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Proteomic profiling of the cancer microenvironment by antibody arrays

Critical changes in protein expression that enable tumors to initiate and progress originate in the local tissue microenvironment, and there are increasing indications that these microenvironmental alterations in protein expression play critical roles in shaping and directing this process. As a model to better understand how patterns of protein expression shape the tissue microenvironment, we analyzed protein expression in tissue derived from squamous cell carcinoma of the oral cavity through an antibody microarray approach for high-throughput proteomic analysis. Utilizing laser capture microdissection to procure total protein from specific microscopic cellular populations, we demonstrate that quantitative, and potentially qualitative, differences in expression patterns of multiple proteins within epithelial cells reproducibly correlate with oral cavity tumor progression. Furthermore, differential expression of multiple proteins was also found in stromal cells surrounding and adjacent to regions of diseased epithelium that directly correlated with tumor progression of the epithelium. Most of the proteins identified in both cell types are involved in signal transduction pathways, thus we hypothesize that extensive molecular communication involving complex cellular signaling between epithelium and stroma play a key role in driving oral cavity cancer progression.

Keywords: Oral cavity cancer / Antibody array / Microenvironment

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1 Introduction

A local tissue microenvironment consists of functional epithelium, extracellular matrix, and supporting stroma generally composed of fibroblasts, a mixture of immune cells, and blood/lymph vessels. Complex and dynamic interaction between these three components maintains normal, healthy, and functional tissue homeostasis. Unregulated growth of the epithelium can lead to normal

cellular architectural breakdown, enzymatic disruption of the basement membrane, and invasion of the stromal boundaries including the local vasculature. This process is called tumor progression and is characterized by a multistep process involving deregulation of gene expression, genomic rearrangements causing novel protein products, gene/protein disruption due to genomic mutations, altered patterns of protein expression, and context-dependent protein modifications. Most, if not all, of these possible perturbations take place either simultaneously or in a stepwise fashion resulting in selective growth advantages for some epithelial cells. If left unchecked the eventual result of this process is metastasis to distant organ sites of the most aggressively growing cells.

We sought to analyze the microenvironment of cancer progression and identify potential protein markers of this multistep process by utilizing oral cavity cancer as a model. Approximately 13 000 deaths in the US are attributed to squamous cell carcinomas of the head and neck

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Abbreviations: EGFR, epidermal growth factor receptor-non-phospho Y1173; LCM, laser capture microdissection; RAR- α , retinoic acid receptor-alpha; Rb, retinoblastoma tumor suppressor, p110; TSP, thrombospondin

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(HNSCC) each year. This includes cancers of the oral cavity, salivary glands, larynx, and pharynx, and five-year survival rates after diagnosis of HNSCC is 53%, considerably lower than for other cancers such as colorectal, prostate and breast [1–3]. While much is known of risk factors, such as tobacco and alcohol, little is known of the proteins that drive progression of this disease [4–6]. Thus, identification of markers of this disease process could ultimately lead to improved diagnosis and treatment of oral cavity cancer and reduce this high rate of mortality.

Use of the high-throughput cDNA microarray format has proven promising in discovering altered patterns of differential gene expression that correlate with disease [7–9]. However, because protein complexity and versatility stems from cellular location and context-dependent post-translational processes such as phosphorylation, sulfation, or glycosylation, the transcriptional activity of a gene does not necessarily reflect activity of the protein product. A number of new technologies are being introduced for high-throughput protein characterization and discovery [10, 11]. Types of multi-analyte detection systems that may have an impact on high-throughput proteomic analysis include ELISA-like systems, atomic force microscopy, use of fiber optic sensing arrays, and multiplexed capillary-based flow immunosensors [12–18]. We have approached this issue by extending the functional genomic microarray format to the field of proteomics by developing an antibody microarray system aimed at high-throughput analysis of changing patterns of protein expression.

Molecular analysis of cells in their native tissue micro-environment provides the most accurate picture of *in vivo* states of disease, however specific cell populations of interest may constitute only a small fraction of the total organ or tissue volume. Laser capture microdissection (LCM) is capable of procuring specific, pure sub populations of cells directly from diseased tissue [19, 20]. Molecular profiling of human disease tissue, and in particular proteomic analysis of cancer progression within a single patient using selected longitudinal study sets of highly purified normal, premalignant, and invasive carcinoma cells, creates the unique ability to not only ascertain altered patterns of protein expression but also at what point in the progressive process these altered patterns occur. Our approach was to combine LCM for the precise procurement of protein reflecting specific, progressive stages of oral cavity cancer with the technology platform of antibody microarrays for high-throughput proteomic analysis.

Such changes, both quantitative and qualitative, in protein expression that correlate consistently with disease phenotype will undoubtedly be important contributors

to the causes or consequences of disease and may be the most important markers for early detection and therapeutic intervention. Protein markers identified here may prove to be useful markers for oral cavity cancer, and novel experimental principles and technological concepts developed here will translate to future investigations of other diseases. To our knowledge this is the first report utilizing an antibody microarray technology platform to develop a proteomic profile of cancer progression directly from patient-derived tissue.

2 Materials and methods

2.1 LCM and protein purification

Tissues were collected according to standard and approved IRB protocol with informed consent, and were snap-frozen soon after surgical removal. Laser capture microdissection of frozen oral cavity tissue sections was performed as previously described [19–20]. Proteins from LCM procured cells (2500–3500 cells) were eluted from the cap by heating to 65°C for 15 min in 50 μ L of 1% SDS in PBS. A volume of 5 μ L was used to determine total protein concentration by 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) protein quantitation assay as recommended by the manufacturer (Molecular Probes, Eugene, OR, USA).

2.2 Standard protein analysis

For biotinylation of crude protein extract, 0.5 μ g of microdissected protein lysate in a total volume of 50 μ L of 1% SDS in PBS as heated to 95°C for 5 min, cooled to room temperature, and 1 μ L of 10 mg/mL EZ-Link Sulfo NHS-LC-Biotin (Pierce, Rockford, IL, USA) was added. This reaction was incubated for 30 min at room temperature. For visual PAGE analysis of total LCM obtained protein, biotinylated protein was separated on a 15% Tris-HCl/polyacrylamide gel, transferred to a PVDF membrane, tagged with avidin-alkaline phosphatase complex, and visualized by DuoLux ECL substrate (Vector Laboratories, Burlingame, CA, USA). For Western blot analysis, protein sample was separated on a 15% Tris-HCl/polyacrylamide gel, transferred to a PVDF membrane, and probed with the corresponding protein-specific antibodies. Positive antibody/protein interactions were visualized by secondary antibody/alk-phos-ECL substrate according to the manufacturer's recommendations (Vector Laboratories). For immunohistochemistry, frozen section of 5 μ in thickness were fixed in 100% methanol with 1% hydrogen peroxide for 15 min, and processed through methanol gradients to PBS with 0.5% Triton X-100. Slides were blocked in 1.5% donkey serum in PBS with 0.05%

Tween-20 (PBT) for 1 h at room temperature followed by 8–12 h incubation with the anti-RAR- α antibody (SC551, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in blocking solution at a 1:200 dilution. Slides were washed in PBT, incubated with secondary antibody-horseradish peroxidase complex, washed again, developed with diaminobenzidine substrate (Vector Laboratories). Slides were counter stained with eosin.

2.3 Antibody microarray fabrication

Antibody microarrays were constructed with antibodies specific for proteins that have been shown to be involved in cancer cell growth, including many cell signal proteins and many extracellular/intracellular matrix proteins. They were obtained from stocks that exist in our laboratories as well as purchased from various vendors. Since a number of antibodies recognize either phosphorylated or nonphosphorylated forms of cellular proteins, many qualitative changes in protein expression could potentially be determined by this approach. A total volume of 50 μ L of each antibody suspension was allocated to a single well of a 384-well microplate for arraying purposes. The GMS 417 (pin and ring) arraying apparatus (Affymetrix, Santa Clara, CA, USA) was used to array antibodies onto a thin film of nitrocellulose matrix bonded to a standard glass slide (FASTslides, Schleicher & Schuell, Keene, NH, USA). Using 500 micron-sized pins, the arraying apparatus deposited a fraction of a microliter each time it came into contact with the matrix. The volume absorbed instantly into the matrix, dried within seconds in a circular spot (600 microns in width), and the estimated total amount of antibody was 10–50 ng *per* spot.

2.4 Antibody microarray data acquisition and analysis

A total of 0.5 μ g of crude protein lysate was biotinylated as described in Section 2.2 in a volume of 50 μ L. Unbound, free biotin was removed by passing the biotinylation reaction twice through a Centrisep spin column according to the manufacturer's recommendations (Princeton Separations, Adelphia, NJ, USA). The biotinylated protein was incubated over the microarray for 8–12 h at 4°C in 50 mM Tris/pH 8.0, 150 mM NaCl 0.05% Tween-20, 1% BSA, 0.5x COMPLETE™ protein inhibitor cocktail (Roche, Indianapolis, IN, USA) and 0.05% azide. Following incubation, slides were washed twice with TBST (50 mM Tris/pH 8.0, 150 mM NaCl, 0.05% Tween-20), incubated in Vectastain ABC-AmP reagent (Vector Laboratories), and washed with TBST again. Antibody-antigen interaction was visualized by 5-bromo-4-chloro-3-isdoyl phosphate/nitro blue tetrazidom (BCIP/NBT) staining (Vector Laboratories).

Incubated/stained antibody microarrays were scanned on a standard flatbed scanner and tagged image file format (TIFF) image files established for each array. Results were analyzed by the software package P-SCAN, previously developed by us for high-throughput cDNA microarray analysis [21]. P-SCAN functions by placing a grid over a single TIFF image, determines the center location of each spot in the array, and places a circle around each spot. The numerical intensity of each spot is defined as the average of all pixel intensities within each circle. For comparing spots between two images, overall intensity of each spot is first normalized by subtracting the median spot intensity within the image. Final P-SCAN results are displayed in a spreadsheet format. Any protein reproducibly demonstrating either a quantitative or a qualitative change in its pattern of expression from normal to invasive histology across all four replicate experiments warranted further validation.

3 Results

We first determined that sufficient amounts of intact protein could be obtained from frozen sections of oral cavity tissue utilizing LCM. Crude protein lysate was prepared from LCM-collected invasive oral cavity epithelium as described in Section 2.1. Protein was transferred to a PVDF membrane, tagged with avidin-alkaline phosphatase complex, and visualized by ECL substrate. Results demonstrated the capability to visualize protein from microdissected invasive oral cavity epithelium and also suggested that no major proteolysis occurred during LCM (Fig. 1A). We next sought to examine the antigenic status of microdissected, biotinylated protein samples. Western blots were performed on PAGE-separated biotinylated protein with seven different antibodies specific to commonly expressed proteins of divergent size. Results show each antibody detected the correct target proteins. In addition, no degradation of the protein preparation was evident, thus demonstrating that intact protein can be successfully procured from frozen oral cavity tissue and visualized without apparent detriment to protein integrity and antigenicity (Fig. 1B).

Next we developed a prototype antibody microarray to determine if a reverse-Western approach could be successfully applied for large-scale protein expression profiling. A microarray was constructed with 250 antibodies, most of which are specific to proteins involved in cancer progression or cell growth. Crude protein was isolated from oral cavity invasive epithelial cells, biotinylated, and approximately 0.5 μ g of protein based on fluorometry was incubated over the entire array field. Unbound protein was subsequently washed off and bound protein visualized

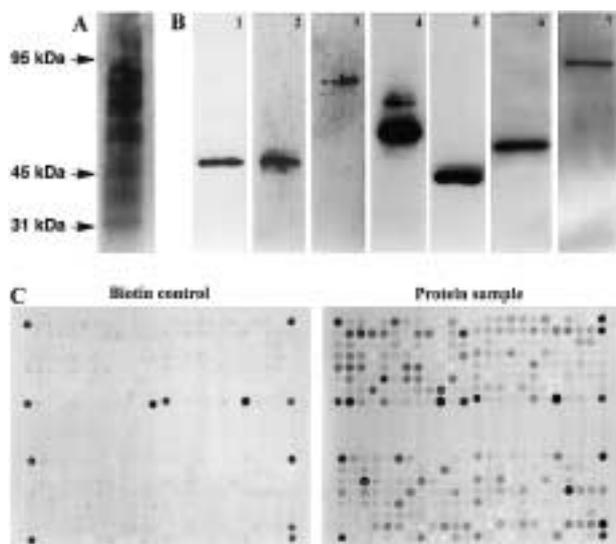


Figure 1. Standard protein analysis of microdissected protein and pilot antibody array experiment; (a) PAGE analysis of crude protein obtained by microdissection of invasive oral cavity epithelial cells. A total of 0.5 μg total protein was isolated from approximately 2500 cells, biotinylated, and visualized by alk-phos/BCIP/NBT substrate. Arrows, indicate size markers; (b) Western blot analysis of parallel protein preparations utilizing antibodies specific to individual proteins. Results were visualized utilizing a secondary antibody with alk-phos/ECL. Compare band sizes to markers in Fig. 1A. The amount of total protein varied from 0.5 to 1.0 μg per lane. Antibodies are as follows; 1, RAR- α 2, Keratin 14; 3, c-myb; 4, Keratin 1; 5, β -actin; 6, α -tubulin; 7, Rsk; (c) Pilot antibody microarray experiment. A total of 0.6 μg of microdissected, biotinylated protein obtained from approximately 2500–3500 invasive oral cavity epithelial cells was incubated on an antibody array consisting of 250 antibodies. The biotin-only control was performed in parallel exactly as the experimental but lacking protein. Results were visualized by BCIP/NBT substrate. Dark spots on the control result from biotinylated protein spotted for visualization and orientation purposes.

utilizing colorimetric means. Comparing biotin-only control to the experimental sample, results demonstrated successful detection of antibody-antigen interaction suggesting the possibility of high-throughput proteomic profiling of microdissected tissue using antibody microarrays (Fig. 1C). All seven antibodies used for Western blots (Fig. 1B) were present on this array and these proteins were positively detected. Results indicated that 51/368, or 14%, of the antibodies gave consistently positive signal, above the biotin-only control, with protein from microdissected oral cavity tissue. Incubation with biotinylated protein obtained by microdissection of invasive breast epithelium demonstrated 17 additional positive antibodies not seen with oral cavity tissue, and experiments with

thyroid-derived microdissected protein gave an additional six positive antibodies not seen with the other two tissue types (data not shown). These results suggest that many of the antibodies showing no positive signal with oral cavity tissue protein may be due, in part, to tissue-specific patterns of protein expression.

To investigate changing patterns of protein expression in oral cavity cancer progression, we developed a second-generation antibody microarray containing 368 antibodies specific to 368 different cellular proteins, 250 of which were on the prototype array. The antibodies chosen for arraying are specific to a variety of classes of cancer-related proteins including intracellular and extracellular matrix proteins, signaling proteins, cell cycle proteins, growth factors, and growth factors receptors. For experimental analysis we chose to collect data from multiple replicate experiments of a single case of oral cavity cancer in order to develop reproducible and consistent results. This was achieved by analyzing, in quadruplicate, the same histologic regions of this case.

Protein lysates were procured, biotinylated, and quantified by fluorometry from approximately 2500–3500 cells of the following histologic compartments; normal epithelium, carcinoma *in situ*, invasive cancer, stroma adjacent to and surrounding normal epithelium, stroma adjacent to and surrounding carcinoma *in situ*, and stroma adjacent to and surrounding invasive cancer. In obtaining stroma, microdissected cells were never greater than 50 microns away from the epithelial cells. The vast majority of stromal cells used for this study were those that lie directly along the basement membrane boundaries between epithelium and stroma. In areas where the epithelium was invading the stroma, the procured stroma cells were less than 25 microns from any area of invasion. No areas of vasculature were dissected and all attempts were made to exclude all blood-borne cells. A total of 24 experimental incubations (0.5 μg protein/incubation) were performed (four replicates across six separate histologic compartments) and processed arrays were scanned as described in Section 2.4. In addition, a biotin-only negative control was performed for each set of six experimental protein incubations. With reference to the level of expression for both normal epithelium and stroma adjacent to and surrounding normal epithelium as baseline, spots were identified that changed consistently in the same direction in either epithelium, stroma, or both, over four replicate experiments. We imparted these rather strict criteria of consistency and reproducibility instead of absolute changes in quantitative levels for a single experiment due to the degree of error from one experiment to the next. In fact, when lesser criteria are used, such as accepting three out of four changes in the same direction, the list of candi-

date protein markers increases. By using these strict criteria, such results impart greater confidence in developing a list of protein markers of disease progression and suggest altered patterns of protein expression between the normal state and progressive stages of oral cavity cancer progression play a key role in this process.

Comprehensive examination by P-SCAN of all replicate experiments identified 11 proteins that consistently changed either relative quantitative levels of expression or relative states of phosphorylation in either epithelium, stroma, or both. Any apparent quantitative degree of change in the same direction across all four replicate experiments was established as a baseline threshold in order to identify reproducible and consistent changes in protein expression levels from one histologic type to the next. Proteins that met this strict criteria of qualitative change are: p90^{rsk} kinase (Rsk), signal transducer and activator of transcription 5A (Stat5A), interferon-alpha (IFN- α), retinoic acid receptor-alpha (RAR- α), progesterone receptor (PR), retinoblastoma tumor suppressor p110 (Rb), Janus kinase 1 (Jak1), signal transducer and activator of transcription 1 (Stat1), and thrombospondin (TSP). Two proteins whose apparent change could be quantitative or qualitative are epidermal growth factor receptor-nonphosphoY1173 (EGFR) and the NR1-Ser896–897 subunit of *N*-methyl-D-aspartate (NR1). Because these antibodies detect specific states of phosphorylation of these proteins, it could not be determined if the differential expression of these two proteins was quantitative or qualitative. Examples of visual analysis of array data indicating either a reproducible increase or reproducible decrease in expression of particular proteins exclusively in epithelium, exclusively in stroma, or in both are shown in Fig. 2.

Nine of the 11 proteins demonstrating changes at the antibody microarray level were further evaluated by Western blot, immunohistochemistry, or both. Results indicated these standard protein methods validated because it was not detected by either Western blot or immunohistochemistry. Stat1 and TSP were not subjected to further evaluation. Cumulative results of antibody microarray, Western blot, and immunohistochemistry data are shown in Table 1. We chose not to describe the absolute, cumulative, or relative numerical value of change because we did not determine the ability of this approach to be quantifiable with respect to the absolute amount of a given protein within a given sample.

An example of experimental confirmation of the microarray results is shown in Fig. 3. Microarray data indicated a pattern of increased RAR- α expression exclusively in stroma surrounding progressing epithelium, and this result

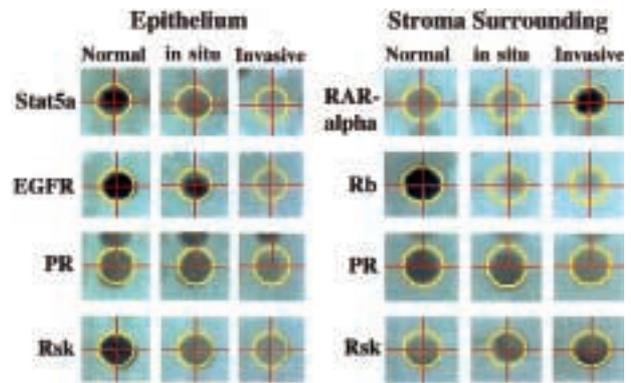


Figure 2. Examples of visual analysis of antibody microarray results. Approximately 2500–3500 cells of each of six histologic types were microdissected four separate times and a total of 0.5 μ g biotinylated protein from each dissection was used for a single incubation on a single antibody array. Examples of P-SCAN analysis indicated that stat5a, EGFR-nonphosphoY1173, PR, and Rsk proteins decreased in expression in epithelium with advancing epithelial disease. Rb and PR proteins decreased in stroma surrounding advancing epithelial disease compartments, while RAR- α and Rsk increased in surrounding stroma with advancing disease. Lines and circles result from the orientation function and data analysis by P-SCAN.

was confirmed by Western blot (Fig. 3A). This pattern of protein expression was also confirmed exclusively in stroma by immunohistochemistry of the same tissue (Fig. 3B). In addition, it is important to note the reproducible increase of RAR- α in stroma with unchanged levels in epithelium indicating the purity of cell procurement by LCM. Collectively, these results demonstrate validity of the antibody microarray data and suggest a powerful approach to high-throughput protein expression screening and disease marker indication. These results also suggest complex molecular communication exists between progressing epithelium and the surrounding stroma mediated by perturbation in proteins involved in cellular signal pathways.

4 Discussion

Molecular interaction and cooperation between cells of different embryonic lineage, for example mesenchymal and epithelial, that reside within the cellular milieu constituting a local tumor tissue microenvironment is neither heavily studied nor well understood [22–24]. Demonstration of the affect of the surrounding non-neoplastic cells that make up organ microenvironments on actively growing neoplastic cells has been reported. These studies show that when tumor cells are injected into athymic

Table 1. Cumulative data indicating changing patterns of protein expression

	Epithelium			Stroma		
	Microarray	Western blot	IHC	Microarray	Western blot	IHC
Rsk	Decrease	Decrease	Decrease	Increase	Increase	Increase
Stat5a	Decrease	ND	Decrease	No change	ND	No change
IFN- α	No change	No change	No change	Increase	Increase	Increase
RAR- α	No change	No change	No change	Increase	Increase	Increase
EGFR	Decrease	ND	ND	No change	ND	ND
NR1	No change	ND	No change	Increase	ND	Increase
PR	Decrease	Decrease	Decrease	Decrease	Decrease	Decrease
Rb	No change	ND	No change	Decrease	ND	Decrease
Jak1	No change	ND	No change	Decrease	ND	Decrease
Stat1	No change	NE	NE	Increase	NE	NE
TSP	No change	NE	NE	Decrease	NE	NE

Proteins were initially identified by antibody arrays and validated by Western blots and immunohistochemistry. Increase, decrease, and no change indicate direction of change with respect to disease progression from normal to invasive with reference to expression in the normal histologic state. ND, not detected; NE, not evaluated; IHC, immunohistochemistry

nude mice, organ-specific fibroblasts found at the site of injection can influence the invasive and metastatic properties of the tumor cells [25–28]. Similarly, a cell co-culture model has indicated that carcinoma-associated fibroblasts can stimulate and promote progression of tumorigenesis, possibly by retarding the apoptotic pathways in tumor cells [29, 30]. Studies of the molecular interplay between stromal-derived cells and epithelial-derived cells in three-dimensional cell culture models has led to some understanding of the involvement of gene and protein expression changes in the microenvironment that can direct and support tumor progression [31–33]. However, for a precise understanding of the factors that permit cellular microenvironments to support epithelial progression to a fully invasive phenotype and reach full metastatic potential, cells need to be studied within a proper 3-D, *in vivo* context of tissue structure. This study combines laser capture microdissection of surgically resected oral cavity cancer tissue with a novel high-throughput proteomics technology platform thus permitting accurate and precise proteomic analysis of the 3-D structural and cellular microenvironment, and the proteins directly responsible for progression of oral cavity cancer.

A number of proteins identified in this study have been previously implicated in tissue culture models of oral cavity cancer including RAR- α , TSP, and Rb [34–36]. In studies involving three-dimensional modeling of tissue structure, TGF- β , INF- α , and β -integrin have been shown to influence the general malignant potential of epithelial cells [31]. This report indicates no change in relative levels

of either TGF- β or β -integrin. In addition, the EGFR signaling pathway has previously been shown to be a major component of the invasive phenotype of oral cavity cancer in cell line models [37]. Because the antibody for EGFR in this study is specific for the nonphosphorylated form of the protein, it is not known if the absolute amount of EGFR is drastically reduced in the invasive epithelium as suggested by the antibody microarray data. If still present in invasive epithelium these results would suggest the EGFR protein is phosphorylated in invasive cancer supporting previous reports of involvement of this signal pathway in progression of oral cavity cancer. Furthermore, this result, as well as that of NR1 expression, suggests the ability of antibody microarrays to ascertain the signaling status of specific proteins, not simply presence or absence of protein. This potential utility has not yet been investigated further. However, by placing antibodies specific for various states of a given protein on the array, for example phosphorylated vs. nonphosphorylated forms, it may be possible to determine qualitative changes (*i.e.* the signaling potential of proteins) thus facilitating high-throughput, functional analysis of signal pathways.

The clinical decision that defines the stage of progression of a particular cancer can be a critical determinant for choice of therapeutic intervention. Since histologic involvement of stroma is one determinant of localized invasive potential of epithelium, reliable markers defining the boundaries of progression are desirable and necessary. This study indicated that RAR- α expression dramati-

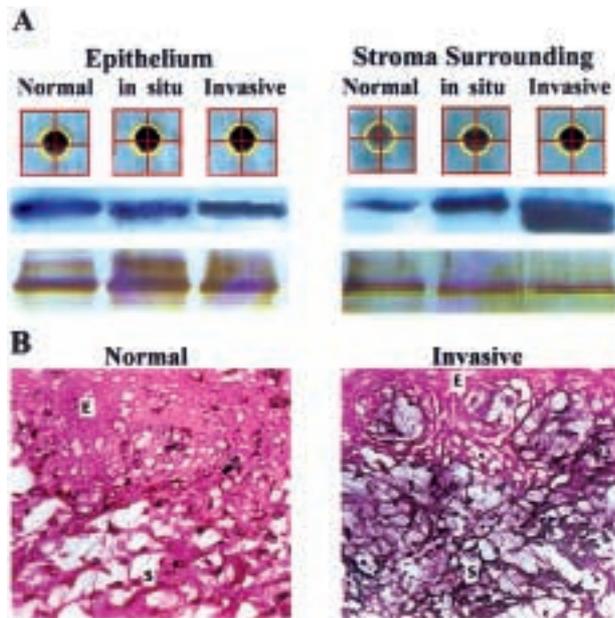


Figure 3. Example of experimental identification and validation. (a) Visual analysis of antibody microarray experiment indicates RAR- α consistently increases exclusively in stroma surrounding epithelium, with no change in epithelium, in response to advancing epithelial disease. As shown directly under microarray data, Western blot analysis using ECL substrate confirmed these results. Silver staining of total protein on a parallel gel demonstrated equal loading of protein; (b) Immunohistochemistry validation of RAR- α microarray and Western blot data. Frozen sections were made from the original tissue used for microdissection. Secondary antibody staining was visualized by HRP/DAB substrate. RAR- α is expressed in apparent equal amounts in both normal and invasive epithelial cells, whereas this protein is dramatically increased in the stroma surrounding invasive epithelium vs. normal epithelium. E, epithelium; S, stroma.

cally increased in stroma (with no change in epithelium) at the point of disease definition from carcinoma *in situ* to invasive cancer, suggesting RAR- α may be a useful marker defining this disease boundary. To our knowledge, this is the first proteomic analysis of human cancer tissue for the identification of molecular boundaries that define the premalignant stage of cancer progression.

Use of LCM and procurement of biomolecules directly from cells existing in their native tissue microenvironment permits accurate study of the molecular events that promote tumor progression. Data presented here on changing patterns of protein expression within a local tissue microenvironment suggests complex cellular communication between epithelial and stromal cells. The majority of proteins implicated by this study are signal transduction proteins. No reproducible and consistent change,

with exception of TSP, was observed in any of the intracellular or extracellular matrix proteins including laminin, fibronectin, vimentin, α -tubulin, β -actin, as well as members of the catenin, collagen, annexin, and keratin families of proteins. In addition, while many cell cycle proteins were detected on the antibody array experiments, no change in relative expression of this class of proteins was evident. These results suggest that altered pattern of signal pathway proteins, either quantitative or qualitative, play a dominant role in cancer progression of the epithelium of the oral cavity. Potentially as important is that many dynamic changes in signal potential were found in cells of the surrounding stroma as well, suggesting elaborate communication between epithelium and stroma. The role stroma plays in supporting or repressing growth of the epithelium is not well understood and may have remarkable future therapeutic implications.

While candidate marker proteins or oral cavity cancer progression identified by this technology platform were validated using standard protein analysis methodologies, this panel of antibodies was not extensively characterized with respect to relative affinities and no attempts were made to optimize the affects of concentration of antibodies on the final results. Consequently no absolute quantitative values of individual protein levels were made. Rather, we concentrated efforts on identifying the few biologically relevant disease-associated changes, from a well-defined cancer progression model, that are consistently and reproducibly found from a relatively invariant or inconsistently changing constellation of analytes.

5 Concluding remarks

To our knowledge this is the first report on an antibody microarray approach for proteomic analysis of the tissue microenvironment of cancer progression. The ability to analyze hundreds to thousands of proteins concomitantly using side-by-side patient-matched material affords the ability to perform this type of direct analysis as a model for rapid proteomic analysis. The utility of these arrays to be multiplexed even further, coupled with the power of LCM to generate biologically relevant material for analysis, will ultimately yield valuable information on patterns of altered protein expression that accurately reflect disease process. Imbedded in this wealth of proteomic information is the ability to generate a protein fingerprint, or a "proteotype", of disease progression. Such a proteotype would consist of well-defined patterns of protein expression characteristic of a specific disease process, as well as individualized patterns of expression specific to the disease profile of a given patient. Ascertainment of

disease proteotypes will ultimately lead to improvements in disease diagnosis, drug development, therapeutic efficacy, and patient-specific tailoring of drug regimens.

This project was funded in whole or in part with Federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. NO1-CO-56000. We thank Dr. Kevin Gardner for helpful discussions and Drs. Mark Raffeld and Vyomesh Patel for antibodies.

Received May 28, 2001

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