Validation of the Lung Subtyping Panel in Multiple Fresh-Frozen and Formalin-Fixed, Paraffin-Embedded Lung Tumor Gene Expression Data Sets

Hawazin Faruki, DrPH; Gregory M. Mayhew, DrPH; Cheng Fan, MSc; Matthew D. Wilkerson, PhD; Scott Parker, BSc; Lauren Kam-Morgan, PhD; Marcia Eisenberg, PhD; Bruce Horten, MD; D. Neil Hayes, MD, MPH; Charles M. Perou, PhD; Myla Lai-Goldman, MD

• ***Context.***—**A histologic classification of lung cancer subtypes is essential in guiding therapeutic management.**

Objective.—**To complement morphology-based classification of lung tumors, a previously developed lung subtyping panel (LSP) of 57 genes was tested using multiple public fresh-frozen gene-expression data sets and a prospectively collected set of formalin-fixed, paraffin-embedded lung tumor samples.**

Design.—**The LSP gene-expression signature was evaluated in multiple lung cancer gene-expression data sets totaling 2177 patients collected from 4 platforms: Illumina RNAseq (San Diego, California), Agilent (Santa Clara, California) and Affymetrix (Santa Clara) microarrays, and quantitative reverse transcription–polymerase chain reaction. Gene centroids were calculated for each of 3 genomic-defined subtypes: adenocarcinoma, squamous cell carcinoma, and neuroendocrine; the latter of which encompassed both small cell carcinoma and carcinoid. Classification by LSP into 3 subtypes was evaluated in both fresh-frozen and formalin-fixed, paraffin-embedded tumor samples, and agreement with the original morphology-based diagnosis was determined.**

Results.—**The LSP-based classifications demonstrated overall agreement with the original clinical diagnosis ranging from 78% (251 of 322) to 91% (492 of 538 and 869 of 951) in the fresh-frozen public data sets and 84% (65 of 77) in the formalin-fixed, paraffin-embedded data set. The LSP performance was independent of tissue-preservation method and gene-expression platform. Secondary, blinded pathology review of formalin-fixed, paraffin-embedded samples demonstrated concordance of 82% (63 of 77) with the original morphology diagnosis.**

Conclusions.—**The LSP gene-expression signature is a reproducible and objective method for classifying lung tumors and demonstrates good concordance with morphology-based classification across multiple data sets. The LSP panel can supplement morphologic assessment of lung cancers, particularly when classification by standard methods is challenging.**

(*Arch Pathol Lab Med.* doi: 10.5858/arpa.2015-0113-OA)

Lung cancer is the leading cause of cancer death in the United States, and more than 220 000 new lung cancer cases are identified each year.[1] It is a heterogeneous disease with subtypes determined by genetics and morphology (ie, small cell carcinoma, carcinoid, adenocarcinoma [ACA], and squamous cell carcinoma [SQC]). Classification of morphologic subtypes of lung cancer is essential in guiding optimal patient management,[2] and complementary molecular testing is used to identify mutated oncogene therapeutic targets. Variability in morphology and the need for assessment of a growing list of therapeutically targeted markers pose challenges to the current diagnostic standard because tissue amounts and quality are often limiting features.

Although new therapies are increasingly directed toward specific subtypes of lung cancer (eg, bevacizumab[3] and pemetrexed[4]), studies of histologic diagnosis reproducibility have shown limited intrapathologist agreement[5] and even

Accepted for publication August 11, 2015.

Supplemental digital content is available for this article. See text for hyperlink.

From the Clinical Development Department, GeneCentric Diagnostics, Durham, North Carolina (Drs Faruki, Mayhew, and Lai-Goldman); the Lineberger Comprehensive Cancer Center (Mr Fan and Drs Hayes and Perou) and the Department of Genetics (Drs Wilkerson and Perou), University of North Carolina, Chapel Hill; and the Center for Molecular Biology and Pathology, Laboratory Corporation of America Holdings, Research Triangle Park, North Carolina (Mr Parker and Drs Kam-Morgan, Eisenberg, and Horten).

All funding for this study was provided by GeneCentric Diagnostics (Durham, North Carolina). Drs Perou and Hayes are members of the board of directors, equity stock holders, and consultants for GeneCentric Diagnostics. They each are also named as inventors on a US patent for lung cancer subtyping. Drs Faruki, Mayhew, and Lai-Goldman are employees of GeneCentric Diagnostics, which has sublicensed the rights to the granted lung cancer subtyping patent from the University of North Carolina. Mr Parker and Drs Kam-Morgan, Eisenberg, and Horten are employees of Laboratory Corporation of America Holdings (Research Triangle Park, North Carolina offering HistoPlus, an assay based on technology underlying the lung cancer subtyping gene signature. Mr Fan and Dr Wilkerson have no other relevant financial interest in the products or companies described in this article.

Presented in part at the annual meeting of the American Society Clinical Oncology, Chicago, Illinois, May 29–June 3, 2014; and at the annual meeting of the Association Molecular Pathology, National Harbor, Maryland, November 12–15, 2014.

Reprints: Hawazin Faruki, DrPH, Clinical Development Department, GeneCentric Diagnostics, 280 S Mangum St, Ste 350, Durham, NC 27701 (e-mail: Hawazin@genecentric.com).

Table 1. Data Sources for Lung Cancer Gene-Expression Data Sets, Including Normalization Methods Applied to Each Data Set

Source	Platform	No.	Subtype	Normalization Method Used	Source, y (Accession Viewer)
TCGA	RNASeq	528	ACAs	RSEM expression estimates were upper-quartile normalized and log2 transformed; data matrix was row (gene) median-centered and column (sample) standardized.[28]	TCGA,[16] 2014
TCGA	RNASeq	534	SQC		TCGA,[15] 2012
UNC	4X44K[a]	56	SQC	Ratios of the 2-channel intensities were log2 transformed and LOWESS-normalized; data matrix was row (gene) median-centered and column (sample) standardized.[29]	Wilkerson et al,[19] 2010 (GSE17710)
UNC	4X44K[a]	116	ACAs		Wilkerson et al,[20] 2012 (GSE26939)
NCI	4X44K[a]	172	ACA, SQC, large cell carcinoma		Roepman et al,[22] 2009 (Agendia)
Korea	HG-U133+2[b]	138	ACA, SQC	MAS5 normalized 1-channel intensities are log2 transformed; data matrix was row (gene) median-centered and column (sample) standardized.[30]	Lee et al,[23] 2008 (GSE8894)
Expo	HG-U133+2[b]	130	All histology subtypes		IGC,[24] 2015 (GSE2109)
French	HG-U133+2[b]	307	All histology subtypes		Rousseaux et al,[25] 2013 (GSE30219)
Duke	HG-U133+2[b]	118	ACA, SQC		Bild et al,[26] 2006 (GSE3141)
UNC	FFPE tissue qRT-PCR	78	ACA, SQC, small cell carcinoma, carcinoid	ΔCt gene-expression data scaled to align gene variance with Wilkerson et al[21] data; gene-specific scaling factors applied to adjust for label-frequency differences between the data sets.	Faruki et al,[27] 2014 (see also Supplemental Table 1)

Abbreviations: ACA, adenocarcinoma; Ct, cycle threshold; FFPE, formalin-fixed, paraffin-embedded; HG, human genome; LOWESS, locally weighted scatterplot smoothing; MAS5, MicroArray Suite 5; NCI, National Cancer Institute; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; RSEM, RNAseq by Expectation Maximization; SQC, squamous cell carcinoma; TCGA, The Cancer Genome Atlas; UNC, University of North Carolina.

[a] Agilent 4X44K array, Agilent Technologies, Santa Clara, California.
[b] Affymetrix HG-U133+2 array, Affymetrix, Santa Clara, California.

less interpathologist agreement.[6,7] The introduction of the immunohistochemistry (IHC) markers thyroid transcription factor and cytokeratin 7 for ACA, and p63/p40 plus cytokeratin 5/6 for SQC are expected to improve the classification of non–small cell lung cancer, thereby reducing the number of samples that would otherwise be reported as non–small cell lung cancer, not otherwise specified.[8–10] Nevertheless, poorly differentiated tumors, conflicting IHC results, and small-volume biopsies, in which only a limited number of IHC stains can be performed, continue to pose challenges to the current diagnostic standard.[11–13] Simultaneously, new genomic data are providing molecular features that enhance the classification of lung tumors beyond what was feasible by morphology alone.[14–16] Therapeutic molecular targets, such as *EGFR* mutations, *ALK* rearrangements, *ROS1* fusions in ACA, and *FGFR1* amplification in SQC, have been found to be histologic subtype specific, thereby emphasizing the need for accurate subtype classification.[15,16] Further classification within the ACA and SQC subtypes is also being investigated to provide a greater understanding of tumor biology and associated clinical implications.[17–20]

The lung subtype panel (LSP), a 57-gene messenger RNA (mRNA) expression signature (52 classification genes plus 5 housekeeping genes), was developed to address the need for a more-objective means of classifying tumors into the known biologic/histologic subtypes. This method can thus be used to assist the pathologist in classifying lung tumors.[21] In this study, the ability of the LSP gene signature to reliably subtype lung tumor samples using gene expression data from any one of multiple platforms, including Affymetrix (Santa Clara, California) and Agilent (Santa Clara, California) DNA microarrays, RNA sequencing, and quantitative reverse transcription-polymerase chain reaction (qRT-PCR), was investigated. The 57-gene signature was also evaluated in both fresh-frozen (FF) and a new set of formalin-fixed, paraffin-embedded (FFPE) lung tumor samples comprising multiple subtypes of lung tumors, including ACA, SQC, and neuroendocrine (NE) (comprising small cell and carcinoid tumors). Concordance of gene expression-based tumor-subtype classification with the original morphologic diagnosis was evaluated.

METHODS

Multiple data sets, comprising 2177 samples, were assembled to evaluate a previously published 57-gene LSP gene-expression classifier.[21] The data sets included several publically available lung cancer gene-expression data sets, including 2099 FF lung cancer samples (The Cancer Genome Atlas [TCGA, Bethesda, Maryland],[15,16] University of North Carolina [Chapel Hill],[19,20] National Cancer Institute (NCI, Bethesda, Maryland),[22] Korea [Samsung Medical Center, Seoul],[23] Expo [International Genomics Consortium, Phoenix, Arizona],[24] France [Institut National de la Santé et de la Recherche Médicale (INSERM), Paris],[25] and Duke University [Durham, North Carolina][26]), as well as newly collected gene-expression data from 78 FFPE samples.[27] Data sources are provided in Table 1, including normalization methods applied to each data set.[28–30] The 78 FFPE samples were archived residual lung tumor samples, primarily surgical resections, collected at the University of North Carolina, Chapel Hill, under an institutional review board–approved protocol. Only samples with sufficient residual tissue and a definitive diagnosis of ACA, carcinoid, small cell carcinoma, or SQC were used in the analysis. A total of 4 categories of genomic data were available for analysis: Affymetrix U133+2 (n = 693), Agilent 44K (n = 344), Illumina RNAseq (San Diego, California) (n = 1062), and newly collected qRT-PCR (n = 78) data.

FFPE Sample Expression Analysis

Archived FFPE lung tumor samples (n = 78) were analyzed using a qRT-PCR gene expression assay as previously described,[21] with the following modifications. RNA was extracted from one 10-μm section of FFPE tissue using the High Pure RNA Paraffin Kit (Roche

Table 2. Sample Characteristics and Lung Cancer Diagnoses From the Data Sets Used to Validate the Lung Subtyping Panel

Characteristics	TCGA RNAseq	Agilent[a]	Affymetrix[b]	UNC qRT-PCR
Total samples, No.	1062	344	693	78
Tissue preservation method	FF	FF	FF	FFPE
Tumor specimen histology				
Adenocarcinoma	468	174	264	21
Carcinoid	0	0	23	15
Small cell carcinoma	0	0	24	16
Squamous cell carcinoma	483	148	227	25
Other (excluded from analysis)	111	22	155	1
Gender				
F/M/NA	285/366/300	87/85/150	151/386/1	NA
Age at diagnosis				
Median (range)	67 (38–88)	66 (37–90)	65 (13–85)	NA
Age NA	323	0	2	NA
Stage				
I	355	NA	NA	NA
II	146	NA	NA	NA
III	119	NA	NA	NA
IV	26	NA	NA	NA
Stage NA	305	322	538	77
Smoking				
Smoker	386	NA	NA	NA
Smoking status NA	526	322	538	77

Abbreviations: FF, fresh frozen; FFPE, formalin-fixed, paraffin-embedded; NA, not available; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; TCGA, The Cancer Genome Atlas; UNC, University of North Carolina.

[a] Agilent 4X44K array, Agilent Technologies, Santa Clara, California.
[b] Affymetrix HG-U133+2 array, Affymetrix, Santa Clara, California

Applied Science, Indianapolis, Indiana). Extracted RNA was diluted to 5 ng/μL, and first-strand cDNA was synthesized using gene-specific 3′ primers in combination with random hexamers (Superscript III, Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts). An ABI 7900 (Applied Biosystems, Thermo Fisher Scientific) was used for qRT-PCR with continuous SYBR green fluorescence (530 nm) monitoring. The ABI 7900 quantitation software generated amplification curves and associated cycle threshold values. Gene expression results for these 78 FFPE samples (changes in cycle threshold after imputing, 52 genes) can be found in Supplemental Table 1 (the supplemental digital content contains 5 tables), and original clinical diagnoses gathered with the samples are in Supplemental Table 2.

Pathology Review

Pathology review was only possible for the FFPE lung tumor cohort in which additional sections were collected and imaged. One section from each of 2 blocks from the same case was stained with hematoxylin-eosin and were scanned using an Aperio ScanScope slide scanner (Aperio Technologies, Vista, California). Virtual slides were viewable at magnifications equivalent to ×32 to ×320 objectives (×340 magnifier). Pathologist review (blocks 1 and 2) was blinded to the original clinical diagnosis, to the link of the blocks to a single case, and to the gene expression–based subtype classification. Pathology review-based, histologic-subtype calls were compared with the original clinical diagnosis (n = 78). Agreement for pathology reviews was defined as those samples for which slides from both blocks were assigned the same subtype as the original diagnosis. The IHC staining, including TTF-1, p63, synaptophysin, and/or Ki-67, were available for postgenomic assay testing to help resolve disagreements among the pathology review, gene expression, and original clinical diagnosis.

Data Analysis

All statistical analyses were conducted using R software (version 3.0.2; http://cran.R-project.org, accessed October 15, 2013). Data analyses were conducted separately for FF and FFPE tumor samples.

FF Data Set Analysis.—Data sets were normalized, as described in Table 1. The Affymetrix data set served as the training set for calculation of ACA, carcinoid, small cell carcinoma, and SQC gene centroids, according to methods described previously.[20,21] Affymetrix training-gene centroids are provided in Supplemental Table 3. The training-set gene centroids were tested in normalized TCGA RNAseq gene expression and Agilent microarray gene-expression data sets. Because of missing data from the public Agilent data set, the Agilent evaluations were performed with a 47-gene classifier, rather than a 52-gene panel with exclusion of the following genes: *CIB1*, *FOXH1*, *LIPE*, *NCAM1* (*PCAM1*), and *TUBA4A* (*TUBA1*).

Evaluation of the Affymetrix data was performed using Leave One Out (LOO) cross-validation. Spearman correlations were calculated for tumor test sample to the Affymetrix gene-expression training centroids. Tumors were assigned a genomic-defined histologic type (ACA, SQC, or NE) corresponding to the maximally correlated centroids. Correct predictions were defined as LSP calls matching the tumor's original histologic diagnosis. Percent agreement was defined as the number of correct predictions divided by the number of total predictions and an agreement kappa statistic was calculated.

qRT-PCR From FFPE Sample Analysis.—Previously published training centroids,[21] calculated from qRT-PCR data of FFPE lung tumor samples, were cross-validated in this new sample set of qRT-PCR gene expressions from FFPE lung tumor tissue. The ACA and SQC centroids were used as published.[21] The NE gene centroids were calculated similarly using published gene-expression data (n = 130).[21] The Wilkerson et al[21] gene centroids for the FFPE tissue evaluation are included in Supplemental Table 4. The FFPE sample gene-expression data were scaled to align gene variance with Wilkerson et al[21] data. A gene-specific scaling factor was calculated that accounted for label-frequency differences between the data sets. Gene expression data were then median-centered, sign-flipped (high threshold cycle = low abundance), and scaled using

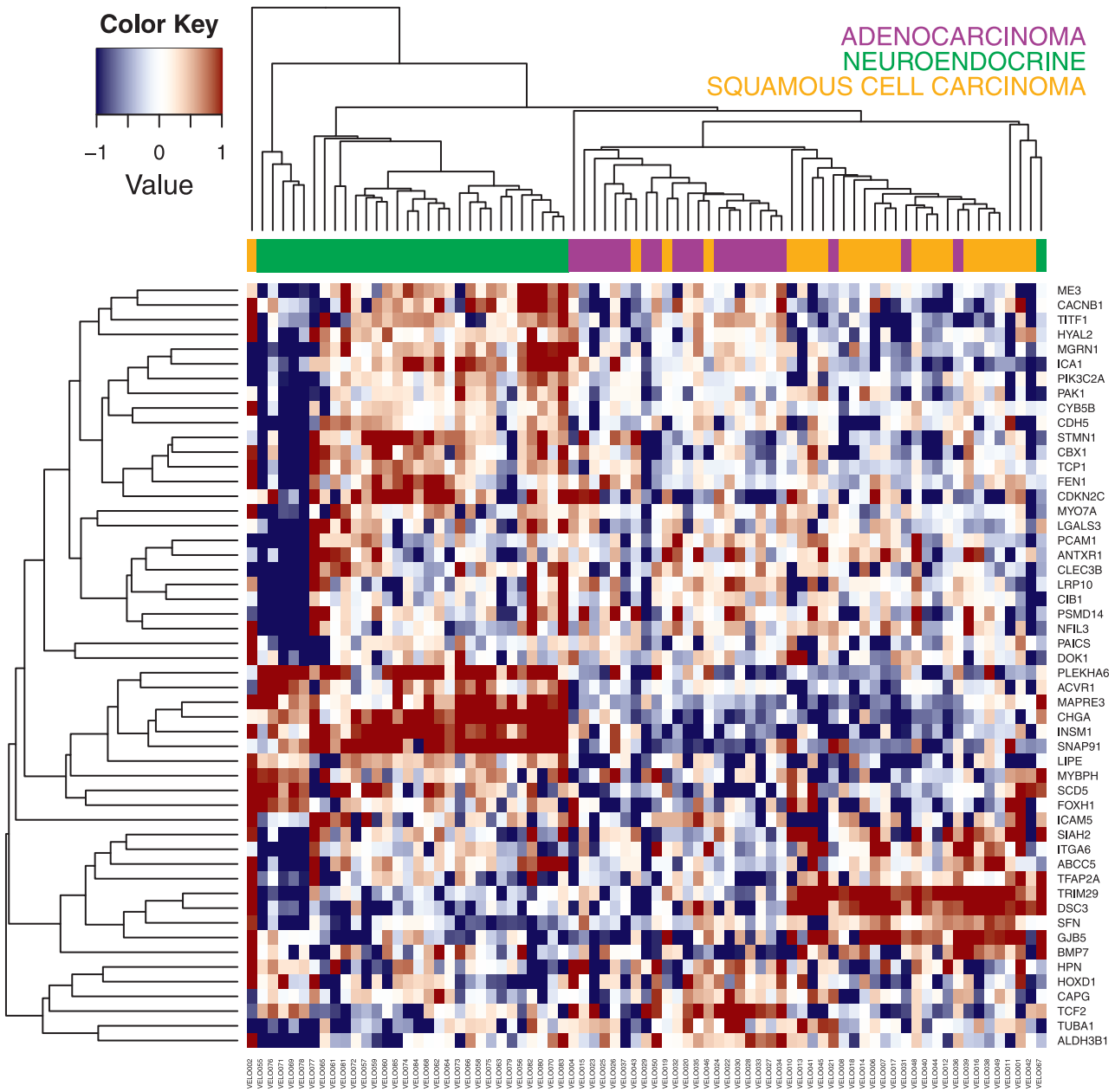

Figure 1. *Heatmap of gene expression hierarchic clustering for formalin-fixed, paraffin-embedded reverse transcription-polymerase chain reaction gene expression data set.*

the gene-specific scaling factor. Subtype was predicted by correlating each sample with the 3 subtype centroids and assignment of the subtype with the highest-correlation centroid (Spearman correlation).

RESULTS

Ten lung tumor gene-expression data sets, including 9 FF data sets plus 1 new FFPE qRT-PCR gene-expression data set, were combined into 4 platform-specific data sets (Affymetrix, Agilent, Illumina RNAseq, and qRT-PCR). For the data sets in which clinical information was available, the patient population was diverse and included smokers and nonsmokers with tumors ranging from stage 1 to stage IV. Sample characteristics and lung cancer diagnoses from the data sets used in this study are included in Table 2. After exclusion of samples without a definitive diagnosis of ACA, SQC, small cell carcinoma, or carcinoid, and exclusion of 1 FFPE sample that failed qRT-PCR analysis, the following samples were available for further data analysis: Affymetrix (n = 538), Agilent (n = 322), Illumina RNAseq (n = 951), and qRT-PCR (n = 77). Samples excluded from analysis included non–small cell lung cancer, not otherwise specified; carcinoma, not otherwise specified; large cell carcinoma; large cell NE carcinoma adenosquamous carcinoma, and normal tissue.

As a means of de novo evaluation of the new FFPE data set, we performed hierarchic clustering of LSP gene expression from the FFPE archived samples (n = 77); as expected, this analysis demonstrated 3 clusters/subtypes corresponding to ACA, SQC, and NE (Figure 1). The predetermined LSP 3-subtype centroid predictor was then applied to all 4 data sets, and results were compared with

Table 3. Percentage of Agreement and Fleiss κ Statistics Calculated for Each Data Set

Histologic Diagnosis	Prediction															
	TCGA RNAseq, n = 951				Agilent,[a] n = 322				Affymetrix,[b] n = 538				FFPE qRT-PCR, n = 77			
	ACA	NE	SQC	Sum	ACA	NE	SQC	Sum	ACA	NE	SQC	Sum	ACA	NE	SQC	Sum
ACA	419	21	28	468	131	6	37	174	248	0	16	264	13	2	6	21
NE	NA	NA	NA	NA	NA	NA	NA	NA	2	43	2	47	1	29	1	31
SQC	22	11	450	483	27	1	120	148	26	0	201	227	1	1	23	25
Sum	441	32	478	951	158	7	157	322	276	43	219	538	15	32	30	77
Agreement, No. (%)	869 (91)				251 (78)				492 (91)				65 (84)			
Fleiss κ	0.83				0.57				0.85				0.76			

Abbreviations: ACA, adenocarcinoma; NA, not available; NE, neuroendocrine; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; SQC, squamous cell carcinoma; TCGA, The Cancer Genome Atlas.

[a] Agilent 4X44K array, Agilent Technologies, Santa Clara, California.
[b] Affymetrix HG-U133+2 array, Affymetrix, Santa Clara, California.

tumor morphologic classifications. Percentage of agreement and Fleiss κ statistics were calculated for each data set (Table 3). The percentage of agreement ranged from 78% (251 of 322) to 91% (492 of 538), with a Fleiss κ range of 0.57 to 0.85.

As another means of assessing independent pathology agreement, the agreement of the blinded pathology review of the 77 FFPE lung tumors with the original morphologic diagnosis was found to be 82% (63 of 77). In 12 of 77 cases (16%), blinded slides from the 2 blocks of the same case provided conflicting results, and in 10 of 77 cases (13%), at least 1 of the blocks had a nondefinitive pathologic subtype classification of adenosquamous, large cell carcinoma, or high-grade, poorly differentiated carcinoma. Comparison of the original morphologic diagnosis, blinded pathology review, and gene-expression LSP subtype determinations for each of the 77 samples is shown in Figure 2.

For the 20 cases in which any discrepancy from the original clinical diagnosis was identified (Figure 2), slides were examined following specific IHC staining (TTF-1, p63, synaptophysin, Ki-67) to help resolve the differences. Residual slides were available for IHC staining in 17 of 20 FFPE cases (85%). The LSP results were confirmed by IHC testing in 11 of 17 cases (65%) and disagreed with IHC in 2 of 17 cases (12%), including 1 ACA and 1 SQC. In 4 of 17 cases (24%), a definitive diagnosis was not possible, despite the use of multiple IHC stains. Full details of IHC testing and discrepancy resolution are provided in Supplemental Table 5. Agreement of the LSP assay with the original and/or the revised morphology diagnosis (with incorporation of multiple IHC stains) was at least as good as the concordance between any 2 pathologists,[5–7] thus suggesting that the LSP assay performed at least as well as a trained pathologist.

COMMENT

The classification of a lung cancer into various histologic subtypes is increasingly important because therapeutic development and patient management have become more-specifically targeted to unique features of each tumor. Emerging data from multiple studies suggest that molecular profiles have the potential to improve the morphologic diagnoses of lung tumors and to better characterize tumors for therapeutic decision making.[14–16,19,20] Several recent studies now suggest that large cell carcinoma and large cell NE tumors represent a mixture of tumor types, which may actually be more similar to ACA, SQC, or small cell lung cancer, when examined by IHC and/or molecular features.[14,31,32] The value of accurately defining the tumor histologic subtype, while preserving as much tissue as possible for molecular assays, has clear therapeutic implications, as is evident in practice management guidelines and drug prescribing information in which histologic subtype dictates many of the drugs that a patient is to recieve.[2,13]

The LSP gene-expression signature has previously been shown to provide reliable classifications of lung cancer subtype.[21] In this study of many additional data sets, the LSP assay provided reliable subtype classifications, validating its performance across multiple gene-expression platforms, even when using FFPE specimens. Hierarchic clustering of the newly assayed FFPE samples demonstrated good separation among the 3 subtypes (ACA, SQC, and NE) based on expression of 52 genes. Concordance with morphology diagnosis when using the LSP centroids was greatest in the TCGA RNAseq data set (agreement, 91%; 869 of 951), possibly because of the extensive pathology review and accuracy of the histologic diagnosis associated with TCGA samples as compared with other data sets. Agreement was lowest (78%; 251 of 322) in the Agilent data set, which may have been affected by the reduced number of genes that were available for that analysis. Overall, the LSP assay displayed a higher concordance with the original morphology diagnosis than the pathology review in all data sets, except in the Agilent data set, in which only 47 genes, rather than 52, were present for the analysis.

In the FFPE samples, for which blinded pathology review was possible, results suggest that pathology calls were not always consistent in duplicate blocks from the same case nor were they necessarily consistent with the original diagnosis. In this study, IHC testing improved the pathology review concordance with the original clinical diagnosis from 63 of 77 (82%) to 66 of 77 (86%); however, 4 samples could still not be resolved, despite the use of multiple IHC stains, and 3 samples could not be resolved because of insufficient tissue material for IHC testing. The IHC testing, although helpful in some cases, was not able to universally resolve ACA versus SQC cases. Studies have shown that 30% of ACA samples may be immunoreactive to p63 and 10% of ACA may not be immunoreactive to TTF-1.[8] These findings highlight the challenges of lung cancer classification and are consistent with several other publications documenting the variability in lung tumor histologic morphology–based diagnoses,[5–7,33] as well as the limits of IHC staining.[8,11,12]

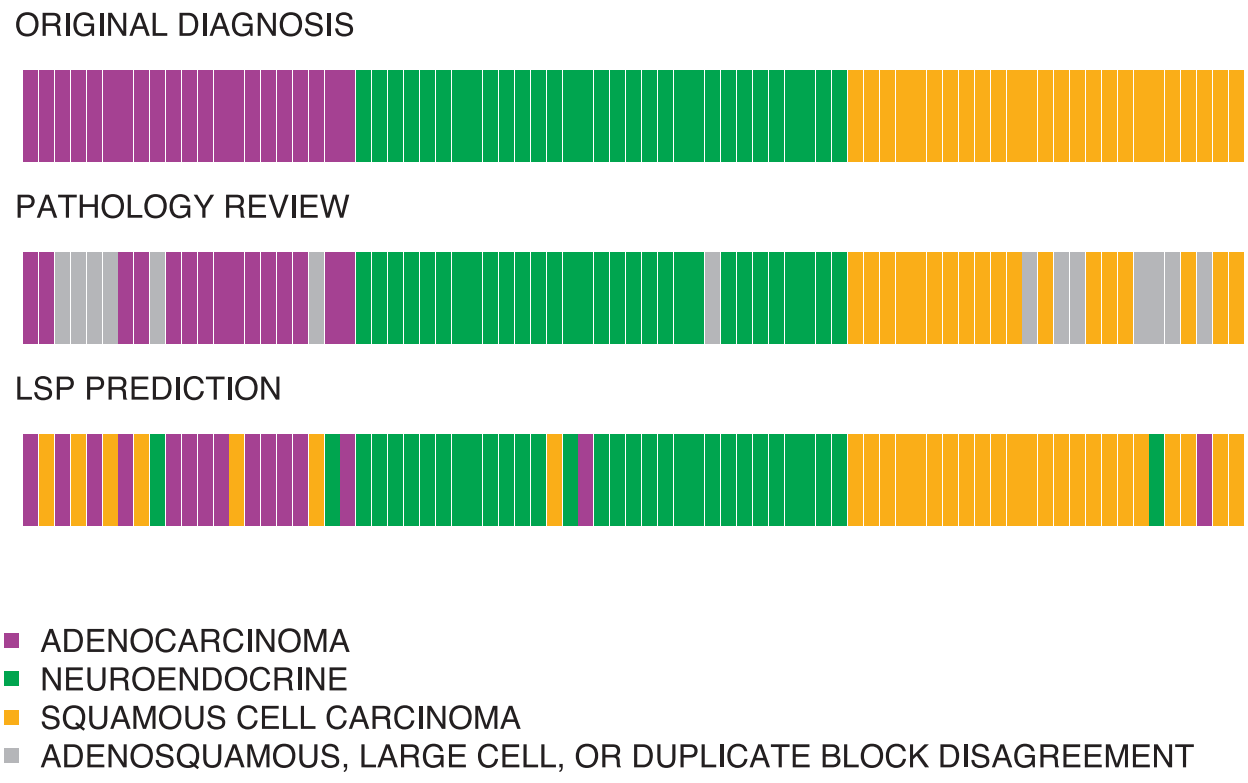

Figure 2. *Comparison of path review and lung subtype panel (LSP) prediction for 77 formalin-fixed, paraffin-embedded samples. Each rectangle represents a single sample ordered by sample number.*

This study was limited by a number of features, including a low number of NE tumor samples in the Affymetrix data set and the absence of NE samples in both the Agilent and TCGA data sets. This limitation was partially overcome by a relatively high number of NE samples in the FFPE sample set (31 of 77; 40%), thus providing a good test of the ability of the LSP gene signature to identify NE samples. Another limitation of the study relates to the blinded pathology rereview. The blinded pathology review was based on single-imaged sections from 2 separate blocks of a single case and did not reflect the usual histology standard practice in which multiple sections or blocks and potentially multiple IHC stains would have been available to make a single diagnosis. The addition of IHC testing to resolve the discrepancies was helpful in addressing the deficiency in this study, but it also demonstrated some the limits of IHC testing in this setting. Final results suggest that our level of pathology agreement was very similar to several past studies[5–7] and was only marginally improved with IHC testing. A recent example involving expert pathology rereview of lung cancer samples submitted to the TCGA Lung Cancer Genome Project led to the reclassification of 16% to 20% (SQC, 35 of 213; ACA, 59 of 289 ACA) of lung tumors submitted,[15,16] confirming similar levels of disagreement and the ongoing challenge of morphology-based diagnoses.

Expanded use of molecular testing in characterizing lung tumors is inevitable, and we feel, is desirable because genomics drives biologically based classifications, which lead to improved therapeutics and more-personalized oncology treatment plans. Despite the limitations of this study, molecular profiling using the LSP assay was validated in more than 2000 samples from multiple data sets, from multiple institutions, and from multiple technology platforms. Molecular profiling compared favorably to light-microscopy–derived diagnoses and showed at least as high a level of agreement with the original diagnosis as did the pathologist reassessments. Gene expression–based tumor subtyping can provide valuable information in the clinic and may be especially helpful when tissue is limited, IHC results are inconclusive, and/or when the morphologic diagnosis remains unclear. This study was primarily restricted to samples from surgical resections in which sufficient residual tissue and a definitive diagnosis was available for analysis. Ongoing initiatives are now specifically focusing on the more difficult task of classifying samples, including small-volume biopsies and/or cytology samples. Independent of the tissue preservation or the gene expression method used, the LSP panel of biologically relevant genes provided a reliable adjunct to standard histopathology.

We thank the many patients who donated their tissues for medical research, both those coming from the new 78-sample University of North Carolina set and for the other 2099-sample data in the public domain.

References

1. American Cancer Society. Cancer facts and figures, 2014. http://www.cancer.org/research/cancerfactsstatistics/cancerfactsfigures2014/index. Accessed July 12, 2015

2. [NCCN] National Comprehensive Cancer Network. *Clinical Practice Guideline in Oncology, Non–Small Cell Lung Cancer*. Version 1. Fort Washington, PA: NCCN; 2015.

3. Genentech. Avastin (bevacizumab) prescribing information. http://www.avastin-hcp.com/indications/nsclc. Accessed July 12, 2015.

4. Eli Lilly. Alimta (pemetrexed disodium) prescribing information. http://www.alimta.com/treatment-for-advanced-nonsquamous-NSCLC.html. Accessed July 12, 2015.

5. Grilley-Olson JE, Hayes DN, Moore DT, et al. Validation of interobserver agreement in lung cancer assessment: hematoxylin-eosin diagnostic reproduc-

ibility for non–small cell lung cancer—the 2004 World Health Organization classification and therapeutically relevant subsets. *Arch Pathol Lab Med*. 2013; 137(1):32–40.

6. Thunnissen E, Boers E, Heideman DA, et al. Correlation of immunohistochemical staining p63 and TTF-1 with EGFR and K-ras mutational spectrum and diagnostic reproducibility in non small cell lung carcinoma. *Virchows Arch*. 2012;461(6):629–638.

7. Thunnissen E, Beasley MB, Borczuk AC, et al. Reproducibility of histopathological subtypes and invasion in pulmonary adenocarcinoma: an international interobserver study. *Mod Pathol*. 2012;25(12):1574–1583.

8. Rekhtman N, Ang DC, Sima CS, Travis WD, Moreira AL. Immunohistochemical algorithm for differentiation of lung adenocarcinoma and squamous cell carcinoma based on large series of whole-tissue sections with validation in small specimens. *Mod Pathol*. 2011;24(10):1348–1359.

9. Travis WD, Brambilla E, Riley GJ. New pathologic classification of lung cancer: relevance for clinical practice and clinical trials. *J Clin Oncol*. 2013; 31(8):992–1001.

10. Thunnissen E, Noguchi M, Aisner S, et al. Reproducibility of histopathological diagnosis in poorly differentiated NSCLC: an international multiobserver study. *J Thorac Oncol* 2014;9(9):1354–1362.

11. Travis WD, Rekhtman N. Pathological diagnosis and classification of lung cancer in small biopsies and cytology: strategic management of tissue for molecular testing. *Semin Respir Crit Care Med*. 2011;32(1):22–31.

12. Travis WD, Brambilla E, Noguchi M, et al. Diagnosis of lung adenocarcinoma in small biopsies and cytology: implications of the 2011 International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society classification. *Arch Pathol Lab Med*. 2013;137(5):668–684.

13. Tang ER, Schreiner AM, Pua BB. Advances in lung adenocarcinoma classification: a summary of the new international multidisciplinary classification system (IASLC/ATS/ERS). *J Thorac Dis*. 2014;6(suppl 5):S489–S501.

14. [CLCGP and NGM] Clinical Lung Cancer Genome Project and Network Genomic Medicine. A genomics-based classification of human lung tumors. *Sci Transl Med*. 2013;5(209):209ra153; doi: 10.1126/scitranslmed.3006802.

15. [TCGA] The Cancer Genome Atlas Research Network. Comprehensive genomic characterization of squamous cell lung cancers [published correction appears in *Nature*. 2012;491(7423):288]. *Nature*. 2012;489(7417):519–525.

16. [TCGA] The Cancer Genome Atlas Research Network. Comprehensive molecular profiling of lung adenocarcinoma [published correction appears in *Nature*. 2014;514(7521):262]. *Nature*. 2014;511(7511):543–550.

17. Hayes DN, Monti S, Parmigiani G, et al. Gene expression profiling reveals reproducible human lung adenocarcinoma subtypes in multiple independent patient cohorts. *J Clin Oncol*. 2006;24(31):5079–5090.

18. Director's Challenge Consortium for the Molecular Classification of Lung Adenocarcinoma, Shedden K, Taylor JM, et al. Gene expression–based survival prediction in lung adenocarcinoma: a multi-site, blinded validation study. *Nat Med*. 2008;14(8):822–827.

19. Wilkerson, MD, Yin X, Hoadley KA, et al. Lung squamous cell carcinoma mRNA expression subtypes are reproducible, clinically important, and correspond to normal cell types. *Clin Cancer Res*. 2010;16(19):4864–4875.

20. Wilkerson M, Yin X, Walter V, et al. Differential pathogenesis of lung adenocarcinoma subtypes involving sequence mutations, copy number, chromosomal instability, and methylation. *PLoS One* 2012;7(5):e36530. doi: 10.1371/journal.pone.0036530.

21. Wilkerson MD, Schallheim JM, Hayes DN, et al. Prediction of lung cancer histological types by RT-qPCR gene expression in FFPE specimens. *J Mol Diagn*. 2013;15(4):485–497.

22. Roepman P, Jassem J, Smit EF, et al. An immune response enriched 72-gene prognostic profile for early-stage non–small-cell lung cancer. *Clin Cancer Res*. 2009;15(1):284–290.

23. Lee ES, Son DS, Kim SH, et al. Prediction of recurrence-free survival in postoperative non–small cell lung cancer patients by using an integrated model of clinical information and gene expression. *Clin Cancer Res*. 2008;14(22):7397–7404.

24. [IGC] International Genomics Consortium. The Expression Project for Oncology http://www.intgen.org/research-services/biobanking-experience/expo/. Accessed July 11, 2015.

25. Rousseaux S, Debernardi A, Jacquiau B, et al. Ectopic activation of germline and placental genes identifies aggressive metastasis-prone lung cancers. *Sci Transl Med*. 2013;5(186):186ra66. doi: 10.1126/scitranslmed.3005723

26. Bild AH, Yao G, Chang JT, et al. Oncogenic pathway signatures in human cancers as a guide to targeted therapies. *Nature*. 2006;439(7074):353–357.

27. Faruki H, Miglarese M, Mayhew G, et al. Validation of a RT-PCR gene expression assay for subtyping lung tumor samples. Poster presented at: the Association of Molecular Pathology Annual Meeting; November 12–15, 2014; Baltimore, MD. Abstract 4222.

28. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics*. 2011;12:323. doi: 10.1186/1471-2105-12-323

29. Yang YH, Dudoit S, Luu P, et al. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. *Nucleic Acids Res*. 2002;30(4):e15.

30. Hubbell E, Liu W, Mei R. Robust estimators for expression analysis. *Bioinformatics*. 2002;18(12):1585–1592.

31. Rekhtman N, Tafe LJ, Chaft JE, et al. Distinct profile of driver mutations and clinical features in immunomarker-defined subsets of pulmonary large-cell carcinoma. *Mod Pathol*. 2013;26(4):511–522.

32. Rossi G, Mengoli MC, Cavazza A, et al. Large cell carcinoma of the lung: clinically oriented classification integrating immunohistochemistry and molecular biology. *Virchows Arch*. 2014;464(1):61–68.

33. Travis WD, Brambilla E, Noguchi M, et al. International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society international multidisciplinary classification of lung adenocarcinoma. *J Thorac Oncol*. 2011;6(2):244–285.

cap ARCHIVES
of Pathology & Laboratory Medicine

EARLY ONLINE RELEASE

Note: This article was posted on the *Archives* Web site as an Early Online Release. Early Online Release articles have been peer reviewed, copyedited, and reviewed by the authors. Additional changes or corrections may appear in these articles when they appear in a future print issue of the *Archives*. Early Online Release articles are citable by using the Digital Object Identifier (DOI), a unique number given to every article. The DOI will typically appear at the end of the abstract.

The DOI for this manuscript is doi: 10.5858/arpa.2015-0113-OA

The final published version of this manuscript will replace the Early Online Release version at the above DOI once it is available.