Real-Time Reverse-Transcription Quantitative Polymerase Chain Reaction Assay Is a Feasible Method for the Relative Quantification of Heregulin Expression in Non–Small Cell Lung Cancer Tissue

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ABSTRACT: In preclinical studies, heregulin (HRG) expression was shown to be the most relevant predictive biomarker for response to patritumab, a fully human anti-epidermal growth factor receptor 3 monoclonal antibody. In support of a phase 2 study of erlotinib + patritumab in non–small cell lung cancer (NSCLC), a reverse-transcription quantitative polymerase chain reaction (RT-qPCR) assay for relative quantification of HRG expression from formalin-fixed paraffin-embedded (FFPE) NSCLC tissue samples was developed and validated and described herein. Test specimens included matched FFPE normal lung and NSCLC and frozen NSCLC tissue, and HRG-positive and HRG-negative cell lines. Formalin-fixed paraffin-embedded tissue was examined for functional performance. Heregulin distribution was also analyzed across 200 NSCLC commercial samples. Applied Biosystems TaqMan Gene Expression Assays were run on the Bio-Rad CFX96 real-time PCR platform. Heregulin RT-qPCR assay specificity, PCR efficiency, PCR linearity, and reproducibility were demonstrated. The final assay parameters included the Qiagen FFPE RNA Extraction Kit for RNA extraction from FFPE NSCLC tissue, 50 ng of RNA input, and 3 reference (housekeeping) genes (HMBS, IPO8, and EIF2B1), which had expression levels similar to HRG expression levels and were stable among FFPE NSCLC samples. Using the validated assay, unimodal HRG distribution was confirmed across 185 evaluable FFPE NSCLC commercial samples. Feasibility of an RT-qPCR assay for the quantification of HRG expression in FFPE NSCLC specimens was demonstrated.

KEYWORDS: Real-time reverse-transcription polymerase chain reaction (RT-qPCR), heregulin, non–small cell lung cancer, validation

Introduction

Lung cancer is currently the leading cause of cancer-related death in the United States,1 and non–small cell lung cancer (NSCLC) accounts for more than 83% of all primary lung cancers.2 Treatment options may include tumor resection (for resectable disease), radiation therapy, and/or chemotherapy or targeted and immune therapy (ie, immunotherapy).2 Although immunotherapy offers the promise of durable responses for a subset of patients,3,5 there are still unmet needs for patients who either do not respond to immunotherapy or who eventually relapse. Furthermore, data show that disease recurrence occurs in 18% to over 70% of all cases in which patients were treated with modalities such as tumor resection, curative-intent radiotherapy, and chemotherapy.6-9 Treatment efficacy and risk of developing toxicities to various cancer treatments also vary among patients and may be related to individual genetics.10 It is therefore crucial to establish reliable tests for predictive biomarkers that can help match patient tumor characteristics with appropriate drugs (ie, personalized medicine)10 to maximize the benefit to risk ratio, as well as guide clinical decision making.

Elevated expression of human epidermal growth factor receptor 3 (HER3) and its ligand heregulin (HRG) has been identified in various solid tumors, including NSCLC,11-15 and in NSCLC, HER3 and HRG may play a role in the mediation of resistance to anti–epidermal growth factor receptor (anti–EGFR) treatment.16,17 Although HER3 upregulation and
reactivation may play a role in resistance to EGFR tyrosine kinase inhibitors,\textsuperscript{16-19} in preclinical models, HRG has been shown to reverse EGFR sensitivity,\textsuperscript{20} suggesting possible synergism between HER3 and EGFR inhibition to more completely block HER signaling. Given that checkpoint inhibitors do not significantly increase survival in patients with EGFR-mutant NSCLC, combining an HER3 inhibitor with anti-EGFR treatment may provide another therapeutic option.

Patritumab, a fully human anti-HER3 monoclonal antibody, was studied in the phase 2 HERALD (HER family Antagonism in Lung cancer, Daiichi-sankyo) trial in combination with erlotinib in EGFR treatment–naive patients with advanced NSCLC who had failed at least 2 prior chemotherapies (NCT02350712).\textsuperscript{21} Patritumab has been shown to inhibit HRG-mediated signaling through the HER3 pathway.\textsuperscript{22-24} Preclinical studies showed that HRG expression, compared with HER3 and pH3R expression, was the most relevant predictive biomarker for patritumab response.\textsuperscript{25,26} It was hypothesized that high messenger RNA (mRNA) levels of HRG in tissue would correspond to greater clinical benefit to patritumab treatment.

Therefore, a sensitive and specific real-time reverse-transcription quantitative polymerase chain reaction (RT-qPCR) assay for use in the HERALD study was developed for the relative quantification of HRG expression in formalin-fixed paraffin-embedded (FFPE) NSCLC tissue samples. Herein, the development and validation of the HRG RT-qPCR assay in NSCLC are described.

**Materials and Methods**

Feasibility and assay development studies were performed to determine an optimal RNA extraction method (kit) for FFPE NSCLC tissues, optimal RNA input for RT-qPCR, primer/probe selection, selection of optimal reference (housekeeping) genes, and HRG expression levels in FFPE NSCLC specimens. The final expression assays selected for HRG RT-qPCR analysis were then validated for specificity, PCR efficiency, PCR linearity, and reproducibility. The validation was intended to demonstrate the performance and define the parameters of TaqMan Gene Expression Assays for HRG and reference genes to be used for RT-qPCR analysis of FFPE NSCLC samples.

**Cell lines and tissue samples**

Cell line cryovial stocks T47D (HRG negative, from ductal carcinoma) and A549 (HRG positive, from lung cancer) were cultured in 1:1 media (F12-K-Dulbecco Modified Eagle Medium). Cells were processed as either fresh (RNA extracted from frozen cell pellet) or to create a simulated FFPE sample. Cells were grown, pelleted, fixed, embedded, and sectioned, and sections were used for RNA extraction.

Matched frozen NSCLC, FFPE normal lung, and FFPE NSCLC tissue specimens were purchased from Asterand Bioscience (Detroit, MI, USA) and BioServe (Beltsville, MD, USA). Non–small cell lung cancer specimens (≥60% tumor cell content) were sectioned to a 5-µm thickness and were used for hematoxylin-eosin (H&E) staining and RNA extraction. Only those FFPE tissues that yielded RNA of acceptable purity (1.5–2.2 using a spectrophotometric absorbance ratio of A260/280) and acceptable functional performance (cycle threshold [Ct] < 35) via reference gene assay analysis were used.

**Identification of an optimal RNA extraction method**

RNA yields and quality from FFPE NSCLC samples were compared using the Qiagen FFPE RNA Extraction Kit (Qiagen, Germantown, MD, USA), the Ambion PureLink FFPE RNA Isolation Kit (purchased from Life Technologies, Carlsbad, CA), and a modified method using the BIOstic FFPE Extraction Kit developed by MO BIO (Carlsbad, CA, USA). Deoxyribonuclease treatment was not included in any of these methods. RNA yields were determined by A260/A280 ratios.

**Identification of optimal RNA input for RT-qPCR**

To identify the optimal RNA input for analysis of HRG expression levels in FFPE NSCLC patient samples, 3 different amounts of RNA (20, 50, and 100 ng) were tested. The 20-ng quantity was chosen as the starting level as this is the lowest RNA input amount recommended by the manufacturer for the complementary DNA (cDNA) synthesis kit. The 100-ng quantity was chosen as the final amount as this is the upper limit of cDNA reaction mix input recommended by the manufacturer for RT-qPCR reactions. Input amounts of cDNA generated from the Qiagen and MO BIO RNA extraction kits were tested using 20, 50, or 100 ng cDNA volumes in a 20-µL reaction mix. All RT-qPCR assays were performed in triplicate.

**HRG primer/probe selection**

For assay optimization experiments, 3 HRG primer/probe sets with amplicon sizes of 93 base pairs (bp) (HS00247620_m1 [primer/probe “A”]), 90 bp (HS01108479_m1 [primer/probe “B”]), and 72 bp (HS01101537_m1 [primer/probe “C”]) were analyzed. The 3 assays (primer/probe sets) were chosen due to their detection of HRG-α and HRG-β isoforms, which are important for the HER3 pathway, as well as for their small amplicon sizes. Because RNA in FFPE specimens is heavily fragmented, small amplicons are important for successful qPCR.\textsuperscript{27}
Identification of optimal reference genes for HRG data normalization

Preliminary reference gene screening. To select reference genes, preliminary screening was conducted across 32 potential genes using RNA extracted from fresh frozen A549 cells.

Final reference gene selection. The purpose of the final gene selection was to identify reference genes that are appropriate to be used for normalization of HRG expression levels in FFPE NSCLC tissue samples. Optimal reference genes should be expressed in the range of HRG levels found in HRG-positive FFPE NSCLC samples and should not be affected by sample matrix or tissue type (ie, normal versus tumor).

Matched frozen NSCLC versus FFPE NSCLC tissue. The expression levels of 8 reference genes (from the original 32 genes) and HRG were measured in frozen NSCLC tissue samples to confirm that their expression levels were close to HRG expression levels. The Ct value shift between matched frozen NSCLC and FFPE NSCLC tissue was expected to be similar among HRG and housekeeping genes.

Matched FFPE NSCLC versus FFPE normal lung tissue. To show that the reference gene expression was not biased between FFPE normal lung and FFPE NSCLC tissue, RNA was extracted from FFPE normal lung and FFPE NSCLC samples using the Qiagen FFPE RNA Extraction Kit. A preliminary assessment of HRG expression levels in FFPE normal lung tissue was also performed using 5 samples of FFPE normal lung tissues matched to the FFPE NSCLC cases and 3 nonmatched (random) FFPE normal samples.

HRG RT-qPCR assay validation

The purpose of the validation was to verify that the required performance characteristics of the RT-qPCR assays were met and determine whether the assays were suitable for relative quantification of HRG expression in FFPE NSCLC tissue samples.

Reverse-transcription quantitative polymerase chain reaction. RNA was extracted from FFPE normal lung and FFPE NSCLC samples using the Qiagen FFPE RNA Extraction Kit. Reverse transcription was completed with 1000 ng of RNA input in 40 μL total volume. First strand cDNA synthesis was performed using the Applied Biosystems High Capacity cDNA Synthesis Kit (Thermo Fisher Scientific, Carlsbad, CA, USA) according to manufacturer’s instructions. Complementary DNA reaction was further diluted to 80 μL to create a 12.5 ng/μL working stock. The RT-qPCR analysis was completed using 4 μL (50 ng cDNA) input in 20 μL total volume. All qPCR reactions were run in triplicate. The qPCR analysis was performed using Applied Biosystems predesigned TaqMan Gene Expression assays and TaqMan Gene Expression Master Mix on the Bio-Rad CFX96 real-time PCR machine. Cycling was per manufacturer’s instructions. Data analysis was performed using the Bio-Rad CFX96 version 1.5 software set with a threshold of 100 and an auto baseline.

Target specificity. Bioinformatic analysis was performed by Applied Biosystems, using proprietary primers and probes, to show that HRG assays specifically targeted HRG. Functional testing of each assay was performed using genomic DNA (gDNA) and cDNA from A549 cells (gDNA and cDNA derived from fresh cell pellets and FFPE samples, respectively), and target specificity was confirmed by visualization of PCR amplicons from HRG and the 3 selected reference gene assays on a 2% agarose ethidium bromide gel. The gene expression assays were run in triplicate using gDNA and cDNA templates.

PCR efficiency and linearity. To determine whether the target and reference gene assays could be used for relative quantification experiments, PCR efficiency and linearity were assessed to verify similarity across a range of template input amounts (0.01, 0.1, 1, 5, 10, 25, 50 and 100 ng) using Universal Human Reference RNA from Stratagene (La Jolla, CA, USA). Acceptance criteria for PCR efficiency were 90% to 110% for each assay, corresponding to a slope of ~3.6 to ~3.1. The coefficient of determination (R²) was calculated to determine the correlation between RNA input and Ct value for each assay. Each individual assay, HRG, HMBS, EIF2B1, and IPO8, must have an R² ≥ 0.99 to distinguish a 2-fold difference in template concentration. For each assay, 6 curves were run, in duplicate, over 3 days. The RT-qPCR analysis was completed with the gene expression assays HRG (primer/probe C), HMBS (Hs00609297_m1), EIF2B1 (Hs00426752_m1), and IPO8 (Hs00183533_m1) using the Bio-Rad CFX96 instrument platform.

Intra-run and inter-run reproducibility. For intra-run reproducibility, 6 RNA extractions were performed within 1 day for each FFPE sample. The number of tissue sections equivalent to 1.5 cm² tissue area from each case was used for each RNA extraction. The RT-qPCR analysis was completed within a single day. The mean ΔCt and standard deviation were calculated across intra-run replicates.

For inter-run reproducibility, the same set of FFPE NSCLC samples was subject to 5 separate RNA extractions (each set comprised 6 FFPE NSCLC samples) that were completed over 3 days. RNA extraction and RT-qPCR analysis were completed by 2 operators. The Universal Human Reference RNA was run as a positive control. No template controls were composed of qPCR master mix and water for confirmation of contaminant-free reagents.

Data analysis

Heregulin expression levels were normalized according to the following formula: ΔCt = Mean Ct HRG − Mean Ct (reference genes).
To further evaluate the precision of intra- and inter-run reproducibility, the fold difference in $\Delta Ct$ values was obtained from the set of 5 or 6 extractions per sample by the following formula (lower values represent higher expression):

$$2^{\frac{\Delta Ct (deviation)}{\Delta Ct (highest) - \Delta Ct (lowest)}} = \text{fold difference}$$

**HRG distribution in NSCLC tumor specimens**

In total, 200 NSCLC commercial tissue blocks were purchased from multiple commercial tissue depository vendors, and newly sectioned slides were used to test for HRG gene expression using the validated RT-qPCR assay. The distribution of NSCLC subtypes in the commercial samples was matched to the distribution of NSCLC subtypes observed in the HERALD study. Biopsies were performed between December 2009 and August 2013, and specimens were analyzed between 1 and 46 months post biopsy (time between biopsy and analysis was staggered to detect any possible variation in the integrity of the stored specimens over approximately 4 years).

**HRG mRNA distribution**. Shames et al. showed a bimodal distribution of HRG gene expression in squamous cell carcinoma of the head and neck tissue samples. In the HERALD study, HRG gene distribution was shown as a unimodal distribution among 103 samples. To confirm the unimodal distribution observed in a larger sample set, the NSCLC FFPE commercial tissue blocks were analyzed to show the distribution of HRG expression.

**Results**

**Identification of an optimal RNA extraction method**

When the Qiagen FFPE RNA and Ambion PureLink extraction kits were compared, better RNA yield and purity were obtained using the Qiagen kit (Supplemental Table S1). Subsequently, 7 FFPE NSCLC tissue samples and 2 FFPE cell line samples (A549 and T47D) were used in the comparison between the Qiagen FFPE RNA Extraction Kit and MO BIO–purified RNA kit. RNA yield and purity of RNA extractions were compared (Table 1). In all, 6 out of 7 NSCLC samples had higher RNA yield and better RNA purity with the Qiagen kit. The Qiagen FFPE RNA Extraction Kit was selected for use in the final assay validation studies.

**Identification of optimal RNA input for RT-qPCR and HRG primer/probe selection**

Replicate and mean Ct values were obtained for HRG assay performed with 20, 50, and 100 ng of RNA (Table 2). Among the 3 tested primer/probe sets (A, B, and C), C generated the smallest amplicon (72 bp) and had the best performance (per detection of HRG transcripts) and was thus selected for further validation studies.

**Identification of optimal reference genes for HRG data normalization**

Premature reference gene screening. During preliminary screening, most reference genes had higher expression levels than HRG expression in frozen A549 cells (data not shown). Seven reference genes (HMBS, PUM1, ABL1, IPO8, **Table 1.** Final RNA extraction comparison.

| RNA ORIGIN | RNA CONCENTRATION, ng/µL | TOTAL RNA YIELD, ng | A260/A280 |
|------------|---------------------------|---------------------|-----------|
|            | QIAGEN        | MO BIO             | QIAGEN        | MO BIO             | QIAGEN        | MO BIO             |
| FFPE NSCLC |                                           |                     |                     |                     |                     |
| 1173692B   | 119.7         | 74.6               | 3590             | 2239             | 1.78               | 1.84               |
| 1173127B   | 82.3          | 69.2               | 2470             | 2076             | 1.80               | 1.89               |
| 1173740B   | 154.7         | 191.2              | 4640             | 5735             | 1.88               | 1.95               |
| 2YHODAZN   | 104.1         | 80.3               | 3122             | 2409             | 1.90               | 2.05               |
| AF8ALAN9   | 45.5          | 35.7               | 1364             | 1070             | 1.81               | 2.03               |
| QISIZA1M   | 88.9          | 69.7               | 2666             | 2090             | 1.91               | 1.99               |
| TRQR7A81   | 258.1         | 167.5              | 7742             | 5025             | 1.94               | 1.76               |
| Cell lines |                                           |                     |                     |                     |                     |
| FFPE HRG+  | 65.4          | 91.2               | 1963             | 2735             | 1.90               | 2.06               |
| FFPE HRG−  | 215.9         | 103.6              | 6476             | 3108             | 1.94               | 1.95               |

Abbreviations: FFPE, formalin-fixed paraffin-embedded; HRG, heregulin; NSCLC, non–small cell lung cancer.

*HRG-positive cell line = A549 and HRG-negative cell line = T47D.*
EIF2B1, GADD45A, and TBP) that demonstrated similar expression levels to HRG in frozen A549 cells were selected for further testing; 1 additional reference gene with higher levels of expression (UBC) was also included, for a total of 8 reference genes.

For the primer/probe set C in frozen NSCLC samples, mean HRG Ct values ranged from 25 to 32 (mean 28.2) (Table 3). When RNA from frozen NSCLC tumor samples was analyzed, the mean Ct values for HMBS (24.5), GADD45A (25.1), and TBP (25.3) were closest to levels of HRG expression in frozen NSCLC samples (Table 4) and were therefore selected as the initial reference genes for subsequent analysis.

When the mean difference in Ct between matched frozen NSCLC and FFPE NSCLC samples for HRG and the 3 reference genes (HMBS, GADD45A, and TBP) was compared using 7 paired samples, the data indicated that the Ct value shift of TBP and GADD45A was significantly higher than the Ct value shift of HRG (data not shown). Therefore, HMBS, EIF2B1, IPO8, PUM1, and ABL1 were selected for further consideration.

Final reference gene selection

Matched frozen NSCLC versus FFPE normal lung tissue. The mean difference in Ct between matched FFPE NSCLC and FFPE normal lung tissue for the 5 reference genes (HMBS, EIF2B1, IPO8, PUM1, and ABL1) ranged from 6.7 to 9.2 (Table 5). The 3 reference genes with a difference closest to that observed for HRG (6.7) were IPO8 (6.7), EIF2B1 (7.7), and HMBS (7.9). These assays also demonstrated the lowest standard deviation (1.06, 1.31, and 1.32, respectively) across samples.

Matched FFPE NSCLC versus FFPE normal lung tissue. The mean difference in Ct between matched FFPE NSCLC and FFPE normal lung tissue for the 5 reference genes (HMBS, EIF2B1, IPO8, PUM1, and ABL1) ranged from 1.5 to 0.5 (PUM1) to -0.5 (IPO8) (Table 6). There did not appear to be bias in the expression level of the 5 reference genes due to tissue type (ie, normal versus tumor). The 3 genes with the smallest mean difference in Ct values were EIF2B1 (-1.2), ABL1 (-0.8), and IPO8 (-0.5). The lowest standard deviation across samples was observed for IPO8 (0.68), HMBS (0.72), EIF2B1 (0.75), and ABL1 (0.72).

Overall, IPO8, EIF2B1, and HMBS generated the lowest difference in standard deviation values for matched frozen NSCLC, FFPE normal lung, and FFPE NSCLC tissue, across all FFPE NSCLC samples (suggesting that they can perform well across varying RNA quality and are suitable for normalization of HRG Ct values), and were thus selected as the final 3 reference genes.
### Table 3. HRG gene expression assay analysis in frozen NSCLC samples.\(^a\)

| RNA ORIGIN | Ct VALUES FOR HRG PRIMER/PROBE C | MEAN | SD  |
|------------|---------------------------------|------|-----|
| Frozen NSCLC |                                 |      |     |
| 1173692F-30 | 28.2                            | 0.05 |     |
| 1173127F-30 | 29.8                            | 0.05 |     |
| 1173740F-30 | 32.0                            | 0.19 |     |
| 2YHOD      | 28.6                            | 0.02 |     |
| AF8AL      | 27.8                            | 0.06 |     |
| Q151Z      | 25.4                            | 0.02 |     |
| TRQR7      | 25.6                            | 0.09 |     |
| Mean       | 28.2                            | 2.29 |     |
| SD         |                                 |      |     |

**Fresh cell lines\(^b\)**

|                      |                   |     |     |
|----------------------|-------------------|-----|-----|
| HRG+                 | 25.5              | 0.05|     |
| HRG (−)              | 38.8              | NC  |     |

Abbreviations: Ct, cycle threshold; HRG, heregulin; NC, could not be calculated; NSCLC, non–small cell lung cancer.

\(^a\)Three HRG gene expression assays (only primer/probe C is shown) were tested in triplicate on 7 frozen NSCLC samples and 2 cell line RNA controls. Cell line RNA controls were included to correlate with previous work and not used in the final calculations for the mean and SD.

\(^b\)Primer/probe C = Hs01101537_m1.

\(^c\)HRG-positive cell line = A549 and HRG-negative cell line = T47D.

### Table 4. Reference gene expression analysis in frozen NSCLC tumor samples.\(^a\)

| RNA ORIGIN | UBC Ct | TBP Ct | GADD45A Ct | ABL1 Ct |
|------------|--------|--------|------------|--------|
| Frozen NSCLC |        |        |            |        |
| 1173692F-30 | 19.5   | 0.06   | 25.1       | 0.15   | 25.4 | 0.19 | 23.8 | 0.04 |
| 1173127F-30 | 20.2   | 0.03   | 26.1       | 0.12   | 24.6 | 0.13 | 24.3 | 0.07 |
| 1173740F-30 | 20.9   | 0.19   | 24.0       | 0.10   | 26.0 | 0.02 | 24.2 | 0.17 |
| 2YHOD      | 19.9   | 0.14   | 25.1       | 0.04   | 25.8 | 0.04 | 23.7 | 0.08 |
| AF8AL      | 19.4   | 0.19   | 25.8       | 0.19   | 24.0 | 0.08 | 23.2 | 0.09 |
| Q151Z      | 19.8   | 0.09   | 25.1       | 0.12   | 24.8 | 0.13 | 22.6 | 0.08 |
| TRQR7      | 20.6   | 0.09   | 26.1       | 0.07   | 24.9 | 0.08 | 23.9 | 0.11 |
| Mean       | 20.0   |        | 25.3       |         | 25.1 |    | 23.7 |    |
| SD         | 0.57   |        | 0.76       |         | 0.71 |    | 0.58 |    |

**Fresh cell lines\(^b\)**

|                      |        |        |            |        |
|----------------------|--------|--------|------------|--------|
| HRG+                 | 21.3   | 0.07   | 27.6       | 0.14   | 27.1 | 0.14 | 25.9 | 0.15 |
| HRG (−)              | 21.7   | 0.12   | 28.6       | 0.11   | 29.2 | 0.12 | 26.1 | 0.10 |
Table 4. (Continued)

| RNA ORIGIN   | Ct VALUES                      | PUM1 | HMBS | IP08 |
|--------------|--------------------------------|------|------|------|
|              | EIF2B1 MEAN SD                 | MEAN SD | MEAN SD | MEAN SD |
| Frozen NSCLC |                                |       |       |       |
| 1173692F-30 | 23.2 0.16 21.5 0.08 24.2 0.07 | 23.9 0.16 |       |       |
| 1173127F-30 | 25.0 0.21 22.2 0.06 25.0 0.16 | 24.7 0.09 |       |       |
| 1173740F-30 | 23.3 0.10 21.6 0.10 24.4 0.09 | 24.1 0.10 |       |       |
| 2YHOD       | 23.5 0.05 20.9 0.07 24.2 0.05 | 24.4 0.02 |       |       |
| AF8AL       | 23.2 0.16 21.1 0.09 23.8 0.12 | 23.5 0.12 |       |       |
| Q151Z       | 23.1 0.09 21.4 0.08 24.4 0.09 | 23.6 0.17 |       |       |
| TRQR7       | 24.4 0.02 22.5 0.03 25.3 0.08 | 24.6 0.18 |       |       |
| Mean        | 23.7 21.6 24.5                 | 24.1   |       |       |
| SD          | 0.72 0.58 0.53                 | 0.48   |       |       |
| Fresh cell lines |                               |       |       |       |
| HRG+        | 25.7 0.25 24.7 0.11 24.6 0.01 | 25.9 0.17 |       |       |
| HRG(−)      | 25.5 0.04 24.7 0.12 24.5 0.03 | 25.6 0.12 |       |       |

Abbreviations: HRG, heregulin; NSCLC, non–small cell lung cancer.

Eight reference gene assays were tested in triplicate on 7 extracted RNA samples and 2 cell line RNA control samples. Cell line RNA controls were included as controls for comparison across experiments. The cell line values were not used in the final calculations for the mean and SD.

Table 5. Comparison of Ct values in matched frozen to FFPE NSCLC tissue with HRG plus 5 reference genes.

| MEAN Ct VALUE                                | HRG (72 bp) | HMBS (64 bp) | EIF2B1 (75 bp) |
|----------------------------------------------|-------------|--------------|---------------|
|                                              | FROZEN NSCLC | FFPE NSCLC | ΔCt FFPE VS FROZEN NSCLC | FROZEN NSCLC | FFPE NSCLC | ΔCt FFPE VS FROZEN NSCLC | FROZEN NSCLC | FFPE NSCLC | ΔCt FFPE VS FROZEN NSCLC |
| FFPE NSCLC                                   |             |              |               |
| TRQR7A81                                     | 25.6        | 28.3         | 2.7           | 25.3        | 30.9        | 5.6           | 24.4        | 29.6        | 5.2           |
| 2YHODAZN                                     | 28.6        | 36.8         | 8.2           | 24.2        | 31.8        | 7.6           | 23.5        | 31.1        | 7.6           |
| QI51ZM                                       | 25.4        | 33.8         | 8.3           | 24.4        | 32.0        | 7.7           | 23.1        | 31.5        | 8.3           |
| AF8ALAN9                                     | 27.8        | 33.4         | 5.6           | 23.8        | 31.2        | 7.4           | 23.2        | 30.0        | 6.8           |
| 1173692B                                     | 28.2        | 36.5         | 8.4           | 24.2        | 34.0        | 9.8           | 23.2        | 32.1        | 9.0           |
| 1173127B                                     | 29.8        | 37.5         | 7.8           | 25.0        | 33.9        | 8.9           | 25.0        | 33.2        | 8.2           |
| 1173740B                                     | 32.0        | 37.9         | 5.9           | 24.4        | 32.4        | 8.0           | 23.3        | 31.9        | 8.5           |
| Mean                                         | 28.2        | 34.9         | 6.7           | 24.4        | 32.3        | 7.9           | 23.7        | 31.3        | 7.7           |
| SD                                           | 2.29        | 3.38         | 2.10          | 0.53        | 1.21        | 1.32          | 0.72        | 1.25        | 1.31          |

(Continued)
### Table 6.
Comparison of Ct values in FFPE NSCLC tissues and FFPE normal lung tissue for HRG plus 5 reference genes.

| Abbreviations: Ct, cycle threshold; FFPE, formalin-fixed paraffin-embedded; NSCLC, non–small cell lung cancer. |

| **MEAN Ct VALUE** | **IPO8 (71 bp)** | **ABL1 (91 bp)** | **PUM1 (89 bp)** |
|-------------------|------------------|------------------|------------------|
|                   | FROZEN NSCLC | FFPE NSCLC | ΔCt FFPE VS FROZEN NSCLC | FROZEN NSCLC | FFPE NSCLC | ΔCt FFPE VS FROZEN NSCLC | FROZEN NSCLC | FFPE NSCLC | ΔCt FFPE VS FROZEN NSCLC |
| **FFPE NSCLC**     |                 |                 |                     |                 |                 |                     |                 |                 |                     |
| TRQR7A81          | 24.6           | 29.3           | 4.6                 | 23.9           | 28.9           | 5.1                 | 22.5           | 28.3           | 5.8                 |
| 2YHODAZN          | 24.4           | 31.5           | 7.0                 | 23.7           | 32.4           | 8.7                 | 20.9           | 30.1           | 9.1                 |
| QISIZA1M          | 23.6           | 31.1           | 7.5                 | 22.6           | 31.7           | 9.1                 | 21.4           | 31.2           | 9.8                 |
| AF8ALAN9          | 23.5           | 29.5           | 6.1                 | 23.2           | 29.9           | 6.7                 | 21.1           | 29.3           | 8.2                 |
| 1173692B          | 23.9           | 31.6           | 7.7                 | 23.8           | 33.1           | 9.3                 | 21.5           | 32.0           | 10.5                |
| 1173127B          | 24.7           | 31.9           | 7.2                 | 24.3           | 32.3           | 8.1                 | 22.2           | 32.5           | 10.2                |
| 1173740B          | 24.1           | 30.9           | 6.8                 | 24.2           | 33.7           | 9.5                 | 21.6           | 32.1           | 10.4                |
| Mean              | 24.1           | 30.8           | 6.7                 | 23.7           | 31.7           | 8.1                 | 21.6           | 30.8           | 9.2                 |
| SD                | 0.48           | 1.03           | 1.06                | 0.58           | 1.70           | 1.63                | 0.58           | 1.60           | 1.70                |

### Table 5. (Continued)
HRG RT-qPCR assay validation

**Target specificity.** Polymerase chain reactions (HRG, HMBS, EIF2B1, and IPO8) produced amplification from a cDNA template, and no amplification was detected from the gDNA template. When PCR products were run on a 2% agarose ethidium bromide gel, a single expected band for HRG, HMBS, EIF2B1, or IPO8 was observed from HRG, HMBS, EIF2B1, or IPO8 PCR reactions, respectively, when cDNA was used as template; no band was observed from PCR reactions when gDNA was used as template (expected, because of the use of primers that are designed to span exon-exon boundaries; data not shown). These data demonstrated specificity of the primer/probes for HRG and the 3 reference gene assays.

**PCR efficiency and linearity.** For HRG and the 3 housekeeping genes (HMBS, EIF2B1, and IPO8), based on the mean values for slope and $R^2$ across 6 runs, the average PCR efficiency and linearity across 6 runs met the predefined PCR efficiency (90%-110%; slope −3.6 to −3.1) and coefficient of determination ($R^2 ≥ 0.99$) requirements (Table 7 and representative graphs in Figure 1).

**Intra-run and inter-run reproducibility.** The intra-run reproducibility experiments demonstrated that normalized HRG expression levels could vary from 1.1-fold to 5.4-fold between independent extractions (Table 8). In FFPE specimens, inter-run reproducibility experiments demonstrated that normalized HRG expression levels could vary from 1.1-fold to 2.5-fold between independent extractions, independent RT-qPCR runs, and 2 operators (Table 9). The fold difference in normalized expression levels for the positive control sample was 1.1. The results also support data from the PCR efficiency and linearity experiments which demonstrated that mean Ct values greater than 35 for either HRG or reference gene expression assays trend toward a higher SD. Variability in HRG ΔCt values can also be attributed to the use of serial tissue sections for each independent extraction due to tumor heterogeneity.

Results of the HRG gene expression assay validation study are summarized in Table 10; the performance of the validation met predefined acceptance criteria and the assay was deemed to be validated and suitable for use for analysis of clinical samples.

**HRG Distribution in NSCLC Tumor Specimens**

Of the 200 NSCLC commercial tissue blocks freshly sectioned and analyzed, most (59.0%) were adenocarcinoma subtypes (Table 11). The distribution of NSCLC subtypes in 200 NSCLC commercial samples was matched to the distribution of NSCLC subtypes observed in the HERALD study. Tumor stage was known for 197 of the 200 matched HERALD specimens in patients with histologically confirmed stage I (26.8%), stage II (37.1%), stage III (30.4%), and stage IV (5.7%) NSCLC; most of the specimens (52.5%) had ≥85% tumor content (91.5% had ≥65% tumor content).

**HRG mRNA distribution.** Figure 2 illustrates a unimodal distribution of HRG expression levels across 185 evaluable samples, with a median ΔCt of 4.6. Approximately, 43% to 44% of NSCLC samples could be classified as HRG “high expressors” ([HRG-high] high and low HRG expression was defined by the median ΔCt of blinded data from the RT-qPCR assay in the HERALD study).29

**Discussion**

An RT-qPCR assay was developed and validated for the relative quantification of HRG expression in FFPE NSCLC tissue samples. Assay feasibility was demonstrated by comparison of data from fresh and FFPE cell line pellets, matched frozen NSCLC and FFPE NSCLC samples, and FFPE normal lung and FFPE NSCLC. Performance was demonstrated and parameters were defined for the Applied Biosystems TaqMan Gene Expression assays for the target gene, HRG, and 3 reference genes (HMBS, IPO8, and EIF2B1). This validated RT-qPCR assay was subsequently used to measure HRG mRNA from FFPE tissue in the phase 2 HERALD study and in commercial samples, in patients with NSCLC.
Table 7. PCR efficiency and linearity for HRG and the 3 housekeeping genes (HMBS, EIF2B1, and IPO8).

| GENE TARGET | RUN 1 | RUN 2 | RUN 3 | RUN 4 | RUN 5 | RUN 6 | MEAN |
|-------------|-------|-------|-------|-------|-------|-------|------|
| PCR efficiency, % |       |       |       |       |       |       |      |
| HRG         | 93.3\(^a\) | 97.6\(^a\) | 92.6\(^a\) | 91.0\(^a\) | 98.1\(^a\) | 88.5\(^a\) | 93.5\(^a\) |
| HMBS        | 94.8   | 93.3   | 91.2   | 97.8   | 99.0   | 102.3 | 96.4 |
| EIF2B1      | 93.3   | 93.6   | 94.7   | 92.5   | 93.8   | 98.4  | 94.4 |
| IPO8        | 100.0  | 101.0  | 99.1   | 99.8   | 96.1   | 99.9  | 99.3 |

| R\(^2\)     |       |       |       |       |       |       |      |
|-------------|-------|-------|-------|-------|-------|-------|------|
| HRG         | 0.9966\(^a\) | 0.9922\(^a\) | 0.9986\(^a\) | 0.9954\(^a\) | 0.9958\(^a\) | 0.9972\(^a\) | 0.9960\(^a\) |
| HMBS        | 0.9988 | 0.9925 | 0.9925 | 0.9987 | 0.9986 | 0.9977 | 0.9965 |
| EIF2B1      | 0.9987 | 0.9994 | 0.9988 | 0.9986 | 0.9982 | 0.9979 | 0.9986 |
| IPO8        | 0.9992 | 0.9986 | 0.9989 | 0.9991 | 0.9989 | 0.9984 | 0.9989 |

Abbreviation: HRG, heregulin.

\(^a\)No amplification was seen in the HRG assay at 0.01 ng complementary DNA. The calculation was based on 7 data points instead of 8 data points.

Figure 1. PCR efficiency and linearity for HRG and 3 housekeeping genes: (A) HRG\(^a\)\(^b\) (B) HMBS\(^b\), (C) EIF2B1\(^b\), and (D) IPO8\(^b\). Ct values as a function of input template. Ct indicates cycle threshold.

\(^a\)The HRG figure represents a 7-point curve analysis due to no amplification seen in lowest point of 0.01 ng RNA.

\(^b\)Each point represents the mean Ct across 6 runs.
The RT-qPCR assay was used to analyze 2 sets of samples. The first set included 186 FFPE NSCLC tissue samples procured from commercial vendors. When plotted, the distribution of expression levels of 185 evaluable samples was representative of a unimodal distribution (unimodal HRG distribution was confirmed in a separate analysis; data not shown). This finding is in contrast to a study by Shames et al., in which data showed a bimodal distribution of HRG (on a log10 scale) in squamous cell carcinoma of the head and neck. This may be due to an insufficient number of samples used to define the bimodal distribution, different assay parameters, different tumor samples (primary vs metastasis), or different tumor histology. In addition, these results were comparable with the distribution observed in the samples from the phase 2 HERALD trial.

Acceptable RT-qPCR results were obtained from tissue samples ranging in age from 1 month to approximately 4 years from the time of collection. A limitation to the RT-qPCR assay is that, compared with immunohistochemistry (IHC), multiple tissue sections are required for RNA extraction. The availability of sufficient tissue sections (1000 ng RNA needed for a final 50-ng yield for assay input) may pose a challenge in the clinical trial

Table 8. Intra-run reproducibility: normalized Ct values for HRG (ΔCt) for each RNA extraction and FFPE sample and mean ΔCt deviation comparison.

| SAMPLE ID | INTRA-RUN ΔCt | INTRA-RUN MEAN ΔCt DEVIATION COMPARISONa |
|-----------|----------------|------------------------------------------|
|           | RUN 1 | RUN 2 | RUN 3 | RUN 4 | RUN 5 | RUN 6 | MEAN | SD | ΔCt HIGHEST | ΔCt LOWEST | X = HIGHEST – LOWEST | ΔCt DEVIATION (2Δ) |
| FFPE NSCLC |       |       |       |       |       |       |       |    |              |              |                              |                        |
| 1173692B  | 3.6   | N/A   | 3.3   | 3.0   | 2.4   | 3.0   | 0.43  |    | 3.6          | 2.4          | 1.2                           | 2.3                      |
| 1173127B  | 5.5   | 4.3   | 4.7   | 3.6   | 4.2   | 4.1   | 0.65  |    | 5.5          | 3.6          | 2.0                           | 3.8                      |
| 1173740B  | 7.4   | 7.0   | N/A   | 7.2   | 6.6   | 7.0   | 0.27  |    | 7.4          | 6.6          | 0.7                           | 1.7                      |
| 2YHODAZN  | 5.9   | 5.8   | 5.4   | 5.3   | 7.7   | 5.7   | 0.89  |    | 7.7          | 5.3          | 2.4                           | 5.4                      |
| TRQR7A81  | −1.6  | −1.7  | −1.5  | −1.4  | −1.7  | −1.6  | 0.11  |    | −1.4         | −1.7         | 0.3                           | 1.2                      |
| FFPE normal lungb | 3.3 | 3.0 | 3.1 | 3.0 | 3.4 | 3.1 | 0.16 |    | 3.4 | 3.0 | 0.4 | 1.3 |
| Positive controlc | 3.7 | 3.7 | 3.6 | 3.6 | 3.6 | 3.6 | 0.04 |    | 3.7 | 3.6 | 0.1 | 1.1 |

Abbreviations: Ct, cycle threshold; Ext., extraction (RNA); HRG, heregulin.
aΔCt deviation calculations using highest and lowest HRG expression values for each patient extraction set.
bTRQR7N58.
cUniversal Human Reference RNA.

Table 9. Inter-run reproducibility: normalized Ct values for HRG (ΔCt) for each run and FFPE sample and mean ΔCt deviation comparison.

| SAMPLE ID | INTER-RUN ΔCt | INTER-RUN MEAN ΔCt DEVIATION COMPARISONa |
|-----------|----------------|------------------------------------------|
|           | RUN 1 | RUN 2 | RUN 3 | RUN 4 | RUN 5 | MEAN | SD | ΔCt HIGHEST | ΔCt LOWEST | X = HIGHEST – LOWEST | ΔCt DEVIATION (2Δ) |
| FFPE NSCLC |       |       |       |       |       |       |    |              |              |                              |                        |
| 1173692B  | 3.5   | 3.8   | 3.7   | 4.2   | 3.6   | 3.7   | 0.27  |    | 4.2          | 3.5          | 0.7                           | 1.7                      |
| 1173127B  | 5.3   | 4.7   | 5.4   | 4.1   | 5.4   | 5.0   | 0.58  |    | 5.4          | 4.1          | 1.3                           | 2.5                      |
| 1173740B  | 6.6   | 7.9   | 7.1   | 6.9   | 6.6   | 7.0   | 0.53  |    | 7.9          | 6.6          | 1.3                           | 2.5                      |
| 2YHODAZN  | 5.4   | 5.5   | 5.2   | 6.0   | 6.2   | 5.7   | 0.43  |    | 6.2          | 5.2          | 1.0                           | 2.0                      |
| TRQR7A81  | −1.2  | −1.2  | −1.2  | −1.3  | −1.1  | −1.2  | 0.06  |    | −1.1         | −1.3         | 0.2                           | 1.1                      |
| FFPE normal lungb | 3.0 | 2.9 | 3.0 | 2.8 | 3.1 | 3.0 | 0.09 |    | 3.1 | 2.8 | 0.2 | 1.2 |
| Positive controlc | 3.5 | 3.6 | 3.6 | 3.6 | 3.6 | 3.6 | 0.04 |    | 3.6 | 3.5 | 0.1 | 1.1 |

Abbreviations: Ct, cycle threshold; HRG, heregulin.
aΔCt deviation calculations using highest and lowest HRG expression values for each patient extraction set.
bTRQR7N58.
cUniversal Human Reference RNA.
Table 10. Summary of HRG gene expression assay validation and final assay conditions for HRG gene expression assay analysis.

| MEASURE OR REQUIREMENT | FINAL RESULT |
|------------------------|--------------|
| **Assay validation**   |              |
| Tissue samples used    | FFPE NSCLC samples |
|                        | FFPE normal lung tissue |
| RNA sample used        | Universal Human Reference RNA |
| Cell lines used        | T47D (fresh and FFPE): HRG negative |
|                        | A549 (fresh and FFPE): HRG positive |
| Specificity            | Single amplicon generated with cDNA template |
|                        | No amplification with gDNA template |
| PCR efficiency and linearity | Target and reference gene expression assays amplify with an efficiency of 100 ± 10% and a coefficient of determination (R²) >0.99 across 7 or 8 RNA input amounts, respectively |
| Reproducibility        | Complete intra-run extractions and analysis within 1 day and inter-run extractions and analysis over 3 days |
| Intraplate             | Fold difference between highest and lowest ΔCt values <5.4 |
| Interplate             | Fold difference between highest and lowest ΔCt values <2.5 |

Abbreviations: cDNA, complementary DNA; FFPE, formalin-fixed paraffin-embedded; gDNA, genomic DNA; HRG, heregulin; NSCLC, non–small cell lung cancer; PCR, polymerase chain reaction.

Table 11. Characteristics of NSCLC commercial specimens used for RT-qPCR assay validation (N = 200).

| CHARACTERISTIC                        | N   | %    | ΔCt (N = 185)** | MEDIAN | RANGE |
|---------------------------------------|-----|------|-----------------|--------|-------|
| **NSCLC tumor subtype**               |     |      |                 |        |       |
| N = 200                               |     |      |                 |        |       |
| Adenocarcinoma                        | 118 | 59.0 | 4.9             | 1.7–9.4 |       |
| Squamous cell carcinoma               | 62  | 31.0 | 2.9a            | 0.4–8.1a |       |
| Large cell carcinoma                  | 18  | 9.0  | 6.2             | 1.1–9.2 |       |
| Adenosquamous carcinoma               | 2   | 1.0  | [4.3]f          | [4.3]f  |       |
| **Tumor stage**                       | N = 194d |    |                 |        |       |
| I                                     | 52  | 26.8 | 4.3             | 1.2–8.5 |       |
| II                                    | 72  | 37.1 | 4.6             | 0.4–9.2 |       |
| III                                   | 59  | 30.4 | 4.2a            | 1.1–9.4a|       |
| IV                                    | 11  | 5.7  | 3.6             | 0.6–8.1 |       |
| **Tumor content, %**                  | N = 200 |    |                 |        |       |
| 50–60                                 | 17  | 8.5  | 4.3             | 2.6–7.9 |       |
| 65–70                                 | 36  | 18.0 | 3.9             | 0.4–8.1 |       |
| 75–80                                 | 42  | 21.0 | 4.6             | 0.6–8.1 |       |
| 85–90                                 | 73  | 36.5 | 4.4a            | 0.5–9.4a|       |
| >95                                   | 32  | 16.0 | 5.9             | 1.3–8.9 |       |
| **Time from biopsy to analysis, mo**  | N = 197w |    |                 |        |       |
| 1–6                                   | 31  | 15.7 | 4.1             | 2.0–7.4 |       |
| 7–12                                  | 55  | 27.9 | 4.1             | 0.5–7.1 |       |
| 13–18                                 | 7   | 3.6  | 2.9             | 1.3–6.9 |       |
Table 11. (Continued)

| CHARACTERISTIC | N   | %   | ΔCt (N = 185)*a,b |
|----------------|-----|-----|-------------------|
| 19–24          | 31  | 15.7| 3.97              |
| 25–30          | 16  | 8.1 | 4.4              |
| 31–36          | 31  | 15.7| 4.8              |
| 37–42          | 15  | 7.6 | 5.8              |
| 43–46          | 11  | 5.6 | 5.6              |

Abbreviations: Ct, cycle threshold; NSCLC, non–small cell lung cancer.

*One specimen (squamous cell carcinoma, stage IIIA) was excluded from the analysis due to an outlier ΔCt value of −7.2.
†ΔCt results missing for 10 adenocarcinoma, 2 squamous cell carcinoma, 1 large-cell carcinoma, and 1 adenosquamous carcinoma specimen.
§Stage I includes stage IA, stage II includes stages IIA and IIB, and stage III includes stage IIIA.
N = 194: tumor stage was unknown for 6 specimens.
N = 197: biopsy dates were missing for 3 specimens.
ΔCt value available from only 1 patient with adenosquamous carcinoma.

Data from preclinical studies (mouse xenograft model) have demonstrated that HRG mRNA is the best biomarker for correlation of in vivo response to treatment with single-agent patritumab.25,26 A secondary objective of HERALD was to identify which patients would most likely benefit from the addition of patritumab to erlotinib treatment by defining a primary biomarker hypothesis prior to data unblinding and then testing.23 In HERALD, patients were randomized to treatment with erlotinib plus either placebo or patritumab (high or low dose), and levels of mRNA from HRG and the 3 reference genes (HMB5, EIF2B1, and IPO8) identified herein were measured by RT-qPCR. In 2 separate analyses (including a simulation analysis to account for any possible imbalances between arms in patients with sensitizing EGFR mutations [6.6%]),29 data indicated significant clinical benefits for patritumab-treated patients with high HRG mRNA levels.21,29 Subgroup analysis by HRG mRNA expression levels demonstrated clinical benefit as judged by hazard ratio (HR) for progression-free survival from patritumab in patients with high HRG mRNA levels, including those patients in the patritumab high-dose (HR: 0.37; P = .0283) and low-dose (HR: 0.29; P = .0027) treatment arms.21,29

Overall, data from this study demonstrated the feasibility of a RT-qPCR assay for the quantification of HRG expression in RNA-extracted FFPE NSCLC specimens. Data from the HRG distribution study in 200 NSCLC FFPE commercial tumor specimens further indicated a unimodal distribution of HRG levels (median ΔCt: 4.6). Previously published data (median ΔCt: 4.1) included the 186 FFPE samples from the current analysis plus 93 fresh frozen tissue samples.26 The slight difference between median ΔCt values is caused by different sample sets. The HRG RT-qPCR assay was analytically validated prior to clinical sample analysis in the HERALD phase 1/2b study in patients with advanced NSCLC, and, based on the results of HERALD, may have identified a subgroup of patients who will receive clinical benefits from patritumab therapy.

Figure 2. Unimodal HRG distribution in NSCLC commercial samples using the validated RT-qPCR assay. Ct indicates cycle threshold; HRG, heregulin.

*N = 185* with ΔCt = −7.2 excluded from the analysis.

setting, in which biopsy tissue, which may be used for multiple purposes, including morphological interpretation and IHC, may be inadequate for molecular studies.30 Another limitation is that, compared with IHC or RNAscope in situ hybridization (a commercially available branched in situ hybridization method31), higher tumor content is required for RT-qPCR to limit the inference of RNA extracted from normal cells. Immunohistochemistry can also show variations between individual cells in expression level, whereas RT-qPCR gives an average value across the sample. However, an overall advantage of RT-qPCR is an objective data output, in contrast to the subjective nature of IHC or RNAscope in situ hybridization data interpretation.
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Author Contributions
KN, MS, CS, and WF designed the study, JK and KS collected and assembled the data for the study. XJ, RAB, DF, CS, and WF performed data analysis and interpreted the data. All authors wrote the report and approved the final version of the manuscript.

Statement of Ethical Assurance
WF is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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