REVIEW

Immunohistochemical markers of prognosis in non-small cell lung cancer: a review and proposal for a multiphase approach to marker evaluation

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Characteristics of the tumour that affect and predict the survival outcome of patients with cancer are prognostic markers for cancer. In non-small cell lung carcinoma (NSCLC), stage is the main determinant of prognosis and the basis for deciding options for treatment. Patients with early-stage tumour are treated by complete surgical resection, which is curative in 40–70% of patients. That there are other factors important in determining the biology of these tumours, especially genes that have a role in metastasis, is indicated. Such factors could potentially be used to further classify patients into groups according to substages that may be treated differently. During the past decade, a large number of proteins that are putatively important in carcinogenesis and cancer biology have been studied for their prognostic value in NSCLC, but none of them have been proved to be sufficiently useful in clinical diagnosis. Several markers (epidermal growth factor receptor, human epidermal growth factor receptor 2, Ki-67, p53 and Bcl-2) have been studied exhaustively. Ki-67, p53 and Bcl-2 are suggested to be important but weak prognostic markers, by meta-analyses of the results. Cyclin E, vascular endothelial growth factor A, $p16^{INK4A}$, $p27^{kip1}$ and β-catenin are promising candidates, but require further study in large randomised clinical trial samples by using standardised assays and scoring systems. Some issues and inconsistencies in the reported studies to date are highlighted and discussed. A guideline for a multi-phase approach for conducting future studies on prognostic immunohistochemistry markers is proposed here.

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Lung cancer is the leading cause of cancer death in North America and throughout the world. In North America, annual deaths from lung cancer are greater than the next three most common cancers combined (breast, prostate and colon). Non-small cell lung carcinoma (NSCLC) accounts for about 80% of all lung cancers. The current management of NSCLC is largely guided by tumour stage. Patients with early stage (I and II) tumour are treated by surgical resection with or without adjuvant chemotherapy, and stage III patients require combined modality approaches that may include chemotherapy, radiation and surgery. Nevertheless, the overall 5-year survival rates of these patients remain relatively poor, ranging from 70% for stage IA patients to 25% for stage IIIA patients whose tumours are surgically resectable.[1] Most deaths are caused by metastatic recurrence. Differing survival outcomes among patients within a stage suggests the existence of other tumour factors affecting prognosis.

Cancer cells manifest complex genetic aberrations that occur during multi-stage carcinogenesis. Genomic instability or selection leads to aberrations that can be grouped into six essential pathways: the acquisition of (1) self-sufficient or autonomous growth signals; (2) insensitivity to growth-inhibitory signals; (3) resistance to signals of apoptosis; (4) unlimited proliferation potential; (5) sustained angiogenesis; and (6) invasion and metastasis.[2 3] Each of these pathways is regulated by further sets of interacting subpathways, which result in redundancy and additional complexities on the roadmap to malignancy. Despite this, some molecular aberrations are more likely than others to influence the clinical behaviour of a cancer, including the risk of metastasis. Such aberrations, once identified, could potentially serve as prognostic markers, which are tumour (or patient) characteristics that may influence and predict the clinical outcome of a cancer patient.

Molecular prognostic markers could potentially be represented by changes in gene copy number, messenger RNA (mRNA) expression or protein expression levels. Immunohistochemistry (IHC) is the most practical method of assessing protein expression changes in histopathology. IHC not only provides a semiquantitative assessment of protein abundance but also defines the cellular localisation of expression. It may also detect functionally important post-translational protein modifications, such as phosphorylation. These

Abbreviations: ADC, adenocarcinoma; CCN, cyclin; CDK, cyclin-dependent kinase; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; FHIT, fragile histidine triad; HER, human epidermal growth factor receptor; HGF, hepatocyte growth factor; hTERT, human telomerase reverse transcriptase; IHC, immunohistochemistry; MMP, matrix metalloproteinase; mRNA, messenger RNA; NSCLC, non-small cell lung carcinoma; PCNA, proliferating cell nuclear antigen; SQCC, squamous cell carcinoma; TGF-β, transforming growth factor β; TIMP, tissue inhibitors of metalloproteinase; VEGF, vascular endothelial growth factor

considerations have led to the extensive use of IHC in studies on prognostic markers for tumours. In this review, we shall summarise to the best of our ability the results of these studies on NSCLC.

MATERIALS AND METHODS

We searched PubMed with the MeSH terms ''non-small cell lung carcinoma'' *and* ''immunohistochemistry'' *and* ''prognosis'', with the search limited to ''humans'' and ''English language''. This search produced 462 papers and 12 reviews dating from May 1987 to October 2005. For individual markers, additional and confirmatory searches were carried out with ''gene of interest'' *and* ''non-small cell lung cancer'' *and* ''immunohistochemistry''. When the same group of investigators published multiple manuscripts on a marker and used overlapping patient cohorts, only the most recent one or the one directly dealing with its prognostic value was emphasised. If meta-analyses or reviews on a specific marker were published, these were considered in place of the original reports (table 1). To limit the scope of this review, we have discussed in more detail only markers that have figured in four or more independent studies. Reports in journals that are difficult to access were also excluded from detailed review. A summary of results from these studies has been compiled and is available at http://www.uhnres.utoronto.ca/labs/tsao/ under supplimentary data.

The prognostic significance of a marker is normally evaluated by Cox proportional regression analysis or log-rank testing for its association with disease-free or overall survival. Independent prognostic markers are identified by multivariate analysis, which adjusts for other clinical and pathological factors.

RESULTS

Self-sufficiency for growth pathway

Cells require mitogenic growth signals to enter into the cell cycle. Neoplastic cells invariably show reduced dependence on such signals. This may result from altered expression of soluble growth factors or their receptors, altered cell–cell or cell–stroma interactions and intrinsic activation of growth signal transducers.[2] Interactions may be mediated by autocrine, paracrine or endocrine mechanisms.[4] In an autocrine loop, cells express both the receptor and its ligand(s). Interactions between tumour and host stromal cells exemplify the paracrine interactions, whereas hormones are the prototype of endocrine signalling.

Epidermal growth factor receptor family

The epidermal growth factor receptor (EGFR)/ErbB family of trans-membrane receptor tyrosine kinases consists of four members: EGFR/human epidermal growth factor receptor-1 (HER-1)/ErbB-1, HER-2/c-*neu*/ErbB-2, HER-3/ErbB-3 and HER-4/ErbB4.[5] These proteins share 40–50% amino-acid homology and have a common domain organisation. On activation by the ligand, these receptors signal through the RAS–RAF–mitogen-activated protein kinase and phosphatidylinositol 3′-kinase pathways, which play critical roles in regulating cell proliferation and survival.

Meert *et al*[6] systematically reviewed 16 studies published between 1989 and 2001, of which 14 were based on immunohistochemistry (IHC). The overall EGFR expression rate in NSCLC was 51%. Expression was less frequent in adenocarcinoma (ADC, 46.2%) than in squamous cell carcinoma (SQCC, 82.6%). Three quarters of the studies (12/16) did not find EGFR expression to be prognostic. A quantitative meta-analysis of eight evaluable IHC studies showed that EGFR IHC expression was marginally significant as a negative (poor) prognostic marker (table 1). In another review, Nicholson *et al*[7] covered reports published during 1985–2000, and found that only 20% and 10% of studies associated increased EGFR levels with decreased recurrence-free survival and overall survival, respectively. Seven additional studies have since confirmed that EGFR IHC is not a prognostic marker for NSCLC,[8–14] whereas one study with questionable analytical methods reported a prognostic significance.[15] Recent, albeit preliminary, evidence, however, suggests that EGFR IHC may yet assume an important role as a predictive marker for response and survival benefit in patients with advanced NSCLC treated by the small-molecule EGFR inhibitors gefitinib and erlotinib.[16 17]

A review of 44 reports (39 studies before August 2004) on HER-2 expression in NSCLC showed an overall positive staining rate of 35%, using cut-offs chosen by the various investigators.[18] HER-2 overexpression differed significantly between histological subtype (38% in ADC, 16% in SQCC and 18% in large cell carcinoma, $p<0.0001$). Among 20 studies (including two that used non-IHC assays) with data evaluable for meta-analysis, eight IHC studies reported a significant detrimental effect of HER-2 expression on survival, whereas 10 reported no significant prognostic value. A meta-analysis of these studies suggested that HER-2 overexpression was a relevant poor prognostic marker for 3-year and 5-year survivals, especially among patients with ADC (table 1). The authors, however, warned that several minor studies were excluded from the meta-analysis as they failed to meet the eligibility criteria for meta-analysis; thus the results require caution in interpretation. Two additional studies were published after the meta-analysis. A study on tissue microarray of samples from 284 patients reported that HER-2 expression was a significant predictor of poor survival

Table 1 Summary of meta-analyses of the results of studies on candidate immunohistochemistry markers for survival of patients with non-small cell lung carcinoma

Marker	First author	Published studies	Number of eligible studies	Number of patients	Overall HR*	95% CI	Significance/comment
EGFR	Meert[6]	Until July 2001	8	1987	1.13	1.00 to 1.28	Weak significance
P21RAS	Mascaux[29]	Until July 2003	7	989	1.08	0.86 to 1.34	Not significant
HER-2	Nakamura[18]	Until August 2004	18	2579	1.32	1.14 to 1.65	17/27 negative studies excluded for lack of detailed survival data
P53	Steels[108]	Until July 1999	8 (Pab1801) 16 (DO-7)	1035 2067	1.57 1.25	1.28 to 1.91 1.09 to 1.43	True significance requires prospective and multivariate confirmation by a standardised technique, scoring criteria and cut-offs
Ki-67	Martin[61]	Until December 2002	16	1863	1.55	1.34 to 1.78	
Bcl-2	Martin[129]	1993 to December 1999	18	2909	0.72	0.64 to 0.82	

CI, confidence interval; EGFR, epidermal growth factor receptor; HER, human epidermal growth factor receptor; HR, hazard ratio of death for high expression of the marker.
*HR>1 implies worse survival for the group with increased expression of the marker, whereas HR<1 (Bcl-2) indicates better survival for the group with higher expression. For p53, the prognostic significance of studies that used Pab1801 and DO7 antibodies were separately analysed.

in patients with lung ADC, but only 2 of 80 patients expressed this protein.[19] A second study on 345 patients also included a rather extensive comparative analysis of various scoring criteria, but reported no noteworthy association between HER-2 expression and prognosis.[20] Overall, the prognostic significance of HER-2 has not been established.

Hepatocyte growth factor and Met receptor

Hepatocyte growth factor (HGF) is also known as scatter factor, a multifunctional cytokine with putative roles in cell proliferation, motility, angiogenesis and morphogenesis.[21] Met is the tyrosine kinase receptor for HGF/scatter factor. The prevalent concept regarding HGF-Met signalling is that it is paracrine, with the Met receptor expressed by epithelial cells and the HGF ligand secreted by stroma fibroblasts. Tsao *et al*,[22] however, showed common expression of HGF mRNA and protein in tumour cells, indicating that HGF-Met autocrine signalling also plays an important role in NSCLC. The reported rates of Met expression or overexpression in NSCLC were 40–60%.[23–25] Takanami *et al*[25] reported that Met expression was an independent poor prognostic marker in ADC. In contrast with other studies that did not find considerable Met expression in tumour stroma cells,[24 25] Tokunou *et al*[26] found Met stromal overexpression in 53% of lung ADC, and this staining was associated with shorter survival for the patients. Takanami *et al*[25] also reported that tumour expression of HGF in ADC was a poor prognostic marker, but only in univariate analysis. Overall, the prognostic roles of Met and HGF in NSCLC remain uncertain, and additional studies using well-characterised and specific antibodies are warranted.

Growth signal transducers

RAS is a small G-protein with intrinsic GTPase activity. It is a critical regulator of signalling downstream of cell surface receptors.[27] Point mutations on codon 12, 13 or 61 of the *ras* family genes result in their oncogenic activation, whose significance in NSCLC has recently been proved by a transgenic mouse model.[28] *Ras* mutations are found in approximately 20% of NSCLC, and they mainly occur in ADC or large-cell carcinoma. The prognostic significance of *ras* mutation remains indeterminate, but the frequency and significance of RAS protein overexpression in lung cancer has been poorly investigated. A meta-analysis of seven evaluable IHC studies showed the absence of prognostic value for RAS protein expression in NSCLC.[29] One possible lack of enthusiasm to study RAS protein expression could be the lack of antibodies with confirmed specificity and reactivity for use in IHC.

Cell cycle checkpoint promoters

Cell cycle includes the G0 (quiescent), G1 (resting), S (DNA synthesis), G2 (pre-mitotic gap) and M (mitotic) phases. Progression between these phases is an orderly process tightly regulated by complex yet redundant mechanisms that include multiple checkpoints, which are used to assess growth signals, cell size and DNA integrity.[30] Cell cycle progression is promoted by cyclins (CCNs) and CCN-dependent kinases (CDKs). The constitutive and persistent high expression of these regulators may cause failure of checkpoint arrests and may lead to uncontrolled proliferation. The CCN family has 18 members (A–I, L and T), each of which may have multiple isoforms (eg, CCND1–3). The CDKs are also composed of several family members, from CDK1 (cdc2) to CDK11. The key CCNs and CDKs are CCNA (S and G2 phases), CCNB (G2/M transition), CCND and CCNE (G1/S transition), as well as CDK1 (G2/M transition), CDK2 (S phase), CDK4 and CDK6 (G1/S transition).

CCND1 is the most studied CCN in NSCLC. Among 15 reports reviewed,[19 31–44] CCND1 expression or overexpression was noted in approximately 50% of NSCLC, but its overall prognostic value remains uncertain. Five studies identified CCND1 overexpression as a negative prognostic marker,[32 33 38 40 42] whereas three others associated it with better prognosis.[19 35 37] Only two poor prognostic reports remained significant in multivariate analyses.[38 42] The remaining seven reports reported no association.[31 34 36 39 41 43 44]

The overexpression of CCNE is also common (~45%) in NSCLC.[35 39 40 45–49] Five of eight studies showed a significant association between CCNE overexpression and worse prognosis,[39 45–49] and three of them found it to be a significant independent marker after adjusting for stage.[39 45 46] By using reverse transcription-polymerase chain reaction assay, Muller-Tidow *et al*[50] also reported that CCNE mRNA overexpression was an independent poor prognostic marker in 70 patients with NSCLC.

Proliferating cell nuclear antigen (PCNA) is the subunit of DNA polymerase δ responsible for its proofreading activity, which has a marked role in maintaining the fidelity of mammalian DNA replication. Thus, overexpression of PCNA may potentially influence cell cycle progression and modulate the effectiveness of radiation and chemotherapy. At least 12 studies have evaluated the prognostic value of PCNA overexpression in NSCLC,[42 44 51–60] with more studies reporting no prognostic value of PCNA overexpression. Ki-67, another nuclear antigen that is expressed only in proliferating cells, preferentially during the late G1, S, G2 and M phases of the cell cycle, is commonly used as a marker to evaluate proliferation of tumour cells. Almost all studies have used the MIB1 antibody to evaluate Ki-67 by IHC. Martin *et al*[61] recently conducted a meta-analysis of studies published before 2003 and identified only 15 of 37 studies (41%) that reported a negative effect of Ki-67 overexpression on prognosis for patients with NSCLC. In all, 16 studies on 1863 patients with NSCLC were evaluable for the meta-analysis. The aggregated survival data showed that Ki-67 immunoreactivity was associated with poorer survival (hazard ratio (HR)1.55, 95% confidence interval (CI) 1.34 to 1.78) and the HR remained significant even within histological subtypes. Four of nine additional studies reported since 2002 also reported that Ki-67 expression was associated with a poorer prognosis.[8 48 62–68]

Insensitivity to growth inhibition pathway

A balance between stimulators and inhibitors of cell proliferation maintains growth homeostasis in normal cells. Resistance to growth-inhibitory factors is an essential step in carcinogenesis. Inhibitory signals may originate from the tumour cells themselves or from their microenvironment. These factors could be soluble molecules, the actions of which are mediated by autocrine, paracrine or endocrine mechanisms. Transforming growth factor β (TGF-β) is generally an antiproliferative factor for epithelial cells and is produced mainly by the stromal cells. Evidence, however, suggests that TGF-β may promote tumour progression.[69] Few studies have explored the prognostic significance of TGF-β expression in NSCLC.[70–72] These studies have provided contradictory results in both the prognostic significance and cellular localisation of TGF-β expression.[72 73]

CCN-dependent kinase inhibitors are negative regulators of CCNs and CDKs.[74] Two major groups exist: the CIP/KIP family genes (p21, p27 and p57), which inhibit all CDKs, and the INK4 family genes (p16, p15, p18 and p19) that inhibit CDK4/6. The loss of CCN-dependent kinase inhibitors should theoretically, therefore, lead to restraint on the proliferative stimuli of tumour cells, thereby leading to tumour progression and poorer prognosis. Less than half the studies on the prognostic effect of $p21^{waf1/cip1}$ in NSCLC have reported significant differences, although this may be owing to the

small sample sizes of the individual studies.[19 41 70 75–78] By contrast, seven of nine studies have reported a noticeable adverse survival effect on the loss of $p27^{kip1}$ protein expression in NSCLC.[19 48 79–85] Loss of p27 expression has been reported in approximately 30% of NSCLC. It is worth noting that unlike many proteins whose protein levels are tightly regulated by gene transcription, the protein level of p27 is mainly regulated by post-translational phosphorylation at the threonine 187 site and degradation through the ubiquitin/26S proteasome pathway.[86] Overall, p27 appears worthy of further evaluation as a potential prognostic marker in clinical trial samples of large cohorts.

The INK4 family proteins bind to CDK4 or CDK6 individually, thereby inhibiting their kinase activities at the mid-G1 phase.[87] The loss or inactivation of $p16^{INK4A}$ results in unchecked activity of CDK4 that phosphorylates retinoblastoma protein, thus releasing the E2F to exert its transcriptional activity. $P16^{INK4A}$ is inactivated in approximately 50% (range 25–60) of NSCLC.[34 38 41 76 83 85 88–94] In approximately 30% of tumours, inactivation is by homozygotic deletion, whereas inactivation by mutation is rare.[95–97] The others are inactivated mainly by promoter hypermethylation. Among 12 studies that have assessed the clinical effect of p16 protein expression, six reported that retention of p16 staining in tumour cells was a good prognostic marker,[38 76 88 89 92 93] although only two of them showed significance in multivariate analysis.[76 93] Two additional studies found p16 loss to be a poor prognostic marker, but only in early-stage tumours or SQCC.[34 90] Thus, despite some discordance, p16 represents a promising marker for further evaluation.

Retinoblastoma protein inactivation may achieve the same results as p16 inactivation and serves as an alternate pathway to promote G1-S cell cycle progression. Loss of retinoblastoma protein expression at variable levels has been reported in approximately 30% of NSCLC samples, but there is no convincing evidence to suggest that it is a significant prognostic marker.[34 38 41 88 91 94 97–104]

Resistance to the apoptosis pathway

Apoptosis is orchestrated by the sequential activation of caspases, a family of cysteine proteases with specificity for aspartic acid residues. Two pathways may mediate apoptosis: (a) the death receptors pathway (tumour necrosis factor, Fas ligand or TRAIL receptors) that activates caspase 8 and 10 and (b) the mitochondrial pathway that activates caspase 9. Both pathways lead to effector caspases (3, 6 and 7).[105] Bcl-2 is antiapoptotic for the mitochondria pathway, whereas Bax is a proapoptotic factor activated by p53.

p53 gene is the most studied gene in all types of cancer, including lung cancer. IHC can detect missense mutant p53 proteins, but may not detect the protein products of nonsense, deletion or truncation mutants. This is partially responsible for the incomplete concordance between p53-positive IHC and sequencing results.[106 107] At least three meta-analyses have been carried out on the prognostic value of p53 gene alterations in NSCLC.[108–110] The frequency of p53 mutation or positive IHC staining is consistently higher in SQCC than in ADC. Although more studies have reported no prognostic significance of p53 IHC than studies that associated it with poor prognosis, the overall conclusion of these meta-analyses is that p53 staining has some adverse prognostic effect in patients with NSCLC (table 1). The strength of this significance, however, remains to be confirmed prospectively and in clinical trials on large patient cohorts with more homogeneous and well-defined patient populations. Since these meta-analyses, more than 20 additional retrospective small cohort studies have been reported. Unfortunately, overall, they provided similarly contradictory results as those reviewed in the meta-analyses.[8 19 42 60 65 75 77 85 93 111–119]

MDM2 is the E3 ubiquitin ligase that targets p53 for nuclear export and degradation. MDM2 and p53 form a feedback loop that is also modulated by $p14^{ARF}$, the alternate spliced product of *INK4* gene that also produces $p16^{INK4A}$. $P14^{ARF}$ binds MDM2, thus the overexpression of p14 would lead to a sequestration q MDM2 and prevents its binding with p53, thus resulting in stabilisation of the p53 protein. This is the postulated mechanism wherein p53 protein overexpression is found in the absence of mutation.[120] The precise role of MDM2 in tumour progression, however, remains uncertain.[121] Although MDM2 gene amplification has been associated with poor prognosis,[122] MDM2 mRNA expression has also been reported to be a favourable prognostic marker in patients with NSCLC.[123] Five studies have evaluated the prognostic value of MDM2 IHC in patients with NSCLC.[123–127] Whereas three studies did not find MDM2 expression to be prognostic, two groups have reported MDM2 staining to be a favourable prognostic marker in p53-negative patients with NSCLC.[124 127]

At least 20 members in the Bcl-2 family share at least one conserved Bcl-2 homology domain.[128] These are subdivided into prosurvival (antiapoptosis) family members, including Bcl-2, Bcl-x_L and Bcl-w, and proapoptosis families, including Bax, Bak and Bok. There are also Bcl-2 homology 3-only proteins that promote cell death, including Bid, Bim, Bik, Bad, Bmf, Hrk, Noxa and Puma. The balance between prosurvival and proapoptosis factors determines whether a cell will respond to apoptotic signals. A meta-analysis of publications from 1993 to 1999 examined the prognostic significance of Bcl-2 in lung cancer (table 1).[129] Among 21 studies on NSCLC, 10 reported Bcl-2 expression as a good prognostic factor, whereas one reported it as a poor prognostic marker. The expression of Bcl-2 was found in approximately 35% of NSCLC, being more common in ADC (61%) than in SQCC (32%). Counter-intuitive to the antiapoptotic function of Bcl-2, a meta-analysis of 18 studies with evaluable data and comprising 2909 patients showed a better survival outcome for patients with Bcl-2-positive tumours (HR 0.72, 95% CI 0.64 to 0.82). The results were similar for the two antibodies most commonly used (clone 100 or 124) and the four ranges of thresholds used as cut-off (table 1). Since the meta-analysis, there have been 27 additional independent IHC studies,[8 44 60 103 112 113 117 119 130–145] most of them using the clone 100 and 124 antibodies. The mean Bcl-2 expression rate (34%) was remarkably similar to those included in the meta-analyses, and approximately 40% of the studies reported Bcl-2 expression to be a good prognostic marker.

No prognostic significance seems to exist for Bax in NSCLC.[67 92 119 130 140 146–149] Only one of seven studies indicated an association of Bax expression with better survival,[119] whereas the others failed to show significance, including one study that evaluated only patients treated by chemotherapy.[147]

Unlimited growth potential pathway

Telomerase complex, which contains an RNA subunit human telomerase RNA gene (hTERC) and a protein catalytic subunit human telomerase reverse transcriptase (hTERT), is responsible for the maintenance of integrity of chromosomal telomeres and genomic stability. IHC detection of hTERT expression is frequent in NSCLC (50–90%)[150–152] and has been correlated with increased telomerase activity,[150 152] but is not predictive of survival. The specificity and sensitivity of hTERT antibody for IHC has not proved to be optimal. Even with the best available monoclonal antibody 44F12 that gives a single band in western blot, the concordance rates of IHC staining

and western blot, telomerase activity and mRNA expression as detected by in situ hybridisation were 76%, 70% and 70%, respectively.[152]

Sustained angiogenesis pathway

Angiogenesis is crucial for primary tumour growth and metastasis, and comprises interactions between tumour cells, endothelial cells and stroma cells. Growth factors and cytokines have been implicated in angiogenesis, as well as proteases that break down extracellular matrix (ECM). Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is a key regulator of angiogenesis in physiological and pathological conditions.[153] To date, seven family members of VEGF have been identified, including VEGF-A (also known as VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E, placenta growth factor 1 and placenta growth factor 2.[154] Overexpression of VEGF has been associated with tumour progression and poor prognosis in most cancer types, and novel antiangiogenic agents have recently been approved or are in various phases of clinical trials for cancer treatment.[155] VEGF expression or overexpression has been reported in approximately 60% of NSCLC. Most studies have indicated a significant positive correlation between VEGF immunoreactivity and tumour vascularity.[67 156–159] To date, seven of 12 studies have reported that higher VEGF protein expression predicts poorer survival for patients with NSCLC, and four of these studies identified it as an independent prognostic marker.[67 150 156–158 160–166] Four of five studies have also shown that VEGF-C is a predictor of poor prognosis, but only one found it to be an independent marker.[67 164 167–169]

Invasion and the metastasis pathway

Aside from angiogenesis, the breakdown of intercellular junctional complexes and ECM proteins by tumour cells is also an important process in tumour progression. Integrins, cadherins, selectins, the immunoglobulin super gene family (IgSF) and CD44 are families of adhesion or junctional molecules with different genetic and biochemical properties. Factors that alter the abundance or function of ECM proteins and adhesion or junctional molecules may influence the ability of tumour cells to invade and metastasise, and thus cancer prognosis.

E-cadherin and catenins are components of adherens junction protein, with crucial roles in the maintenance of intercellular junctions in epithelial cells. Thus, reduced catenin and E-cadherin expression could potentially affect tumour differentiation, metastasis and prognosis. In all, three family members of catenin exist, α, β and γ. Whereas α-catenins and γ-catenins strictly participate in junctional complex formations, β-catenin is also associated with the regulation of transcription through the wingless-type MMTV integration site family pathway.[170 171] Expression of catenins has usually been assessed in conjunction with that of their partner protein E-cadherin. Eight studies have evaluated the prognostic role of β-catenin in NSCLC[172–179]; three of these simultaneously investigated the significance of α-catenins and γ-catenins.[172 176 177] Reduced expression of β-catenin was found in about 30% of NSCLC, and five studies reported the association of reduced staining with poor prognosis.[172 175–178] Interestingly, two of three studies that investigated the α-catenins and γ-catenins also found that their reduced expression was a poor prognostic marker.[176 177] Four of seven studies reviewed also reported favourable prognosis for NSCLC tumours that retained normal E-cadherin expression, with two studies remaining significant in multivariate analysis.[173 175–177 179–181] Thus, β-catenin and E-cadherin are worthy candidates for further investigation.

Matrix metalloproteinases (MMPs) comprise of almost 20 family members and are the only enzymes that can degrade fibrillar collagens. There are also four tissue inhibitors of metalloproteinase (TIMPs), which are endogenous anti-MMP molecules.[182] Although the expression of many of these MMPs and TIMPs has been studied by IHC, most have focused on the gelatinases MMP-2 and MMP-9.[163 166 183–188] Results from most of these studies have suggested that high expressions of MMP-2 and MMP-9 are poor prognostic markers for NSCLC, especially the expression of MMP-2. Most of these studies have identified tumour cell expression of the MMPs as prognostic,[163 183–185 187] whereas Ishikawa *et al*[186] reported that the stroma, but not tumour cell expression of MMP-2, was prognostic. Increasing evidence resulting from in situ hybridisation and laser capture microdissection coupled with real-time reverse transcription-polymerase chain reaction technique suggests that most MMPs and TIMPs are predominantly expressed in the stroma rather than in the tumour cells,[189] whereas in nearly all published studies the focus has been almost exclusively on tumour cell expression, with few studies reporting on both tumour and stroma cells. Overall, the role of MMP IHC in the prognosis of NSCLC should be studied further, but these need to be accompanied by a very vigorous validation of the specificity of the antibodies.

Other candidate markers

Our discussion is restricted to markers that have been studied in four or more independent studies. A large number of markers exist, however, which have been less studied but have shown either promising or controversial results as prognostic indicators (fig 1). Markers in which expression changes have been associated with worse prognosis in at least two studies, and which have no reports of an opposite association, are considered to be promising candidate markers. These include CCNA (two studies),[49 59] CCNB1 (two of three studies),[168 190 191] carbonic anhydrase IX (two studies)[166 192] and fragile histidine triad (FHIT; two of four studies).[116 193–195] In these markers positive expression is associated with poorer prognosis, although retained (positive) FHIT expression is associated with better outcome.

DISCUSSION AND CONCLUSION

Of 17 markers that have been investigated by eight or more groups (fig 1), none have shown consistent results in all studies. There are, however, six markers (overexpression of CCNE and VEGF-A, and loss of $p16^{INK4A}$, $p27^{kip1}$, β-catenin, and E-cadherin) that have shown correlation with poor prognosis in 50% or more of the studies, without contrary results. Interestingly, markers that have been studied most exhaustively (EGFR, HER-2, Ki-67, p53 and Bcl-2) have all reported inconsistent results, perhaps suggesting that their prognostic values are at best weak. In addition to the above markers, there are other promising markers that have been less studied, yet have shown significant results more often than non-significant results. These may also be worthy of further validation, and include HGF/Met, CCNA, CCNB1, VEGF-C, carbonic anhydrase (CA) IX, α-catenin and γ-catenin, MMP-2, MMP-9 and FHIT.

Our review has largely confirmed what is well known among pathologists and oncologists—that studies on prognostic markers, particularly, but not exclusively, those including immunohistochemical assays, often give rise to inconsistent or contradictory results. The issue has become especially urgent as oncology is entering the dawn of personalised medicine with targeted treatments.[196 197] Recently, the Statistics Subcommittee of the NCI-EORTC Working Group on Cancer Diagnostics has published the REporting recommendations for tumour MARKer prognostic studies (REMARK) guidelines.[198] The data we collected during this review (http://www.uhnres.utoronto.ca/labs/

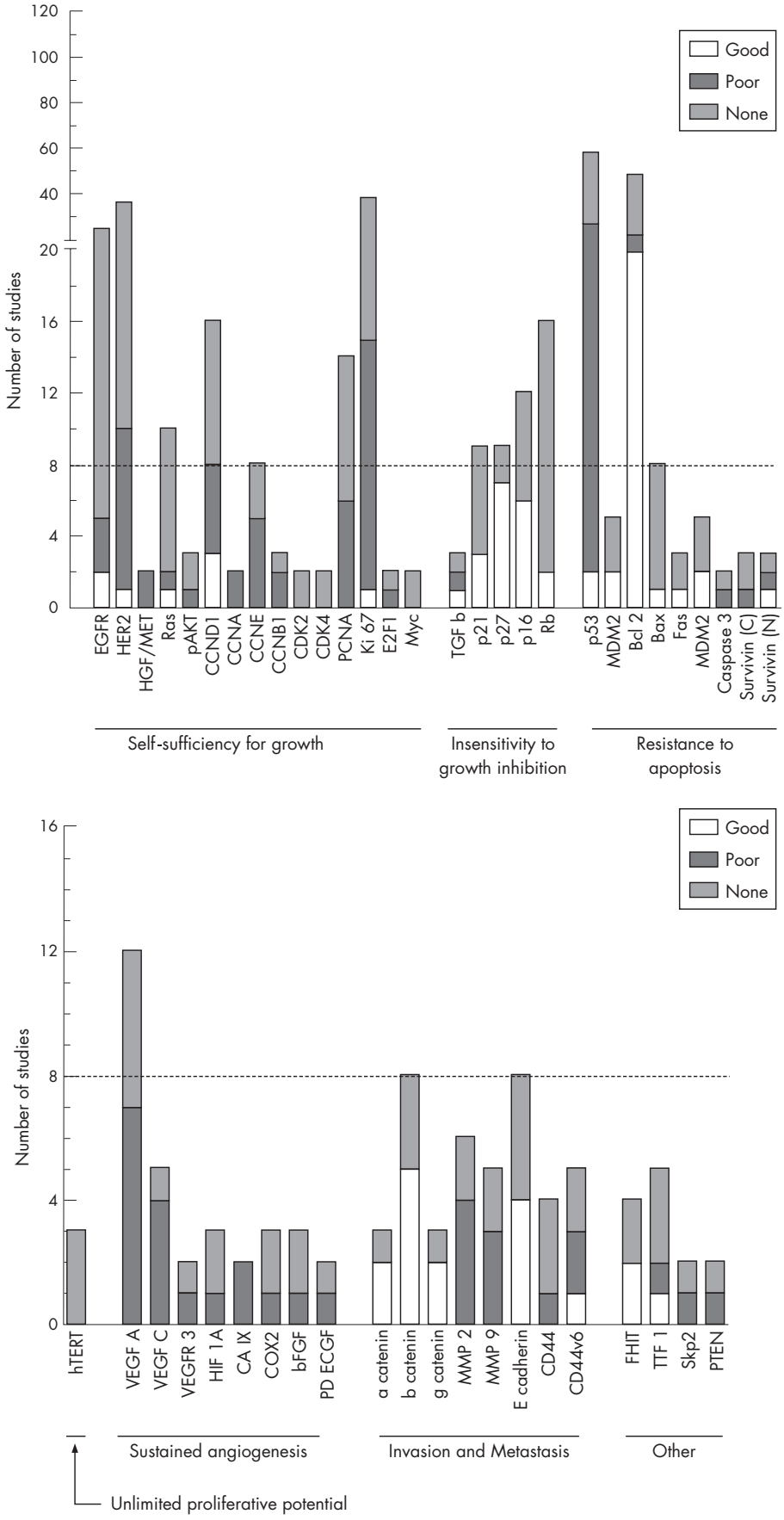

Figure 1 Number of reports for markers that have been studied by two or more independent groups of researchers. For each marker, the graphs also indicate the relative distribution of studies that showed significant association of high expression or overexpression with good (favourable) prognosis, poor prognosis or no prognostic value. The broken line is to facilitate identifying markers that have been studied by eight or more groups. The detailed summaries of markers and their deregulations are available at http://www.uhnres.utoronto.ca/labs/tsao/index.php.

tsao/supplementary-data.php) clearly highlight the multiple study design factors that have contributed to the inconsistencies and contradictions. Many of these issues require serious discussion by the pathologist community.

For almost all markers, diverse sources and types of antibodies have been used. Multiple antibodies per se should not become an issue as long as each antibody has been well characterised for its specificity and sensitivity. For many markers, especially new ones, the quality assurance for the antibody is often not adequately described. Specification of the method used to determine the antibody titre that is finally adopted is often neglected. Including representative low and high photomicrographs of the staining would also go a long way in convincing readers that the staining is probably specific.

It should be recognised that IHC scoring is at best a semiquantitative exercise for which no standard criteria have as yet been proposed or adopted. The heterogeneity in scoring methods possibly partly reflects our difficulty in coming to grips with genotypic and phenotypic (expression) heterogeneity in tumours. Unfortunately, our understanding of the cause and effect of these heterogeneities, including immunostaining, is almost non-existent, thus making it difficult to design rational and biologically meaningful scoring criteria. The scoring methods that have been used included estimating the grade of staining intensity (numerically from absent to strong), percentage of tumour cells stained, cellular localisation of the antigen and systems that combined these parameters. The approaches to combining the intensity and percentage of cells stained have also been divergent, including a direct multiplication of the percentage of stained cells with staining intensity score, or of grouping the staining percentage into 3–4 scores and then summing this with the intensity score. Although superficially the two methods could lead to comparable final scores, in reality, the former could unreasonably overweight the percentage score. The only scoring system that has been generally accepted is the one used to assess HER-2/c-erbB2 staining. Perhaps a more important confusion that permeates many biomarker correlative studies, especially IHC, is the method of determining cut-offs for dichotomising scores in log-rank tests. Cut-offs were often arbitrary and sometimes selected to obtain the desired effect by the ''minimum p value'' approach.[199] Such approaches have led to the existence of diverse but poorly justified cut-offs, which may contribute to non-reproducibility of the results. In general, taking the median as cut-offs for log-rank tests provides the most unbiased, albeit stringent, criteria for analysis, but selecting multiple rationally justified cut-offs is also worthy of performing.[199]

Unlike predictive markers for the efficacy of specific treatment, prognostic markers in NSCLC are most relevant for early-stage or resectable tumours. By including patients with advanced-stage tumour into cohorts of patients with primarily early-stage tumour, we may introduce unwanted effects. Some studies have reported that specific markers could have different prognostic significance in patients with early-stage tumours as compared with those with advanced-stage tumours, or in different histological subtypes.[101] Unfortunately, subgroup analysis will reduce the number of patients in each subgroup, and thus may underpower the statistical analysis. Biased selection of patient cohorts may also lead to results that will be difficult to reproduce in other studies.

The most relevant end point for assessing the prognostic value of a marker is its association with overall or disease-free survival. Correlations with stage or tumour grade may provide insights into the tumour biology, but are weak and inadequate parameters for assessing the significance of the outcome. As marker expression can be correlated with other clinical and pathological parameters (eg, age, stage, sex), which may also influence prognosis, only multivariate analysis that adjusts for these factors is more likely to become clinically useful. Studies with small sample size (<100 cases) more often than not will fail such stringent statistical tests.[196]

Last but not least, we have limited our review to the prognostic significance of individual markers, although most of them were published in the context of studies on multiple

Table 2 Proposed multiphase evaluations of immunohistochemical prognostic markers

mPhase	Objectives	Suggested sample size	Data to include in publication
1	To verify: 1. Specificity of antibody and assay technique 2. Cellular localisation of expression 3. Expression changes in tumour cells compared with normal cells	10–20	Source of antibody and dilution Technical details Nature of samples Pattern of expression changes
2	To investigate the prognostic significance of markers in samples from a single institution	50–300	Details of scoring system Cut-offs for log-rank test of survival and their justification Type of survival (disease-free or overall) correlation Univariate and multivariate HR, 95% CI, p value Kaplan–Meier plot
	Meta-analyses of mPhase 2 studies to determine markers that show evidence and potential strengths as prognostic markers	⩾10 studies	Inclusion and exclusion criteria Details of meta-analysis results Effects of assay technique, cut-off Potential for publication bias Overall strength of HR and significance
3	To validate the significance of best candidate markers in phase III clinical trials with tumour samples of limited availability	00s–000s	Demographics of studied patients compared towith patients in the overall trial patients Results of univariate and multivariate survival analysis results
4	To test prospectively the performance of markers in phase III randomised clinical trials by incorporating markers as stratification factors	00s–000s	

CI, confidence interval; HR, hazard ratio.

markers. Many of these studies have reported that whereas an individual marker was not prognostic, a combination phenotype could be prognostic, but such analyses could raise many issues and caveats that are beyond the scope of the current review.

In accordance with the above caveats, we suggest that perhaps studies on tumour biomarkers should adopt a similar multi-phase approach as that being used in clinical trials for new therapeutics (table 2). Most studies may combine marker Phase (mPhase) 1 and 2 studies but even in this early stage of marker evaluation, a conceptual multi-phase approach would provide a more consistent reporting of the studies. We believe that all results, including the full data from well-conducted mPhase 1 and 2 studies, should be reported to avoid publication biases.[200] Meta-analyses on markers for which 10 or more mPhase 2 studies have been reported would provide important insights on whether such markers are worthy of further mPhase 3 studies on samples of patients participating in phase III controlled and randomised clinical trials. These samples are usually of limited availability, and thus should be reserved only for advanced validation of the best candidate markers. The ultimate (mPhase 4) test for the clinical effect of a marker will be when it becomes a stratification factor in prospectively designed phase III clinical trials. A rigorous implementation of such a multi-phase approach to studies on prognostic markers can improve our chances of identifying true prognostic markers that may be reliably applied in our clinical practice.

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