Characterization of $p53$ Mutations in Colorectal Liver Metastases and Correlation with Clinical Parameters$^1$

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ABSTRACT

The presence and type of mutations of the $p53$ tumor suppressor gene were determined in 40 patients undergoing curative hepatic resection for metastatic colorectal carcinoma. This represents the largest series in the literature on the screening of $p53$ mutations for liver metastases. The analysis was performed in exons 5–9 by denaturing gradient gel electrophoresis followed by direct sequencing. Forty-five percent of tumors showed mutation in $p53$, and this was observed only in exons 5–8. Mutations at codon positions 167, 196, 204, 213, 245, 281, 282, 286, and 306; deletion of codon 251 and of the first nucleotide of codon 252; and Leu residue (CTC) insertion downstream codon 252 are reported for the first time in colorectal liver metastasis. Mutations at codon positions 163, 248, and 273 have been reported previously. Correlation of $p53$ status with clinical parameters showed that patients with mutated $p53$ had a statistically higher number of lesions when compared with patients with wild-type $p53$ ($P < 0.050$). In particular, of patients with mutated $p53$, 41% had three or more metastases compared with 14% of patients with wild-type $p53$. Synchronous metastases were present in 70% of the patients with $p53$ mutations and in only 29% of patients with wild-type $p53$ ($P < 0.025$). In addition, patients with $p53$ mutations are more likely to develop recurrence (73%) compared with patients with wild-type $p53$ (33%; $P < 0.001$). Other factors considered, including preoperative carcinoembryonic antigen level, bilobar distribution, and size of the lesion(s), did not show significant correlation with $p53$ status. These results suggest that $p53$ status might be an important prognostic indicator to predict the pattern and likelihood of treatment failure after hepatic resection.

INTRODUCTION

Liver metastases from colorectal cancer are a major health concern in westernized countries. A small proportion of patients are likely to benefit from curative resection, but only $\sim 30\%$ will survive more than 5 years (1). There have been numerous attempts, based largely on clinical and pathological criteria, to predict the prognosis after resection. However, a more accurate way to define the behavior of a tumor is to study directly at the molecular level the mutations that are associated with carcinogenesis.

Oncogenes, tumor suppressor genes, and DNA damage recognition and repair genes are believed to play a fundamental role in the initiation and progression of most neoplasms (see Refs. 2 and 3 for review). In colorectal cancer, one of the most common solid tumors affecting people around the world, the $DCC^3$ and $APC^3$ genes, on 18q and 5q, respectively, have been found to be lost or mutated in a large proportion of cases (2). Moreover, somatic mutations in other proto-oncogenes are frequently present in K-RAS and less frequently in N-RAS, MYC, MYB, and HER-2/neu, and appear to be critical in colorectal carcinogenesis. It is thought that inactivation of DNA damage recognition and repair genes (e.g., $MSH2$ and $MLH1$) may lead to the more rapid acquisition of mutations in oncogenes such as K-RAS or tumor suppressor genes such as $p53$ and $DCC$.

Loss or mutation of the $p53$ tumor suppressor gene on chromosome 17p in colorectal cancer is well documented, with allelic losses on chromosome 17p detectable in more than 75% of cases. Wild-type $p53$ alleles are presumed to be targeted for inactivation by allelic losses because the remaining allele is very frequently mutated in cases with 17p loss of heterozygosity often in codon 175, 248, or 273. Only a very small subset of colorectal tumors that have not suffered 17p loss of heterozygosity have a mutant $p53$ allele.

Although primary colorectal carcinoma has been studied extensively, the genetic changes occurring in metastases are not well understood. There is a surprising degree of specificity in the organs targeted by metastases from a particular type of tumor. The metastatic process is not random but is based on multiple specific factors such as adhesion molecules, growth factors, and invasive enzymes, which must be produced by the metastatic cell.

The survival of patients with colorectal carcinoma depends primarily on the presence of liver metastases, which often become apparent after resection of the primary. However, it is
difficult to predict the risk of liver metastasis by analyzing conventional tumor markers or pathological findings. Recently, it has been demonstrated that genetic alterations might be a reliable biological marker for assessing the risk of liver metastasis after colorectal resection (4, 5). In particular, it has been reported that an increased incidence of p53, DCC, and Ki-Ras mutations are associated with secondary lesions of colorectal tumors. This suggests that mutation(s) in these genes may play an important role in the establishment of colorectal liver metastases (6).

It has been shown that angiogenesis is required for the growth and metastasis of human solid tumors (7), and recent studies have demonstrated that the p53 tumor suppressor gene plays an important role in controlling tumor angiogenesis (8, 9). p53 mutation is thought to enhance angiogenesis, favoring the growth of the hepatic metastases. The combined analysis of p53 and expression of vascular endothelial growth factor, a well-characterized angiogenic inducer, might be useful for predicting the occurrence of liver metastasis in patients with this disease (10). With regard to the use of new therapy, work is in progress on the study of the efficacy of angiogenesis inhibitor on metastatic potential. In animal models, it has been shown that TNP-470 has excellent antitumorstatic and antiproliferative effects on aggressive primary colorectal tumors in which angiogenesis may be dominant (11).

A previous study from this unit showed that the frequency of allelic loss in colorectal liver metastases has a similar high frequency of loss of heterozygosity on chromosomes 5q, 17p, and 18q as has been reported for primary colorectal tumors (12). The use of liver resection for metastatic colorectal cancer is now well established with long-term survival in ~30% of cases. Nevertheless, the selection of patients likely to benefit from surgery remains controversial and subjective (13). There have been several studies that have attempted to establish a prognostic scoring system to improve case selection (14–19). These studies investigated the prognostic value of different factors including age, size of largest metastasis, preoperative CEA level, stage of the primary tumor, disease-free interval, number of liver nodules, and resection margin.

With the aim to update these criteria with an additional prognostic indicator, we performed the molecular characterization of the mutational pattern of p53 in 40 patients with resected colorectal liver metastases. This report represents the largest series in the literature regarding the screening of p53 mutations and is the first study to report correlation between p53 status and clinical factors in relation to hepatic resection for colorectal metastases.

PATIENTS AND METHODS

Patients. Forty patients undergoing resection of their colorectal liver metastases were studied. Retrospective review of the records was performed to analyze the following factors: age, size of liver lesion(s), number of lesions, synchronous or metachronous lesion(s), preoperative CEA level, and recurrence. Statistical analyses was performed using Student’s t test, and differences were considered significant at P < 0.050.

Tumor tissue samples as well as surrounding normal liver tissue samples were obtained at the time of operation, “snap frozen” in liquid nitrogen, and stored at −80°C until DNA extraction. The remaining liver resection specimen was fixed in 10% neutral formalin and subjected to routine histopathological examination.

PCR and DGGE Study. DNA was prepared from tissue samples by the standard phenol/chloroform method (20). Primers flanking p53 exons 5–9 were used according to a previous report (21). One μg of genomic DNA was mixed with 50 pmol of each appropriate oligonucleotide primer, 0.2 μmol of each deoxyribonucleotide triphosphate, and 1.5 units of Taq DNA polymerase (Bioline) in 50 μl of standard KCl buffer. Samples were incubated in a DNA thermal cycler (GeneAmp PCR system 2400; Perkin-Elmer) for a total of 40 cycles at 94°C, 58°C, and 72°C for 30 s at each temperature. Four μl of the PCR products were subjected to electrophoresis on a 2% agarose gel to examine successful amplification of each fragment.

The optimum gradient for each PCR product was determined with perpendicular DGGE according to the manufacturer’s instructions (D Gene Denaturing Gel Electrophoresis System; Bio-Rad). The ranges of denaturant of parallel DGGE and the optimum conditions of electrophoresis used are reported in Beck et al. (21).

Gels were stained with ethidium bromide.

Sequencing. Exons showing an altered migration in DGGE were sequenced both with forward primers and with reverse primers to confirm the mutation on both strands. To overcome some inherent difficulties in the sequencing of double-stranded PCR product because of the rapid reassociation of the short linear template, we prepared single-stranded templates by asymmetric PCR. The primer ratio used for asymmetric PCR was 100:1. The single-stranded template was sequenced with the limiting primer. Sequence compression problems, due to the high G-C content in the gene, were solved by using modified nucleotides such as deazaanucleotides and inosine and controlled temperature conditions in electrophoresis.

Sequencing was performed using reagents and protocols of Ampli-Cycle Sequencing kit (Perkin-Elmer) and 35S-labeled dATP. Sequence reactions were electrophoresed on a denaturing polyacrylamide-7 M urea gel.

RESULTS

p53 Molecular Characterization. Forty patients who had undergone curative hepatic resection for metastatic colorectal carcinoma were analyzed.

The patients were screened for p53 gene mutation in exon 5–9 by DGGE analysis. Nineteen patients (47.5%) showed an altered migration compared with the control, which indicates the presence of base substitutions, confirmed by sequencing analysis on both strands of samples from normal and tumoral tissues. Table 1 lists all of the mutations and Fig. 1 shows the most common and rare mutations we have detected.

In exon 9, no mutations were identified. Only two mutations were present in exon 5 (codons 163 and 167), and the remaining nucleotide substitutions were fairly well distributed between exons 6, 7, and 8. In exon 6, four tumor samples (cases 3, 4, 6, and 7) showed mutations. In exon 7, six patients (cases 8–13) and in exon 8, six patients (cases 14–19) showed sequence alterations.
As to the type of mutation, the 4-bp deletion in case 12, causing the loss of the codon 250 and of the first base of codon 251, results in a frameshift mutation with the formation of a different amino acid sequence at the COOH terminus of the p53 protein. The CTC insertion in case 13 is in frame and results in the addition of a Leu residue between codons 252 and 253.

Of the 19 substitutions, 10 were missense mutations leading to amino acid change and 6 were nonsense mutations resulting in the insertion of a stop codon. Only one was a silent substitution of the third position of codon 213 (CGA → CGG), resulting in no amino acid change (case 5; not reported in Table 1). All patients showed a single mutation.

Of all mutated samples, three showed a single mutant band in sequencing gel (cases 4, 12, and 13), which most probably represents a homozygous state resulting from the pairing of a mutant allele with a deletion in the remaining allele.

In the remaining cases, the sequencing gels revealed both the mutant as well as the wild-type bands. These tumors may have a p53 mutation but no deletion of the remaining wild-type p53 allele; alternatively the wild-type band may be derived from the contamination of tumor DNA by the nontumoral stroma or liver DNA.

**Evaluation of p53 Status and Clinical Data.** The median length of follow-up was 28 months (range, 3–78 months), and the mean age of the patients was 60 years (range, 43–79 years). The 40 patients were divided into two groups according to their mutated (18) or wild-type (22) p53 status. The two groups were comparable with regard to sex and age.

The available clinical details of the patients are shown in Table 2. The number of metastases was significantly related to p53 status (0.025 < P < 0.050). Patients with wild-type p53 had up to four metastases, whereas patients with mutated p53 had up to eight metastases. Of the patients with wild-type p53, 14% had three or more metastases and 86% had one or two metastases. In comparison, of the patients with mutated p53, 41% had three or more metastases and 59% had one or two metastases. Twenty-nine percent of the patients with wild-type p53 had synchronous lesions (0.010 < P < 0.025), and 33% of patients with wild-type p53 have had recurrence versus 73% of patients with mutated p53 (P < 0.001).

The size of the lesion(s), bilobar distribution, and the preoperative CEA level were not related to p53 status (P > 0.4).

**DISCUSSION**

Liver metastases from colorectal carcinoma are common, and a small proportion of cases may benefit from curative resection. Even after careful case selection, long-term survival can be expected in only ~30% of patients (1). In an attempt to improve the selection of patients for surgery, there has been considerable interest in identifying potential prognostic factors. In addition, if patients with poor prognosis could be reliably identified after resection, there could be more effective use of adjuvant therapies.

This study reports the results of screening of p53 gene mutations in exons 5–9 in 40 patients with colorectal cancer metastatic to the liver. The results were correlated with the clinical profiles to analyze the effect of this biological factor on the presentation and patterns of failure after hepatic resection for colorectal metastases.

The incidence of mutations in the p53 coding sequence is 45%, which is a much higher figure than that observed in primary liver cancers in our previous studies: 24% in hepatocellular carcinoma (22), and 13% in cholangiocarcinoma.⁴

Of all of the mutations we have characterized, 11 involve an Arg. Structurally, Arg takes part in basic interactions. Its side chain participates in Van der Waals, electrostatic, and hydrogen bonding interactions with other side chains with backbone carbonyl groups. We have found that the “hot spot” codons in liver metastasis are 213 (exon 6), 248 (exon 7), and 273 (exon 8). These residues, in particular Arg248 and Arg273, are involved in DNA binding, and these mutations inactivate p53 by eliminating critical DNA contacts.

We have compared our p53 mutational pattern with the only two reports available in literature on colorectal metastasis (6, 23). The exon distribution of the mutation is different. Kastrinakis et al. (6), analyzing 18 samples, did not find mutations in exon 6, and Yao et al. (23), analyzing 8 samples, did not find mutations in exon 8. We report for the first time mutations at codon positions 167, 196, 204, 213, 245, 281, 282, 286, and 306; deletion of codon 251 and of the first nucleotide of codon 252; and Leu residue (CTC) insertion between codons 252–253 in colorectal metastases. Mutations at codon positions 273 and 163 (but with different amino acid substitutions) have been reported. Only the mutation at codon position 248 is common to the three sets of patients.

The detected mutations were searched against the p53 specialized databases (24, 25). In the databases, no entries relative to p53 mutation in colorectal metastasis were available. We have compared the mutational pattern we characterized in colorectal metastasis against those of primary colorectal carcinoma contained in these databases. Fig. 2 compares the distribution of p53 mutations in colorectal liver metastasis with those in primary colorectal carcinoma.

**Table 1** p53 mutational pattern

<table>
<thead>
<tr>
<th>Case</th>
<th>Exon</th>
<th>Codon</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>163</td>
<td>TAC (Tyr) → AAC (Asn)</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>167</td>
<td>CAG (Glu) → TAG (Stop)</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>196</td>
<td>CGA (Arg) → TGA (Stop)</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>204</td>
<td>GAG (Glu) → TAG (Stop)</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>213</td>
<td>CGA (Arg) → TGA (Stop)</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>204</td>
<td>GAG (Glu) → TAG (Stop)</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>213</td>
<td>CGA (Arg) → TGA (Stop)</td>
</tr>
<tr>
<td>8</td>
<td>7</td>
<td>245</td>
<td>GCC (Gly) → AGC (Ser)</td>
</tr>
<tr>
<td>9</td>
<td>7</td>
<td>248</td>
<td>CGG (Arg) → TGG (Try)</td>
</tr>
<tr>
<td>10</td>
<td>7</td>
<td>248</td>
<td>CGG (Arg) → TGG (Try)</td>
</tr>
<tr>
<td>11</td>
<td>7</td>
<td>248</td>
<td>CGG (Arg) → TGG (Try)</td>
</tr>
<tr>
<td>12</td>
<td>7</td>
<td>251–252</td>
<td>ATC C deletion</td>
</tr>
<tr>
<td>13</td>
<td>7</td>
<td>252–253</td>
<td>CTC (Leu) insertion</td>
</tr>
<tr>
<td>14</td>
<td>8</td>
<td>273</td>
<td>CGT (Arg) → CAT (His)</td>
</tr>
<tr>
<td>15</td>
<td>8</td>
<td>273</td>
<td>CGT (Arg) → TGT (Cys)</td>
</tr>
<tr>
<td>16</td>
<td>8</td>
<td>273</td>
<td>CGT (Arg) → CAT (His)</td>
</tr>
<tr>
<td>17</td>
<td>8</td>
<td>281</td>
<td>GAC (Asp) → CAC (His)</td>
</tr>
<tr>
<td>18</td>
<td>8</td>
<td>282</td>
<td>CGG (Arg) → TGG (Try)</td>
</tr>
<tr>
<td>19</td>
<td>8</td>
<td>306</td>
<td>CGA (Arg) → TGA (Stop)</td>
</tr>
</tbody>
</table>

⁴ Manuscript in preparation.
Codons 248 and 273 are the most frequently mutated positions in primary tumor and liver metastases. In our analysis, the metastases did not show any mutations at codon 175, although codon 213 frequently was altered. A possible explanation of the different mutational patterns may be related to a selective advantage for some \( p53 \)-altered cells in establishing a secondary lesion.

Differences between the mutational patterns in matched primary/hepatic metastases from the same individuals have been reported by Kastrinakis \textit{et al.} \cite{6} and Yao \textit{et al.} \cite{23}. They observed a higher rate of \( p53 \) point mutations in hepatic metastasis compared with the primary tumor and that some mutational events in hepatic metastases were not detected in the primary tumor. They hypothesize that metastatic lesions can arise from subpopulations of cells that remain undetected in the sample of the primary lesion. Moreover, the increased incidence of \( p53 \) mutations in secondary lesions compared with the primary colorectal tumors is suggestive of a role for \( p53 \) in the establishment of colorectal hepatic metastases.

Fig. 1 Direct sequencing of the amplified exon products. The most frequently mutated codons are 213, 248, and 273 (cases 6, 9, and 14, respectively), whereas insertions and deletions are considered rare events. Cases 12 and 13 show a homozygous mutation in tumoral DNA.

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<thead>
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<th>Table 2 ( p53 ) status and frequency of clinicopathological features</th>
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<tr>
<td>Mutated ( p53 )</td>
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<td>Wild-type ( p53 )</td>
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<td>Wild-type ( p53 )</td>
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</tbody>
</table>

* Up to eight with mutated \( p53 \); up to four with wild-type \( p53 \).
It cannot be excluded that these differences between liver metastases and the primary tumor could be due to the small number of liver metastases we have analyzed compared with the extensive database on p53 mutations in primary colorectal tumors.

It has been observed that the incidence of transition mutations (purine to purine or pyrimidine to pyrimidine) and transversion mutations (purine to pyrimidine and vice versa) is a function of different cancers. In particular, mutations appear as the result of endogenous processes or the action of exogenous, physical, or chemical carcinogens that damage the genome in a characteristic way, leaving “mutagen fingerprints” in DNA. For example, mutation patterns induced by defined exogenous carcinogens include a G-T transversion at codon 249 in hepatocellular carcinoma due to aflatoxin B1 and at codons 247–250 in tobacco-related cancers due to benzo(a)pyrene.

In this study, we observed that the percentage of transition (74%) is higher than that of transversion, which is similar (80%) to reported studies of primary colon carcinoma. The percentage of transitions is 26% G→A and 47% C→T in liver metastases and 41.5% G→A and 29% C→T in primary colon carcinomas. Transversions, deletions, insertions, or splice mutations are considered very rare events.

Interestingly, in our analysis, most transitions, 11 of 14, involve CpG dinucleotides as observed in primary colorectal cancer. This suggests that most of the mutations that alter the p53 gene in these cancers are probably due to endogenous processes. CpG sites are preferential targets for point mutations in different mammalian genes during the process of DNA replication, presumably because of spontaneous deamination of methylated cytosine residues at these nucleotides (26). Recently, it has been suggested that mutational hot spots at methylated CpG sequences in the p53 gene may be a consequence of preferential carcinogen binding at these sites (27).

The relationship between p53 status and the clinical parameters that appear to determine long-term survival was reviewed. Patients with p53 mutations have a statistically higher number of lesions, compared with patients with wild-type p53. In the former group, 41% of the patients had between three and eight metastases, and in the latter group, 14% of the patients had three or four metastases. Numerous studies have reported that the number of hepatic metastases is one of the most important prognostic factors and that the prognosis of patients with four or more metastatic nodules in the liver is poor (16, 17). On the basis of this, most authorities suggest that when there are more than three lesions, surgical resection is not indicated (17, 28–30).

Synchronous metastases were present in 70% of patients with p53 mutations and in only 29% of patients with wild-type p53. Conversely, metachronous metastases occurred in 30% of patients with p53 mutations and in 71% of patients with wild-type p53. It has been reported that the results of curative hepatic resection for metachronous lesions are significantly better than those for synchronous lesions (15). Moreover, patients with p53 mutations are more likely to suffer recurrence (73%) than patients with wild-type p53 (33%), and it is notable that all of the patients with homozygous mutations have had recurrence.

The present study did not find any correlation between p53 status and CEA, whose level is considered a significant prognostic indicator (17). A negative association between the accumulation of mutant p53 protein and CEA has been reported in breast tumors (31). Other factors, including bilobar distribution and the size of the lesion(s), did not show significant correlation with p53 status.

For patients with unresectable liver metastases from colorectal cancer, the outcome generally is poor. However, chemotherapy can induce tumor regression (32) and may lead to improved overall survival (33). Knowledge of the p53 status would be helpful in selecting patients who may benefit from chemotherapy because it has been reported that there is a relationship between p53 mutations and survival in patients with unresectable colorectal hepatic metastases undergoing regional chemotherapy with 5-fluorouracil (34). Tumors with mutated p53 had either a weak or absent response to chemotherapy (34).

In conclusion, the results of the present study suggest that the determination of p53 status may be a useful prognostic indicator for patients undergoing resection of colorectal liver metastases.
p53 Mutations in Colorectal Liver Metastases

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REFERENCES


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