Microdissection and the Study of Cancer Pathways

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Abstract: The study of genetic alterations in tumors and their precursor lesions is often hampered by the presence of a heterogeneous background of non-neoplastic elements such as stromal cells, inflammatory cells, and angiogenic elements. Microdissection involves the extraction of specific populations of cells under direct visualization. In this article, we will discuss the currently available techniques of microdissection, and briefly review how this material is being utilized in the study of cancer pathways. Microdissected tissue is amenable for the study of cancer genomics, expression analysis and most recently, cancer proteomics. The purity of reagents obtained from microdissected material has resulted in the successful identification of tumor suppressor genes as well as novel transcripts and proteins that are altered in neoplastic cells. Improved techniques of tissue fixation and microdissection, supplemented with ancillary technology such as pre-amplification, have permitted the use of increasingly smaller quantities of material for the study of cancer pathways. Importantly, it is now possible to analyze many of the genetic changes that precede cancer, thereby identifying populations "at risk" for developing malignancies in the future.

INTRODUCTION

Most cancers evolve by a multistage pathway wherein there is progressive accumulation of genetic changes in existing normal cells prior to malignant transformation. An astounding array of genetic "hits" involving silencing of tumor suppressor genes and activation of tumor promoting oncogenes can be seen in a given cancer phenotype. Although cancers in general can be considered to be clonal populations, the same is not true for the non-neoplastic elements native to the tissue in which they arise. Histopathologic examination of most tumors reveal an intimate intermix of neoplastic and non-neoplastic cells, the latter partly incited by the cancer [63]. One of the greatest challenges in the study of human tumors has been the isolation of pure populations of neoplastic cell types from a heterogeneous background of normal epithelium, desmoplastic stroma, inflammatory cells and blood vessels. In the absence of prior cell enrichment, the accurate analysis of molecular changes associated with tumors and their precursor lesions requires is confounded by genetic material not derived from the cancer cells alone [105].

LASER-BASED MICRODISSECTION: A REVOLUTION IN CELL ENRICHMENT

Microdissection is a technique that involves isolation of specific subpopulations of cells from a heterogeneous background, usually obtained under direct visual inspection. Although the present discussion will primarily focus on one aspect of microdissection, i.e. laser-based instruments, a variety of other techniques have been used by cancer researchers to extract pure cell populations from heterogeneous tissues (Table 1). The current prototypes of laser-based instruments include the laser microbeam microdissection coupled with laser pressure catapulting (LMM/LPC) and laser capture microdissection [LCM), both of which allow extraction of small numbers of cells, including single cells from archival tissue in a non-contact based manner. LMM/LPC uses tissue that has been mounted on a 6 µm membrane and placed on a glass slide, onto which the operator directs an ultraviolet laser beam under direct visualization [12, 74, 75]. The laser beams burns a rim of membrane and ablates the underlying unwanted tissue around the area of interest, leaving the desired cell population intact. The latter is then isolated by catapulting under pressure onto an overhanging cap, followed by isolation of reagents (DNA, RNA and protein) in an Eppendorf tube. The use of an ultraviolet laser rather than infrared laser (***vide infra***) means that the membrane mounted tissue is cut away by the high photon density ("cold" laser) and the heat generated during microdissection is minimal, which at least theoretically reduces the risk of damaging the extracted reagents [74].

LCM, which has become the prototype laser-based technique in the United States, was devised at the National Institutes of Health (NIH) in 1996 [13]. A second generation commercial version of this instrument is now available (http://www.arctur.com). The LCM technique uses

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Table 1. Microdissection techniques.

Technique	Principle
Selective Ultraviolet Radiation Fractionation (SURF) [78]	UV radiation-induced damage of "unwanted" DNA; ink-dots protect desired cells
Selective laser ablation [8]	UV laser-induced damage of "unwanted" DNA
Manual microdissection [38, 39, 65, 104]	Extraction of desired cells using blades, needles or with the use of a mechanical micromanipulators and modified Pasteur pipettes or tungsten wire needles
Laser-based microdissection	Laser pulses to "capture" cells of interest
Laser capture microdissection (LCM) [13]	Infrared laser beam
Laser microbeam microdissection/ laser pressure catapulting (LMM/LPC) [74, 75]	UV laser beam

100µm thick ethylene vinyl acetate (EVA) film impregnated with a dye that absorbs light in the near-infrared spectrum, and is attached to a rigid 6mm laser cap [82]. The cap is lowered in exact apposition to the area of interest on the tissue section, and a pulse of near-infrared laser beam is directed from above. The cap absorbs the energy from the laser, momentarily heats to 90 ^{0}C, and melts and adheres to the underlying tissue. Varying the spot size of the laser within a narrow range (7.5µm to 30 µm) ensures the specificity of dissection. The laser cap can be moved around on the tissue by means of a joystick and can be used to select multiple areas on the same cap. Up to 3000-5000 cells can be isolated from a single slide in this fashion [83]. Since the laser cap absorbs most of the energy from the pulse, there is minimal transfer of energy to the tissue, decreasing the possibility of heat-induced damage to extracted reagents. The slides used are without cover slips, which makes visualization fuzzy. Hence the newer versions of the LCM have a built-in optical system that allows the operator to confirm the histology of the area to be microdissected without transferring the slide. Once the cells of interest have been captured using LCM, the cap containing the dissected cells are placed in an 0.5 ml Eppendorf tube containing lysis buffer, that forms an air tight junction with the laser cap. The long chain polymers that compose the EVA film and thus surround and tightly hold the cells, are designed in such a way that they dissolve under the effects of the lysis buffer such that the cells are released into the solution. The protocols used for molecular analysis from LCM-captured tissue are fairly standardized and are available for use by the public-at-large on the NIH web site (http://dir.nihcd.nih.gov/lcm/lcm.htm).

Table 2a. Selected tumor suppressor genes identified by loss of heterozygosity analysis.

Gene	Locus	Cancer [Ref.]
APC	5q21	Colon [48]
BRCA1	17q21	Breast, Ovary [28, 87]
BRCA2	13q12	Breast, Ovary [102]
DCC	18q21	Colon [33]
DPC4/smad4	18q21	Pancreas [40]
FHIT	3p14.2	Lung, Breast, Cervix, Head and neck [68]
Multiple endocrine neoplasia 1(MEN) 1	11q13	Endocrine tumors [30]
PTEN/MMAC	10q23	Brain, Prostate, Breast [58]
RASF1	3p21.3	Lung, Breast [23]
vonHippel Lindau (VHL)	3p25	Kidney [54]

Table 2b. Putative tumor suppressor gene loci identified by loss of heterozygosity analysis.

Locus	Cancer [Ref.]
1p36	Brain [85] Prostate [11] Neuroblastoma [67]
Multiple 3p sites (3p12, 3p14.2, 3p21.3, 3p22-24.3,3p25)	Lung [45, 98] Breast [2, 64, 76]
4q32-33 4q25-26	Mesothelioma [81] Breast [80] Colon [79]
6q	Prostate [88] Breast [37]
8p12-23	Lung [99] Breast [103] Prostate [22]
19q13.3	Brain [86]
22q13	Breast [17] Colon [18] Ovary [15]

GENOMIC ANALYSIS OF MICRODISSECTED CANCERS

The study of normal and neoplastic genomes has been the most widely used application of microdissected material in the study of cancer pathways. Genetic changes in the multistep progression of cancer can involve amplification or gain of function mutations in dominant oncogenes, or they may involve loss of function by deletion, mutation or methylation in recessive tumor suppressor genes. In Knudson's classical two hit hypothesis of tumor suppressor gene function, one parental allele is lost by deletion, while the second is inactivated by mutation [49]. Thus if tumors are analyzed with respect to the integrity of their parental alleles at a given polymorphic locus, both alleles would be present in the constitutional DNA while one allele would be lost in the tumor (a phenomenon called "loss of heterozygosity or LOH) [63]. Microdissection has made a remarkable difference in the application of LOH analysis to the study of cancer pathways [63]. The logistics of LOH analysis are such that virtually pure populations of tumor cells or preneoplastic foci are required, since contamination by even a few unwanted cells will mean the second allele deleted in the cell population of interest will be amplified by the PCR reaction. LOH analysis has been invaluable for mapping of tumor suppressor genes (TSGs), localization of putative chromosomal "hot spots" and the study of sequential genetic changes in preneoplastic lesions (Tables 2 and 3). The use of microdissected material has unraveled that the true incidence of allelic losses involving numerous TSGs is actually significantly higher than what has been previously assumed [37, 52]. Not surprisingly, the study of preneoplastic lesions has shown that many of the genetic alterations found in cancers actually begin in histologically "benign" tissue (Table 3).

Besides LOH analysis, other studies that can be performed on microdissected DNA include analysis of X-chromosome inactivation to assess clonality [106], single strand conformation polymorphism (SSCP) analysis for mutations in critical genes [61, 62, 72, 89], comparative genomic hybridization (CGH) [4, 5, 53] and the analysis of promoter hypermethylation [9, 41, 92]. All of these methods are applicable to formalin-fixed archival tissues, with no more than 50-100 sectioned cells required per PCR reaction and even fewer cells if material from cryostat sections or methanol-fixed specimens

Table 3. Genetic changes in microdissected preneoplastic Lesions – selected references.

Organ	Gene / Allelic Locus	Reference
Colon		
Early adenoma	5q21-22 (APC)	[34, 35, 93]
	Ki-ras	
Late adenoma	18q21 (DCC)	
	17p13 (p53)	
Lung		
Hyperplasia / metaplasia	3p LOH	[97, 98, 99, 100]
	9p21 (p16)	
	8p21-23	
Dysplasia	17p13 (p53)	
Prostate		
Prostatic intraepithelial neoplasia	8p12-22	[32, 73]
	16q22	
Cervix		
Cervical intraepithelial neoplasia I-III	3p LOH	[19, 101]
	(3p14.2, 3p12, 3p25)	
	Human papilloma virus integration	[43]
Esophagus		
Barrett's esophagus	17p13 (p53)	[7, 96,106]
	9p21 (p16)	
	5q21-22 (APC)	

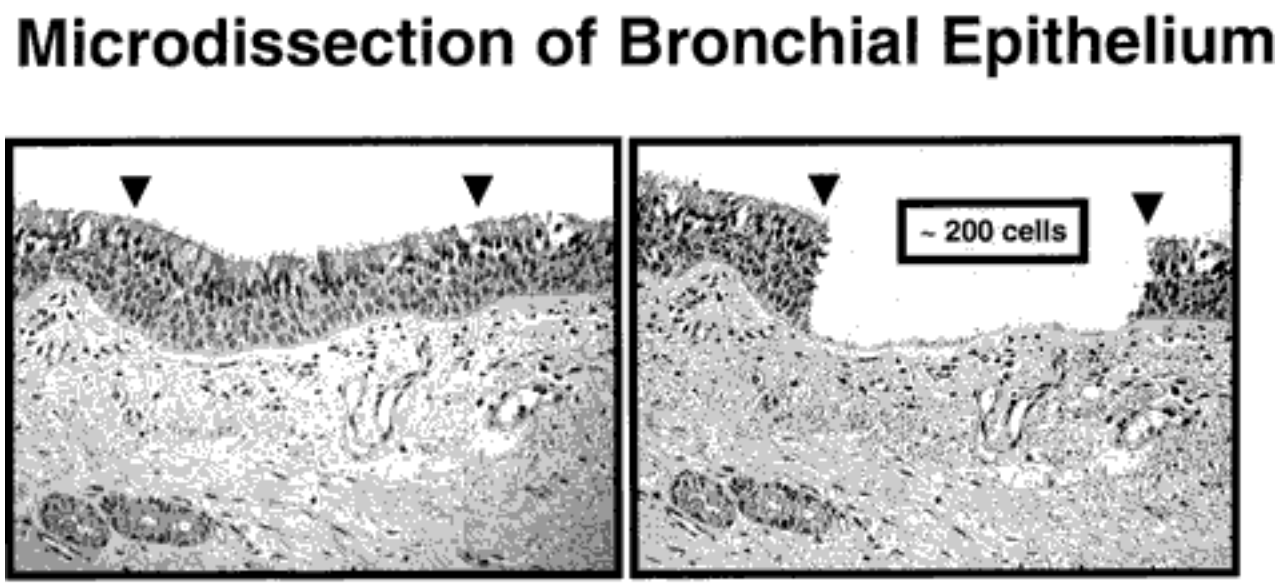

Figure 1. Manual microdissection of a patch of approximately 200 cells from non-neoplastic bronchial epithelium using a Narishige(micromanipulator. Left panel: Hematoxylin-eosin stained tissue section of bronchial epithelium, obtained by fluoroscopic-guided biopsy. Right panel: following microdissection of overlying bronchial epithelium, the underlying basement membrane and stroma remain intact.

is used [63]. CGH, which until recently was limited by large quantities of fresh/frozen DNA, has been successfully performed using 20-100 microdissected cells [4, 5, 53]. Similarly, quantitative analysis of ***her-2/neu*** and ***toposiomerase II*** α gene amplification using 5'-exonuclease-based-real time PCR has been performed on 50-100 cells in LCM-microdissected archival breast carcinomas [57]. Other novel approaches to the study of cancer genomes using laser-assisted microdissection include determination of DNA ploidy, flow cytometric analysis of surface antigen expression and fluorescence in situ hydridization ("LCM-FISH") [25]. The combination of microdissection with newer technologies such as primer extension preamplification and whole genome amplification [24, 95, 107] has made genomic analyses possible on smaller and smaller quantities of cells, thereby permitting the study of even microscopic preneoplastic lesions.

EXPRESSION ANALYSIS OF MICRODISSECTED CANCERS

Differential gene expression is a useful parameter to determine how tumors differ from the normal tissues they are derived from. Gene expression can be studied by a variety of available methods such as expressed sequence tag (EST) sequencing [1], differential display [60], subtractive hybridization [44], serial analysis of gene expression (SAGE) [91] and microarray hybridization [50] (See Table 4). Of these techniques, differential displays, SAGE and microarrays have the particular benefit of being high throughput assays, meaning that a large number of samples can be analyzed over a short period of time [16]. SAGE has been used to study gene expression differences between normal and neoplastic pancreatic cells [91], and between human bronchial epithelial cell cultures and non-small cell lung carcinoma [42]. Similarly differential display analysis has been used to identify genes that are differentially expressed in prostate cancers [94] or are involved in the progression of breast carcinomas [59]. Microarrays (popularly known as “gene chips”) have generated the most excitement of all current expression technologies [14, 26]. Gene expression monitoring using microarrays was first described using radioactive targets hybridized onto filter-immobilized cDNA clones. Subsequently, DNA printed on glass and hybridized with fluorescence-labeled cDNA have been developed that allow simultaneous analysis of independent biological samples by using different fluorochromes [47]. The biggest impediment with application of gene expression technologies has been the ability to collect material in a fashion that preserves RNA. Most expression analysis technologies require a substantial input of mRNA that is hard to obtain from microdissected tissue [16]. Nevertheless, the analysis of gene expression in tumors and their preneoplastic lesions is equally prone to be confounded by the presence of contaminating inflammatory and stromal cells as is DNA analysis. Therefore, there has been an increasing need to apply available enrichment techniques to expression studies as well. Microdissection combined with RT-PCR to study the expression of one or two analytes comprised some of the early works in this field (Table 5). The results from these studies were important because they demonstrated that a) expression analysis is feasible in

Table 4. Analysis of gene expression : current methods.

Technique	Basic Principle	Comments	Minimum RNA [16]
Expressed sequence tag (EST) sequencing [1]	Creates cDNA libraries from tissues of interest, followed by random selection of clones and sequencing. "Normalized" cDNA libraries – each transcript expressed more or less equal numbers, reduces redundant sequencing of highly expressed genes	Discovery of novel genes from selected cells or tissues is possible. Quantitation of differences in levels of expression between tissue types is difficult. Low throughput.	1.0-5.0 µg polyA RNA
Differential display [60]	RT-PCR and sequencing of mRNA from two populations of cells using pairs of oligonucleotide primers, one of which is bound to polyA-tail and other to arbitrary oligonucleotide sequences at varying distances.	Not all differences are discovered using a single arbitrary primer. Quantitation of differences in levels of expression between tissue types is difficult. High throughput.	10-100 ng polyA RNA
Subtractive cloning [44]	Double stranded cDNA created from two cell populations of interest, one designated "tester" (from which unique clones are desired) and the other "driver" (used for subtraction). Driver-driver and driver-tester hybrids removed by affinity separation or digestion with exonucleases.	Limited sensitivity, subtle quantitative differences between two populations may be missed. Restricted to a pair of samples in a given analysis. Low throughput.	10-100 ng polyA RNA
Serial analysis of gene expression (SAGE) [91]	Unique ten to eleven base pair long sequence "tags" produced from each transcript, with concentration of tags being proportional to level of mRNA in original sample.	Eliminates sequence to sequence variation in translation rate inherent in PCR. Specialized bioinformatics required for analysis of SAGE data. High throughput.	1.0-5.0 µg polyA RNA
Microarray hybridization [50]	Radioactive or fluorescent-labeled normal and tumor mRNA samples hybridized to cDNA clones or oligonucleotides spotted on a test surface(eg. silicon)	High throughput.	1.0 µg or more polyA RNA

microdissected tissue, down to the single cell level, and b) frozen and alcohol fixed tissues are probably the best substrates to perform these studies. The application of more sophisticated technologies such as cDNA microarray analysis of microdissected tumor tissue is still in its infancy, but represents one of the most exciting frontiers in cancer research. Already, cDNA libraries have been constructed and analyzed from microdissected prostate, head and neck, and breast carcinomas, and similar work is underway with other common tumors [51, 71]. The Cancer Genome Anatomy Project (CGAP) at the National Cancer Institute (http://www.ncbi.nlm.nih.gov/ncicgap) has been initiated with the objective of identifying genetic differences between normal, preneoplastic and cancer tissues by comparing and contrasting expression profiles from microdissected regions in the same patient. The availability of such cDNA libraries will permit identification of novel genes that are either over-or under expressed in the multistage pathogenesis of cancer, leading to the construction of focussed cancer-specific and organ-specific microarrays for genetic "profiling" of individual patient samples in the future.

PROTEOMIC ANALYSIS OF MICRODISSECTED CANCERS

Proteomic analysis of biological specimens aims at determining the overall set of proteins that are important in normal cellular physiology or altered by a disease process such as cancer [27]. The analysis of the entire protein complement within a cell or a tissue type (the "proteome") can be performed by a variety of techniques such as Western blotting, mass spectrometry, and peptide sequencing. High-resolution two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) is a useful technique to analyze populations of proteins in different cell types [66]. In 2-D PAGE, individual proteins from cell extracts are first separated by charge and then by size, using sodium dodecyl sulfate-PAGE. Protein analysis is a powerful complementary approach to DNA and RNA-based

Table 5. Expression analysis from microdissected normal and tumor tissues.

Author (Ref)	Transcript and tissue analyzed
Jin et al [46]	Prolactin expression in anterior pituitary cells (single cells)
Maitra et al [63]	CK-19 expression in prostate carcinoma (50 cells) Podoplanin expression in kidney (6 glomeruli)
Chuaqui et al [21]	Novel transcripts in prostate carcinoma (5000 –10,000 cells)
Chuaqui et al [20]	Complete transcript amplification from cervical cytologic ("Pap") smears (2000 cells)
To et al [90]	BRCA1, $p21^{waf1}$ expression in breast carcinoma (200 cells)
Fend et al [36]	Lineage-specific transcripts (CD4, CD19) from immunostained lymphocytes (500 cells) ["Immuno-LCM"]
Ansari-Lari et al [3]	Estrogen receptor expression in breast cancer
Bernsen et al [10]	Tyrosinase and MART-1 expression in malignant melanoma metastasis (1-10 cells)
Krizman et al [51]	cDNA microarray analysis of prostatic intraeoithelial neoplasia (5000 - 10,000 cells)
Sgroi et al [77]	cDNA microarray analysis of normal, invasive and metastatic breast cell populations
Leethanakul et al [55, 56]	cDNA microarray analysis of normal and neoplastic squamous cell carcinomas of head and neck (5000 cells)

investigations in the pathogenesis of cancer. Protein is more stable than RNA, and has the distinct advantage of reflecting both post-transcriptional control as well as post-translational modifications. The identification of proteins that are dysregulated in cancer could be an important step in formulating treatment and intervention strategies. However, an important factor limiting the application of proteomics to the study of human cancers has been the difficulty in obtaining pure populations of cells to study. All of the current techniques require tissue homogenization and

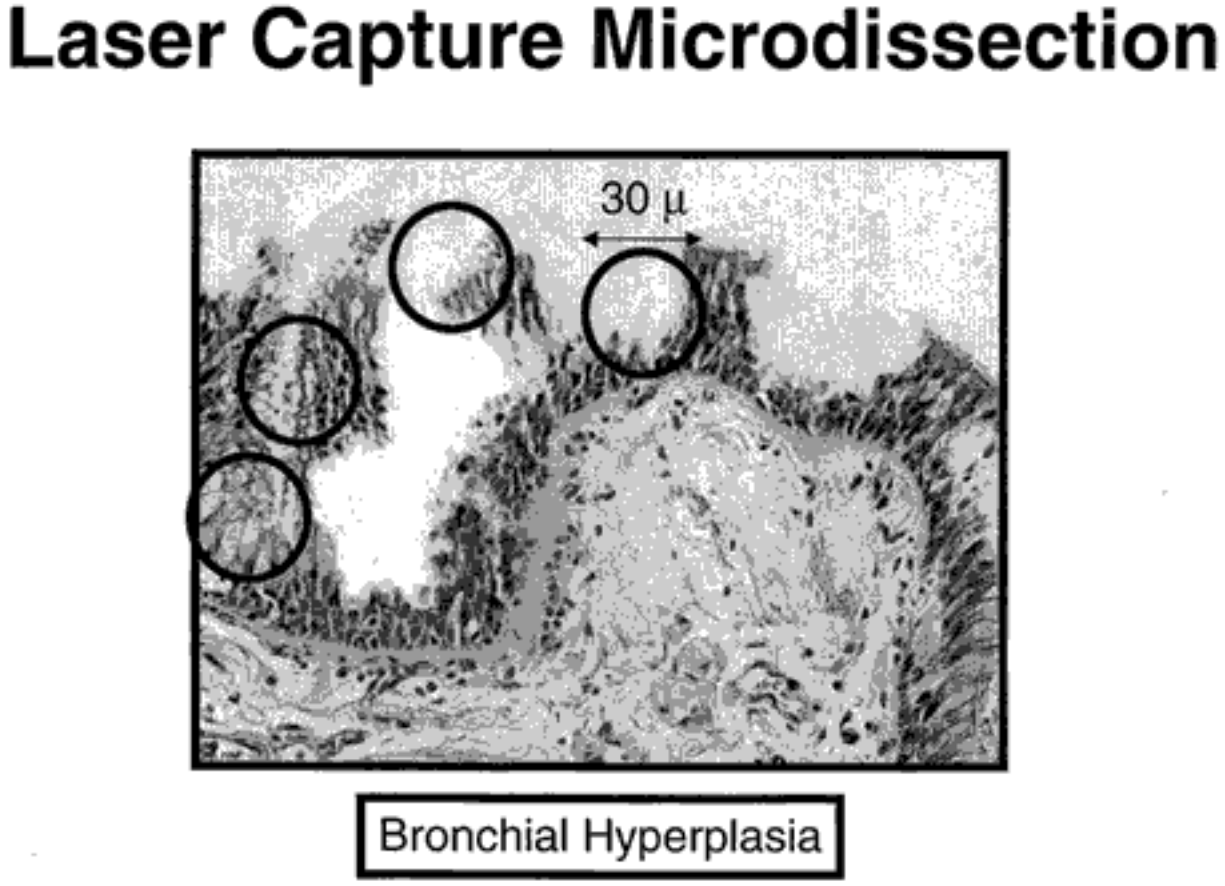

Figure 2. Laser capture microdissection of non-neoplastic bronchial epithelium using the PixCell II LCM instrument. The diameter of the laser beam is approximately 30µm, and only epithelial cells of interest are retrieved.

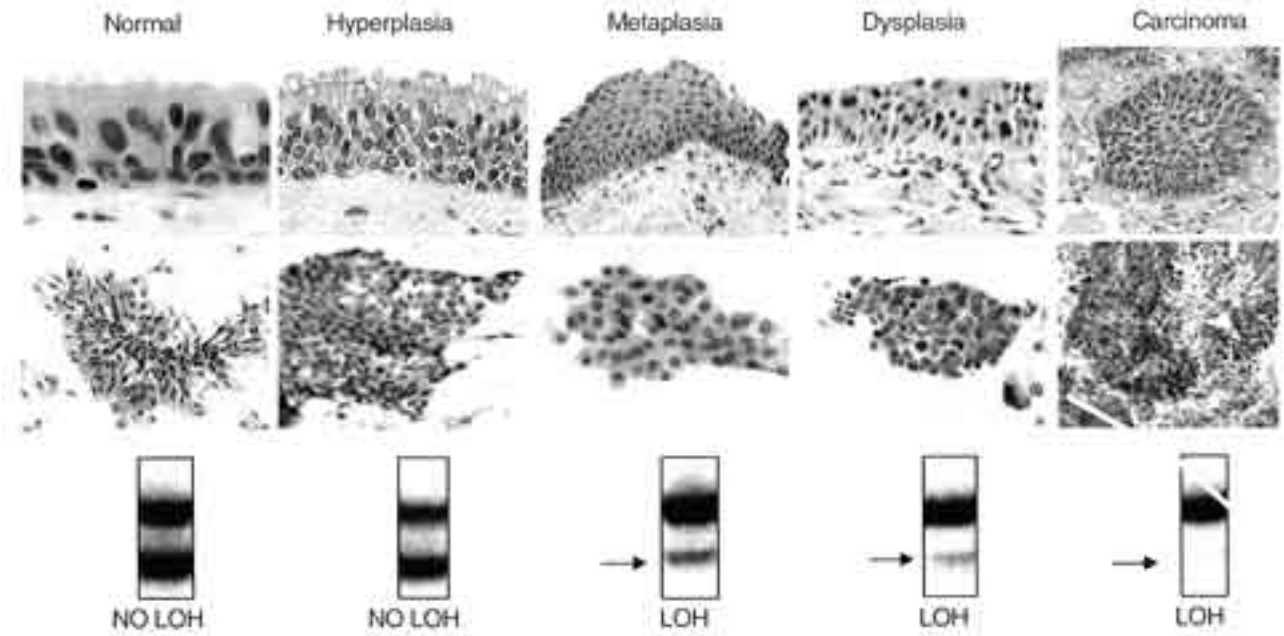

Figure 3. Use of tissue microdissection in the study of multistage pathogenesis of lung cancer. Top panel: Histologic sections of normal, hyperplastic, metaplastic, dysplastic and neoplastic foci from a resected squamous cell carcinoma; center panel corresponding methanol-fixed epithelial cell clusters prepared by the epithelial cell aggregate separation and isolation (EASI) technique (Maitra et al, Nature Med, 5:459; 1999). Bottom panel: The polymorphic microsatellite marker used for loss of heterozygosity (LOH) analysis is the pentanucleotide repeat marker of p53. Both parental alleles are retained in normal and hyperplastic epithelium, while metaplastic, dysplastic and tumor samples demonstrate partial or complete loss of lower allele (arrows). (Reprinted with permission from Nature Medicine, USA)

hence do not account for the cell of origin contributing the measured protein content. This is a major drawback, since normal and neoplastic epithelium may share >98% identity in protein profiles, and aberrantly expressed proteins so few as to be masked by the normal population of cells [69]. ***In vitro*** propagated cell lines are poor models of studying protein deregulation in cancer, since they vary widely in expression profiles from ***in vivo*** tumor specimens [70]. The availability of LCM has greatly facilitated the study of protein alterations in pure populations of tumor cells and their preneoplastic lesions. Reassuringly, preliminary studies have shown little change in electrophoretic mobility patterns of proteins following LCM, with retention of identical mass spectrometric sequencing profiles as well as stable functional characteristics such as binding to carrier molecules [6]. 2-D PAGE analyses using approximately 50,000 cells are sufficient to resolve more than 600 proteins or their isoforms and identify dysregulated products in cancer cells [29]. Novel tumor specific alterations can be identified by sequencing of the altered peptide products unique to the tumor population. For example, six differentially expressed proteins, including prostate specific antigen, were detected by proteomic analysis of microdissected prostate cancers and benign prostatic epithelium [70]. Similarly, increased levels of gelatinase and cathepsin B, both putatively implicated in cancer invasion and metastasis, have been detected in microdissected colon cancer specimens [31]. A rapid, sensitive and quantitative chemiluminescent assay has been developed to measure prostate specific antigen (PSA) levels, applicable to microdissected tissue [84]. Hopefully, this quantitative technique can be commercialized and extended to detect a variety of aberrantly expressed proteins in tumor cells. The era of proteomics represents an exciting frontier in cancer research, and microdissection techniques have greatly facilitated the refinement of this approach.

Over the last few years, microdissection instruments have ceased to be within the purview of a few specialized laboratories, and become more widely available to cancer researchers. As we continue to unravel the genetic pathways implicated in carcinogenesis, there will be an increasing demand to detect these changes at the earliest stages for screening and chemoprevention purposes. The judicious combination of operator-friendly microdissection techniques, improved nucleic acid amplification technologies and high throughput assays will make it possible to successfully determine the molecular profiles in progressively smaller numbers of cells over shorter periods of time.

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