Short reports

Whole genome amplification of single cells from clinical peripheral blood smears

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Abstract

Molecular analysis of clinical samples has been hampered by the lack of fresh or frozen specimens and the presence of contaminating background cells within samples obscuring the molecular analysis of the pathological cells of interest. Routine cytology specimens are a ubiquitous and abundant, yet largely untapped, source of clinical samples for molecular analysis. Morphologically defined single cells from peripheral blood smears can be microdissected from contaminating background cells and their whole genome amplified by primer extension preamplification, followed by polymerase chain reaction analysis of the specific DNA of interest. Thus, molecular information can be traced back to the cell of origin in these clinical specimens. This should allow studies on clonality, loss of heterozygosity, mutation, or amplification of multiple loci from one single cell in haematological smears and possibly other clinical cytology specimens.

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Keywords: single cell; whole genome amplification; cytology

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Molecular analysis of clinical specimens is restricted by several factors. Normally, fresh or frozen samples are needed and, usually, the more abundant clinical cytology specimens contain only a small number of cells. Furthermore, many clinical specimens have to be preserved for future reference. Owing to contamination with normal cells, nucleic acid extraction from these specimens results in a mixture of normal and abnormal DNA and RNA, which is likely to interfere with the molecular analysis of the cells of interest. Conventional extraction of nucleic acids also destroys the histological context of the molecular information and leads to a loss of nucleic acids.

A method for the molecular analysis of defined single cells allowing the use of the abundant and virtually indestructible clinical cytology specimens would be useful. Therefore, we adapted the technique of microdissec-

tion to the retrieval of single cells from haematological smears. We then applied the technique of whole genome amplification that has been described for fresh cells to this archival tissue. We demonstrated that single cells from these stained clinical cytology specimens identified by light microscopy can be isolated and their whole genome amplified for subsequent molecular analysis.

Materials and methods

PATIENTS AND SAMPLES

Two diagnostic smears of peripheral blood from two children with acute lymphoblastic leukaemia (ALL) diagnosed by standard morphological, immunological, and cytogenetic criteria were used. The blood smears were made using a standard technique, air dried, fixed, and stained with modified May-Gruenwald's eosin-methylene blue solution, followed by Giemsa staining. The glass slides had not been coverslipped at any stage during their storage before use in this study. Leukemic blasts conforming to standard morphological criteria were identified by light microscopy using $\times 1600$ magnification and oil immersion.¹ Five single blasts and their surrounding areas from different regions of each slide were sketched or photographed by a video camera. Residual oil was washed off with xylol.

MICRODISSECTION

Microdissection was performed using an inverted microscope (Leitz DM IL; Leitz, Leica, Germany) and a mechanical micromanipulator (Singer Instrument Co, Watchet, UK). Alternatively, laser microdissection was used as described previously, with some modifications.² The laser microdissection device was equipped with a liquid dye laser (rhodamine 6G, 590 nm), excited by a nitrogen laser through a microscope connected to a TV camera and computer. Using the sketch or videoprint as a guide, the lymphoblast was re-identified without oil immersion. All surrounding cells were microdissected from the lymphoblast using a sterile 27G3/4 needle (Microlance 3; Becton Dickinson, Heidelberg, Germany) connected to the micromanipulator. When using laser microdissection, the surrounding cells were evaporated by sending a laser beam with one impulse per second

through a $\times 10$ or $\times 16$ objective lens. The blast, together with a few surrounding erythrocytes, was picked up with a new needle and transferred to a microtube containing 5 μ l lysis buffer (200 mM KOH, 50 mM dithiothreitol). As a negative control, all cells of an area were microdissected away from the slide, the cleared area scraped with a sterile needle, and the needle tip rinsed in a microtube containing 5 μ l lysis buffer. Negative controls were done repeatedly before and once during the actual analysis of the slide.

WHOLE GENOME AMPLIFICATION

Whole genome amplification of single cells was performed by primer extension preamplification as described previously.³ The single cell in lysis buffer was incubated at 65°C for 10 minutes. A 5 μ l volume of neutralisation buffer (900 mM Tris-HCl, pH 8.3, 300 mM KCl, 200 mM HCl) was added, followed by 5 μ l of 400 μ M random primers (Operon Technologies, Alameda, California, USA), 6 μ l 10 $\times$ K⁺ free polymerase chain reaction (PCR) buffer (25 mM MgCl₂, 1 mg/ml gelatin, 100 mM Tris-HCl, pH 8.3), 0.75 μ l of each dNTP at 2 mM, 1 μ l of 4 U/ μ l Taq polymerase (MBI Fermentas, St Leon-Rot, Germany), and 35 μ l distilled water. Primer extension was performed using an MJ Research thermocycler (Cambridge, Massachusetts, USA). An initial five minute denaturing step at 92°C was followed by 50 cycles of denaturation at 92°C for one minute, annealing at 37°C for two minutes, ramping from 37°C to 55°C with 0.1°C/second, and primer extension at 55°C for four minutes.

PCR

A 4 μ l aliquot of the whole genome amplification reaction was used as the template for a

PCR reaction with primers derived from the published CD95 (APO-1/Fas) sequence: forward primer (intronic) 5'-CATTAGAA AAACAAATTTTCAGAC-3', reverse primer (exonic) 5'-CAGAACTGAATTTGTTGTT TTTCAC-3'.⁴ Amplification was performed with 300 nM of each primer, 50 μ M of each dNTP, 75 mM KCl, 3.5 mM MgCl₂, 10 mM Tris, pH 8.3, and 1.2 U Taq DNA polymerase (MBI Fermentas). The samples were denatured at 95°C for three minutes, amplified for 15 cycles with each cycle consisting of 45 seconds at 95°C and one minute of 70°C with a decrease of 1.0°C per cycle, followed by 30 cycles with each cycle consisting of 45 seconds at 95°C, 30 seconds at 50°C, and one minute at 72°C, followed by five minutes at 72°C. A second, seminested PCR was performed with the same forward primer and a nested reverse primer (exonic): 5'-GGACATTGTCATTCT TGATCTC-3'. The PCR conditions were the same as for the first PCR. Polymerase chain reaction products were visualised using a 2% agarose gel with ethidium bromide.

Results

Single cells of interest, identified microscopically in clinical peripheral blood smears, such as a lymphoblast (fig 1A), could be microdissected from background cells consistently and efficiently by mechanical or laser microdissection (figs 1B and C). The isolated single cell could then be picked up by electrostatic forces with a needle, without the risk of contamination with nucleated background cells (fig 1D).

After transfer into reaction tubes, the whole genome was amplified successfully by primer extension preamplification in four of seven single cells in one of two blood smears from two different patients. This was demonstrated by

Figure 1 Mechanical microdissection of a single lymphoblast from a peripheral blood smear. Modified May-Gruenwald and Giemsa stain. (A) Lymphoblast; $\times 1600$ magnification with oil immersion. (B) Microdissection needle placed near a lymphoblast; $\times 400$ magnification. (C) Surrounding cells microdissected from the lymphoblast. (D) The single lymphoblast has been picked up.

Figure 2 Nested PCR amplification of a 175 base pair sequence from the CD95 gene after whole genome amplification of a single cell microdissected mechanically from a peripheral blood smear. Ethidium bromide stained 2% agarose gel. Lane M, 100 base pair marker; Lanes 1–7, CD95 amplification of 4 μ l aliquots from different whole genome amplified single cells; Lane 8, CD95 amplification after a whole genome amplification reaction was performed with pick ups from an area cleared of all cells (negative control); Lane 9, CD95 amplification after whole genome amplification reaction without DNA or single cell (negative control); Lane 10, CD95 amplification after whole genome amplification of 75 ng human genomic DNA (positive control). There is an additional 407 base pair band resulting from carry over of the outer primers.

PCR amplification of a 175 base pair sequence from the CD95 gene using only 4 μ l aliquots of the 60 μ l whole gene amplification reaction (fig 2, lanes 1–7). Amplification of the CD95 sequence did not occur because of chance sequestration of the single (non-amplified) genome into the aliquot since other aliquots produced the same result (data not shown). Only genomic DNA of the picked single cell was amplified, without contamination from DNA contained in microscopically undetected remaining debris of the eliminated surrounding cells. This was demonstrated by the absence of CD95 amplification in areas made devoid of cells by scraping (fig 2, lane 8).

Discussion

Whole genome amplification of single cells has been reported using sperm,^{3,5} amniocytes and chorionic villus cells,⁶ blastomers,^{6,7} peripheral lymphoblasts,⁶ and tumour cells.⁸ All these cells were isolated from fresh rather than archival tissue. In this study we show that not only cell clusters⁹ but also defined single cells can be microdissected from archival clinical specimens such as peripheral blood smears. Furthermore, we demonstrate that whole genome amplification of single cells can be applied to peripheral blood smears. Unlike fresh or frozen tissue, these specimens are readily available, do not require an elaborate infrastructure, and are stable. Because only an extremely small amount of material is needed, even reference specimens can be used. Because cellular morphology and histological context is observed before picking cells, molecular findings can be traced back to specific cells. This should facilitate studies on clonality, loss of heterozygosity, mutation, or amplification, even in these clinical specimens, with their marked cellular heterogeneity and occasional scarcity of pathological cells. Whole genome amplification enables sufficient DNA copies to be produced 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 analyses. Unlike many other cytology

specimens, haematological smears are suited exceptionally well for microdissection because their nucleated cells are spread well apart. However, the limitations of our study have to be kept in mind. As the glass slides had not been coverslipped, potential interference of single cell PCR by xylene based solvents and mountants could not be assessed. These chemicals are used in the preparation of many cytology specimens. Finally, the number of samples studied was low.

Initially, we used a laser for microdissection as it efficiently destroys cells adjacent to the cell of interest. However, this technique requires sophisticated equipment, similar to the laser capture microdissection technique described recently.¹⁰ Subsequently, we used mechanical microdissection, which is as efficient as laser microdissection. This requires only common equipment and harvests single cells in less than five minutes.

In our experiments we used the CD95 sequence as the gene of interest because we are studying mutations of this gene in leukaemias. The CD95 sequence was amplified in most, but not all, of the whole gene amplification aliquots from one slide and not at all in the aliquots from a second slide. This may have been caused by the absence of genome amplification owing to loss of the cell on its way from pick up to the reaction tube, sequestering of the cell on the wall of the reaction tube, insufficient lysis, or inaccessibility of the genome to amplification. The average DNA copy number after whole gene amplification is 60,³ but if amplification is less effective, there may be no copies present in a small aliquot, despite amplification of the genome.

The probability of amplifying any sequence in the genome to a minimum of 30 copies by whole gene amplification has been estimated to be not less than 0.78 (95% confidence).³ Therefore, it is possible that not all genes are equally amenable to amplification.

Nested PCR is needed to amplify specific genes efficiently after whole gene amplification. The exquisite sensitivity of nested PCR makes strict avoidance of contamination, and appropriate controls to detect it, mandatory. The latter should also include amplifications of scrapings from areas made devoid of cells.

Taken together, the technical advance described in this paper may provide a new method for the molecular analysis of haematological smears of blood and bone marrow and, potentially, of other cytology specimens.

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A PCR-SSP method for detecting the Cys282Tyr mutation in the HFE gene associated with hereditary haemochromatosis

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Abstract

Hereditary haemochromatosis is a common genetic disorder that causes hyper-absorption of dietary iron, leading to increased deposition and various organic diseases. Early diagnosis is important if effective treatment is to be applied and the iron overload corrected before the onset of clinical symptoms. Recently, a candidate gene has been identified in which a single point mutation shows a very close association with hereditary haemochromatosis. A polymerase chain reaction method using sequence specific primers (PCR-SSP) is described that, in conjunction with a simple DNA extraction method, would provide a specific diagnostic test or rapid screening procedure for this putative haemochromatosis associated mutation.

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Keywords: haemochromatosis; PCR-SSP; HFE gene; rapid screen

Haemochromatosis is transmitted as an autosomal recessive trait with a homozygosity frequency of 0.005-0.008 and an estimated carrier frequency of 10% among individuals of North European origin. Although a link between haemochromatosis and the human leucocyte antigen (HLA) allele, HLA-A3, was described over 20 years ago,¹ a much closer association has now been found with a mutation in a newly recognised gene, assigned the name HFE (the accepted representation for the haemochromatosis locus approved by the Genome Database Nomenclature Committee).² This gene belongs to the major histocompatibility complex (MHC) class I related gene family and is situated 4.5 Mb telomeric to HLA-A. The mutation, whereby

G is replaced by A at nucleotide position 845 in the cDNA sequence (845G→A), results in the substitution of tyrosine for cysteine at amino acid position 282 in the transcribed protein (Cys282Tyr); this critically affects an intramolecular disulphide bond.³ It is possible that the products of MHC class I related genes are involved in iron metabolism and that this function is compromised by a mutation that affects the structure of the gene product.

Over 83% of haemochromatosis patients are homozygous for the Cys282Tyr mutation in the HFE gene,³⁻⁶ compared with ~0.2% in normal controls. The PCR-SSP method described here allows rapid and specific typing for this haemochromatosis associated mutation.

Methods

DNA was extracted from 2 ml anticoagulated blood by a simple salting out method⁷ and DNA concentration adjusted to 50 ng/μl.

Polymerase chain reaction (PCR) amplification was carried out in a reaction volume of 10 μl, consisting of 20 mM ammonium sulphate, 75 mM Tris-HCl (pH 9.0), 0.01% Tween, 1.5 mM MgCl₂, 200 μM dNTPs, 1.0 μM specific primers (Oswel, Southampton,

Figure 1 Gel electrophoresis of PCR products from three individuals (I-III). Lanes 1-4 (individual I), nucleotide 845G/G, Cys282Tyr -/-; Lanes 6-9 (individual II), nucleotide 845G/A, Cys282Tyr +/-; Lanes 11-14 (individual III), nucleotide 845A/A, Cys282Tyr +/+; Lanes 5 and 10, molecular markers (100 base pair ladder).