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ORIGINAL ARTICLE

# Using Whole Genome Amplification (WGA) of Low-Volume Biopsies to Assess the Prognostic Role of *EGFR*, *KRAS*, *p53*, and *CMET* Mutations in Advanced-Stage Non-small Cell Lung Cancer (NSCLC)

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**Background:** Progression of non-small cell lung cancer (NSCLC) from early- to late-stage may signify the accumulation of gene mutations. An advanced-stage tumor's mutation profile may also have prognostic value, guiding treatment decisions. Mutation detection of multiple genes is limited by the low amount of deoxyribonucleic acid extracted from low-volume diagnostic lung biopsies. We explored whole genome amplification (WGA) to enable multiple molecular analyses.

**Methods:** Eighty-eight advanced-stage NSCLC patients were enrolled. Their low-volume lung biopsies underwent WGA before direct sequencing for epidermal growth factor receptor (*EGFR*), *KRAS* (rat sarcoma virus), *p53*, and *CMET* (mesenchymal-epithelial transition factor) mutations. Overall survival impact was examined. Surgically-resected tumors from 133 early-stage NSCLC patients were sequenced for *EGFR*, *KRAS* and *p53* mutations. We compared the mutation frequencies of both groups.

**Results:** It is feasible for low-volume lung biopsies to undergo WGA for mutational analysis. *KRAS* and *CMET* mutations have a deleterious effect on overall survival, hazard ratios 5.05 ($p = 0.009$) and 23.65 ($p = 0.005$), respectively. *EGFR* and *p53* mutations, however, do not have a survival impact. There also does not seem to be significant differences in the frequency of mutations in *EGFR*, *KRAS*, and *p53* between early- and advanced-stage disease: 20% versus 24% ($p = 0.48$), 29% versus 27% ($p = 0.75$), 10% versus 6% ($p = 0.27$), respectively.

**Conclusions:** In advanced-stage NSCLC, *KRAS*, and *CMET* mutations suggest poor prognosis, whereas *EGFR* and *p53* mutations do not seem to have survival impact. Mutations in *EGFR*, *KRAS* and *p53* are unlikely to be responsible for the progression of NSCLC from early- to late-stage disease. WGA may be used to expand starting deoxyribonucleic acid from low-volume lung biopsies for further analysis of advanced-stage NSCLC.

**Key Words:** Non-small cell lung cancer, Advanced stage, Early stage, Whole genome amplification, Low-volume biopsies, KRAS mutations, CMET mutations, EGFR mutations, p53 mutations, Prognosis.

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Lung cancer remains the leading cause of cancer mortality, comprising 17.6% of cancer deaths worldwide, with an average 5-year survival of 9 to 15%.[1] Non-small cell lung cancer (NSCLC) is more common (85% versus 15% being small cell lung cancer), and is the focus of this report.

The poor survival statistics can be largely attributed to the advanced stage of disease at presentation in the majority of NSCLC cases. Currently, the tumor, node, metastasis (TNM) stage at presentation still has the greatest impact on prognosis, as shown by the 5-year survival data collected for the proposed 7th TNM staging system[2]: for pathologic stage I, II, III, and IV, they were 58 to 73%, 36 to 46%, 9 to 24%, and 13%, respectively; for clinical stage I, II, III, and IV, they were 43 to 50%, 25 to 36%, 7 to 19%, and 2%, respectively.

To improve overall clinical outcomes requires consideration of several factors, including effective measures for early disease detection (not dealt with here), as well as elucidating molecular processes underlying carcinogenesis and disease progression. Expanding on the concept that a

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tumor cell acquires and accumulates molecular events that increasingly affect its invasive and metastatic nature, it is not unreasonable to envisage this evolution in cell behavior to coincide with the clinically manifested disease stage. Therefore, we may expect molecular profiles that distinguish early- from late-stage disease. Identifying these differences may lead to the devising of measures to arrest these changes.

The systematic evaluation of advanced-stage NSCLC tumors, however, has been hampered by the relative lack of tissue for molecular analysis. A lot of the data on NSCLC molecular genetics has been garnered from early-stage disease, where there is ample tissue from surgical resections, but which only comprise the minority of cases (20–30%). For advanced-stage NSCLC, available tissue is usually restricted to the low-volume biopsies obtained during the diagnostic procedure. The low abundance of tissue precludes guaranteeing sufficient cells leftover for paraffin-embedment and subsequent molecular analysis for every single case. In Shih et al.'s[3] retrospective study of lung cancer patients on gefitinib treatment, 75 of 139 (54%) patients did not have paraffin-embedded cells from their diagnostic biopsies for deoxyribonucleic acid (DNA) sequencing and were therefore excluded from analysis. In the TRIBUTE trial,[4] of the 710 patients who gave consent to releasing their archival tumor samples for research, 479 (67%) had samples available, of which 274 (39%) contained enough tumor cells to attempt DNA sequencing. This dropout rate needs to be reduced, especially in the context of developing personalized medicine, where the intention is to use the molecular data from the individual's malignant tissue to guide ensuing treatment. We previously proposed an alternative strategy of obtaining separate biopsies during the diagnostic procedure and tested its feasibility in the clinical context.[5–7]

Here, we hypothesize that a profile of a tumor's gene mutations can prognosticate and guide treatment decisions in advanced NSCLC cases, e.g., whether to initiate treatment in the context of borderline performance status. Additionally, if drugs are developed that tend to induce tumor response in the context of particular gene mutations, e.g., tyrosine kinase inhibitors (TKI) and epidermal growth factor receptor (*EGFR*) mutations,[8–10] then knowing the mutation profile of the tumor would be a prerequisite. Hence, developing a system that achieves this from low-volume lung biopsies is clinically relevant.

We had previously explored and tested various ways to maximize the yield of molecular data from these low-volume biopsies, as a means to gaining insight on advanced-stage NSCLC.[5–7] We attempted to define the limits of mutational analysis of these samples through direct sequencing.[6] We decided to test whole-genome amplification (WGA) as a means of pushing these technical boundaries. Ideally, WGA amplifies DNA to sufficient levels for reliable laboratory analysis, and still retains an accurate representation of the original sample. Several methods exist to enhance the replication fidelity.[11–13] We used the multiple displacement amplification method in this study, which had previously been tested on clinical samples.[14]

Apart from testing the feasibility of gene mutation detection in WGA-amplified genomic DNA from low-volume lung biopsies, we wanted to evaluate the mutation status of *EGFR*, *KRAS* (rat sarcoma virus), *p53*, and *CMET* (mesenchymal-epithelial transition factor) in advanced-stage NSCLC and their relation to overall survival. This assesses the suitability of their being included in a panel of mutations of putative clinical significance, as mentioned earlier.

Here, we report the feasibility of using WGA on low-volume lung biopsies for increasing the potential yield of mutation data, the survival analysis of 88 patients with corresponding mutational status obtained via direct sequencing of WGA-amplified genomic DNA from low-volume lung biopsies, and a comparison of mutation frequencies between early- and advanced-stage disease.

## METHODS

### Patients and Sample Collection

Approvals for this study were obtained from the Institutional Review Boards of the National University Hospital, Tan Tock Seng Hospital and National Cancer Centre, Singapore. Samples were obtained from patients with informed consent. For patients undergoing surgery, tumor tissue (1 $cm^3$) was obtained from each patient ex vivo. For patients undergoing nonsurgical diagnostic procedures, study samples were obtained if the preceding sample was assessed by a cytologist to contain sufficient cells for diagnosis. Therefore, study samples were acquired in the same sitting as the diagnostic procedure, but were separate from the diagnostic biopsies, thereby not compromising the usual diagnostic process. Bronchoscopic biopsies were obtained with a 22-gauge Wang cytology needle (Bard Endoscopic Technologies, Billerica, MA) or forceps (Olympus America Inc., Center Valley, PA). Percutaneous needle aspirates were obtained with computed tomography/fluoroscopic guidance using an 18- or 20-gauge Quick-Core needle (Cook Inc., Bloomington, IN), or 20-gauge Chiba or Westcott needles (Medical Device Technologies Inc., Gainesville, FL). As these were diagnostic procedures, a range of pathologies was included. NSCLCs of various histologic subtypes were covered, including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. Each aspirate and surgical sample was collected in 80 $\mu$l and 1 ml of ribonucleic acid (RNA) later (Ambion, Austin, TX), respectively.

### Preparation of Genomic DNA

Genomic DNA was extracted from fresh surgical tissues with Puregene DNA purification kits (Gentra Systems Inc., Minneapolis, MN), using isopropanol for DNA precipitation. To maximize yield for the low-volume samples, DNA precipitation was achieved with oyster glycogen (Sigma-Aldrich, St. Louis, MO) added to the last step to a final concentration of 0.02 $\mu$g/$\mu$l, then overnight incubation at −20°C before centrifugation.[15] DNA extracted from each sample was resuspended in 15 $\mu$l of TE (Tris-EDTA, pH 7.5) buffer. Quantification of extracted DNA was performed using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE).

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## Whole Genome Amplification

Whole genome amplification was done with the Qiagen REPLI-g Midi Kit (cat 150045, Qiagen, Hilden, Germany). Briefly, 20 ng of extracted genomic DNA in a 5 μl volume was mixed with 5 μl of denaturation buffer (D1) and incubated for 3 minutes at room temperature. This was followed by adding 10 μl of neutralization buffer (N1). Thirty microliters of the master mix (REPLI-g Midi Reaction Buffer + REPLI-g Midi DNA Polymerase) was added to the denatured DNA and incubated at 30°C for 16 hours to achieve maximum DNA yield (40 μg). REPLI-g Midi DNA polymerase was inactivated by heating at 65°C for 3 minutes. Amplified DNA was stored at −20°C.

## PCR and Purification of PCR Products

Exons 18–21 of *EGFR*, exons 4–9 of *p53*, exon 2 of *KRAS* and exons 13–22 of *CMET* were amplified by polymerase chain reaction (PCR). Primer sequences (forward and reverse, respectively) and PCR conditions are as follows:

*EGFR*-Exon18F: -CAAATGAGCTGGCAAGTGCCGTGTC-
*EGFR*-Exon18R: -GAGTTTCCCAAACACTCAGTGAAAC-
*EGFR*-Exon19F: -GCAATATCAGCCTTAGGTGCGGCTC-
*EGFR*-Exon19R: -CATAGAAAGTGAACATTTAGGAT-GTG
*EGFR*-Exon20F: -CCATGAGTACGTATTTTGAAACTC-
*EGFR*-Exon20R: -CATATCCCCATGGCAAACTCTTGC-
*EGFR*-Exon21F: -CTAACGTTCGCCAGCCATAAGTCC-
*EGFR*-Exon21R: -GCTGCGAGCTCACCCAGAAT-GTCTGG-
*p53*-Exon5–6F: -CTAGCTCGCTAGTGGGTTG-
*p53*-Exon5–6R: -AGGAGAAAGCCCCCCTACTG-
*p53*-Exon7F: -TGCTTGCCACAGGTCTCC-
*p53*-Exon7R: -AAGCTCCAGCTCCAGGTAGG -
*p53*-Exons8–9F: -TTCCTTACTGCCTCTTGCTT-
*p53*-Exons8–9F: -GAAAACGGCATTTTGAGTG-
*KRAS*-Exon2F: -TTCTTAAGCGTCGATGGAGG–
*KRAS*-Exon2R: -ACAGAGAGTGAACATCATGGAC-
*CMET*-Exon13F: -GGCAGTTATGCCATTTGTAGAAT-
*CMET*-Exon13R: -AGCGAACTAATTCACTGCCC-
*CMET*-Exon14F: -CCATGAGTTCTGGGCACTG-
*CMET*-Exon14R: -GCAGAGGTAAATACTTCCTTTAG-GTTT-
*CMET*-Exon15F: -AGCATGGCTTTTTGCTATTGA-
*CMET*-Exon15R: -GCTCTGTCAGTTGCTTTCACC-
*CMET*-Exon16F: -CACACCTACGTACCTATAGTGG-TATTG-
*CMET*-Exon16R: -TTTTCCACAAGGGGAAAGTG-
*CMET*-Exon17F: -AAACCCTCAGGACAAGATGC-
*CMET*-Exon17R: -AGGGATGGCTGGCTTACAG-
*CMET*-Exon18F: -AGGCTTGAGCCATTAAGACC-
*CMET*-Exon18R: -ATCCCCAGGGCTTACACATC-
*CMET*-Exon19F: -TGGCAATGTCAATGTCAAGC-
*CMET*-Exon19R: -TGAAGAAAACTGGAATTGGTG-
*CMET*-Exon20F: -TGTTGCCCAAAACAGAAACC-
*CMET*-Exon20R: -AAGGCAGGCATTTCTGTAAAAG-
*CMET*-Exon21F: -TCCTACAACCCGAATACTGC-
*CMET*-Exon21R: -CCCAGAAGGAGGCTGGTC-
*CMET*-Exon22F: -TGTCAAAGCAACAGTCCACAC-
*CMET*-Exon22R: -TGGGTGAATGACACCATCAG-

All PCR assays were carried out in a 20 μl volume that contained 30 ng of DNA, 1 unit of Platinum *Taq*DNA polymerase (Invitrogen, CA) and 0.3 mM of dNTP (Bioline Ltd, London, UK). Each PCR reaction volume was subjected to 95°C for 5 minutes, then DNA was amplified for 35 cycles at 95°C for 30 seconds, 56°C for 30 seconds, 72°C for 1 minute, with a final extension time of 10 minutes at 72°C. QiaQuick PCR purification kits (Qiagen, Hilden, Germany) were used.

## DNA Sequencing

Purified PCR products were sequenced in forward and reverse directions using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Version 3) and ABI PRISM 3730 Genetic Analyzer (Applied Biosystems, CA). Chromatograms were analyzed by SeqScape V2.5 and manual review.

## Statistical Analysis

Survival distribution functions were estimated using the Kaplan-Meier product limit method. Standard errors of the Kaplan Meier estimates were computed by using Greenwood's formula. The effects of *EGFR*, *KRAS*, *p53*, and *CMET* mutational status on the survival time of the patient were studied via the Cox proportional hazards model. As additional confounding variables such as the patient's age and sex were deemed likely to influence survival time, these variables were taken into account when assessing the extent of any exposure difference. We included these variables in the Cox regression models. Likelihood ratio tests were used to compare alternative models. Fisher's exact test tests were used to compare the *EGFR* mutation rates between genders, adenocarcinoma and nonadenocarcinoma subtypes and smoking versus nonsmoking status. Independent two-sample tests for proportions were used to compare the mutation proportions between the early-stage patients and the advanced-stage patients.

Statistical analyses were performed in SAS software (version 9.2, SAS institute, Cary, NC). Graphs were produced in R system, available from Comprehensive R Archive Network (http://www.CRAN.R-project.org).

# RESULTS

## Similar Sensitivity and Accuracy in Detecting *EGFR*, *p53*, and *KRAS* Mutations in WGA-Amplified Versus Unamplified Samples of Surgically Resected Lung Tumors

Several commercially available WGA kits were initially assessed using genomic DNA extracted from cell lines, which were either mutant or wildtype for particular genes (data not shown). Through a series of mixing experiments, where mutant and wildtype DNA were present in differing quantities, we selected the WGA kit that was most sensitive in subsequent mutation detection via direct sequencing. This kit was used for all subsequent WGA-based analyses.


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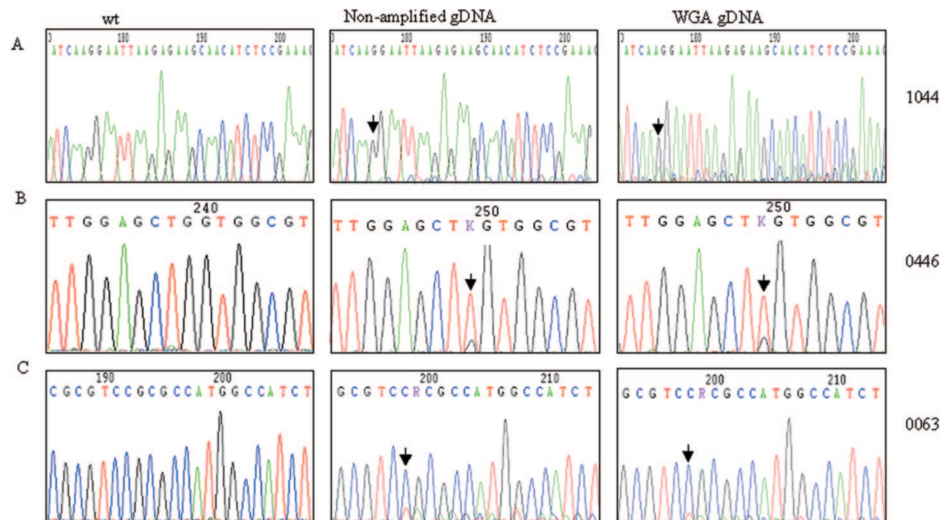


**FIGURE 1.** Detection of deoxyribonucleic acid (DNA) mutations in unamplified and whole genome amplification (WGA)-amplified genomic DNA (gDNA) extracted from surgically-resected lung tumors. Left column, Sequence chromatograms of wildtype tumors using unamplified gDNA. Middle column, Sequence chromatograms of mutation-bearing tumors using unamplified gDNA. Right column, Sequence chromatograms of mutation-bearing tumors, same as in the middle column, but using WGA-amplified gDNA. Identity numbers of the mutation-bearing tumors are listed on the right. *A*, epidermal growth factor receptor (*EGFR*) exon 19: the start of the heterozygous in-frame deletion (delE746-A750) is indicated by the arrows. *B*, *Kras* exon 2: the heterozygous missense mutation (G12C) is indicated by the arrows. *C*, *p53* exon 5: the start of the heterozygous in-frame deletion (delR158-Y163) is indicated by the arrows.

We proceeded to assess the rate of fidelity of mutation detection in WGA-amplified versus unamplified genomic DNA from surgically resected lung tumor tissues. Figure 1 illustrates the comparison of DNA sequence chromatograms of genomic DNA extracted from fresh frozen surgical lung tissue resections, known to be wildtype or mutant for *EGFR*, *KRAS*, and *p53*. For the lung samples that possessed mutations, we compared the DNA sequence variation obtained from unamplified and WGA-amplified genomic DNA. As shown, the mutations were not lost after the WGA process. The heterozygous *EGFR* exon 19 deletion (delE746-A750), heterozygous *KRAS* exon 2 point mutation (G12C) and heterozygous *p53* exon 5 deletion (delR158-Y163) present in unamplified genomic DNA are similarly detected in WGA-amplified genomic DNA, with similar peak-to-peak ratios.

## Similar Sensitivity and Accuracy in Detecting *p53* Mutations in WGA-Amplified Versus Unamplified Samples of Low-Volume Lung Biopsies

We analyzed low-volume lung biopsies known to be wildtype or mutant for *p53* from our previous work.[6] The

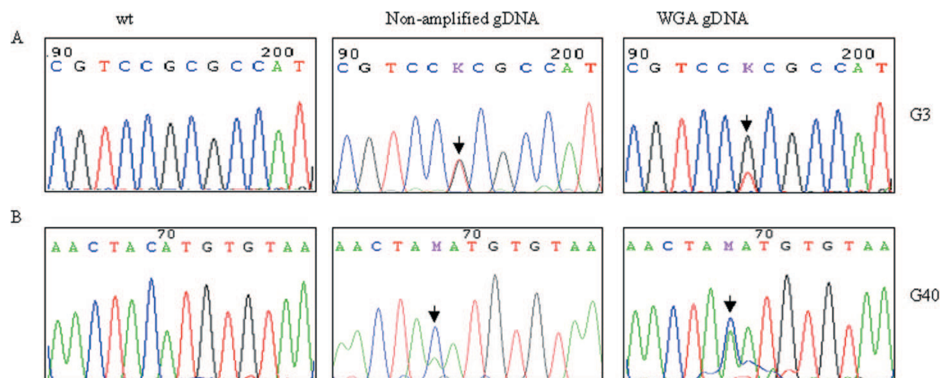


**FIGURE 2.** Detection of deoxyribonucleic acid (DNA) mutations in unamplified and whole genome amplification (WGA)-amplified genomic DNA (gDNA) extracted from low-volume lung biopsies. Left column, Sequence chromatograms of wildtype tumors using unamplified gDNA. Middle column, Sequence chromatograms of mutation-bearing tumors using unamplified gDNA. Right column, Sequence chromatograms of mutation-bearing tumors, same as in the middle column, but using WGA-amplified gDNA. Identity numbers of the mutation-bearing tumors are listed on the right. *A*, *p53* exon 5: the heterozygous missense mutation (R158L) is indicated by the arrows. *B*, *p53* exon 7: the heterozygous missense mutation (Y236stop) is indicated by the arrows.

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mutant samples were G40 and G3, the former being a fine-needle aspirate, and the latter being a core biopsy. Owing to the tissue acquisition technique, G40 is of lower tissue quantity than G3. Extracted DNA leftover from our previous analysis was used here. As shown in Figure 2, the mutations were still present after the WGA process. The heterozygous *p53* exon 5 (R158L) and exon 7 (Y236stop) point mutations are preserved in the WGA-amplified DNA, albeit of varying peak-to-peak ratios, as compared with unamplified DNA. Figure 2 shows 1:1 versus 1:3 mutant-to-wildtype allele peak-to-peak ratios for unamplified and WGA-amplified G3 samples, respectively. Conversely, the peak-to-peak ratios are 2:5 and 4:5 for unamplified versus WGA-amplified G40 samples.

## Characteristics of Patients and Tissue Samples for Sequence Analysis from WGA-Amplified Genomic DNA

A total of 96 ethnic Chinese patients were included in this study. The low-volume lung biopsy study samples were not microscopically examined, but their corresponding diagnostic biopsy reports showed that four were lung secondaries (primary tumors were breast, colon, pancreas, and tonsil), one was a malignant mesothelioma, one was a low-grade MALT lymphoma, two were nonmalignant with inflammatory and/or necrotic changes. The remaining 88 were biopsies of lung primaries, and their corresponding diagnostic biopsy samples were cytologically assessed as NSCLC. They were of advanced-stage disease. All samples were grouped according to the acquisition technique, via (1) bronchoscopy, (2) percutaneous core biopsy, and (3) percutaneous fine needle aspiration (Table 1).

**TABLE 1.** Characteristics of Patients and Lung Biopsies

| | Bronchoscopic Biopsy | Needle Aspirate | Core Biopsy | Overall |
|---|---|---|---|---|
| No. of patients | 15 | 30 | 51 | 96 |
| Age, yr | | | | |
| Median | 69 | 67 | 71 | 69 |
| Range | 48–73 | 44–89 | 29–92 | 29–92 |
| Gender | | | | |
| Male | 12 (80%) | 20 (67%) | 40 (78%) | 72 (75%) |
| Female | 3 (20%) | 10 (33%) | 11 (22%) | 24 (25%) |
| Smoking status | | | | |
| Smokers and ex-smokers | 11 (73%) | 21 (70%) | 39 (76%) | 71 (74%) |
| Never smokers | 4 (27%) | 9 (30%) | 12 (24%) | 25 (26%) |
| Histology | | | | |
| NSCLC | 13 | 29 | 46 | 88 |
| Adenocarcinoma | 1 (8%) | 15 (52%) | 26 (57%) | 42 (48%) |
| Squamous cell carcinoma | 9 (69%) | 2 (7%) | 8 (17%) | 19 (22%) |
| Adenosquamous | 0 | 1 (3%) | 0 | 1 (1%) |
| Large cell carcinoma | 1 (8%) | 0 | 1 (2%) | 2 (2%) |
| Unspecified | 2 (15%) | 11 (38%) | 11 (24%) | 24 (27%) |
| Malignant (non-NSCLC) | 2 | 1 | 3 | 6 |
| Nonmalignant | 0 | 0 | 2 | 2 |

NSCLC, non-small cell lung cancer.

The patient populations are demographically homogeneous across the three groups, dominated by males (72%) and smokers (71%). The histologic subtype distribution reflects the intrinsic clinical presentation and tissue acquisition technique used: adenocarcinomas and squamous cell carcinomas are usually situated peripherally and centrally, respectively, and therefore tend to be biopsied via the percutaneous and endoscopic routes, respectively. The larger amount of tissue obtained through bronchoscopy and core biopsy, as compared with fine needle biopsy, likely accounts for the a lower proportion of samples that cannot be confidently subtyped (NSCLC unspecified: bronchoscopy 15% and core biopsy 24% versus fine needle aspirate 38%).

## Detection of EGFR, KRAS, p53, and CMET Mutations in 88 Samples of Advanced Stage NSCLC

Table 2 shows the numbers, types and distribution of mutations detected in 43 of 88 samples of advanced stage NSCLC using direct sequencing of WGA-amplified genomic DNA from low-volume lung biopsies. Mutations were identified regardless of the mode of tissue acquisition: 21 of 46 core biopsies (46%), 3 of 13 bronchoscopic biopsies (23%), and 19 of 29 fine needle aspirates (66%).

The proportion of samples bearing *EGFR*, *p53*, *KRAS*, and *CMET* sequence variations were 21 of 88 (24%), 24 of 88 (27%), 5 of 88 (6%), and 1 of 88 (1%), respectively. The two commonest *EGFR* mutations, exon 19 deletion and exon 21 L858R missense mutation, were present in 33% ($n = 7$) and 48% ($n = 10$), respectively, of samples with *EGFR* mutations. There were three samples that had double *EGFR* mutations. As previously described elsewhere, *EGFR* and *KRAS* mutations were mutually exclusive. However, there were 7 samples with dual *EGFR* + *p53* mutations, and 1 with *KRAS* + *p53* mutations.

We observed the well-known correlation of *EGFR* mutations with clinical phenotypes of female gender ($p = 0.0014$), adenocarcinoma subtype ($p = 0.00003$) and non-smoking history ($p = 0.0002$), using the Fisher's exact test.

## Assessing Association of Mutational Status and Survival

Figure 3*A* shows a Kaplan Meier probability plot of overall survival in the 88 NSCLC patients whose tumors were subjected to mutational analysis as described above. At last observation, 56 were alive. The censoring proportion was considerably high (approximately 64%).

To determine the impact on survival by mutational status, we considered the Cox proportional hazards regression model. Other variables, e.g., age, gender, were also taken into account. The results are shown in Table 3. Overall, there was a survival advantage for patients who received systemic treatment (hazard ratio: 0.312, $p = 0.03$); they comprised 56% (49 of 88) of the group. There were 10 patients treated with TKI, of which 7 were *EGFR* mutation positive. Treatment decisions were made independent of knowledge of


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**TABLE 2.** Clinicopathological Factors and Mutations Detected in Advanced-Stage NSCLC Tumors by Direct Sequencing of WGA-Amplified Genomic DNA from Low-Volume Lung Biopsies

| Sample Identity | Gender | Age, yr | Cytology | Smoking Status | *EGFR* | *p53* | *Kras* | c-met | Biopsy Procedure |
|---|---|---|---|---|---|---|---|---|---|
| G73 | F | 58 | Adenosquamous carcinoma | NS | Exon 18: G719A (G>C) Exon 20: R776H (G>A) | Exon 8: C275F | | | Needle aspirate |
| V153 | M | 67 | Adenocarcinoma | S | Exon 18: E709A (A>C) Exon 21: L858R (T>G) | Exon 5: V157F | | | Core biopsy |
| V83 | M | 79 | Adenocarcinoma | S | Exon 19: 9bp del (L747-E749) Exon 19: A750P (G>C) | | | | Core biopsy |
| G75 | F | 44 | Adenocarcinoma | NS | Exon 19: 15bp del (E746-A750) | | | | Needle aspirate |
| V31 | F | 53 | Adenocarcinoma | NS | Exon 19: 15bp del (E746-A750) | Exon 7: R248W | | | Core biopsy |
| V71 | F | 70 | Adenocarcinoma | NS | Exon 19: 15bp del (E746-A750) | | | | Core biopsy |
| V139 | M | 76 | Adenocarcinoma | NS | Exon 19: 15bp del (E746-A750) | | | | Core biopsy |
| G66 | F | 60 | Adenocarcinoma | NS | Exon 19: 15bp del (L747-T751) | Exon 5: V172F | | | Needle aspirate |
| H13 | F | 81 | Adenocarcinoma | NS | Exon 19: 15bp del (K745-E749) | | | | Needle aspirate |
| G45 | M | 61 | NSCLC unspecified | S | Exon 20: M766T (T>C) | | | | Needle aspirate |
| V162 | F | 63 | Adenocarcinoma | NS | Exon 20: 6bp ins P774-H775 | | | | Core biopsy |
| V17 | M | 50 | Adenocarcinoma | NS | Exon 21: L858R (T>G) | Exon 8: R273C | | | Needle aspirate |
| P92 | M | 52 | Adenocarcinoma | S | Exon 21: L858R (T>G) | Exon 5: 4 bp ins W146 | | | Needle aspirate |
| V43 | 82 | M | Adenocarcinoma | S | Exon 21: L858R (T>G) | | | | Core biopsy |
| V104 | 63 | F | Adenocarcinoma | NS | Exon 21: L858R (T>G) | | | | Core biopsy |
| V141 | 76 | F | Adenocarcinoma | NS | Exon 21: L858R (T>G) | | | | Core biopsy |
| V180 | 68 | M | Adenocarcinoma | S | Exon 21: L858R (T>G) | | | | Core biopsy |
| B127 | 62 | M | Adenocarcinoma | S | Exon 21: L858R (T>G) | Exon 7: N239S | | | Forceps |
| V80 | 79 | F | Adenocarcinoma | NS | Exon 21: L858R (T>G) | | | | Core biopsy |
| V169 | 42 | M | Squamous cell carcinoma | S | Exon 21: L858R (T>G) | | | | Core biopsy |
| V197 | 69 | M | Adenocarcinoma | S | Exon 21: L858R (T>G) | | | | Core biopsy |
| B107 | 70 | M | Squamous cell carcinoma | S | | Exon 8: 1 bp del E297 | | | Forceps |
| G72 | 82 | M | Squamous cell carcinoma | S | | Exon 5: V173M | | | Needle aspirate |
| H4 | 59 | M | NSCLC unspecified | S | | Intron 6: 5 bp del | | | Needle aspirate |
| P78 | 72 | M | Adenocarcinoma | S | | Exon 6: 18 bp del E198 Exon 6: E204A | | | Needle aspirate |
| P81 | 67 | M | Squamous cell carcinoma | S | | Exon 6: E224D | | | Needle aspirate |
| T21 | 83 | M | NSCLC unspecified | S | | Exon 5: 4 bp del S149 | | | Needle aspirate |
| V20 | 78 | M | Adenocarcinoma | S | | Exon 7: G244C | | | Needle aspirate |
| V55 | 87 | M | Adenocarcinoma | S | | Exon 8: R273H | | | Needle aspirate |
| V56 | 59 | M | NSCLC unspecified | S | | Exon 5: 1 bp ins P152 | | | Core biopsy |
| V107 | 65 | M | Adenocarcinoma | S | | Exon 6: Y205F | | | Core biopsy |
| V112 | 76 | M | Squamous cell carcinoma | S | | Exon 7: E258D | | | Core biopsy |
| V113 | 76 | M | Adenocarcinoma | S | | Exon 8: R273L | | | Core biopsy |
| V121 | 47 | M | Adenocarcinoma | S | | Exon 7: G245V | | | Needle aspirate |
| V144 | 73 | M | Adenocarcinoma | S | | Exon 5: V173M Exon 6: V216L | | | Core biopsy |
| V152 | 72 | M | Large cell carcinoma | S | | Exon 5: 1 bp del P152 | | | Core biopsy |
| V154 | 49 | M | NSCLC unspecified | S | | Exon 7: R248W | | | Needle aspirate |
| V159 | 76 | F | NSCLC unspecified | S | | Exon 5: R158L | Exon 2: G12C (G>T) | | Needle aspirate |
| B151 | 63 | M | NSCLC | S | | | Exon 2: G12D (G>C) | | Forceps |
| P70 | 63 | F | Adenocarcinoma | S | | | Exon 2: G12C (G>T) | | Needle aspirate |
| V177 | 92 | F | Adenocarcinoma | S | | | Exon 2: G12C (G>T) | | Core biopsy |
| G80 | 78 | F | NSCLC unspecified | S | | | Exon 2: G12C (G>T) | | Needle aspirate |
| V138 | 70 | M | NSCLC unspecified | S | | | | 17 bp del splice region | |

NSCLC, non-small cell lung cancer; M, male; F, female; NS, neversmokers; S, smokers; EGFR, epidermal growth factor receptor; WGA, whole genome amplification; DNA, deoxyribonucleic acid.

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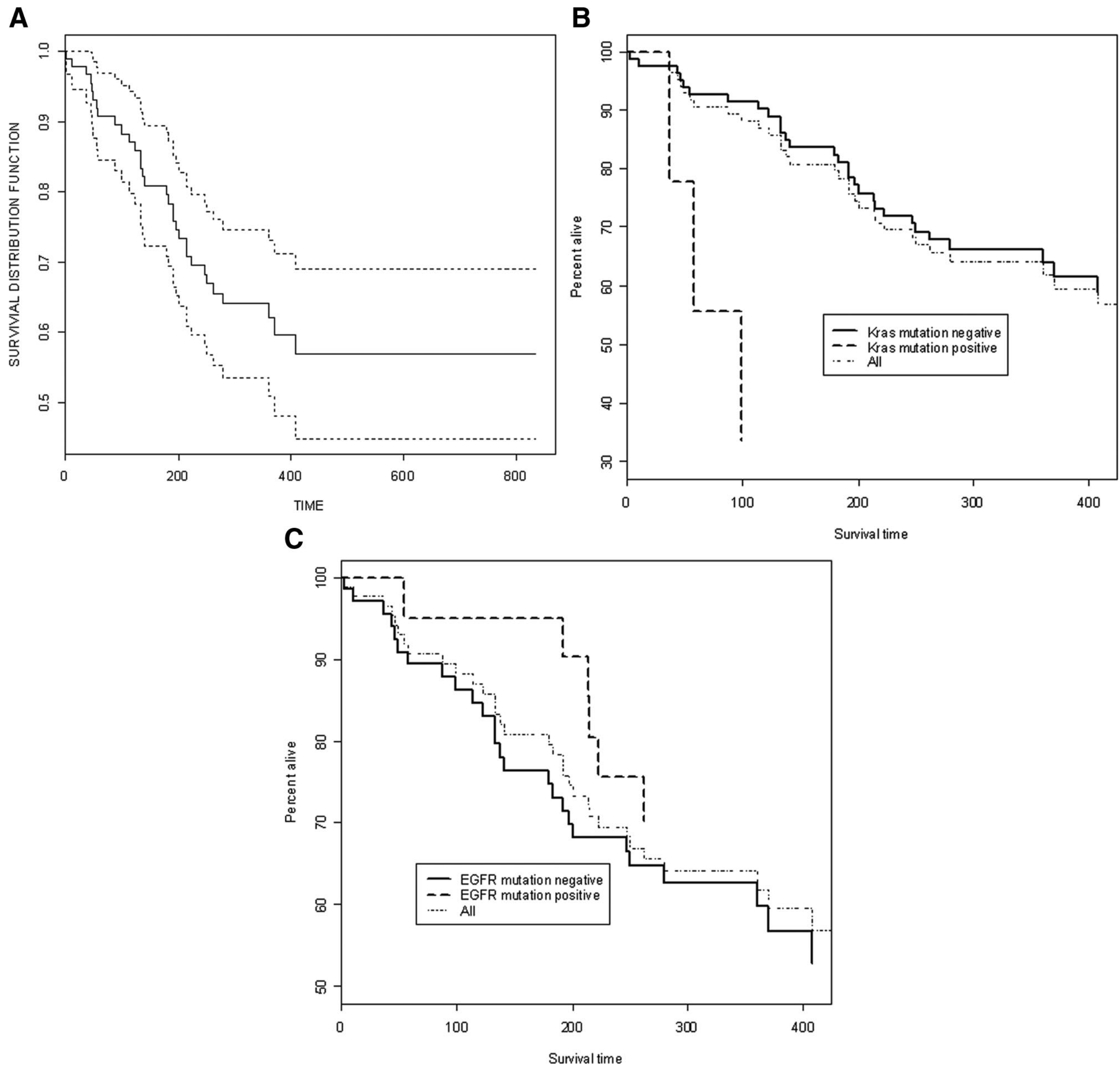


**FIGURE 3.** *A*, Overall survival of 88 non-small cell lung cancer (NSCLC) patients (survival time in days). The dotted lines indicate 95% confidence intervals. *B*, Survival curves of patients with and without *KRAS* mutations (survival time in days). *C*, Survival curves of patients with and without epidermal growth factor receptor (*EGFR*) mutations (survival time in days).

**TABLE 3.** Association of *KRAS*, *CMET*, *p53*, and *EGFR* Mutations with Overall Survival Using Cox proportional Hazards Regression Model

| | Coefficient (Standard Error) | Hazard Ratio | *p* |
|---|---|---|---|
| *KRAS* | 1.618 (0.619) | 5.05 | 0.009 |
| *CMET* | 3.163 (1.120) | 23.65 | 0.0048 |
| *p53* | −0.250 (0.415) | 0.778 | 0.55 |
| *EGFR* | −0.5379 (0.5881) | 0.584 | 0.360 |
| Age × *EGFR* | −0.0826 (0.0389) | 0.921 | 0.034 |

EGFR, epidermal growth factor receptor.

**TABLE 4.** Comparing *EGFR*, *KRAS*, and *p53* Mutation Frequencies Between Advanced-Stage and Early-Stage NSCLC

| | Advanced Stage NSCLC ($n$ = 88) | Early Stage NSCLC ($n$ = 133) | *p* |
|---|---|---|---|
| *EGFR* | 24% | 20% | 0.48 |
| *p53* | 27% | 29% | 0.75 |
| *Kras* | 6% | 10% | 0.27 |

NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor.


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mutational status. Whether patients received systemic treatment or not was also controlled for in the subsequent correlative studies on survival and gene mutations.

It seems that *KRAS* and *CMET* mutations are independently associated with decreased survival (Figure 3*B*, similar curve for *CMET*). Mutations in *EGFR* had no significant impact on survival, nor did mutations in *p53* (Figure 3*C*, similar curve for *p53*). Disruptive versus nondisruptive *p53* mutations also did not significantly affect survival (see Discussion). However, there was significant interaction between age and *EGFR* mutations, i.e., as age increases by 1 year, the hazard decreases to 92.1% of the original hazard. Hence, there is a protective effect due to *EGFR* mutations, which becomes stronger as the age at diagnosis is greater.

We also considered the effect on survival of combined mutations, i.e., *EGFR* + *p53*, *KRAS* + *p53*, but this was nonsignificant, using the likelihood ratios test.

## Comparison of Mutation Frequencies Between Advanced- and Early-Stage NSCLC

Table 4 shows the comparison in mutation frequencies between 133 surgically-resected early-stage disease, and this set of 88 patients with advanced-stage disease. There is no significant difference between both groups for mutations in *EGFR* ($p = 0.48$), *p53* ($p = 0.75$) and *KRAS* ($p = 0.27$), by using independent two-sample tests of proportions. The histologic distribution of the surgically-resected samples is 55% (73 of 133) adenocarcinoma and 45% (60 of 133) nonadenocarcinoma. All 27 of the *EGFR* mutations were found in the adenocarcinoma specimens. Of the 13 *KRAS* mutations, 12 were detected in adenocarcinomas and 1 in squamous cell carcinoma. The *p53* mutations were more evenly distributed, where 23 of 38 (61%) were found in adenocarcinomas. *EGFR* and *KRAS* mutations were mutually exclusive. The squamous cell carcinoma specimen that possessed the *KRAS* mutation also bore a *p53* mutation. All the other double mutations occurred in adenocarcinomas, 12 with *EGFR* + *p53* mutations, and 3 with *KRAS* + *p53* mutations.

## DISCUSSION

The scarcity of genomic DNA from clinical samples has been a limiting factor in the comprehensive molecular analysis of advanced-stage NSCLC. Many investigations, including array-based technology for genome-wide association studies, require DNA of sufficiently high biologic integrity, quality and quantity. The development of whole genome amplification (WGA) has allowed for high-fidelity in vitro reproduction of quality template DNA.[11–14] Many commercially available WGA kits use the multiple displacement amplification technology, which carries out isothermal genome amplification with a uniquely processive DNA polymerase capable of replicating up to 100 kb without dissociating from the genomic DNA template. The DNA polymerase has a 3′ to 5′ exonuclease proofreading activity to maintain high fidelity during replication and is used in the presence of exonuclease-resistant primers to achieve high yields of DNA product (40 $\mu$g). The average product length is typically greater than 10 kb, with a range between 2 kb and 100 kb. There is resultant highly uniform amplification across the entire genome, with negligible sequence bias. We used the Qiagen REPLI-g whole genome amplification kit in this study.

From the technical perspective, demonstrating the feasibility of WGA in multiple mutational analysis in low-volume lung biopsies opens the door to further array-based analysis that require a greater amount of starting DNA material, e.g., DNA copy number variations, methylation. In fact, WGA-amplified genomic DNA has been subjected to array-based comparative genomic hybridization.[15] With WGA, early- and advanced-stage disease can be meaningfully compared on a full complement of standardized analysis platforms.

We had previously attempted to address the molecular differences between early- and advanced-stage NSCLC by first exploring the approaches towards maximizing the amount of molecular information obtainable from advanced-stage disease with current clinical procedures. The main drawback of analyzing low-volume lung biopsies is the paucity of starting DNA and RNA material. We had assessed the feasibility of RNA amplification of low-volume lung biopsy tissues separately acquired during the diagnostic procedure, from which we obtained RNA expression profiles.[5] The results supported the feasibility of the methods used. We also assessed the typical amounts of genomic DNA extractable from low-volume lung biopsies similarly acquired, and the limits of DNA mutation data (via direct sequencing) that could be eked out from them.[6] Here, we have shown that sequencing of WGA-amplified genomic DNA can reproduce mutations detected from unamplified genomic DNA. This concordance in sequence data was not extended beyond one mutation in the two low-volume samples analyzed (Results subsection two) because of the limited amount of extractable DNA per sample.

The clinical premise of this work was to assess the feasibility of WGA in analyzing a panel of mutations in advanced-stage NSCLC, which may in turn guide treatment decisions in future on a more individualized basis. Although only a few genes were assessed in this study, the number of genes that could be analyzed is potentially limitless, given the micrograms of amplified genomic DNA obtained. Although we are unlikely to ever be able to comprehensively test the fidelity of WGA-amplified genomic DNA, as we do not surgically resect primary tumors of advanced-stage disease for comparison's sake, the results presented here are encouraging. This technique allows the future evaluation of more gene mutations and their impact on prognosis and treatment decisions. If new treatments are developed that are targeted towards specific gene mutations, then this analysis platform is relevant, especially if treatment is considered in a combinatorial fashion. If the prognostic value of a set of mutations is validated, this may guide decisions on treatment initiation in patients with borderline performance status.

In this study, the statistically calculated deleterious prognostic influence of *KRAS* and *CMET* mutations is tempered by the small numbers of these mutations, hence diluting their possible clinical significance. Nonetheless, the de-

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creased survival associated with *KRAS* mutations in our study is partially consistent with other published work in both the early- and advanced-stage setting. In the TRIBUTE trial looking at advanced NSCLC treated with chemotherapy versus chemotherapy plus erlotinib, a retrospective subgroup analysis found that *KRAS* mutations did not have prognostic significance.[4] For resected NSCLC, a worse prognosis associated with *KRAS* mutation-containing lung tumors was initially described by Rodenhuis et al.[16] A subsequent meta-analysis[17] concluded that patients with *KRAS* mutations had a significantly poorer prognosis compared with *KRAS* wildtype, with a combined hazard ratio of 1.35 (95% confidence interval 1.16–1.56). However, analysis of *KRAS* mutational status in the JBR10 trial patients where adjuvant chemotherapy (cisplatin and vinorelbine) was compared with observation did not demonstrate prognostic nor predictive value.[18,19] Tumors bearing *KRAS* mutations also seem to be resistant to EGFR TKI therapy.[20–22] The TRIBUTE patients with *KRAS* mutations who received erlotinib with chemotherapy had poorer clinical outcomes than those treated with chemotherapy alone.[4] At present, there is no effective therapy targeting *KRAS*-bearing tumors, although an immunotherapeutic approach seems interesting.[23]

The MET receptor tyrosine kinase and its cognate ligand hepatocyte growth factor (HGF) promote cell proliferation, invasion, motility and angiogenesis in in vitro studies.[24–31] The clinical significance of the HGF/MET oncogenic signaling was initially described by Siegfried et al.[32], where high levels of HGF indicated worse clinical outcomes in early-stage NSCLC patients. In cell line studies, mutations of *CMET* have been identified that lead to its increased expression.[33] In our study, although *CMET* mutations did not occur frequently, it was significantly correlated with decreased survival. Drugs targeting MET have been developed that induce response in tumor xenograft models,[34] as well as being synergistic with other targeted agents,[35] further enhancing its potential relevance in clinical therapeutics.

The prognostic significance of p53 mutations and expression in NSCLC had been examined previously, with a few meta-analyses inclining towards abnormal p53 status being associated with poorer prognosis.[36–40] A recent analysis of p53 mutational status in resected NSCLC in the JBR10 trial did not support its prognostic value.[19] Our results also do not support the role of p53 as a prognostic indicator in advanced-stage NSCLC. We also did not discern any survival difference between disruptive versus nondisruptive p53 mutations, which did have a significant impact on prognosis in head and neck cancer.[41]

The lack of significant association between *EGFR* mutations and survival in our study was interesting. Several prospective trials have shown that tumors with *EGFR* mutations (most commonly being the exon 19 deletion and exon 21 L858R mutation) have a response rate of 75% when treated with EGFR TKI such as gefitinib and erlotinib.[10] In the subgroup analysis of the TRIBUTE trial, patients with *EGFR* mutations had significantly better clinical outcomes than *EGFR* wildtype, whether or not they received erlotinib. However, other studies of early-stage disease have not found significant differences in overall survival between patients with *EGFR* mutations and *EGFR* wildtype.[42,43] The interim results of the INTEREST trial looking at second-line treatment with gefitinib or docetaxel in advanced-stage NSCLC were presented at the World Congress on Lung Cancer in September 2007.[44] It seemed that there was no correlation between survival and EGFR abnormal status, i.e., mutations, gene copy number and expression, which was consistent with our results. The interaction between age of diagnosis and *EGFR* mutation was thought-provoking. We can only surmise that other larger-scale genomic changes associated with increasing age and cancer, e.g., global hypomethylation and the ‘spreading’ phenomenon[45,46] affecting gene expression, may interact with *EGFR* mutation-related molecular signals, giving rise to a net effect of survival benefit.

The comparison of mutation frequencies of *EGFR*, *KRAS*, and *p53* between early- and advanced-stage disease have not yielded significant differences in this study. This suggests the presence of these gene mutations in the early stages of carcinogenesis. The evolution of disease from early-stage to advanced-stage would probably be attributable to the acquisition of mutations in other genes, emphasizing the need to interrogate other genes responsible for increased invasive cell behavior with high metastatic potential. The rapid advancement of genome sequencing technology enhances greater efficiency in this endeavor, and WGA-amplified genomic DNA enables a comprehensive evaluation of low-volume lung biopsies from advanced-stage NSCLC. As mentioned earlier, WGA-amplified genomic DNA allows for concurrent analysis of advanced-stage NSCLC on multiple platforms, facilitating comparisons with similar analyses in resected early-stage NSCLC, thereby elucidating and refining the identification of genes implicated in disease progression from early- to late-stage.

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