Review Article

Prognostic and predictive biomarkers in early stage NSCLC: CTCs and serum/plasma markers

Philip A.J. Crosbie[1,2], Rajesh Shah[3], Yvonne Summers[4,5], Caroline Dive[2], Fiona Blackhall[2,5]

[1]North West Lung Centre, University Hospital of South Manchester NHS Foundation Trust, University of Manchester, Manchester Academic Health Science Centre, Wythenshawe, M23 9LT, UK; [2]Clinical and Experimental Pharmacology Group, Paterson Institute for Cancer Research, University of Manchester, M20 4BX, UK; [3]Department of Thoracic Surgery, [4]Pulmonary Oncology Unit, University Hospital of South Manchester NHS Foundation Trust, Wythenshawe, M23 9LT, UK; [5]Department of Medical Oncology, The Christie NHS Foundation Trust, Manchester, M20 4BX, UK

Corresponding to: Dr. Fiona Blackhall. Department of Medical Oncology, The Christie NHS Foundation Trust, Wilmslow Road, Manchester, M20 4BX, UK. Email: fiona.blackhall@christie.nhs.uk.

Abstract: Resection of early stage non-small cell lung cancer (NSCLC) offers patients the best hope of cure, however recurrence rates post-resection remain high suggesting the presence of micro-metastatic disease at the time of surgery undetected by standard staging methods. A critical step in the metastatic cascade is the entry of tumor cells into the circulation enabling their distribution to and seeding of distant organs. This review explores the evidence for predictive and prognostic circulating biomarkers in the early stage NSCLC population. We summarize studies that have explored a variety of targets including circulating proteins, nucleic acids and more recently circulating tumor cells (CTCs) as potentially clinically relevant biomarkers in the early stage setting. Circulating biomarkers may add clinically relevant information about the biological behavior of tumors over and above that provided by pathological staging. Improvement in the stratification of patients according to the likelihood of metastatic relapse after radical treatments such as surgical resection could allow more effective targeting of systemic therapies such as adjuvant chemotherapy.

Key Words: Early stage; non-small cell lung cancer (NSCLC); prognostic; predictive; circulating; biomarker

Submitted Sep 08, 2013. Accepted for publication Sep 27, 2013.
doi: 10.3978/j.issn.2218-6751.2013.09.02
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Introduction

Lung cancer is the most common cause of cancer related mortality in the world, responsible for 1.4 million deaths/year (1). A striking feature of lung cancer is its poor survival, with 5-year survival less than 10% in the UK and less than 15% in the USA. One major contributing factor to poor survival is the late clinical presentation of the majority of patients, 80% present with locally advanced or distant metastatic disease at which stage treatments are generally much less effective. The benefit of early diagnosis was underlined by the National Lung Screening Trial which investigated the effect of low dose CT screening in an at risk population and reported a 20% reduction in lung cancer specific mortality (2). However, achieving long-term survival even after curative intent surgery is a major challenge with recurrence occurring in 50% of cases overall and five-year survival rates of 58% to 73% for stage I, 36% to 46% for stage II and 24% for stage IIIA reported (3). Recurrence most commonly occurs at distant sites indicating the presence of micro-metastatic disease undetected by current staging strategies (4,5). Trials of platinum based adjuvant chemotherapy to treat micro-metastases have shown increases in 5-year survival in the order of 5% in patients with stage II-IIIA disease (6). The adjuvant treatment of stage I is more controversial as definitive evidence of efficacy is lacking (7). There is therefore a pressing clinical need to develop both more effective adjuvant therapy but also target the current therapies in a more effective

manner. Pathological stage is the most robust methodology of selecting patients for adjuvant chemotherapy however recurrence rates even in stage I disease are in the order of 25% to 40% suggesting that an additional marker/markers that enable accurate stratification of recurrence risk over and above that provided by pathological stage are necessary for more accurate prognostication. In such a way it may be possible to stratify high-risk stage I patients who may benefit from adjuvant chemotherapy and low risk stage II who may avoid chemotherapy. Furthermore, lung cancer is a heterogenous disease and the molecular profile and/or biological behavior of disease recurring after surgery may be different to that of the primary tumor. Consequently, there may be added benefit of examining circulating factors, which may reflect the behavior and molecular profile of metastatic disease more accurately than primary tumor sampling, which could result in sampling error because of tumor heterogeneity.

Method

A literature search was performed using PubMed/Medline. Search limits set included human studies, 1990 to the present day (June 2013) and articles written in English. Initial searches using the terms: circulating biomarkers, non-small cell lung cancer (NSCLC) and prognostic/predictive were followed by more targeted searches. For circulating tumor cells (CTCs) additional search terms included: CTCs, CellSearch (CS), ISET and pulmonary vein. Nucleic acid searches used additionally: circulating nucleic acids, or DNA, or RNA or microRNA and protein searches included specific proteins including: carcinoembryonic antigen (CEA), or CYFRA 21-1, or neuron specific enolase and other identified proteins. Finally reference lists were screened for additional studies.

Results

Standard clinical measures in blood

Standard biochemical and hematological measures, taken routinely as part of the assessment for radical treatment, may in themselves provide additional prognostic information. The results of several trials are discussed below.

Hemoglobin

Tomita *et al.* studied 240 patients who underwent surgical resection of NSCLC (8). Classification of low hemoglobin level was <13.0 g/dL in men and <12.0 g/dL in women. Overall, 88 patients (36.7%) were classified as having a low pre-operative hemoglobin. Five-year survival was significantly lower in patients with a low hemoglobin (43.0% *vs.* 73.5%; P<0.0001). After multivariate analysis pre-operative hemoglobin level remained a significant poor prognostic factor.

White blood cells

The impact of peripheral pre-operative white blood cell count has been investigated in several reports (9-12). A total white blood cell count above the median has been reported to be a poor prognostic factor (9). Kobayashi *et al*. examined the outcomes of 237 patients with resected node negative NSCLC (10). Raised neutrophil count and low lymphocyte count were associated with survival after univariate analysis; however only a low lymphocyte count remained an indicator of poor prognosis after multivariate analysis. Similarly Zhang *et al*. reported elevation of peripheral lymphocytes to be a favorable prognostic factor (11). Sarraf *et al*. examined the ratio of neutrophil and lymphocyte counts as a prognostic marker. This measure was significantly associated with stage, however after multivariate analysis the ratio remained an independent prognostic factor (12).

Platelets

A study of 510 patients by Yu *et al*. examined pre-operative platelet count and outcomes in patients newly diagnosed with NSCLC (13). All patients were treated with surgical resection [clinical stage I n=234 (45.9%), stage II n=128 (25.1%) and stage III n=148 (29.0%)]. Three-year overall survival (OS) was 75.3% for patients with a normal count ($\leq 300\times10^9$/L, n=449; 88.0%) and 59.2% for those with an elevated count ($>300\times10^9$/L, n=61; 12.0%); the risk of disease progression was also increased (HR 1.57, 1.02-2.45). Multivariate analysis showed age and platelet count to be the only independent prognostic markers. Tomita *et al*. also reported that pre-operative thrombocytosis ($>40\times10^4$/mm^3) was a poor prognostic marker in a study of 289 patients undergoing resection for NSCLC. Five-year survival was 30.8% in patients with elevated platelets (n=13/289) compared with 68.7% in patients with a normal count (276/289) (14).

These studies suggest that routinely measured clinical parameters in blood may provide prognostic information. The advantages of this approach are that no additional cost is required for the assays and the assays themselves are

robust. The disadvantages are that measured changes are not necessarily specific to the cancer but may be reflective of other co-morbidities or co-existing acute illnesses such as infection.

Circulating proteins

Historically circulating proteins have been most intensely investigated as prognostic biomarkers in the early lung cancer setting. The most commonly investigated proteins are CEA and CYFRA 21-1 and these studies are reviewed below.

CEA

CEA is a cell adhesion glycoprotein that is expressed in a limited number of tissues and at very low levels in healthy individuals (15). A large number of studies over the past two decades have examined the prognostic value of CEA in serum/plasma in early stage NSCLC [reviewed (16) and summarized in *Table 1*]. A majority of studies have found elevated levels of CEA to be associated with poor prognosis in resected NSCLC including stage I (17-21,23,27,28,30,32-35,37,40-47). A limited number of studies have reported no association (25,29,31,38,48,49). The largest study by Okada *et al*. examined pre and post-operative serum CEA levels in 1,000 patients undergoing resection for NSCLC (33). Patients with elevated levels of pre-operative CEA (368/1,000) had significantly lower 5-year survival compared to patients with normal levels (53.8% *vs*. 75.2%; P<0.0001). In patients with resected pathological stage I disease, those with persistently elevated post-operative CEA levels had significantly worse 5-year survival (48.6%) than patients whose CEA level normalized post-operatively (74.2%) and those who had normal pre-operative levels (84.2%) (34). Several studies have confirmed the association with persistently elevated CEA in the post-operative period and marked reduction in survival (17,18,30,33,39). As an example, Kosu *et al*. retrospectively analyzed pre and post plasma CEA levels in 263 patients with resected pathological stage I NSCLC (18). A majority had adenocarcinoma and half the population had less than 5 pack years smoking history. Patients with normal CEA before and after surgery had a 5-year survival of 95.5% compared with 85.5% (4-year survival) in patients with a pre-operative high CEA that normalized post-operatively and 59.3% in patients whose CEA was elevated both pre and post-operatively. After multivariate analysis tumor diameter greater than 30 mm, the presence of visceral pleural invasion and persistent CEA elevation were independent poor prognostic indicators. The authors conclude that patients with high post-operative CEA may benefit from adjuvant chemotherapy.

One important aspect of many of the studies investigating CEA as a prognostic marker is that the prevalence of smoking is relatively low with upwards of half the lung cancer cases being diagnosed in never smokers. Significantly smoking may increase CEA expression in lung tissue (50). In the study by Okada *et al*. CEA measurement was not prognostic in current smokers potentially reducing the clinical use of CEA in populations with a higher burden of smoking induced lung carcinogenesis (34). In a majority of studies it is also unclear what role co-existing inflammatory conditions such as chronic obstructive pulmonary disease may have in modifying CEA measurements.

CYFRA 21-1 (serum cytokeratin 19 fragment)

Cytokeratins are filamentous proteins ubiquitously expressed by epithelial cells which can be used as markers of epithelial origin (51). Cytokeratin 7, 8, 18 and 19 are the most commonly seen types associated with carcinomas and these proteins can leak into the circulation and be detected as degraded complexes and as such may act as circulating biomarkers of malignancy (52,53). Degradation fragments of cytokeratin 19 can be detected in the circulation, referred to as CYFRA 21-1, released from tumor cells by necrosis or cell lysis. This protein fragment has been assessed as a prognostic marker in several studies (25,32,38,45,46,54-58). A meta-analysis of 2,063 patients with newly diagnosed NSCLC showed an elevated pre-treatment CYFRA 21-1 fragment level (>3.6 ng/mL) to be an independent poor prognostic factor at 12 months (HR 1.88, 1.64-2.15) (56). Raised CYFRA 21-1 was of borderline significance in a subpopulation of patients treated with surgical resection (survival HR 1.41, 0.99-2.03; n=437). In a study of 85 patients with squamous cell carcinoma (stages I-IIIA), the risk of death after five years of follow up was doubled in patients with CYFRA 21-1 levels above 3.6 ng/mL (HR 2.05; 1.09-3.83) (54).

Several studies have examined the combined prognostic value of pretreatment CEA and CYFRA 21-1 as a 'tumor marker index' (TMI) (25,32,57). Blankenburg *et al*. reported no prognostic value of either single or combined measures in a study of 240 patients. In contrast, studies by Tomita (57) and Muley (32) both reported improved prognostic value to the combined data. Muley *et al*. reported reduced 3-year survival in 153 stage I patients with raised CYFRA 21-1

Table 1 Summary table of studies that have investigated the prognostic value of circulating CEA levels in patients undergoing radical lung cancer surgery

Author	No. patients	Stage [%]	Pathology[1] [%]	CEA level ng/mL	Smoking status [% NS][2]	CEA result[3] n [%]	Outcome[4]	Prognostic
Kato 2013 (17)	177	I	Ad [80]; Sq [20]	>2.5 Ad; >3.0 Sq	Ad [50]; Sq [3]	Ad: HH 29 [23.1], HN 36 [28.6], N 61 [48.4], Sq: H 15 [41.7]	5 ys DFS: Ad: HH 49.4%, HN 65.1%, N 93.4%, Sq: H 51.9%, N 81.0%	Yes
Kozu 2013 (18)	263	I	Ad [81]; Sq [18]	>3.5	<5 pack yr [49]	N 198 [75.3], HN 44 [16.7], HH 21 [8.0]	5 ys: N 95.5%, HN 85.5%, HH 59.3%	Yes
Lin 2012 (19)	169	IB-IIIA after 2 cycles adj chemo	Ad [37]; Sq [49]	>4.7	–	H 63[37.3], N 98 [58.0]	Median DFS: H 34 *vs.* N 53 months	Yes
Nagashima 2012 (20)	71	I [70]	Sq [100]	>4	SI low [44]	H 19 [26.8], N 52 [73.2]	DFS: H 63.5%, N 88.2%	Yes
Tomita 2012 (21)	197	I [75]; II-III [24]	Ad [80]	>5	–	H 48 [24.3], N 149 [75.6]	5 ys: H 51.1%, N 82.7%	Yes
Hanagiri 2011 (22)	341	I	Ad [77]; Sq [16]	>2.5	–	H 88 [25.8], N 253 [74.2]	5 ys: H 76.3%, L 88.3%	Yes
Takahashi 2011 (23)	649	I [76]; II [10]	Ad [71]; Sq [24]	>3	–	HH 145 [22.3], HN 149 [23.0], N 297 [45.8]	5 ys: HH 51.1%, HN 85.5%, N 78.4%	Yes
Tomita 2009 (24)	220	I [70]; II [15]	Ad [75]	>2.5	NS [43]	–	5 ys all: H 62%, N 79.6%; 5 ys Ad: H 71.1%, N 88.2%	Yes
Blankenburg 2008 (25)	240	I	Ad [38]; Sq [42]	>6.7	–	–	5 ys: H 49% *vs.* N 66%	No
Kashiwabara 2008 (26)	136	I	Ad [100]	>2.5	NS [65]	H 16 [11.8], N 120 [88.2]	DFS: H 43.8%, N 95%	Yes
Matsuguma 2008 (27)	455	I	Ad [69]; Sq [25]	>5	NS [35] FS [25]	HH 20 [4.4], HN 112 [24.6], N 323 [71.0]	5 ys: HH 43.1%, HN 56.2%, N 85.9%	Yes
Hsu 2007 (28)	163-f	I	Ad [83]	>6	NS [93]	H 47 [29], N 115 [71]	5 ys: H 59.6%, N 75.7%	Yes
Kobayashi 2007 (29)	163	I <2 cm	Ad [83]	>5.0	NS [48]	H 20 [12.5], N 140 [87.5]	HR 1.03 (0.3-3.1)	No
Matsuoka 2007 (30)	275	I	Ad [70]; Sq [26]	>5	NS [38]	H Ad 51, Sq 13, N Ad 133, Sq 50	5 ys Ad: H 54.6%, N 86.9%; Sq: H 82.1%, N 78.5%	Yes
Mizuguchi 2007 (31)	137	I	Ad [63]; Sq [34]	>7.8	–	–	Not prognostic after multivariate analysis	No
Muley 2004 (32)	153	I	Ad [49]; Sq [39]	>9.8	–	–	3 ys: H 41.6% *vs.* N 79.2%	Yes

Table 1 *(continued)*

Table 1 (continued)

Author	No. patients	Stage [%]	Pathology[1] [%]	CEA level ng/mL	Smoking status [% NS][2]	CEA result[3] n [%]	Outcome[4]	Prognostic
Okada 2004 (33)	1,000	I [70]	Ad [69]; Sq [26]	>5	–	HH 126 [12.6], HN 242 [24.2], N 632 [63.2]	5 ys: HH 35.2%, HN 62.6%, N 75.2%	Yes
Okada 2004 (34)	954	I [69]	Ad [73]; Sq [27]	>5	Ad [50]; Sq [8]	smoking increases CEA	5 ys: Ad 83.7/57.4, Sq 76.6/28.0	Yes
Sakao 2004 (35)	100		Ad [100]	>5	–	H 24 [24], N 76 [76]	DFS: H 33.3%, N 63.2%	Yes
Tomita 2004 (36)	313	I-II [70]	Ad [70]; Sq [30]	>5	–	–	5 ys: Ad H 42.5%, N 77.6%; Sq H 57.8% N 63.3	Ad-yes, Sq-no
Buccheri 2003 (37)	118	I [57]; II [14]	Ad [49]; Sq [40]	>10	–	H [55]	Stage I + II 1yr rec: H 67%, N 12%	Yes
Reinmuth 2002 (38)	67	I [64]; II-III [36]	Ad [25]; Sq [46]; Lg [24]	>5	–	–	No significant difference in survival	No
Sawabata 2002 (39)	297	I	–	>7	–	HH 15 [5.1%], HN 41 [13.9%] Ad, N 241 [81%]	5 ys: HH 18%, HN 68%, N 72%	Yes
Hotta 2000 (40)	39	I	Ad [67]; Sq [28]	>6.7	SI low [49]	H 9 [23.1], N 30 [76.9]	H: Lower OS + DFS	Yes
Suzuki 1999 (41)	365	I	Ad [100]	>5	<5 pack yr [40]	H 89, N 180	5 ys: H 53%, N 78.5%	Yes
Rubins 1998 (42)	50	I [56]; II [14]; III [30]	Ad [42]; Sq [44]	variable	–	–	Reduced survival with increasing CEA level	Yes
Icard 1994 (43)	152	I [34]; II [23]; IIIa [36]	Ad [43]; Sq [28]	all >10; Int 10-30; H >30	–	Stage I: Int 35, H 7; stage II: Int 17, H 12; stage IIIa: Int 28, H 17	5 ys: stage I: H 0%, Int 40%; stage II: H O%, Int 44%; stage IIIa: H 9%, Int 0%	Yes

[1]Ad, adenocarcinoma; Sq, squamous cell carcinoma; Lg, large cell carcinoma; [2]NS, non-smoker; FS, former smoker; SI, smoking index; [3]H, high preoperatively; HH, high pre and post-operatively; N, normal pre-operatively; HN, high pre-operatively and normal post-operatively; Int, intermediate; [4]abbreviations as per [3], DFS, disease free survival; OS, overall survival; 5 ys, 5-year survival

baseline levels prior to surgical resection (3-year survival: high 60.2% *vs.* normal 78.4%). Patients were categorized according to high, intermediate and low levels of geometric mean CEA and CYFRA 21-1 and 3-year survival analyzed. Survival was 96.7% in patients with low levels, 77.2% in the intermediate group and 55.7% in patients with high levels. Tomita *et al*. reported reduced 5-year survival in patients with elevated pre-treatment CYFRA 21-1 (5-year survival: high 40% *vs.* normal 67%), in a cohort of 291 patients of stage I-III. An analysis of 5-year survival by TMI, defined as positive by the presence of elevated CYFRA 21-1 and or CEA, showed reduced survival with TMI positive patients (37.1%) compared to TMI negative patients (72.3%).

A study by Mizuguchi *et al.* measured Sialyl Lewis^x (an important cell surface carbohydrate antigen) in addition to CYFRA 21-1 and CEA pre-operatively in 137 patients with completely resected stage I NSCLC (31). Thirty of the patients recurred and after multivariate analysis pre-operative CYFRA 21-1 and SXL levels, but not CEA, were independent prognostic markers. Patients with high

Table 2 Summary table of studies to have combined CYFRA 21-1 and CEA/SLX to determine a tumor measure index (TMI)

Author	No. patients	Stage [%]	Pathology[1] [%]	Measure	Level ng/mL	Smoking status (% NS)[2]	Result[3] n [%]	Outcome[4]	Prognostic
Tomita 2010 (57)	291	I [64], II-III [36]	Ad [72]	CYFRA 21-1	>2.4	–	H 58, N 233	5 ys: H 40%, N 67%	Yes
				CEA + CYFRA = TMI	<1= both N, >1=1or 2 H		<1=202, >1=89	5 ys: <1 72.3%, >1 37.1%	Yes
Blankenburg 2008 (25)	240	I	Ad [38], Sq [42]	CYFRA 21-1	>3.3	–	–	5 ys: H 64% N 64%	No
				CEA + CYFRA = TMI	Geometric mean		H 71 [30], Int 87 [36], N 82 [34]	5 ys: H 63.1%, Int 63.5%, N 65.1%	No
Mizuguchi 2007 (31)	137	I	Ad [63], Sq [34]	CYFRA 21-1	>3.2	–	–	OS: HR 3.5 (2.9-4.1)	Yes
				SLX	>36 U/mL			OS: HR 4.1 (3.4-4.8)	Yes
				SLX + CYFRA	<1= both N, 1=1H +1N, >1= both H		>1 [6], 1 [31], <1 [63]	5 ys: >1-13%, 1-52%, <1-80%	Yes
Muley 2004 (32)	153	I	Ad [49], Sq [39]	CYFRA 21-1	>3.3	–	H [21], N [79]	3 ys: H 60.2% *vs.* N 78.4%	Yes
				NSE	>14.5		–	Not related to outcome	No
				CEA + CYFRA = TMI	Geometric mean		H 49 [23], Int 60 [42], N 32 [35]	3 ys: H 55.7%, Int 77.2%, N 96.7%	Yes

[1]Ad, adenocarcinoma; Sq, squamous cell carcinoma; Lg, large cell carcinoma; [2]NS, non-smoker; FS, former smoker; SI, smoking index; [3]H, high preoperatively; HH, high pre and post-operatively; N, normal pre-operatively; HN, high pre-operatively and normal post-operatively; Int, intermediate; [4]abbreviations as per [3], DFS, disease free survival; OS, overall survival; 5 ys, 5-year survival

levels of both markers were five times more likely to recur than those with normal values (*Table 2*).

Neuron specific enolase

Enolases are integral to the glycolysis pathway by linking 2-phosphoglycerate and phosphor-enolpyruvate metabolism. Neuron specific enolase has been associated with small cell lung cancer as a clinical biomarker (59). However several studies have examined NSE as a circulating biomarker in NSCLC (38,49,54,55,60,61). In three of the studies NSE was not prognostic (38,49), including a study of 164 patients with resected stage I disease (60). Two studies reported an association with poor prognosis in advanced disease (54,55). However, the largest study to date by Yu *et al.* looked at 481 patients with operable NSCLC and measured pre-operative serum levels of NSE, SCC and carbohydrate antigen 125 (CA125) (61). Both elevated levels of NSE and CA125 were associated with reduced disease free survival (DFS) and OS. In a multivariable Cox regression model advanced clinical stage and both elevation of CA125 and NSE were independent prognostic factors associated with reduced survival. Elevation of more than one circulating biomarker was associated with a worse outcome.

C-reactive protein (CRP)

Elevated CRP has been associated with poorer outcomes after surgery in two studies (62,63). O'Dowd *et al.* reported elevated CRP but not total white cell count to be an independent prognostic factor after multivariate analysis (63). Median survival was 75.9 months in the normal CRP group

and 26.2 months in the high CRP group. Similarly Hara *et al*. showed reduction in 5-year disease specific (56.2% *vs*. 77.6%; P=0.003) and OS (50.2% *vs*. 74.2%; P=0.001) in the high CRP arm.

Fibrinogen

Sheng *et al*. report the prognostic significance of fibrinogen, an important protein in the clotting cascade, in a study of patients with operable NSCLC (n=567; 69.3% were stage I + II) (64). Normal serum fibrinogen levels (<4 g/L) were recorded in 343 patients (60.4%) compared with 224 patients (39.5%) with elevated fibrinogen levels (>4 g/L). Patients with higher baseline fibrinogen levels had lower 3-year progression-free survival (49.2% *vs*. 63.3%) and lower OS rates (66.0% *vs*. 80.9%) than patients with normal serum fibrinogen concentrations. Fibrinogen level was an independent poor prognostic marker after Cox proportional regression analysis.

Other circulating proteins

Squamous cell carcinoma antigen has been investigated in several studies without showing prognostic significance (31,54,60,65). CA125 is a standard clinical measure used in the diagnosis and monitoring of patients with ovarian cancer, but its prognostic potential has also been investigated in a small number of studies looking at patients undergoing curative resection of NSCLC. Three studies have reported elevation of pre-operative CA125 to be associated with a worse prognosis (65-67) with one study reporting no association (60).

Despite the multiple studies of circulating proteins described above none have proved sufficiently robust to incorporate into routine practice either to alter the intensity of follow up or direct adjuvant therapy.

Circulating nucleic acids

Circulating DNA can be detected in healthy individuals and in significantly higher concentrations in patients with cancer (68). Tumor specific RNA and DNA can enter the circulation through processes including necrosis and apoptosis from both primary and metastatic sites and may also be detected in plasma/serum (69).

Total circulating free DNA

Total circulating plasma DNA concentration was investigated as a potential biomarker in a population of 1,035 heavy smokers over the age of 50 who were taking part in an annual low dose CT screening study (70). A total of 38 patients were diagnosed with lung cancer during the study period. Study participation also included annual blood tests and no difference in median DNA concentration was seen between cases and controls at baseline. However, in 33 lung cancer cases who went on to have surgical resection, and who also had repeat blood samples, median DNA concentration was significantly higher within the 12-month period immediately prior to diagnosis and surgery (4.6 ng/mL) compared with samples taken over a year prior to diagnosis (2.4 ng/mL). This finding suggests that longitudinal changes in DNA concentration may predict for the development of malignancy in smokers rather than a fixed threshold concentration. In addition, five-year survival was noted to be significantly worse in patients with DNA concentrations in the highest tertile (33%) when compared to the lowest tertile. A similar association with outcome was reported by van der Drift *et al*., in a study of 46 patients with newly diagnosed NSCLC (29/46 were stage I to III). Significantly reduced survival was seen in patients with DNA concentrations in the highest tertile (median survival 11.8 months) compared to the lowest tertile (median survival 21.5 months) (71).

Conversely, baseline circulating free DNA concentration was not correlated with prognosis, either DFS or OS, in a study of 76 patients undergoing curative resection (stage I-II n=60, stage III n=16) (72). However in patients who relapsed early (within 3 months; n=9) increasing circulating DNA concentration was seen in a repeat blood sample taken 3 months post-operatively. By comparison patients who did not relapse showed a reduction in DNA concentration by the same time point. A similar finding was reported by Szpechcinski *et al*. who demonstrated reduction (n=11) or stability (n=7) in circulating DNA levels in patients who had undergone resection of NSCLC between 3 to 6 months post-surgery except for 2 individuals with early relapse whose DNA concentrations increased significantly (73). On this basis cfDNA might have an application to monitor patients for relapse prior to symptom development or radiological change and thereby inform on the intensity of follow up.

Circulating microRNAs

MicroRNAs are small non-coding single stranded RNA molecules that serve a regulatory purpose in controlling the function of messenger RNA (74,75). Tissue expression of microRNAs has been shown to be prognostic in NSCLC (76). MicroRNAs can also be detected in the

circulation, where they are stable (77), and several studies have examined their prognostic significance in early stage lung cancer. Hu *et al*. developed a panel of 4 miRNAs (high miR-486 and miR30d; low miR1 and miR499) whose differential expression was associated with poor survival (78). Testing of the panel in 243 patients with stage I-IIIa NSCLC treated with both surgery and adjuvant chemotherapy showed the miRNAs to be an independent predictor of survival. Indeed the authors reported a dose effect dependent on how many markers in the panel were affected and increasing risk of cancer death (2 measures: HR 3.14, 1.7-6.0; 3 measures HR 16.5, 8.6-31.7; 4 measures 34.1, 16.3-71.6).

Boeri *et al*. examined the predictive value of miRNAs in plasma taken from two CT screening trials (79). Patients who attended for annual CT screening also had an annual blood test. A panel of 15miRNAs (mir-660, mir-140-5p, mir-451, mir-28-3p, mir-30c, and mir-92a most commonly deregulated) was able to correctly categorize 30 out of 35 patients who developed lung cancer, using plasma taken over 12 months and upwards of 28 months prior to diagnosis. Misclassification occurred in 1 out of 5 control pools. A further signature was developed (containing mir-486-5p) to explore the use of miRNA expression and prognosis at the time of diagnosis. The panel correctly classified 9 out of 11 patients with poor prognosis but misclassified 2 out of 10 patients with good prognosis.

Chen *et al*. examined the prognostic significance of a specific miRNA (miR-17-5p) in the serum of 221 patients newly diagnosed with lung cancer (80). Aberant expression of miR-17-5p has previously been demonstrated in lung cancer and serum miR-17-5p levels were significantly higher in this study than normal controls. Patients were stratified according to expression levels (stage I-III: high expression n=99 and low expression n=109) and survival compared. High expression was associated with a lower median survival of 33 months compared with 40 months. This difference remained after a Cox proportional hazard model (HR 1.8, 1.04-3.01). Reduced survival in patients with stage I disease (n=180) was also reported by Heegaard *et al*. when the population was stratified according to miR-233 levels (reduced levels associated with worse survival) (81). Silva *et al*. demonstrated a reduction in DFS for patients undergoing surgery (n=37) who had low levels of miR-30e-3p (DFS rate at 50 months =13%, 14-52) compared with high levels (DFS rate 50%, 23-77) (82). Measures of plasma let-7f and miR-20b were not associated with DFS. Survival was associated with differential expression of three miRNAs (miR-96, miR-182, and miR-183) in a study of 70 patients with NSCLC treated with surgical resection (76).

Circulating mRNA

Cheng *et al*. explored the prognostic value of relative levels of circulating cMET mRNA in blood from 45 patients undergoing resection using RT-PCR (83). Over-expression of cMET, a proto-oncogene implicated in angiogenesis and metastasis, was recorded in 23 patients and after a mean follow up period of 23 months 18 (78.3%) of these patients had recurred and 8 died. This compared with 4 (18.2%) recurrences and no deaths in the cMET negative group. After multivariate analysis cMET positivity was the strongest predictor of recurrence (HR 3.9, 1.2-13.3). Hepatocyte growth factor (HGF), the ligand for cMET, has been variably associated with outcome in early stage NSCLC; reported to be prognostic in studies with small samples sizes (84,85) but not in a much larger study of 196 patients (86).

CTCs

CTCs or Circulating Tumor Microemboli (CTMs, cells in contiguous groups) are postulated to have a critical role in the development of metastatic disease (87). The detection of CTCs in patients with lung cancer has been described in numerous clinical studies and has in general been associated with a poorer prognosis (88). A number of different methods have been used [reviewed (89)] and the field is rapidly evolving; current methodologies can be broadly divided into assays that physically isolate individual cells/groups of cells for further characterization and nucleic acid based techniques, which infer the presence of CTCs.

Cell based detection

The most robustly developed platform is CellSearch (CS), which is fully validated and approved for clinical use in advanced breast, bowel and prostate cancer. CTC enrichment from blood is performed automatically (CellTracks AutoPrep) through immunomagnetic selection of epithelial cell adhesion molecule (EpCAM) expressing cells, using ferrofluid particles coated with an anti-EpCAM antibody, which are then separated from blood by magnets. Selected cells are then stained with phycoerythrin-conjugated anti-cytokeratin (8,40,44) antibodies (epithelial cell marker), allophycocyanin-conjugated anti-CD45 antibodies (white blood cell marker) and a nuclear stain (4',6-diamidino-2-phenylindole;

DAPI). Analysis is undertaken using a semi-automatic fluorescence microscope (CellTracks Analyser II) and CTCs categorised using morphological (round or oval, visible nucleus in cytoplasm, cells at least 4 μm in diameter) and immunofluorescent criteria (CK^+, $DAPI^+$ and $CD45^-$). The automated processing and semi-automated analysis produce low inter and intra-assay variability (90). Using this method, our group has shown that CTC number is an independent prognostic marker in advanced NSCLC; in univariate analysis, patients ≥5 CTCs had an OS of 4.3 months compared with 8.1 months in those with <5 CTCs (P<0.001). Indeed, CTC number was the strongest predictor of OS (HR 7.9, 2.9-22.0; P<0.001) after multivariate analysis. However, ≥2 CTCs were detected in only 32% of stage IV patients (19/60) and rarely in patients with stage IIIB (7%, 2/27) and not at all in stage IIIA (0%, 0/14) (91). One possible disadvantage of the CS approach is the reliance on EpCAM positive selection. EpCAM expression is commonly found in tumours of epithelial origin (92), but not necessarily on all CTCs (93).

The theory of epithelial to mesenchymal transition proposes that cells develop a more metastatic phenotype, reflected by down regulation of epithelial markers and upregulation of mesenchymal markers, that facilitates migration from the primary tumour into the circulation (94). Selection of CTCs based purely on epithelial markers may underestimate the CTC burden and therefore lower the sensitivity of this approach; this may be especially relevant in NSCLC (95). Selection of CTCs using non-epithelial based markers may therefore have advantages; one example is ISET (Isolation by Size of Epithelial Tumour cells) (96). ISET technology involves the filtering of blood through a membrane with 8 μm pores to isolate cells or groups of cells larger than this size independently of cellular protein expression. Cells may then be characterized morphologically and by protein expression. Using this method in a direct comparison with CS we have shown that ISET identifies significantly more CTCs and CTMs in a study of 40 patients with advanced lung cancer (97). In this study ISET demonstrated CTCs in 80% (32/40) of patients compared with 23% (9/40) using CS; CTCs were detected by both methods in only 7 patients and although CTMs were detected in 38% (15/50) by ISET, CS identified no CTMs.

In the early non-small cell cancer setting, Hofman *et al*. investigated the prognostic value of CTCs detected in peripheral blood drawn from 210 patients prior to surgical resection of NSCLC using both CS and ISET (98,99). Pathological stage was I or II in 62% (131/210) and III or IV in the remainder (38%, 79/210). CTCs were standardly defined (CK^+, $DAPI^+$, $CD45^-$) with CS and with ISET as cells with positive immunocytochemical staining for cytokeratin and/or vimentin with morphological features of non-hematological cells. CTCs were detected in 69% of patients overall [ISET 50% + ve (104/210); CS + ve: ≥1 in 39% (82/210) and ≥2 in 21% (44/210)]. CTC number was not related to disease stage or histological subtype. CTC counts were higher using ISET (mean 34, range, 1 to 23) compared with CS (mean 12, range, 1 to 150). In the 144 patients where CTCs were detected only one in five (20%, 42/210) were detected using both CS and ISET. ISET cells stained with cytokeratin alone in 26.0% (27/104), cytokeratin and vimentin in 52.9% (55/104) and vimentin alone in 22.1% (23/104). Multivariate Cox proportional hazard regression analysis showed the presence of CTCs to be a poor prognostic factor, associated with reduced DFS after a median follow up of 15 months, irrespective of method used for CTC detection (CS: HR 1.6, 1.3-4.7; P=0.008. ISET: HR 1.4, 1.1-3.3; P=0.006).

A small study by Sawabata *et al*. examined CTC count using CS in peripheral blood immediately pre and post operatively and 10 days after surgical resection of NSCLC. CTCs were detected in 1 of 9 before, 3 out of nine patients immediately after and in no patients 10 days after surgery. After a median follow up of 14 months no patient had evidence of relapse (100).

Nucleic acid based detection

Nucleic acid based methodologies to detect CTCs have been used in the early NSCLC setting. A study by Yie *et al*. applied a RT-PCR ELISA technique to the cellular fraction of peripheral blood (2 mL) taken from patients with NSCLC to determine the concentration of survivin mRNA (101). Survivin is a protein commonly overexpressed in malignancy and has a role in tumor progression (102), a recent meta-analysis reported expression of survivin in NSCLC to be a poor prognostic factor (103). The authors determined an upper limit of normal for survivin mRNA concentration in blood by examining 172 healthy volunteers. Levels above the highest in this cohort were then classed as abnormal and designated as CTC positive. Using this classification, 44.1% of patients with NSCLC taking part in the study had detectable CTCs (n=63/143), corresponding to 26% of stage I + II (n=13/50) and 47.9% (n=35/73) of stage III patients. Follow up for a median period of 36 months was performed on 67 patients who were treated with surgical resection; of these 26 were

CTC positive (38.8%). A total of 18 relapses occurred in the CTC positive cohort (n=18/26; 60% stage I + II, 75% stage III) compared with 4 in the CTC negative cohort (n=4/41; 15.9% stage I + II, 8.3% stage III). The likelihood of relapse was significantly higher in CTC positive patients (RR 43.5, 2.7-70.9; P=0.008).

A panel of 4 marker genes (homo sapiens keratin 19, ubiquitin thiolesterase, highly similar to HSFIB1 for fibronectin, tripartite motif-containing 28) was developed by Sher *et al*. and tested (RT-PCR) in a cohort of 54 patients undergoing curative resection of NSCLC (104). The panel was determined by examining genes with large differential expression ratios between lung cancer cell lines and white blood cells. Detection of any of the marker genes classified a sample as CTC positive; the detection rate was 72% (39/54). The method was further developed to allow semi-quantitative measurement of the relative expression of marker genes to determine a circulating tumor cell load; which was validated using spiked tumor cell experiments. Patients were categorized into high and low cancer cell load for analysis and those with a low cancer cell load had better prognosis when matched for stage.

Yamashita *et al*. examined the blood of 103 patients with NSCLC pre and post-surgery (105). CTCs were defined by the detection of mRNA for CEA, which is expressed in epithelial cells. Patients with detectable CEA in the pre-operative blood sample had a significantly worse prognosis. Median survival in patients with a positive pre-operative test was 14 months compared to over 26 months in those with a negative test. Multivariate analysis showed that stage and CEA mRNA detection were independent prognostic factors (P=0.0004, RR 0.21). Yoon *et al*. sampled blood from patients before and after resection of NSCLC (n=79) and defined CTC status by the presence of thyroid transcription factor-1 (TTF-1) and cytokeratin19 (CK19) mRNA using real time RT-PCR (106). TTF-1 was detected in 36.1% (22/61) and 37.5% of patients before and after surgery respectively. CK19 mRNA positive samples were detected in 42.6% (26/61) and 25.0% (12/48) before and after surgery. Post-surgery positivity for both measures was most strongly correlated with shorter disease-free survival (P=0.006). Patients who only had post-surgery positive TTF-1 samples had a significantly shorter disease-free survival (P<0.001); this was not the case for CK19, which was not prognostic when measured independently.

Pulmonary vein sampling

In addition to analysis of peripheral blood, a small number of studies using various methodologies have explored the presence of CTCs within the pulmonary vein at the time of surgical resection. The pulmonary veins drain blood directly from the lungs and deliver oxygenated blood to the heart for distribution to the systemic circulation; their proximity to the primary tumor and the ability to take blood before the filtering effect of the capillary bed, which has been reported to remove 90% of CTCs (107), makes pulmonary vein sampling potentially advantageous. In a study of 30 patients undergoing resection of lung cancer, Okumura *et al*. detected CTCs in 96% (n=29/30) of pulmonary compared with 17% (n=5/30) of matched peripheral vein samples (using CS) (108). Pulmonary vein CTC count was not prognostic after a median follow-up period of 13 months (2 deaths from lung cancer-one patient with high and one with low pulmonary vein CTC count).

A novel method of CTC detection, involving a CD45 negative enrichment step followed by density gradient centrifugation, was developed by Funaki *et al*. who reported a pulmonary vein CTC prevalence of 72% (n=68/94) (109). Circulating tumor micro-emboli were detected in over half of patients with a CTC positive blood sample (n=35/68). Recurrence occurred in a total of 16 patients at local (n=4), local and distant (n=7) and distant only (n=5) sites after a median follow up period of 13 months. Of these 16 patients 15 had detectable CTCs or CTMs in the pulmonary vein at the time of surgery. Multivariate analysis showed the presence of pulmonary vein CTMs (RR 8.9, 1.7-21.0; P=0.006) and tumor stage III and IV (RR 9.8, 3.4-40.4; P=0.002) to be the only significant predictors of relapse.

Sienel *et al*., using an anti-cytokeratin antibody to define CTCs, detected pulmonary vein CTCs in 18% of patients (11/62) (110). There was an overall trend towards poor prognosis in patients with detectable CTCs, 7/11 died from lung cancer compared to 13/39 patients with no CTCs detected (P=0.054). Subgroup analysis of patients with no mediastinal nodal involvement (i.e., N0 or N1) showed CTC detection to be a significantly adverse prognostic marker (RR 4.2, 1.6-11.1; P=0.004) after multivariate analysis. Pulmonary vein CTCs were also shown to have adverse prognostic significance by Dong *et al*. using flow cytometry to detect CTCs (defined as $CD45^-$, CK^+, $2F7/S5A^+$) (111). Of 31 patients included in the study 15 had detectable CTCs (48.4%). Median follow up was 30 months and patients with CTCs in the pulmonary vein had significantly worse prognosis [2-year survival 62.5% (CTC – ve) *vs*. 26.7% (CTC + ve); P=0.023]. Multivariate analysis showed that only disease stage and CTC positive test (RR 2.8, 1.1-7.2;

P=0.03) were the only independent prognostic tests. By contrast, Franco *et al*. reported the presence of CTCs in the pulmonary vein of 23.9% of patients undergoing resection of NSCLC (n=11/45) but CTC number was not related to prognosis (112).

In these studies the prevalence of pulmonary vein CTCs was markedly different (18% to 96%). The interpretation of these results needs to take into account not only methodological differences in CTC isolation and characterization but also the exact timing of pulmonary vein sampling with respect to the operation itself. Surgical manipulation of lungs peri-operatively has been investigated as a potential cause of increased CTCs. Two studies examining the sequence of vessel ligation at the time of surgery concluded that initial pulmonary vein ligation was associated with a lower post-operative release of CTCs than pulmonary artery ligation (113,114). However, studies by Yamashita *et al*. and Kozak *et al*. reported that the sequence of vessel ligation had no significant impact on prognosis (105,115). In three of the studies pulmonary vein sampling was performed after lung resection, potentially artificially elevating CTC numbers. Nevertheless, collectively the results are provocative for CTC analysis from pulmonary vein samples to inform on prognosis and thereby therapeutic decision making.

In summary, these studies overall have demonstrated the presence of CTCs in the surgical setting and have associated the finding of CTCs with a poorer prognosis. The utility of CTC detection has not yet been developed to a point that could guide clinical decision making and further prospective studies are required for this purpose.

Conclusions and future directions

Achieving long-term survival in patients with radically treated early stage lung cancer remains a major challenge with recurrence rates overall of 50%. Several different circulating biomarkers show promise as indicators of prognosis in patients with resected NSCLC. As an example, CEA has been studied most extensively and recent studies measuring pre and post-operative CEA levels have identified a small proportion of stage I adenocarcinoma patients with a particularly poor prognosis who may benefit from adjuvant chemotherapy. Evidence is lacking however for the predictive value of CEA in this setting. Some studies were performed prior to the routine use of adjuvant therapy or in stage I patients who are not routinely treated. In addition, the prognostic value of CEA may be more applicable to countries with a significant proportion of non-smoking lung cancer cases (up to 50% in some reports). The clinical value in European and American populations is less clear-cut, where there is a much heavier burden of smoking induced lung cancer and as a consequence common co-existence of inflammatory lung conditions such as chronic obstructive pulmonary disease.

A panel of biomarkers may be more reliable in predicting prognosis than a single measure, e.g., combined CYFRA 21-1 and CEA, which have been shown in several studies to be more strongly associated with prognosis than each individual measure taken alone. Newer markers such as circulating nucleic acids (DNA or microRNAs) or CTCs have the potential to reflect directly the biological behavior and provide molecular insights into the tumor biology itself. However, due to the ready availability and low cost of protein based markers in clinical laboratories, it would be prudent to include for example CEA or CYFRA 21-1 in any prospective study of a novel biomarker requiring more sophisticated and costly technology.

There are a multitude of studies that have examined circulating biomarkers and prognosis post-surgical resection. Results do indicate that it may be possible to differentiate patients with similar pathologies and stage into high and low risk categories based on the probability of recurrence on the basis of a convenient blood based test. Adjuvant therapy trials of the late 1990s/early 2000s did not incorporate translational studies of circulating biomarkers; focusing instead on tissue markers in resected tumor tissue. With the availability of new technologies and the opportunities they provide it may be opportune to revisit adjuvant trials with prospective evaluation of circulating biomarkers.

Acknowledgements

Disclosure: The authors declare no conflict of interest.

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Cite this article as: Crosbie PA, Shah R, Summers Y, Dive C, Blackhall F. Prognostic and predictive biomarkers in early stage NSCLC: CTCs and serum/plasma markers. Transl Lung Cancer Res 2013;2(5):382-397. doi: 10.3978/j.issn.2218-6751.2013.09.02

