Micron 67 (2014) 125–131

Contents lists available at ScienceDirect

Micron

journal homepage: www.elsevier.com/locate/micron

# Digital differentiation of non-small cell carcinomas of the lung by the fractal dimension of their epithelial architecture

Lik Hang Lee[a], Mauro Tambasco[b,c,1], Shannon Otsuka[c], Allison Wright[c], Alexander Klimowicz[d], Stephanie Petrillo[d], Don Morris[c], Anthony Magliocco[a,b,2], D. Gwyn Bebb[c,*]

[a] Department of Pathology and Laboratory Medicine, University of Calgary, 1403 29 Street NW, Calgary, AB, Canada T2N 2T9
[b] Department of Physics and Astronomy, University of Calgary, 2500 University Drive NW, Calgary, AB, Canada T2N 1N4
[c] Department of Oncology, University of Calgary and Tom Baker Cancer Centre, 1331 29 Street NW, Calgary, AB, Canada T2 N 4N2
[d] Functional Tissue Imaging Unit, Translational Research Laboratory, Tom Baker Cancer Centre, 1331 29 Street NW, Calgary, AB, Canada T2 N 4N2

ARTICLE INFO

*Article history:*
Received 13 April 2014
Received in revised form 14 June 2014
Accepted 21 July 2014
Available online 28 July 2014

*Keywords:*
Digital microscopy
Digital imaging
Histology
Fractal analysis
Lung
Carcinoma

ABSTRACT

*Introduction:* In recent years, differences have emerged in the treatment of squamous and non-squamous non-small cell lung carcinomas (NSCLCs). This highlights the importance of accurate histopathologic classification. However, there remains inter-observer disagreement when making diagnoses based on histology. Fractal dimension (FD) is a mathematical measure of irregularity and complexity of shape. We hypothesize that the FD of carcinoma epithelial architecture can assist in differentiating adenocarcinoma (ADC) from squamous cell carcinoma (SCC) of the lung.
*Methods:* 134 resected (88 ADC and 46 SCC) cases of resected early-stage NSCLC were analyzed. Tissue micro arrays were generated from formalin-fixed paraffin-embedded tissue, stained with pan-cytokeratin, and digitally imaged and the FD of the epithelial structure calculated. Mean FD of ADC and SCC were compared using the independent *t*-test, partial correlations, and receiver operating characteristic (ROC) analyses.
*Results:* A statistically significant difference ($p < 0.001$) between the mean FD of ADC ($M = 1.70$, $SD = 0.07$) and SCC ($M = 1.78$, $SD = 0.07$) was found. Significance remained ($p < 0.001$) when controlling for several possible confounders. ROC analysis demonstrated an area-under-the-curve of 0.81 ($p < 0.001$).
*Conclusions:* The epithelial structure FD of NSCLC has potential as a reproducible and automated measure to help subtype NSCLCs into ADC and SCC. With further image analysis algorithm improvements, fractal analysis may be a component in computerized histomorphological assessments of lung cancer and may provide an adjunct test in differentiating NSCLCs.



## 1. Introduction

Non-small cell lung cancer (NSCLC) continues to be the leading cause of cancer related deaths globally, with the majority of patients presenting with advanced stage disease. While the division of pulmonary malignancies into small cell and non-small cell carcinoma was historically done for management purposes, there has until recently been no histologically based difference in management of NSCLC subtypes. However, the last few years have seen the emergence of level I evidence challenging that paradigm placing new emphasis on accurate histological subtyping.

Trials of the antiangiogenic agent Bevacizumab in combination with standard chemotherapy first showed a histologically based difference in adverse events. The unexpected finding of catastrophic hemoptyses in the early trials was linked to

*Abbreviations:* ADC, adenocarcinoma; ALK, anaplastic lymphoma kinase; AUC, area-under-the-curve; DAPI, 4′,6-diamidino-2-phenylindole; EGFR, epidermal growth factor; FD, fractal dimension; H&E, hematoxylin and eosin; HR, hazard ratio; IRQ, interquartile range; *M*, mean; NSCLC, non-small cell lung carcinoma; ROC, receiver operating characteristic; SCC, squamous cell carcinoma; SD, standard deviation; SE, standard error; TMA, tissue micro array.

* Corresponding author. Tel.: +1 403 521 3166; fax: +1 403 283 1651.
*E-mail address:* Gwyn.Bebb@albertahealthservices.ca (D.G. Bebb).
[1] Present address: Department of Physics, San Diego State University, 5500 Campanile Drive, San Diego, CA 92182-1233, USA.
[2] Present address: Department of Anatomic Pathology, H Lee Moffitt Cancer Center and Research Institute, 12902 Magnolia Drive, Tampa, FL 33612, USA.

http://dx.doi.org/10.1016/j.micron.2014.07.005
0968-4328/

squamous histology so that subsequent accrual in the Phase III trial was limited to patients with non-squamous cancers (Sandler et al., 2006). A difference in efficacy between squamous and non-squamous NSCLCs was clearly demonstrated in a phase III study comparing cisplatin/pemetrexed to cisplatin/gemcitabine in the treatment of advanced/metastatic NSCLC. Patients with adenocarcinoma and large-cell histology showed significantly higher overall survival when treated with cisplatin/pemetrexed (adenocarcinoma: $n = 847$, 12.6 vs. 10.9 months, large-cell: $n = 153$; 10.4 vs. 6.7 months). Pointedly, patients with squamous cell histology showed improved survival with cisplatin/gemcitabine ($n = 473$; 10.8 months vs. 9.4 months) (Scagliotti et al., 2009a, 2008).

Meanwhile, improved knowledge of molecular signaling pathways has led to the development of biomarkers and targeted therapies. There is now convincing data that targeted treatments improve overall survival in selected populations. The small molecule epidermal growth factor (EGFR) inhibitors, gefitinib and erlotinib now have distinct roles in patient treatment (Cappuzzo et al., 2010; Mok et al., 2009; Rosell et al., 2012; Zhou et al., 2011). Likewise, a growing body of knowledge has confirmed a role for anaplastic lymphoma kinase (ALK) inhibitors in ALK positive cases. The ALK inhibitor crizotinib prolonged progression-free survival to 7.7 months compared to 3.0 months in patients receiving standard chemotherapy (HR 0.49; 95% CI 0.37–0.64; $p < 0.0001$) in a recent Phase III trial (Kwak et al., 2010). EGFR and ALK mutations occur almost exclusively in adenocarcinomas and very rarely in squamous cell carcinomas. The availability of targeted agents for patients, whose tumors bear these mutations, highlights the absolute importance of accurate histopathologic classification (Giaccone and Rodriguez, 2005; Shaw et al., 2009).

Given the therapeutic implications, reliable and accurate histological subtyping of NSCLC has assumed a new importance. This in turn has created new challenges. Minimally invasive diagnostic procedures are increasingly common while the information we expect from tissue samples is increasing. Multiple immunohistochemistry stains may be required to differentiate histology; however, it is now suggested that immunohistochemistry staining be minimized to preserve tissue for molecular analysis (Travis et al., 2011). The need to develop more objective techniques to distinguish histologic subtypes of NSCLC is therefore greater than ever.

A method to quantify the irregularity and complexity of the cells and the histological architecture may provide the reproducible objectivity needed to help classify NSCLC tumors. One mathematical concept that lends itself to this task is the fractal dimension (FD). FD is a mathematical measure of detail contained in a structure at different scales. It is particularly useful in nature, since many natural structures are irregular in shape and detail, making FD an ideal descriptor for biological structures, such as histological architecture (Tambasco et al., 2009). Several studies have shown that it can be used to describe complex pathological structures including colon, prostate and breast cancer, and can be used for diagnosis, staging and prognosis (Baish and Jain, 2000; Braverman and Tambasco, 2013; Cross, 1997, 1994; Cross et al., 1994; Esgiar et al., 2002; Tabesh et al., 2007; Tambasco and Magliocco, 2008; Tambasco et al., 2010, 2009). However, it should be noted that actual fractals do not exist in nature because there is a fundamental limitation to the scaling behavior of natural objects. Even real image renderings of mathematical fractals cannot be truly fractal because of the finite resolution of the rendering (Braverman and Tambasco, 2013). Hence, as in previous studies, the concept of FD in this study refers to the complexity of an object over a very finite range of scales. As such, in this study FD represents a pragmatic tool to extract clinically meaningful information, and it does not imply that the objects in question are actually fractal in nature.

We hypothesize that the complexity of the epithelial architecture, mathematically quantified by the FD over a finite range of scales, differs between NSCLC subtypes. There have been few previous studies performed to computationally differentiate lung cancer subtypes (Bigras et al., 1996). The objective of this preliminary assessment is to evaluate the potential of computing the architectural complexity of NSCLCs to differentiate adenocarcinoma (ADC) from squamous cell carcinoma (SCC) in resected specimens.

## 2. Methods and materials

### 2.1. Sample selection and preparation

Patients with resected NSCLC tumors from the years 2003 to 2006 were identified from the Glans-Look Lung Cancer Database at the Tom Baker Cancer Centre. This included all patients diagnosed during this period within the provincially legislated health region. Early stage resections that had sufficient formalin-fixed, paraffin embedded tissue specimens available were assessed and re-diagnosed by a pathologist. All cases of ADC and SCC were selected. Tumor areas for tissue micro array (TMA) were identified by a pathologist from whole sections stained with hematoxylin and eosin (H&E). Three 0.6 mm cores per patient were chosen randomly from tumor areas, to reduce selection bias. They were assembled in triplicate into each TMA which contained 40–50 patients using a Beecher Manual Tissue Microarrayer (Beecher Instruments Inc., Sun Prairie, WI).

Four-micrometers thick sections were cut from each TMA block, deparaffinized in xylene, rinsed in ethanol, and rehydrated. These were then stained with 1:500 dilution of anti-pan-cytokeratin mouse monoclonal antibody (Dako, Glostrup, Denmark). Secondary antibodies were applied for 60 min at room temperature: a goat anti-rabbit antibody conjugated to a horseradish peroxidase1-decorated dextran polymer backbone from the DAKO EnVision TM + system (Dako) with a 1:200 dilution of Alexa-555 conjugated goat anti-mouse antibody Pan-cytokeratin was chosen in order to highlight the epithelial structure of the carcinoma and exclude other cells such as connective tissues.

### 2.2. Image acquisition

The TMAs were digitally imaged using fluorescence-based quantitative immunohistochemistry on the HistoRx PM-2000 automated image acquisition platform (HistoRx, Inc., Branford, Connecticut) at 20× magnification. High-resolution monochromatic 8-bit digital images (resulting in 256 discrete intensity values per pixel of an acquired image) were obtained for every histospot on the TMAs using filters specific for DAPI to define the nuclear compartment and Cy3 to define cytokeratin-positive NSCLC cells and the tumor cytosolic compartment. Pixels were written to image files as a function of power (Power $[P]$ = [Pixel Intensity/256]/exposure time) to help compensate for experimental variations in staining intensity and saved at a resolution of $2048 \times 2048$ pixels.

### 2.3. Fractal analysis

A computer-programmed fractal analysis technique, developed in previous work (Tambasco et al., 2009), was applied to each histospot image to quantify the architectural complexity of the epithelial structures in the core. The immunofluorescent image for the image was first converted to grayscale. A series of intensity thresholds were used to convert each grayscale image into a series of binary images from which the outlines of the epithelial structures were extracted (Fig. 1). The FD of each of the resulting series of outlines generated for each image was calculated