P53 Alteration and Microsatellite Instability Have Predictive Value for Survival Benefit from Chemotherapy in Stage III Colorectal Carcinoma[1]

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ABSTRACT

Purpose: **We recently presented evidence for tumor site and gender-specificity in the survival benefit from adjuvant chemotherapy in Stage III colorectal cancer (CRC). In the current study, we examined whether *p53* alteration or the microsatellite instability (MSI) phenotype provide additional predictive information in CRC patients.**

Experimental Design: **A retrospective series of 891 Stage III CRC patients with negative surgical margins was investigated. Thirty percent (270 of 891) received postoperative adjuvant chemotherapy with curative intent and comprising of 5-fluorouracil/levamisole. Adjuvant treatment and nontreatment patient groups were well matched for tumor site, grade, *p53* alterations, and MSI. Surgical tumor specimens were investigated for *p53* overexpression using immunohistochemistry and for *p53* mutation and MSI using single-strand conformation polymorphism analysis. The predictive value of these markers was evaluated by comparing the survival of adjuvant-treated and nonadjuvant treated patients.**

Results: **A strong inverse correlation was observed between *p53* alteration and MSI ($P < 0.0001$). In univariate analysis, the factors of sex, site, *p53* alteration, and MSI were each strong predictors of a survival benefit from chemotherapy. Multivariate analysis revealed that chemotherapy provided maximal survival benefit for female patients ($P = 0.005$) and for patients whose tumors contained normal *p53* ($P = 0.041$). Males whose tumors contained a *p53* alteration and were negative for MSI appeared not to benefit from chemotherapy.**

Conclusions: **Our findings suggest that *p53* alteration and MSI could be clinically useful molecular predictive markers for the identification of CRC patients who might benefit from 5-fluorouracil-based chemotherapy.**

INTRODUCTION

Patients with stage III CRC[3] obtain a 10–15% absolute survival benefit from the use of 5-FU-based adjuvant chemotherapy (1). Recent work (2) from our laboratory suggests that the site of tumor origin and patient gender may be important determinants of the benefit obtained from this treatment. Female patients and patients with right-sided tumors were found to derive the most survival benefit, whereas male patients with left-sided tumors obtained the least. The MSI phenotype, found almost exclusively in right-sided tumors, also appears to be associated with excellent survival in patients who receive chemotherapy (2, 3). In addition to tumor site, sex, and MSI status, tumor-specific genetic alterations such as mutations in the *p53* and Ki-*ras* genes and epigenetic alterations such as DNA methylation may also have independent predictive significance. This can be evaluated by comparing the survival of adjuvant-treated and nonadjuvant-treated patients harboring a specific molecular alteration. Ideally, this should be performed on tumors obtained from prospective trials where patients have been randomized to receive adjuvant chemotherapy. Unfortunately, such trials are almost always multicentered and, consequently, the tumor blocks are dispersed among many different pathology laboratories, making it difficult to retrieve such specimens for molecular analysis. Furthermore, 5-FU-based chemotherapy for Dukes' stage C CRC found widespread introduction in the early to mid-1990s, particularly for younger patients. This has made it difficult to obtain age-matched, nonadjuvant-treated patients for comparison with the adjuvant-treated group in retrospective studies of predictive molecular markers.

Attributable largely to its central role in tumorigenesis, alteration of the *p53* gene has held the most promise as a molecular prognostic and predictive factor with potential clinical utility (4). Several *in vitro* and animal studies (5, 6) have shown that tumor cells with inactivated or mutant *p53* are more resistant to cytotoxic agents including 5-FU; however, this has not been a universal finding (7), possibly because of tumor-type differences in the effects of *p53* aberration. In clinical studies, *p53* gene mutation has been associated with poor prognosis in two relatively small CRC series in which patients received

Received 11/20/00; revised 1/29/01; accepted 1/29/01.
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[1] Supported by the Cancer Foundation of Western Australia and the Raine Medical Research Foundation.
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[3] The abbreviations used are: CRC, colorectal cancer; 5-FU, 5-fluorouracil; MSI, microsatellite instability; IHC, immunohistochemistry; SSCP, single-strand conformation polymorphism; CI, confidence interval.

chemotherapy with curative (8) or palliative (9) intent. The survival rates of patients with or without this genetic alteration were compared and found to be significantly worse for those with *p53* mutation. It is impossible to determine from these results whether patients with *p53* mutant tumors derive a survival benefit from adjuvant therapy. To investigate this, the survival of patients with a *p53* alteration must be compared between adjuvant- and nonadjuvant-treated groups. We are aware of only one study that has carried out such a comparison in CRC (10). In 104 stage III CRCs from the Southwest Oncology Group, it was observed that patients whose tumors overexpressed the *p53* gene derived no significant survival benefit from chemotherapy, whereas those without overexpression did. Overexpression of the *p53* protein was presumed to signify the presence of an underlying gene mutation.

In the present study, we assessed the predictive values of both *p53* overexpression and *p53* gene mutation in a retrospective series of 891 consecutive cases of stage III CRC having negative surgical margins. These were obtained from a single institute over a 14-year period during a time in which chemotherapy was being introduced for the routine management of stage III CRC. The large number of cases analyzed has allowed evaluation of the predictive significance of *p53* alteration and MSI.

PATIENTS AND METHODS

Patients. The retrospective CRC series studied here is described in earlier reports from our laboratory (2, 11). A total of 891 consecutive cases of stage III CRC treated surgically at the Sir Charles Gairdner Hospital, Perth, Australia, between 1985–1998 were identified from histopathology records. Tumor blocks were retrieved from archives, and sections were cut for IHC and PCR-based molecular analysis. Care was taken to select blocks with at least 25% tumor cell content. Tumors with positive circumferential margins were excluded. Right-sided tumors were classified as those originating proximal to the splenic flexure, and left-sided tumors were classified as those located distal to or at this site and including rectal carcinomas. The median age for all of the patients was 67.6 years (range, 19–93 years). Beginning in 1991, 270 patients received adjuvant chemotherapy with curative intent. Two patients included in our previous study (2) but originating from a different institute were excluded in the present series. The standard regimen used in Western Australia comprised of 5-FU/levamisole (1), and in 85% of cases, the patients completed at least six monthly cycles. Information on disease-specific patient survival was obtained from the death registry of the Western Australian Health Department and from hospital records. The median follow-up time was 6.5 years (range, 1–15 years). Patients who died perioperatively (within 1 month of surgery) were not included in the series. At the end of the study period (November 1999), 487 patients (55%) had died as a result of recurrence of their disease and 42 (5%) from unrelated causes. Ethical approval for this project was obtained from Sir Charles Gairdner Hospital Human Research Ethics Committee.

Analysis of p53 Protein Overexpression and Gene Mutation. Tumors were analyzed for overexpression of *p53* protein using the DO-7 monoclonal antibody (Dako, Australia) and an identical IHC protocol to that described earlier (12). Antigen retrieval techniques were not used for any of the samples. Scoring for nuclear staining was carried out by two observers who were unaware of patient outcomes. A threshold of 5% of tumor cells showing nuclear staining was used as the cutoff score for positive IHC staining. This threshold has been shown previously (12) to give the best concordance with *p53* mutation. Tumors from 248 patients (92%) who received chemotherapy and from 331 patients (53%) who did not receive chemotherapy were analyzed by IHC. The latter were evenly distributed between site and gender subgroups. Limitations on resources prevented the analysis of all of the tumor samples.

Screening for mutations within exons 4–8 of the *p53* gene was carried out using PCR-SSCP mutation protocols described previously by our laboratory (12–14). Excellent concordance between the various isotopic, silver stain, and fluorescent modifications of the SSCP technique for *p53* mutation detection has been demonstrated (13, 14). Sensitivity of the SSCP technique for the detection of *p53* mutations has been estimated at 90–95% (15). DNA for PCR-SSCP analysis was extracted from 10-μm archival tumor sections cut serially to those used for IHC. All of the mutations were confirmed at least once by separate PCR and SSCP runs. Results on *p53* mutation were obtained for 246 patients who received chemotherapy (91%) and for 519 patients who did not receive this treatment (84%). Results were not available for all of the tumors because of PCR failure and limitations on tumor DNA. MSI was evaluated in all of the 336 right-sided tumors and in 396 (73%) left-sided tumors using previously described PCR-SSCP methods (16) based upon the detection of deletions within the BAT-26 mononucleotide microsatellite marker. This has been shown to establish MSI status with greater than 99% accuracy (17).

Statistical Analysis. Associations between clinicopathological features, adjuvant treatment, and *p53* alterations were evaluated using the χ^2 test. Survival was defined as the time from surgery until death. Patients dying from causes other than recurrence of CRC were censored at the time of death. Surviving patients were censored at the end of follow-up. Univariate survival analysis was conducted using the method of Kaplan and Meier, with the difference between curves evaluated by the log-rank test. Cox proportional hazards regression was used to estimate the effect of adjuvant treatment and interactions of this effect with those associated with clinicopathological factors and with the presence or absence of *p53* alterations. Statistical analysis was performed using Stata Statistical Software (Stata Corporation, College Station, TX).

RESULTS

Fig. 1 shows the percentage of CRC patients who received chemotherapy in each of the clinicopathologically defined subgroups (sex, age, tumor site, histological grade, and nodal involvement). As expected, younger patients received this treatment more frequently than older patients. Slightly fewer females received chemotherapy compared with males, perhaps relating to the fact that the median age of female CRC patients in this series was 4 years older than male patients (70 *versus* 66 years). No difference was observed for the use of chemotherapy in patient groups defined by tumor p53 or MSI status. These results

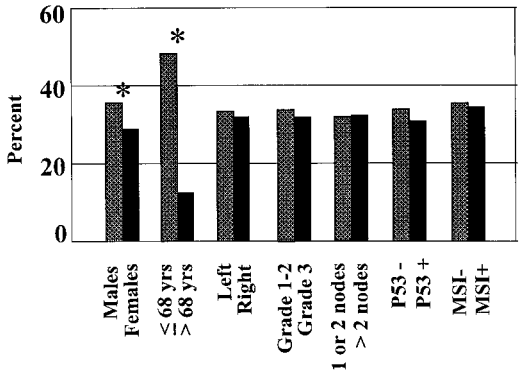

Fig. 1 Use of adjuvant chemotherapy in stage III CRC patient subgroups defined by clinicopathological and molecular features. Significant differences were observed within age and sex subgroups. The *p53* results shown are for patient groups defined by overexpression, as detected using IHC technique. *, $P < 0.05$.

Table 1 Clinicopathological characteristics and MSI status of stage III tumors with *p53* alterations

Feature	Percentage of *p53* overexpression	Percentage of *p53* mutation
Total	40 (233 of 579)	38 (289 of 765)
Age		
≤67.6 years	38 (126 of 336)	34 (135 of 392)
>67.6 years	44 (107 of 243)	41 (154 of 373)
	P = NS[a]	P = NS
Sex		
Male	41 (122 of 297)	31 (151 of 391)
Female	39 (111 of 282)	37 (138 of 374)
	P = NS	P = NS
Tumor site		
Right-sided	28 (59 of 207)	32 (89 of 278)
Left-sided	47 (173 of 366)	42 (198 of 476)
	$P < 0.0001$	$P < 0.0001$
Histological grade		
Well differentiated	52 (30 of 58)	37 (41 of 110)
Moderate differentiated	39 (147 of 376)	38 (177 of 464)
Poorly differentiated	38 (47 of 122)	38 (67 of 175)
	P = NS	P = NS
Microsatellite instability		
No	47 (186 of 389)	40 (226 of 562)
Yes	8 (3 of 34)	16 (7 of 43)
	$P < 0.0001$	$P < 0.0001$

[a] NS, not significant.

demonstrate that chemotherapy and nonchemotherapy groups were well matched for all of the features except age and sex.

Table 1 shows the frequency of *p53* overexpression and mutation in age, sex, site, grade, and MSI subgroups. Similar to several previous studies (18–21), *p53* alterations were more frequent in left-sided tumors compared with right-sided tumors. No associations were observed between the frequency of *p53* alterations and age, sex, or histological grade. As reported in earlier work (22–24), a strong inverse correlation was found between the presence of *p53* alteration and the MSI phenotype ($P < 0.0001$). In the present study, this was more pronounced for *p53* overexpression than mutation. Only 8% of MSI+ tumors showed staining for *p53* compared with 47% of MSI− tumors.

Kaplan-Meier analysis of the prognostic significance of *p53* overexpression and MSI in adjuvant-treated and nontreated groups is shown in Fig. 2. As reported previously by our group (11) and others (10), *p53* overexpression was associated with improved prognosis in patients who were treated with surgery alone (Fig. 2*A*). In the adjuvant-treated patient group, *p53* status was not prognostic (Fig. 2*B*). The MSI+ phenotype showed prognostic significance in adjuvant-treated (Fig. 2*D*) but not in nonadjuvant-treated (Fig. 2*C*) patients.

We recently reported evidence (2) for tumor site and patient gender differences in the survival response of stage III CRC to adjuvant chemotherapy. In the present study, we included an additional 235 unselected cases diagnosed between 1985–1990, for which *p53* overexpression and mutation data were already available (11). None of these additional cases received chemotherapy, thereby providing a better matched patient group for comparison with the adjuvant-treated patients. Cox univariate analysis for the survival benefit from chemotherapy in various CRC subgroups is shown in Table 2. A highly significant survival benefit from chemotherapy is apparent for the overall patient group. Subgroups that appeared to derive the most benefit from this treatment were female patients and patients with right-sided, poorly differentiated, normal *p53* (IHC− or SSCP−) or MSI+ tumors. No significant difference was apparent for the predictive values of the two types of *p53* alteration examined.

Multivariate analysis using Cox proportional hazards regression was performed to estimate the effect of adjuvant chemotherapy on the risk of death from CRC after adjustment for other factors likely to be associated with mortality. The effect of chemotherapy was modeled after adjustment for sex, site, age, grade, *p53* alteration (IHC), and MSI. Interactions between the effect of chemotherapy and each of these factors were also included in the model. Survival benefit from chemotherapy was not significantly modified by age, site, or grade, indicating that these were not confounding factors. The final model shows the effect of chemotherapy after adjustment for sex, *p53* alteration (IHC), and MSI (Table 3). Care must be taken in its interpretation, however, because interactions between chemotherapy and each of these factors are also included in the model. MSI+ was not an independent predictive factor, presumably because of its strong inverse relation to *p53* mutation (Table 1).

Results on the survival benefit conferred by chemotherapy for each combination of sex, *p53*, and MSI factors are shown in Table 4. The large CIs seen for the MSI+ groups are likely to be attributable to the relatively small number of MSI+ tumors ($n = 63$). In all of the four subgroups defined by sex and *p53* status, patients with MSI+ tumors appeared to derive more survival benefit from chemotherapy than those with MSI− tumors. All of the patient groups defined by sex, *p53*, and MSI obtained a survival benefit from chemotherapy with the exception of male patients having *p53* mutant/MSI− tumors. The latter group comprised approximately 20% of all of the CRC cases in this series.

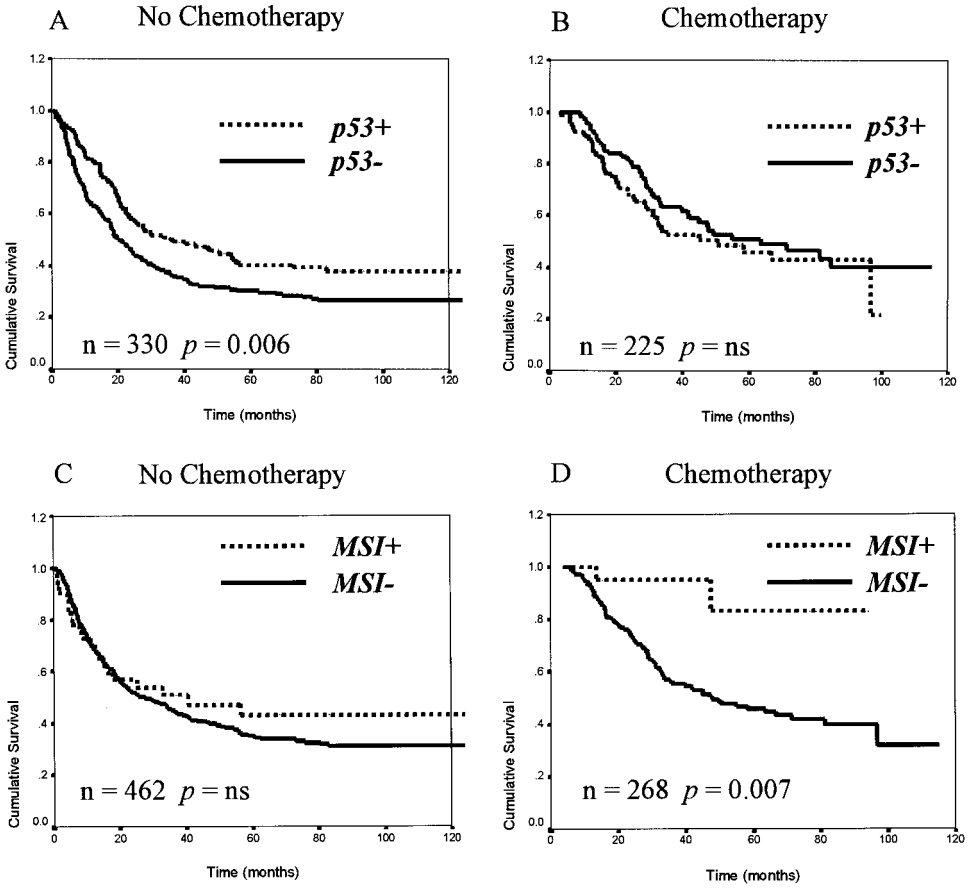

Fig. 2 Kaplan-Meier survival analysis of stage III CRC patients with or without *p53* mutations (*A*, *B*) or MSI (*C*, *D*) in adjuvant-treated and nontreated groups.

DISCUSSION

The present results and those from a recent study by our laboratory (2) suggest that the factors of patient sex and *p53* alteration have significant predictive value for survival benefit from chemotherapy in CRC. Our previous observation of an apparent tumor site effect was shown here using multivariate analysis to be attributable to the associations of this factor with sex, *p53* alterations, and MSI. Right-sided tumors are more frequent in females and are more often *p53* normal (18–21) and MSI+ (22, 25) compared with left-sided tumors. Each of these factors was shown in the present study to be associated with survival benefit from 5-FU-based chemotherapy (Table 2 and Table 4).

Until recently, the factors of site and sex have not been considered as potential predictive factors for CRC. Differences in the embryology and epidemiology (26) and in the frequency of allelic loss (27) between left-sided and right-sided colonic epithelia and tumors, respectively, were first noted more than a decade ago. Site-related differences in the frequency of *p53* mutation (18) and MSI (22, 25) were also reported in the early to mid-1990s. Although with the benefit of hindsight these differences might be expected to translate into differential survival benefits from chemotherapy, the possibility of tumor site specificity was apparently not considered during the evaluation of results from clinical trials. Sex-related differences in the frequencies of *p16* (28) and *hMLH1* (29) gene methylation have been reported recently and may be related to our observation of a gender difference in the response to chemotherapy (Table 3). As suggested previously by our group (2), the apparent gender difference in survival benefit from chemotherapy may be attributable to differences in frequency of the methylator phenotype between tumors from males and females.

The data from our study were derived from analysis of a large number of single stage tumors originating from one institute. However, we caution that the retrospective nature of this study makes it impossible to control for several potentially important and confounding factors that may have influenced our findings. Most of the nonadjuvant-treated patients were from the first half of the study period, whereas all of the adjuvant-treated patients were from the second half. We cannot exclude that pathological assessment, surgical practice, and patient management differed between the first and second halves of the study, thereby influencing the observation of a survival benefit from chemotherapy. However, comparison of nonchemotherapy-treated patients from the first and second halves of the study revealed no difference in survival (data not shown), suggesting that any changes in clinical practice have not influenced the outcome for nonchemotherapy-treated stage III CRC. A further possible confounding issue is that a proportion of rectal cancer patients received post-operative radiation therapy. We did not

Table 2 Univariate analysis for survival benefit from chemotherapy in stage III CRC subgroups

CRC subgroup[a] (*n1*, *n2*)	Relative risk	95% (CI)	*P*
Total (270,621)	0.62	0.51–0.77	<0.00001
Age			
≤67.6 years (209,226)	0.64	0.49–0.84	0.001
>67.6 years (61,395)	0.66	0.44–0.98	0.037
Sex			
Male (157,290)	0.81	0.62–1.06	0.132
Female (113,331)	0.43	0.31–0.61	<0.00001
Tumor site			
Right-sided (97,239)	0.46	0.32–0.65	<0.0001
Left-sided (172,372)	0.77	0.59–1.00	0.048
Histological grade			
Well differentiated (41,81)	0.66	0.37–1.18	0.163
Moderately differentiated (169,381)	0.66	0.50–0.86	0.003
Poorly differentiated (54,140)	0.49	0.32–0.74	0.0007
p53 alteration			
IHC− (145,201)	0.44	0.33–0.62	<0.00001
IHC+ (103,130)	0.86	0.60–1.25	0.438
SSCP− (164,312)	0.52	0.39–0.69	<0.00001
SSCP+ (82,207)	0.85	0.59–1.21	0.358
MSI			
Negative (241,428)	0.64	0.51–0.84	0.0001
Positive (21,42)	0.13	0.03–0.57	0.0004

[a] For each stage III CRC subgroup, the survival of patients who received chemotherapy is compared with that of patients who underwent surgery alone; *n1*, number in chemotherapy group; *n2*, number in nonchemotherapy group.

Table 3 Cox proportional hazards regression analysis for the effect of chemotherapy on the risk of death from stage III CRC with adjustment for the effects of sex, *p53* mutation, and MSI

	Relative risk	SE	z[a]	*P*
No chemotherapy	1.00			
Chemotherapy	0.26	0.064	−5.46	<0.001
Male	1.00			
Female	0.80	0.152	−1.17	0.2
Chemotherapy/sex interaction	2.23	0.638	2.80	0.005
MSI−	1.00			
MSI+	0.41	0.172	−2.12	0.034
Chemotherapy/MSI interaction	0.49	0.409	−0.86	0.4
p53 IHC+	1.00			
p53 IHC−	0.65	0.122	−2.28	0.023
Chemotherapy/*p53* interaction	1.76	0.487	2.04	0.041

[a] Z, standard normal deviate.

Table 4 Risk of death from CRC in patients receiving chemotherapy *versus* those receiving surgery alone in sex, *p53*, and MSI subgroups

	Females HR[a] (95% CI)	Males HR (95% CI)
p53 normal		
MSI+	0.13 (0.02–0.65)	0.28 (0.06–1.15)
MSI−	0.26 (0.16–0.42)	0.58 (0.36–0.93)
p53 mutant		
MSI+	0.22 (0.04–1.23)	0.50 (0.09–2.73)
MSI−	0.46 (0.28–0.76)	1.02 (0.64–1.62)

[a] HR, hazard ratio.

assess the influence of this treatment, however, because the available evidence suggests that post-operative radiation therapy has no impact on overall patient survival (30).

Confirmation of our findings on site and sex differences in the survival benefit from chemotherapy can be achieved by the evaluation of data from previous clinical trials. However, validation of the strong predictive values observed for *p53* and MSI requires molecular analyses of large series of tumors with known adjuvant therapy status and with long-term survival information. Access to tumor specimens from previous clinical trials of adjuvant therapy is difficult because of the large number of institutions that are often involved in such studies. The present study highlights the importance of collecting tumor specimens from prospective trials so that various somatic genetic alterations can be evaluated for their prognostic and predictive significance.

As seen in the results from Table 2, *p53* overexpression and *p53* mutation provide similar predictive information. The concordance between the two techniques (IHC+/SSCP+ and IHC−/SSCP−) was 70% (197 of 280). No additional predictive information was apparent from the use of both markers (both alterations present or either alteration present). Which type of *p53* screening is more suitable for routine analysis? IHC is technically simple and relatively inexpensive; however, there is wide variation in the protocols used in the literature. Our laboratory uses the DO-7 monoclonal antibody without prior antigen retrieval treatments. This gives distinct nuclear staining but is often quite variable in intensity both within and between positive tumors. The "cutoff" threshold used to score positive staining (5% of nuclear cells showing reaction product) is necessarily a subjective assessment. Our experience has been that a 5% threshold gives the strongest prognostic value as well as the best concordance with *p53* mutation (12). A further limitation of IHC is that the different fixatives used in various laboratories can influence the intensity of staining. Standardization and quality control measures would obviously be required if *p53* IHC is to be used as a routine marker. Direct molecular screening for *p53* mutation using relatively simple SSCP-based methods such as those used in the present study (13, 14) may provide a more objective assessment of *p53* status. PCR-SSCP can be carried out on routinely processed tumor specimens (13) and may also be more amenable to standardization (14). DNA sequencing is currently too expensive to be used for routine *p53* mutation screening, but it has the advantage over SSCP of being able to identify the type of mutation. This has been shown in several different tumor types to influence the response to chemotherapy (31, 32).

In view of the strong inverse correlation between MSI+ and *p53* alteration (Ref. 23 and Table 1), it is perhaps not surprising to find that the former is associated with a good survival benefit from chemotherapy (Fig. 2; Table 2). MSI+ tumors are found predominantly in the right-sided colon (22, 23, 25) where they comprise about 20–25% of stage III cases. They are frequently poorly differentiated (23), and it was interesting to note that patients with poorly differentiated tumors appear to derive the most benefit from chemotherapy (Table 2). It has been estimated that approximately 70% of MSI+ tumors belong to a larger "methylator" phenotype (33) that is characterized by

aberrant methylation of CpG-rich promoter regions in genes including *hMLH1* (29, 34) and *p16* (28, 33). This phenotype is found in both left-sided and right-sided tumors, and future work may reveal that it too has important predictive value in CRC.

The present work confirms and extends the results of Ahnen *et al.* (10) who reported no survival benefit from adjuvant chemotherapy for stage III CRC patients with *p53* overexpression. These clinical observations support *in vitro* and animal studies showing that CRC cell lines with inactivated *p53* were strikingly resistant to the effects of 5-FU (6). It remains to be established whether the chemoresistance of CRC patients with *p53* alteration is directly attributable to *p53*-related defects in apoptosis and/or cell cycle checkpoints. An alternate explanation is that *p53* alterations are associated with an as yet unidentified tumor phenotype that is resistant to chemotherapy. The *in vitro* and clinical results observed for CRC suggest that *p53* alterations may have strong predictive value in other tumor types that are also treated with adjuvant therapies. Evidence in support of this has been presented for breast (31, 35), head and neck squamous cell (32), and esophageal (36) carcinomas. Whether cancer patients with *p53*-altered tumors derive any survival benefit from chemotherapy can only be determined by the inclusion of this marker in prospective, randomized trials. A finding of no response would suggest that such patients should be considered for entry into future trials designed to evaluate the efficacy of non-*p53*-dependent cytotoxic agents.

On the basis of results presented in this and previous work (2, 10), we propose that sex, MSI, and *p53* alteration may be important predictive factors in stage III CRC and could be used to identify patient subgroups who derive significant benefit from adjuvant chemotherapy. These factors may also prove useful in identifying subgroups of stage II and IV CRC patients who are currently not being offered chemotherapy on a routine basis and who might stand to gain from this therapy.

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Clin Cancer Res 2001;7:1343-1349.