Prognosis in patients with hepatocellular carcinoma correlates to mutations of *P53* and/or *hMSH2* genes

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Abstract

Association of gene alterations and prognosis has not fully been elucidated in hepatocellular carcinoma (HCC). To clarify the relationship between *p53* and *hMSH2* mutations and prognosis, we analysed these mutations in 83 HCC cases and assessed their association with various clinicopathological factors. The 3-year disease-free survival (DFS) or overall survival (OS) rates in HCC patients with *p53* mutation and *p53* wild/*hMSH2* mutation significantly decreased compared with those without these mutations (14.3% and 37.5% vs. 67.5% for DFS; 35.7% and 50.0% vs. 96.4% for OS, respectively). In the multivariate analysis, categories by *p53* and *hMSH2* mutation status, and liver cirrhosis demonstrated statistically significances for DFS and OS. Moreover, the frequency of patients with *p53* and/or *hMSH2* mutations in intrahepatic metastasis (75.0%) was significantly higher than that in multicentric occurrence (14.3%). Thus, *p53* and *hMSH2* mutations will be useful for identifying subsets of HCC patients with poor prognosis.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers world-wide, especially in Asia and Africa. Hepatocarcinogenesis seems to be a multi-step process that normal hepatocyte is transformed through hepatitis, cirrhosis and adenomatous hyperplasia into malignant tumor and then clinical liver cancer [1]. The various risk factors associated with the development of HCC are well known. They mainly include chronic HCV and HBV infection, heavy alcohol intake, prolonged exposure to aflatoxin B1 (AFB1) and metabolic live diseases such as hemochromatosis. HCC development is closely associated with cirrhosis, and 80-90% of HCC are found in a chronic hepatitis or a cirrhotic liver.

The HCC as well as precursor benign lesions have been extensively studied in terms of genetic alteration in the past 10 years. As in other solid tumors, genetic abnormalities including genomic instability, gene alterations and aberrant expression of genes are accumulated during the carcinogenesis process. Indeed, chromosomal aberrations with loss of heterozygosity have been found in many cirrhotic livers and dysplastic nodules as well as HCC [1-3]. Furthermore, genetic alterations including *p53* family, *Wnt* pathways and DNA mismatch repair genes have been detected in the cirrhotic and dyplastic nodules, and HCC [1, 4-7].

P53 behaves as a multifunctional transcription factor involved in the control of cell cycle, programmed cell death, senescence, differentiation, DNA replication, DNA repair and maintenance of genomic stability. *P53* mutation is shown to be associated with the progression of HCC from an early to a more advanced stage [1]. In HCC developed from populations exposed AFB1, specific *TP53* mutation R249S is observed in more than 50% of the tumors [1]. Microsastellite instability (MSI) occurs in hepatocytes in some cases of chronic hepatitis, cirrhosis, and HCC [8, 9]. Alterations in DNA mismatch repair genes involved in MSI have also been found in HCC, especially HCV-associated HCC [10]. In this way, genetic alterations involved in some of the multi-steps in carcinogenesis have been elucidated to some extent in recent years. Understanding of the molecular mechanisms of HCC development is very important for the improvement in prevention, treatment strategies and prognosis of HCC.

There has been great recent advanced made in treatment of HCC, but the long-term prognosis after curative resection of HCC remains poor. One of the primary reasons for poor prognosis following curative resection is the high recurrence rate –roughly 20-30% within 1 year and 80% in 5 years [11, 12]. Intrahepatic recurrences arise from either intrahepatic metastases or multicentric occurrences. Recurrences due to intrahepatic metastases are generally found to be more aggressive than those from multicentric occurrences possibly because intrahepatic metastases are in a later stage of

hepatocarcinogenesis than those of multicentric occurrences, which can be considered de novo tumors [13, 14]. Distinction of these two types of recurrence is important not only for understanding the biological process of liver carcinogenesis, but also for the determining the optimal treatment for the patient.

Our previous study showed that most mutations of not only *p53* but also *hMSH2* genes occurred in moderately or poorly differentiated HCCs, suggesting that the presence of either a *p53* or *hMSH2* gene mutation is involved in the tumor progression of HCC [4]. It was also suggested that lack of mutations in both *p53* and *hMSH2* closely correlate with the survival in HCC patients treated by surgery [4]. Among DNA mismatch repair genes, there seems close interaction between p53 and hMSH2 proteins during carcinogenesis. First, p53 alterations are associated with altered expression of MMR protein namely hMSH2 protein [7, 15]. Second, MSI inversely correlates with the presence of *p53* mutation in tumors [16]. Third, the presence of p53 response element in the *hMSH2* proximal promoter suggests that p53 regulates hMSH2 expression [17, 18]. Fourth, p53 overexpression was associated with upregulation of hMSH2 protein [7, 15]. Therefore, we hypothesize that alterations of *p53* and *hMSH2* genes may be associated with not only survival in HCC patients but also recurrence of HCC following curative resection

The purpose of this study is to examine the effects of deficiencies of *p53* and *hMSH2* gene function on the development of recurrences of HCC and overall survival following curative resection and establish a relatively simple predictive assay which may be of value in the clinical care of HCC patients.

2. Materials and methods

2.1. Subjects

We obtained tissue samples by surgical resection from 83 HCC patients, in all cases with their informed consent. Histopathologic examination of hematoxylin-eosin stained, paraffin-embedded, sections was performed for all patients. Tumors were histologically classified into well, moderately, and poorly differentiated HCC according to the criteria of Edmondson and Steiner [19]. Histological grading is classified by acidophilic cytoplasm, nuclear / cytoplasm ratio and arrangement of neoplastic cell. No patient was lost to follow-up. The duration of follow-up period is 36 to 105 months. The patient group consisted of 67 men and 16 women (ages 48-77 years; mean 63.0±7.0). Serological testing for serum hepatitis B virus surface antigen was positive in 13 patients (15.7%), and serum anti-hepatitis C virus antibody was present in 64 patients (77.1%); we also found that 2 patients (2.4%) were positive for both markers and 4 patients (4.8%) were negative for both. Fifty of our patients (60.2%) had cirrhosis. Times to relapse and survival were measured from

the date of surgery. For survival analyses, one patients who dies of disease complication within a month of surgery and three patients whose resections were non-curative were excluded.

2.2. DNA isolation

Samples of dissected tumors and surrounding non-cancerous tissues were frozen in liquid nitrogen and stored at –80°C until their DNA was extracted. Genomic DNA was digested with SDS and proteinase K prior to extraction with phenol-chloroform and precipitation with ethanol. After extraction, the purified DNA was stored at 4°C.

2.3. Single-strand conformational polymorphism (SSCP)

To screen the *hMSH2* and *p53* genes for variant sequences, we performed SSCP analysis by the method of Orita and colleagues [20], with particular emphasis on all coding exons of the *hMSH2* gene as well as exons 5-8 of the *p53* gene. The PC primers for amplification of each exon of *hMSH2* and *p53*, and the PCR conditions were as previously described [21, 22]. PCR-amplified fragments were heat denatured at 95°C for 10 min and then loaded on to 8% non-denaturing polyacrylamide gels maintained at 5°C, the gels were dried and exposed to X-ray film. Samples exhibiting altered SSCP migration patterns were subjected to direct nucleotide sequencing.

2.4. Direct nucleotide sequencing

After purification of the PCR products, the products were used as templates for sequencing. For *p53*, the PCR products were denatured to produce single-stranded templates before fluorescence sequencing was performed in an automated sequencing system (ALFred DNA Sequencer, Pharmacia LKB, Uppsala, Sweden). The dideoxy chain-termination method and the Thermo sequenase fluorescent labelled primer cycle sequencing kit were used (Amersham Life Science, Little Chalfont, England).

2.5. Statistics

The Chi-square or Fisher's exact test was used to evaluate the statistical significance of categorical variables. Cumulative disease-free and survival rates were estimated by the method of Kaplan and Meier. The statistical significance of differences in the survival curves of different subgroups was analysed by the log-rank test. The overall survival of the study variables was assessed using the Cox proportional hazards model. Multiple group comparisons were conducted by one-way analysis of variance (ANOVA),

followed by Tukey's honestly significant difference (HSD) test. These statistic analyses were performed with SPSS ver. 11.5J software.

3. Results

3.1. Mutations of the *p53* and *hMSH2* genes in HCC

We screened the genomic DNA from 83 HCC patients for somatic mutations in the *p53* and *hMSH2* genes by both PCR-SSCP and direct sequencing using primer sets shown in Table 1. Tables 2 and 3 summarize results of these mutations. We detected mutations of the *p53* gene in 16/83 patients (19.3%); 12 of the 16 mutations were missense, 3 were deletions and one was nonsense (Table 2). Among these *p53* mutations, we found triple missense mutations in one case (#57) and double ones in another (#69) (Table 2). On the other hand, point mutations of the *hMSH2* gene were found in 9/83 patients (10.8%) (Table 3). Only one patient had both *p53* and *hMSH2* gene mutations in his tumor.

3.2. Clinico-pathological characteristics by mutation status of *p53* and *hMSH2* genes

To assess the association of mutation status of *p53* and *hMSH2* genes with clinico-pathological characteristics, HCC patients were divided into three groups; namely, those with *p53* mutation (n=16) including one case possessing both *p53* and *hMSH2* mutations, with *p53* wild/ *hMSH2* mutation (n=8), and with *p53* wild/ *hMSH2* wild (n=59). Presence or absence of intrahepatic metastasis (im) showed a significant heterogeneity in the distribution of number of patients among three groups (P=0.026, chi-square test) (Table 4). In addition, when compared HCC patients harbouring *p53* and/or *hMSH2* mutations and those without mutations, there was a significant difference only for im (P=0.015, Fisher's exact test) (Table 4). Except for the im, none of other variables showed any difference among these groups.

3.3 Disease-free survival

We conducted univariate analysis for disease-free survival (DFS) in all 79 HCC patients with follow-up data available (Table 5). Presence or absence of *p53* mutation showed a significant difference of DFS period (median: 5 vs. 48 months, P<0.0001, log-rank test). On the other hand, other variables including *hMSH2* mutation did not show any significant differences for DFS. Since *p53* mutation status was such a strong prognostic factor, stratification of HCC patients by *p53* mutation status is required not to overlook other prognostic factors. We then analysed only for 65 HCC patients without *p53* mutation. Notably, HCC patients with *hMSH2* mutation showed a significantly shorter DFS than

those without the mutation (18 vs. 58 months, P=0.019). In addition, liver cirrhosis showed a marginal significance among 65 HCC patients with wild *p53* (P=0.075, log-rank test).

Comparisons of DFS among three groups of HCC patients with *p53* mutation (n=14), those with *p53* wild/*hMSH2* mutation (n=8) and those without mutation (n=57) are shown using Kaplan-Meier survival curves (Fig.1). The 3-year DFS rates of HCC patients were 14.3%, 37.5% and 67.5%, respectively.

In the multivariate analysis, categories by *p53* and *hMSH2* mutation status and liver cirrhosis that showed P<0.1 in univariate analysis were included as explanatory variables (Table 6). HCC patients with *p53* wild/*hMSH2* mutation and those with *p53* mutation showed 2.9- and 7.3-fold risks as compared with those without mutation (P=0.014, P<0.001, respectively). In addition, liver cirrhosis also showed a statistically significant difference (P=0.025) in the analysis.

3.4. Association of recurrent patterns and mutation status of *p53* and *hMSH2* genes

Recurrence was detectable in 45 of 79 patients (57.0%). As shown in Table 7, frequency of recurrence in *p53* and/or *hMSH2* mutation group was significantly higher than that in the *p53* wild/ *hMSH2* wild group (87.0% vs. 44.6%, p= 0.001). Moreover, interestingly, the frequency of patients with *p53* and/or *hMSH2* mutations in intrahepatic metastasis (75.0%) was significantly higher than that in multicentric occurrence (14.3%) (P=0.001, respectively, Tukey's HSD test).

3.5. Overall survival

We also conducted univariate analysis for overall survival (OS) in all 79 HCC patients (Table 5). HCC patients with *p53* mutation demonstrated a significantly shorter OS than those without (24 vs. 98 months, P<0.0001). In addition, liver cirrhosis also showed a significant difference (P=0.037), while a marginal significance of OS was found for HCV status (p=0.068).

We next analysed OS using only 65 HCC patients without *p53* mutation as described above. Remarkably, HCC patients with *hMSH2* mutation showed a significantly shorter OS compared with those without the mutation (30 vs. 98 months, P=0.011). Comparisons of OS among HCC patients with *p53* mutation (n=14), those with *p53* wild/*hMSH2* mutation (n=8) and those without mutation (n=57) are also shown in Fig.2. The 3-year OS rates of HCC patients were 35.7%, 50.0% and 96.4%, respectively.

In the multivariate analysis, we included categories by *p53* and *hMSH2* mutation status, HCV and liver cirrhosis as explanatory variables that showed P<0.1 in univariate

analysis (Table 8). HCC patients with *p53* wild/*hMSH2* mutation and those with *p53* mutation showed 6.8- and 14.5-fold risks compared with those without mutation (P=0.003, P<0.001, respectively). In addition, liver cirrhosis also revealed a statistically significant difference (P<0.001). On the other hand, HCV did not show significance for OS in the analysis.

4. Discussion

This study presents the results of an analysis of the clinical features of hepatocellular carcinoma in 83 HCC patients with and without *p53* and/or *hMSH2* mutations. Interestingly, HCC patients without mutations of these genes showed a better prognosis including recurrence and survival than those with gene mutations. Furthermore, *p53* or *hMSH2* mutation status was also found to be associated with the pattern of recurrence. These findings suggest that mutations of these genes may be deeply involved in not only the progression of HCC but also the recurrence development of HCC.

A *p53* or *hMSH2* gene mutation may accelerate the progressional development of HCC by a 100-600 fold increase in the rate at spontaneous mutations and accumulation due to defects in mismatch repair [23, 24]. It is of course also possible that the loss of a mismatch repair function is likely to lead to high frequencies of ectopic recombination [25, 26]; this may then lead to genomic rearrangement(s) and, as a result, activation of an oncogene or inactivation of a tumor suppressor gene, resulting in accelerating of progression of HCC.

On the other hand, although *p53* or *hMSH2* mutation were not detected in non-malignant cells in cancer tissue, prognosis of the patients was worse than that without the mutations. This may be due to that genome in normal cells around tumors is more instable in HCCs with *p53* or *hMSH2* mutation than those without these mutation, influencing the recurrence or survival. The possibility cannot be excluded that a few normal cells with *p53* or *hMSH2* mutation have existed within cancer tissue though we cannot detect these mutations by our assay system.

Cells in a tumor may well become resistant to chemotherapeutic agents following their acquisition of mutations that affect the *p53* or *hMSH2* genes [27-29]; this is because it should be much easier for cells that have a disrupted mismatch repair system to acquire a drug resistance phenotype by virtue of the increased mutability which stems from their genomic instability. Thus there may be significant differences in responsiveness to chemotherapy in HCC patients with *p53* and/or *hMSH2* mutations and without these mutations, and this could well lead to their experiencing significantly different survival periods following a relapse.

The recurrent tumors identified in this study were classified in accordance with the recommendations of the Liver Cancer Study Group of Japan in their paper on the classification of primary liver cancers [30]. Thus the term intrahepatic metastasis is used to describe 1) tumors that can be clearly seen has having grown from portal vein tumor thrombi, 2) tumors that surround a large main tumor with multiple satellite nodules, and 3) a small solitary tumor which is close to the main tumor and is either histologically similar to or less differentiated than the main tumor. Multiple HCC lesions that cannot be described as metastases under the above criteria are believed to represent potential multicentric occurrences; such tumors are separately recorded as "*de novo carcinogenesis*". However, there may often be recurrent tumors that are difficult to classify as either intrahepatic metastases or multicentric occurrences. Several studies have tried to examine the possible histologic [14] and genetic [31-33] differential diagnoses, but this is still impossible. In our present study, almost all of the cases in which we found a *p53* and/or an *hMSH2* gene mutation appeared to relapse within two years of surgery as a consequence of intrahepatic metastasis. Although we noted that the outcomes were reasonably good in the great majority of the *p53* wild / *hMSH2* wild cases, there were a few cases involving multicentric occurrence that led to relapses occurring 3-plus years after surgery. Frequency of multicentric occurrence was also higher in *p53* wild / *hMSH2* wild cases than in these mutation positive cases. These findings suggest that gene alterations other than mutations of *p53* and *hMSH2* may be involved in recurrence and survival. So, gene alterations, especially on DNA repair system including other mismatch repair genes, need to be analysed.

Figure 3 summarizes the situation as we found it in patients with HCC. There seemed to be relatively few mutations in either *p53* or *hMSH2* in early-stage HCCs. The early-stage HCCs then developed into advanced-stage HCCs, which were more than 2cm in diameter and were either poorly or moderately de-differentiated. The outcome tended to be pretty poor in those HCC patients whose tumors showed clear evidence of *p53* or *hMSH2* mutations, whereas in patients whose tumors had no mutation of either gene the outcome tended to be somewhat better.

Conflict of interest statement

None declared

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Figure Legends

Fig. 1. The Kaplan-Meier curves for disease-free survival for the three groups of HCC patients after a curative resection are shown: patients with *p53* mutation, those with *p53* wild/*hMSH2* mutation and those with *p53* wild/*hMSH2* wild.

Fig. 2. The Kaplan-Meier curves for overall survival for the three groups of HCC patients after a curative resection are shown: patients with *p53* mutation, those with *p53* wild/*hMSH2* mutation and those with *p53* wild/*hMSH2* wild.

Fig. 3. A proposed model of hepatocellular carcinogenesis with emphasis on progression in relation to mutations of *p53* and *hMSH2* genes

Table 1. Oligonucleotide primer sequences used for detection of alterations of *p53* and *hMSH2* genes

p53	Sense (5'→3')	Antisense (5'→3')	Length (bp)
Exon 5	TCCTACAGTACTCCCCTGCC	GCCCCAGCTGCTCACCATC	207
Exon 6	ACTGATTGCTCTTAGGTCTG	AGTTGCAAACCAGACCTCAG	143
Exon 7	AGGTTGGCTCTGACTGTACC	CTCCTGACCTGGAGTCTTCC	120
Exon 8	CTATCCTGAGTAGTGGTAATC	GTCCTGCTTGCTTACCTCGC	165

hMSH2	Sense (5'→3')	Antisense (5'→3')	Length (bp)
Exon 1	TCGCGCATTTTCTTCAACCA	TCCCTCCCCAGCACG	284
Exon 2	TTTTTTGAGCAAAGAATCTGC	ACCTTATATGCCAAATACCAATC	162
Exon 3	TTAGGCTTCTCCTGGCAATC	CCTTTCCTAGGCCTGGAATC	332
Exon 4	CTTATTCCTTTTCTCATAGTAGT	TTGTAATTCACATTTATAATCCATG	221
Exon 5	GCTATAGGAAATCTTCGATTTTTA	TACCTAAAAAGGTTAAGGGCTC	193
Exon 6	TGAGCTTGCCATTCTTTCTATT	TGGGTAACTGCAGTTACATAAA	225
Exon 7	TTCAGATTGAATTTAGTGGAAGC	ACCTTCATGTTTTTCCAGAGC	207
Exon 8	TTTGTTTTACTACTTTCTTTTAGG	AAGTATATTGCATACCTGATCC	148
Exon 9	TAATTTCTGTCTTTACCCATTATTT	CAACCTCCAATGACCCATTC	204
Exon 10	TGGTAGTAGGTATTTATGGAATAC	ATCATGTTTAAGAGCATTTAGGG	264
Exon 11	TACACATTGCTTCTAGTACAC	AGCCAGGTGACATTCAGAAC	202
Exon 12	ATTATTCAGTATTCCTGTGTAC	ACCCCCACAAAAGCCCAAA	326
Exon 13	ATTTATTAGTAGCAGAAAGAAGTT	AAGGGACTAGGAGATGCAC	287
Exon 14	GTTACCACATTTTATGTGATGG	TTCTGAATTTAGAGTACTCC	329
Exon 15	TCTCATGCTGTCCCCTCAC	AAGTTAAACTATGAAAACAAACTG	247
Exon 16	ACTAATGGGACATTCACATGTG	TCAATATTACCTTCATTCCATTAC	232

Table 2. Mutations of *p53* gene in HCC

Case No.	Exon	Codon	Nucleotide alterations	Amino acid substitutions
4	6	220	TAT->TGT	Tyr->Cys
6	7	237	ATG->ATT	Met->Ile
25	5	176	TGC->TAC	Cys->Tyr
31	5	164	AAG->TAG	Lys->Stop
32	7	246	ATG->GTG	Met->Val
35	8	281	GAC->TAC	Asp->Tyr
39	6	189	1 bp deletion (frame shift)	
42	5	146	TGG->TGT	Trp->Cys
44	8	283	1 bp deletion (frame shift)	
52	5	155	ACC->ATC	Thr->Ile
56	6	214	CAT->CGT	His->Arg
57	6	200	AAT->AGT	Asn->Ser
	6	205	TAT->TCT	Tyr->Ser
	6	207	GAT->GCT	Asp->Ala
68	7	238	TGT->CGT	Cys->Arg
69	5	173	GTG->ATG	Val->Met
	5	175	CGC->ATG	Arg->Cys
72	5	154-157	9 bp deletion	
76	5	155	ACC->ATC	Thr->Ile

Table 3. Mutations of *MSH2* gene in HCC

Case No.	Exon	Codon	Nucleotide alterations	Amino acid substitutions
1	1	45	GCG->GTG	Ala->Val
14	7	390	CTT->TTT	Leu->Phe
22	7	390	CTT->TTT	Leu->Phe
	12	629	CAA->CGA	Gln->Arg
33	14	803	ACA->GCA	Thr->Ala
39	3	180	CCA->ACA	Pro->Thr
59	3	180	CCA->TCA	Pro->Ser
64	3	191	CAT->CGT	His->Arg
73	3	180	CCA->ACA	Pro->Thr
81	3	180	CCA->ACA	Pro->Thr

Table 4. Clinico-pathological and epidemiological features stratified by *p53* and *hMSH2* mutation status

		p53 wild/*hMSH2* wild (Reference) (n=59)	*p53* mutation (n=16)	*p53 wild/ hMSH2* mutation (n=8)	P*	*p53* and/or *hMSH2* mutation (n=24)	P**
Gender	Female (n)	11	3	2	0.9	5	1
	Male (n)	48	13	6		19	
Age	≤69 yrs (n)	45	15	5	0.2	20	0.6
	≥70 yrs (n)	14	1	3		4	
Tumor size	< 2 cm (n)	10	0	1	0.2	1	0.2
	≥2 cm (n)	49	16	7		23	
Differentiation	Well (n)	11	0	1	0.2	1	0.2
	Moderately/Poorly (n)	48	16	7		23	
HBs Ab	Negative (n)	48	14	7	0.8	21	0.7
	Positive (n)	11	2	1		3	
HCV	Negative (n)	15	3	1	0.6	4	0.6
	Positive (n)	44	13	7		20	
Liver cirrhosis	Negative (n)	20	9	4	0.2	13	0.1
	Positive (n)	39	7	4		11	
Intrahepatic metastasis	Negative (n)	36	4	3	0.026	7	0.015
	Positive (n)	23	12	5		17	
Portal vein involvement (n)	Negative (n)	39	7	5	0.3	12	0.2
	Positive (n)	20	9	3		12	

*Chi-square test for 2 x 3 table. ** Fischer's exact test for 2 x 2 table (*p53* wild/*hMSH2* wild vs. *p53* and/or *hMSH2* mutation).
im, intrahepatic metastasis; vp, portal vein invasion.

Table 5. Prognostic factors of disease-free and overall survival by univariate analysis

		All (n=79)					Only p53 wild (n=65)				
			DFS		OS			DFS		OS	
Variables		No. of Patients	Median (months)	P*	Median (months)	P*	No. of Patients	Median (months)	P*	Median (months)	P*
Gender	Female (n)	16	36	0.9	67	0.5	13	41	0.9	98	0.9
	Male (n)	63	44		-		52	48		-	
Age	≤69 yrs (n)	61	40	0.4	78	0.8	48	47	0.8	-	0.9
	≥70 yrs (n)	18	48		98		17	48		98	
Tumor size	<2 cm (n)	10	44	0.2	-	0.3	10	44	0.5	-	0.6
	≥2 cm (n)	69	38		78		55	48		98	
Differentiation	Well (n)	12	44	0.5	67	0.7	12	44	0.9	67	0.7
	Moderately/Poorly (n)	67	41		98		53	58		98	
HBs Ag	Negative (n)	65	40	0.9	78	0.3	53	48	0.9	98	0.4
	Positive (n)	14	44		-		12	47		-	
HCV	Negative (n)	18	58	0.2	-	0.068	16	58	0.2	-	0.1
	Positive (n)	61	32		67		49	44		98	
Liver cirrhosis	Negative (n)	33	47	0.3	-	0.037	24	-	0.075	-	0.008
	Positive (n)	46	32		61		41	41		67	
im	Negative (n)	42	44	0.5	98	0.7	38	47	1.0	98	1.0
	Positive (n)	37	36		78		27	48		-	
vp	Negative (n)	49	44	0.7	-	0.1	43	47	0.6	-	0.4
	Positive (n)	30	20		78		22	-		98	
p53	Wild (n)	65	48	<0.0001	98	<0.0001					
	Mutated (n)	14	5		24						
hMSH2	Wild (n)	70	44	0.3	98	0.3	57	58	0.019	98	0.011
	Mutated (n)	9	21		-		8	18		30	

DFS, disease-free survival; OS, overall survival; im, intrahepatic metastasis; vp, portal vein invasion .

A dash (-) indicates that the median survival could not be calculated because the last cumulative survival was greater than 50%.

Table 6. The multivariate analysis of disease-free survival by means of the Cox's proportional hazard model

Variables		Risk ratio (95% CI)	*P*
Gene mutations	*p53* wild / *MSH2* wild	1 (reference)	
	p53 wild / *MSH2* mutation	2.929 (1.241-6.915)	0.014
	p53 mutation	7.328 (3.470-15.474)	<0.001
Liver cirrhosis	Negative	1 (reference)	
	Positive	2.106 (1.097-4.044)	0.025

Table 7. Recurrent pattern in HCC patients by status of *p53* or *hMSH2* mutation

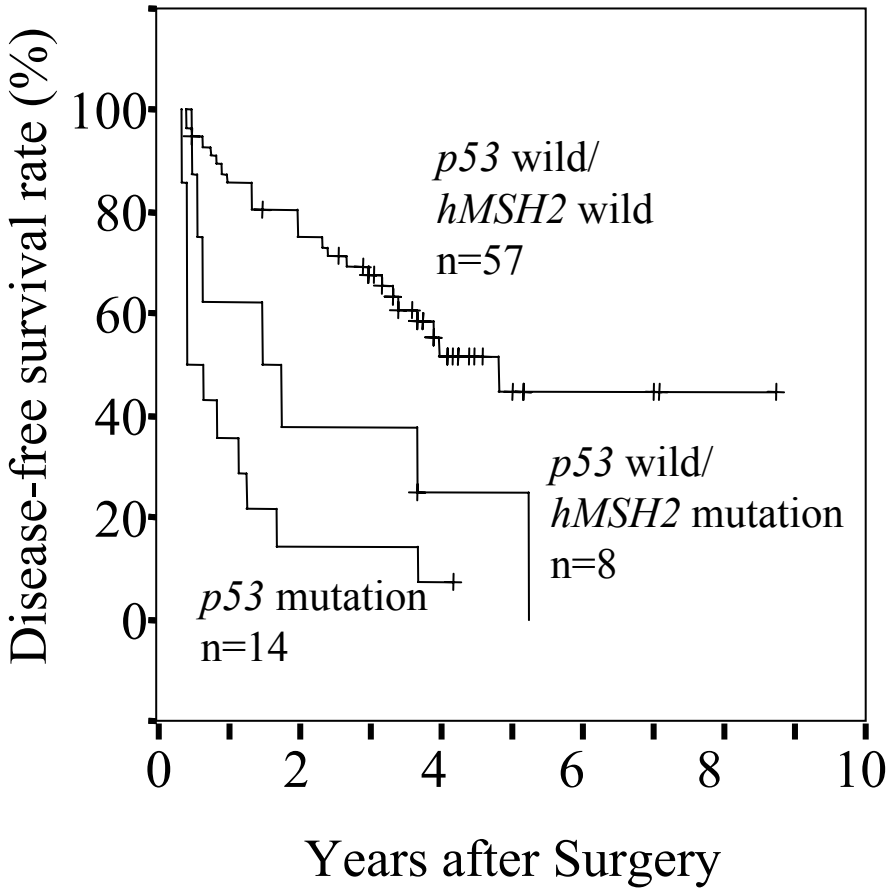
		p53 and/or *hMSH2* mutations	*p53* wild/ *hMSH2* wild	Frequency of mutated patients (%)	*P*
Recurrence	Intrahepatic recurrence				0.001 *
	Intrahepatic metastasis (n)	12	4	75.0	0.001 †
	Multicentric occurrence (n)	2	12	14.3	
	Unclassified (n)	1	4	20.0	
	Distant metastasis (n)	5	5	50.0	
	Total (n)	20	25	44.4	<0.001 **
No recurrence		2	32	5.9	

*One way ANOVA for patients with intrahepatic recurrence. **Fisher's exact test for recurrence vs. no recurrence. †Tukey's HSD test for intrahepatic metastasis vs. multicentric occurrence.

Table 8. The multivariate analysis of overall survival by means of the Cox's proportional hazard model

Variable		Risk ratio (95% CI)	*P*
Gene mutations	*p53* wild / *MSH2* wild	1 (reference)	
	p53 wild / *MSH2* mutation	6.813 (1.894-24.505)	0.003
	p53 mutation	14.504 (5.240-40.147)	<0.001
Liver cirrhosis	Negative	1 (reference)	
	Positive	6.544 (2.298-18.637)	<0.001
HCV	Negative	1 (reference)	
	Positive	3.060 (0.764-12.255)	0.11

Figure 1.

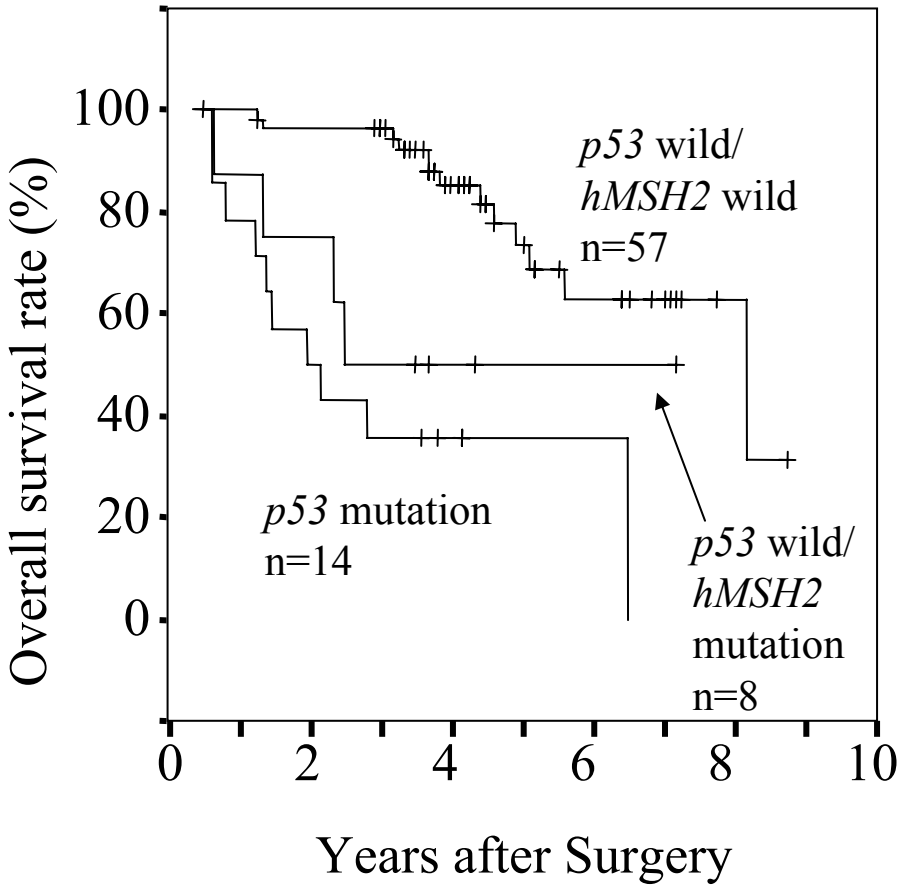


Log-rank test

p53 wild/*hMSH2* wild vs.
p53 wild/*hMSH2* mutated
P=0.019

p53 wild/*hMSH2* wild vs.
p53 mutation
P<0.0001

Figure 2.

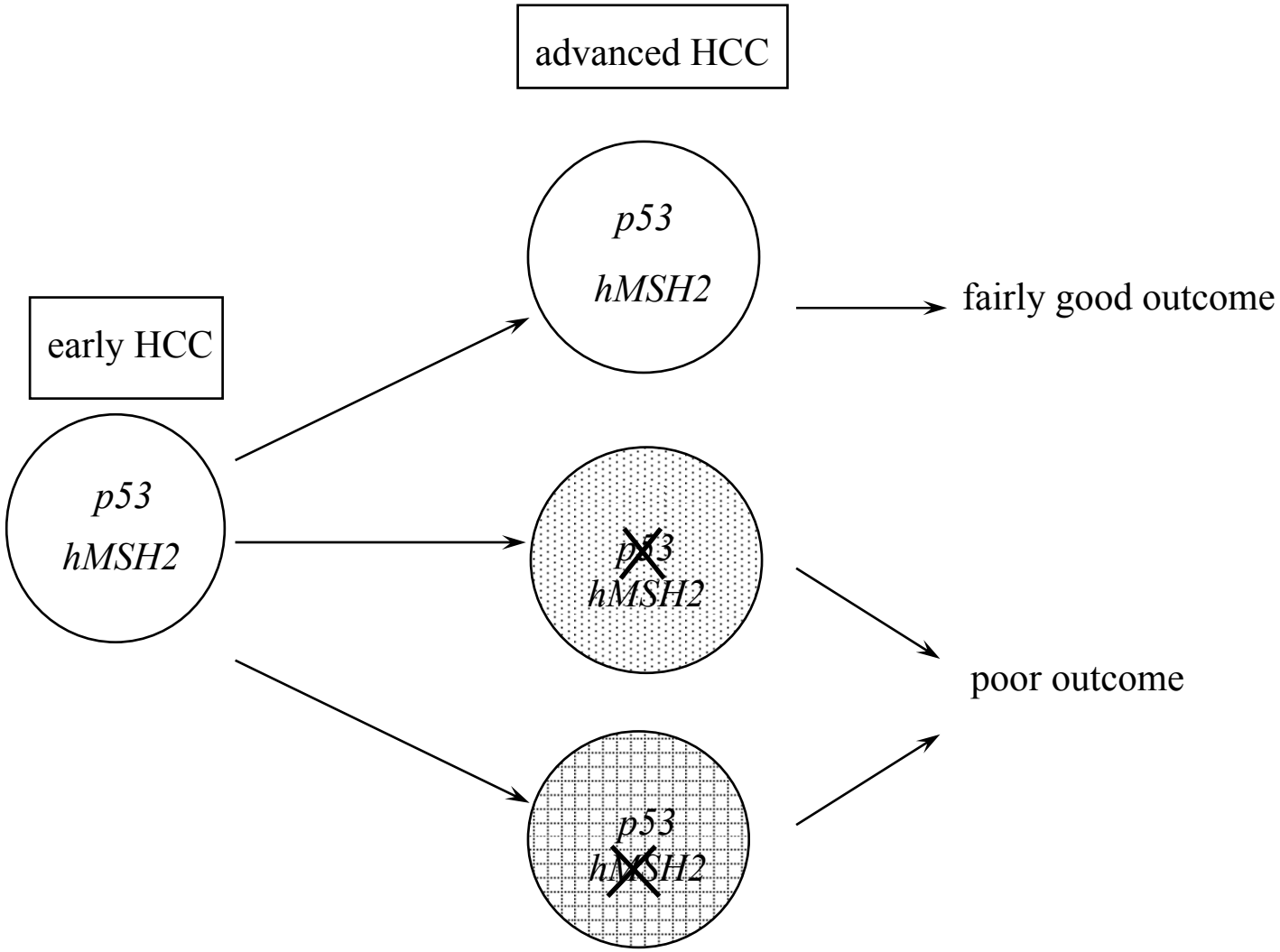


Log-rank test

p53 wild/*hMSH2* wild vs.
p53 wild/*hMSH2* mutation
P=0.011

p53 wild/*hMSH2* wild vs.
p53 mutation
P<0.0001

Figure 3.


advanced HCC
p53
hMSH2
fairly good outcome
early HCC
p53
hMSH2
p53
hMSH2
poor outcome
p53
hMSH2