Telomerase reactivation is a hallmark of human carcinogenesis. Increased telomerase activity may result from gene amplification and/or overexpression. This study evaluates the prognostic value of hTERT gene amplification and mRNA overexpression in 144 resectable non-small-cell lung cancer (NSCLC) specimens. The hTERT gene copy number was assessed by quantitative polymerase chain reaction (qPCR) on laser-capture microdissected tumour cells of 81 tumours, and by fluorescence in situ hybridisation (FISH) on a subset of 59 tumours. hTERT mRNA level was determined by reverse transcription (RT)–qPCR in 130 tumours. In total, 57% of (46 out of 81) primary NSCLC specimens demonstrated hTERT amplification, which was significantly more common (P < 0.001) in adenocarcinoma (30 out of 40) than in squamous cell carcinoma (13 out of 37). The hTERT mRNA overexpression was noted in 74% (94 out of 130) of tumours; it was more frequent in squamous cell than in adenocarcinoma (87 vs 68%, P = 0.03). Overexpression was significantly associated with amplification (P = 0.03), especially in adenocarcinoma. The hTERT gene amplification was prognostic for shorter recurrence-free survival (hazard ratio = 2.16, P = 0.03). These data indicate that gene amplification is an important mechanism for hTERT overexpression in lung adenocarcinoma and is an independent poor prognostic marker for disease-free survival in NSCLC.

Amplification of telomerase (hTERT) gene is a poor prognostic marker in non-small-cell lung cancer

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British Journal of Cancer (2006) 94, 1452–1459. doi:10.1038/sj.bjc.6603110 www.bjcancer.com

Keywords: hTERT; telomerase; laser-captured microdissection; qPCR; FISH

Telomerase is a ribonucleoprotein reverse transcriptase complex containing an RNA subunit hTERC and a protein catalytic subunit hTERT (Nakamura et al., 1997). The hTERT RNA is expressed universally in eukaryotic cells and hTERT expression has been correlated with activation of the telomerase complex (Nakamura et al., 1997; Kolquist et al., 1998; Arinaga et al., 2000). Telomerase activity is absent in most human adult somatic cells. With continuous cell proliferation, there is a progressive loss of telomeric DNA that ultimately may trigger replicative senescence. The forced expression of hTERT cDNA in normal human cells has led to extension of the replicative lifespan (Vaziri and Benchimol, 1998). An alternative mechanism for cellular immortalisation is by the telomerase-independent ALT pathway (Newbold, 2002). Activations of the hTERT or ALT pathways are obligate for senescence bypass and for neoplastic transformation of normal cells (Newbold, 2002). Telomerase activity and/or hTERT expression are increased in cancers and are prognostic factors in various cancer types (Harada et al., 1999; Bieche et al., 2000; Lee et al., 2001; Marchetti et al., 2002; Wang et al., 2002; Fujita et al., 2003; Krams et al., 2003; Ohali et al., 2003; Tchirkov et al., 2003; Lantuejoul et al., 2004). However, the clinical impact of hTERT expression or activity in non-small-cell lung cancer (NSCLC) remains controversial (Albanell et al., 1997; Taga et al., 1999; Hirashima et al., 2000; Komiya et al., 2000; Toomey et al., 2001; Marchetti et al., 2002; Wang et al., 2002; Fujita et al., 2003; Wu et al., 2003; Hsu et al., 2004; Lantuejoul et al., 2004). The clinical significance of increased hTERT gene copy number has not been investigated.

Multiple mechanisms may regulate hTERT expression and activity. There is considerable evidence that transcriptional activation plays a major role in regulating hTERT mRNA expression (Ducrest et al., 2002), and the latter is correlated with telomerase activity (Arinaga et al., 2000; Marchetti et al., 2002; Saretzki et al., 2002). However, post-translational modifications may also contribute to the regulation of hTERT activity (Kang et al., 1999). Increased expression of hTERT resulting from gene amplification was recently reported in embryonal brain tumours (Fan et al., 2003) and cervical carcinoma (Zhang et al., 2002). The hTERT gene is located on chromosome 5q15, a chromosomal arm that is commonly overrepresented or amplified in lung cancer (Luk et al., 2001). Amplification of the hTERT gene and a concomitant increase in telomerase activity has been reported in lung cancer cell lines and other cancer types (Zhang et al., 2000; Saretzki et al., 2002). In this study, we have investigated the frequency and prognostic significance of hTERT gene amplification and overexpression in NSCLC.
**MATERIALS AND METHODS**

**Patients and clinical samples**

Patients included in this study had undergone lobectomy or pneumonectomy for resection of their primary lung cancer but had not received prior radiation or chemotherapy. Altogether, 169 tissue samples from 144 patients were used; these included 144 tumours and a corresponding subset of 30 non-neoplastic lung tissues. The latter were used to define the normal ranges for hTERT mRNA expression levels and gene copy number. Tissues were collected within 30 min after resection, snap-frozen and stored in liquid nitrogen until used; all were verified by histopathology. The collection of tissue and clinical and follow-up data was carried out in accordance with guidelines established by the Research Ethics Board (REB) of the University Health Network (UHN), which also approved this study.

**DNA isolation and laser-captured microdissection**

DNA was isolated from tumour cells micro-dissected using the Arcturus Pixcell II (Mountain View, CA, USA) laser capture microdissection (LCM) system. This includes 40 adenocarcinomas (ADC), 37 squamous cell carcinomas (SQCC), three adenosquamous carcinomas (AD/SCC) and one large cell carcinoma (LCC). In addition, DNA was also extracted from 19 non-neoplastic lung tissues. The tumour cells micro-dissected using LCM system or incubated in DNA extraction buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.1 mg ml⁻¹ gelatin, 0.45% Nonidet P-40, 0.45% Tween 20 and 0.4 mg ml⁻¹ proteinase K. DNA was subsequently extracted by the phenol–chloroform method (Zhu et al., 2004).

**Quantitative polymerase chain reaction**

The quantitative polymerase chain reaction (qPCR) was performed using the SYBR Green technique in an ABI Prism 7700 Sequence Detection System (Applied Biosystem, Foster City, CA, USA). The primer sequences were: hTERT sense 5'-tta aat tat cca cat ggc tca cgt-3', antisense 5'-cct ggg aac cag cac aa ggg-3'; PIK3R1 sense 5'-atc tgc cac tgg ctc tt-3', antisense 5'-cag tct ttc ctt gat tga acc-3'. The PCR conditions were optimized as reported (Zhu et al., 2004). The PIK3R1 (Sqi3.1) gene is used as the reference nonamplified gene in NSCLC (Massign et al., 2002), and the hTERT gene copy number was estimated using comparative CT method. DNA from normal male lymphocyte (Novagen, San Diego, CA, USA) was used as the reference DNA. With this method, DNA from normal male lymphocyte (Novagen, San Diego, CA, USA) was used as the reference DNA. The CT method was used to account for sample-to-sample variation in RNA/cDNA quantity using the 18s ribosomal RNA as the housekeeping gene (Zhu et al., 2004).

**Fluorescence in situ hybridisation**

Archival paraffin blocks of 59 tumours that had been studied by qPCR were retrieved for fluorescence in situ hybridisation (FISH) analysis. Sections (4 μm) were mounted on positively charged slides and baked flat for 12–16 h at 56°C. Slides were dewaxed in three changes of xylene for 10 min each, followed by two changes in 100% ethanol for 5 min each. After air-drying, slides were treated in 2 × SSC for 20 min at 75°C, then for 5 min at room temperature (RT). The sections were then treated with 0.25 mg ml⁻¹ proteinase K (Roche, Laval, QC, Canada) in 2 × SSC at 45°C for 20 min, followed by washing in 2 × SSC at RT for 5 min and serial dehydration through 70, 90 and 100% ethanol, and then left to air dry.

The hTERT75q dual-colour FISH probe cocktail (Biogenex, Montreal, QC, Canada) was applied at 0.02–0.06 μl mm⁻² and sealed with rubber cement. The probe and target DNA were co-denatured by heating to 80°C for 10 min in a Hybrite slide incubator (Vysis/Abbott Laboratories, Markham, ON, Canada). Hybridisation was for 16–20 h at 37°C in a moist light-sealed chamber in a dry oven. The slides were washed in two changes of 2 × SSC with 0.1% SDS at 45°C for 5 min each, followed by 5 min in 2 × SSC at RT. Slides were partially air-dried and 20–30 μl of DAPI mounting medium with antifade (Vector Labs, Burlingame, CA, USA) was applied, then cover slipped without sealing. Slides were stored at low light in the dark at 20°C prior to imaging.

The FISH images were captured using the Axiolmage system (Zeiss, Göttingen, Germany) with Z-stacking capabilities. Tumour cell nuclei identified using a DAPI filter and Z-stacked three-channel colour images (DAPI, FITC and Rhodamine/Cy3) were captured at ×63 or ×100 under oil immersion. Intact, nonoverlapping tumour cell nuclei (minimum 50 per case) without juxtaposed FISH signals were scored for the number of green (Sp15.33) hTERT locus and red (Sp31) control signals. The surrounding nontumour cells provide baseline estimation of the normal FISH signals (two green and two red signals). For survival analysis, high gene copy number cases included tumours with high polysomy (>4 hTERT gene copy in more than 40% of the tumour cells) or amplification (presence of tight hTERT gene clusters and a ratio of hTERT to chromosome of ≥2 or >15 copies of gene per cell in ≥10% of analysed tumour cells), as defined by Cappuzzo et al. (2005) for their study of the role of epidermal growth factor receptor (EGFR) gene copy number in EGFR inhibitor therapy.

**Reverse transcription–qPCR**

The mRNA expression was assayed using reverse transcription (RT)–qPCR on total RNA of 130 primary NSCLC and 18 corresponding non-neoplastic lung tissues using the ABI PRISM 7700 Sequence Detection System (Zhu et al., 2004). Total cellular RNA was isolated from the frozen tissues, as previously described (Tsao et al., 1998) and purified by the RNeasy Mini kit (Qiagen Inc., Mississauga, ON, Canada). The quality of the RNA preparations was confirmed by the Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). In total, 5 μg of RNA was reverse transcribed using the Taqman reverse transcription reagent (Applied Biosystems, Branchburg, NJ, USA) in 100 μl reaction solution according to the manufacturer’s instruction. After appropriate dilution, duplicate of 10 ng of cDNA was used as template for qPCR analysis of each sample. Primers were designed to span two adjacent exons to avoid amplification of contaminating genomic DNA sequences. The primers for hTERT were: sense 5'-cctgctgctgccaggtct-3', antisense 5'-agt gctgctgctgctgctg-3'. The ACT method was used to normalize the sample-to-sample variation in RNA/cDNA quantity using the 18s ribosomal RNA as the housekeeping gene (Zhu et al., 2004).

**Statistical analysis**

The Spearman correlation, χ² tests or Fisher’s exact test were used appropriately to assess association within and between molecular indices and the pathological or clinical factors. The end points for analyses were overall survival (from date of surgery to date of death) and recurrence-free survival (from date of surgery to date of recurrence). Cox proportional hazards regression was used in univariate and multivariate analyses. For Kaplan–Meier analysis, gene copy number and mRNA expression level were dichotomized using the upper limits of 95% confidence interval (95% CI) (mean ± 2 s.d.) for normal samples into nonamplified vs amplifying or normal expression vs overexpression groups. Kaplan–Meier
analysis estimates the survival of patient groups, and significant differences were determined by the log-rank test.

RESULTS

Patient characteristics

Table 1 shows the demographics of patients in the studies of hTERT gene copy assessment by qPCR (n = 81) or FISH (n = 59), and hTERT mRNA expression (n = 130) by RT–qPCR. There were no significant differences in the age, gender, stage and tumour differentiation grade among the three groups, but the mRNA expression study included more ADC patients. More than 90% of patients were stage I–II. The median follow-up was 3.19 (0.24–7.95) years, and 11 patients died without a relapse.

hTERT gene amplification

Figure 1A shows the distribution of relative gene copy of hTERT in normal and NSCLC. Using the upper limit of 95% CI (mean + 2 s.d.) for normal samples as cutoff, hTERT gene amplification was found in 57% (46 of 81) of NSCLC patients. Gene copy loss was not observed. Amplification was more common in ADC compared to SQCC (Figure 1A), but was not correlated with tumour stage or differentiation grade (Table 2). The Kaplan–Meier survival estimation showed that patients with hTERT amplification by qPCR had poorer recurrence-free survival (log rank test P = 0.02, Figure 2A). An analysis using 2 × mean of normal + 2 s.d. as the cutoff to identify highly amplified patients showed statistically not significant separation of the survival curves of amplified vs unamplified patients, but further analysis showed that patients with gene copy changes between ≥mean of normal + 2 s.d. and <2 × mean of normal + 2 s.d. showed similar survival outcome as the highly amplified (≥mean of normal + 2 s.d.) group, indicating that low amplification patients also experienced poorer survival outcome (Supplementary Figure 1). A similar trend of poorer overall survival for patients with amplified hTERT gene was noted, but this did not reach significance (log rank test P = 0.15, Figure 2B).

Cox proportional hazards regression also showed a significant association of hTERT amplification with increased risk for death from recurrence (hazard ratio (HR) 2.16, 95% CI 1.07–4.37; P = 0.03), but the correlation with poorer overall survival did not reach significance (HR 1.70, 95%CI 0.82–3.52; P = 0.16). The hTERT amplification remained a significant prognostic marker for shorter recurrence-free survival (HR 2.06, 95%CI 1.01–4.2; P = 0.05) after adjusting for the patient age, tumour stage and differentiation grade.

The P-values were calculated using the χ² test. ADC = adenocarcinoma; SQCC = squamous cell carcinoma; WD = well differentiated; MD = moderately differentiated; PD = poorly differentiated; qPCR = quantitative polymerase chain reaction; FISH = fluorescence in situ hybridisation.
Because there was a high frequency (38%) of patients who were lost to follow-up at greater than 3 years after surgery, the 3-year survival rates were also estimated (Table 3). hTERT amplification was significantly associated with poorer recurrence-free survival (HR 2.96, 95% CI 1.27–6.90, \( P = 0.01 \)) and overall survival (HR 2.04, 95% CI 0.89–4.66, \( P = 0.09 \)) at 3 years. Multivariate analysis...
 Validating of qPCR data with FISH

Fluorescence in situ hybridisation was performed on 59 tumours that had been studied for hTERT gene copy by qPCR (Figure 3). The ratios between the hTERT (green probe) and 5q13 reference locus D5S89 (red probe) signals were significantly correlated with the qPCR ratios of hTERT/PIK3R1 gene content (Spearman correlation coefficient $r = 0.43$, $P = 0.0006$). A better correlation was found for ADC ($r = 0.61$, $P = 0.0003$) than for SQCC ($r = 0.34$, $P = 0.086$). hTERT gene amplification by FISH was found in 73% (43 out of 59) of tumours and there were significant correlations between qPCR and FISH results for all tumours ($P = 0.008$, Table 4) and for ADC ($P = 0.004$). Although patients with high hTERT gene copy number (high polysomy and amplification) by FISH were more likely to have early recurrence compared to those with lower gene copy numbers (low polysomy, trisomy or disomy), the difference was not statistically significant (HR 1.51, 95% CI 0.61 – 3.76, $P = 0.37$).

hTERT mRNA expression

Reverse transcription–qPCR did not detect hTERT mRNA expression in several non-neoplastic lung samples; therefore, the expression level of each sample was arbitrarily represented relative to the median of the entire data set. We used the mean + 2 s.d. of the non-neoplastic lung expression levels as the cutoff to dichotomise tumours into hTERT normal expression and overexpression groups. Overexpression occurred in 72% (94 out of 130) of NSCLC, but was significantly more frequent in SQCC (87%) compared to ADC (68%) (Figure 1B and Table 2). Overexpression was also associated with higher tumour stages (Table 2). Among tumours with expression data, hTERT gene copy results by qPCR were also available for 67 cases (Table 5). Overexpression correlated with amplification ($P = 0.03$) but only among the ADC ($P = 0.05$). There were only 54 tumours with both FISH and expression results; high hTERT gene copy by FISH was not correlated with mRNA overexpression (data not shown).

| Table 4 Correlation between gene copy by qPCR and by FISH |
|---------------------------------------------------------|
| **Gene copy changes** | **FISH** | **P-value** |
| | **qPCR Nonamplified** | **Amplified$^a$** | |
| All | 22 | 37 | |
| Nonamplified | 13 | 9$^e$ | 0.008 |
| Amplified | 9 | 28 | |
| Adenocarcinoma | Nonamplified | 6 | 1 | 0.004 |
| | Amplified | 5 | 18 | |
| Squamous cell carcinoma | Nonamplified | 7 | 7 | 0.5 |
| | Amplified | 4 | 8 | |

$^a$Amplified tumours were those showing presence of tight hTERT gene clusters or hTERT to chromosome ratio of $\geq 2$, or $\geq 15$ copies per cell in $\geq 10\%$ of tumour cells, as defined by Cappuzzo et al (2005). $^e$P-values calculated using the two-sided Fisher’s exact test. One case was a large-cell carcinoma. qPCR = quantitative polymerase chain reaction; FISH = fluorescence in situ hybridisation.

| Table 5 Correlation between gene copy increases by qPCR and hTERT mRNA expression |
|---------------------------------------------------|
| **Gene copy changes** | **Nonamplified** | **Amplified** | **P-value$^a$** |
| All | 27 | 40 | 0.03 |
| Normal expression | 9$^e$ | 4 | |
| Overexpression | 18 | 36 | |
| Adenocarcinoma | Normal expression | 4 | 4 | 0.05 |
| | Overexpression | 4 | 24 | |
| Squamous cell carcinoma | Normal expression | 4 | 0 | 0.12 |
| | Overexpression | 14 | 12 | |

$^a$P-values calculated using the two-sided Fisher’s exact test. $^e$One case was a large-cell carcinoma. qPCR = quantitative polymerase chain reaction.

![Figure 3](image-url) Representative fluorescent in situ hybridisation (FISH) images. (A) A tumour with diploid genotype showing most tumour cell nuclei containing two green signals (hTERT, 5p15.33) and two red signals (control locus, 5q31). (B) A polysomy tumour showing several signals of both the hTERT and 5q. (C) Tumour with high-level amplification with their nuclei containing 10–30 hTERT signals and two or more 5q signals.
Kaplan–Meier estimation revealed only trends for association between hTERT mRNA overexpression with recurrence-free survival or overall survival (log rank $P=0.24$ and $P=0.13$, respectively, Figure 2C and D), but it was significant for reduced overall survival at the 3-year follow-up time (log rank $P=0.03$, Table 3). Cox proportional hazards regression also showed that overexpression was an independent prognostic marker for overall survival at 3-year follow-up (HR 2.29, 95% CI 1.06–4.96, $P=0.04$) after adjusting for age, tumour stage and differentiation.

**DISCUSSION**

We have evaluated the clinical and pathological significance of hTERT gene amplification and mRNA overexpression in NSCLC patients who were treated primarily by surgical resection. The hTERT gene amplification occurred in 57% of NSCLC, but this was more common among ADC (75%) than SQCC (35%). Among ADC, hTERT mRNA overexpression was significantly correlated with gene amplification ($P=0.005$). However, 87% (33 of 38) of SQCC also showed overexpression. These findings suggest that amplification is responsible for hTERT mRNA overexpression in a majority of ADC, while epigenetic factors at the transcriptional or post-transcriptional levels significantly affect hTERT expression. Most importantly, we have demonstrated that hTERT amplification is an independent prognostic marker for shorter recurrence-free survival in NSCLC patients.

Although many studies have examined the prognostic significance of hTERT mRNA/protein expression or activity in NSCLC (Table 6), to our knowledge, this is the first study that examined the prognostic value of hTERT gene amplification in lung cancer patients. The hTERT gene amplification was common in cell lines and primary tumours of lung, cervix, breast and in neuroblastoma (Zhang et al., 2000). Using $\geq 5$ copies of hTERT gene copy per nucleus in at least 20% of the cells to define amplification, Zhang et al. (2000) reported hTERT amplification in 38% (eight out of 21) of lung carcinomas. Using qPCR that defines amplification as tumours with hTERT gene content greater than that of PIF3R1 (5q13.1), we found amplification in 57% of NSCLC. We also found a significant concordance between hTERT gene copy number assayed by qPCR and FISH (Spearman correlation coefficient $r=0.43$, $P=0.0006$), indicating that qPCR may serve as an alternative method to assay amplification.

**Table 6 Previous reports on the prognostic significance of telomerase**

| Prognostic significance | Case | Activity | hTERT expression (assay)* | TRFLRβ |
|-------------------------|------|---------|--------------------------|--------|
| Albanell et al. (1997)  | 99   | No      | —                        | No     |
| Taga et al. (1999)     | 103  | Yes     | —                        | —      |
| Komiya et al. (2000)   | 58   | —       | Yes (RT–qPCR)            | —      |
| Hirashima et al. (2000)| 72   | No      | —                        | Yes    |
| Arinaga et al. (2000)  | 92   | —       | No (RT–qPCR)             | —      |
| Kumaki et al. (2001)   | 115  | Yes     | No (mISH, IHC)           | —      |
| Hara et al. (2001)     | 62   | —       | Yes (RT–semi-qPCR)       | —      |
| Toomey et al. (2001)   | 115  | —       | No (IHC)                 | —      |
| Wang et al. (2002)     | 153  | —       | Yes (mISH)               | —      |
| Marchetti et al. (2002)| 90   | Yes     | YES (RT–qPCR)            | —      |
| Wu et al. (2003)       | 56   | Yes     | No (RT–qPCR)             | —      |
| Fujita et al. (2003)   | 146  | —       | Yes (mISH)               | —      |
| Hsu et al. (2004)      | 48   | No      | —                        | —      |
| Lantuejoul et al. (2004)| 122 | —       | Yes (mISH, IHC)          | —      |
| Lu et al. (2004)       | 94   | —       | No (mISH)                | —      |

*— not studied; RT–qPCR = reverse transcription–quantitative polymerase chain reaction; mISH = mRNA in situ hybridization; IHC = immunohistochemistry. *Telomere terminal restriction fragment length ratio (tumour vs normal).
need to be developed. Since hTERT reactivation is a mechanism for cancer cells to avoid senescence (Shay and Roninson, 2004) and the latter could be induced by chemotherapy, the predictive value of hTERT amplification for benefit to adjuvant chemotherapy also needs evaluation (Winton et al., 2005). Recently, telomerase has been intensively studied as a target for novel cancer gene therapy and therapeutics (reviewed in Shay and Wright, 2002; Keith et al., 2004). Our finding that different types of NSCLC may alternate regulate hTERT overexpression suggests that patients with hTERT amplification could have different responses to telomerase-based therapies. The possible differential role of hTERT gene dosage in the diagnosis and treatment of lung cancer patients should be further investigated.

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ACKNOWLEDGEMENTS

This work was supported by Grant # 015184 from the Canadian Cancer Society through funding by the National Cancer Institute of Canada. Dr Tsao holds the M Qasim Choksi Chair in Lung Cancer Translational Research. Dr Squire holds the JC Boileau Grant Chair in Oncologic Pathology. Dr Shepherd holds the Scott Taylor Chair in Lung Cancer Research. Dr Cuiz was a Fellow in the CIHR Training Programme (STF-53912) for Clinician Scientist in Oncologic Pathology.

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

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need to be developed. Since hTERT reactivation is a mechanism for cancer cells to avoid senescence (Shay and Roninson, 2004) and the latter could be induced by chemotherapy, the predictive value of hTERT amplification for benefit to adjuvant chemotherapy also needs evaluation (Winton et al., 2005). Recently, telomerase has been intensively studied as a target for novel cancer gene therapy and therapeutics (reviewed in Shay and Wright, 2002; Keith et al., 2004). Our finding that different types of NSCLC may alternately regulate hTERT overexpression suggests that patients with hTERT amplification could have different responses to telomerase-based therapies. The possible differential role of hTERT gene dosage in the diagnosis and treatment of lung cancer patients should be further investigated.

ACKNOWLEDGEMENTS

This work was supported by Grant # 015184 from the Canadian Cancer Society through funding by the National Cancer Institute of Canada. Dr Tsao holds the M Qasim Choksi Chair in Lung Cancer Translational Research. Dr Squire holds the JC Boileau Grant Chair in Oncologic Pathology. Dr Shepherd holds the Scott Taylor Chair in Lung Cancer Research. Dr Cuiz was a Fellow in the CIHR Training Programme (STF-53912) for Clinician Scientist in Oncologic Pathology.

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)
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