PTEN and PIK3CA gene copy numbers and poor outcomes in non-small cell lung cancer patients with gefitinib therapy

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) have provided a novel way to treat advanced non-small cell lung cancer (NSCLC; Shepherd et al, 2005; Thatcher Lancet 2002; Lou et al, 2003; Bianco et al, 2003; Lou et al, 2003; Antonia et al, 2007; Engelman, 2009). The expression of these enzymes has also been related to EGFR TKI resistance in preclinical models (Janmaat et al, 2003; Engelman et al, 2005; Yamasaki et al, 2007). Earlier work suggested an association between patient outcomes and the combination of high chromosome 7 copy number (CEN7; a surrogate for EGFR copy number gain due to polisomy) and PTEN expression in advanced NSCLC patients treated with gefitinib (Buckingham et al, 2007). The objective of this study is to evaluate potential relationships between gene copy numbers for PIK3CA (the catalytic subunit of PI3K), PTEN and EGFR, and outcomes for NSCLC patients treated with gefitinib.

MATERIALS AND METHODS

This was a retrospective analysis of specimens from 82 patients in the gefitinib Expanded Access Trial, treated for >1 week, and collected as described previously. (Buckingham et al, 2007) Eligibility criteria and methods for establishing clinical data were also described. Chart review and this study’s analyses were approved by the Rush University Medical Center Institutional Review Board.

Keywords: EGFR; PTEN; PI3KCA; lung cancer; gefitinib
In general, patients were previously treated with at least one chemotherapy regimen or were considered ineligible for chemotherapy.

Based on previous work showing higher survival with PTEN expression by immunohistochemistry (IHC), an exploratory analysis was undertaken to study the influence of PTEN copy number loss and PIK3CA copy number gains in conjunction with EGFR alterations by fluorescent in situ hybridisation (FISH) on the same gefitinib cohort previously analysed. As no prospectively validated EGFR FISH analysis has been published to date, EGFR gene copy number status was assessed using multiple measures: the average number of EGFR copies per cell (EGFR/cell), the average number of chromosome 7 copies per cell (CEN7/cell), the average number of EGFR copies per chromosome 7 copies (EGFR/CEN7), the percentage of cells with more copies of EGFR than chromosome 7 (EGFR/CEP7 gain), the percentage of cells with >2 copies of EGFR (EGFR gain), and the percentage of cells with >2 copies of chromosome 7 (CEN7 gain). Fluorescence in situ hybridisation analyses were carried out as follows. The formalin-fixed paraffin-embedded lung tumour tissues and cell pellets were analysed with a dual colour probe set (Abbott Molecular Inc, Desplains, IL, USA) comprising SpectrumOrange LSI EGFR and SpectrumGreen CEP 7 and a four-colour probe set comprising a probe spanning PTEN (LSI PTEN) labelled with SpectrumRed, a probe spanning PIK3CA labelled with Spectrum-Gold, and probes containing peri-centromeric repeat sequences specific for chromosomes 3 (CEN 3) and 10 (CEN 10) (Spectrum-Green CEP 3 and SpectrumAqua CEP 10). Probes with CEP and LSI designations were obtained from Abbott Molecular Inc.,

### Table 1 Patient characteristics

| Characteristic        | Number of patients (%) | Objective response (%) |
|-----------------------|------------------------|------------------------|
| Total                 | 82 (100)               | 12 (15)                |
| Age (years)           |                         |                        |
| ≥ 60                  | 62 (77)                | 8 (13)                 |
| < 60                  | 20 (23)                | 4 (20)                 |
| Gender                |                         |                        |
| Male                  | 37 (46)                | 5 (14)                 |
| Female                | 44 (54)                | 7 (16)                 |
| Smoking status        |                         |                        |
| Yes                   | 70 (85)                | 5 (7)                  |
| Never smoked          | 12 (15)                | 7 (58)                 |
| Histopathological subtype |                 |                        |
| Adenocarcinoma        | 56 (69)                | 10 (18)                |
| Other                 | 26 (32)                | 2 (8)                  |
| Performance status    |                         |                        |
| (0 – 1)               | 46 (57)                | 6 (13)                 |
| (2 – 4)               | 34 (43)                | 6 (17)                 |
| Previous chemotherapy |                         |                        |
| None                  | 14 (17)                | 2 (14)                 |
| One                   | 39 (49)                | 7 (18)                 |
| Two or more           | 28 (34)                | 3 (11)                 |

### Table 2 Univariate analyses

| Biomarkers          | Patients (%) | Median PFS months | Hazard ratio | P-value (log-rank test) | Median survival months | Hazard ratio | P-value (log-rank test) |
|---------------------|--------------|-------------------|--------------|------------------------|------------------------|--------------|------------------------|
| EGFR/cell Low       | < 4.5        | 59 (73)           | 2.53         |                        | 7.27                   |              |                        |
| EGFR/cell High      | ≥ 4.5        | 22 (27)           | 4.31         | 0.78                   | 9.44                   | 0.74         | 0.24                   |
| CEN7/cell Low       | < 4          | 67 (83)           | 2.86         |                        | 6.9                    |              |                        |
| CEN7/cell High      | ≥ 4          | 14 (17)           | 4.04         | 0.78                   | 17.2                   | 0.49         | 0.02                   |
| EGFR/CEN7 Low       | ≤ 1          | 24 (27)           | 2.04         |                        | 5.95                   |              |                        |
| EGFR/CEN7 High      | > 1          | 57 (73)           | 4.04         | 0.54                   | 8.78                   | 0.66         | 0.11                   |
| EGFR/CEN7 gain Low  | < 34%        | 41 (51)           | 2.47         |                        | 6.61                   |              |                        |
| EGFR/CEN7 gain High | ≥ 34%        | 40 (49)           | 4.32         | 0.58                   | 11.2                   | 0.68         | 0.1                   |
| EGFR gain Low       | < 70%        | 52 (64)           | 2.53         |                        | 7.27                   |              |                        |
| EGFR gain High      | ≥ 70%        | 29 (36)           | 4.31         | 0.82                   | 11.5                   | 0.73         | 0.2                   |
| CEN7 gain Low       | < 80%        | 68 (84)           | 3.22         |                        | 7.27                   |              |                        |
| CEN7 gain High      | ≥ 80%        | 13 (16)           | 4.32         | 0.73                   | 17.2                   | 0.62         | 0.12                   |
| PTEN loss Low       | < 20%        | 15 (21)           | 5.92         |                        | 20                     |              |                        |
| PTEN loss High      | ≥ 20%        | 58 (79)           | 3.25         | 1.47                   | 6.9                    | 2.13         | 0.01                   |
| PI3KCA gain Low     | < 40%        | 33 (46)           | 3.52         |                        | 9.6                    |              |                        |
| PI3KCA gain High    | ≥ 40%        | 39 (54)           | 3.61         | 1.46                   | 6.64                   | 1.31         | 0.27                   |

Abbreviations: CEN7 = chromosome 7; EGFR = epidermal growth factor receptor; PFS = progression-free survival; PI3KCA = phosphatidylinositol 3-kinase catalytic subunit alpha; PTEN = phosphatase and tensin homologue.

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and the four-colour probe set has been previously described. (Morrison et al, 2007) Fluorescence in situ hybridisation signals were enumerated in >40 cells per specimen to obtain copy numbers for each locus, and 72 specimens yielded results for all six probes. Gene copy number gain for EGFR, PTEN, and PIK3CA was defined as >2 gene copies per cell. Conversely, gene loss was defined as <2 copies per cell. Epidermal growth factor receptor/CEN7 was deemed high if the ratio was >1. The response variables considered include, PFS and overall survival (OS). The status of EGFR mutation (exons 19 and 21) was obtained for 55 of the specimens (as described in Buckingham et al, 2007). Statistical analyses were carried out on the total population of patients with FISH analyses and repeated for the EGFR wild-type and mutant populations. Descriptive statistics were obtained and Fisher’s exact test was used to measure the association among recurrence, survival, and categorised covariates. For purposes of tabular and time-to-event analyses, the biomarker measurements were divided into two classes (high/low) using optimally chosen marker specific thresholds in the absence of prior published cutoffs for PTEN and PI3KCA FISH testing. The differences in OS and PFS between the low and high biomarker groups were assessed by the log-rank test and Kaplan–Meier method was used to obtain estimates of OS and PFS curves. Predictors that were statistically significant or marginally significant in univariate analyses or were deemed to be clinically or biologically important were included as candidate covariates in multivariate Cox proportional hazards (PHs) regression models. Statistical analyses were performed using Version 9.2 of the SAS software, Version 7.0 of the JMP software (SAS Institute, Cary, NC, USA) and the statistical software R. All reported P-values are two sided and P-values between (0.05–0.10), (0.01–0.05), and (0.001–0.01) are reported as marginally significant, significant, and strongly significant, respectively.

RESULTS

The majority of patients included in this study were over the age of 60 (77%). In all, 54% were female, 85% had a smoking history, 69% had adenocarcinoma, 57% had Eastern Oncology Cooperative Group performance status of 0–1, and 83% had received previous chemotherapy as detailed in Table 1. The gain of PIK3CA is marked as >2 PIK3CA copies and high PIK3CA gain is measured PTEN-loss <20 or PIK3CA gain <40, n=45 PTEN-loss ≥20 and PIK3CA gain ≥40, n=27 Log-rank P=0.002 PTEN-loss <20 or PIK3CA gain <40, n=45 PTEN-loss ≥20 and PIK3CA gain ≥40, n=27 Log-rank P<0.001 Figure 1 Progression free survival (PFS) and OS by PTEN loss and PIK3CA gain. (A) Progression free survival in all patients. (B) Overall survival in all patients.

Figure 2 Progression-free survival (PFS) and OS by PTEN loss, PIK3CA gain and chromosome 7 (CEN7) polysomy. (A) Progression free survival in all patients. (B) Overall survival in all patients.
as the percentage of cells with PIK3CA gain being ≥40%. Similarly, a cutoff of 20% is used for PTEN loss (percentage of cells with <2 PTEN copies). The associations of PIK3CA gain, PTEN loss and the six EGFR-related parameters with PFS and OS were assessed by univariate analyses in Table 2. Chromosome 3 and CEN10 copy numbers did not provide useful associations with outcomes and were not included in the table. Of the six EGFR-related parameters examined, only CEN7/cell, cutoff = 4.0, provided statistically significant classification with respect to OS (P = 0.02). Epidermal growth factor receptor/CEN7 gain, cutoff = 34% (P = 0.02), and EGFR/CEN7, cutoff > 1.0 (P = 0.04), were the only EGFR-related parameters to provide statistically significant classification with respect to PFS. Individually, PIK3CA did not show a statistically significant relationship with the above endpoints, whereas high PTEN loss did correlate with worse OS (P = 0.01).

Thirty-eight percent of 72 patients had the double combination of high levels of PIK3CA gain (cutoff = 40%) and high levels of PTEN loss (cutoff = 20%) and had significantly shorter PFS (P = 0.002) and OS (P < 0.001) than the remaining patients (3.02 vs 4.27 months median PFS, 4.93 vs 12.3 months median OS).

Corresponding PFS and OS curve estimates are plotted in (Figure 1A and B), respectively. Thirty-one percent of the 72 patients had the triple combination of low CEN7/cell (cutoff = 4), high PIK3CA gain (cutoff = 40%) and high PTEN loss (cutoff = 20%), and experienced further shortened PFS (P < 0.001) and OS (P < 0.001) than the remaining patients (2.04 vs 4.21 months median PFS, 4.34 vs 12.3 months median OS). Corresponding PFS and OS curve estimates are plotted in (Figure 2A and B), respectively, for this three variate combination. Several EGFR-related parameters other than CEN7/cell provided highly significant associations in combination with PTEN loss and PIK3CA gain. Low EGFR gain (cutoff = 75%), high PTEN loss, and high PIK3CA gain provided highly significant associations with PFS (P < 0.001) and OS (P < 0.001), and grouping low EGFR/CEN7 gain (cutoff = 34%) with the same PTEN and PIK3CA parameters also provided high association with PFS (P = 0.002) and OS (P < 0.001). See Table 3 for double and triple covariate analyses.

Seventeen of 55 patients tested had an EGFR activating gene mutation in exon 19 or exon 21. Patients with EGFR gene mutation had superior PFS compared with EGFR wild-type patients (13.6 vs 3.25 months median PFS, P = .003) but the association with OS

### Table 3: Two and three markers analyses

| Biomarkers | Patients | % | Median PFS months | Hazard ratio | P-value (log-rank test) | Median survival months | Hazard ratio | P-value (log-rank test) |
|------------|----------|---|-------------------|--------------|------------------------|-----------------------|--------------|------------------------|
| **High PTEN loss and high PIK3CA gain** |          |   |                   |              |                        |                       |              |                        |
| No         | 45       | 62 | 4.27              | 2.23         | 0.002                  | 4.93                  | 2.38         | <0.001                 |
| Yes        | 27       | 38 | 3.02              | 2.02         |                        |                       |              |                        |
| **Low CEN7/cell, high PTEN loss, and high PIK3CA gain** |          |   |                   |              |                        |                       |              |                        |
| No         | 50       | 69 | 4.21              | 2.54         | <0.001                 | 4.34                  | 4.04         | <0.001                 |
| Yes        | 22       | 31 | 2.04              | 2.02         |                        |                       |              |                        |
| **Low EGFR/CEN7 gain, high PTEN loss, and high PIK3CA gain** |          |   |                   |              |                        |                       |              |                        |
| No         | 56       | 78 | 4.04              | 2.04         | 0.002                  | 4.93                  | 3.37         | <0.001                 |
| Yes        | 16       | 22 | 4.04              | 2.04         |                        |                       |              |                        |
| **Low EGFR, high PTEN loss, and high PIK3CA gain** |          |   |                   |              |                        |                       |              |                        |
| No         | 55       | 76 | 4.04              | 2.04         | 0.003                  | 4.93                  | 3.35         | <0.001                 |
| Yes        | 17       | 24 | 4.04              | 2.04         |                        |                       |              |                        |
| **Wild Type (n = 37)** |          |   |                   |              |                        |                       |              |                        |
| **High PTEN loss and high PIK3CA gain** |          |   |                   |              |                        |                       |              |                        |
| No         | 20       | 54 | 3.55              | 1.84         | 0.09                   | 5.49                  | 2.41         | 0.012                  |
| Yes        | 17       | 46 | 3.02              | 3.02         |                        |                       |              |                        |
| **Low CEN7/cell, high PTEN loss, and high PIK3CA gain** |          |   |                   |              |                        |                       |              |                        |
| No         | 24       | 65 | 3.35              | 2.41         | 0.02                   | 4.18                  | 6.77         | <0.001                 |
| Yes        | 13       | 35 | 3.35              | 3.04         |                        |                       |              |                        |
| **Low EGFR/CEN7 gain, high PTEN loss, and high PIK3CA gain** |          |   |                   |              |                        |                       |              |                        |
| No         | 27       | 73 | 3.35              | 1.94         | 0.098                  | 5.49                  | 3.97         | 0.001                  |
| Yes        | 10       | 27 | 3.35              | 2.04         |                        |                       |              |                        |
| **Low EGFR, high PTEN loss, and high PIK3CA gain** |          |   |                   |              |                        |                       |              |                        |
| No         | 26       | 70 | 3.25              | 1.84         | 0.13                   | 5.49                  | 3.97         | 0.001                  |
| Yes        | 11       | 30 | 3.25              | 2.04         |                        |                       |              |                        |
| **EGFR Mutant (n = 15)** |          |   |                   |              |                        |                       |              |                        |
| **High PTEN loss, high PIK3CA gain yes/no** |          |   |                   |              |                        |                       |              |                        |
| No         | 12       | 80 | 16.31             | 5.01         | 0.02*                  | 5.57                  | 8.14         | 0.002                  |
| Yes        | 3        | 20 | 4.54              | 4.54         |                        |                       |              |                        |
| **Low CEN7/cell, high PTEN loss, and high PIK3CA gain** |          |   |                   |              |                        |                       |              |                        |
| No         | 13       | 87 | 16.31             | 3.07         | 0.02*                  | 11.5                  | 4.35*        | 0.07                   |
| Yes        | 2        | 13 | 9.07              | 9.07         |                        |                       |              |                        |

Abbreviations: CEN7 = chromosome 7; EGFR = epidermal growth factor receptor; PFS = progression-free survival; PI3KCA = phosphatidylinositol 3-kinase catalytic subunit alpha; PTEN = phosphatase and tensin homologue. *P-values, though statistically significant, are reflective of a small sample size.
was not significant (23.8 vs 7.98 months OS, \( P = 0.18 \)). In the 37 patients with wild-type \textit{EGFR} and complete FISH data, patients with both high PIK3CA gain (cutoff = 40%) and high PTEN loss (cutoff = 20%) showed marginally significant shortening of PFS (3.02 vs 3.55 months median PFS, \( P = 0.09 \)) and significant shortening of OS (5.49 vs 12.3 months OS, \( P = 0.01 \)). Corresponding PFS and OS curve estimates are plotted in (Figure 3A and B), respectively. Wild-type patients with the triple combination of low CEN7/cell (cutoff = 4), high PIK3CA gain (cutoff = 40%), and high PTEN loss (cutoff = 20%) showed significant shortening of PFS (2.04 vs 3.35 months, \( P = 0.02 \)) and strongly significant shortening of OS (4.18 vs 12.5 months, \( P < 0.001 \)). Corresponding PFS and OS curve estimates for patients with and without this triple combination are plotted in Figure 3C and D, respectively. Triple combinations using EGFR/CEN7 gain and EGFR gain were also significant and are presented in Table 3. Of note, \( \sim 30\% \) of wild-type patients presented with an unfavourable triple combination and had shortened PFS and OS. In the small group of 15 patients with activating \textit{EGFR} mutations and complete FISH data, the combination of low CEN7/cell (cutoff = 4), high PIK3CA gain (cutoff = 40%), and high PTEN loss (cutoff = 20%) showed marginally significant shortening of OS (\( P = 0.07 \)).

The results of multivariate Cox PHs regression analysis are listed in Table 4 for the subset of patients tested for EGFR mutations with full FISH data (\( N = 52 \)). Chromosome 7/cell (cutoff = 4), PTEN loss (cutoff = 20%) and PIK3CA gain (cutoff = 40%) continued to have strongly significant association with OS after adjusting for gender, smoking status and histology, and PIK3CA gain was marginally associated with PFS. Epidermal growth factor receptor mutation did not show a significant association with OS, but had strongly significant association with PFS.

**DISCUSSION**

Patients with \textit{EGFR} mutations in exons 19 and 21 have been shown to have significantly higher response rates and improved PFS when treated with frontline gefitinib, and \textit{EGFR} mutation has become an

|                | PFS HR | P-value | OS  | P-value |
|----------------|--------|---------|-----|---------|
| Female         | 1.19   | 0.6     | 0.77| 0.45    |
| Smoker         | 1.48   | 0.35    | 1.32| 0.53    |
| Adenocarcinoma | 1.49   | 0.35    | 1.06| 0.9     |
| EGFR mutation  | 0.32   | 0.006   | 0.74| 0.47    |
| CEN7/cell <4   | 0.99   | 0.97    | 3.36| 0.006   |
| PTEN loss >20% | 1.62   | 0.26    | 4.06| 0.002   |
| PIK3CA gain >40% | 2     | 0.06*   | 3.02| 0.003   |

Abbreviations: CEN7 = chromosome 7; \textit{EGFR} = epidermal growth factor receptor; HR = hazard ratio; PFS = progression-free survival; PI3KCA = phosphatidylinositol 3-kinase catalytic subunit alpha; PTEN = phosphatase and tensin homologue.

Figure 3  (A) Progression-free survival in \textit{EGFR} wild-type patients (two covariates). (B) Overall survival in \textit{EGFR} wild-type patients (two covariates). (C) Progression-free survival in \textit{EGFR} wild-type patients (three covariates). (D) Overall survival in \textit{EGFR} wild-type patients (three covariates).
established criterion for selecting an EGFR TKI as first-line therapy in stage IV NSCLC patients. (Mok et al., 2009; Maemondo et al., 2010; Mitsudomi et al., 2010). In the absence of an EGFR-activating gene mutation, it seems likely that a functional EGFR pathway is necessary for EGFR TKIs to be effective. Much work has been done that correlates retrospectively applied EGFR gene copy number with outcomes in NSCLC patients treated with EGFR TKIs (Cappuzzo et al., 2005; Tsao et al., 2005; Zhu et al., 2008). A recent meta-analysis showed that high EGFR gene copy number was associated with longer survival in NSCLC patients treated with an EGFR TKI (Dahebreh et al., 2011). The most commonly applied criteria for FISH positivity is complex, has yet to be validated when prospectively applied to a clinical trial and seems to have less importance when outcomes in patients treated with TKIs vs second-line chemotherapy (Douillard et al., 2010). In this study, an exploratory analysis was conducted measuring EGFR copy number in several different ways. Both high chromosome 7 copy number and a high ratio of EGFR to chromosome 7 copy numbers were associated with either prolonged OS or PFS.

Preclinical work suggests that the striking benefit of EGFR TKI therapies in mutation-positive tumours is related to massive apoptosis (Sordella et al., 2004) involving PI3K and PTEN. Recently, PTEN loss has been suggested as a potential mechanism of EGFR TKI resistance in NSCLC, which contain activating EGFR mutations (Sos et al., 2009). In addition, multivariate analysis in our initial studies showed that PTE expression, detected by IHC, was significantly related to OS in gefitinib-treated patients (Buckingham et al., 2007). These considerations suggested PTEN might also be a determinant of the efficacy of EGFR TKis in EGFR wild-type tumours and prompted us to evaluate PTEN and PIK3CA gene copy number in our gefitinib-treated patients. Although PTEN gene copy number alone was significantly related to OS, this was not the case for PIK3CA. However, the combination of gene copy data for PTEN and PIK3CA was strongly associated with both PFS and OS, and may be a more useful stratification than PTEN alone (Table 3).

The most powerful correlate of improved survival was the combination of EGFR or chromosome 7 copy number with PIK3CA and PTEN copy numbers. Although our results were obtained in a relatively small, single arm study, gefitinib-treated patients whose tumours contained low CEN7/cell (or low EGFR/ CEN7 gain or low EGFR gain), high PTEN loss, and high PIK3CA gain, had significantly shorter PFS and OS than other patients. The poor outcome with this molecular signature was seen both in the entire group and in the wild-type EGFR subset. The subset of patients treated with gefitinib who are unlikely to benefit from treatment with EGFR TKIs.

In addition to the clinical implications of excluding patients who are unlikely to benefit from treatment with EGFR inhibitors, this selection strategy might have significant economic impact. Bradbury et al. (2010) recently reported that the cost–benefit ratio for erlotinib was marginal.Subset analysis showed that the cost–benefit ratio was more favourable in never smokers and in patients with high EGFR gene copy numbers. The authors recommended increasing efforts to identify the most cost-effective way to use EGFR TKIs.

Our observations might also be useful in designing combination regimens targeting both the EGFR TKIs and downstream pathways. Preclinical studies have shown that reduced PTEN expression increases cancer cell survival and proliferation, and has been associated with resistance to EGFR inhibitors in NSCLC and colon cancers, and resistance to trastuzumab in breast cancer (Bianco et al., 2003b; Fujita et al., 2006; Berns et al., 2007; Sierra et al., 2010). It was somewhat surprising to find that a relatively high percentage of tumours (80%) had PTEN gene loss (defined as ≥ 20% of cells with <2 copies of PTEN), and that relatively subtle PTEN loss was associated with significantly shorter PFS and OS. If additional studies yield similar results, relatively minor alterations in wild-type PTEN gene copy number might have prognostic and therapeutic implications for NSCLC patients in identifying a practical patient group likely to benefit from multi-targeted therapy.

Our observation that the combination of PTEN and PIK3CA gene copy number more strongly related to outcome than either marker alone is consistent with recent results reported in breast cancer patients treated with trastuzumab Berns et al. (2007), observed higher rates of disease progression on trastuzumab in breast cancer patients with either PIK3CA mutations or with low PTEN expression. Further, they suggested that assessing both molecular markers might be required for optimal prediction of disease progression during the treatment with trastuzumab. These preliminary observations might be particularly pertinent in defining the roles of PI3K inhibitors and mTOR inhibitors in NSCLC.

In summary, if our results with CEN7, EGFR, PIK3CA, and PTEN FISH analyses are confirmed in larger groups of patients, this molecular profile could have clinical and economic implications for patients being considered for EGFR TKI treatment. Similarly, if our ongoing PTEN and PIK3CA gene copy number study in erlotinib-treated patients shows results consistent with our observations in gefitinib-treated patients, evaluation of PTEN and PIK3CA gene copy numbers should be considered in single agent and combination trials testing PI3K and mTOR inhibitors in NSCLC patients.

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