SPANDIDOS PUBLICATIONS

ONCOLOGY REPORTS 26: 49-54, 2011

Rare *MDM4* gene amplification in colorectal cancer: The principle of a mutually exclusive relationship between *MDM* alteration and *TP53* inactivation is not applicable

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Received December 9, 2010; Accepted February 15, 2011

DOI: 10.3892/or.2011.1270

Abstract. MDM4, a homolog of MDM2, is considered a key negative regulator of p53. Gene amplification of *MDM4* has been identified in a variety of tumors. *MDM2* or *MDM4* gene amplification is only associated with the wild-type *TP53* gene in retinoblastomas, thus the amplification of the two genes is mutually exclusive. Previously, we demonstrated that *MDM2* amplification and *TP53* alteration were not mutually exclusive in colorectal cancer, and we identified a subset of colorectal cancer patients without alterations in either the *TP53* or the *MDM2* gene. In this study, we investigated the gene amplification status of *MDM4* in the same set of colorectal cancer cases. Unexpectedly, *MDM4* amplification was rare, detected in only 1.4% (3 out of 211) of colorectal cancer cases. All the three gene-amplified tumors also harbored *TP53*-inactivating mutations. This contradicts the simple mutually exclusive relationship observed in retinoblastomas. Surprisingly, two of the three *MDM4*-amplified tumors also demonstrated *MDM2* amplification. Paradoxically, the MDM4 protein levels were decreased in the tumor tissue of the gene-amplified cases compared with levels in the matched normal mucosa. We speculate that MDM4 might play a role in colorectal carcinogenesis that is not limited to negative regulation of p53 in combination with MDM2. The functional significance of MDM4 is still unclear and further studies are needed.

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Key words: MDM4 (MDMX), MDM2, p53, colorectal cancer, gene amplification

Introduction

The p53 tumor suppressor protein is an important mediator in the response to DNA damage and activated oncogene products, and executes cell cycle arrest, senescence or apoptotic cell death as a guardian against tumorigenesis. Approximately half of all human tumors carry alterations of the *TP53* gene (1), and mutations in this gene have been described in 40 to 70% of human colorectal cancers (CRC) (2,3). Other genetic alterations, such as mutations of *KRAS* or DNA mismatch repair (MMR) genes, microsatellite instability (MSI) and changes in the regulators of *TP53*, are also identified as being involved in CRC tumorigenesis (4,5).

The intracellular levels of p53 are partly regulated by MDM2 that promotes p53 degradation through an ubiquitin-dependent pathway (6,7). Gene amplification and overexpression of *MDM2* are observed in a variety of tumor types (8,9). We and another group have reported that amplification of the *MDM2* gene occurs in ~10% of CRC (10,11). Although a mutually exclusive relationship was speculated from MDM2 function, *MDM2* gene amplification and a *TP53*-inactivating mutation were independent events in CRC, and *MDM2* amplification was generally associated with progression of the disease. In our Japanese CRC study, the increase in *MDM2* copy number was mainly caused by gain of the whole chromosome 12, on which *MDM2* is located, and the amplification levels were low (11).

MDM4 (also known as MDMX) shares strong homology with MDM2, and is considered another key negative regulator of p53 function (12). Unlike MDM2, MDM4 lacks ubiquitin ligase activity and is not able to directly ubiquitinate p53 (13,14). On the other hand, it has been reported that MDM4 forms stable hetero-oligomers with MDM2 through its C-terminal RING domain, and that these hetero-oligomers can ubiquitinate p53 more effectively than MDM2 homodimers (15-18). However, the function of MDM4 is still controversial, because it has been reported that MDM4 binds directly to p53 and inhibits its transcriptional activity (12), and also that

overexpression of MDM4 interferes with the ubiquitin ligase activity of MDM2 (13,14).

Gene amplification of *MDM4* was found in 25% of 533 tumor specimens including brain tumors, breast cancers and soft tissue tumors (9). MDM4 protein overexpression was found in 17% of a variety of tumors (9). For retinoblastomas, *MDM4* gene amplification was reported to occur in 65% of tumors, without association with *TP53* gene alterations (19). *MDM2* amplification was also found in 10% of the tumors (19). Retinoblastomas retain wild-type *TP53*, and *MDM4* or *MDM2* amplification was considered to abrogate the p53 pathway, which would inevitably lead to tumorigenesis. Similarly, the subset of CRC with wild-type *TP53* and diploid *MDM2* in our previous study may have alterations in *MDM4* (3,11). In this study, we focused on the gene amplification of *MDM4* in 211 CRC cases and characterized their clinicopathological and molecular features including the *TP53* mutation status.

Materials and methods

Patient population and tissue specimens. The study population comprised 211 patients who had surgical resection of sporadic colorectal cancers at Kanagawa Cancer Center Hospital between 2002 and 2004. We have previously described the demographic and clinicopathological characteristics of these patients (3). The pathologic stage for each patient was determined using Dukes' staging system (20), while the histologic types and differentiation of the adenocarcinomas were evaluated according to the TNM classification (21). The Ethics Committee of Kanagawa Cancer Center approved the present study.

Quantitative real-time polymerase chain reaction. Genomic DNA for quantitative real-time polymerase chain reaction (qPCR) was isolated from tumor tissues using a DNA mini kit (Qiagen KK, Tokyo, Japan) according to the manufacturer's instructions. qPCR was performed using a LightCycler® 480 System II (Roche Diagnostics GmbH, Mannheim, Germany) to analyze the copy number of the *MDM4* gene. The hybridization probe for qPCR was selected from the Universal ProbeLibrary Probes (Probe #1; Roche Diagnostics GmbH). qPCR was performed using LightCycler 480 Probes Master mix in combination with Universal ProbeLibrary assays (Roche Diagnostics GmbH). PCR amplification was carried out in a total volume of 20 μl; the reaction mixture comprised 3 ng of DNA, 500 nM of each PCR primer and 100 nM of probe. PCR amplification was performed with an initial denaturation step at 95˚C for 10 min, followed 45 cycles of denaturation at 95˚C for 10 sec, annealing at 60˚C for 20 sec and extension at 72˚C for 1 sec. The nucleotide sequences for the *MDM4*-specific primers were: 5'-TTGGAGGTGGC TGACCATA-3' and 5'-ACTCCAGGAGGACCAAATGA-3'. To obtain the gene dosage, the albumin gene *(ALB)* was used as an endogenous reference gene. The relative quantification method for the calculation of gene copy number has been described previously (11).

TP53 and KRAS mutation and microsatellite instability analyses. The *TP53* mutation status for all 211 CRC specimens has already been evaluated, and the details of all individual mutations are available in our previous study (3). Determination of the *KRAS* nucleotide sequence at codons 12 and 13 was performed as described previously (22), and the *KRAS* mutation status of all 211 CRC specimens will be described elsewhere. For the determination of microsatellite instability, PCR amplification was performed using the mononucleotide repeat of five microsatellite markers for colorectal cancer (BAT-25, BAT-26, *TGFβRII*, *BAX* and *MRE11*) (23,24). The precise information for the PCR conditions and the primers is available on request.

Table I. Molecular and immunohistochemical analysis of *MDM4*-amplified tumors from colorectal cancer patients.

	Case 1	Case 2	Case 3
MDM4 copy no.	5.30	4.13	4.10
MDM2 copy no.	6.50	4.12	3.74
TP53 mutation	R282W	V143G	P250L
KRAS mutation	G12C	wt	wt
Microsatellite markers	Stable	Stable	Stable
Immunohistochemical staining			
MLH1	+	+	+
MSH2	+	+	+
p53	+	+	+
RB1	++	+	++
MDM4	+++	+++	+++

p53, +: >10% positive cells, -: <10%; RB and MDM4, -: no positive cells, +: <10%, ++: 10–50%, ++: >50%; wt, wild-type.

Immunohistochemistry. The mismatch repair proteins (MLH1 and MSH2) and tumor suppressor proteins (p53 and RB1) were subjected to immunohistochemical staining using an avidin-biotin complex protocol; the staining and calculation procedures for thin sections of CRC specimens were described in our previous study (11). The following primary antibodies were used for immunohistochemical staining: anti-mouse MLH1 monoclonal antibody (G168-15; GeneTex, Inc., Irvine, CA), anti-mouse MSH2 monoclonal antibody (25D12; GeneTex, Inc.), anti-mouse p53 monoclonal antibody (DO-7; Nichirei Biosciences, Inc., Tokyo, Japan), anti-mouse Rb1 monoclonal antibody (1F8; Thermo Fisher Scientific Inc., Fremont, CA) and anti-rabbit MDMX polyclonal antibody (NB110-40639; Novus Biologicals, LLC, Littleton, CO).

Fluorescent in situ hybridization. Fluorescent *in situ* hybridization (FISH) was performed on 4-μm sections of formalin-fixed, paraffin-embedded CRC specimens, as described previously (11) with minor modifications. Two bacterial artificial chromosome (BAC) clones containing the *MDM4* or the *MDM2* gene locus were obtained from the BAC Clone Collection (RP11-430C7 and RP11-611O2; Invitrogen, Carlsbad, CA). A CL1/CL2 Alu-PCR product was prepared from the BAC DNA and was used to generate a FISH probe by nick translation with digoxigenin (DIG)-11-dUTP or biotin-16-dUTP (Roche Diagnostics GmbH), as previously

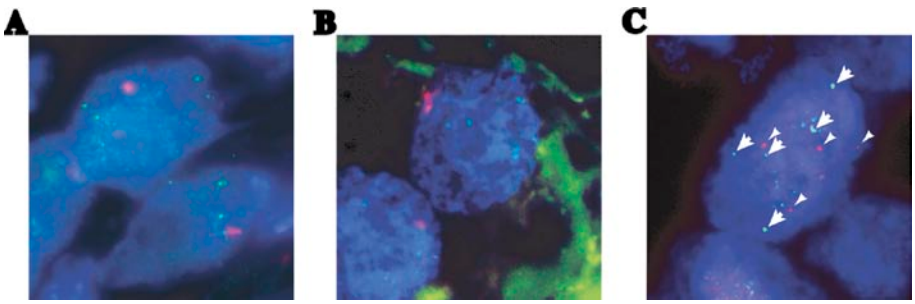

Figure 1. Representative images of the fluorescence *in situ* hybridization (FISH) analyses. (A and B) Dual-color FISH analysis of *MDM4* (green) and centromere 1 (red) in paraffin sections of the MCF7 cell line (A) and a CRC tissue specimen (B). (C) Double amplification of *MDM4* (green) and *MDM2* (red) occurred in the same tumor cell. Arrows and arrowheads indicate the location of *MDM4* and *MDM2* signals, respectively.

described (25). For the two-color FISH of the *MDM4* gene locus and the centromeric satellite DNA locus on chromosome 1, an *MDM4* probe and a centromere 1 (CEP® 1, Satellite II/III) DNA probe (Abbott Molecular, Inc., Des Plaines, IL) were applied to the sections. After denaturation and hybridization, the sections were incubated with fluorescein-conjugated anti-DIG Fab fragments (Roche Diagnostics GmbH) in 4% bovine serum albumin (BSA)/4X saline-sodium citrate (SSC). For the two-color FISH of the *MDM4* gene locus and the *MDM2* gene locus, a biotinylated *MDM4* probe and a digoxigeninylated *MDM2* probe were applied to the sections. After denaturation and hybridization, the signals were amplified by biotinylated anti-avidin D (Vector Laboratories, Inc., Burlingame, CA), and were detected by fluorescein avidin DN (Vector Laboratories, Inc.) and rhodamine-conjugated anti-DIG Fab fragments (Roche Diagnostics GmbH) in 4% BSA/4X SSC. The sections were counterstained with SlowFade® Gold Antifade Reagent with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen).

A tumor xenograft in the severe combined immunodeficiency (SCID) mouse of human breast cancer cell line MCF7, which is known to have *MDM4* amplification, was prepared, fixed in formalin, embedded in paraffin and used as a positive control for the FISH analysis.

Cell culture and transfection of small interfering RNA. The human embryonic kidney cell line 293T was maintained in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum. To create the MDM4 expression vector, full-length human *MDM4* cDNA was amplified by PCR with the following primers; the forward primer containing a *Bam*HI site and a Kozak consensus sequence, 5'-GAAGGATCCGCCACCATGACATCATTTTCCAC CTCTGC-3' and the reverse primer containing a *Bst*BI site, 5'-GGGTTCGAATGCTATAAAAACCTTAATAAC CAGC-3'. The PCR product was cut with *Bam*HI and *Bst*BI, and was ligated into the *Bam*HI/*Bst*BI sites of the pEF4/*Myc*-His A vector (Invitrogen). A small interfering RNA (siRNA) cocktail targeting human *MDM4* (siMDM4) was purchased from B-Bridge International, Inc. (Mountain View, CA; SHF27A-2066). The sequences for the three siRNA duplexes were: 5'-guggagaucuuuugggagaTT-3' (sense) and 5'-ucucccaaaagaucuccacTT-3' (antisense), 5'-gaagcaaag ugcagaggaaTT-3' (sense) and 5'-uuccucugcacuuugcuucTT-3' (antisense), 5'-gcaguuagguguuggaauaTT-3' (sense) and 5'-uauuccaacaccuaacugcTT-3' (antisense). A negative control cocktail was also purchased from B-Bridge International, Inc. (S6C-0126). siRNA duplexes and the MDM4 expression vector were co-transfected into 293T cells using Lipofectamine™ 2000 reagent (Invitrogen).

Western blot analysis. To detect proteins by Western blotting, the transfected cells and the tumor and adjacent normal tissue pairs were lysed in lysis buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1% NP40, 1 mM Na_3VO_4, 25 mM NaF supplemented with a protease inhibitor cocktail (Roche Diagnostics GmbH). Whole lysates of 10 μg protein were boiled in NuPAGE LDS Sample Buffer (Invitrogen), and were fractionated by 4-12% NuPAGE Novex Bis-Tris gel electrophoresis (Invitrogen) and subsequently transferred to polyvinylpyrrolidone membranes (GE Healthcare UK, Ltd., Little Chalfont, UK). After protein transfer, membranes were blocked for 1 h with Tris-buffered saline containing 5% nonfat dry milk or 5% BSA, 0.05% Tween-20. The following primary antibodies were used: anti-MDM4 monoclonal antibody (MDMX-82; Sigma-Aldrich, Inc., St. Louis, MO), anti-Mdmx/Hdmx p-Ser367 monoclonal antibody (#15; BioAcademia Inc., Ibaraki, Osaka, Japan), and anti-β-Actin monoclonal antibody (AC-15; Sigma-Aldrich, Inc.). Primary antibodies were detected with anti-mouse IgG horseradish peroxidase-conjugated antibody (GE Healthcare UK, Ltd.), using Amersham ECL Plus™ Western blotting detection reagents (GE Healthcare UK, Ltd.)

Results

Copy number assessment of MDM4 and MDM2 genes in CRC. The copy number of the *MDM4* gene was first evaluated by qPCR, and a calculated copy number of >4.0 was considered positive for gene amplification. Three cases out of the 211 CRC cases examined (1.4%) were considered to show *MDM4* amplification, with a copy number of 4.1, 4.1 and 5.3, respectively (Table I). To confirm this result, we further evaluated the specimens by FISH. The FISH signal copy numbers for *MDM4* of the three tumors were 2.4, 2.3 and 2.7, respectively, and the numbers were increased compared with that of centromere 1, on which chromosome *MDM4* is located (1.7, 1.8 and 1.9, respectively). We have previously demonstrated that the signal range of centromere 1 in these tumor cells is normal diploid (11); therefore, we considered these increases in copy number to reflect amplification of the *MDM4* locus (Fig. 1A and B).

Table II. Histological and clinical features of *MDM4*-amplified tumors from colorectal cancer patients.

	Case 1	Case 2	Case 3
Gender	Male	Female	Female
Age (years)	55	89	73
Primary tumor location	Upper rectum	Canalis analis	Lower rectum
Dukes' stage	B	C	C
Histopathological grade	G2	G1	G1
Invasion depth	pT4	pT2	pT4
Lymph node metastasis	N0	N2	N1
Lymphatic invasion	-	-	+
Venous invasion	+	-	+
Recurrence	-	-	-

+, positive; -, negative.

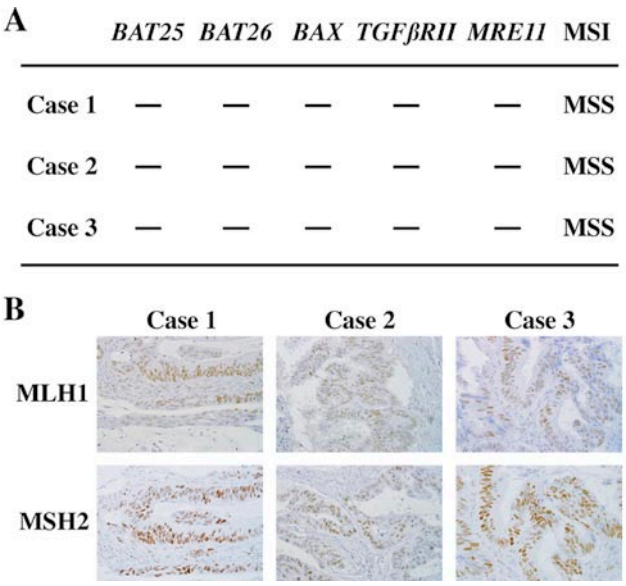

Figure 2. Analysis of microsatellite markers and DNA mismatch repair protein expression. (A) All three *MDM4*-amplified tumors (cases 1-3) showed stable microsatellites for five markers. (B) Immunohistochemical staining for MLH1 and MSH2 of the three *MDM4*-amplified tumors. These proteins were stably conserved in the tumors. MSI, microsatellite instability; MSS, microsatellite stable.

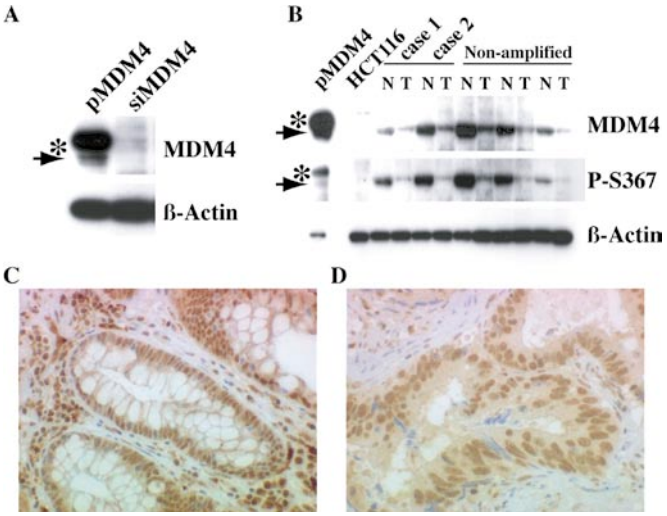

Figure 3. Protein expression analysis in *MDM4*-amplified tumor specimens. (A) By Western blotting, the MDM4 expression vector in 293T cells (pMDM4) showed two different bands at ~75 kDa. The larger form (asterisk) was more abundant than the smaller form (arrow). In addition, treatment with *MDM4* siRNA (siMDM4) significantly suppressed both bands. (B) Although the HCT116 cells showed weak expression to mainly the larger form of MDM4, only the smaller form (arrow) was detected in both tumor tissue and paired normal mucosa. The expression level of MDM4 was decreased in the tumor tissue regardless of the presence of gene amplification. The phospho-serine antibody of MDM4 did not show a different migration in these specimens from the MDM4 antibody. The image was generated using a membrane transferred from a single gel. T, tumor tissue; N, normal mucosa. (C and D) Immunohistochemistry for MDM4 in matched normal mucosa (C) and a tumor specimen (D).

Cases 1 and 2 of the *MDM4*-amplified cases corresponded to the *MDM2*-amplified cases reported in our previous study (11) (Table I). To investigate whether this double amplification occurs in the same tumor cells or in different cells, we performed two-color FISH for *MDM2* and *MDM4*. This clarified that the amplification of the two genes occurred in the same tumor cell (Fig. 1C).

Histopathological and clinical information for the *MDM4*-amplified tumors is provided in Table II. Because of the small number of cases, statistical analysis was not appropriate. All three tumors were located in the lower rectum or the canalis analis, and not in the colon. None of patients received postoperative chemotherapy, and they are all alive without any relapses after a follow-up period of up to 5 years.

Molecular characteristics of MDM4-amplified CRC. All three *MDM4*-amplified tumors contained mutated *TP53*, but *KRAS* mutation was detected only in case 1 (Table I). MSI was evaluated with the cognate five markers, and none of the three specimens showed instability (Fig. 2A). Since MSI is generally associated with defects of MMR proteins, we also

examined MLH1 and MSH2 protein expression by immunohistochemistry. The two MMR proteins were conserved in these tumors (Fig. 2B).

Evaluation of MDM4 protein expression. To clarify whether the expression of MDM4 protein was increased in parallel with the *MDM4* gene amplification in the tumor tissues, we analyzed MDM4 expression by Western blotting and immunohistochemistry. Because of studies of multiple bands of different molecular sizes on Western blotting for MDM4 (26,27), we first evaluated 293T cells after transfection of a human MDM4 expression vector. The anti-MDM4 antibody detected two different MDM4 bands: a larger, abundant form migrating at ~75 kDa and a slightly smaller, less abundant form (Fig. 3A, lane 1). These two bands were both derived from MDM4, because they were barely detectable when siMDM4 was simultaneously transfected (Fig. 3A). The HCT116 cells showed weak expression of MDM4, and mainly detected the larger form. The protein expression of MDM4 was then evaluated in tumor tissue and paired noncancerous mucosa in *MDM4*-amplified cases 1 and 2 (case 3 was not available for this assay) and three *MDM4* non-amplified cases. Unexpectedly, MDM4 protein was less abundant in tumor tissue than in the paired normal mucosa even in *MDM4*-amplified cases (Fig. 3B). Furthermore, the MDM4 detected in tissues corresponded to the smaller, minor form, which was much less abundant in 293T cells (Fig. 3B). Western blotting with anti-MDM4 antibody, specific to phospho-Ser-367, gave a similar result to that using the nonspecific MDM4 antibody; thus, phosphorylation of the serine residue was not responsible for the difference in migration (Fig. 3B). Immunohistochemical staining of tumor tissue and paired normal mucosa clearly identified MDM4 proteins in the nuclei of both cancer cells and normal mucosal epithelial cells. The nuclei of cells in the stroma, mainly infiltrating blood-derived cells, were also positive for MDM4 (Fig. 3C and D).

Discussion

In the present study, we investigated *MDM4* gene amplification in CRC for the first time, and examined the correlation with *TP53* gene mutation status and clinicopathological features of the tumors. The incidence of amplification was unexpectedly quite infrequent at 1.4% (3 out of 211 CRC cases). Furthermore, all three *MDM4*-amplified tumors also harbored *TP53*-inactivating mutations; this does not comply with the mutual exclusivity demonstrated for retinoblastomas and other tumor types (19,28). Two of the three *MDM4*-amplified tumors also contained *MDM2* amplification. The mode of amplification was different; that is, *MDM4* amplification was caused by regional copy number gain of chromosome 1, but *MDM2* amplification was associated with gain of the whole chromosome 12. Both *MDM4*-specific amplification and co-amplification with *MDM2* have been reported in astrocytic gliomas (29). We speculate that MDM4 may play a role in carcinogenesis, at least in CRC, which is not limited to negative regulation of p53 in combination with MDM2.

We demonstrated that the MDM4 protein levels in the gene-amplified tumors were not elevated in comparison with the non-amplified cases. In fact, the protein level was decreased in tumor tissues compared with the adjacent normal mucosal tissues, independent of the gene amplification. Phosphorylation of MDM4 through CHK2 and 14-3-3 has been reported to be partly responsible for degradation of the protein through an MDM2-dependent pathway (30,31). However, the level of phosphorylated MDM4 was no different from the total MDM4 protein level, and the reasons for the decrease in MDM4 in the tumor tissues, even in the gene-amplified cases, are unclear. We may have to consider the content of MDM4-positive non-epithelial normal cells such as infiltrating cells in the stroma. On the contrary, an immunohistochemical analysis of MDM4 in CRC has been reported previously, which demonstrated that MDM4 expression was increased in aggressive tumors and that it was independent of the *TP53* mutation status (32). The roles of MDM4 in CRC tumorigenesis and progression remain to be elucidated.

In this study, we also characterized the pathological and molecular biological features of the tumors with *MDM4* gene amplification. Chromosomal instability (CIN) and MSI are recognized as major mechanisms of colorectal carcinogenesis, and some cases of CRC display both (4). CIN is the presence of multiple structural or numerical chromosome changes in tumor cells, and patients with CIN-positive tumors have a worse prognosis than those with CIN-negative tumors. In the present study, all *MDM4*-amplified tumors showed stable microsatellites and chromosomal instability. These tumors were located in the rectum or canalis analis, which is consistent with previous studies demonstrating that the incidence of MSI in distal colonic and rectal tumors is lower than that in proximal tumors (33). It has also been reported that MDM4 expression is induced in CRC by activated KRAS and insulin-like growth factor 1 (IGF1) and that the expression is activated through extracellular signal-related kinase (ERK) phosphorylation (32). Although wild-type *KRAS* was detected in two of the three *MDM4*-amplified tumors (cases 2 and 3), the level of MDM4 protein was only able to be assessed in one of these. Therefore, it will be necessary to analyze more specimens to clarify the relationship between activated KRAS and *MDM4* gene amplification in tumors.

In conclusion, we demonstrated in the present study that *MDM4* gene amplification is a rare event in CRC, at least among the Japanese. Gene amplification and the *TP53* mutation status were not mutually exclusive, and two of the three *MDM4*-amplified cases also demonstrated *MDM2* amplification. Paradoxically, we found that MDM4 protein levels were decreased in the gene-amplified cases. The functional significance of MDM4 is still unclear and further studies are needed.

Acknowledgements

This study was supported by grants from the Kanagawa Cancer Research Fund. The authors thank Ms. Yukiko Yamazaki, Ms. Kumiko Ohrui and Ms. Akiko Sekiyama for their technical assistance.

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