

Elevated Expression of Poly(ADP-Ribose) Polymerase-1 is Associated with Liver Metastasis in Colorectal Cancer.

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Activation of poly(ADP-ribose) polymerase-1 (PARP-1) and its subsequent cleavage are early markers of apoptosis. PARP-1 is associated with DNA repair and so chromosome stability, cell cycle regulation, as well as tumorigenesis. To investigate the role of PARP-1 expression in colorectal carcinoma and its metastasis of liver, we compared the expression of PARP-1 in primary colorectal cancers with (n=15) and without liver metastasis (n=17) using a semi-quantitative reverse transcription - polymerase chain reaction. We also examined the expressions of poly(ADP-ribose) (PAR) and p53 in these tumors by immunohistochemistry. A significantly higher PARP-1 mRNA expression was noted in tumors with liver metastasis than those without liver metastasis ($p < 0.01$). Colorectal cancers positively stained for p53 exhibited significantly higher PARP-1 mRNA expression than p53-negative tumors ($p < 0.01$). The PAR labeling index (LI) of tumors with metastasis (0.33 ± 0.33) was not significantly different ($p = 0.35$) from that of tumors without liver metastasis (0.38 ± 0.19). p53-positive tumors tended to have higher PAR LI levels than p53-negative tumors ($p = 0.08$). Our findings suggest that PARP-1 may contribute to liver metastasis due to its DNA repair activity, resulting in survival of the tumor cells with accumulation of metastatic-related gene's damages. Detailed analysis of PARP-1 may be useful in cancer research and/or cancer therapy.

ACTA MEDICA NAGASAKIENSIA 47 : 111–115, 2002

Key Words: poly(ADP-ribose) polymerase-1, poly (ADP-ribose), p53, colorectal cancer, liver metastasis

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Introduction

Poly(ADP-ribose) polymerase-1 (PARP-1) is a nuclear protein found in most of the eukaryotes, with the exception of yeast¹⁾. PARP-1 gene is mapped on chromosome 1q41-42 in human¹⁾. PARP-1 activation and subsequent cleavage are well known as early markers of apoptosis²⁾. Intranuclear expression of PARP-1 is marginal in cells without DNA damages¹⁾, but it is explosively increased when DNA strand breaks are induced by some DNA damaging agents or γ -irradiation³⁾. PARP-1 binds to the site of DNA breaks, and catalyzes the production of poly(ADP-ribose) (PAR). PARP-1 is thought as a DNA strand break sensing molecule, and PAR is as a regulator of various nuclear proteins, for example, p53⁴⁾, histon H1 and H2B⁵⁾, topoisomerase I^{4,5)}, PARP-1⁶⁾, DNA-dependent protein kinase⁷⁾, DNA ligase⁸⁾, and DNA polymerase⁸⁾. Therefore, PARP-1 is supposed to be associated with DNA repair system and so chromosome stability.

PARP-1 was reported to be involved in cell cycle regulation⁹⁾ and in malignant transformation¹⁰⁾. In immortalized fibroblasts originally derived from PARP-1^{-/-} mice, p53 expression was reduced, and PAR, which covalently bound to p53, was absent¹¹⁾. Inhibition of PARP activity by chemical inhibitors and ultraviolet light rendered mice or rats susceptible to carcinogenic agents, and resulted in the occurrence of various tumors: hepatocellular carcinoma¹¹⁾, skin cancer¹²⁾, adenoma, and sarcoma¹³⁾. These associations suggest a role of PARP-1 in suppressing tumorigenesis. However, the study of PARP-1 and PAR in human cancer tissue has been rarely seen, and their relevance of these molecules to metastasis has not been investigated.

In this study, we hypothesized that, among several clones that compose primary colorectal cancer, a PARP-1 deficient clone might metastasize because the damages of metastatic-related genes could not be repaired due to its insufficient DNA repair capacity. To study the relation between DNA repair capacity

correlated with PARP-1 expression and liver metastasis in human cancer, we investigated the expression of PARP-1, PAR, and p53 protein in primary colorectal cancers with or without liver metastasis.

Materials and Methods

Tumor samples

Fifteen primary tumor specimens obtained from colorectal cancer patients with liver metastasis were examined. Thirteen patients had synchronous liver metastasis, and two had metachronous liver metastasis. As controls, we examined 17 specimens of primary colorectal cancers obtained from patients who did not develop liver metastasis during a period of more than 5 post-operative years. All patients underwent surgical resection at the Division of Surgical Oncology, Department of Translational Medical Sciences, Nagasaki University Graduate School of Biomedical Sciences. Samples were frozen at -80°C immediately after surgery. The patients ranged in age between 41 and 81 years (mean, 62 years), and included 22 males and 10 females. All surgical samples were examined histopathologically¹⁴⁾, and classified by the American Joint Committee on Cancer Classification¹⁵⁾. There were 9 well-differentiated and 22 moderately-differentiated adenocarcinomas, and one was poor differentiated. In the patients, 13 were with stage IV, 6 with stage III, and 11 with stage II disease. No patient had other distant metastasis at the time of surgery and none of the patients had received radiation therapy or chemotherapy prior to surgery.

Semi-quantitative reverse transcription - polymerase chain reaction (RT-PCR) for PARP-1 mRNA

Total RNA was extracted from frozen tissues using TRIzol Regent (Life Technologies, Rockville, MD) following the procedures recommended by the manufacturer. One step RT-PCR was performed from the prepared total RNA (about $1\mu\text{g}$) using an RT-PCR High-Plus- kit (TOYOBO, Tokyo) according to the instructions provided by the manufacturer. Semi-quantitative RT-PCR was used to analyze PARP-1 expression. Primer sequences used for RT-PCR are as follows: PARP-1: forward primer, 5'-GAGCGATGCCTATTACTGCAC-3', reverse primer, 5'-GAACAACCTCCTGAAGGCTCTTG-3';¹⁶⁾ glyceraldehyde-3-phosphate dehydrogenase (G3PDH): forward primer, 5'-TCCACCACCTGTTGCTGTA-3', reverse primer, 5'-ACCACAGTCCATGCCATCAC-3'. One step RT-PCR was performed at 60°C for 30 min and 94°C for 2 min for 19-40 cycles using a step cycle

program at 94°C for 1min and 62°C for 1.5 min in a Perkin-Elmer thermal cycler (Foster City, CA), with a 25- μl reaction mixture containing 2 mM $\text{Mn}(\text{OAc})_2$, 300 μM each deoxynucleotide, 400 nM each primer, and 2.5 units rTth DNA polymerase in the appropriate buffer. After PCR, 9 μl of the reaction mixture were electrophoresed with 1 μl of the 10x loading dye on a 2% Nusive 3:1 agarose gel (Biocompare, Inc., Burlingame, CA) containing ethidium bromide. Estimation of the band intensity of PCR fragments was performed using a scanner and Scion image software (Scion Corporation, Frederick, MD). Agarose gels were photographed and their digital images were captured using the scanner. The scan data were saved and imported into the Scion image software for subsequent analysis. Band intensities were compared between PARP-1 and G3PDH for quantification after the same number of PCR cycles, at a point at which the PCR reaction had not reached a plateau (Figure 1).

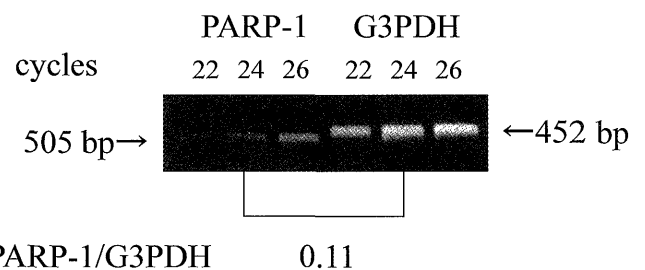


Figure 1. Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) for poly(ADP-ribose) polymerase-1 (PARP-1) mRNA. PCR was performed for three consecutive cycles. The band intensity of PARP-1 was compared with that of glyceraldehyde-3-phosphate dehydrogenase (G3PDH), a house keeping gene, after the same number of cycles, before PCR reaction reached a plateau.

Immunohistochemistry for PAR and p53

Immunohistochemical studies were performed on 5- μm thick sections of formalin-fixed, paraffin-embedded specimens. An immunoperoxidase method, using a LSAB2 kit (Dako, Glostrup, Denmark), was used to detect p53 and PAR in deparaffinized tissue sections. After microwave processing for 10 min in 10 mM citric acid buffer (pH 6.0), the tissue sections were incubated for 5 min at room temperature in hydrogen peroxide (H_2O_2) to inhibit endogenous peroxidase activity. Tissue sections were then incubated for 5 min at room temperature in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA). Anti-human p53 protein (clone DO7) (Dako), that recognizes an epitope located between amino acids 19 to 26 in both wild-type and mutant p53 protein, and monoclonal antibody to PAR (clone 10H) (Alexis Biochemicals, Lausen,

Switzerland) were used as primary antibodies. Tissue sections were incubated overnight with a 1:50 dilution of DO7 monoclonal antibody or a 1:200 dilution of 10H monoclonal antibody in a moist chamber at 4°C. As a negative control, we added normal mouse IgG instead of the primary antibody. After incubation, the specimens were treated by biotin-labeled goat anti-mouse immunoglobulin and streptavidin-conjugated horseradish peroxidase. The peroxidase reaction was performed using diaminobenzidine as chromogen and H₂O₂. Tissue sections were counterstained with hematoxylin. We examined at least 1000 tumor cells and designated the sample as positive for p53 protein when at least 10% of tumor cells displayed strong nuclear staining. For PAR expression, the PAR labeling index (LI) was defined as the percentage of positively stained nuclei observed on counting more than 1000 tumor cells.

Statistical analyses

Statistical analyses were performed using the computer program STATISTICA™ (StatSoft, Tulsa, OK). Data are expressed as mean ± SD. Data were processed using the Mann-Whitney U test, Welch's T test, Fisher's exact test, and likelihood ratio test. The correlation between PARP-1 mRNA and PAR LI was analyzed by Spearman's rank correction. A P value of less than 0.05 denoted the presence of a statistically significant difference.

Results

There were no significant differences in age ($p=0.57$), gender ($p=0.89$) or histological type ($p=0.38$) between patients with colorectal cancers and liver metastasis ($n=15$) and those without liver metastasis ($n=17$).

PARP-1 mRNA expression

PARP-1 mRNA expression did not show any significant differences according to age, gender or histological type. The mean expression level of PARP-1 mRNA in tumors with liver metastasis (0.91 ± 2.10) was significantly higher than those without liver metastasis (0.04 ± 0.07 , $p<0.01$, Figure 2A). The tumors with liver metastasis had significantly higher PARP-1 mRNA expression than those without liver metastasis ($p<0.01$). p53 immunoreactivity was localized in the nuclei of tumor cells (Figure 3A). Fifteen of 32 (47%) colorectal cancers were p53-positive. Five of 15 (33%) tumors with liver metastasis were p53-positive, compared with 10 of 17 (59%) tumors without metastasis ($p=0.14$). Colorectal

cancers positive for p53 ($n=15$) exhibited significantly higher PARP-1 mRNA expression than p53 negative tumors ($n=17$, 0.79 ± 2.24 vs. 0.32 ± 0.84 , $p<0.01$, Figure 2B).

PAR expression

PAR immunoreactivity was localized in the nuclei of tumor cells and infiltrating lymphocytes with homogeneous intensity, but we only counted tumor cells in this study (Figure 3B). PAR expression did not correlate significantly with age, gender, histological type or PARP-1 mRNA expression. The PAR LI of tumors with liver metastasis (0.33 ± 0.33 , $n=15$) was not significantly different from that of tumors without liver metastasis (0.38 ± 0.19 , $n=17$, $p=0.35$). The PAR LI of p53-positive tumors (0.42 ± 0.19 , $n=15$) tended to be higher than

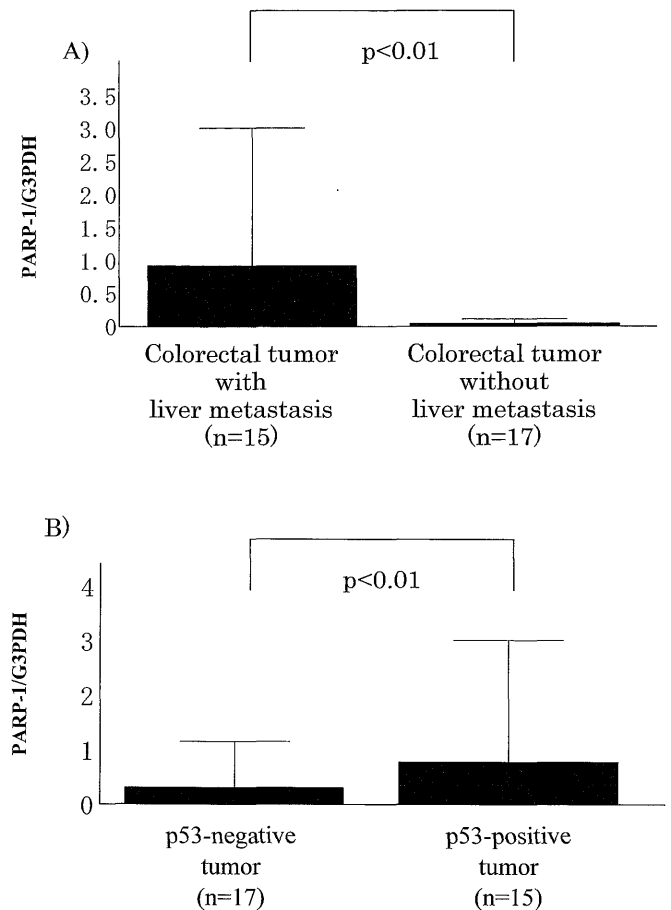


Figure 2. Expression of poly(ADP-ribose) polymerase-1 (PARP-1) mRNA in primary colorectal tumors. (A) PARP-1 expression in tumors with liver metastasis ($n=15$) and without liver metastasis ($n=17$) (0.91 ± 2.1 vs. 0.04 ± 0.07 , $p<0.01$). (B) PARP-1 expression in p53-negative ($n=17$) and p53-positive ($n=15$) tumors (0.32 ± 0.84 vs. 0.79 ± 2.24 , $p<0.01$). The ordinate represents the relative band intensity of PARP-1/G3PDH. Data are mean ± SD.

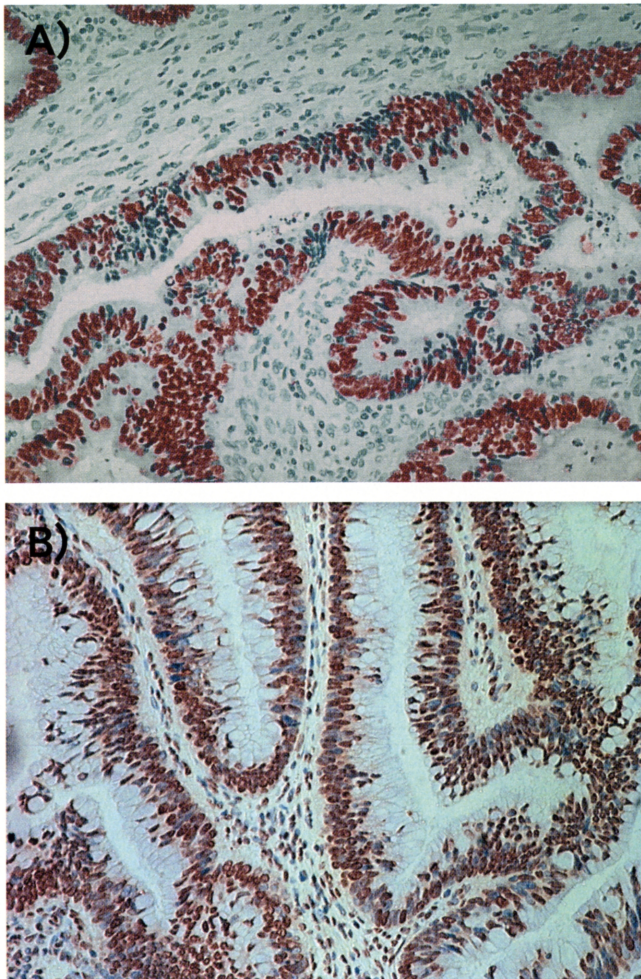


Figure 3. Immunohistochemical staining of p53 and poly (ADP-ribose). (A) p53 immunoreactivity (brown) was localized in the nuclei of tumor cells ($\times 200$). (B) Poly(ADP-ribose) immunoreactivity (brown) was localized in the nuclei of tumor cells and infiltrating lymphocytes with homogeneous intensity ($\times 200$).

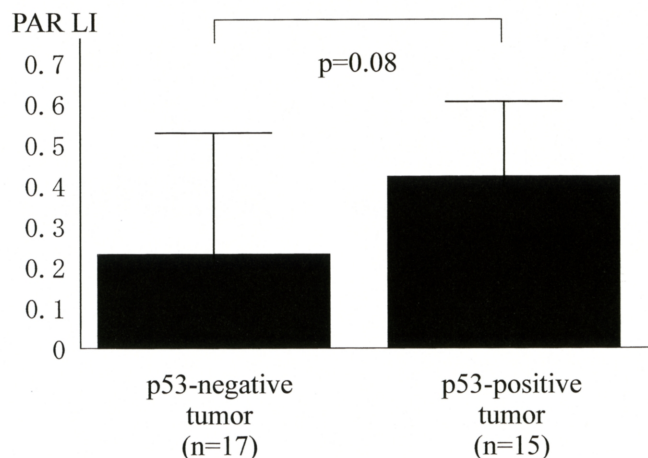


Figure 4. Poly(ADP-ribose) labeling index (PAR LI) in p53-negative (n=17), and p53-positive (n=15) tumors (0.23 ± 0.30 vs. 0.42 ± 0.19 , $p=0.08$). Data are mean \pm SD.

that of p53-negative tumors (0.23 ± 0.30 , $n=17$, $p=0.08$, Figure 4).

Discussion

In this study, we focused on an intra-nuclear multi-functional enzyme, PARP-1, and investigated the association among PARP-1 and PAR expression and cancer metastasis in primary colorectal cancers with or without liver metastasis. Contrary to our expectation, the mean expression level of PARP-1 mRNA in primary tumors with liver metastasis was significantly higher than that of tumors without liver metastasis.

DNA-dependent protein kinase (DNA-PK) is required for the rejoining of double-strand DNA breaks, and plays a critical role in DNA repair. PARP-1 adds PAR to the DNA-PK catalytic subunit, and activates DNA-PK⁷⁾. Thus, PARP-1 is considered to facilitate double-strand DNA break repair by stimulating the protein kinase activity of DNA-PK. Our finding of a significantly high PARP-1 mRNA in primary tumors with liver metastasis could be explained by the following mechanism. In fact, liver is supposed to be an unfavorable environment for colorectal cancer cells¹⁷⁾, especially at early phase of liver metastasis. A report using melanoma cells suggested most of tumor cells reached liver were apoptotic, and finally they could survive only 36%¹⁸⁾. Therefore, colorectal cancer cells with high PARP expression may survive despite genetic damage due to the high DNA repair activity, whereas those cells with low levels of PARP expression may succumb to apoptosis. Moreover, because the mRNA expression of cell cycle regulators, such as cyclin A and B1 and B2, were decreased in PARP-1^{-/-} mouse fibroblasts⁹⁾, PARP-1 expression may contribute to cell cycle progression mediated by cyclins. Therefore, it might be possible to speculate that colorectal cancer cells with high PARP expression could proliferate even in the liver. In this regard, Bieche et al. reported that PARP-1 mRNA expression was higher in human breast cancer tissue than in adjacent normal breast tissues¹⁹⁾. Nomura et al. found that PARP-1 mRNA expression was elevated in hepatocellular carcinoma but not in normal liver²⁰⁾. These data suggest an important role of enhanced PARP-1 expression in human cancers, in accordance with our finding that PARP-1 may contribute to liver metastasis by increasing survival of the cells with metastatic potential.

In the present study, the expression of PARP-1 mRNA and PAR was not in parallel. PAR production was controlled not only by PARP-1, but also by many factors, such as the amount of catalytic substrate

nicotinamide adenine dinucleotide (NAD⁺), the degree of DNA damage, and the rapid degeneration of PAR by poly(ADP-ribose) glycohydrolase³¹. Therefore, PAR production was not always dependent upon the amount of PARP-1. Moreover, although PARP-1 has a most powerful PARP activity²¹¹, other PARP enzymes, such as PARP-2 and tankylase²²¹, may be involved.

With respect to p53, Simbulan-Rosenthal et al. reported that p53 protein in PARP-1^{-/-} immortalized mouse cells decreased, compared to that of wild-type cells, and that p53 expression level was partially restored by the reintroduction of PARP-1 cDNA²³¹. These results suggested that PARP-1 might be involved in p53 stabilization and accumulation. In the present study, we found that p53 expression was significantly correlated with the expression of PARP-1, and tended to be associated with PAR labeling index, confirming the relationship between p53 and poly(ADP-ribosyl)ation in human tissues, at least partly. It is most likely that direct binding of PAR to p53 protein in the nucleus may contribute to prolongation of the half life of p53 protein, and thus enhance p53 accumulation. Despite the link between PARP-1 mRNA and liver metastasis or PARP-1 and p53, why p53 did not correlate liver metastasis? The contradiction may be ascribed to the small number of samples in this study and numerous samples are required for further investigation.

It is possible that PARP-1 inhibition may be beneficial for cancer therapy. PARP-1 inhibits topoisomerase I activity by poly(ADP-ribosyl)ation in the presence of NAD⁺, the catalytic substrate of PARP-1²⁴¹. It has been recently reported that the combining use of PARP-1 inhibitor and topoisomerase I inhibitor show the synergistic cytotoxic effect²⁵¹. Therefore, assessment of PARP-1 activity in tumors may be useful for cancer chemotherapy.

In conclusion, we found the possible relationship between PARP-1 expression and liver metastasis in colorectal cancer. The detailed analysis of PARP-1 may be useful for further cancer study and also cancer therapy.

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