Clinical Observations

p53 MUTATIONS AND PROTEIN OVEREXPRESSION IN PRIMARY COLORECTAL CANCER AND ITS LIVER METASTASIS

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ABSTRACT

Objective: To compare p53 status in primary and hepatic metastatic colorectal cancer in 34 patients. Methods: p53 gene status (exons 5–9) was examined by PCR, denaturing gradient gel electrophoresis (DGGE) and automated sequencing. P53 protein was detected by immunohistochemistry using monoclonal antibody DO-7. Results: p53 mutations were found in exons 5 through 9 in 21 of 34 patients (61.8%). Among them, 5 patients had mutation in liver metastasis but not in their primary tumors while in the other patients the same mutations were found in both primary and metastatic colorectal cancers. In no patients was p53 mutation exclusively found in the primary colorectal tumors. Moreover, additional mutation was detected in the metastatic lesions in two cases. Of the 37 mutations within the exons examined, 73% was missense mutation and 16% was nonsense mutation. There were 4 microinsertions. p53 protein was overexpressed in both primary and metastatic colorectal cancers with p53 gene mutations. The presence of p53 mutation significantly correlated with p53 protein accumulation (r=0.96, $P< 0.001$). However, in 4 patients with p53 nonsense mutation, immunohistochemical staining was negative. In three patients who showed no p53 mutation of the primary tumor, p53 protein was consistently overexpressed. Conclusion: In colorectal cancers, p53 gene mutation usually appears first in the primary tumor and maintains as such but is more prominent when metastasized to the liver. However, p53 gene mutation may occur only after being metastasized. Although p53 gene mutation and p53 protein overexpression correlate with each other, either parameter examined alone may lead to false positive or negative results.

Key words: Colorectal cancer, Liver metastasis, p53 protein

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Although there have already been many studies reported about p53 status on primary colorectal cancer and / or hepatic metastases,[1, 2] we have not found any report that compares p53 status between primary and metastatic lesions in each patient on an individual level. 34 patients with colorectal cancer and liver metastases were chosen for this study. P53 status in primary and liver metastatic tumor lesions of every individual were investigated, in order to understand that p53 gene status during tumor development and metastasis, and its meaning.

MATERIALS AND METHODS

Tumor Samples

Specimens from primary and hepatic metastatic lesions of colorectal cancer were obtained from 34 patients who underwent radical resection for their primary colorectal cancers and after words partial hepatectomy for hepatic metastases. Specimens were formalin fixed and paraffin embedded and were then checked by microscopy to confirm the histologic diagnosis of colorectal adenocarcinoma. The tumor cell amount exceeded 90% in all examined tumor specimens.

DNA Extraction

Paraffin-embedded sections were deparaffinized in xylene and rehydrated in graded ethanol. Genomic DNA

from samples were prepared by proteinase K/SDS digestion at 48°C followed by subsequent phenol/ chloroform extraction and ethanol precipitation. DNA was amplified by standard PCR-technique.

Polymerase Chain Reaction (PCR)

DNA samples were heated at 94°C for 4 minutes before they were added for a PCR reaction in order to inactivate proteases. Exons 5–9 of the p53 gene were selectively amplified in a DNA thermocycler (Perkin-Elmer, Cetus). The reactions were performed at a temperature cycle condition as below: 35 cycles at 95°C for 30 sec, 58°C for 30 sec and 72°C for 90 sec. The last cycle was followed by a 7 min 72°C extension step. The PCR products were electrophoresed on a 2.7% agarose gel to verify amplification.

A universal 5' 28-bp long GC-rich sequence was added at 5'-end of sense primers for the purpose of DGGE analysis.

DGGE

DGGE electrophoresis on GC-clamped DNA fragment was performed as described by Cottu et al..[3, 4] Briefly, 10% polyacrylamide gel containing a linear gradient of DNA denaturants was prepared (100% was equal to 7 mmol Urea/40% formamide). Appropriate gradient conditions were determined experimentally with perpendicular DGGE. For each exon, the optimum DGGE conditions were evaluated (Table 1). DNA samples were electrophoresed at 56°C, 150V for various times (Table 1). The gel was then stained in ethidium bromide (0.4 μg/ml) for 20 min and photographed by UV transillumination with Polaroid type 667 pack film.

DNA Sequencing

The p53 PCR products showing a mobility shift in the DGGE were analysed with DNA sequencing. The same primers as for PCR amplification and DGGE analysis but without GC-clamp were chosen for sequencing. Around 100 ng of purified PCR fragments were used as templates in 20 μl of reactive solution containing 3.2 pmole of primer and 8.0 μl of the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Mix (Perkin Elmer, USA). The cycle condition was 96°C, 50°C and 60°C for 30 sec, 15 sec and 4 min, respectively during 25 cycles. After ethanol precipitation, samples were resuspended in 25 μl of the Template Suppression Reagent (Perkin Elmer, USA) and analysed by an automated sequencer (ABI PRISM™ 310 Genetic Analyser, Perkin Elmer, USA). Each sample was sequenced both in the sense and antisense direction.

When tumor specimens contained p53 mutations, the result was confirmed by repeating the entire procedure including amplification of genomic DNA, fragment purification, and sequencing.

Immunohistochemical of Staining of p53

Tissue sections of 4 μm thickness were deparaffinized, rehydrated, and microwave treated in citrate buffer solution, at pH 6.0. Immunohistochemical staining (IHC) was performed all by automatic equipment (Dakopatts Techmate 500), using ABC method followed by reaction with diaminobenzidine (DAB) and hydrogen peroxide solution. Tissue sections were incubated with a primary antibody p53 (DAKO, monoclonal, mouse antihuman clone DO-7, 1:500) for 25 minutes. Appropriate positive and negative controls were included in each staining process. The slides were counterstained with Mayer's hematoxylin. Samples exhibiting definitive nuclear staining in >5% of the tumor cells in a tissue section were considered positive.

Statistical Analyses

T-test was used for comparing the ratio of mutated/normal base peak between primary and metastatic lesion. Association between p53 mutation and p53 overexpression was tested with the chi-square test. P-values less than 0.05 were considered statistically significant.

RESULTS

p53 Mutation Analyses

PCR failed in two samples. Thus, PCR products in the rest 32 patients sample were analysed by electrophoretic DGGE and subsequent sequencing analyses. Out of the 32 patients, 19 were demonstrated mutations in exons 5 through 9 (59.4%). Among them, fourteen displayed the same mutation in both the primary and the secondary hepatic metastasis, and two had one additional mutation in their metastatic lesions compared to the primary tumors. Five had mutations only in their liver metastases, while their primary tumors displayed wild type p53. The mutations were mainly point mutations, most frequently observed in exon 8 (codon 263 and 273), exon 7 (codon 248) and exon 6 (codon 196).

Of the 37 mutations in both the primary and secondary tumors 73.0% (27/37) were missense, 16.2% (6/37) nonsense and 4 microinsertions. The most of these point mutations were base-pair transitions mainly G-->A and C-->T, while 6 mutations were transversion.

Sequence curves showed that in 15 patients who had been identified single base alterations in both the primary

and the metastatic tumors, 13 displayed an increased molar ratio of the mutated base *vs* the wild type base in DNA from their liver metastases compared to the corresponding base ratio in the primary tumors ($P<0.001$). This observation may indicate increased cellular homogeneity among the metastases compared to the primaries.

p53 Immunostaining

Immunostaining of tumor material showed p53 protein overexpression in 17 of the 34 patients. All the tumors displayed overexpression in both the primary colorectal tumors and the corresponding hepatic metastatic lesions. An overall correlation was found between the presence of p53 mutations determined by sequencing and p53 protein overexpression, ($r=0.96$, $P<0.001$). In all liver metastatic lesions with p53 protein overexpression, mutations could be detected by DGGE and sequencing. In primary colorectal samples with p53 protein overexpression, no mutation was found by either DGGE or sequencing in 3 patients. Altogether, there were 6 samples (primary plus metastases) showing negative p53 immunostaining while sequencing confirmed mutations. Among these, 4 were stop codon creating mutations and 2 were silent type mutations.

Table 1. Conditions for denaturing gradient gel electrophoresis(DGGE)

Exon	Fragment size	DGGE conditions*	
		Denaturant range (%)	Running time (h)
5	239	50–80	3–4
6	213	40–70	6–8
7	167	40–70	7–8
8	228	35–65	4–6
9	130	20–60	4–5

* 150 V and 56°C

DISCUSSION

Available information on the role of p53 in the development of malignant transformation and progression of cancer diseases has increased enormously during recent years.[1, 5, 6] But those investigations were mostly limited to the comparison between group and group. In this study p53 gene alterations were analysed in primary colorectal tumors and compared to subsequent mutations in both synchronous and metachronous metastases in the liver. This approach differs from previously published articles. The goal of this study was to investigate whether p53 alteration occurs first in primary colorectal lesions; whether and how p53 mutations change during a metastatic process; and what is its meaning.

Gene analyses demonstrate that in fourteen patients who displayed a p53 mutation in primary colorectum cancer, the same mutations were also confirmed in corresponding hepatic metastases. In addition, two patients had an additional mutation in their metastatic lesions. Five had mutations only in their liver metastases, while their primary tumors displayed wild type p53. This indicates that p53 mutation, for the colorectal carcinomas followed by hepatic metastases, mostly start in primary lesion and are brought into the liver organ. Their characters do not change during metastatic processes. The presence of additional point mutations in liver suggests that the p53 mutation might also be produced in the metastatic lesions. We did not find a mutation in primary tumor which was lost or replaced in the metastases.

Sequence curves showed that in 14 patients who had been identified single base alterations in both the primary and the metastatic tumor, 13 displayed an increased molar ratio of the mutated base *vs* the wild type base in DNA from their liver metastases compared to the corresponding base ratio in the primary tumor. Although this has to be considered first, whether more normal tissue cells from colorecta were involved in DNA samples extracted, since every tissue section had been checked and confirmed that the tumor cell amount was more that 90 per cent of total tissue cells. The possibility and influence of normal tissue contamination for colon samples ought to be rare. This result has to be explained as the amount of tumor cells or DNA with p53 mutations in metastatic lesions was incremental. This increment perhaps promotes abnormal proliferation and development of metastatic tumor cells by losing negative regulation of wild-type p53 on the cell cycle. This can help us from molecular genetics' point of views to understand why a metastatic tumor grows faster.

The mutations were mainly point mutations, most frequently observed in exon 8 (codon 263 and 273), exon 7 (codon 248) and exon 6 (codon 196). Most of these

point mutations were base-pair transitions mainly G-->A and C-->T.

In 3 individuals of this study, p53 protein over-expression without gene mutation detected by DGGE and sequencing was found in primary colorectal tumors, while their metastatic lesions presented p53 accumulation and mutation. The reason perhaps is that amount of mutant p53 was still low in the primary colorectal lesions. Beck[7] reported that for DGGE, if mutant gene is <10%, the mutant band may not be visible (perhaps it is the same for sequencing). This result also indicates that we can not ignore immunohistochemical technique and just rely on the DGGE and automatic sequencing though they are rather modern techniques. That is not just due to the fact that immunohistochemical staining can reflect the alteration of p53 on protein level, but also because the immuno-method is more sensitive in some cases, especially in the case when p53 mutant tumor cells are much less than non-mutant cells.

On the other hand, unlike p53 missense mutation (which result in a stable mutant protein detectable by IHC), other mutations such as stop or silent mutations do not result in a stable protein and therefore cannot be detected with IHC approaches. In our data, in 4 nonsense mutation cases, none led to a detectable p53 accumulation. This is because that p53 overexpression detected by IHC is the increased expression of mutant p53. Nonsense mutation may lead to inactivity of wild type p53 but is unable to cause the increasing of expression. That means IHC also has a possibility to lead a false negative. IHC and gene examination must be combined in use.

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REFERENCES

[1] Levine AJ. The p53 tumor suppressor gene. Nature 1991; 351:453.

[2] Sutliff K. How cells cycle toward cancer. Science 1994; 263:319.

[3] Masahiro W. Allelic loss of chromosome 17P, mutation of the p53 gene, and micro-satellite instability in right-and left-sided colorectal cancer. Cancer (Suppl) 1996; 77:1688.

[4] Masataka kato. Detection of DCC and Ki-ras gene alterations in colorectal carcinoma tissue as prognostic markers for liver metastatic recurrence. Cancer (Suppl) 1996; 77:1729.

[5] Pocard M. Different p53 mutations produce distinct effects on the ability of colon carcinoma cells to become blocked at the G1/S boundary after irradiation. Oncogene 1996; 12:875.

[6] Diez M, Camunas J, Enriquez JM, et al. A Comparative study on the prognostic value of the nuclear expression of protein p53 vis-a-vis histopathology in colorectal cancer. An-Med-Interna 1996; 13:222.

[7] Cottu PH, Muzeau F. Inverse correlation Between RER+ status and p53 mutation in colorectal cancer cell lines. Oncogene 1996; 13:2727.