCR for further molecular diagnostic analysis. Despite successful retrieval and transfer of single cells, the PCR amplification often fails, as shown for patient B and some single cells of patient A. However, this is a (well known) problem of single cell PCR and not of single cell microdissection and aspiration. A potential problem is the contamination by debris in the oil that has escaped visual detection. This problem can be solved by decreasing damage to cells surrounding the cell of interest by crafting needle tips more suitable to dissection than conventional cannulae. Even more efficient in this regard would be capillaries designed like the oocyte holding pipettes used for in vitro fertilisation: the mobilised cell would stick to the tip of the capillary after suction has been applied, thus occluding it and preventing the aspiration of potentially contaminated oil into the capillary. Appropriate experiments are underway in our laboratory.

Taken together, the cytopicker might make molecular analysis of cytology specimens practicable for the routine cytology laboratory.

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