Review

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Microdissection of Tissue Sections: Application to the Molecular Genetic Characterisation of Premalignant Lesions

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Abstract
The characterisation of the early molecular genetic events of tumor development depends on the selective procurement of histopathologically defined small cell populations from premalignant tissue. In order to obtain high-quality DNA, mRNA and proteins from these small tissue samples and even from single cells, tissue microdissection is one of the most useful techniques, becoming increasingly important for molecular pathologists. Using different microdissection techniques which allow the isolation of morphologically defined cell populations under direct visualisation, it is now feasible to study molecular genetic events that drive the multistep evolution in tumours. This review aims to present the current techniques of tissue microdissection and these techniques are discussed in the light of their ability to isolate premalignant cell populations in particular. Furthermore, we describe the subsequent application of several multiplex molecular analyses for characterising the microdissected premalignant cells. Applying these advanced techniques, alterations in the cellular DNA or the fluctuation of expressed genes that correlate with a particular stage of carcinogenesis can ultimately be compared within or between individual patients. Thus, these new technologies will have an enormous impact on molecular pathology with several diagnostic, prognostic and therapeutic implications.

Introduction

Tumour development and progression are dynamic processes accompanied by the accumulation of molecular genetic alterations [1, 2]. So far, various distinct cytogenetic and molecular genetic changes have been identified in most tumour types. However, the exact timing of these events during the early steps of tumorigenesis are not well characterised. In the past, molecular genetic studies on premalignant cells have been limited for several reasons. These difficulties result mainly from the impossibility to define and localise these lesions without microscopic examination. Moreover, the premalignant cells in most organs form tiny lesions within or nearby non-neoplastic

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tissue, e.g. stroma or residual parenchyma. Therefore, a close relation to morphology is the prerequisite for molecular genetic studies of premalignant cells.

The recently developed techniques of tissue microdissection can be applied to histological and cytological routine preparations and allow for the isolation of morphologically well-defined cells or cell groups that can be further processed for molecular genetic analyses. This combined approach of microdissection and molecular genetic analyses is particularly prone to investigate premalignant lesions. Under microscopic control, the premalignant cells can be exactly defined and dissected from the surrounding non-neoplastic tissue by means of a micromanipulator or, more recently, with the help of a laser beam. The isolated cells represent purified pools of histologically well-defined cells with no or minimal contamination by non-neoplastic cells. These samples can be further investigated especially by various modifications of the polymerase chain reaction (PCR) which allow the detection of molecular genetic alterations in small cell groups, and even at the level of single cells.

This review aims to present the different techniques of tissue microdissection. They are discussed in the light of their ability to isolate premalignant cell populations in particular. Furthermore, we decribe the application of several molecular-genetic techniques for the characterisation of microdissected premalignant tissues.

Tissue Sources for Microdissecting Premalignant Lesions

Paraffin-Embedded and Formalin-Fixed Tissue

For various cancers, e.g. carcinomas of the gastrointestinal tract, the bladder, the prostate and the breast, premalignant lesions are histologically well defined. Therefore, formalin-fixed and paraffin-embedded biopsies provide the main source of tissue for molecular analysis. Routine sections (5 μm) stained with hematoxylin and eosin are commonly used for tissue microdissection. Other histologic stains such as methyl green or nuclear fast red may also be used [3]. The sections can be mounted on routine glass slides for most microdissection techniques. Immunohistochemical staining of the tissue sections prior to microdissection offers an additional phenotypic characterisation [4]. Unstained tissue sections, however, are unsuitable for the assessment of the exact histomorphological graduation of precursor lesions. It is helpful to increase the histo- and cytomorphology by covering the stained sections with a thin layer of xylene or 2-propanol which improves by wetting and refractive index matching the morphology of premalignant cells on the unmounted slides. Xylene or 2-propanol evaporates quickly before cell procurement.

Frozen Sections

Sections from fresh frozen tissue can also be used for tissue microdissection [5, 6]. For an immunophenotypical characterisation, immunohistochemical staining procedures can also be applied to frozen sections [7]. However, the exact histomorphological graduation of premalignant lesions may be hampered in frozen sections.

Cytological Preparations

The examination of cytological preparation from several organs such as the uterine cervix is well-established for the identification of premalignant cells. Routinely prepared cell smears stained with Papanicolaou can be used for microdissection and subsequent PCR analysis, even after several years' storage [8]. Other cell preparations, e.g. cytospin samples, are also suitable to isolate cells or cell groups by microdissection. In general, the isolation of premalignant lesions by microdissection requires a well-preserved histo- or cytomorphology and a trained pathologist.

Techniques for Microdissecting Premalignant Lesions

Precision, avoidance of contamination and efficiency of the procedure are the most important parameters in tissue microdissection. The spectrum of techniques ranges from manual microdissection to single-cell preparations based on laser- and computer-assisted systems. Due to the small tissue samples which are typical for premalignant lesions, techniques with an inherent high precision are preferable. An overview of the most common microdissection techniques in molecular pathology is given in table 1.

Manual Tissue Dissection

This technique can be performed on routinely stained slides using 5- to 15-μm-thick sections placed on non-coated glass slides. Manual tissue dissection requires histologically homogeneous premalignant lesions and the areas should have a diameter of at least 1 mm [9]. Using a sterile needle, the selected lesions can be procured [10]. It is helpful to perform the tissue dissection under an inverted microscope which offers more space for the han-

Table 1. Overview of the most common microdissection techniques in molecular pathology

	Manual tissue dissection	Laser microbeam microdissection	LPC	Microdissection of membrane-mounted tissue	LCM
Function principle	procurement of large tissue areas using a sterile needle or a scalpel with/without an inverted microscope	'cold ablation' of unwanted cells using an UV laser (337 nm); procurement of remaining cells with/without a micromanipulator under an inverted microscope	'cold ablation' of unwanted cells using an UV laser (337 nm); procurement with 'non-contact' laser pressure catapulting	polyethylene foil as supporting membrane allows to cut out single cells or cell groups	melting effect between selected tissue and a transfer film due to local heating by an IR laser (980–1,064 nm)
Minimum sample size/preferential spectrum of use	≅50–100 µm large and homogeneous cell areas (>10^4 cells)	<1 µm small lesions (<50 cells), single cells (also suitable for chromosome microdissection)	<1 µm single cells	1 µm single cells or small cell groups (<50 cells)	>7 µm small cell groups (5–20 cells), large single cells
Specimen specification	FFPE, FS	FFPE, FS, fixed cells after cytocentrifugation, cell smear, chromosomes or metaphase spreads	FFPE, FS, fixed cells after cytocentrifugation, cell smear	FFPE, FS, fixed cells after cytocentrifugation, cell smear, chromosomes or metaphase spreads	FFPE, FS, fixed cells after cytocentrifugation, cell smear
Sample procurement	manually (sterile needle, scalpel), micromanipulator	computer-assisted micromanipulator	'non-contact' laser pressure catapulting directly into the sample tube	'non-contact' laser pressure catapulting directly into the sample tube	thermoplastic transfer film
Preparation time	5–10 min for >10^4 cells	5–10 min for 1–20 cells	<3 min for 1–10 cells	<3 min for 1–10 cells	<10 s for 1–30 cells
Costs	low	high	high	high	high
Advantages	easy and quick method for large homogeneous tissue areas	high precision, in particular for 1–10 cells	minimalised risk contamination under procurement	rapid method to procure clear-cut cells with minimalised risk of contamination	very rapid method to procure homogeneous small cell groups
Disadvantages	high risk of contamination, not suitable for heterogeneous tissue types, not suitable for small lesions (<50 cells)	time-intensive	danger of destruction of selected cells under procurement	sophisticated preparation of tissue sections, exclusively for use in membrane-mounted tissue sections	cost-intensive transfer tubes are necessary

FFPE = Formalin-fixed and paraffin-embedded tissue; FS = fresh frozen tissue sections.

dling of the needle. In order to obtain material from lesions smaller than 1 mm, a micromanipulator can be used [11]. However, manually based techniques may result in contamination of the microdissected material and are therefore insufficient for most of the premalignant lesions which usually consist of several hundreds of cells or less.

Laser Microbeam Microdissection

The principle of laser cutting is a locally restricted ablative photo-decomposition process without heating the direct environment of the laser beam [12]. Within the diffraction-limited focus of the laser beam obtained by a high numerical microscope lens, a very high energy density is available, and if the pulse duration is shorter than the relaxation time of the biological material (range of microseconds), heat transfer is avoided [13]. In this way, a pulsed UV laser microbeam can be used to cut or ablate stromal, inflammatory or residual parenchymal cells surrounding the tumour cells of histological sections without destruction of genetic information of the remaining cells as shown in different experiments [14]. At the site of laser exposure and ablation, no amplifiable material is left behind [15]. To retrieve the cells from the slide, a computer-controlled micromanipulator and conventional sterile needles are used to pick and transfer the cells into a tube for further molecular analysis. As an alternate and rapid method for cell procurement, it is possible to obtain the cells with a single laser shot as described below (laser pressure catapulting). Laser isolation and cell pick-up proce-

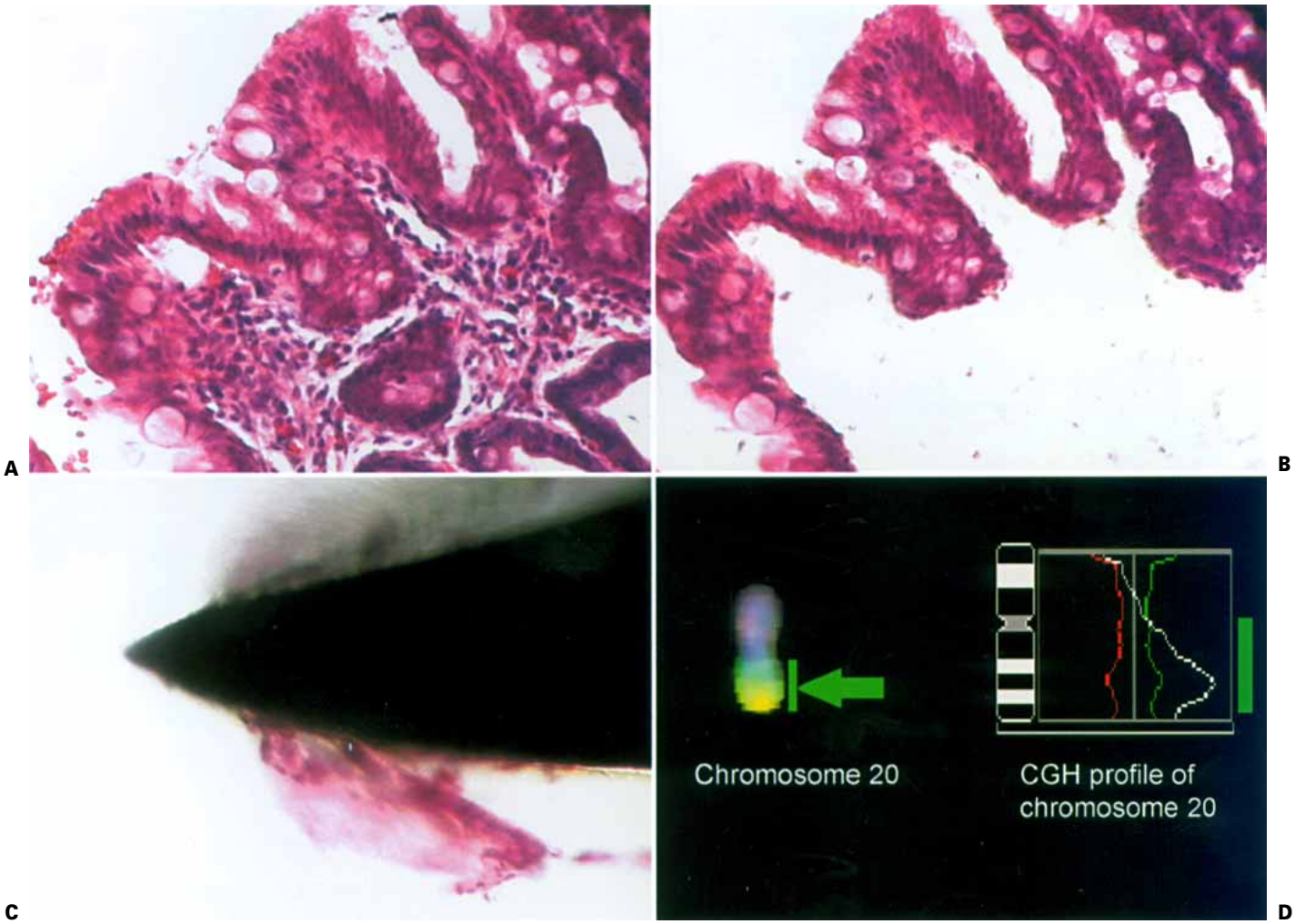

Fig. 1. UV laser microdissection of an intestinal metaplasia of Barrett's oesophagus and subsequent molecular-genetic characterisation by CGH. **A–C** × 400. A routine tissue section (5 μm) stained with haematoxylin and eosin containing a metaplasia before (**A**) and after (**B**) ablation using an UV laser microbeam. Note the stromal and inflammatory cells in the lamina propria (**A**) and the remaining pure cell population (**B**) after laser ablation. To retrieve the cells from the slide, a computer-controlled micromanipulator and sterile needles are used to pick and transfer the cells into a tube for further molecular analysis (**C**). After universal amplification and labelling of DNA, CGH was performed on metaphase chromosome spreads. (**D**) Details of the detected changes on chromosome 20. The resulting CGH profile obtained from the microdissected metaplastic cells indicates a DNA amplification on chromosome 20q (**D**).

dures are quick (within few minutes) and are easily performed. Thus, this technique is suitable for accurate microdissection of premalignant lesions. Figure 1 illustrates the subsequent steps of laser microbeam microdissection (LMM) of an intestinal metaplasia in Barrett's esophagus using an UV laser microbeam.

Laser Pressure Catapulting

Laser pressure catapulting (LPC) allows to catapult an isolated cell or cell group out of its surroundings with a single precisely aimed laser shot [16]. The ejected dissectates are either caught on a small piece of cover glass, or directly catapulted into the cap of a common PCR tube. The greatest advantage of this method is the procurement of the material in a 'non-contact' manner, which minimises the risk of contamination. Thus, this technique is also suitable for premalignant lesions due to the inherent problem of contamination in small cell samples.

Microbeam Microdissection of Membrane-Mounted Native Tissue

The tissue sections are mounted onto a 1- to 3-μm polyethylene foil (P.A.L.M., Bernried, Germany) which is attached to a slide by nail polish. After the final preparation step, the membrane may be turned upside down [17]. With an UV laser microbeam, tissue areas can be cut out with high precision. Combining this method with LPC, one single laser shot makes it possible to catapult cell groups or tissue areas of up to 1,000 μm in diameter. Although this method is suitable to procure small cell groups and single cells, the use of this technique implicates a special slide preparation with polyethylene foils which excludes the use of routinely processed glass slides.

Laser Capture Microdissection

Laser capture microdissection (LCM) is helpful to select and procure cell clusters from tissue sections by use of a laser pulse. Once the cells are captured, the DNA, RNA or protein can be easily extracted from the isolated cells. In LCM, a thermoplastic polymer coating attached to a rigid support is placed in contact with a tissue section. The polymer over microscopically selected cell clusters is precisely activated by a near-infrared laser pulse and then bonds to the targeted area. Removal of the polymer and its support from the tissue section procures the selected cell aggregates for molecular analysis. The spectrum of application of this technique is wide, and it allows fast procurement of histologically homogeneous tissue areas or single cells [18]. A great advantage is the well-preserved morphology of the transferred cells which can be readily visualised under the microscope. Ease and rapidity of the use has been achieved by the commercial LCM microscope (Arcturus Engineering, Mountain View, Calif., USA; http://www.arctur.com). However, the focal spot of the melting laser cannot reach below 7 μm in diameter and there is no possibility to selectively destroy unwanted cells or tissue, neither adjacent nor within the selected area.

Processing of Small Microdissected Tissue Samples

DNA

From the microdissected cells, DNA isolation according to standard procedures is possible, if the samples contain at least 10^5 cells. However, microdissection of premalignant lesions most often results in smaller samples. Thus, a simple one-step DNA preparation is recommended [15]. The resulting DNA preparation is not 'clean', but is sufficient for PCR-based analysis.

RNA

RNA from microdissected tissue can be obtained by standard methods using commercially available RNA isolation kits. The microdissection procedure for RNA isolation is more intensive compared to that for DNA since a large number of cells (10^5–10^6 cells) are required. In addition, microdissection by UV laser-based techniques must be carefully performed to eliminate all bystander cells because high copy mRNA transcripts from contaminating cells can produce erroneous results. In general, frozen tissues yield more reverse transcription PCR (RT-PCR) products than paraffin-embedded tissues. Precipitative fixatives, such as ethanol and acetone, consistently produce more RT-PCR amplification products than cross-linking fixatives such as formaldehyde [19]. For less than 10^5 cells, representing the amount of most premalignant cell populations within a tissue section, RNA amplification techniques should be applied. Using a T7-based RNA amplification in combination with cDNA microarrays, Luo et al. [20] were able to demonstrate gene expression profiles from small cell samples of rat neurones. RNA was extracted from sets of 1,000 neurones obtained with LCM and linearly amplified an estimated 10^6-fold using T7 RNA polymerase. Alternate procedures for mRNA and DNA isolation from small tissue samples isolated by laser-assisted microdissection are described by Bernsen et al. [21].

Proteins

Proteomics-based studies offer a powerful complementary approach to DNA/RNA-based investigations and are now being applied to investigate aspects of many diseases including cancer. Proteins are more stable than mRNA in many tissues and the disparity in stability has important consequences for the measurement of these molecules in biological materials. A very recent study demonstrated the successful combination of LCM and proteomic analysis using hematoxylin- and eosin-stained frozen sections [22]. In this study, however, a large number of cells were required (many pooled LCM samples are currently needed to run a gel, since a minimum of 50 μg of protein has to be loaded for silver-staining detection). Therefore, the application of a combination of proteomic analysis and tissue microdissection on premalignant lesions is so far a difficult approach depending on the availability of the appropriate amount of cell material.

Table 2. Some recent molecular-genetic approaches applying microdissection techniques on premalignant lesions

Tumor type	Precursor lesions	Tissue	Method	Reference
Prostate cancer	PIN	FFPE	CGH	Zitzelsberger et al. [25]
	atypical adenomatous hyperplasia	FFPE	LOH	Cheng et al. [41]
	PIN	FS	LOH	Emmert-Buck et al. [42]
Laryngeal squamous carcinoma	dysplasia, CIS	FS	LOH	El-Naggar et al. [43]
Breast cancer	normal lobules adjacent to breast cancer	FFPE	LOH	Deng et al. [34]
	normal lobules	FFPE	LOH	Larson et al. [35]
	DCIS	FFPE	CGH	Aubele et al. [29]
	DCIS	FFPE	CGH	Kuuskasjärvi et al. [27]
	atypical hyperplasia, DCIS, LCIS	FFPE	LOH	Chuaqui et al. [33]
	ductal hyperplasia, DCIS	FFPE	CGH	Werner et al. [28]
	ductal hyperplasia	FFPE	CGH	Aubele et al. [30]
Barrett's adenocarcinoma	metaplasia, dysplasia	FFPE	LOH	Zhuang et al. [32]
	metaplasia, dysplasia	FS	LOH	Gleeson et al. [44]
	metaplasia, dysplasia	FFPE	CGH	Walch et al. [31]
Cervical cancer	dysplasia, CIS	FFPE	CGH	Heselmeyer K. [45]
	squamous intraepithelial lesion	cell	CGH	Aubele et al. [8]
	dysplasia	smear		
Oral squamous cell carcinoma	dysplasia, CIS	FFPE	CGH	Weber et al. [26]
Neuroendocrine lung tumors	pulmonary tumourlets	FFPE	sequence analysis	Finkelstein et al. [39]
Lung cancer	squamous metaplasia, dysplasia, type II cell hyperplasia, atypical adenomatous hyperplasia	FFPE	PCR-RFLP sequence analysis	Sagawa et al. [38]
Squamous cell cancer of skin	dysplasia, CIS	FS	sequence analysis, LOH	Ren et al. [37]
Melanoma	dysplastic nevi	FFPE	sequence analysis, LOH	Lee et al. [46]

PIN = Prostatic intraepithelial neoplasia; CIS = carcinoma in situ; DCIS = ductal carcinoma in situ; LCIS = lobular carcinoma in situ; FFPE = formalin-fixed and paraffin-embedded tissue; FS = fresh frozen tissue sections.

Molecular Analysis of Microdissected Premalignant Lesions

Comparative Genomic Hybridization

A genome-wide screening for DNA sequence copy number aberrations became possible with the development of comparative genomic hybridization (CGH) [23]. CGH has already been applied to various cancer types and has increased our knowledge on genetic aberrations in several solid tumours [24]. The standard CGH protocol requires 0.5–1 μg of genomic DNA, corresponding roughly to 5–10 $\times$ 10^4 diploid cells from the tumour sample, and it can be applied to both fresh frozen and paraffin-embedded tissue specimens. Microdissection prior to CGH can be used to obtain pure populations of tumour cells, which increases the sensitivity for detecting DNA gains and losses. Microdissected specimens of premalignant lesions are, however, often too small to provide enough DNA for standard CGH. Thus, universal amplification of all DNA sequences using PCR with degenerate oligonucleotide primers (DOP-PCR) and low stringency PCR cycle conditions has been introduced. Recently, several studies were published demonstrating the successful application of this technique to premalignant lesions of prostate [25], uterine cervix [8], oral cavity [26] and breast [27–30] (table 2). We also applied this technique for the first comprehensive analysis of DNA gains and losses associated with the metaplasia-dysplasia-carcinoma sequence in Barrett's esophagus. Recurrent chromosomal changes were identified in metaplastic (gains on 8q, 6p, 10q, 20q losses on 13q #Y, 9p) and dysplastic epithelium (gains on 8q, 20q, 2p 10q, 15q, losses on #Y, 5q, 9p, 13q, 18q). Figure 1 illustrates the UV laser microdissection of an intestinal metaplasia in Barrett's esophagus and the detected changes on chromosome 20. Furthermore, novel amplified chromosomal regions on chromosomes 2p and 10q were detected both in Barrett's adenocarcinoma and premalignant lesions [31].

Loss of Heterozygosity

Loss of heterozygosity (LOH) is a key pointer to the existence of tumour suppressor genes (TSG). By screening paired morphologically normal and neoplastic tissue samples for allelic imbalance of polymorphic DNA sequences spaced across the genome, it became possible to discover candidate locations for TSG. As with CGH, microdissection of premalignant lesions is helpful to increase the sensitivity for detecting allelic imbalances (LOH). This approach with respect to a specific TSG has been successfully applied to several premalignant lesions such as Barrett's esophagus where *APC* has been described in dysplasia and even in intestinal metaplasia [32].

In breast cancer, several genetic alterations are also detectable in the histologically proposed models of multistep carcinogenesis such as LOH at 11q13 in atypical ductal hyperplasia showing a distinct lower frequency of LOH at the same locus compared with ductal carcinoma in situ [33]. In our series, we were able to detect several chromosomal loci displaying genetic abnormalities in ductal hyperplasia, e.g. DNA loss on chromosome 9p12–22 [29]. Deng et al. [34] detected LOH even in morphologically normal lobules adjacent to breast cancer in which the most frequent aberration was found at chromosome 3p22–25. A further recent study demonstrated genetically abnormal clones in 21/95 (22%) seemingly normal breast tissue samples using nine microsatellite markers [35]. Taken together, these data indicate that genetic abnormalities potentially critical to breast tumorigenesis accumulate already before phenotypic changes occur and are detectable in microdissected tissue that is histologically normal.

Mutation Analysis

Recently, methods of whole genome amplification from small cell groups were optimised for reliable mutation analysis by restriction fragment length polymorphism (RFLP)-PCR and conventional sequencing studies even at the single cell level [36]. For mutational analysis of preamplified genomic DNA, the intrinsic error rate of *Taq* polymerase (error rate: 10–4/bp) and the high total number of PCR cycles during whole genomic amplification and subsequent PCR must be taken into consideration. So far, reports of conventional sequencing of DOP- or primer-extension-preamplification-reamplified DNA are rare [36]. However, reliable whole genome amplification techniques in combination with tissue microdissection provide a powerful new tool to study the molecular-genetic changes underlying both precursor lesions and invasive cancer.

Combining immunohistochemistry, microdissection and DNA sequencing, Ren et al. [37] identified mutations in the p53 gene within synchronous dysplasia, carcinoma in situ and invasive cancer of sun-exposed skin, a strong indication that they all derive from the same originally transformed clone. Furthermore, p53 mutations were observed in morphologically normal p53 immunoreactive keratinocytes.

Sagawa et al. [38] reported the detection of a K-*ras* point mutation in a type II cell hyperplasia in the context of lung carcinogenesis combining microdissection by selective ultraviolet radiation fractionation, RFLP-PCR and direct sequencing. A very recent study investigated pulmonary tumourlets which are minute neuroendocrine cell proliferations believed to be precursor lesions to pulmonary carcinoids [39]. Genotyping on microdissected tissue was designed to detect allelic imbalance of the *int*-2 gene and involved DNA sequencing of two closely spaced deoxynucleotide polymorphisms. *int*-2 allelic imbalance was shown to be an early event in carcinoid tumour formation.

Microarrays and Microdissection

The current process of whole genome sequencing forms the basis for the construction of DNA arrays that permit genome-wide studies of DNA copy number, DNA sequence and RNA expression. The possible highly parallel data acquisition and data analysis on DNA arrays allows the exact determination of complex genetic changes. Several methods have been devised to study gene expression on a large scale: cDNA subtraction, differential display, representational difference analysis, expressed sequence tags sequencing, serial analysis of gene expression, and differential hybridization on either high-density-spotted nylon filters or glass microarrays. Profiles of gene expression can only be determined if pure populations of neoplastic cells can be obtained, which laser-based microdissection has made possible. An inherent problem of gene expression profiling of premalignant lesions are the small amounts of RNA. This problem can be overcome by the method of linear amplification. However, until now there is only one report on successful combination of LCM and microarray technology. Luo et al. [20] showed that RNA amplification is reproducible between individual LCM capture cells. Furthermore, these authors demonstrated a differential gene expression between large- and small-sized neurones in the dorsal root ganglia. For this study, two sets of 1,000 large neurones and three sets of 1,000 small neurones were captured for cDNA microarray analysis. So far, no investigation on precursor lesions using microarray technology has been published.

Conclusions

Tissue microdissection of neoplastic cell populations in premalignant lesions is a prerequisite for identifying the early molecular-genetic changes occurring during the multistep process of carcinogenesis. The ongoing improvement of the accuracy of microdissection techniques as well as the increasing sensitivity of various molecular-genetic analyses will allow investigators to determine specific genetic changes in human cancer and precursor lesions. In the near future, it will be possible to trace the gene pattern along the length of a prostate gland, a breast duct or Barrett's esophagus, in order to examine the progression of neoplastic development. Thus, these new technologies will have an enormous impact on molecular pathology with several diagnostic, prognostic and therapeutic implications.

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