The American Journal of Pathology, Vol. 182, No. 2, February 2013

The American Journal of
PATHOLOGY
ajp.amjpathol.org

TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Three-Dimensional mRNA Measurements Reveal Minimal Regional Heterogeneity in Esophageal Squamous Cell Carcinoma

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Accepted for publication October 15, 2012.

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The classic tumor clonal evolution theory postulates that cancers change over time to produce unique molecular subclones within a parent neoplasm, presumably including regional differences in gene expression. More recently, however, this notion has been challenged by studies showing that tumors maintain a relatively stable transcript profile. To examine these competing hypotheses, we microdissected discrete subregions containing approximately 3000 to 8000 cells (500 to 1500 μm in diameter) from *ex vivo* esophageal squamous cell carcinoma (ESCC) specimens and analyzed transcriptomes throughout three-dimensional tumor space. Overall mRNA profiles were highly similar in all 59 intratumor comparisons, in distinct contrast to the markedly different global expression patterns observed in other dissected cell populations. For example, normal esophageal basal cells contained 1918 and 624 differentially expressed genes at a greater than twofold level (95% confidence level of <5% false positives), compared with normal differentiated esophageal cells and ESCC, respectively. In contrast, intratumor regions had only zero to four gene changes at a greater than twofold level, with most tumor comparisons showing none. The present data indicate that, when analyzed using a standard array-based method at this level of histological resolution, ESCC contains little regional mRNA heterogeneity.*(Am J Pathol 2013, 182: 529–539; http://dx.doi.org/10.1016/j.ajpath.2012.10.028)*

Esophageal squamous cell carcinoma (ESCC) is the predominant histological subtype of esophageal cancer, ranking as the eighth most common neoplasm in the world, with the sixth highest mortality.[1,2] Detection at a late stage and a paucity of effective chemotherapeutic and other clinical modalities make ESCC difficult to treat, and overall survival is poor. Today, only 19% of patients live for 5 years or longer after diagnosis.[3] Better understanding of the molecular etiology of this type of cancer is needed, both to identify detection markers for early diagnosis and to identify new therapeutic targets for advanced disease. Moreover, given that ESCC shares several histopathological and molecular features with other SCCs (eg, head and neck, and lung), greater understanding of ESCC may improve our overall understanding of SCCs, the class that results in the largest number of cancer deaths worldwide.[4–6]

ESCC contains extensive genomic alterations.[7–10] At present, however, little is known about the amount of transcriptomic heterogeneity that exists within this tumor type. One theory of cancer progression postulates that molecular heterogeneity is a dominant mechanism of tumor development, with unique subclones differentiating from the parent neoplasm in three-dimensional space over time, producing

Supported in part by the Cohen-Reinauch BATTLE-2 Fund, the Novartis-IPCT BATTLE Program, and the NIH Intramural Research Program of the Center for Cancer Research and the Division of Cancer Epidemiology and Genetics, National Cancer Institute.

Disclosures: M.R.E.-B. is an inventor on all NIH patents covering laser capture microdissection and expression microdissection and is entitled to receive royalty-based payments through the NIH Technology Transfer Program.

http://dx.doi.org/10.1016/j.ajpath.2012.10.028

a heterogeneous collection of spatially distinct tumor subfields that differ in molecular profile.[11] In this model, clonal evolution based on molecular status is an important process that underlies the progression of tumors into aggressive, faster-growing forms, ultimately conferring metastatic capability on certain subclones within the tumor that then disseminate via the bloodstream or lymphatic system.

An alternative view of mRNA profiles in tumor progression is that tumors arise *de novo* with their transcriptome more or less intact, including the key changes that mediate metastasis.[12–17] In this model, tumor cells share essentially the same transcript profile throughout the three-dimensional space of a neoplasm, and the rise of heterogeneous regional subclones with altered transcript profiles is not a fundamental feature of cancer progression. To date, studies examining gene expression patterns in cancer have tended to support the latter view, that there is only a limited amount of intratumor transcript profile variation.[12–17]

To address the issue of mRNA heterogeneity in ESCC, we measured global gene expression levels in microdissected tumor cell populations from discrete three-dimensional microanatomical regions. Two separate analyses were performed: the central region of tumors versus the peripheral area, and a three-dimensional comparison of multiple subregions throughout tumors.

Materials and Methods

Tissue Specimens

All cases and samples were obtained from subjects residing in the Taihang Mountain region of north-central China, and the study was approved by the Institutional Review Boards of the collaborating institutions [Shanxi Cancer Hospital and Institute, Taiyuan, Shanxi Province, China (Single Project Assurance no. S-12118-01) and the National Cancer Institute, NIH, Bethesda, Maryland]. After informed consent was obtained, patients were interviewed to obtain information on demographics, cancer risk factors (smoking, alcohol drinking, and detailed family history of cancer), and clinical information (Supplemental Table S1). None of the patients had prior therapy, and none of the ESCC cases were from patients with a family history of the disease. Using accepted inclusion criteria,[18] nine cases having sufficient tumor and matched normal epithelium were evaluated and selected by a pathologist (J.R.-C). Resected specimens were fresh frozen, optimal cutting temperature media embedded and stored in liquid nitrogen according to standard practices[19] until assays could be performed.

Tissue Processing

Immediately before use, the paired normal and tumor samples were cut into sections (8 μm thick) using a cryostat (Leica Microsystems, Wetzlar, Germany), placed onto glass slides, and stored for <2 weeks at −80°C. Before dissection, each section was individually removed from storage and immediately stained and dehydrated using an H&E protocol designed for microdissection.[20–22]

Laser Capture Microdissection

The peripheral and central tumor regions from each of the nine cases were morphologically selected and dissected by laser capture microdissection with a PixCell IIe system (Arcturus Engineering, Mountain View, CA) using 4000 medium-sized laser shots (laser spot size, 15 μm; approximately two cells per laser pulse), procuring approximately 8000 cells in total (Supplemental Table S2). Because molecular comparison between the regions was a critical aspect of the study, overlapping dissection of the two areas was avoided (Figure 1A). In addition, three-dimensional microdissections were performed on tumor regions from five of the same cases (Figure 1B). Approximately 3000 cells were procured from each dissection using large-sized laser shots (laser spot size, 30 μm; approximately three cells per laser pulse) and two to four serial recut slides per case (Supplemental Table S2).

RNA Isolation and Assessment

The time from slide removal from the freezer to completion of laser capture microdissection did not exceed 30 minutes. After laser capture microdissection, RNA was isolated using a PicoPure RNA isolation kit (Arcturus Engineering) and digested with DNase. The quantity and quality of the RNA was measured as described previously,[20,22] using a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA) and a NanoDrop ND-100 spectrophotometer (Thermo Scientific, Wilmington, DE), respectively.

RNA Amplification and Microarray Hybridization

Total RNA was used as the template for amplification, because of bias reduction compared with using mRNA alone.[23] To ensure that all samples contained a similar overall representation of transcriptome, 50 ng of total RNA was used for each sample. Two rounds of linear amplification were performed by combining reagents supplied in the Ambion MessageAmp II biotin-enhanced, single-round a(ntisense)RNA amplification kit (AM1791; Life Technologies, Foster City, CA) and the Ambion MessageAmp II aRNA amplification kit (AM1751; Life Technologies), resulting in biotin-labeled antisense complimentary (c) RNA.[24] For each sample 15 μg of biotin-labeled cRNA sample was fragmented and processed for hybridization to a GeneChip human genome U133A 2.0 array (Affymetrix, Santa Clara, CA) according to the expression kit user manual. Arrays were washed and stained using the Midi euk2v3 protocol (version 4) on a GeneChip Fluidics Station 450 (Affymetrix). The fluorescent intensity emitted by the labeled target was measured using a GeneChip 3000 7G scanner (Affymetrix). The microarray gene expression data

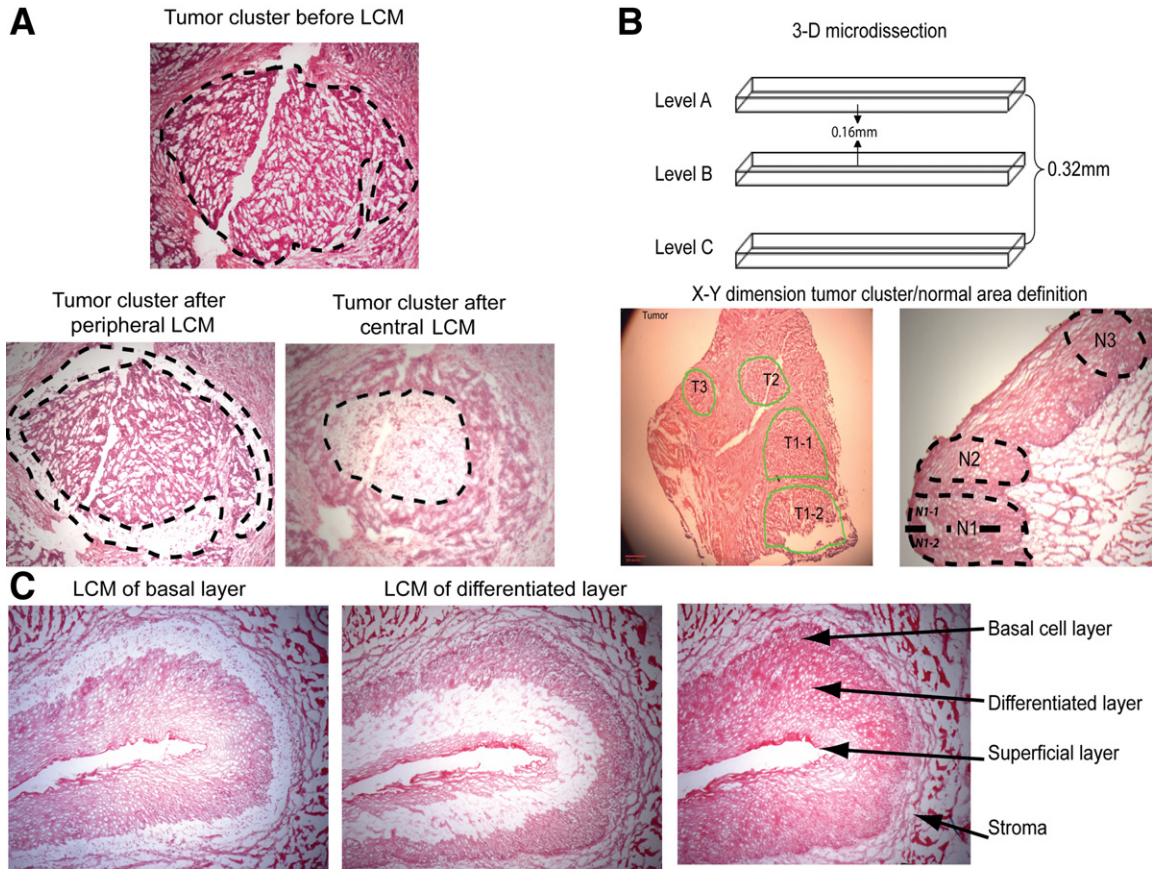

Figure 1 Microdissection design. **A:** H&E staining showing an ESCC focus before and after dissection of the tumor periphery and center. **B:** Three-dimensional microdissection design and definition of the tumor cluster and normal area. Dissected areas are outlined in green. **C:** Histology of normal esophageal epithelium compartment before and after microdissection.

discussed in this publication have been deposited in the NCBI Gene Expression Omnibus (*http://www.ncbi.nlm.nih.gov/geo*; accession number GSE33426).

For Affymetrix GeneChip studies, a single-round linear amplification is the gold standard for processing labeled aRNA.[25] In earlier experiments, we compared various quality metrics after one round of amplification (using 1 μg total RNA) versus lower RNA concentrations using two and even three rounds of amplification. These data suggest that integrity and the detection percentage of genes were similar to one round of amplification, down to concentrations as low as 1 ng (Supplemental Table S3). The overall signal intensity (ie, scaling factors) was closely related, and the gene-present percentage varied by only approximately 8% to 9%. Moreover, scatter-plot analysis of the samples showed significant correlation in probe-set signaling (Supplemental Figure S1).

Data Analysis and Statistics

Microarray Data Quality Control, Preprocessing, and Gene Filtering

Quality assessment of each microarray was done using probe-level, model-based quality statistics: developed by Richard Simon and the BRB-ArrayTools Development Team; (NUSE) and relative log expression (RLE).[26,27] Arrays with their respective median NUSE and RLE values exceeding the upper control limits were considered as poor quality and excluded from analysis. Robust multiarray analysis with quantile normalization was completed for the good-quality arrays, using the Bioconductor suite of array analysis tools (*http://www.bioconductor.org*) under R version 2.8.0 (*http://www.r-project.org*). Different probe-set filtering criteria were applied to the two analyses. For the comparison of gene expression between different cell types (central tumor, peripheral tumor, normal basal, and normal differentiated cells), probe sets showing minimal variation across the arrays were excluded from the analysis. Probe sets were selected if their expression differed by $\geq$1.5-fold from the median in $\geq$20% of the arrays. Overall, 10,725 probe sets were retained and used for the analysis. Because the goal was to assess the homogeneity in expression among the three-dimensional samples, no filtering criterion was applied for the three-dimensional comparison of tumor subregions.

Class Comparison

For the analysis of gene expression from different cell types, because a large number of differentially expressed genes

between tumor and normal cells were expected, a stringent multiple testing procedure was used to identify differentially expressed genes while controlling for the proportion of false positives. Specifically, a multivariate permutation test[28,29] was used to provide a false positive proportion of <5% with 95% confidence. The test statistics used were paired *t*-statistics for each probe set. Although paired *t*-statistics were used, the multivariate permutation test is nonparametric and does not require the assumption of normal distributions for gene expression measurements. For the analysis of comparing gene expression among tumor three-dimensional samples, because a much smaller number of differentially expressed genes were expected, and to allow for higher false positive rate, probe sets with expression difference at $P < 0.001$ based on the paired *t*-test were identified. The multivariate permutation test was performed using BRB-ArrayTools version 3.8.0 software (*http://linus.nci.nih.gov/BRB-ArrayTools.html*).

Multidimensional Scaling

Multidimensional scaling (MDS) was used to evaluate gene expression profiles of specimens in three dimensions. The data points were generated based on the principal coordinate analysis, using one minus the correlation coefficient ($1 - r$) as the distance metric.

Pathway Analysis

Genes identified by class comparison as described above were used for network and gene ontology analyses. Data were analyzed through the use of IPA (Ingenuity Systems, Redwood City, CA). Gene accession numbers were imported into the Ingenuity Pathways Analysis system, and networks were algorithmically generated based on their connectivity. Biochemical pathway analysis was performed using the ratio of the number of molecules in a given pathway to the total number of molecules that make up that pathway, to generate networks in which the differentially regulated genes are related to known associations between genes or proteins but are independent of established canonical pathways.

Intratumor and Intertumor Correlation Coefficients Analysis

Intratumor versus intertumor variability for microarray gene expression data can be assessed in two ways: one assesses the Pearson correlation coefficient across all genes on different arrays and the other assesses the intratumor heterogeneity on a gene-by-gene basis by one minus the intraclass correlation coefficient ($1 - \text{ICC}$). The ICC is calculated by fitting a variance component model for each gene. Two variance components were estimated: variance between cases (ie, intertumor variance, denoted by B) and variance within a case (ie, intratumor variance, denoted by W). The ICC is defined as $B/(W + B)$, and intratumor heterogeneity is defined as $1 - \text{ICC} = W/(W + B)$. For the variance component analysis, gene expression data of tumor specimens in both experiments were filtered to remove genes that were invariant (<20% of the arrays with expression differing from median by >1.5-fold).

Using these analytic and statistical methods, we have previously demonstrated reproducible and valid gene expression array and quantitative RT-PCR measurements with microdissected specimens.[20,22,24]

Results

Technical Parameters

A total of 74 microdissected samples from nine patients were generated: 18 samples were procured from the tumor center (TC) or tumor periphery (TP) of a neoplastic focus (Figure 1A), 44 came from a set of dissections performed as three-dimensional tumor maps (Figure 1B), and 12 samples were from a control three-dimensional map of normal squamous epithelium (Figure 1B). The recovered RNA had a preamplification mean RNA integrity number of 6.1 (range, 3.6 to 8.6) (Supplemental Table S2). One of the 74 samples did not generate enough cRNA after two rounds of linear amplification, and two samples failed NUSE and RLE array quality testing (Supplemental Figure S2); however, the remaining 71 samples were of sufficient quality for analysis.

Tumor Heterogeneity

Central versus Peripheral Regions

We first analyzed the expression profiles of cells located in the TC versus those at the TP (Figure 1A and Supplemental Table S4). On average, the microdissected TC was 660 μm in diameter, whereas the TP was two to four cell layers thick (40 to 80 μm) and was in direct contact with the surrounding stroma.

Striking similarity in global transcript levels was observed between the two tumor areas (Supplemental Table S4); thrombospondin 2 (*THBS2*) was the only differentially expressed gene, with a 2.4-fold increase in the TP. As a metric for determining relative transcriptome heterogeneity, we compared and contrasted the present TC and TP data with those from normal squamous epithelium and cancer from a previous study.[30] That analysis showed that expression profiles of microdissected normal basal (NB) squamous epithelium, adjacent normal differentiated (ND) cells, and ESCC cells from the same patient were significantly different from each other, with a large number of differentially expressed genes. For example, comparison of dissected NB cells versus adjacent ND cells (Figure 1C) revealed 4994 gene alterations at false positive ≤ 5% with 95% confidence level, including 1918 with fold change ≥ 2 and 202 with fold change ≥ 4 (Supplemental Table S4). Similarly, comparison of both TP versus NB cells and TC versus ND cells showed

large numbers of differentially expressed genes, in sharp contrast to the single differentially expressed gene in the TP versus TC analysis (Supplemental Table S4).

The data from the dissected cell populations are illustrated in both hierarchical (Figure 2) and graphical (Supplemental Figure S3) format. The dendrogram (Figure 2) illustrates the separation of the three different cell types (NB, ND, and ESCC) into distinct groups after unsupervised clustering. In contrast, the TC and TP samples were closely aligned, because of the similarity of their transcript profiles. This dendrogram also shows that the intrapatient TC and TP profiles were more closely aligned than the patient−patient comparisons, which were less than the distance between normal and tumor cells. This same pattern is seen with MDS (Supplemental Figure S3), in which the two sets of intratumor dissections are admixed but are distinct from the NB and ND cells.

Three-Dimensional Mapping

We next analyzed tumor transcriptome heterogeneity by microdissecting multiple regions throughout the three-dimensional anatomical field of five neoplasms. In each tumor, four regions were dissected in the $x-y$ plane and, depending on the thickness of the tissue block, in two or three levels in the z dimension (Figure 1B). For each $x-y$ dissection, the tumor areas were selected according to the following criteria: T1-1 and T1-2 were located within a single tumor focus and were dissected as two separate but closely associated areas; T2 was an independent tumor region, separated from T1 by stroma; T3 was an independent cluster, located as far from the T-1 and T-2 regions as possible. The same three-dimensional microdissection procedure was performed on a specimen of normal esophageal epithelium from one case in the study, which served as a normal tissue comparator.

The number of differentially expressed genes varied among the several tumor subregions (Table 1). As with the TC versus TP measurements, three-dimensional transcriptome mapping identified relatively little heterogeneity in ESCC. At the $P < 0.001$ level, the number of genes was slightly under 20 per comparison (range, 2 to 54; false discovery proportion, 24.7% to 100%), whereas at the twofold level or greater there were only a few differentially expressed genes, with most comparisons showing none.

The expression profiles are illustrated using both hierarchical clustering (Figure 3) and MDS (Supplemental Figure S4). Again, as with the TC versus TP comparison, the case-to-case heterogeneity was greater than the intratumor heterogeneity, which showed little difference across the three-dimensional field. The normal case also exhibited minimal heterogeneity among the three-dimensional dissections of esophageal squamous epithelium (Supplemental Figure S4).

We next compared the three-dimensional ESCC data with microdissected normal and tumor prostate cells from a previous study,[24] as a technical assessment of relative transcriptome heterogeneity. The $1 - r$ average linkage distance of prostate cells versus esophageal cells was approximately an order of magnitude greater than that of the largest inter-ESCC comparison (Supplemental Figure S5), indicating that the methodological approach used in the study was capable of detecting large differences present in transcriptome profiles.

Overall, the expression data from both the TC versus TP analysis and the three-dimensional mapping measurements

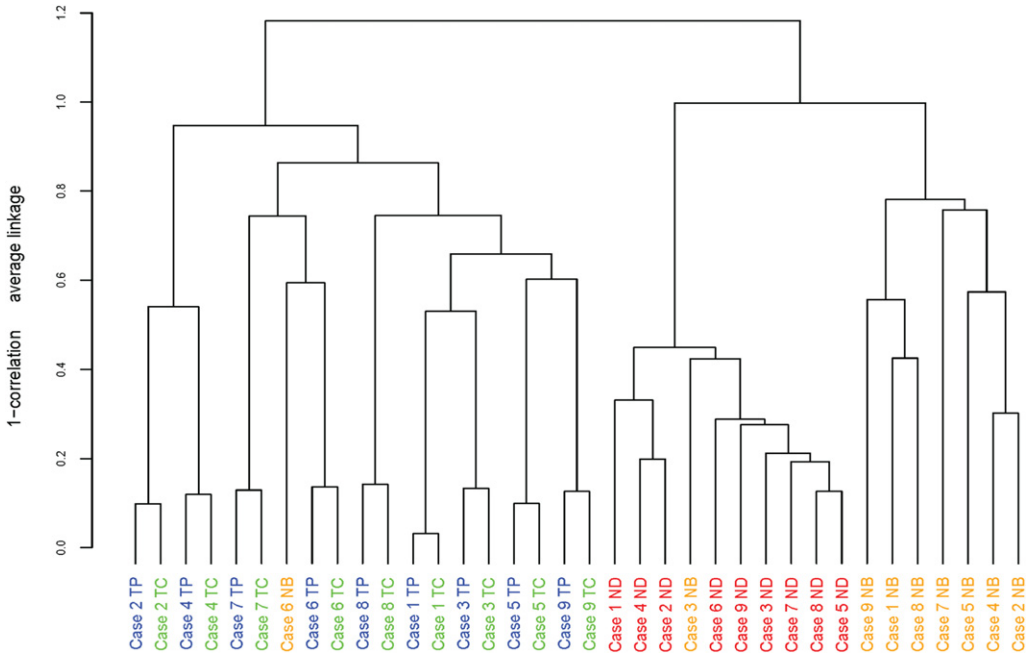

Figure 2 Differential gene expression comparisons of TP/TC (blue/green) versus NB/ND (orange/red) in nine cases by hierarchical cluster analysis. Each bar presents one case.

Table 1 Class Comparisons of Three-Dimensional Microdissected Intratumor Subregions

Comparison	Comparison definition*	Differentially expressed genes (No.)	
		$P < 0.001$	Fold change ≥ 2
X-Y dimension			
A1	T1-1 vs T1-2 at level A	24	1
A2	(T1-1 + T1-2) vs T2 at level A	10	0
A3	(T1-1 + T1-2) vs T3 at level A	5	0
A4	T2 vs T3 at level A	3	0
C1	T1-1 vs T1-2 at level C	2	0
C2	(T1-1 + T1-2) vs T2 at level C	30	0
C3	(T1-1 + T1-2) vs T3 at level C	33	0
C4	T2 vs T3 at level C	21	0
AC1	T1-1 vs T1-2 across level A and B	7	0
AC2	(T1-1 + T1-2) vs T2 across level A and B	19	0
AC3	(T1-1 + T1-2) vs T3 across level A and B	24	0
AC4	T2 vs T3 across level A and B	10	0
Z dimension			
V1.1	T1-1 at level A vs T1-1 at level C	32	2
V1.2	T1-2 at level A vs T1-2 at level C	10	0
V2	T2 at level A vs T2 at level C	54	4
V3	T3 at level A vs T3 at level C	27	0
Overall difference between level A and level C		93	0

*Tumor subregions (T) and levels (A—C) are as shown in Figure 1B.

showed that a relatively stable transcriptome is present in ESCC. We found little evidence of a significant departure from this profile with respect to the number of expression differences in any tumor subregion, in contrast to the relatively large number of differentially expressed mRNAs that characterized other dissected cell populations.

Intratumor and Intertumor Correlation Coefficients

The intratumor versus intertumor variability of microarray gene expression data were assessed in two different ways. We first determined the Pearson correlation coefficient across all genes on the arrays and found a higher correlation between intratumor specimens than intertumor specimens,

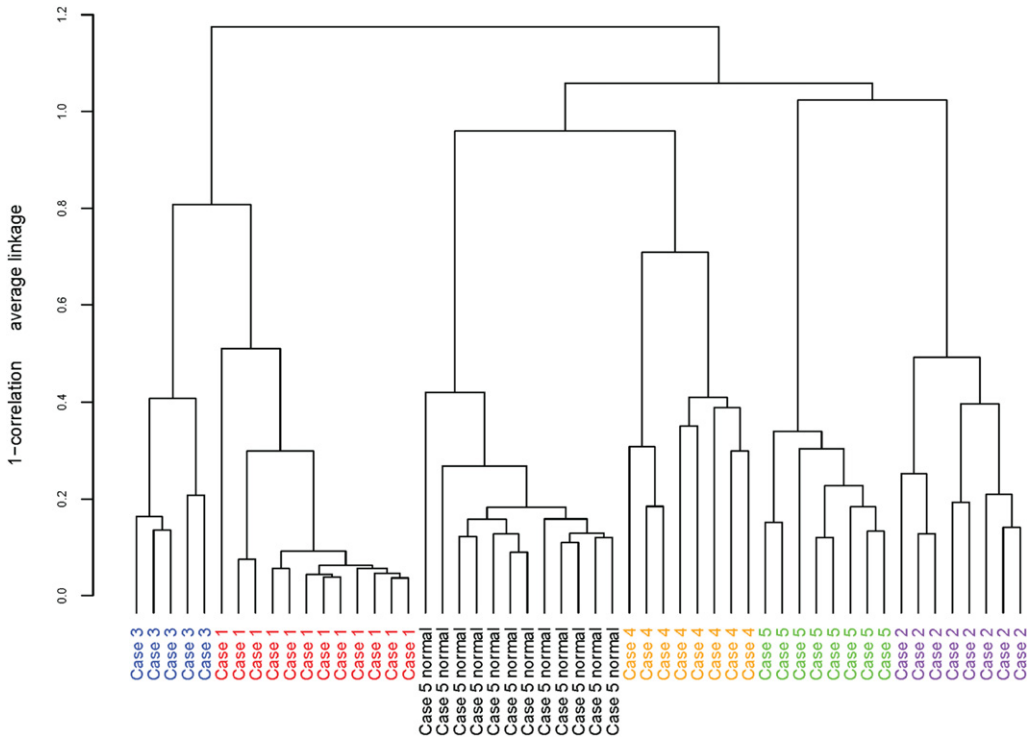

Figure 3 Differential gene expression comparisons of three-dimensional-microdissected ESCC in five cases by hierarchical cluster analysis. Each bar represents one dissection (including normal epithelium for case 5). Microdissection was performed as shown in Figure 1B.

implying low intratumor heterogeneity. The intratumor heterogeneity was less than the intertumor heterogeneity (Figures 2 and 3). All of the intratumor specimens clustered together, and the distance as measured by $1 - r$ was shorter between the intratumor specimens than between the intertumor specimens.

We then assessed intratumor heterogeneity on a gene-by-gene basis, using 1 − ICC. For one set of experiments (nine TP and nine TC specimens, taken from nine cases), 10,301 probe sets were included for the variance component analysis. For the second set of experiments (41 three-dimensional specimens taken from five cases), 9834 probe sets were included. For both analyses, the histogram of intratumor heterogeneity was plotted (Supplemental Figure S6). Intratumor heterogeneity was less than intertumor heterogeneity in 97% of the genes, with a median intratumor heterogeneity of 0.13 (ICC = 0.87). Thus, for almost all genes, there was a greater variation between tumors (cases) than within a tumor. In the second analysis, intratumor heterogeneity was less than intertumor heterogeneity in approximately two thirds of the genes (67.2%), with a median intratumor heterogeneity of 0.37 (ICC = 0.63). Overall, both analyses demonstrated that intratumor heterogeneity was less than intertumor heterogeneity.

Individual Genes and Pathways

To determine whether there were small but biologically consequential expression changes among the intratumor regions, we examined expression of individual genes and the status of specific biochemical pathways. Thrombospondin 2 (*THBS2*) was the only gene significantly differentially expressed in TP versus TC comparison. Transcripts that were altered in one or more of the three-dimensional mapping comparisons are listed in Table 2. Seven genes (*CEP55*, *CDK5R1*, *RGS5*, *GGH*, *DICER1*, *SET*, and *MAP4K3*) showed fold change ≥ 2 in 1 of the 16 subregion comparisons, and six genes (*YYI*, *RYBP*, *LAX1*, *PGGT1B*, *CDC42EP4*, and *LARS2*) were differentially expressed (fold change ≥ 2) in 2 of the 16 subregions (Table 2). However, no one individual mRNA was altered in more than two comparisons. Thus, the individual gene-level measurements mirrored the transcriptome overall, in that little intratumor heterogeneity was observed.

The biochemical pathway analysis also showed minimal differences among the three-dimensional intratumor comparisons. Only two pathways, the mitochondrial dysfunction pathway and the valine, leucine, and isoleucine biosynthesis pathway, showed an alteration in any tumor subregion, and they were identified in only 1 or 2 of the 16 comparisons (Table 3). Moreover, the top networks from the 16 comparisons typically had a score of 2 (Supplemental Table S5). In contrast, the scores of the top networks in other dissected cell populations, including normal versus tumor, and NB versus ND, ranged from 10 to 28 (data not shown), much larger than the intratumor comparisons.

Discussion

ESCC was selected as a tumor type to evaluate transcriptome heterogeneity in patients, in part because of the high degree of genomic alterations that are present,[7–10] potentially providing fertile ground for finding any associated mRNA heterogeneity. However, little evidence of regional intratumor heterogeneity at the mRNA level was observed in ESCC, at least at the dissection resolution used here (3000 to 8000 cells), suggesting that clonal evolution that significantly affects transcriptomes is not a predominant mechanism in this cancer type and that a relatively stable expression profile is maintained throughout three-dimensional tumor space.

Table 2 Individual Genes Differentially Expressed within the Three-Dimensional Intratumor Comparisons

Gene symbol	UniGene ID	Probeset	Chromosomal location	Gene name	Comparison*
Fold change ≥ 2 in 1 of the 16 subregion comparisons					
CEP55	Hs.14559	218542_at	10q23.33	Centrosomal protein 55kDa	A1
CDK5R1	Hs.93597	204995_at	17q11.2	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)	V1.1
RGS5	Hs.24950	209071_s_at	1q23.1	Regulator of G-protein signaling 5	V1.1
GGH	Hs.78619	203560_at	8q12.3	Gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase)	V2
DICER1	Hs.87889	206061_s_at	14q32.13	Dicer 1, ribonuclease type III	V2
SET	Hs.436687	213047_x_at	9q34	SET nuclear oncogene	V2
MAP4K3	Hs.655750	218311_at	2p22.1	Mitogen-activated protein kinase kinase kinase kinase 3	V2
Fold change ≥ 2 in 2 of the 16 subregion comparisons					
YY1	Hs.388927	200047_s_at	14q	YY1 transcription factor	A2, AC2
RYBP	Hs.7910	201844_s_at	3p13	RING1 and YY1 binding protein	AC3, V2
LAX1	Hs.272794	207734_at	1q32.1	Lymphocyte transmembrane adaptor 1	A2, AC2
PGGT1B	Hs.254006	216288_at	5q22.3	Protein geranylgeranyltransferase type I, beta subunit	AC2, V1.2
CDC42EP4	Hs.3903	218063_s_at	17q24∼q25	CDC42 effector protein (Rho GTPase binding) 4	AC3, C4
LARS2	Hs.526975	34764_at	3p21.3	Leucyl-tRNA synthetase 2, mitochondrial	V1.2, V3

*Comparisons are as defined in Table 1.

Table 3 Pathway Analysis of Three-Dimensional Intratumor Comparisons

Top canonical pathways	Comparison*	*P* value
Mitochondrial dysfunction	A1, C3	0.0151, 0.0337
RAN signaling	A1	0.0285
Wnt/β-catenin signaling	A1	0.0302
cAMP-mediated signaling	A1	0.0463
Cell cycle control of chromosomal replication	A2	0.0229
Semaphorin signaling in neurons	A2	0.0395
Glioma invasiveness signaling	A2	0.0445
DNA methylation and transcriptional repression signaling	A3	0.00683
Glycine, serine, and threonine metabolism	A3	0.0223
Tyrosine metabolism	A3	0.0230
Melatonin signaling	A3	0.0245
Phospholipid degradation	A3	0.0249
FXR/RXR activation	C2	0.0117
Taurine and hypotaurine metabolism	C2	0.0187
DNA double-strand break repair by homologous recombination	C2	0.0269
Pyrimidine metabolism	C2	0.0269
BMP signaling pathway	C3	0.0159
TGF-β signaling	C3	0.0171
Oxidative phosphorylation	C3	0.0354
AMPK signaling	C3	0.0455
T helper cell differentiation	C4	0.00477
IL-10 signaling	C4	0.00556
RhoA signaling	C4	0.0106
mTOR signaling	C4	0.0201
Corticotropin releasing hormone signaling	V1.1	0.00264
CDK5 signaling	V1.1	0.0181
Neuregulin signaling	V1.1	0.0208
Rac signaling	V1.1	0.0272
Valine, leucine, and isoleucine biosynthesis	V1.2, V3	0.00910, 0.0271
Ascorbate and aldarate metabolism	V1.2	0.0129
Aminoacyl-tRNA biosynthesis	V1.2	0.0248
Histidine metabolism	V1.2	0.0323
Regulation of actin-based motility by Rho	V2	0.0377
Estrogen receptor signaling	V3	0.0303
Aryl hydrocarbon receptor signaling	V3	0.0312
Folate biosynthesis	V3	0.0337
C21-steroid hormone metabolism	V3	0.0381

*Comparisons are as defined in Table 1.

To assess heterogeneity, we examined mRNA levels in several ways: tumor center versus tumor periphery, three-dimensional mapping, overall transcriptome profiles, individual gene differences, and biochemical pathways. None of the comparisons identified any pronounced intratumor expression differences (which is consistent with the findings from six of seven previous studies that have examined this question in cancer[12–17,31]), thus challenging the theory that cancer progression produces subclones with significantly different mRNA profiles[11] and supporting the alternative view that ESCC arises with and then maintains an intact and relatively stable transcriptome.[32]

Analysis of heterogeneity of DNA mutations and gene expression in nonmicrodissected samples represents the majority of the intratumor heterogeneity literature to date.[12–17,32–34] In contrast to the heterogeneity observed in DNA, multiple RNA samples from a single patient tumor have demonstrated low intratumor heterogeneity, irrespective of organ type, irrespective of whether the RNA was procured from core needle biopsies or samples as large as 1-cm^3 tissue chunks, and irrespective of whether RNA amplification was performed before hybridization.[12–17] To further investigate this apparent discrepancy of genomic variability but transcriptional stability, more powerful molecular discovery tools for analyzing small numbers of cells need to be used. For example, once next-generation deep-sequencing technologies are validated for use with small quantities of DNA and RNA from dissected cells, these technologies should allow for the combined in-depth analysis of genomic and transcriptomic intratumor heterogeneity necessary to examine this issue in more detail.

The 14 genes identified as differentially expressed in 1 or 2 of the 16 ESCC three-dimensional comparisons have been reported in previous gene expression profiling studies of ESCC and other tumors. Although none are known to be influenced by genomic instability,[35] a new mechanism involving the SEI1/SET/NM23H1 pathway in ESCC has been identified. In this pathway, SEI1 is able to up-regulate SET expression and promote translocation of NM23H1 to the nucleus from the cytoplasm.[36] In three-dimensional peripheral zone analysis of our ESCC samples, THBS2 was significantly up-regulated, which may indicate that cells in the tumor periphery were under the transcriptional influence of endogenous angiogenesis and tumor growth inhibitor signaling.[37] In an analysis of two-dimensional peripheral and core specimens from leiomyosarcoma, minimal intratumor heterogeneity was observed, with only 13 genes differentially expressed between the two zones.[16] Comparison of these genes with those altered in ESCC identified *RGS5* as common to both tumor types. The RGS5 protein is a member of the regulator of G-protein signaling group, and its expression has been associated with clinical outcome in renal cell carcinoma[38] and with chemoresistance in ovarian cancer.[39] Recent global gene expression profiling of ESCC demonstrated that CEP55 was significantly up-regulated in tumor compared with normal tissue.[40] CEP55 expression has also been shown to be related to tumor progression in head and neck SCC.[41] Although we do not have human papillomavirus infection data on the ESCC cases used in the current study, it is interesting to note the differential expression of transcription factor YYI, which is known to bind to human papillomavirus in head and neck SCC[42] and is highly correlated with human papillomavirus infection in cervical cancers.[43]

Zonal gene expression heterogeneity was observed in a microdissected xenograft pancreatic carcinoma study.[31] However, other analyses of mRNA levels from clinical specimens demonstrating minimal heterogeneity[12–17] have not used microdissection, and thus these measurements represent an aggregate profile from multiple different cell types, including tumor, stroma, and inflammatory cells.[32,34,44] Our strategy for overcoming tissue heterogeneity and potential masking of tumor specific profiles was to use microdissection[45,46] of discrete tumor areas. This is particularly important, because previous microdissection-based studies by our research group showed that tumor cells and adjacent tumor stroma have opposite changes in the directionality of gene expression, with tumor cells decreasing the number of differentially expressed genes and stroma increasing them; this characteristic of the tumor microenvironment is missed in bulk specimen analysis, which can mask important biological phenomena.[24,30] Thus, to ensure that we were accurately and fully measuring tumor cell heterogeneity, we procured and analyzed carefully dissected samples.

Five caveats to the current study require brief mention. First, the question of what constitutes heterogeneity versus homogeneity is somewhat arbitrary. Indeed, the intratumor comparisons were not identical, but were judged to be relatively similar, compared with other dissected cell populations. Second, the microdissection process was performed blindly, in that the tumor cells were procured based only on spatial location and not on either a priori knowledge of molecular status or on phenotypic features of the targeted cells. Thus, it is possible that one or more hidden tumor regions with a significantly altered transcriptome were present but were missed during the dissection process; however, the marked similarity of more than 50 individual tumor areas analyzed to date does not provide evidence for this phenomenon. In future studies, it will be possible to address this question in more detail by using molecularly targeted laser dissection methods (eg, expression microdissection[47] or immunoguided laser capture microdissection[48]) to prescreen for and then dissect tumor regions that are markedly different from each other based on expression of a target protein or transcript. Third, the study did not examine heterogeneity at the level of a single cell or a few cells; approximately 10,000 cells were procured in each regional dissection to provide a deep and robust transcriptome analysis. This issue also can be addressed in future studies, as the reproducibility of single-cell measurements improves. Fourth, we used standard bioinformatics tools in the present study, and it is possible these methods were incapable of detecting subtle but important mRNA-level heterogeneity. We hope that the publicly available data sets from our work will be analyzed in unique ways by other investigators to examine these results from various perspectives.

Finally, we did not independently validate the present results at the mRNA level, because we and others have previously shown that expression array technology provides robust and reliable mRNA measurements from microdissected tissue.[24,30,40,49] To date, the vast majority of transcripts that we have evaluated by both array and quantitative RT-PCR have shown the same result, although the expression differences are often less on the array, given the complex nature of the hybridization reaction. We did attempt to validate four differentially expressed genes at the protein level, but we were not successful because of technical challenges. This is a frequent limitation of immunohistochemical staining of tissue, especially for proteins (and these are a majority) that are not highly abundant in a given cell type. The same difficulty has also been observed previously in a larger cohort of ESCC cases.[40]

The ESCC data have implications for molecular diagnostics, because tumor heterogeneity can have important clinical consequences. For example, DNA clonal diversity measures are known to be strong predictors of cancer progression in Barrett's esophagus,[50] and are used in guiding treatment decisions.[33,51,52] Moreover, it has been demonstrated experimentally that clonally heterogeneous tumors can either inhibit or increase the therapeutic efficacy of cytotoxic drugs[33,53] and can contribute to drug resistance.[54] In the clinic, however, obtaining specimens from multiple regions of a patient's tumor to assess heterogeneity would be difficult and in many cases impractical. Thus, the question of whether a small core needle biopsy is representative of an entire tumor is coming to the forefront of molecular diagnostics and personalized medicine, such as with the clinical trial Biomarker-Integrated Approaches of Targeted Therapy for Lung Cancer Elimination (BATTLE).[55] The feasibility of making precise microarray-based predictions of clinical outcome or tumorigenesis from a single core has been demonstrated in breast cancer,[13] but in cervical cancer the number of cores necessary for obtaining reliable gene expression estimates is dependent on the intratumor heterogeneity level for the probe sets or genes of interest.[12] The predominantly homogeneous transcriptomic expression data from microdissected tumor epithelium in ESCC suggest that molecular diagnostic transcriptomic profiles obtained from clinical core needle biopsies will indeed be sufficient to accurately represent a patient's entire tumor, which supports prior ESCC analysis of two-dimensional nonmicrodissected tumor core needle biopsies.[56]

In summary, global expression measurements of ESCC indicate that regional mRNA heterogeneity is not a prominent feature, and that a relatively stable transcriptome is maintained throughout three-dimensional space, at least at a resolution on the order of 500 to 1500 μm in diameter. This finding may extend to SCCs in other organs, which as a group produce significant cancer morbidity and mortality worldwide. Future studies that measure molecular profiles in smaller cell numbers and in other geographic regions, and that include DNA mutations and proteomic status, are needed to further assess what degree of molecular heterogeneity exists in this clinically aggressive histological class of tumors.

Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2012.10.028*.

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