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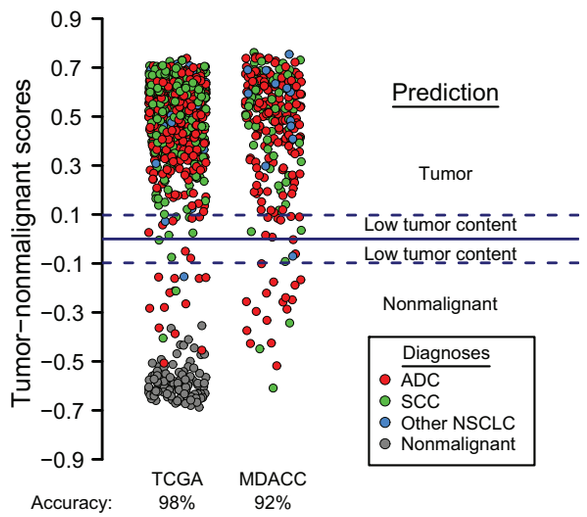

Figure 4.

The tumor–nonmalignant signature is validated on the TCGA dataset, which consists of 979 lung tumors and 108 nonmalignant lung tissues. The prediction accuracies are 98% for tumors and 100% for nonmalignant lung (overall: 98%, Table 1). Many tumor samples have negative scores which could be due to larger stromal infiltration. A score plot for the MDACC dataset, which has tumor samples only, shows an accuracy of 92% (Table 1).

known genes with different mutational spectra in ADCs and SCCs to see whether their mutation (or amplification) was associated with a higher score. For ADC, we indeed observed significantly higher scores for cases with mutations in *EGFR*, *CTNNB1*, *HER2*, *BRAF*, or *KRAS* (Supplementary Fig. S3B). For SCC, we found significant differences for *SOX2* amplification and for *NFE2L2* or *PIK3CA* mutations (Supplementary Fig. S3C). Hence, lung tumors with histology-specific mutations (which in some cases may be more differentiated) tended to have higher ADC-SCC scores.

Finally, one of us (AFG) looked at a random subset of the TCGA pathology slides and graded them using a modification of the standard grading system (23). Specifically, we selected 50 ADC slides and 50 SCC slides randomly but with a uniform score distribution, and their degree of differentiation (grading) was assessed in a blinded fashion, that is, without knowledge of their prediction score. Grading was then compared with prediction scores using two statistical tests. Significant associations with ADC and SCC scores were found ($P < 0.03$ for ADC and $P < 10^{-4}$ for SCC; Supplementary Fig. S4). Together, these data strongly suggest that the signature scores are correlated with the degree of differentiation.

ADC-SCC score and prognosis prediction

As tumor grading is also correlated with patient survival (high grade tumors, i.e. poorly differentiated tumors, having worse prognosis; refs. 23, 24), we hypothesized that a similar relationship between ADC-SCC score and survival would exist. We therefore looked at three cohorts with available clinical information: the MDACC set (non-neoadjuvant cases only: 145 ADCs and 64 SCCs), the Director's Challenge set (ADC only; $n = 423$; ref. 25), and a SCC study (GSE4573; SCC only; $n = 106$; ref. 26). In most of these cases, high ADC or SCC scores were indeed associated with better prognosis (Supplementary Fig. S5).

Application of the classifier to FFPE-resected tumor specimens and small biopsies

In preliminary studies, the 62-gene signature was adapted to the HTG EdgeSeq technology (HTG Molecular Diagnostics) and was used to classify ADC from SCC and tumor from nonmalignant lung using FFPE sections from surgically resected tumors and core-needle biopsy specimens. We applied our Classifier to these data and found that 34 of 35 FFPE-resected samples (97%) were correctly classified as ADC or SCC and 32 of 36 FFPE core-needle biopsies (89%) were also correctly classified (Table 1). Interestingly, almost all discrepant cases had low scores (< 0.1 in absolute value; Supplementary Table S3) and had been independently diagnosed as poorly differentiated, thus explaining the majority of the discrepancies. In addition, the ADC-SCC score correlation between FFPE resected tumors and matched CNB samples from the same patients was $r = 0.98$, demonstrating the ability to use small, clinically relevant samples with this assay. Taken together, these results indicate that our Classifier, adapted to the HTG EdgeSeq platform, can also determine NSCLC subtype in fixed tissue, both from resected and CNB specimens.

Discussion

The advent of "precision medicine" has made accurate classification of NSCLC a necessity for the clinical management of these tumors. However, currently the majority of diagnostic specimens (~70%) are small biopsies or cytologic specimens (27), greatly increasing the difficulty of accurately diagnosing poorly differentiated tumors. On the basis of the anticipated 225,000 new cases of lung cancer for 2016 (28), of which an estimated 85% will be NSCLC, this amounts to over 130,000 NSCLC cases per year in the United States that will be diagnosed from small biopsies or cytology specimens. Cases without definitive diagnoses, and those wrongly classified, may not receive optimal therapy or may not be eligible for histology classification–restricted clinical trials. While the use of small panels of immunostains has greatly aided this task, about 5%–10% of small biopsy cases at major medical centers will still be signed out as NSCLC-NOS. Examination of the SEER data registry suggests that the high diagnostic standards present at major medical centers may not extend to the medical community as a whole. Thus, 14% of the lung cancer cases in the SEER registry were not further classified, amounting to an estimated 22,000 cases per year in the United States. In addition, an unknown percentage of cases will be misclassified or subject to arbitrary diagnosis by pathologists using varying pathologic criteria or interpretation. A recent European interobserver study examined the diagnostic accuracy on lung cancer small biopsies for the distinction between ADC and SCC and related these to immunostaining and mutation analysis (29). The study was performed on prospectively collected biopsies obtained by bronchoscopy or transthoracic needle biopsy of patients with NSCLC. Eleven experienced pulmonary pathologists independently read H&E-stained slides of 110 cases, resulting in a kappa (κ) value of 0.55 ± 0.10 and the diagnosis of NSCLC-NOS was given on average to 29.5% of the biopsies. This indicates that even experienced pathologists at major medical centers may disagree on interpretation or may not be able to fully classify a relatively high percentage of small biopsy specimens without the use of immunostains or other adjunct tests.

The widespread use of immunostains for the classification of NSCLC has greatly reduced the number of cases in the NOS

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category (30) and most of these tumors can now be classified with a single SCC and a single ADC marker (1). These findings led the new WHO Classification to recommend using immunostaining for SCC markers such as TP63 or its isoform p40 (deltaNp63) and high molecular weight keratins as well as ADC markers such as NKX2-1 (TTF-1) and Napsin A to classify poorly differentiated lung cancers including NSCLC-NOS (1, 31). However, interpretation of immunostains is not uniform and alternative approaches to lung cancer classification are being explored as adjunct tools to aid the pathologic diagnosis of lung cancers. These methods include digital nuclear imaging, mutation analysis, copy number variations, and various other molecular methods, either singly or in combination (32–34).

In this report, we developed and validated a gene expression classifier from a training set consisting of 263 surgically resected tumors to accurately and nonsubjectively separate ADC from SCC. The list of top differentially expressed genes heavily favored SCC, possibly reflecting the greater pathologic heterogeneity and molecular complexity of ADCs and their multiple subtypes (3, 11). Thus, we selected an equal number of top genes significantly overexpressed in ADCs ($n = 21$) and SCCs ($n = 21$) so as not to bias the selection in favor of one NSCLC type. Not surprisingly, many of the selected genes are among the most frequently used and reliable immunostains in routine pathologic practice (Fig. 1B, red arrows) or are known to play a role in lung cancer or in one of the major subtypes (Fig. 1B, blue labels). We validated the Classifier using the TCGA lung cancer datasets, which were available on a different platform (RNAseq) than our training set (Illumina BeadArray). We obtained very high prediction accuracies (95%) in spite of the fact that a fraction of the TCGA diagnostic materials were found to be of less than optimal quality (e.g. frozen sections instead of permanently fixed H&E slides) and in spite of the partially subjective nature of pathologic diagnosis (29). In fact, a significant limitation to the TCGA project was that the materials for immunostaining were not always available. Nevertheless, N. Rekhtman and W.D. Travis, who are the TCGA reference pathologists, reviewed the discrepancies, and this resulted in even better classification accuracy (Table 1, "Revised histopathologic diagnosis").

Interestingly, several nonmalignant lung TCGA specimens were classified as ADC by the signature, so we used the EDRN/Canary dataset to develop another classifier, containing 20 genes, that separated tumor cells from nonmalignant lung with high accuracy. The combined 62-gene signature could now segregate ADC, SCC, and nonmalignant lung in this TCGA test set.

There are no squamous cells in the normal lung. Squamous metaplasia arises as the result of noxious stimuli such as tobacco exposure, mechanical trauma, inflammation, or infection. Many of the SCC-associated classifier genes are involved in squamous differentiation, including basal (stem) cell proliferation, expression of high molecular weight keratins, desmosome formation, calcium regulation or cornified envelope formation (35–37). ADCs demonstrate considerable heterogeneity of morphologic and biologic subtypes (31). However, most of the ADC genes had relevance to lung cancer or were known to be ADC-specific. Both *NKX2-1* and *NAPSA* are routinely used in many pathology classification schemes; however the latter, with a rank of 177, was not part of the top 21 genes overexpressed in the ADC group, and was not used in the Classifier. Our data indicate that other genes in the Classifier, such as the trypsin inhibitor *SPINK1* which is already known to be overexpressed in lung ADCs (38), may represent good candidates for new immunostains in pathologic diagnosis, provided sensitive and specific antibodies are available. For SCC identification, the Classifier selected several high molecular weight *KRTs* as well as *TP63* among the top genes, but excluded *SOX2*, a gene frequently amplified in SCCs, although it was also significantly overexpressed in SCC (rank = 46; refs. 9, 39, 40).

The Classifier can also provide a score that reflects the degree of differentiation. In support of this, we observed that NSCLCs with mutations that are specific for ADCs (*EGFR*, *KRAS*, and others) or SCCs (*SOX2* amplification, *NFE2L2* mutation) tended to have higher magnitude scores than tumors that were wild-type for these mutations or amplifications (Supplementary Fig. S3B and S3C). In addition, the lepidic subtype of ADCs which is believed to be more differentiated also had a relatively higher score (Supplementary Fig. S3A). Finally, evaluation of tumor grade from TCGA histopathology slides revealed a strong concordance between prediction score and histologic grading (high score was associated with better differentiation). Thus, our Classifier can be interpreted both qualitatively and quantitatively.

Consistent with the association between histologic grading and survival, our signature turned out to have prognostic value as well (high scores being associated with better survival). Thus, this Classifier has the additional advantage of being of prognostic importance and may be useful in selecting the subpopulation of curative resected lung cancer patients that will benefit from adjuvant therapy.

Previous ADC-SCC gene signatures have been reported (41–46) and about 10%–45% of the genes in these signatures overlap with ours. Two of these signatures were formally developed as classifiers, with external tumor set validation. The first, from Hou and colleagues (44), comprises 50 unique genes (15 of which overlapped with our signature) and were validated in one external dataset with a prediction accuracy of 84%. To directly compare this classifier with our own, we tested it in TCGA RNAseq data using the class centroids provided by the study and Pearson correlation to predict the class. The resulting prediction had an accuracy of 92% (sensitivity, 99%; specificity, 84%) while our Classifier showed 95% accuracy (97% sensitivity, 93% specificity). The second study, from Wilkerson and colleagues (46), had 15 genes (4 overlapping with our signature) and a reported prediction accuracy of 81% in external validation. Using the TCGA validation test, this corresponded to an accuracy of 92% (sensitivity: 90%, specificity 95%). Our current study thus offers the following advantages over prior ones: (i) a slightly better overall accuracy; (ii) a balance between sensitivity and specificity; (iii) the ability to distinguish nonmalignant from lung cancer; (iv) validation in a larger number of public NSCLC expression datasets with high prediction accuracies (93% for the ADC-SCC classification); (v) the quantitative aspect of our Classifier and its correlation with differentiation and prognosis (this point also supports removing the term LCC and replacing it with poorly differentiated NSCLC); (vi) the ability, as mentioned below, to classify small biopsy samples and FFPE materials, using technology that can be transferred to a CLIA-certified environment.

While some pathologists may question the necessity for a molecular classification of NSCLC, the large number of nonclassified cases, and the potential lack of diagnostic reproducibility even among experienced lung cancer pathologists, point to the value of a nonsubjective test. This may even be a necessity in institutions or countries where immunostains are not routinely used and where staff pathologists may apply highly variable

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diagnostic criteria. An especially relevant use of a molecular classification would be for large multinational clinical trials where no central pathology review is available. We will also need further evaluation in a set of cases that have been diagnosed utilizing established immunohistochemical methods recommended by the 2015 WHO Classification. Unfortunately, these new criteria could not be applied to the datasets evaluated in this study.

Finally, to demonstrate the potential clinical applicability of our Classifier, we have developed an extraction-free, highly sensitive, automated, and cost-effective NGS version based on the HTG EdgeSeq technology and have shown that its accuracy is similar to the original microarray-based Classifier (Table 1). In fact, the NGS classifier can be reproducibly applied to commonly available clinical specimens, including FFPE materials and core-needle biopsies.

In summary, we have developed and validated a sensitive and specific gene expression classifier for NSCLC that distinguishes ADC from SCC, and lung cancer from normal lung. The Classifier was shown to be largely independent of the major gene expression platforms in common usage. Most of the genes in the Classifier are relevant to lung cancer or are known to be differentially expressed in NSCLC. The development and further validation of a practical and cost effective FFPE-based CLIA-certified version has the potential to lead to a widespread clinical application of the Classifier.

Disclosure of Potential Conflicts of Interest

D.M. Thompson and I.W. Botros have ownership interest (including patents) in HTG Molecular Diagnostics. No potential conflicts of interest were disclosed by the other authors.

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