Low-depth whole genome sequencing reveals copy number variations associated with higher pathologic grading and more aggressive subtypes of lung non-mucinous adenocarcinoma

Zheng Wang1, Lin Zhang1, Lei He1, Di Cui1, Chenglong Liu1, Liangyu Yin1, Min Zhang2, Lei Jiang2, Yuyan Gong3, Wang Wu3, Bi Liu3, Xiaoyu Li3, David S Cram3, Dongge Liu1

1Department of Pathology, Beijing Hospital, National Center of Gerontology, Beijing 100730, China; 2Department of Radiology, Beijing Hospital, National Center of Gerontology, Beijing 100730, China; 3Berry Genomics Corporation, Beijing 102206, China

Correspondence to: Dongge Liu. Department of Pathology, Beijing Hospital, Beijing 100730, China. Email: 13661275182@163.com; David S Cram. Berry Genomics Corporation, Beijing 102206, China. Email: david.cram@berrygenomics.com.

Abstract

Objective: Histology grade, subtypes and TNM stage of lung adenocarcinomas are useful predictors of prognosis and survival. The aim of the study was to investigate the relationship between chromosomal instability, morphological subtypes and the grading system used in lung non-mucinous adenocarcinoma (LNMA).

Methods: We developed a whole genome copy number variation (WGCNV) scoring system and applied next generation sequencing to evaluate CNVs present in 91 LNMA tumor samples.

Results: Higher histological grades, aggressive subtypes and more advanced TNM staging were associated with an increased WGCNV score, particularly in CNV regions enriched for tumor suppressor genes and oncogenes. In addition, we demonstrate that 24-chromosome CNV profiling can be performed reliably from specific cell types (<100 cells) isolated by sample laser capture microdissection.

Conclusions: Our findings suggest that the WGCNV scoring system we developed may have potential value as an adjunct test for predicting the prognosis of patients diagnosed with LNMA.

Keywords: Lung adenocarcinoma; lung non-mucinous adenocarcinoma (LNMA); histological grading; TNM staging; copy number variations (CNVs); whole genome copy number variation (WGCNV) score

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Introduction

Based on recommendations from the International Association for the Study of Lung Cancer (IASLC), the American Thoracic Society (ATS), the European Respiratory Society (ERS) and the 2015 World Health Organization (WHO), novel classification concepts were introduced for lung adenocarcinoma, including adenocarcinoma in situ (AIS) and minimally invasive adenocarcinoma (MIA). Moreover, invasive adenocarcinoma (IA) is now classified as lepidic, acinar, papillary, micropapillary or solid subtypes according to their predominant growth pattern (1-3). This classification system has subsequently been shown to be important for predicting prognosis and survival (4-7). In some studies and clinical practices, a three tiered architectural grading system has been applied, with grade 1 inclusive of AIS, MIA and lepidic predominant IA patterns, grade 2 inclusive of papillary and acinar predominant IA patterns and grade 3 inclusive of micropapillary and solid predominant IA patterns (8-11).

Chromosome instability is a common cytogenetic event in the evolution of most cancers, including lung adenocarcinomas (12-15). In recent molecular studies of lung adenocarcinoma, both oncogenic mutations and copy
number variations (CNVs) were observed in the early stages of tumor development with a tendency for CNVs to increase in frequency as tumorigenesis progresses (16-19). Based on these studies and similar observations of progressive genetic changes in other organ-specific carcinomas (20,21), we hypothesize that molecular subtyping may provide additional diagnostic and prognostic value in patients with lung adenocarcinoma.

To test this hypothesis, we selected lung non-mucinous adenocarcinoma (LNMA) as a model system to evaluate the clinical utility of CNV analysis. First, we established an accurate technique for whole genomic CNV (WGCNV) detection in laser capture micro-dissected adenocarcinoma samples, and second, developed a new scoring algorithm to assess the pathological significance of these WGCNV changes. Utilizing this approach for 91 LNMA patients, we further explored correlations between pathologic grading and subtyping of adenocarcinoma cells and WGCNV scores, and then assessed the diagnostic and prognostic value of WGCNV scoring.

**Materials and methods**

**Patients**

The retrospective study (Figure 1) was approved by Beijing Hospital Ethics Committee (approval number 2020BJYXEC-065-02). Patients provided written informed consent for collection and analysis of tissue samples. Pathologic diagnostic and subtypes determination for LNMA were based on 2011 IASLC/ATS/ERS/2015 WHO criteria (22-24). In addition, the 8th edition of the American Joint Committee for TNM stage was adopted to assess cancer staging. Cases were enriched for AIS, MIA and the different subtypes of IA in surgically resected samples collected from the Department of Pathology, Beijing Hospital between January 2017 and March 2018. Malignant pleural effusion (MPE) and metastatic nodules (MN) originating from LNMA were selected as stage IV samples. Patients were excluded from the study if they had either a history of neoadjuvant therapy, other lung cancer surgeries or discrepancies of diagnosis existing among

![Figure 1](image-url)  
**Figure 1** Study design. LNMA, lung non-mucinous adenocarcinoma; AIS, adenocarcinoma in situ; MIA, minimally invasive adenocarcinoma; IA, invasive adenocarcinoma; MPE, malignant pleural effusion; MN, metastatic nodules; LCM, laser capture microdissection; WGCNV, whole genome copy number variation; WGA, whole genome amplification; seq, sequencing; TUSON, Tumor Suppressor and Oncogene.
pathologists/radiologists.

**Histological and radiological evaluation**

All pathological hematoxylin and eosin (H&E) stained slides were reviewed independently by two pulmonary pathologists (co-authors Z.W, and D.G.L). In addition, images of the enhanced chest CT scan were assessed by two chest radiologists (M.Z and L.J). The predominant histological subtype grading system was adopted in this study using a three tiered system and divided into grade 1 (AIS, MIA, lepidic predominant IA), grade 2 (acinar or papillary predominant IA) and grade 3 (solid or micropapillary predominant IA) (4,7). For all cases, pathologists and radiologists were blinded to the results of WGCNV scores.

**Laser capture microdissection (LCM)**

LCM was used to isolate pure predominant histological subtype tissue from each LNMA patient for WGCNV analysis. One H&E staining slide was selected based on the presence of the adenocarcinoma in situ components in AIS, the invasive components in MIA, a predominant histological subtype in IA and the adenocarcinoma component in MPE or MN samples. Formalin-fixed paraffin-embedded (FFPE) histological and cytological blocks corresponding to the selected H&E staining slides were then cut as 4–5 μm tissue sections (25,26). The individual sections were deposited on FrameSlides PEN-Membrane slides (Leica MicroDissect GmbH, Herborn, Germany) and H&E stained. LCM was performed with a Leica LMD 6500 (Leica Microsystems, Wetzlar, Germany). An average of 3–4 pieces containing homogeneous histological subcategory or subtype were collected by LCM into the caps of 200 μL Eppendorf tubes. As controls, 20 para-cancerous matching lung tissue samples (non-tumor lung tissue) were also randomly collected from the 91 enrolled LNMA cases.

**CNV sequencing (CNV-seq) of germline genomic DNA**

To identify background germline CNVs, CNV-seq was performed following previously published protocols (27,28). In brief, 50 ng genomic DNA extracted from peripheral blood lymphocytes was fragmented and purified using the QiAquick PCR Purification Kit (QIAGEN). After an end repair step, the sequencing adapter was ligated to the input DNA. To distinguish different libraries, index sequences were introduced by a PCR step. Next-generation sequencing (NGS) of purified libraries was performed using the Illumina HiSeq 2500 sequencer, and approximately 5 million single end 36 bp reads were obtained for CNV analysis.

**Whole genome amplification (WGA) and CNV-seq of LCM cells**

A centrifugation step was performed to ensure tissue samples collected by LCM were placed down at the bottom of the Eppendorf tubes. Samples (test and control) were subjected to WGA and sequencing library construction following previously published protocols (27,28). Next, the libraries were purified by AMPure XP beads (Beckman Coulter) and the concentration measured by Qubit 3.0 Fluorometer with Qubit dsDNA HS assay kits (Thermo Fisher Scientific, Waltham, USA). Finally, sequencing was performed using the HiSeq 2500 to obtain approximately 5 million 36 bp single end reads for CNV analysis.

**CNV calling**

The 20 batched test para-cancerous control tissues were internally compared with each other as reference using the data processing and analysis algorithms described previously (29). To analyze chromosomal abnormalities, we applied our previously reported pipeline for identifying, mapping and quantitating chromosomal aneuploidies, CNVs or mosaic variants. The copy number of different chromosomal regions was calculated using the fused lasso algorithm (30). Considering that cells of multiple genotypes may be present in one sample, a more relaxed threshold was used with copy number >2.3 marked as a duplication and a copy number <1.7 marked as a deletion.

**Determination of resolution of CNV detection from WGA templates**

To evaluate whether the WGA step affected copy number calls, we firstly collected 8 germline DNA samples harboring different known CNVs, which were previously detected by CNV-seq with the 50 ng genomic DNA input. We then performed WGA and CNV-seq from 100 pg of the corresponding genomic DNA and compared the CNVs detected by two different methods (Supplementary Figure S1). Validation experiments showed that both deletions and duplications larger than 2 Million bases (Mb) were accurately identified by CNV-seq from WGA templates,
whereas several other smaller CNVs not detected in the original genomic DNA samples were additionally detected and these were considered to be false positives. The sensitivity and specificity for detecting CNVs larger than 2 Mb was 100% (9/9) and 75% (9/12), respectively, whereas both sensitivity and specificity was 100% for detecting CNVs larger than 5 Mb. Therefore, for CNV calling, we defined the resolution of CNV detection at 5 Mb. Thus, in this study, only CNVs larger than 5 Mb in size were analyzed and scored.

**WGCNV scoring and essential gene scoring algorithm**

A scoring algorithm was developed to evaluate the cytogenetic abnormality of each sample (Supplementary Figure S2, selected example). The value was calculated by the formula below. The summed value was named “WGCNV score”.

\[ \text{WGCNV score} = \sum \frac{\text{CNV length}}{\text{Located chromosome length}} \times \left| \text{CN}_{\text{test}} - \text{CN}_{\text{ref}} \right| \]

The presence of CNVs involving tumor suppressor genes (TSGs) and oncogenes (OGs) was screened using Tumor Suppressor and Oncogene (TUSON) Explorer software. This method quantifies the likelihood that a gene within the CNV interval is a TSG or OG according to the pattern of mutational signature in tumors. Genes were predicted as TSGs or OGs based on the combined p value and q value of the selected parameters using the Liptak method (31) and these genes were scored using a transformation value equal to \(-\log(q \text{ value})\) range from 0 to 100.

**Statistical analysis**

One-way analysis of variance (ANOVA)/analysis of covariance (AN(C)OVA) was used as the statistical method to compare the means of two or more groups of samples. To further determine significant differences between every two groups of samples, we used the Tukey’s test for multiple comparisons. Chi-squared test was also used to determine whether there was a significant difference in distribution of low to high WGCNV scores between two groups. Pearson’s correlation coefficient was used to measure the association between the WGCNV score and TUSON score and statistical significance was judged by the P value of the correlation test. A receiver operating characteristic (ROC) curve was used to elucidate any diagnostic value of the WGCNV score, a binary classifier created by plotting the true positive rate (sensitivity) against the false positive rate (1–specificity) at various threshold settings. The best cut-off was set by balancing sensitivity and specificity. The area under the ROC curve (AUC) was used as a measure of how well a threshold can diagnostically distinguish between two classes of samples. All P values were based on two-tailed statistical analysis and \(P<0.05\) was considered as statistically significant. P values for all Tukey’s test and Chi-squared test comparisons in this study are shown in Supplementary Table S1. Statistical analyses were conducted using R software (Version 3.2.5; R Foundation for Statistical Computing, Vienna, Austria).

**Results**

**Clinicopathologic characteristics of LNMA patients**

A total of 91 LNMA (tumor) and 20 para-cancerous (non-tumor) lung tissue specimens were collected by LCM from 91 patients (Figure 1). Of these specimens, 76 of 91 (84%) were surgical resection samples, including 11 (12%, 11/91) AIS, 7 (8%, 7/91) MIA and 58 (64%, 58/91) IA. In addition, 13 of 91 (14%) were MPE and 2 of 91 (2%) were MN biopsy samples. The predominant histological subtypes of 58 IA included 14 lepidic, 8 acinar, 10 papillary, 10 solid and 16 micropapillary subtypes. Of the 91 patients, 11 (12%) were in stage 0, 43 (47%) in stage I, 9 (10%) in stage II, 13 (14%) in stage III and 15 (17%) in stage IV. There were approximately equal numbers of females (47, 52%) and males (44, 48%) with an average age of 62 (range, 24–81) years. All patients were of Chinese ethnicity, with 30 (33%) classified as smokers (11 former smokers and 19 current smokers) and 61 (67%) were non-smokers. Patient clinicopathologic characteristics data are shown in Supplementary Table S2.

**WGCNV scores versus histologic subtypes and predominant subtype grading**

The presence of confounding germline CNVs was adjusted by subtraction of control germline CNVs (pool of 20 control samples) from the test CNVs, thus only the somatic CNVs were used for calculating the WGCNV scores. The WGCNV score algorithm was then applied to evaluate the extent and pattern of WGCNVs in tumor tissue from the 91 LNMA samples. The median WGCNV score of the 91 LNMA test samples was 2.70 (range, 0–9.95) whereas the
median WGCNV score of the para-cancerous control samples was 0.36 (range, 0–0.80) (Supplementary Figure S3). The median WGCNV scores of histological subcategories, subtypes, grades and TNM stages are shown in Table 1. Trends of positive correlation were observed in the median WGCNV scores among LMNA histological subtypes lepidic, acinar, papillary, solid and micropapillary and also among the three tiered grades. There were statistically significant differences between the WGCNV scores of lepidic and solid (P=0.026), lepidic and micropapillary (P<0.001), acinar and micropapillary (P=0.031) subtypes groups, as well as between grade 1 and grade 2 groups (P=0.004), grade 1 and grade 3 groups (P<0.001) and grade 2 and grade 3 groups (P=0.002) (Figure 2A, B). However, lepidic and acinar, lepidic and papillary, acinar and papillary and, acinar and solid patterns showed no significant differences in WGCNV scores.

A higher degree of invasiveness and a more advanced TNM stage are associated with a more complex spectrum of CNVs

Based on the trends observed with increased WGCNV scores and higher levels of pathological subtypes and grades, we further hypothesized that LNMA tumor samples with a higher degree of invasiveness may harbor larger CNV types or more complex CNV patterns. To test this hypothesis, we conducted more in depth comparisons of WGCNV scores from the different LNMA histological subcategories groups (from AIS, MIA, to IA and MPE&MN) based upon the degree of invasiveness (Figure 2C). While histological subcategories with a higher degree of invasiveness were associated with higher median WGCNV scores, the median WGCNV scores between the

**Table 1** WGCNV scores of diversified groups of pathologic subcategories, subtypes, grades and TNM stages

| Variables                  | WGCNV score-low (0–1.74) | WGCNV score-medium (1.75–4.23) | WGCNV score-high (4.24–9.95) | WGCNV scores [median (range)] | Significance* |
|----------------------------|---------------------------|--------------------------------|-------------------------------|--------------------------------|----------------|
| **Pathological subcategories (n/N)** |                           |                                 |                               |                                |                |
| AIS (11/91)                 | 100 (11/11)               | 0 (0/11)                       | 0 (0/11)                      | 0.25 (0–1.63)                  | a              |
| MIA (7/91)                  | 71.4 (5/7)                | 28.6 (2/7)                     | 0 (0/7)                       | 1.12 (0–2.50)                  | a              |
| IA (58/91)                  | 20.7 (12/58)              | 37.9 (22/58)                   | 41.4 (24/58)                  | 3.50 (0–9.95)                  | b              |
| MPE&MN (15/91)              | 20.0 (3/15)               | 40.0 (6/15)                    | 40.0 (6/15)                   | 3.51 (0–9.20)                  | b              |
| **Histological subtypes in IA (n/N)** |                           |                                 |                               |                                |                |
| Lepidic type (14/58)        | 35.7 (5/14)               | 57.1 (8/14)                    | 7.1 (1/14)                    | 2.02 (0–5.31)                  | a              |
| Acinar type (8/58)          | 25.0 (2/8)                | 50.0 (4/8)                     | 25.0 (2/8)                    | 2.28 (1.36–5.80)               | a              |
| Papillary type (10/58)      | 30.0 (3/10)               | 30.0 (3/10)                    | 40.0 (4/10)                   | 3.82 (0.31–8.86)               | a              |
| Solid type (10/58)          | 20.0 (2/10)               | 30.0 (3/10)                    | 50.0 (5/10)                   | 4.69 (1.30–9.95)               | ab             |
| Micropapillary type (16/58) | 0 (0/16)                  | 25.0 (4/16)                    | 75.0 (12/16)                  | 5.22 (2.99–9.46)               | b              |
| **Subtype grades in resection (n/N)** |                           |                                 |                               |                                |                |
| Grade 1 (32/76)             | 65.6 (21/32)              | 31.3 (10/32)                   | 3.1 (1/32)                    | 0.73 (0–5.31)                  | a              |
| Grade 2 (18/76)             | 27.8 (5/18)               | 38.9 (7/18)                    | 33.3 (6/18)                   | 2.57 (0.31–8.86)               | b              |
| Grade 3 (26/76)             | 7.7 (2/26)                | 26.9 (7/26)                    | 65.4 (17/26)                  | 5.22 (1.30–9.95)               | b              |
| **8th AJCC TNM stages (n/N)** |                           |                                 |                               |                                |                |
| Stage 0 (11/91)             | 100.0 (11/11)             | 0.0 (0/11)                     | 0.0 (0/11)                    | 0.25 (0–1.63)                  | a              |
| Stage I (43/91)             | 37.2 (16/43)              | 30.2 (13/43)                   | 32.6 (14/43)                  | 2.14 (0–8.95)                  | b              |
| Stage II (9/91)             | 0 (0/9)                   | 77.8 (7/9)                     | 22.2 (2/9)                    | 3.11 (2.03–9.46)               | c              |
| Stage III (13/91)           | 7.7 (1/13)                | 30.8 (4/13)                    | 61.5 (8/13)                   | 4.59 (0.31–9.95)               | bc             |
| Stage IV (15/91)            | 20.0 (3/15)               | 40.0 (6/15)                    | 40.0 (6/15)                   | 3.59 (0–9.20)                  | bc             |

WGCNV, whole genome copy number variation; AIS, adenocarcinoma in situ; MIA, minimally invasive adenocarcinoma; IA, invasive adenocarcinoma; MPE&MN, malignant pleural effusion & metastatic nodule biopsy; AJCC, American Joint Committee on Cancer. *, Chi-squared test for distribution, P<0.05 is statistically significant, and same letters indicate no significant difference.
MPE&MN and IA group were considered similar. The WGCNV scores were significantly higher among the IA group and the MPE&MN groups compared to the AIS group (P<0.001 and P=0.010, respectively) and in the IA group compared to the MIA group (P=0.019). However, there was no significant difference in WGCNV scores between AIS and MIA groups (P=0.930), MIA and MPE&MN groups (P=0.148), IA and MPE&MN groups (P=0.878).

Trends of positive correlation were also observed between the median WGCNV scores and stage groups, from 0 to III, but not in stage IV group (Figure 2D). The WGCNV scores were significantly higher for stages I–IV compared to stage 0 (P<0.05) and for stage I compared to stage III (P=0.025). However, there were no significant differences of WGCNV scores between stage I and II (P=0.831), I and IV (P=0.983), II and IV (P=0.984), III and IV (P=0.222) groups.
Higher pathologic subcategories, subtypes, grades and TNM stages of LNMA tend to distribute into higher WGCNV scores groups

The WGCNV scores of 91 tumor samples were evenly distributed between 0 and 9.95 (refer to scatter diagram of the 91 tumor samples distribution in Supplementary Figure S3). For a more global view, we first sorted the 91 test cases based on their WGCNV scores and then equally divided them into three groups as score-low (31 cases, 0–1.74), score-medium (30 cases, 1.75–4.23) and score-high (30 cases, 4.24–9.95).

Firstly, when investigating the distributions of WGCNVs according to four pathological subcategories (Table 1), it was found that all 11 AIS samples (100%) fell in the score-low group. Similarly, 5 of 7 MIA samples (71.4%) fell in the score-low group and 2 of 7 (28.6%) fell in the score-medium groups. In contrast, the majority of IA and MPE&MN samples (58/73, 79%) fell into either the score-medium (28/58, 48%) or score-high (30/58, 52%) groups. Chi-squared test confirmed statistically significant differences for the distributions in IA vs. AIS (P<0.001), in MPE&MN vs. AIS (P<0.001), in MIA vs. IA (P=0.010), and in MIA vs. MPE&MN (P=0.040).

Secondly, we further investigated the distributions of histological subtypes in IA and three tiered grades of LNMA in three WGCNV score groups. Significant differences of the distributions into WGCNV score low to high groups were observed not only among lepidic, acinar, papillary and solid cells with micropapillary subtypes, but also among grade 3 samples compared to grade 1 (P=0.004) and grade 2 (P<0.001) samples. All these observations indicated that LNMA samples with a higher grading in the pathologic subcategories, histological subtypes or grading system, had tendencies to distribute into higher WGCNV score groups (Table 1). However, we found some exceptions where there were differences in distributions within one histological subtype or grade group, for example, 10 cases with predominant papillary type IA, and 3, 5, and 2 cases were distributed into the low, medium and high WGCNV score groups, respectively.

Thirdly, we also investigated the distribution of TNM stages in the WGCNV score from low to high groups (Table 1). All stage 0 (11/11, 100%) samples fell into the score-low CNV group whereas the 43 stage I samples almost equally fell into the three SCORE CNV groups. In contrast, the majority (7/9, 77.8%) of stage II samples fell into the score-medium CNV group and a minority (2/9, 22.2%) fell into the score-high. There were significant differences among the distributions from stage 0 vs. I–IV (P<0.001, P<0.001, P<0.001, P<0.001, respectively), stage I and stage II (P=0.018) but not in stage I and III (P=0.082) or stage I and IV (P=0.471) and stage III and IV (P=0.461) group.

Association of TUSON scores with histological LNMA subtypes and grading

To assess the overall effect of CNVs on tumorigenesis, we further evaluated the full spectrum of tumor CNVs in each of the 91 samples according to specimen histologic grade/subtype (Figure 3A). For CNV deletions, the TSG scores in IA and MPE&MN samples were generally higher than OG scores. However, for CNV duplications there was an opposite trend. The median TUSON score was 0 (–143.2–1.6) for AIS samples, 8.3 (–57.7–61.6) for MIA samples, 134.53 (–70.3–534.6) for IA samples and 116 (0–397.3) for MPE&MN samples. In regard to the predominant histological grade system, the TUSON scores of grade 2 and grade 3 were significantly higher than those of grade 1 (P<0.001). TUSON scores were significant higher in IA samples than in AIS samples (P=0.005) and in MIA samples (P=0.033). There was no significant difference in TUSON scores between MPE&MN and IA samples (P=0.874). There was a general trend for selective enrichment for TSG in deletion CNVs and for OG in duplication CNVs and these may all contribute to tumorigenesis (Figure 3B). By Pearson correlation (R=0.53, P<0.001), there was a moderate association of TUSON and WGCNV scores, indicating that a majority of the total CNVs detected affected expression of tumorigenesis genes or oncogenes (Figure 3D).

Assessment of diagnostic value of WGCNV scores

WGCNV scores of all the 111 samples in this study (91 cancerous and 20 para-cancerous lung tissue specimens) were calculated to determine the differential diagnostic value for LNMA from non-tumor lung tissue (LNMA differentiated diagnosis). By ROC curve analysis of WGCNV scores we were able to define a cut-off value of 0.02 to separate LNMA and para-cancerous non-tumor lung samples. The diagnostic sensitivity and specificity was 95.0% and 97.8%, and the positive and negative predictive value of this classifier was 99% and 90%, respectively (Figure 4A). The AUC was 0.981, indicating WGCNV...
Figure 3 Distribution and score of OGs or TSGs on tumor CNV. (A) In the first and second row of the grid diagram, TSGs and OGs scores in loss and gain CNV regions for all 91 tumor samples were separately calculated. TSGs score was generally higher than OGs in deletion areas in IA and MPE&MN tissues; a contrary trend was shown in duplication areas. This trend was not seen in AIS and MIA. The third and fourth row of the grid diagram show TUSON score and WGCNV score of all 91 tumor samples, dots of different colors represent different types of samples. There is a rising trend in TUSON score and WGCNV score based upon the degree of invasiveness from AIS, MIA to IA and MPE&MN; (B) Box-plot of TUSON score of samples from different gradings. TUSON scores in grade 1 were significantly lower than that in the grade 2 and grade 3. Letters (a,b,c) above the box-plot diagrams are results of Tukey’s test comparisons. Same letters indicate no significant difference between the two groups WGCNV scores.; (C) Scatter plot of TSGs and OGs scores in loss and gain CNV regions. There is a trend for selective enrichment for TSG in deletion CNVs and for OG in duplication CNVs, DEL, deletion; DUP, duplication; (D) Moderate correlation was shown in Pearson correlation analysis for WGCNV score and TUSON score (r=0.53, P<0.001, Pearson correlation). OG, oncogene; TSG, tumor suppressor gene; CNV, copy number variation; IA, invasive adenocarcinoma; MPE&MN, malignant pleural effusion & metastatic nodule biopsy; AIS, adenocarcinoma in situ; MIA, minimally invasive adenocarcinoma; TUSON, Tumor Suppressor and Oncogene; WGCNV, whole genome copy number variation.
scores are a very good separator of cancerous and non-cancerous tissue. To further evaluate whether the WGCNV scores could distinguish AIS and MIA samples from IA and MPE&MN samples, a cut-off value of 1.85 was set to be the threshold to classify the two groups. The sensitivity and specificity was 78.1% and 94.4%, respectively, and the positive and negative predictive value of this classifier was 98% and 52%, respectively. The AUC was 0.896 (Figure 4B).

**Discussion**

In this present study, we developed a new scoring algorithm to assess WGCNV changes and found positive correlation trends of median WGCNV scores with the diversified LNMA histological subcategories (from AIS, MIA to IA and MPE&MN), five histologic subtypes (from lepidic, acinar, papillary, solid to micropapillary subtype) of IA, and three tiered predominant histologic subtype grades. In addition, both a higher degree of invasiveness and histological grade in LNMA were associated with a more complex spectrum of CNVs. Based on statistical analyses of tumor samples from LNMA patients, there was a general trend for low histological pathologic subcategories, subtypes, grading and TNM stages with lower WGCNV scores and a trend for higher histological pathologic subcategories, subtypes, grading and TNM stages with higher WGCNV scores. For both IA and MPE&MN samples, TSG and OG scores based on a subset of deletions and duplications, respectively, displayed a general trend for selective enrichment.

More recently, some research studies have concluded that chromosomal instability is a driver not only for tumor evolution but also for metastasis (19,32). Higher CNV levels were associated with cancer recurrence and death. In this study, we revealed significant correlations between WGCNV scoring with important pathological diagnostic and clinical prognostic criteria, suggesting that the WGCNV scoring algorithm developed here may have potential clinical value as a more objective biomarker for predicting prognosis as well as diagnostic value to differentiate diversified histological subcategories in LNMA patients. For the first time, to our knowledge, the findings reported here build on the correlations between evaluation for the number of copy number aberrations and chromosome abnormality score with pathological morphological diagnostic and clinic-pathological prognostic parameters in LNMA.

Although a positive correlation was found between WGCNV score levels and the degree of invasiveness in LNMA (from AIS, MIA to IA and MPE&MN), there were no significant differences between AIS and MIA groups for median WGCNV score levels and the distribution in low to high score groups. Recently, the clinical and prognostic meaning of the IASLC/ATS/ERS and WHO classification (as AIS, MIA and IA) was challenged (33-35), because the

**Figure 4** Diagnostic value of WGCNV score. ROC curve shows diagnostic performance of WGCNV score in distinguishing LNMA from para-cancerous non-tumor lung samples [cut-off value, 0.02; sensitivity, 95.0%; specificity, 97.8%; AUC=0.981]. (A) and in distinguishing AIS, MIA from IA and MPE&MN samples [cut-off value, 1.85; sensitivity, 78.1%; specificity, 94.4%; AUC=0.896] (B). WGCNV, whole genome copy number variation; ROC, receiver operating characteristic; LNMA, lung non-mucinous adenocarcinoma; AIS, adenocarcinoma in situ; MIA, minimally invasive adenocarcinoma; IA, invasive adenocarcinoma; MPE&MN, malignant pleural effusion & metastatic nodule biopsy; AUC, the area under the ROC curve.
results of individual center studies and large meta-analyses showed no significant differences in the survival rates between AIS and MIA patients with lung adenocarcinoma. Based on our finding that chromosome abnormalities mentioned above were not significantly different between AIS and MIA groups, this challenge to the classification may have a genuine basis.

Although there is still no internationally accepted grading system for lung adenocarcinoma, the new histological classification has at least given the impetus to develop the grading systems based more on both architectural and nuclear morphology (4,8). From this study, a positive correlation trend and significant differences of median WGCNV score levels were identified amongst the three-tiered predominant subtype grades. Additionally, the predominant histological grading system adopted in this study was supported by our findings, and thus the WGCNVs scoring algorithm we developed may prove to be a useful ancillary system for molecular grading of LNMA.

A diversified distribution in WGCNV-low to -high score groups from one histological subtype was found in our data. For example, of the 10 cases with predominant papillary type IA, 3, 5 and 2 cases distributed into the low, medium and high WGCNV score groups, respectively. In one study, the papillary subtype IA was separated into 3 different types on the basis of the degree of papillary architectural and cytological pleomorphism and patient follow-up showed that this subclassification of papillary was associated with a prognostic impact with type 3 having a worse prognosis (36). According to the 2011 IASLC/ATS/ERS/2015 WHO classification, the cribriform and fused glands are regarded as acinar subtype in LNMA (9), but some reports suggest that the cribriform and fused glands should be distinguished from the acinar type as a subtype with worse prognosis (36-38). We reviewed the H&E staining slides from the 10 predominant papillary IA cases, and found 2 high WGCNV score cases had the morphological characteristics of type 3 papillary tumor previously proposed (36). This finding suggested that the tumor morphologic heterogeneity may be linked to different molecular abnormalities. Thus establishing a WGCNV scoring system may be more objective and practical than a simple architectural grading system and thus more useful as a prognostic evaluator in LNMA patients. Nonetheless, WGCNV scores need to be further assessed in association with survival analysis in a larger number of patients to determine prognostic value.

Based on the previous suggestions of the relationship between chromosomal instability and tumor metastasis (32,39-41), it has been proposed that the invasive ability of tumor cells may be enhanced by a rising degree of increasing CNV changes, and specific cell subclones with the most severe extensive CNVs changes may obtain the capacity to invade out of organs and form local or distant metastasis. In general, our findings underlined the positive correlation trend with median WGCNV scores among AIS, MIA and IA within the lung and supported our hypothesis. The exception was for the MPE&MN samples, which showed almost the same score with IA, even in the distributions analysis of the low to high score groups. Also, similar to the results of histologic subcategories, in the analysis of the relationship between WGCNV scores and TNM stages, the median WGCNV scores were generally increasing with higher TNM stages. Thus, within the organ (lung), the hypothesis about the relationship between invasion and WGCNV scoring was verified. Further research is therefore needed to confirm our findings and determine whether different mechanisms of tumor aggression exist within and outside of an affected organ.

Frequent CNV accumulation may be the most striking characteristic of cancer genomes, though ostensibly random, they may follow a pattern. Based on our results, we identified a non-random pattern of somatic CNVs which gradually increased with the degree of invasion (Figure 3A). Scores of TSG and OG were basically the same either in loss or gain CNVs for AIS, MIA and predominant lepidic IA, whereas a trend of selective enrichment for TSG in deletion CNVs and for OG in duplication CNVs was more pronounced in other IA and MPE&MN samples, and such changes are likely to promote tumorigenesis. Whether the non-random accumulation of CNV mainly occurs in clonal or subclonal lines or not needs to be verified and the relationship of CNV and oncogenic mutation enrichment in different clonal lines remains obscure.

Our molecular studies suggested that the WGCNV score may have diagnostic value for LNMA patients. ROC curve analysis indicated that WGCNV scores may be valuable in differentiated diagnosis of LNMA from non-tumor lung tissue (AUC=0.981), and of AIS and MIA from IA and MPE&MN (AUC=0.896). WGA of LCM samples combined with CNV-seq was shown to be a relatively simple technique for detecting CNV changes in less than 100 cells, and may add diagnostic value to guide treatment options. In addition, in cases where limited biopsy or low
tumor content cytological samples are all that is available, it will be possible to obtain additional information to give a more precise pathologic diagnosis.

The main limitations of introducing a WGCNV scoring system into a routine pathology laboratory are the time and resources needed to identify and assess the CNVs and derive a score. For this purpose, a next generation sequencing platform would be required to perform CNV sequencing which normally takes 2–3 days for sample preparation, library construction, sequencing and data analysis. Further, staff training would be required so that the molecular and histology results could be interpreted and integrated into a final report, so that the information can be used to guide treatment. In addition, the whole procedure would add additional cost to the patient for molecular assessment of the tumor.

There were also some limitations of this study that may have influenced the association studies with WGCNV scores. First, in some cases there were only small sample numbers for assessing the relationship between WGCNV scores with histological subtypes and TNM stages, and this may have introduced statistical bias in some comparisons. Thus, further studies on a larger cohort of LNMA patients are still needed to establish more confidently some of the associations reported in this study. Second, for obtaining pure histological subtype material, the LCM technique was used in our study to examine the predominant tumor type. However, in cases whether there is significant tumor heterogeneity, it would be interesting to compare WGCNV scores between different tissue regions. Third, for the purpose of avoiding poor DNA quality and reducing test failure, FFPE histologic and cytologic cell blocks less than 2 years old were selected and so no meaningful patient survival data linking with WGCNV scores could be obtained. Fourth, our research only focused on LNMA samples, and so additional studies are necessary to determine if similar associations are found in lung mucinous adenocarcinoma and other types of lung cancer or in other organ-specific tumors. Fifth, it will be important to perform CNV and oncogenic mutation analyses in parallel to identify a more holistic set of genetic markers. Lastly, genome-wide low-depth analysis cannot determine other genetic changes such as genomic doubling reported in lung adenocarcinoma.

Conclusions

In this cohort study of LNMA patients, we showed a positive correlation of WGCNV score with morphological diagnostic and clinicopathological prognostic parameters. Accordingly, we propose that WGCNV scoring may have intrinsic value as a robust molecular parameter in pathological diagnosis and could be used to better evaluate the prognosis of LNMA patients and, potentially other patients with lung adenocarcinoma.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Figure S1 Sensitivity of CNV-seq for detecting CNVs. CNV, copy number variation; seq, sequencing; WGA, whole genome amplification.
**Figure S2** Calculation of WGCNV scores from CNV-seq profiles. WGCNV, whole genome copy number variation; CNV, copy number variation; seq, sequencing.

**Figure S3** Distribution of WGCNV scores for LNMA tumor samples (N=91). Median is indicated by the dotted line. WGCNV, whole genome copy number variation; LNMA, lung non-mucinous adenocarcinoma.
| Tukey's test | 8th AJCC TNM stages (WGCNV score) | Chi-squared test | 8th AJCC TNM stages |
|--------------|-----------------------------------|-----------------|-------------------|
| **Histological subtypes in IA (WGCNV score)** | | | |
| Acinar–Lepidic | 0.895 | 0.012 | Acinar–Lepidic | 0.491 | I–0 | 0.001 |
| Papillary–Lepidic | 0.518 | 0.011 | Papillary–Lepidic | 0.134 | II–0 | <0.001 |
| Solid–Lepidic | 0.026 | <0.001 | Solid–Lepidic | 0.057 | III–0 | <0.001 |
| Micropapillary–Lepidic | <0.001 | 0.016 | Micropapillary–Lepidic | <0.001 | IV–0 | <0.001 |
| Papillary–Acinar | 0.983 | 0.831 | Papillary–Acinar | 0.671 | II–I | 0.018 |
| Solid–Acinar | 0.362 | 0.025 | Solid–Acinar | 0.543 | III–I | 0.082 |
| Micropapillary–Acinar | 0.031 | 0.983 | Micropapillary–Acinar | 0.026 | IV–I | 0.471 |
| Solid–Papillary | 0.640 | 0.669 | Solid–Papillary | 0.856 | III–II | 0.088 |
| Micropapillary–Papillary | 0.084 | 0.984 | Micropapillary–Papillary | 0.048 | IV–II | 0.148 |
| Micropapillary–Solid | 0.821 | 0.222 | Micropapillary–Solid | 0.146 | IV–III | 0.461 |
| **Subtype grades in resection (WGCNV score)** | Pathological subcategories (TUSON score) | Subtype grades in resection (WGCNV Score) |
| Grade 2–1 | 0.004 | MIA–AIS | 1.000 | Grade 2–1 | 0.004 |
| Grade 3–1 | <0.001 | IA–AIS | 0.005 | Grade 3–1 | <0.001 |
| Grade 3–2 | 0.002 | MPE&MN–AIS | 0.106 | Grade 3–2 | 0.072 |
| Pathological subcategories (WGCNV score) | | | |
| MIA–AIS | 0.930 | MPE&MN–MIA | 0.213 | MIA–AIS | 0.267 |
| IA–AIS | <0.001 | MPE&MN–IA | 0.874 | IA–AIS | <0.001 |
| MPE&MN–AIS | 0.010 | MPE&MN–AIS | <0.001 | IA–AIS | <0.001 |
| MPE&MN–MIA | 0.019 | IA–MIA | 0.010 | MPE&MN–MIA | 0.040 |
| MPE&MN–IA | 0.148 | MPE&MN–IA | 0.989 | MPE&MN–IA | 0.989 |

IA, invasive adenocarcinoma; WGCNV, whole genome copy number variation; AIS, adenocarcinoma in situ; MIA, minimally invasive adenocarcinoma; MPE&MN, malignant pleural effusion & metastatic nodule biopsy; AJCC, American Joint Committee on Cancer; TUSON, Tumor Suppressor and Oncogene.
| No. | Sex | Age (year) | TNM stage | Neoadjuvant therapy | Smoking status |
|-----|-----|------------|-----------|---------------------|----------------|
| 1   | M   | 71         | TIS       | N0                  | M0             | No | Former |
| 2   | F   | 50         | TIS       | N0                  | M0             | No | Never  |
| 3   | M   | 64         | TIS       | N0                  | M0             | No | Never  |
| 4   | F   | 46         | TIS       | N0                  | M0             | No | Never  |
| 5   | M   | 24         | TIS       | N0                  | M0             | No | Current |
| 6   | F   | 66         | TIS       | N0                  | M0             | No | Never  |
| 7   | F   | 53         | TIS       | N0                  | M0             | No | Never  |
| 8   | M   | 54         | TIS       | N0                  | M0             | No | Never  |
| 9   | M   | 54         | TIS       | N0                  | M0             | No | Current |
| 10  | F   | 56         | TIS       | N0                  | M0             | No | Never  |
| 11  | F   | 58         | TIS       | N0                  | M0             | No | Never  |
| 12  | M   | 65         | T1a       | N0                  | M0             | No | Never  |
| 13  | F   | 53         | T1a       | N0                  | M0             | No | Never  |
| 14  | F   | 53         | T1b       | N0                  | M0             | No | Never  |
| 15  | F   | 62         | T1b       | N0                  | M0             | No | Never  |
| 16  | M   | 61         | T1a       | N0                  | M0             | No | Current |
| 17  | F   | 56         | T1a       | N0                  | M0             | No | Never  |
| 18  | F   | 46         | T1a       | N0                  | M0             | No | Never  |
| 19  | F   | 73         | T1a       | N0                  | M0             | No | Never  |
| 20  | M   | 56         | T1a       | N0                  | M0             | No | Former |
| 21  | F   | 50         | T1a       | N0                  | M0             | No | Never  |
| 22  | M   | 62         | T1a       | N0                  | M0             | No | Never  |
| 23  | M   | 60         | T1a       | N0                  | M0             | No | Current |
| 24  | F   | 56         | T1a       | N0                  | M0             | No | Current |
| 25  | F   | 53         | T1a       | N0                  | M0             | No | Never  |
| 26  | F   | 77         | T1a       | N0                  | M0             | No | Never  |
| 27  | M   | 65         | T1a       | N0                  | M0             | No | Never  |
| 28  | F   | 53         | T1a       | N0                  | M0             | No | Never  |
| 29  | F   | 60         | T1a       | N0                  | M0             | No | Never  |
| 30  | F   | 60         | T1a       | N0                  | M0             | No | Never  |
| 31  | F   | 55         | T1b       | N0                  | M0             | No | Never  |
| 32  | M   | 56         | T1b       | N0                  | M0             | No | Never  |
| 33  | F   | 56         | T1b       | N0                  | M0             | No | Never  |
| 34  | F   | 60         | T1b       | N0                  | M0             | No | Never  |
| 35  | M   | 75         | T1b       | N0                  | M0             | No | Former |
| 36  | M   | 75         | T1b       | N0                  | M0             | No | Former |
| 37  | M   | 68         | T1b       | N0                  | M0             | No | Never  |
| 38  | F   | 56         | T1b       | N0                  | M0             | No | Never  |
| 39  | F   | 49         | T1b       | N0                  | M0             | No | Never  |
| 40  | M   | 51         | T1c       | N0                  | M0             | No | Former |
| 41  | M   | 71         | T1c       | N0                  | M0             | No | Former |

Table S2 (continued)
| No. | Sex | Age (year) | TNM stage | TNM stage | Neoadjuvant therapy | Smoking status |
|-----|-----|------------|-----------|-----------|---------------------|----------------|
|     |     |            | Tumor     | Nodes     | Metastasis          |                |
| 42  | M   | 71         | T1c       | N0        | M0                  | IA3            | No             | Former         |
| 43  | F   | 56         | T1c       | N0        | M0                  | IA3            | No             | Never          |
| 44  | F   | 56         | T1c       | N0        | M0                  | IA3            | No             | Never          |
| 45  | M   | 64         | T1c       | N0        | M0                  | IA3            | No             | Current        |
| 46  | M   | 81         | T1c       | N0        | M0                  | IA3            | No             | Current        |
| 47  | M   | 64         | T1c       | N0        | M0                  | IA3            | No             | Never          |
| 48  | M   | 64         | T1c       | N0        | M0                  | IA3            | No             | Never          |
| 49  | F   | 66         | T1c       | N0        | M0                  | IA3            | No             | Never          |
| 50  | F   | 72         | T2a       | N0        | M0                  | IB             | No             | Never          |
| 51  | M   | 47         | T2a       | N0        | M0                  | IB             | No             | Never          |
| 52  | M   | 47         | T2a       | N0        | M0                  | IB             | No             | Never          |
| 53  | M   | 58         | T2a       | N0        | M0                  | IB             | No             | Former         |
| 54  | M   | 58         | T2a       | N0        | M0                  | IB             | No             | Current        |
| 55  | M   | 54         | T2a       | N1        | M0                  | IIB            | No             | Current        |
| 56  | F   | 55         | T1c       | N1        | M0                  | IIB            | No             | Never          |
| 57  | M   | 63         | T2a       | N1        | M0                  | IIB            | No             | Never          |
| 58  | M   | 63         | T2a       | N1        | M0                  | IIB            | No             | Never          |
| 59  | F   | 75         | T3        | N0        | M0                  | IIB            | No             | Never          |
| 60  | M   | 75         | T3        | N0        | M0                  | IIB            | No             | Former         |
| 61  | M   | 54         | T1b       | N1        | M0                  | IIB            | No             | Current        |
| 62  | F   | 66         | T2a       | N1        | M0                  | IIB            | No             | Never          |
| 63  | F   | 79         | T2a       | N1        | M0                  | IIB            | No             | Never          |
| 64  | F   | 70         | T4        | N0        | M0                  | IIIA           | No             | Never          |
| 65  | F   | 70         | T4        | N1        | M0                  | IIIA           | No             | Never          |
| 66  | F   | 57         | T1c       | N2        | M0                  | IIIA           | No             | Never          |
| 67  | M   | 61         | T3        | N2        | M0                  | IIIB           | No             | Former         |
| 68  | F   | 49         | T1c       | N2        | M0                  | IIIA           | No             | Never          |
| 69  | F   | 49         | T1c       | N3        | M0                  | IIIB           | No             | Never          |
| 70  | M   | 61         | T4        | N0        | M0                  | IIIA           | No             | Current        |
| 71  | M   | 77         | T4        | N0        | M0                  | IIIA           | No             | Current        |
| 72  | M   | 65         | T4        | N1        | M0                  | IIIA           | No             | Current        |
| 73  | F   | 62         | T4        | N1        | M0                  | IIIA           | No             | Never          |
| 74  | M   | 65         | T4        | N2        | M0                  | IIIB           | No             | Never          |
| 75  | M   | 65         | T4        | N2        | M0                  | IIIB           | No             | Current        |
| 76  | M   | 70         | T4        | N3        | M0                  | III C          | No             | Current        |
| 77  | F   | 52         | Tx        | Nx        | M1                  | IV             | No             | Never          |
| 78  | F   | 62         | Tx        | Nx        | M1                  | IV             | No             | Never          |
| 79  | M   | 68         | Tx        | Nx        | M1                  | IV             | No             | Current        |
| 80  | M   | 81         | Tx        | Nx        | M1                  | IV             | No             | Current        |
| 81  | F   | 46         | Tx        | Nx        | M1                  | IV             | No             | Never          |
| 82  | F   | 81         | Tx        | Nx        | M1                  | IV             | No             | Never          |
Table S2 (continued)

| No. | Sex | Age (year) | TNM stage | Tumor | Nodes | Metastasis | TNM stage | Neoadjuvant therapy | Smoking status |
|-----|-----|------------|-----------|-------|-------|------------|-----------|--------------------|----------------|
| 83  | F   | 80         | Tx        | Nx    | M1    | IV         | No        | Never              |
| 84  | F   | 74         | Tx        | Nx    | M1    | IV         | No        | Never              |
| 85  | F   | 54         | Tx        | Nx    | M1    | IV         | No        | Current            |
| 86  | F   | 68         | Tx        | Nx    | M1    | IV         | No        | Never              |
| 87  | F   | 77         | Tx        | Nx    | M1    | IV         | No        | Never              |
| 88  | M   | 77         | Tx        | Nx    | M1    | IV         | No        | Never              |
| 89  | M   | 77         | Tx        | Nx    | M1    | IV         | No        | Never              |
| 90  | M   | 67         | Tx        | Nx    | M1    | IV         | No        | Current            |
| 91  | M   | 79         | Tx        | Nx    | M1    | IV         | No        | Never              |

LNMA, lung non-mucinous adenocarcinoma; M, male; F, female.