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Genetic Markers of Survival and Liver Recurrence after Resection of Liver Metastases from Colorectal Cancer

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Abstract. A significant number of patients with liver metastases from colorectal cancer (CRC) achieve 5-year survival after liver resection. Increased expression of genetic markers in the primary tumor are known to predict outcome after colonic resection, but the predictive value of such markers after resection of hepatic metastases is unknown. The objective of this study was to evaluate whether DNA content and multiple genetic markers, separately or expressed together, can predict patient outcome (liver recurrence and survival) after resection of hepatic metastases. We studied the paraffin-embedded liver tissue of 71 consecutive patients who had undergone a potentially curative resection of hepatic metastases from CRC. Using DNA flow cytometry and immunohistochemical staining techniques we determined the DNA content and the level of co-expression of seven tumor-associated proteins: proliferating cellular nuclear antigen (PCNA), epidermal growth factor receptor (EGFr), p53, c-erbB-2, H-ras, c-myc, and nm23. Three endpoints (liver recurrence, cancer specific, overall survival) were correlated with these tumor markers. The 5-year overall survival of the group was 31.2%. There was no correlation detected between the DNA aneuploidy and overall or cancer-specific survival. Similarly, expression of the individual tumor-associated proteins did not predict survival. Patients whose tumors co-expressed multiple markers had survivals similar to those whose tumors expressed fewer markers. However, a significant difference in hepatic recurrence was found between the p53-positive and p53-negative patients ($p = 0.007$), with marker-negative tumors having decreased recurrence. In conclusion, this study demonstrates that the DNA content and genetic markers c-myc, c-erbB-2, EGFr, H-ras, p53, PCNA, and nm23 do not predict survival after potentially curative resection of hepatic metastases from CRC. However, the immunoreactivity of p53 may be an important marker of local recurrence in the liver, which may be useful if re-resection of metastatic liver tumors is considered a viable management option in this disease.

Hepatic resection of patients with limited metastases from colorectal cancer is now an established modality, with 5-year survival rates of up to 30% [1, 2]. Unfortunately, most patients undergoing resection receive minimal long-term survival benefit, suggesting that the standard criteria for selecting patients for hepatic resection is poor. As well as the standard clinicopathologic factors, many other factors thought to influence the natural history of colorectal liver metastases have been studied to identify adverse prognostic factors [3–5]. They mainly include histologic factors, such as the grade of the primary tumor and biologic factors such as carcinoembryonic antigen (CEA), gastrin, and CA 19-9 concentrations. DNA ploidy has also been studied but to date has not been found to predict survival consistently after resection of metastases [6, 7].

Only a few oncoproteins, known to be predictive of survival when measured in primary tumors, have been assessed in liver metastases [8–10]. For example, although K-*ras* mutations are known to be important in primary colorectal tumourigenesis, the presence or absence of K-*ras* mutations in metastases of long-term survivors after liver resection does not predict outcome[11]. This is not surprising considering that the metastatic process involves an accumulation of many genetic abnormalites. Although some animal studies suggest that correction of a single genetic defect can affect tumor morphology and subsequent metastatic ability, there is no evidence to date that this applies in humans [12]. We hypothesized that metastases that have accumulated many genetic abnormalities may have a worse prognosis than those with fewer abnormalities. Our previous work has suggested that this hypothesis may be true when primary tumors are examined [13]. The aim of this study was to investigate the possible significance of DNA ploidy and the expression of seven genetic markers, together and individually, known to be involved in carcinogenesis or in the development of metastases, as predictors of liver recurrence and cancer-specific and overall survival in patients undergoing hepatic resection for metastatic colorectal cancer (CRC).

Materials and Methods

Patients and Paraffin-fixed Tissue

Paraffin-embedded tissue blocks of metastatic liver tumors from 71 consecutive patients with CRC were studied. These patients had undergone a potentially curative resection of liver metastases from CRC between 1982 and 1992 (Table 1) and were selected for resection using standard clinical and pathologic criteria. Detailed medical histories including follow-up data (mean 6.7 years, range 1.5–14.2 years), liver recurrence, and survival were available on all patients. CEA was not available in enough patients for appropri-

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Table 1. Pathologic properties of hepatic metastases from colorectal cancer patients ($n = 71$).

Characteristic	No. of patients	%
Metastases		
Synchronous	27	38
Metachronous	44	62
Microscopic margins		
Negative	64	84.50
"Clear"	28	
< 1 cm	24	
> 1 cm	12	
Positive	7	9.90
Number of metastases		
Solitary	55	78.90
Two	7	9.85
Three or more	7	9.85
Inadequate data	1	1.40
Size of metastases (cm)		
< 3	28	39.40
3–5	20	28.20
> 5	22	31.10
Distribution of metastases		
Unilobar	66	94.40
Bilobar	2	2.80
Inadequate data	2	2.80
Survival[a]		
2 Years		66.4
5 Years		31.2

[a]Survival was calculated from 70 patients.

ate analysis. The follow-up protocol was the same in all patients. No patients received chemotherapy as treatment for their liver disease. The patients' primary tumors were Dukes B ($n = 27$) or Dukes C ($n = 44$). Patients who died within 30 days of operation were excluded from follow-up analysis. Follow-up was obtained from the operating surgeon and general practitioner if necessary. Our practice is to review patients every 4 months for 2 years then every 6 months for 5 years, or more frequently when symptoms demand investigation. Liver recurrence was detected by ultrasonography (US) or computed tomography (CT) scan, which was generally performed every 6 to 12 months or earlier when symptoms developed. Endpoints were local recurrence in the liver and cancer-specific and overall survival. The study was approved by the Research Ethics Committee of the Eastern Sydney Area Health Service.

Immunohistochemical Assay

Paraffin-embedded sections were cut, dewaxed, and stained using a method for immunohistochemistry described elsewhere [13]. Pretreatment was given in either the antigen retrieval solution (Bio Genex, USA) heated in a microwave oven for c-myc, PCNA, and p53 or in 0.05% saponin (Sigma, USA) solution for c-erbB2, EGFr, and H-ras. No pretreatment was given for detection of nm23. Various concentrations (from 0.05 to 20.0 μg/ml) of the primary monoclonal antibodies: 6E10 (against c-myc; Cambridge Res. Biochem.), 3B5 [against c-erbB2 (c-neu); OSI], C11 (against EGFr; Cambridge Res. Biochem.), Y13-259 (against H-ras; OSI), PC10 (against PCNA; OSI), PAb1801 (against p53; OSI) and #56 (against nm23-H1 and nm23-H2; Transduction Lab.) were compared to determine the optimal concentrations for staining. These concentrations were 10 μg/ml for antibodies against c-myc, EGFr, H-ras, and p53; 5 μg/ml for anti-PCNA antibody; 1 μg/ml for anti-c-erbB2 antibody; and 0.08 μg/ml for anti-nm23 antibody. Biotinylated secondary linked antibody was either a horse anti-mouse immunoglobulin G (IgG) or an anti-rat IgG (BA-2000, Vector, USA). The streptavidin-biotin-peroxidase (ABC-HRP) reagent (PK-4002, Vector) and diaminobenzidine (DAB) were used for signal amplification and staining. The sections were finally examined for frequency and intensity of staining using a semiquantitative scale (0 indicating no staining to + 4 indicating intense staining of > 50% of cells) with + 2 or higher considered positive as per previous studies [13].

DNA Flow Cytometry

DNA content was analyzed by standard techniques using a commercial flow cytometer (FACScan, Becton Dickinson, USA). Flow cytometry was performed within 2 hours of cell staining. The singlet data were analyzed using CellFIT Software after removing doublets and higher cell aggregates by the Gate and the Doublet Discrimination System. The data obtained were analyzed using our already reported interpretation system [7].

Statistical Analysis

Statistical analysis was performed using the SPSS/Windows 6.0 statistical package (SPSS, Chicago, IL, USA). Pearson's correlation coefficiency was used to detect the association between any two variables. A nonparametric Mann-Whitney U-test or Kruskal-Wallis method was performed to assess the significance of the differences between variables where applicable. Log-rank test, and univariate and multivariate Cox proportional harzards model were applied for liver recurrence and survival analysis; and Kaplan-Meier curves were constructed for the selected variables. Statistical values of $p < 0.05$ (two-tailed) were considered significant.

Results

The mean age of the group was 58 years (range 30–80 years), and there were 41 men and 30 women. One patient died during the perioperative period and was excluded, leaving 70 patients for survival analysis. The pathologic characteristics of the resected specimens and survival data are shown in Table 1. The mean survival of the group was 35 months (median 29 months). Altogether, 34 (48%) patients developed recurrence in the liver and 28 developed distant recurrence. All but three deaths were due to metastatic colorectal cancer.

Only two patients had bilobar disease, and none had extrahepatic disease. In 28 cases the surgical margin was classified only as "clear," with no indication of the size of the margin (Table 1). An involved surgical margin ($n = 7$) significantly affected liver recurrence ($p < 0.00001$), as did the size of the lesion and age ($p < 0.02$). Age ($p < 0.0001$) and the presence of mesenteric lymph node involvement at the time of resection of the primary ($p < 0.00001$) were the only clinical factors that significantly affected overall survival (log-rank test and Cox proportional hazards model).

The frequency of positive expression of the seven markers is shown in Figure 1. There was no correlation between DNA ploidy and the expression of any of the individual tumor-associated markers and the cancer-specific survival (Cox regression). As

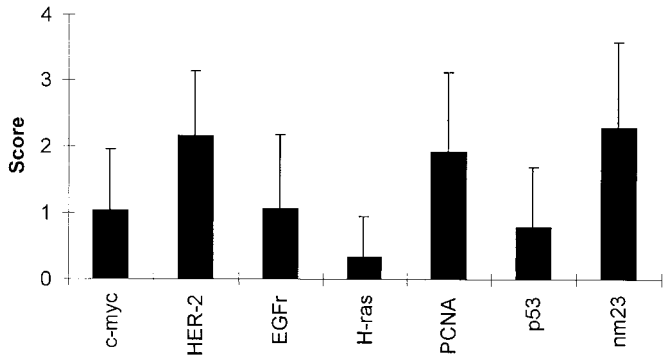

Fig. 1. Frequency of positive expression of proliferating cellular nuclear antigen (PCNA), epidermal growth factor receptor (EGFr), p53, c-erbB-2, H-ras, c-myc, and nm23 in resected liver metastases. Values are means + SD.

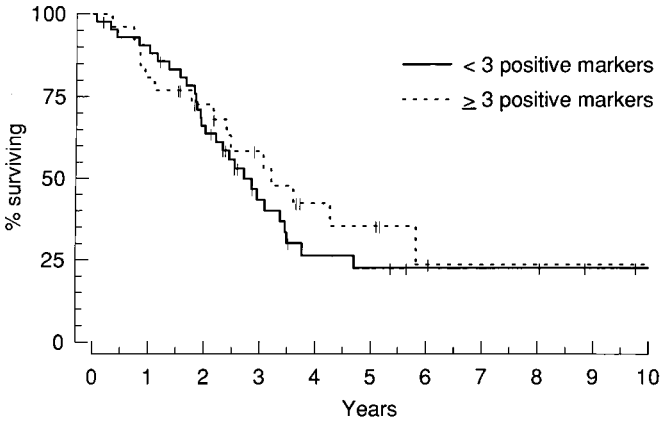

Fig. 3. Overall survival in the patients whose tumors expressed three or more tumor-associated markers compared to those whose tumors expressed fewer than three markers.

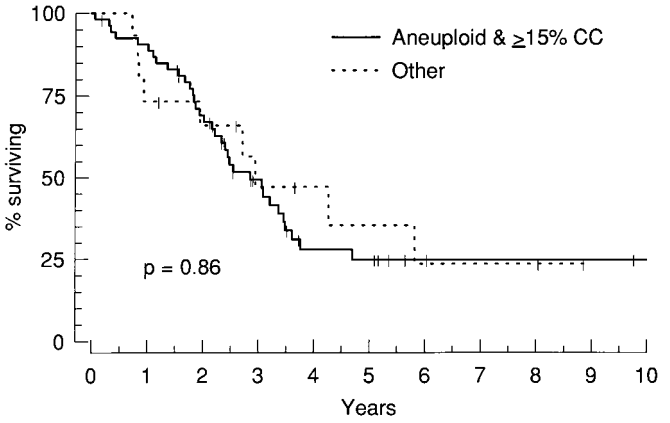

Fig. 2. Overall survival in patients whose tumors had aneuploidy and > 15% cycling cells (CC) compared to those whose tumors had diploidy and < 15% cycling cells.

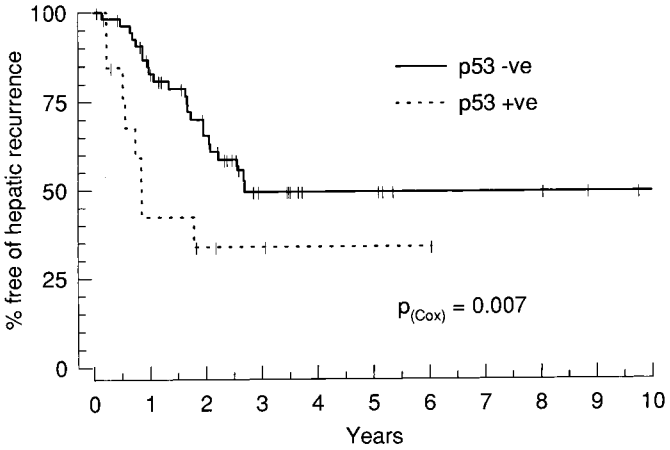

Fig. 4. The p53 status in patients with hepatic recurrence after liver resection. -ve: negative; +ve: positive.

metastases tend to accumulate more genetic changes than primary tumors, tumors with both aneuploidy and more than 15% cycling cells were assessed and correlated with hepatic recurrence and survival (Fig. 2). Patients whose tumors had aneuploidy and > 15% cycling cells had survivals similar to those whose tumors were diploid and had < 15% cycling cells. A similar analysis was performed on tumors that expressed three or more of the tumor-associated antigens (Fig. 3). Patients whose tumors expressed multiple markers had survivals similar to those whose tumors expressed fewer markers. However, a significant difference in hepatic recurrence was found between the p53-positive and p53-negative patients (univariate/multivariate Cox model, $p = 0.007$) (Fig. 4) When the analysis was repeated examining only tumors with a negative liver margin, hepatic recurrence was still less in tumors that were p53-positive ($p = 0.006$).

Discussion

The metastatic process is complex and characterized by many and varied genetic abnormalities that are likely to be different from the genetic aberrations of tumorigenesis. Hepatic metastases from CRC therefore may not have the same profile of tumor-associated proteins as the primary colon cancer, nor the same correlation with survival. In a previous study examining the co-expression of four tumor-associated proteins in primary CRC, we have shown that those primary tumors that express a larger number of markers are more likely to develop metastases [13]. The cells in metastases that recur after an otherwise "curative" resection may have also accumulated more genetic abnormalities than cells that are less likely to recur—hence the rationale for this study.

The clinicopathologic factors that predict survival, such as the number of metastases, the absence of extrahepatic disease, the size of the primary lesion, the surgical margin, and unilobar disease have been well studied [2, 14–16]. None of these factors, except the margin, affected outcomes in this study, as patients with bilobar disease, more than four tumors, and extrahepatic disease were generally not offered resection; thus only a small number of patients with these characteristics were in this study. Despite this fact, only 31% of the patients lived more than 5 years, highlighting the need for better prognostic markers.

Several authors have examined other biologic or genetic factors that might help predict survival after liver resection. The preoperative CEA level may predict outcome after both initial hepatic resection [17] and after re-resection of recurrent liver disease, a procedure that appears to be gaining acceptance [18–20]. Although flow cytometry appears to predict survival when measured in primary colorectal cancers [6, 7], its prognostic significance in liver metastases is likely to be minimal. We did not find any survival advantage among patients with diploid tumors compared to nondiploid tumors, and this was unchanged when the percentage of cycling cells in each tumor was considered. Other studies provide both supportive and conflicting data but must be interpreted with caution owing to the relatively small numbers of patients studied [3, 21–23]. Although our study is one of the largest, even larger studies are required to better assess ploidy status. Based on the present present data, it is unlikely to have major prognostic significance.

Few studies of hepatic metastases have assessed the prognostic significance of the expression of oncogenes, tumor suppressor genes, or other proteins indicative of proliferation. Studies in primary CRC have shown that proliferating cellular nuclear antigen (PCNA) expression is associated with Dukes' stage and independently predicts prognosis [24]. Although our own work [13] and that of others [25] suggests a poorer outcome for patients with primary tumors overexpressing PCNA, this was not the case in hepatic metastases. Other oncogenes such as c-*myc* and c-*erbB2* are also thought to be involved in the progression of colorectal malignancy [13, 24]. However, based on our data, the expression of these oncoproteins did not predict survival after resection of hepatic secondary lesions. Similarly, the *ras* gene, and specifically K-*ras* mutations, are known to be involved in colorectal tumorigenesis, although in this study it does not appear to affect the prognosis of patients with resected hepatic metastases. Although H-*ras* is important for tumor progession, we could not demonstrate any prognostic significance in tumors overexpressing H-ras, a close relative of K-*ras*.

Overexpression and the presence of *p53* mutations appear to be associated with poor survival in patients with primary CRC [26, 27]. Although we found no survival advantage, a finding noted by others [28], liver recurrence was significantly less with hepatic tumors that did not overexpress p53. Other studies have suggested that p53 overexpression may be associated with local recurrence of disease, particularly in rectal cancer [29, 30]. Ding et al. [31] found that allelic losses on chromosome 17p (*p53* gene), 5q (*APC* gene), and 18q (*DCC* gene) correlated with such clinical features as the size and number of liver metastases and the presence or absence of involved lymph nodes. However, these genetic abnormalities were not correlated with outcome possibly due to the small numbers examined. Thus it is possible that *p53* abnormalities may be important in local tumor growth and recurrence of metastases, as they appear to be for recurrence of primary CRC [30]. As hepatic re-resection is being increasingly employed, measurement of p53 may help identify patients at risk of recurrent liver tumors.

The *nm23* gene is a candidate metastatic suppressor gene known to be involved in the formation of basement membrane and to have growth inhibitory effects [32]. The only studies on liver metastatic tissue reported so far showed reduced expression of nm23 protein [33] but overexpression at the mRNA level[34] in liver metastases of colorectal cancer. Although nm23 protein was overexpressed in more than 70% of liver metastases, which suggests that in CRC *nm23* may not be a metastasis-suppressor gene but may be associated with tumor progression, its expression did not predict survival after liver metastases resection. Overexpression of nm23 may be caused by mutation or partial sequence deletion of the *nm23* gene, resulting in the increased expression of a nonfunctional mutant-type nm23 protein [35].

We hypothesed that metastases that overexpress many known tumor-associated proteins might indicate a worse prognosis than those that expressed fewer proteins. Although none of the tumor-associated proteins discussed above predicted survival, individually or when co-expressed, the strategy of examining co-expression of a number of oncogenes, tumor suppressor genes, and other tumor-associated genes appears to have some merit [35]. A larger prospective study of a wider range of genetic abnormalities in metastases is likely to provide more definitive information. However as p53 expression was increased in patients who developed liver recurrence, this study provides further evidence that p53 expression plays an important role in detecting the behavior of CRC. This may have clinical relevance when following patients after liver resection, particularly if re-resection is considered a viable management option.

Résumé

La survie à cinq ans après résection de métastases hépatiques d'un cancer colorectal (CCR) est notable. On sait qu'une augmentation des marqueurs d'expression génétique de la tumeur primitive prédit l'évolution après résection colique mais la valeur prédictive de ces marqueurs après résection des métastases hépatiques n'est pas connue. Le but de cette étude a été d'évaluer si le contenu en ADN et les marqueurs génétiques multiples, soit séparément, ou ensemble, pouvaient prédire l'évolution des patients (récidive hépatique et survie) après résection de métastases hépatiques. Nous avons étudié des prélèvements provenant de 71 patients consécutifs ayant euune résection potentiellement curative de métastases hépatiques d'origine colorectale. Par la cytodébitométrie d'ADN et des techniques de coloration immunohistochimiques, nous avons déterminé le contenu d'ADN et le niveau de co-expression de sept protéines en rapport avec le cancer: l'antigène nucléaire de prolifération cellulaire (PCNA), le facteur récepteur de croissance épithéliale (EGFr), le p53, la c-erbB-2, la H-ras, la c-myc et la nm23. On a observé une corrélation entre ces marqueurs et trois critères que sont la récidive hépatique, la survie globale et la survie sans maladie. Le taux de la survie globale à 5 ans a été de 31,2%. II n'y avait aucune corrélation entre l'aneuploïdie d'ADN et la survie globale ou spécifique. De même, l'expression des protéines tumorales individuelles n'était pas prédictive de survie. La survie des patients dont les tumeurs ont exprimé de multiples marqueurs était similaire à celle des patients dont l'expression tumorale a été moindre. Cependant, on a remarqué une différence significative en ce qui concerne les récidives hépatiques des patients p53 positifs et p53 négatifs ($p = 0{,}007$), les patients ayant des marqueurs négatifs avaient moins de récidives. En conclusion, cette étude démontre que le contenu en ADN et les marqueurs génétiques c-myc, c-erbB-2, EGFr, H-ras, p53, PCNA et nm23 ne sont pas prédictifs de survie après une résection potentiellement curative de métastases hépatiques provenant d'un cancer colorectal. Cependant, l'immunoréactivité de p53 pourrait être un

marqueur important pour la récidive métastatique hépatique, surtout si on envisage une re-résection de ces tumeurs.

Resumen

Un número significativo de pacientes con metástasis hepáticas de cáncer colo-rectal (CCR) alcanza supervivencia de cinco años luego de resección hepática. La expresión aumentada de marcadores genéticos es reconocida como factor de predicción del resultado luego de resección del colon, pero se desconoce el valor predictivo de tales marcadores luego de la resección de metástasis hepáticas. El propósito del presente estudio fue determinar si el contenido de DNA y los múltiples marcadores genéticos, bien por separado o cuando se expresan conjuntamente, puede predecir el resultado final (recurrencia hepática y supervivencia) después de la resección de las metástasis hepáticas. Estudiamos cortes de higado montados en parafina provenientes de 71 pacientes consecutivos que habian sido sometidos a una resección de metástasis hepáticas de CCR potencialmente curativa. Utilizando citometría de flujo y técnicas inmunohistoquímicas de coloración, se determinó el contenido de ADN y el nivel de co-expresión de 7 proteínas asociadas a tumor: antígeno de núcleo celular proliferante (PCNA), receptor de factor de crecimiento epidermal (EGFr), p53, c-erbB-2, H-ras, c-myc y nm23. Tres resultados finales fueron correlacionados con tales marcadores tumorales: recurrencia hepática, supervivencia específica para cáncer y supervivencia global. La supervivencia global a cinco años del grupo fue 31.2%. No se encontró correlación entre la aneuploida de ADN y la supervivencia específica para cáncer o la supervivencia global. Tampoco se halló que la expresión individual de las proteínas asociadas con cáncer fueran predictoras de supervivencia. Aquellos pacientes cuyos tumores co-expresaron múltiples marcadores tumorales tuvieron igual supervivencia que los que exhibían los tumores que expresaban menos marcadores. Sin embargo, se observó diferencia significativa en la tasa de recurrencia hepática entre los pacientes p53 positivos y los p53 negativos ($p = 0.007$) con menores tasas en los negativos. En conclusión, el estudio demuestra que el contenido de ADN y los marcadores genéticos c-myc, c-erbB-2, EGFr, H-ras, p53, PCNA y nm23 no son predictores de supervivencia luego de una resección potencialmente curativa de metástasis hepáticas de CCR. Sin embargo, la inmunorreactividad del p53 puede ser un marcador importante de recurrencia local en el higado, lo cual puede ser útil cuando se considere que la resección de tumores metastásicos en el higado es una opción viable de manejo de la enfermedad.

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