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A simple, precise and economical microdissection technique for analysis of genomic DNA from archival tissue sections

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Abstract Formalin-fixed and paraffin-embedded tissues are valuable resources for retrospective analysis of the molecular changes in DNA present in tumour tissues. One common problem that precludes an accurate DNA analysis in a human tissue sample is cellular heterogeneity. We have developed a simple and inexpensive, but micrometrically precise, microdissection technique that allows for selective isolation of minute cell clusters and even single cells from archival tissue sections. The features of our technique include use of a 30G1/2 needle affixed to a mechanical micromanipulator as a dissector sharp enough to be used for dissection of even single cells and use of the stage and focus control knobs of the microscope to scrape the target cells instead of moving the needle during microdissection. The main advantages of this technique over the current methods lie in its simplicity, low cost, easy handling and precision.

Key words Microdissection technique · Micromanipulator · Archival tissue · Tissue heterogeneity · Mutations

Introduction

Formalin-fixed and paraffin-embedded tissues are valuable resources for retrospective analysis of the molecular changes in DNA present in tumour tissues. One common problem that precludes an accurate DNA analysis of human tissue sample is the cellular heterogeneity: a mixture of neoplastic and nonneoplastic cells such as supporting stromal cells, inflammatory cells, and pre-existing original tissue. The nature of the heterogeneous cells may obscure the results of molecular assays, depending on the ratio of neoplastic to non-neoplastic genomic DNA and the sensitivity of the tests. Several methods, including selective ultraviolet radiation fractionation [7, 11], starch adhesive gum fractionation [13] and microdissection using hand-held instruments [7, 8, 11, 13–15], have been used in tissue microdissection performed to obtain the genomic DNA derived from pure populations of cells exhibiting the characteristic morphology of the disease. However, lesions less than 1 mm in size are technically difficult to dissect by these methods. More recently, microdissection techniques using laser beams have been described [1–4, 6]. Although these techniques may ultimately improve the speed and precision of dissection, the hardware needed for them is very expensive.

To overcome the drawbacks of current tissue microdissection techniques, we have developed a simple and inexpensive, but micrometerically precise, microdissection device and technique (Fig. 1). The system consists of a conventional light microscope, manual three-axis mechanical micromanipulators, and 30G1/2 disposable hypodermic needles. The basis of the strategy lies in us-

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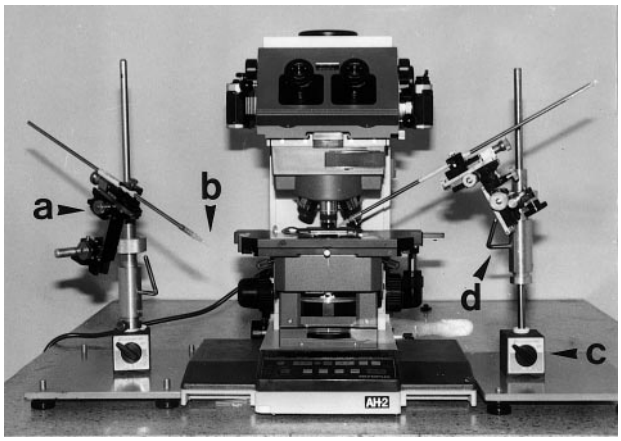

Fig. 1 Microdissection device: *a* micromanipulator, *b* 30G1/2 gauge needle, *e* 'stopping bar', *d* magnetic stand and base plate

ing the stage and focus control knobs of the microscope to scrape the target cells instead of moving the needle affixed to a needle holder belonging to the micromanipulator during microdissection. As will be shown, this simple system is easy to use and also provides a useful tool for contamination-free isolation of target cells from routine histological tissue sections for subsequent molecular analysis. The main advantage of this technique over the current methods lie in its simplicity, low cost and easy handling with no loss of precision.

Materials and methods

The system consists of a conventional light microscope, manual three-axis mechanical micromanipulators (Sam Jung Industry, Seoul, Korea; Fig. 1a) mounted on magnetic baseplates (Fig. 1c), and 30G1/2 disposable hypodermic needles (Becton Dickinson, Franklin Lakes, N.J.; Fig. 1b). Our system was designed to allow a whole micromanipulator to be moved back and forth, rotating horizontally around the central axis bar mounted on a magnetic baseplate in order to facilitate needle changing or sample collection. There also is a 'stopping bar' (Fig. 1d) to prevent over-rotation and to return the needle to the previous dissection area after sample collection.

Specimens were obtained from the files maintained by the Department of Pathology, Catholic University Medical College, Seoul, Korea. Surgical specimens were processed for conventional histological examination (fixed in 10% buffered formalin and embedded in paraffin). Several serial 5-µm tissue sections of paraffin-embedded tissue on the glass slides were stored at room temperature in a covered box.

Unstained 5-µm tissue sections on the glass slides were deparaffinized twice with xylene, rinsed twice with ethanol, briefly stained with haematoxylin and eosin (H&E), and finally air dried. Since the dried H&E-stained tissue sections do not show up fine cytological detail and tend to be too brittle for scraping, the target slides were rinsed in a 2% glycerol in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for 2 min. However, prolonged incubation in a glycerol buffer or the addition of more than 2% glycerol makes the tissue too gummy for microdissection. Wet conditions provide good cytomorphological detail and make it easy to scrape the cells, but then it is difficult to pick up the dissected cells owing to the surface tension of liquid remaining in tissue. Therefore, the best conditions for dissection are just before the tissue becomes completely dried out. When the tissue dried up before the completion of dissection, the target slides were rinsed again briefly in glycerol buffer.

To place the needle tip onto the target cell precisely under ×100 magnification, we proceeded as follows. The *x*- and *y*-axis control knobs of the micromanipulator were used to position the needle in the middle of the microscopic visual field a few millimetres above the tissue sections. Then the needle was brought down by means of the *z*-axis control knob of the micromanipulator to touch the glass slides in a tissue-free area. The stage was also moved down slightly with the fine focus control knob of the microscope, to position the needle so that it was not quite touching the surface of the glass slide, which prevented the tissue from being scraped while the needle was moved to the targeted area; this produced a slightly under-focused image, but still allowed recognition of cytomorphological detail. After the visual field had been moved to the targeted area the image was put back into focus by means of the fine focus control knob, which brought the needle tip in contact the target cell. Finally, the target cell were dissected away from the adjacent tissue with the sharp point of a needle using moved by way of the stage and fine focus control knobs of the microscope. This process was repeated until the target cells had been scraped away completely.

After the completion of dissection, the tissue affixed to the tip of needle was then lifted high above the tissue sections using the focus control knob of the microscope and the *z*-axis control of the micromanipulator. To collect the sample, the micromanipulator was pulled and the tip of the needle was carefully submerged in a 0.5 ml microcentrifuge tube containing 20 µl of DNA extraction buffer (100 mM Tris-HCl, pH 8.0; 1% Tween 20, and 2 mg/ml proteinase K). If a right-handed micromanipulator is difficult to position for dissection, a left-handed device can be used.

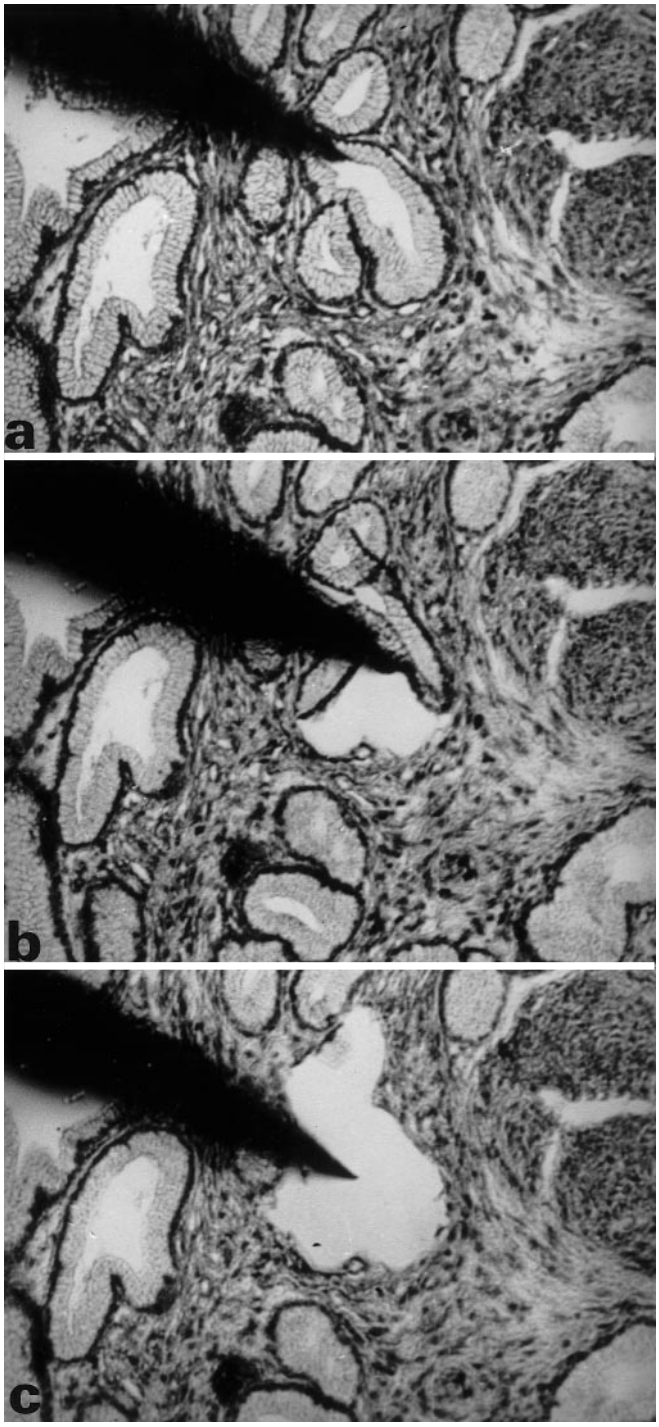

Fig. 2a–c An area of malignant adenoma showing numerous well-differentiated tumour glands. H&E, original magnification ×100. **a** Beginning of microdissection with a 30G1/2 needle at the preselected glands to be dissected. **b, c** The same section of microdissection **b** in progress and **c** after microdissection

DNA extraction was done by a modified single step DNA extraction method [13]. The cells obtained (50 cells/µl) in 20 µl of DNA extraction buffer were incubated at 52° C for 1 or 2 days. The mixture was boiled for 10 min to inactivate the proteinase K, and 1 µl of this solution was used as DNA template for polymerase chain reaction (PCR) amplification.

Tumour DNA and corresponding normal DNA from each slide were amplified by a thermal cycler (MJ Research Ins., Watertown, MA) for LOH analysis with five microsatellite markers (Research Genetic, Huntsville, Ala.), including *D19S883*, *D19S894* and *D19S216* for malignant adenoma and *D9S171* and *IFNA* for malignant melanoma. For SSCP and sequencing analysis of exon 2 of $p16^{INK4a}$, tumour DNA and normal DNA from malignant melanoma were amplified with primer (forward: 5′-AGCCCAACTGCGCCGAC-3′; backward; 5′-CCAGGTCCACGGGCAGA-3′). Each PCR reaction was generally performed under standing conditions in a 10-µl reaction mixture containing 1 µl of template DNA, 0.4 µM of each primer, 1.25 µM of each dNTP, 1.5 mM $MgCl_2$, 0.4 U Taq polymerase, 0.5 mCi [^{32}P]dCTP (Amersham, Bucks., UK), and 1 µl of 10×buffer. The reaction mixture was denatured for 5 min at 95° C and incubated for 35 cycles (denaturing at 95° C for 50 s, annealing at 57° C for 90 s and extension at 72° C for 90 s), with some variations in the annealing temperature. Final extension was continued for 10 min. Reaction products (2 µl) were then denatured and electrophoresed in 6% polyacrylamide gel containing 7 M urea. After electrophoresis, the gels were transferred to 3MM Whatman paper, dried, and subjected to autoradiography using Kodak-OMAT film (Eastman Kodak, Rochester, N.Y.).

Results

We have successfully obtained tumour cells without any surrounding normal cell contamination by the microdissection technique described here. Figure 2 shows an area of malignant adenoma, a well-differentiated adenocarcinoma of the uterine cervix, showing several malignant glandular structures (Fig. 2a) and the same section of the dissection in progress (Fig. 2b) and after microdissection of glandular structures without any normal cell contamination (Fig. 2c). Figure 3 demonstrates allelic losses at a putative tumour suppressor locus on chromosome 19 in tumour cells (T) compared with normal allelotype (N) determined in a corresponding normal cell. The complete absence of signal in deleted alleles of tumour DNA (arrowheads) suggests a total lack of normal cell contamination. Another example of microdissection in Fig. 4

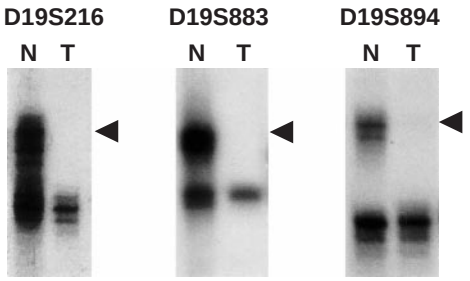

Fig. 3 Demonstration of LOH at the three markers on chromosomal band 19p13.3 in tumour DNA dissected from malignant adenoma as shown in Fig. 2. The complete absence of signal in deleted alleles of tumour DNA (*arrowheads*) indicates a total lack of normal cell contamination

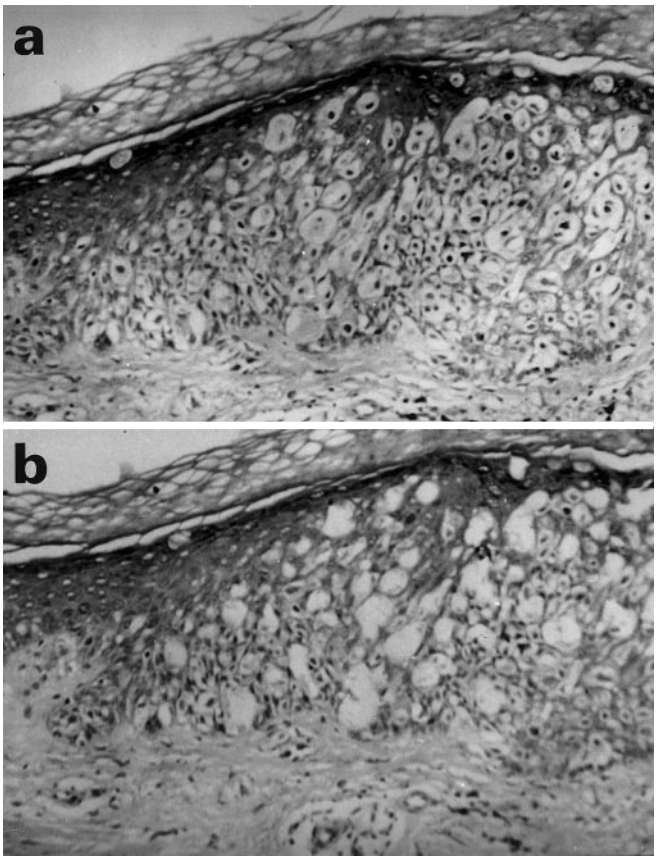

Fig. 4 **a** An area of pagetoid type of malignant melanoma showing numerous individual melanoma cells throughout the epidermis. H&E, original magnification ×100. **b** The same section after microdissection of individual melanoma cells

shows an area of malignant melanoma, pagetoid type, with numerous individual melanoma cells throughout the epidermis (Fig. 4a), and the same section after dissection of individual melanoma cells (Fig. 4b). These examples show the fine control possible with this microdissection technique, which permits precise separation of even individual tumour cells from surrounding normal tissue. The autoradiograms of SSCP (Fig. 5a), sequencing analysis (Fig. 5b) and LOH study (Fig. 5c) also showed a clear lack of normal cell contamination in tumour DNA.

Discussion

The microdissection technique described here is a reliable method of preparing pure genomic DNA present in tumour tissues. The dissection device is very simple and economical, and it is easy to handle with no loss of precision. With this method, the micromanipulator was used only to place the needle tip onto glass slide in a tissue-free area, and the other procedures, including targeting the needle tip on the cells to be dissected and scraping them, were performed by moving the stage of the microscope. This was achieved by adjusting the focus and stage control knobs of microscope with the needle tip in

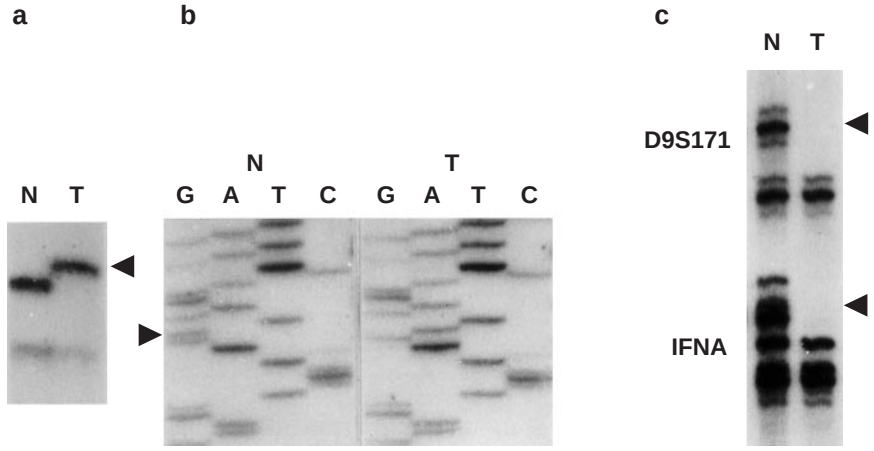

Fig. 5 **a** SSCP analysis of *p16^{INK4a}* in DNA from malignant melanoma as shown in Fig. 3 (*T*) and normal sample (*N*). *Arrowhead* indicates abnormal electrophoresis bands. **b** Cyclic sequencing analysis was performed using DNA eluted from above abnormal band. *Arrowhead* indicates G-to-A mutation of *p16^{INK4a}*. **c** Multiplex PCR was done for 9p21. *Arrowheads* indicate alleles exhibiting LOH

a fixed position. Therefore, the micromanipulator in our system need not be as precise in movement as in other systems equipped with a computer and/or hydraulically controlled micromanipulators [1–5, 10], and its operation does not require much skill. The 30G1/2 disposable needle is very useful and conventient as a dissector. The base of the needle can easily be bent for adjustment of the dissection angle, and the needle tip is less than 1 µm in diameter and is sharp enough to be used for dissection of even individual cells as shown in Fig. 4. In addition, when cell clusters are to be dissected, the tip can be formed into the shape of a hoe by tapping it gently against a clean hard surface; this allows for scrapeing off a group of cells [9]. Furthermore, using the use of disposable needles can minimize cross-tissue DNA contamination. Since the dissection is performed under direct microscopic visualization, investigators can reliably obtain only the selected cell population of interest and be certain that surrounding normal cells are not included.

Malignant adenoma [7, 9, 12] is an extremely well-differentiated adenocarcinoma of the uterine cervix and is composed of glands and cysts lined by a single layer of tall-columnar mucinous epithelium surrounded by cervical stromal cells (Fig. 2). To date, there have been no reports on the genetic alterations involved in carcinogenesis of malignant adenoma, because it is so rare and because of the difficult of selectively procuring malignant glandular cells without using a microdissection technique. Using DNA of malignant glandular cells taken from an archival tissue section of malignant adenoma, we were able to detect allelic deletions on the short arm of chromosome 19. A subsequent study of nine cases of the sporadic form of malignant adenoma was performed. All nine cases were diagnosed from hysterectomy specimens; no patient had a family history of Peutz-Jeghers syndrome. In this study, we showed a distinct region with 100% LOH frequency at marker *D19S216* [9]. This result indicates that a putative tumour suppressor gene for malignant adenoma is located at *D19S216* on chromosomal band 19p13.3 and plays an important part in tumorigenesis in the case of malignant adenoma. Examples of LOH study are displayed in Figs. 4 and 5C. The complete absence of signal in deleted alleles of tumour DNA (arrowheads) suggests that tumour samples are absolutely devoid of normal cell contamination. These findings stress the importance of microdissection when an LOH analysis is studied by a PCR-based technique, since surrounding normal tissue may mask the tumour-specific changes.

In the second study, we investigated LOH and sequencing analysis of the *P16^{INK4a}* in a case of cutaneous malignant melanoma. As shown in Fig. 4, we are able to dissect even single cells by using our dissection technique. And the autoradiograms of SSCP and sequencing analysis (Fig. 5a, b) also showed a clear lack of normal cell contamination in tumour DNA.

Relatively crude manual microdissection techniques [7, 8, 11, 13–15] are of limited value in dissection of lesions less than 1 mm in size. Moskaluk and Kern [10] introduced a microdissection technique using a hydraulic micromanipulator. In contrast to our system, they used glass needles as a dissector prepared by pulling borosilicate glass pipettes in a pipette puller and breaking the tip to form blunt scrapping edges. Since the glass needle tip is 10–30 µm in diameter, it is impossible to dissect single cells because of the girth of a blunt edge. Nevertheless, laser-based techniques, such as the laser capture microdissection method (PXL-100; PixCell Laser Capture Microdissection System, Arcturus, Mountain View, CA 94043 US) [3, 6], microbeam MOMeNT (microdissection of membrane-mounted native tissue) [2], and the UV laser-based single cell dissection method (PALM UV-laser microbeam, Wolfratshausen, D-82515 Germany) [1, 4], seem to be highly accurate in the contamination-free preparation of single cell isolation from stained histological sections. But both PXL-100 [3, 6] and microbeam MOMeNT [2] need a specifically designed ultrathin supporter membrane on the histological section, and all of these system seems to be very complex, requiring a laser source, computer, videoscreen, and micromanipulator, and are therefore very expensive. For example, the price of PXL-100 is about $65,000 US dollars excluding its workstation and software. In contrast, our system is very simple and in-

expensive compared with laser-based techniques, with no loss of precision. Our simple dissection device cost less than $1,000 US dollars not including the light microscope and is currently available in commercial form (Sam Jung Industry, Seoul, Korea).

We have used this dissection method to analyse approximately 200 tissue specimens of tumour or premalignant lesions from various tissues. We have found that 50 cells is sufficient for a single PCR reaction with the PCR products in the 200-bp range. One concern over the use of this technique is the potential allelic dropout that might occur with PCR amplification of a small number of intact nuclei. Among the 200 tissue specimens analysed, we have not seen a single case with inappropriate absence of an allele in a normal sample where the matched tumour sample showed the presence of both alleles.

The main advantages of this technique over the current methods lie in its simplicity, low cost and easy handling with no loss of precision. These advantages should lead to widespread use of the technique for genetic analysis of small lesions.

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