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Review

Molecular prognostic markers in resectable colorectal liver metastases: A systematic review

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ABSTRACT

Background: Determination of prognosis in patients with resectable colorectal liver metastases (CLM) is desirable in order to improve case selection for surgery and tailor adjuvant treatment according to individual recurrence risk. Conventional clinicopathological factors lack the sensitivity to accurately achieve this goal. Consideration of tumour biology and the identification of molecular prognostic markers may allow more accurate risk stratification.
Method: This systematic review examines evidence from published manuscripts looking at molecular markers in resectable colorectal liver metastases and their correlation with disease recurrence and survival following hepatectomy.
Results: Studies have yielded promising results in the search for prognostic molecular markers of CLM. Molecular biomarkers from varied aspects of tumour biology have been examined and a number of these, including proliferation indices, telomerase, thymidylate synthase, microvessel density and thrombospondin-1 appear to have prognostic utility in this context. Validation of other markers, notably p53, has been limited by a failure of methodologies to account for their biological complexity.
Conclusions: A biomarker-based approach may yield significant benefits through informed treatment of resectable metastatic colorectal malignancy. Standardised retrospective analyses are necessary to confirm preliminary findings and identify existing and novel markers for inclusion into prospective studies. Assessment and verification of multiple molecular markers in this manner may allow molecular profiling of metastases and tailoring of therapy according to the biological aggressiveness of individual tumours. The advent of genomic- and proteomic-based technologies will allow the simultaneous analysis of multiple molecular markers and the derivation of disease profiles associated with disease recurrence and poor survival.

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1. Introduction

Despite continuing efforts towards the early detection of colorectal cancer (CRC), up to 35% of patients have hepatic metastasis at the time of operation for the primary lesion.[1,2] A further group, up to 25%, will develop hepatic lesions after resection of the primary tumour.[1,2] Hepatic resection offers the only hope of cure for colorectal liver metastases (CLM), and though possible in only 15–25% of patients, yields 5-year survival rates approaching 30–50%.[3,4] Recurrence in the remnant liver and/or extrahepatic sites is common, however, affecting two-thirds of patients despite optimal metastasectomy.[5,6] Recurrence is not inevitable, however, and certain patients achieve excellent long-term results, with 20-year survival of up to 20% reported.[7] As the indications for hepatectomy for CLM broaden, the ability to predict outcome within these prognostic extremes is urgently required in order to guide surgical and chemotherapeutic treatment according to individual recurrence risk.

Recurrence following optimal metastasectomy results from the growth of residual micrometastatic deposits.[8,9] Host immune surveillance, often aided by chemotherapy, facilitates clearance of this residual disease in a proportion of patients. Recurrence develops when the volume or aggressive phenotype of disease is such that clearance cannot be achieved. Individual prognosis, therefore, depends upon determination of the burden and biological aggressiveness of residual disease.

Clinicopathological factors, including stage of the primary tumour, interval between primary tumour resection and diagnosis of hepatic metastases, number and size of metastases, and pre-operative carcinoembryonic antigen (CEA) level, have been shown to be indicative of disease status and to stratify patients for recurrence risk following metastasectomy.[10,11] They lack the sensitivity for accurate individual prognostication, however, as patients with identical clinicopathological variables may arrive at disparate outcomes.[12] The pressing need for more accurate outcome predictors has led to consideration of the role of specific aspects of tumour biology in determining disease recurrence and a drive to identify molecular markers of poor prognosis.

Fearon and Vogelstein proposed a model for the development and progression of colorectal tumours through the accumulation of somatic mutations of key oncogenes and tumour suppressor genes.[13] Four to five mutations, involving the *adenomatous polyposis coli* (*APC*), *Kirsten-ras* (*K-ras*), *deleted in colorectal cancer* (*DCC*) and *p*53 genes, were considered necessary for malignant transformation. In fact, studies have demonstrated concomitant mutations of these genes to occur in less than 10% of tumours,[14] with alternative pathways to tumourigenesis in most cases.[15,16] Further adaptations are necessary for the acquisition of metastatic capability, including varied alterations in cell–cell and cell–matrix recognition, secretion of proteases and induction of angiogenesis. CLM, therefore, represent a diverse disease state, with significant molecular heterogeneity. This diversity contributes to the variable recurrence risk following metastasectomy, with particular alterations in gene expression driving more aggressive metastatic phenotypes.

The role of prognostic molecular markers following resection of primary CRC has recently been reviewed.[17–19] Generalisation of these results to resected CLM, however, is inappropriate. Distinct cellular adaptations are necessary for acquisition of metastatic competence and the expression of putative biomarkers, including tumour suppressor genes and oncogenes, alters significantly in the transition to metastatic spread.[20–23] Furthermore, the significance of candidate biomarkers may be context dependent, resulting in a different cellular response in the regeneratory milieu following hepatic resection to that occurring after primary tumour excision. Recent years, therefore, have seen extensive investigation of the prognostic role of molecular markers within resected CLM.

Cancer biomarkers are quantifiable molecules involved in the physiological or pathological events occurring between exposure to carcinogens and the development and progression of cancer.[24] Biomarkers may be the consequence of a continuous process, such as increased cell mass, or a discrete event, such as genetic mutation.[25] Criteria for the development of prognostic cancer biomarkers[26] require those for resectable CLM to provide insight above and beyond that afforded by clinicopathological factors. The aim of this review is to assess putative biomarkers of resectable CLM for the ability to predict post-operative prognosis, setting them in context against their role in the colorectal carcinogenesis cascade and their prognostic value for resected primary CRC.

2. Methods

A systematic literature review was undertaken to determine the prognostic significance of biomarkers in resectable CLM. Searches of the Pubmed and Web of Science databases were performed using the following keywords, in varied combinations: colorectal cancer, liver, hepatic, metastasis, resection, hepatectomy, recurrence, prognosis, molecular markers, biomarkers. Individual biomarkers were also included in searches. References cited in identified articles were then used to identify further relevant publications. The search was restricted to English Language publications and was conducted up to 31st October 2005. Studies published in abstract form only, unpublished studies and articles published in non-peer reviewed journals were not included. Studies of molecular markers solely in unresectable CLM were also excluded.

Molecular prognostic markers in the following classes are discussed: oncogenes [*ras*, *epidermal growth factor receptor* (*EGFR*)]; tumour suppressor genes [*p*53, *p*21, *DCC*, *Smad*4]; proliferation indices [S-phase cell fraction (SPF), Ki-67, MIB-1, proliferating cell nuclear antigen (PCNA)]; markers of immortalisation [telomerase]; biochemical markers [thymidylate synthase (TS)]; apoptotic regulators [Fas/CD95; Bcl-2 family]; markers of genomic instability [chromosomal instability(CIN)/aneuploidy, microsatellite instability (MSI), fractional genomic alteration (FGA)]; markers of angiogenesis [microvessel density (MVD), vascular endothelial growth factor (VEGF), thrombospondin-1 (TSP-1)]; and markers of invasion and metastasis [epithelial cadherin (E-cadherin), CD44 variant 6 (CD44v6), non-metastatic protein 23 (nm23), matrix metalloproteinases (MMPs)].

3. Oncogenes

Oncogenes are mutated or upregulated forms of genes that promote normal cellular growth (proto-oncogenes), with aberrant expression typically causing gain-of-function and uncontrolled cellular proliferation. The *ras* and *EGFR* oncogenes have been assessed in resected CLM.

3.1. *K-ras*

The *ras* family of proto-oncogenes (*K-ras*, *N-ras*, *H-ras*) encode inner plasma membrane proteins which transduce signals from extracellular mitogenic ligands to proliferative and anti-apoptotic pathways.[27] *K-ras* mutations occur at codons 13 and 61, but most commonly arise at codon 12, which usually undergoes mis-sense mutation.[28] Mutation stabilises the *ras* protein ($p21^{ras}$) through disruption of its normal intrinsic hydrolytic deactivation,[29] imparting a strong growth signal on mutated cells.[30] Mutation accompanies the transition from small to intermediate adenoma and is present in approximately 50% of CRC.[28]

The prognostic role of *ras* following primary CRC resection is uncertain. Numerous studies have failed to demonstrate a significant association between *ras* status and outcome (reviewed by Kahlenberg et al.[17]) whilst others, including the collaborative RASCAL studies, found decreased patient survival in association with *K-ras* mutation.[31–34] The inclusion of varying tumour stages may account in part for these discrepancies. In those studies identifying an association with prognosis, specific codon mutations have varyingly been found to be of significance.[31–34]

In CLM, Russo et al. found no significant difference in survival between patients with *K-ras* wild-type or mutant metastases as determined by PCR-SSCP (polymerase chain reaction-single strand conformational polymorphism), though a non-significant relationship with poor prognosis was noted for tumours with codon 13 mutations.[35] Kastrinakis et al., however, found neither the presence of *K-ras* mutation in resected metastases, nor the precise nucleotide change, to be predictive of long-term survival.[36] Similarly, Petrowsky et al. found no correlation between *K-ras* codon 12 status and survival following metastasectomy, though mutation at the codon was identified in only 6 of 41 patients.[37] Furthermore, CLM with *K-ras* mutation had no proliferative advantage over wild-type metastases, suggesting that aggressive growth of colorectal metastases may not be mediated via *K-ras* activation.

Current data, therefore, suggest that *K-ras* is not a prognostic marker in resected CLM. Larger studies are necessary for confirmation, particularly if a prognostic role of specific *K-ras* mutations, as demonstrated for primary CRC, is to be ascertained. Mutation of the *H-ras* gene, an infrequent occurrence in CRC, was also found to have no prognostic role following metastasectomy.[38]

3.2. *Epidermal growth factor receptor (EGFR)*

The EGFR superfamily consists of four transmembrane tyrosine kinase receptors (erbB1–erbB4) that bind distinct ligands and induce cellular proliferation. Although significant prognostic indicators for breast carcinoma, their prognostic role following primary CRC resection is, at present, unclear.[39,40] These receptors may have particular relevance in the context of metastasectomy, however, where their expression may enable residual disease to respond to the multitude of growth factors released following hepatic resection.

De Jong et al. examined expression of EGFR (erb-B1) and one of its ligands, transforming growth factor-α (TGF-α), in resected CLM, finding increased EGFR expression to independently predict decreased disease-free survival.[41] Upregulation of TGF-α expression, relative to expression in the corresponding primary tumour, also correlated with decreased survival, though the association was borderline on multivariate analysis. Other studies, however, have shown no prognostic role for EGFR in resected CLM.[38,42] Expression of human epidermal growth factor receptor-2 (HER-2, erb-B2) was also shown to have no prognostic significance following metastasectomy.[38] Further studies are needed to confirm these findings.

4. Tumour suppressor genes

Tumour suppressor genes inhibit tumourigenesis through regulation of cell division and differentiation. Prognostic significance of mutation of tumour suppressor genes *p53* and *p21*, as well as those located at the 18q chromosome locus have been studied in resected CLM.

4.1. *p53*

Mutation of the *p53* tumour suppressor gene, located on chromosome 17p13.1, occurs in over 50% of sporadic CRC and represents a late step in tumourigenesis, occurring at the juncture between adenoma and carcinoma.[43] The gene is central to regulation of cell cycle checkpoints at the G_1/S and G_2/M boundaries[44] and to apoptosis through regulation of Bax activity.[45] Mutations, therefore, permit unchecked replication of defective DNA, genomic instability and progression to cancer. A role for *p53* mutation in the promotion of angiogenesis, through regulation of VEGF[46] and thrombospondin,[47] has also been proposed. *p53* mutation may be important in progression from local to systemic disease, with a higher mutation rate noted in metastasising compared with non-metastasising primary CRC.[48] *p53* mutation was also found to correlate with increased metastatic burden, as well as the development of synchronous, rather than metachronous, metastases.[49] Such observations have raised considerable interest in the potential of *p53* as a prognostic biomarker in resected CLM.

p53 status may be studied by both immunohistochemical and mutational analysis (by sequencing or PCR-SSCP). The short half-life of wild-type p53 protein normally renders it undetectable by immunohistochemistry (IHC). Mutation may cause protein-stabilising conformational alteration,[50] permitting nuclear accumulation and positive staining.[51]

Reports linking *p53* status of resected primary CRC with outcome have yielded conflicting results. Soong et al. found no prognostic role for PCR-determined *p53* status, though noted improved survival in patients with over-accumulation of p53 on IHC.[52] In contrast, Kahlenberg et al. reported *p53* mutation to independently predict disease recurrence.[53] A

Table 1 – Characteristics of studies on the prognostic role of p53 following metastasectomy

Study	Year	Patients (*n*)	Adjuvant therapy following metastasectomy	Method	Cut off for positivity[a]	% positive cases	Correlation with survival
Costa et al. [55]	1997	104	Not reported	IHC	>5%	59	No significant prognostic role
Pocard et al. [56]	1998	102[b]	Not reported	IHC	⩾5%	61	No significant prognostic role
Nitti et al. [57]	1998	69	18 'high risk' patients	IHC	⩾10%	64	p53-positivity independently predicted decreased OS ($P = 0.0079$)
De Jong et al. [41]	1998	45[c]	Not reported	IHC	Not reported	71	p53-negativity independently predicted decreased DFS ($P < 0.03$) p53-negativity associated with decreased OS on UV analysis. No independent prognostic role for OS
Russo et al. [35]	1998	36[d]	If unresectable	PCR-SSCP	p53 mutation	43	No significant prognostic role
Heisterkamp et al. [58]	1999	43	Not reported	IHC	>10%	53	No significant prognostic role
Sturm et al. [59]	1999	41	71% of patients	IHC	>10%	60	No significant prognostic role
				PCR-SSCP	p53 mutation	35	No significant prognostic role
Tullo et al. [49]	1999	40	Not reported	PCR/DGGE	p53 mutation	45	p53 mutation associated with decreased DFS on UV analysis ($P < 0.01$). MV analysis not reported
Yang et al. [60]	2001	41	None	IHC	>5%	51	p53-positivity independently predicted increased OS
				PCR/DGGE	p53 mutation	62	p53 mutation independently predicted increased OS ($P < 0.04$)
Crowe et al. [38]	2001	71	None	IHC	⩾2 on semiquantitative scale	Not reported	p53-positivity independently predicted decreased HDFS ($P = 0.007$). No significant prognostic role for OS
Saw et al. [61]	2002	63	Not reported	IHC	⩾6 on semiquantitative scale	44	No significant prognostic role
				PCR-SSCP	p53 mutation	53	No significant prognostic role
Gonen et al. [42]	2003	144	All patients	IHC	⩾20%	52	No significant prognostic role
Tanaka et al. [62]	2004	75	All patients	IHC	>50%	48	p53-positivity independently predicted decreased OS ($P = 0.011$) p53-positivity associated with decreased HDFS on UV analysis ($P < 0.05$). No independent prognostic value for HDFS
De Jong et al. [63]	2005	44	15 patients	IHC	>10%	61.4	No significant prognostic role
				PCR-SSCP	p53 mutation	36	No significant prognostic role

IHC, immunohistochemistry; OS, overall survival; DFS, disease-free survival; UV, univariate; PCR-SSCP, polymerase chain reaction-single-stranded conformational polymorphism; DGGE, denaturing gradient gel electrophoresis; MV, multivariate; HDFS, hepatic disease-free survival.

a Immunohistochemical cut-offs indicate the percentage of cells with positively staining nuclei.

b Short-term survivors ($n = 10$) and long term disease-free survivors ($n = 11$) only used for analysis.

c 7 unresectable.

d 23 unresectable.

meta-analysis of 28 studies, including 4416 patients, reported only a borderline significant hazard associated with overexpression or mutation of *p*53.[54]

Studies of p53 in resected CLM have also yielded inconclusive results (Table 1), with many showing no significant prognostic value.[35,42,55,56,58,59,61,63] Amongst immunohistochemical studies, three groups found p53-positivity to independently predict decreased disease-free or overall survival.[38,57,62] Tanaka et al. reported 5-year survival rates of 4.4% and 60.6% in patients with p53-positive and p53-negative metastases respectively, with p53-positivity representing a 4-fold increased relative risk of death.[62] Other groups, however, have reported p53-positivity to independently predict increased disease-free and overall survival following hepatic resection.[41,60]

Conflicting results have also been reported with PCR-based mutation analysis of resected CLM. Tullo et al. reported that 33% of patients with wild-type metastases developed disease recurrence, compared to 73% of patients with mutant *p*53.[49] In contrast, Yang et al. found *p*53 mutation in metastases to be associated with significantly improved survival compared to that seen with *p*53 wild-type metastases.[60] The authors proposed that this paradox may be due to the fact that *p*53-mutated cells represent a functionally impaired population, less able to respond to the cascades of regenerative growth factors released following hepatic resection.[64]

The prognostic role of *p*53 in resected CLM, therefore, remains to be confirmed. The conflicting results to date may be explained, in part, by differences in the methodologies employed. Differences in antibodies, detection systems and cut-offs have likely contributed to the divergent results of immunohistochemical studies.[51] Furthermore, it is established that prediction of the presence of *p*53 mutation by immunostaining is unreliable, giving concordance with mutational analysis in the region of only 70% for CRC.[65,66] Frameshift and nonsense mutations do not cause protein stabilisation and, conversely, a significant proportion of carcinomas with stabilised p53 protein on IHC do not contain mutations in exons 5–8.[67] False positives on IHC may arise due to interaction of wild-type p53 with stabilising cellular proteins or upregulation of wild-type expression in response to continuous DNA damage.[68,69] Immunohistochemistry, therefore, provides an unreliable assessment of tumour *p*53 status.

With regard to sequence-based studies, several factors may account for the conflicting results of the relevance of *p*53 mutation in resected CLM, including the area of the gene analysed. Several studies have restricted their analysis to exons 5–8 of the *p*53 gene, though it is now known that around 13% of mutations are located outside this region, particularly on exons 4, 10 and, to a lesser extent, 9.[70] Furthermore, due to the multifunctional nature of the p53 protein, specific mutations may exert distinct effects upon tumour phenotype and, therefore, prognosis. In primary CRC, for example, mutations in the L3 zinc-binding domain were associated with shorter survival than both wild-type *p*53 and mutations elsewhere in the *p*53 gene.[71] Future studies must examine the role of specific *p*53 mutations upon patient outcome following metastasectomy.

4.2. *p21*

The cyclin-dependent-kinase inhibitor p21 represents a key effector of p53 anti-proliferative activity, inactivating the cyclin D-cyclin-dependent-kinase 4 complex that is essential for DNA synthesis and so inducing cell cycle arrest.[72] Positive immunohistochemical staining for p21 was found to be an independent predictor of increased survival in primary CRC.[73] Studies to date, however, have shown no correlation between metastatic *p*21 status and overall survival following hepatic resection.[59,62]

4.3. *DCC/Smad4 (chromosome 18q loss of heterozygosity)*

Loss of heterozygosity (LOH) of chromosome 18q occurs in 60–70% of primary CRC,[28] rising to 90–100% of hepatic metastases,[74] suggesting a role in invasion and distant spread. Primary cancers with 18q LOH are associated with distant metastasis and poor prognosis, independent of tumour stage.[75,76] The candidate tumour suppressor gene *DCC*, located at 18q2.21, was initially proposed as the target of 18q LOH, encoding a transmembrane protein involved in signal transduction from netrin chemoattractant and cell guidance factors.[77] Defective expression of DCC in primary CRC correlated with distant metastasis and poor prognosis.[78,79]

Subsequently, the tumour suppressor genes *Smad2* and *Smad4*, encoding key signalling molecules within the transforming growth factor-β pathway, were also mapped to chromosome 18q.[80,81] The frequency of *Smad4* mutations rises from 0% in adenomas, to 7% in early stage carcinomas and 31% in distant metastases,[82] suggesting a role in the acquisition of an invasive phenotype. *Smad2* mutations are less common and are specifically mutated in a subset of colorectal tumours.[80]

Kocharr et al. examined the prognostic role of 18q LOH in resected CLM, but found no relationship between 18q status and survival, though the majority of patients were uninformative for the 18q markers used.[83] Similarly, Saw et al. found no correlation between DCC immunohistochemical staining and survival following resection of CLM.[61] In patients with unresectable CLM, however, DCC was recently shown to be the single best predictor of survival, despite no significant effect upon response to fluorouracil-based chemotherapy.[84] Two-year survival rates of 8.5% and 42.5% were reported for DCC-negative and DCC-positive tumours respectively. Given these findings and those for early stage CRC, larger studies are imperative to confirm the role of 18q LOH, and mutation of the individual tumour suppressor genes located there, upon prognosis following metastasectomy.

5. Proliferation indices

Proliferative activity, the ratio of cycling to total cells in the tumour population, provides an indication of underlying molecular abnormalities in cell cycle regulation and correlates with aggressive biological behaviour in a wide range of human malignancies.[85–87] Rapid proliferation may account for resistance of residual disease to host immune surveillance following liver resection, resulting in disease recurrence. Techniques employed for the determination of proliferative

Table 2 – Characteristics of studies on the prognostic role of proliferation indices after metastasectomy

Study	Year	Patients (*n*)	Adjuvant therapy following metastasectomy	Marker (method)	Cut off for positivity[a]	% positive cases	Correlation with survival
Costa et al. [55]	1997	104	Not reported	SPF (TLI)	>11%	Not reported (median TLI 8%)	High TLI independently predicted decreased DFS ($P = 0.035$)
De Jong et al. [41]	1998	45[b]	Not reported	Ki-67 (IHC)	Low < 33%. Intermediate 33%–66% High > 67%.	35 22.5 42.5	No significant prognostic role
Russo et al. [35]	1998	36[c]	If unresectable	SPF (flow cytometry)	>20.3%	47	High SPF independently predicted decreased OS ($P < 0.01$)
				Ki-67 (IHC)	>18.8%	50	High Ki-67 associated with decreased OS on UV analysis ($P < 0.01$). No independent prognostic role for OS
Nanashima et al. [99]	1999	27	Not reported	Ki-67 (IHC) AgNOR (Silver staining)	>45% >13.5 dots/cell	Not reported Not reported	No significant prognostic role No significant prognostic role
Weber et al. [100]	2001	221	66% of patients	Ki-67 (IHC)	>50%	50	High Ki-67 independently predicted decreased OS ($P < 0.0001$) and DFS ($P = 0.014$)
Petrowsky et al. [37]	2001	41	71% of patients	Ki-67 (IHC)	>50%	20	High Ki-67 independently predicted decreased OS ($P = 0.036$)
Crowe et al. [38]	2001	71	None	PCNA (IHC)	⩾2 on semiquantitative scale	Not reported (mean 2)	No significant prognostic role
Smith et al. [12]	2004	63	For synchronous metastases	Ki-67 (IHC)	⩾50%	38	High Ki-67 associated with decreased OS on UV analysis ($P = 0.04$). MV analysis not reported
Onodera et al. [101]	2005	85	Not reported	PCNA (IHC)	Not reported	Not reported (mean PCNA LI 59)	No significant prognostic value

SPF, S-phase cell fraction; TLI, ^{3}H-thymidine labelling index; DFS, disease-free survival; IHC, immunohistochemistry; OS, overall survival; UV, univariate; AgNOR, argyrophil nucleolar organiser region associated proteins; PCNA, proliferating cell nuclear antigen; MV, multivariate; PCNA LI, proliferating nuclear antigen labelling index.

a Immunohistochemical cut-offs indicate the percentage of cells with positively staining nuclei.

b 7 unresectable.

c 23 unresectable.

activity include S-phase fraction measurement (by flow cytometry or ^{3}H-thymidine labelling index) and immunohistochemical quantification of proliferation-associated nuclear proteins, such as Ki-67,[88] its epitope MIB-1[89] and the DNA polymerase auxiliary protein PCNA.[90]

Studies of proliferation indices in relation to prognosis of resected primary CRC have yielded inconclusive results. Although some reports suggested a correlation between rapid cell proliferation and poor prognosis,[91,92] most studies have failed to support this finding,[93–95] with some reporting increased proliferation to correlate with improved prognosis.[96,97] Primary colorectal tumours are known to be heterogenous with respect to proliferation rates[98] and this may account in part for these conflicting findings.

CLM demonstrate higher proliferation rates than their corresponding primary tumours,[21] and a more consistent role has been demonstrated for proliferation indices, particularly Ki-67, in the context of resectable CLM (Table 2).[12,35,37,38,41,55,99–101] Though several small studies failed to show a correlation between Ki-67 and prognosis after metastasectomy,[41,99] other studies have consistently found an association with outcome.[35,37,100,12] In the largest of these, Ki-67 was found to be the most significant indicator of overall survival following metastasectomy, amongst a range of clinicopathological variables.[100] On multivariate analysis Ki-67 labelling index in excess of 50% represented a 2.8-fold increased risk of death. Similarly, a recent study found high Ki-67 to correlate significantly with death following metastasectomy and to do so more strongly than clinical prognostic scoring.[12]

Studies assessing S-phase cell fraction support this independent prognostic relationship between cell proliferation and patient survival,[35,55] with Costa et al. showing high ^{3}H-thymidine labelling index to equate to a 1.5-times higher risk of recurrence following metastasectomy compared to tumours with a low labelling index.[55] Post-metastasectomy CEA-doubling time, an alternative measure of tumour proliferation, has also been demonstrated to be an independent prognostic factor for both early disease recurrence and overall survival following metastasectomy.[62,101,102] Proliferation indices therefore appear to hold promise as prognostic markers of resectable CLM.

6. Telomerase

Expansive tumour growth requires increased expression of the ribonucleoprotein enzyme telomerase, in order to prevent chromosome attrition and fusion with repeated cell division. Telomerase activity predicts aggressive behaviour of several gastrointestinal malignancies.[103,104] Smith et al. examined the prognostic role of the human telomerase reverse transcriptase (hTERT) subunit in 58 resected CLM, and found nucleolar expression to predict poor survival better than clinical prognostic scoring.[12] A larger, multicenter study recently demonstrated independent prognostic value, with patients with hTERT-positive CLM found to have a median survival of 23 months compared to 46 months in patients with hTERT-negative metastases ($P < 0.0001$).[105]

7. Thymidylate synthase

Thymidylate synthase activity is the rate-limiting step in the *de novo* synthesis of DNA in proliferating cells, catalysing the methylation of deoxyuridine monophosphate to thymidine monophosphate.[106] It represents the cellular target of 5-fluorouracil (5-FU) and raltitrexed chemotherapy, both widely used in the treatment of colorectal malignancy. Elevated intratumoural TS has been confirmed as a prognostic marker of decreased disease-free and overall survival in primary CRC, irrespective of disease stage (reviewed by Popat et al.[107]). This was initially attributed to TS-induced tumour resistance to 5-FU-based chemotherapy,[108,109] but elevated

Table 3 – Characteristics of studies on the prognostic role of thymidylate synthase following metastasectomy

Study	Year	Patients (*n*)	Adjuvant therapy following metastasectomy	Method	Cut off	% positive cases	Correlation with survival
Bathe et al. [116]	1999	15	3 patients	RT-PCR	Ratio of TS to GADPH >0.6	42	High TS expression associated with decreased OS ($P = 0.0006$) and DFS ($P = 0.004$) on UV analysis. MV analysis not reported
Corsi et al. [117]	2002	48	All patients	IHC	2–3 on semi-quantitative scale (staining intensity and heterogeneity)	33	High TS expression independently predicted decreased OS ($P = 0.038$)
Saw et al. [61]	2002	63	Not reported	IHC	>6 on semi-quantitative scale (staining intensity and heterogeneity)	46	No significant prognostic value
Gonen et al. [42]	2003	144	All patients	IHC	>20%	21	High TS expression independently predicted decreased OS ($P < 0.01$) and HDFS ($P = 0.01$)

RT-PCR, reverse transcriptase polymerase chain reaction; TS, thymidylate synthase; GADPH, glyceraldehyde phosphate dehydrogenase (internal control); OS, overall survival; DFS, disease-free survival; UV, univariate; MV, multivariate; IHC, immunohistochemistry; HDFS, hepatic disease-free survival.

intratumoural TS has since been found to have a negative prognostic influence irrespective of chemotherapeutic treatment.[110,111] This observation was explained in part through demonstration of a direct correlation between TS activity and tumour cell proliferation.[112,113] In addition, TS binds both *c-myc* and *p53* mRNA,[114,115] suggesting a direct involvement in tumour suppressor gene and oncogene expression.

Studies examining the prognostic role of TS in resected CLM are summarised in Table 3.[42,61,116,117] Bathe et al. examined the prognostic value of TS mRNA expression after metastasectomy in the absence of adjuvant chemotherapy, finding a statistically significant difference in disease-free survival despite a small study population; patients with high expression had a median survival of just 5 months, compared to 18 months for patients with low expression.[116] Other studies have confirmed these findings in patients treated with surgery and adjuvant chemotherapy,[42,117] with only one study failing to show a relationship between TS expression and prognosis following metastasectomy.[61] Overall these studies suggest that TS gene expression may be associated with poor prognosis in patients undergoing metastasectomy, whether treated with surgery alone or with additional chemotherapy. Increased TS gene expression appears to be associated with an inherently more aggressive tumour phenotype.

8. Apoptotic regulators

Evasion of apoptosis (programmed cell death) by proliferating tumour cells is key to rapid tumour growth and its assessment may have prognostic significance.[118] Apoptosis may be induced by intracellular events such as DNA damage or by extracellular pro-apoptotic stimuli, which induce signalling via members of the cell surface 'death-receptor' family, including Fas/CD95 and the tumour-necrosis factor receptor (TNFR). Death receptor-mediated signalling impacts upon various pro-apoptotic mediators including initiator caspases and members of the Bcl-2 protein family, the latter of which control mitochondrial permeability and the release of the apoptotic catalyst cytochrome C.[119]

Recent evidence suggests that dysregulation of these apoptotic mediators may have prognostic importance in resected CLM. Loss of expression of Fas/CD95 was an independent prognostic indicator of poor survival following metastectomy.[101] Members of the Bcl-2 family have also been examined in resected CLM, and though the anti-apoptotic protein Bcl-2 was itself found to have no prognostic significance,[55] low expression of the pro-apoptotic mediator Bax was found to be an independent prognostic marker of decreased overall survival.[59] Further studies are required to confirm these findings and examine the impact of other apoptotic mediators upon prognosis following metastasectomy.

9. Genomic instability

9.1. *Chromosomal instability (CIN)/aneuploidy*

Most aggressive human tumours are characterised by genomic instability, a phenomenon that is thought to favour cancer progression and adaptation.[120] Most commonly this constitutes chromosomal instability (CIN) and aneuploidy, which is characterised by the loss or gain of entire chromosomes, as well as gross chromosomal rearrangements, such as amplifications and non-reciprocal translocations.[121] CIN is the hallmark of most CRC,[122] arising early in tumour evolution.[123] Its molecular basis is incompletely understood, but likely involves mutations of mitotic checkpoint genes[124] and APC.[125] Aneuploidy is thought to herald acquisition of a 'mutator phenotype' that drives further mutation and tumour progression.[126] Its determination may, therefore, be of prognostic significance, as it signifies existing genetic alterations and the potential for an increasingly aggressive phenotype.

Though several studies of resected primary CRC found prognostic value for aneuploidy in early stage cancers only,[127,128] some recent studies have demonstrated independent prognostic significance across all tumour stages.[129,130] Only one study of resected CLM, however, has shown an independent prognostic role for aneuploidy, with significantly increased disease-free survival reported in patients with diploid

Table 4 – Characteristics of studies on the prognostic role of tumour ploidy after metastasectomy

Author	Year	Patients (*n*)	Adjuvant therapy following metastasectomy	% cases with aneuploidy	Correlation with survival
Tsushima et al. [132]	1987	88	Not reported	42	Aneuploidy associated with decreased OS on UV analysis ($P < 0.03$). No independent prognostic role for OS
Lind et al. [133]	1992	37	2 patients	59	No significant prognostic role
Cady et al. [131]	1992	51	Not reported	53	Aneuploidy independently predicted decreased DFS ($P < 0.05$)
Yamaguchi et al. [134]	1993	36	Not reported	69	Aneuploidy associated with decreased HDFS on UV analysis ($P < 0.005$) MV analysis not reported
Costa et al. [55]	1997	104	Not reported	86	No significant prognostic role ($P = 0.09$ on UV analysis)
Cady et al. [135]	1998	184	Not reported	Not reported	No significant prognostic role
Russo et al. [35]	1998	36[a]	If unresectable	78	No significant prognostic role ($P = 0.05$ on UV analysis)
Crowe et al. [38]	2001	71	None	Not reported	No significant prognostic role

OS, overall survival; UV, univariate; DFS, disease-free survival; HDFS, hepatic disease-free survival; MV, multivariate.
a 23 unresectable.

compared to non-diploid metastases[131] (Table 4). Two studies found aneuploidy to correlate with adverse outcome on univariate analysis only,[132,134] with other studies reporting no prognostic significance in this context.[35,38,55,133,135] Costa et al., for example, examined 104 patients and, though finding DNA content to be a prognostic discriminator in slowly proliferating lesions, found no overall prognostic value.[55] Similarly, in the largest study of ploidy and prognosis following resection of CLM, no significant relationship was demonstrated amongst 184 patients.[135] Crowe et al. recently also failed to show any significant role for ploidy after metastasectomy, even allowing for the percentage of cycling cells.[38]

Current evidence, therefore, suggests that aneuploidy is unlikely to have a prognostic role following resection of CLM. A correlation between multiploidy (the presence of multiple abnormal DNA clones) and increased tumour aggressiveness has been demonstrated for primary CRC,[130,136] but its significance in resectable CLM remains to be confirmed.

9.2. *Microsatellite instability (MSI)*

In approximately 10–20% of sporadic CRC, genomic instability is the result of microsatellite instability (MSI), arising from mutation or hypermethylation of colonic 'caretaker' mismatch repair genes,[137,138] resulting in a 100- to 1000-fold increase in the rate of point mutations or small insertion/deletions. MSI status is assessed by detection of band shift alterations in tumour microsatellite size of mononucleotide repeat markers, such as BAT25 and BAT26.[139] To date, the majority of studies examining MSI in primary CRC have linked the MSI phenotype with a favourable prognosis (reviewed by Anwar et al.[19]). Three groups have examined the prognostic significance of MSI in resected CLM, finding no association with overall or disease-free survival, though the small numbers of MSI metastases (between 2.5% and 6% of CLM examined) were probably too small to permit meaningful statistical analysis.[83,140,141]

Attempts to correlate genomic instability with prognosis solely by examining either CIN or MSI may be limited due to the complex nature of genomic instability in CRC. It is now established that a proportion of tumours achieve genomic instability via both CIN and MSI,[142] whilst a further group, up to 37%, exhibit features of neither CIN nor MSI, developing genomic alteration through alternative, uncharacterised mechanisms.[142,143] Future prognostic studies, therefore, must account for the heterogenous nature of genomic instability in CRC.

9.3. *Fractional genomic alteration (FGA)*

Array comparative genomic hybridisation (array CGH) has recently been used to assess genomic damage in colorectal malignancy.[144–146] This technique allows high-resolution, genome-wide screening of DNA copy number changes and provides an overview of the extent of genomic damage at both the chromosomal and subchromosomal level, so providing a more robust measure of genomic alteration. Importantly, array CGH detected genetic imbalances in tumours with any combination of CIN and MSI, including tumours with no detectable cause of genomic instability.[146] Increasing genomic alteration determined by array-CGH was found to be a marker of poor outcome in early stage CRC.[147] Mehta et al., however, recently demonstrated resected CLM with an FGA of 20% or more to be associated with a significantly better prognosis than those with an FGA of less than 20%.[141] This finding, together with the negative results of studies of aneuploidy in resected CLM, suggests that whilst genomic instability may be key to the progression of early stage tumours, its relevance may be lost following metastatic spread, when further instability may exceed the threshold for tumour cell viability.[148] Larger studies are needed to confirm this hypothesis.

10. Angiogenesis

Angiogenesis plays a key role in tumour growth and metastatic spread, and the ability of residual micrometastatic deposits to induce a neovascular response may be critical in disease recurrence following metastasectomy. Angiogenesis may be assessed by determination of intratumoural vascular density or through analysis of angioregulatory molecules, such as vascular endothelial growth factor (VEGF) and thrombospondin-1 (TSP-1).

10.1. *Microvessel density (MVD)*

Microvessel density, determined by immunohistochemical labelling of endothelial cell determinants, relates to prognosis for a variety of solid tumours.[149–151] In the context of primary CRC, studies have variably shown a correlation of high MVD with decreased,[152,153] improved,[154,155] or unchanged[156,157] survival. Amongst studies examining the prognostic role of MVD in resected CLM (Table 5), two have reported high MVD to correlate with significantly lower overall survival following metastasectomy,[158,159] with high metastatic MVD associated with a 4.9-times increased risk of death over low MVD metastases on multivariate analysis.[159] Miyagawa et al. also reported high MVD to correlate with poor outcome after CLM resection, but these results were confounded by the inclusion of patients with extra-hepatic metastases, themselves associated with high CLM MVD.[160] Two recent studies, however, have failed to show an association between MVD and patient outcome,[63,101] the latter of which also showed no relationship between endothelial cell apoptosis and survival.[63]

Methodological variations likely contributed to the differing results in these MVD studies. At present, no consensus exists as to which pan-endothelial marker is the most reliable for MVD assessment in colorectal cancer. Studies of breast and prostate carcinoma, however, have identified significant differences in the reliability of these markers in the determination of MVD,[161,162] with Cluster Determinant 34 (CD34) found to be considerably more reliable than Cluster Determinant 31 (CD31) (98% vs. 87% consistency).[162] In addition, variations in tumour sampling site and the number of 'hot spots' used to define the MVD likely influenced the results obtained.

Larger studies with standardised methodology are, therefore, needed to confirm the role of MVD in CLM prognostics. To this end, our laboratory recently examined MVD in 182 resected CLM, using CD34 staining and a computerised image analysis system, and found high tumour edge microvessel density to be an independent prognostic marker of poor survival following CLM resection ($P = 0.038$).[163]

Table 5 – Characteristics of studies on the prognostic role of microvessel density (MVD) after metastasectomy

Author	Year	Patients (*n*)	Adjuvant therapy following metastasectomy	Endothelial cell determinant	Area of metastasis used for measurement	Method of measurement	Cut-off for high MVD	Correlation with survival
Nanashima et al. [158]	1998	44	60% of cases	CD34	Central and marginal areas	Mean count/5 fields (×200 magnification)	>46.4 (=median)	High MVD associated with decreased OS on UV analysis ($P < 0.01$). MV analysis not reported No significant prognostic role for DFS
Nanashima et al. [159]	2001	62	63% of cases	CD34	Marginal areas	Mean count/5 fields (×200 magnification)	>60 (mean = 59.2)	High MVD independently predicted decreased OS ($P = 0.022$). No significant prognostic role for DFS
Onodera et al. [101]	2005	85	All patients	CD31	Most deeply invasive tumour edge	Mean count/3 fields (×200 magnification)	Not stated (mean = 43)	No significant prognostic role
De Jong et al. [63]	2005	44	Not stated	CD34	Liver-tumour interface	Chalkley grid, hot-spot overlap technique (×400 magnification)	3.3 (=median)	No significant prognostic role

CD34, cluster determinant 34; OS, overall survival; UV, univariate; DFS, disease-free survival; MV, multivariate; CD31, cluster determinant 31.

10.2. *Vascular endothelial growth factor*

The VEGF glycoprotein is a key angiogenic stimulator, increasing vascular permeability and inducing serine proteases.[164,165] High VEGF-A expression correlated with increased metastatic spread[166] and poor prognosis[167] for primary CRC. Studies of resected CLM, however, have failed to show any correlation between VEGF expression and survival.[158,168]

10.3. *Thrombospondin-1*

TSP-1 is a multidomain glycoprotein that has been shown to have varying tumour-modulating properties, including pro-angiogenic or anti-angiogenic activity and stimulation or inhibition of migration, depending upon the system examined. This multifunctionality is thought to be the result of the presence of multiple functional domains within the TSP-1 structure.[169] In primary CRC, Maeda et al. found tumour TSP-1 to be associated with increased disease-free survival and to correlate with decreased MVD, suggesting an anti-angiogenic function in this context.[170] Our group recently examined the prognostic role of TSP-1 in 182 resected CLM and, though finding no expression in tumour cells, demonstrated perivascular and/or stromal expression of TSP-1 in 31% of cases. TSP-1 expression significantly correlated with poor survival on multivariate analysis ($P = 0.01$) suggesting a contrasting role for TSP-1 in primary CRC and its hepatic metastases.[171]

Further studies are needed to clarify the prognostic role of markers of angiogenesis, such as MVD and TSP-1, in resected CLM. The prognostic role of other pro- and anti-angiogenic factors, including basic fibroblast growth factor (bFGF), angiopoietins, ephrins and markers of tumour hypoxia remain to be determined.

11. Invasion and metastasis

11.1. *Adhesion molecules*

Alterations in cellular adhesion molecules are key to the metastasis of primary tumours to the liver. Loss of E-cadherin and increased expression of CD44 variant 6 have been associated with hepatic metastasis and poor prognosis for primary CRC.[172,173] Nanashima et al. examined the expression of these adhesion molecules in resected CLM, finding loss of either to be associated with worse prognosis, though these correlations were lost on multivariate analysis.[159,174] The putative metastasis suppressor nm23[175] has also been examined, but was found to have no prognostic role following resection of CLM.[38]

11.2. *Matrix metalloproteinases (MMPs)*

Secretion of proteases such as MMPs enables tumour invasion and metastasis.[176] To date, only Waas et al. have examined the prognostic value of the MMPs in resected CLM, finding increased levels of proform and active MMP-2 and MMP-9 to predict early intrahepatic recurrence following metastasectomy.[177]

12. Predictive molecular markers

In addition to providing information regarding recurrence risk and mortality following metastasectomy per se (prognostic value), molecular markers may also predict response to subsequent chemotherapy (predictive value). Though the exact role of adjuvant treatment following CLM resection remains to be determined, studies of unresectable disease suggest that such interactions may have important clinical implications. *p*53 mutation, for example, is associated with poor metastatic responsiveness to a range of chemotherapeutic agents, secondary to decreased apoptotic reactivity.[178] Similarly, the pattern of expression of the apoptotic regulator mcl-1 correlated with chemotherapeutic response in patients with unresectable disease.[179] Thymidylate synthase is itself the target of chemotherapeutic agents, with increased expression causing decreased responsiveness to 5-FU-based chemotherapy.[180] Notably, hepatic arterial infusion therapy with floxuridine following CLM resection led to a significant improvement in survival compared to systemic therapy alone in patients with high TS-expressing tumours, but no significant change in outcome for patients with low TS tumour expression.[42]

Molecular markers, therefore, have the potential to guide selection of adjuvant agents, as well as their dosing and route of administration. With increasing patient enrolment into trials of adjuvant treatment following metastasectomy, however, delineation of the effect of a molecular marker on outcome per se and additional effects through determining response to chemotherapy presents a challenge for future studies.

13. Conclusions

Biological prognostic markers of resectable CLM have the potential to yield significant improvements in patient outcome through informed allocation of treatment. Biomarkers, in combination with established clinicopathological variables, may permit stratification of patients according to recurrence risk following surgery. This would allow selection of chemotherapy in concordance with the biological characteristics of the individual tumour. Hence, high-risk patients would receive aggressive therapy, whilst the patient at very low risk of recurrence may be spared the morbidity and occasional mortality associated with chemotherapeutic drugs. Choice of chemotherapy regime and intensity of follow-up may also be guided by the molecular nature of the tumour. Furthermore, prognostic molecular markers may themselves represent targets for the development of novel anti-cancer agents.

Studies to date have yielded promising results in the search for prognostic molecular markers of CLM and have confirmed differing prognostic potential for several biomarkers in this context to that demonstrated for primary CRC. A number of markers, including proliferation indices, telomerase, thymidylate synthase and microvessel density have all shown strong evidence of prognostic utility and await prospective validation. Several potential biomarkers, such as 18q LOH and apoptotic regulators, have yet to be adequately examined, whilst identification of the prognostic utility of other markers, notably *p*53, though examined at length, has been limited by a failure of methodologies to account for their biological complexity. Confirmation of their role, and that of novel factors, requires rigorous experimental design, retrospective analysis in a standardised way and subsequent confirmation in large prospective studies. In this manner, combinations of prognostic biomarkers assessing the 'hallmarks' of cancer development (self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, invasion and metastasis)[181] may be identified, allowing the 'molecular staging' of CLM.

Given the continual rise in the number of potential biomarkers of CRC, future studies will increasingly employ genomic and proteomic technologies, which enable the measurement and analysis of numerous potential biomarkers simultaneously. These techniques are able to produce gene or protein 'profiles' associated with clinical outcome, the analysis of which may then yield novel biomarkers with prognostic and/or therapeutic potential. DNA microarray technology has identified gene expression signatures associated with distant metastasis and poor outcome in primary CRC[182,183] and array CGH, in addition to providing an overview of total genomic damage, has identified specific copy number alterations in CRC.[144] In resected CLM, this technique recently identified a set of approximately 100 clones, located predominantly on chromosomes 7 and 20, whose alteration was associated with overall survival.[141] Correlation of such information with sequence data will enable the localisation and identification of cancer causing genes and candidate biomarkers.

As surgical resection currently represents the only hope of cure for CLM, to date neither clinical risk scoring nor molecular markers have been used to exclude patients with resectable disease from metastasectomy. With the advent of more efficacious chemotherapeutic regimes, however, it is possible that patients at highest recurrence risk may achieve longer survival with alternative treatments than that achieved with surgical resection. Analysis of protein 'signatures' in serum and plasma samples may, in time, permit such pre-operative determination of prognosis, guiding patient selection for surgery and choice of chemotherapy, including the selection of neoadjuvant regimes when disease down-staging is required.

The aim of identifying molecular prognostic markers in resectable CLM is the tailoring of therapy according to individual recurrence risk. Though in its infancy, the potential benefits of this research are enormous, not only in terms of improved patient survival, but also the economic benefits that would result from the informed use of effective therapies.

Conflict of interest statement

None declared.

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