MDM2 Gene Amplification Is Correlated to Tumor Progression but not to the Presence of SNP309 or *TP53* Mutational Status in Primary Colorectal Cancers

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Abstract

Mdm2 is the main regulator of p53 and is amplified in ∼7% of all human cancers. *MDM2* gene amplification as well as expression has been correlated to an increased tumorigenic potential. We have analyzed the prevalence of *MDM2* gene amplifications and SNP309 in 284 colorectal tumors using a relatively new highly sensitive PCR/ligase detection reaction method in relation to *TP53* mutational status and genomic instability. We found *MDM2* to be amplified in 9% of the 284 colorectal cancers analyzed and a significantly higher proportion of tumors with high *MDM2* gene amplification retained a wild-type p53 gene (*P* = 0.058). *MDM2* gene amplification was significantly correlated to advanced tumor stage. Several small-molecule MDM2 antagonists have already been identified that either physically inhibit the p53-MDM2 binding or the E3 ligase function of MDM2. Our results suggest that MDM2 is a promising target for this type of cancer therapy in a substantial subgroup of colorectal cancers. (Mol Cancer Res 2008;6(2):205–11)

Introduction

The vast majority of cancers have a deficient p53 pathway. It is estimated that half of all human cancers have a mutation in p53 and in colorectal cancer mutations have been reported in >40% (IARC TP53 Mutation Database, version R11. Accessed October 15, 2006; ref. 1).[7] In response to cellular stress such as DNA damage, telomere shortening, nucleotide depletion, onco-

gene expression, hypoxia, etc., p53 is stabilized and activated. Through its activity as a transcription factor, expression of p53-responsive genes results in numerous biological outcomes such as transient and reversible growth arrest, apoptosis, or senescence as well as differentiation. The p53 tumor suppressor protein is expressed in normal tissues at extremely low levels and has a rapid turnover. Several ubiquitin ligases have been identified to target p53 for degradation through a proteasome-mediated mechanism, and MDM2 (mouse double minute 2) is thought to be the main protein. A fine balance between p53 and MDM2 expression is thought to be important for proper function. This is exemplified by the fact that p53 knockout mice develop multiple cancers at an early age (2) and *MDM2* knockout leads to lethality during embryogenesis, which is probably explained by excessive amounts of activated p53. The lethal phenotype of *MDM2* knockout mice is completely overcome by concomitant inactivation of the p53 gene (3). Regulation of MDM2 expression can be modified by several different mechanisms such as gene amplification (4), the presence of functional single nucleotide polymorphisms (SNP; ref. 5), stabilization by an aberrantly spliced form of HMDX (6), and augmented translation as well as increased expression by activated p53 (7). In this study, we have analyzed two of these factors, the presence of *MDM2* gene amplification and SNP309 in colorectal cancers. The T > G polymorphism in the promoter region of *MDM2* has been shown to increase the expression of MDM2 transcripts and protein and to dramatically increase both cancer incidence and to result in earlier age at onset in Li-Fraumeni population (5).

MDM2 is located on chromosome 12q14-15, which is frequently amplified in many cancers (4, 8), suggesting this as a mechanism for increased expression of the protein. Amplification and overexpression of the *MDM2* gene has been seen to increase the tumorigenic potential of murine cells (9-11). The creation of transgenic mice containing increased copies of the *MDM2* gene and concomitantly displaying an increase in expression level had a 100% incidence of early tumor development (11). Human tumors and cell lines analyzed from a variety

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7http://www.p53.iarc.fr/index.html

of tissue origins show a correlation between *MDM2* gene amplification and increased expression of MDM2, and data from small studies in sarcomas suggests that *MDM2* gene amplification and p53 mutation in general are exclusive events (12, 13). Conversely, overexpression of MDM2 does not always correlate to gene amplification (12, 14). In melanoma, overexpression of MDM2 has been shown in cancers in the absence of gene amplification (15). By the use of a relatively new quantitative method using PCR followed by ligase detection reaction (PCR/ LDR), we have been able to detect small gene copy number changes at high sensitivity and reproducibility (16-18).

Overexpression of MDM2 might be a significant factor in inhibiting the p53 pathway in a subset of cancers and could therefore be a promising therapeutic target in these tumors. By screening chemical libraries for substances that bind and inhibit MDM2 function, several small-molecule MDM2 antagonists have been identified that either physically inhibit the p53-MDM2 binding or the E3 ligase function of MDM2 (19-21). Antagonist Nutlin-3 as well as RITA has been shown to be efficient in activating a p53-dependent stress response both *in vivo* and *in vitro* and to suppress tumor growth in mice with no obvious toxicity (19, 20, 22).

These results suggest that MDM2 antagonists may offer promising agents for cancer therapy in colorectal cancer, especially in a subset of patients with tumors containing wild-type p53 and *MDM2* gene amplification. In this study, we show that there might be a substantial subgroup of patients with primary colorectal cancer that falls into this category and might benefit from MDM2-targeted treatment.

Results

MDM2 *Gene Amplification versus Clinical and Molecular Variables*

The cutoff was set based on both frequency distribution and model-based calculation of the PCR/LDR–derived raw values. Gene amplification results >4-fold were regarded as true amplification (Fig. 1). Two hundred and eighty-four primary colorectal tumors were analyzed for *MDM2* gene amplification. Nine percent (26 of 284) of the primary colon tumors analyzed showed gene amplification. Among the clinical variables, *MDM2* gene amplification showed a statistically significant correlation with overall stage and M stage ($P = 0.011$ and 0.019, respectively), but not with age, gender, location, and T or N stage. Among the molecular variables, *MDM2* gene amplification was not correlated with SNP309 or microsatellite instability (MSI). Tumor samples with high *MDM2* gene amplification (>8-fold) were correlated to the presence of mutated *TP53*. Details are summarized in Table 1. The same conclusions were made when analyzing the correlation of *MDM2* gene amplification to the clinical and molecular variables in Table 1 using multivariate logistic regression. Only overall stage and M stage were statistically significantly correlated to *MDM2* gene amplification with a P value of 0.011 and 0.017, respectively, when adjusting for age, gender, location, SNP309, MSI, and *TP53*.

Fluorescent In situ Hybridization

MDM2 gene amplification detected by PCR/LDR was confirmed by fluorescent *in situ* hybridization (FISH) analysis.

The positive cell line control for *MDM2* gene amplification showed strong amplification by FISH (Fig. 2). In general, FISH showed a good correlation with PCR/LDR results. All samples defined as not amplified showed normal staining and samples with high staining (>10-fold amplification) showed a strong signal for *MDM2*. The samples with *MDM2* gene amplification determined to be between 4- and 10-fold had a mixture of cell populations with very heterogeneous staining. In general, 20 to 30 cells were summarized for each sample, and in these samples, some cells showed dense staining for *MDM2* whereas some cells were normal.

Discussion

In this study, we have found *MDM2* to be amplified in 9% of 284 colorectal cancers analyzed. In the initial study of *MDM2* gene amplification in human cancers by Oliner et al., almost one-third of the sarcomas contained an amplified *MDM2* gene, whereas no amplification in any of the 74 carcinomas (colorectal or gastric) analyzed was detected (4). In contrast to this, we have shown that a substantial proportion of colorectal cancers harbor gene amplification of *MDM2*.

Most amplification analyses of a single gene have previously been done by Southern blotting, FISH, or real-time PCR. We believe that we are able to detect gene amplification by the use of a relatively new method, LDR, at very high sensitivity and reproducibility. The DNA used as a template in the present gene copy analyses was quantified by picogreen instead of commonly used UV absorbance. This is important to emphasize because picogreen specifically measures double-stranded nucleic acids compared with UV absorbance (at 260 nm) in which the signal is significantly influenced by contaminating single-stranded nucleotides and proteins. Therefore, the choice of picogreen as a DNA concentration determination method should add considerably to the correctness of the measurements, which obviously is very important when performing quantitative analyses.

MDM2 gene amplification detected by PCR/LDR was confirmed by FISH in both the osteosarcoma cell line SJSA-1, which is known to have an increased gene copy number of *MDM2*, and in tumor tissue positive for *MDM2* gene amplification. Interestingly, the FISH staining was homogeneous for tumors with no or high amplification, whereas tumors with *MDM2* gene amplification between 4- and 10-fold showed a heterogeneous cell population. Tumor cells might need to reach a certain level of *MDM2* gene copy number before they are selected for, and at that level, they will be present homogenously in the tumor. In human tumors, amplicons often consist of double minutes which can be initiated by DNA double-strand breaks (23, 24). P53 is activated by DNA double-strand breaks and has been shown to inhibit the establishment of gene amplifications and deletions (25). In line with this, *TP53* mutations have been correlated with genome-wide instability in tumors of colorectal origin (26). Genome-wide chromosomal instability with low to medium-high gene amplifications caused by nonfunctional p53, as compared with tumors with high copy gene amplification of *MDM2* and wild-type p53, might develop through different mechanisms (27). The fact that *MDM2* gene amplification in general is not correlated to MSI in this set of tumors ($P = 0.328$), and that only high *MDM2* gene
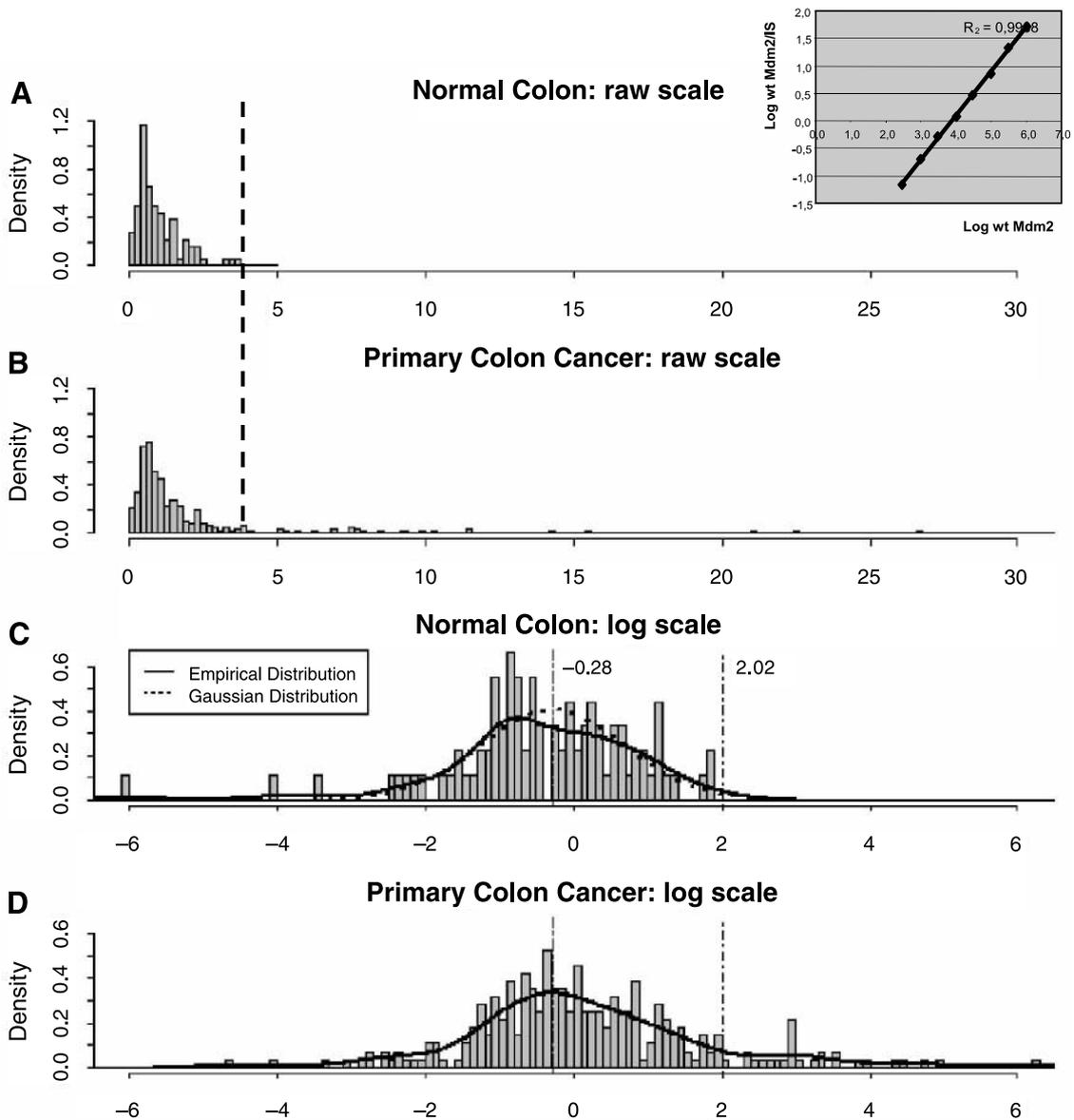
FIGURE 1. Sample of a standard curve (*top right corner*). For every PCR/LDR set, eight samples were devoted to construct a standard curve. The standard curve was generated by mixing a fixed amount of internal standard (1×10^4 copies) to a wide range of wild-type plasmids (3×10^2 to 1×10^6 copies). The LDR peak area ratio of wild-type to internal standard was plotted on the *Y*-axis against wild-type copy number on the *X*-axis. The equation derived from regression of the slope was used to calculate the amount of wild-type *MDM2* copies in samples. The cutoff was set based on both frequency distribution (**A** and **B**) and model-based calculation of the raw values (**C** and **D**). Measurements of normal samples were used to fit a Gaussian distribution (**C**), from which the 99th percentile was estimated. The estimated 99th percentile was then used as a cutoff for *MDM2* amplification and was applied to the measurements for tumor samples. Tumor sample results were normalized against the pooled mean of 90 normal tissue DNA samples. Distribution of normalized samples resulted in a cutoff value of 4 in both normal samples and primary colon samples (**A** and **B**). Based on this, gene amplification results >4-fold were regarded as true amplification.

amplification is correlated to wild-type p53, could indicate that these tumors might represent chromosomal instability caused by different mechanism(s). The heterogenicity of tumors with lower levels of *MDM2* gene amplification (4- to 10-fold), shown by FISH, can explain why there is no correlation between *MDM2* gene amplification, SNP309, p53 mutations, and MSI in this subgroup. It is difficult to predict the cause of genomic instability in these tumors because the correlations may be confounded by the mix of cells containing different types of alterations in different genes.

Because of the ability of MDM2 to bind to and inactivate p53, it has been thought that mutation of *TP53* and amplification of *MDM2* are two different ways to inactivate the p53 stress response pathway (4, 13). In general, when analyzing all tumors included in the study, we found a rate of *MDM2* gene amplification in tumors harboring *TP53* mutations similar to that in tumors with wild-type p53 gene (8% versus 14%, respectively, $P = 0.440$), but when specifically analyzing tumors containing high *MDM2* gene amplification (>8-fold), a markedly higher proportion of tumors retained a wild-type p53

Table 1. Detailed Summary of Clinical and Molecular Correlates in the 284 Samples Analyzed for *Mdm2* Gene Amplification

	Mdm2+ (*n* = 26)	Mdm2− (*n* = 258)	P*
Clinical correlates			
Age (y)			
Median	62.5	67	*P* = 0.128
Range	31-78	17-87	
Gender			
Female	10	131	*P* = 0.419
Male	16	127	
Total	26	258	
Location			
Right	10	96	*P* = 0.560
Left	8	97	
Rectum	8	65	
Total	26	258	
Stage			
I	0	42	**P = 0.011**
II	3	60	
III	9	73	
IV	14	83	
Total	26	258	
T Stage			
I	0	9	*P* = 0.279
II	2	54	
III	21	171	
IV	3	24	
Total	26	258	
N Stage			
0	8	127	*P* = 0.159
1 + 2	17	131	
Total	25	258	
M Stage			
0	12	175	**P = 0.019**
1	14	83	
Total	26	258	
Molecular correlates			
SNP309			
T/T	9	93	*P* = 0.707
T/G	14	123	
G/G	3	42	
Total	26	258	
TP53			
Wild-type	13	79	*P* = 0.440
Mutant	7	82	
Total	20	161	
MSI			
MSI	1	29	*P* = 0.328
MSS	23	203	
Total	24	232	

*P < 0.05 are considered statistically significant and are shown in boldface.

gene ($P = 0.058$). It might be that MDM2 affects p53 in a dose-dependent way, and only tumors with higher copy number of the *MDM2* gene express enough protein to inhibit p53. The increase in rate of tumorigenesis seen in *MDM2*-transgenic mice homozygous for the transgene insertion relative to hemizygous *MDM2*-transgenic mice suggests that the rate of tumorigenesis correlates with the level of *MDM2* gene copies and expression (11).

Still, concordant presence of *MDM2* gene amplification in tumors with mutant p53 indicates an independent tumorigenic role for MDM2 in these tumors. The fact that >40 different splice variants of MDM2 have been isolated from both tumor and normal tissues, may in part explain the potential p53-independent functions of the MDM2 protein (28). Sigalas et al. observed a higher frequency of splice variants in late-stage and high-grade ovarian and bladder carcinomas, as well as the

transforming ability of these variants in NIH3T3 cells (29). Marked overexpression of a lower molecular weight form of MDM2 was also observed in melanoma with high levels of *MDM2* amplification (30), which likely represents one of many known alternatively spliced forms of MDM2. We currently don't know if the tumors that we have analyzed with amplified *MDM2* express wild-type MDM2 or a splice variant thereof. An increase in the expression of either wild-type or splice variant would probably increase the tumorigenic potential in the tumor. Another possibility would be that splice variants are expressed in tumors with no amplification or low amplification (<8-fold). This should be interesting to clarify further in future studies.

In contrast to a study of soft tissue sarcoma (31), we found that the frequency of *MDM2* gene amplification increased with the overall tumor stage ($P = 0.01$). This finding suggests that amplification of the *MDM2* gene is involved in colorectal cancer progression and is probably occurring late rather than early in the tumor evolvement.

Because both the presence of SNP309 and *MDM2* gene amplification are generally expected to increase expression, we hypothesized that these two mechanisms of obtaining MDM2 overexpression would represent two independent ways of increasing the tumorigenic potential of MDM2. In our study, we found a prevalence of the G/G genotype in 16% of the analyzed samples, which is slightly higher than what has been previously seen in normal populations (5, 32). The presence of SNP309 was not correlated to *MDM2* gene amplification or clinical stage in our samples. In colorectal cancer, SNP309 has been associated with an earlier onset of tumors in subsets of patients with wild-type p53, nondominant *TP53* mutations, or women with high estrogen expression (32-34). We could not discern any correlation between SNP309 and p53 status in *MDM2*-amplified cases.

Our findings suggest that MDM2 is involved in colorectal tumor progression, and that the tumorigenic potential of MDM2 is independent of SNP309. MDM2 is correlated to wild-type *TP53* when *MDM2* gene amplification is >8-fold. The fact that *MDM2* is amplified in 9% of colorectal cancers, and is associated with tumor progression, points to MDM2 as a possible target for therapy in these patients. Currently, intensive research is focused on small molecules or peptides that can restore the p53 pathway in cancer cells, either by activating the wild-type function of p53 or inhibiting MDM2 binding to p53 (35, 36). Tumors with high *MDM2* gene amplification have been seen to respond well to MDM2 antagonists, but even tumors with no *MDM2* gene amplification and wild-type p53 may benefit from this treatment (22). Better diagnostic tools to determine the genetic profile in patients, in combination with new target-specific drugs, are needed to optimize the treatment for every single patient. The presence of *MDM2* gene amplifications is one such potential target that, in the future, patients with colorectal cancer should be screened for.

Materials and Methods

Patients and Tumor Samples

Tissue was collected at the time of surgery for colorectal cancer under Institutional Review Board Protocol at Memorial

Sloan-Kettering Cancer Center between 1991 and 2004. Normal and malignant tumors were frozen in optimal cutting temperature compound and stored at −70°C. Colorectal cancers were microdissected using a H&E-stained template prior to DNA extraction (Qiagen, Inc.) or RNA extraction (Invitrogen Corporation). Frozen tumor samples from 284 patients diagnosed with primary colorectal cancer (42 stage I, 63 stage II, 82 stage III, and 97 stage IV) were analyzed for gene amplification of the proto-oncogene MDM2 and the concordant presence of SNP309. In addition to this, a subset of samples was analyzed for *TP53* mutations and MSI. The patient cohort consisted of 141 females and 143 males with a median age of 66 (range, 17-87).

PCR for MDM2 *Gene Amplification Analyses*

PCR primers were selected in intron 1 and exon 2 to specifically amplify a 198-bp genomic fragment of the *MDM2* gene. The sequences of the primers were as follows: forward, 5′-cgt agt ctg ggc ggg att g 3′; reversed, 5′ tcc gtg ccc aca ggt cta cc 3′. Fifty microliters of the PCR mixture contained 40 ng of tumor DNA, 1×10^4 copies of internal standard, 200 mmol/L of Tris-HCl (pH 8.4), 500 mmol/L of KCl, 2.5 mmol/L of MgCl$_2$, 0.2 mmol/L of each flanking primer, 2.5 units of *Thermus aquaticus* YT1 (Taq) DNA polymerase (Invitrogen), 250 mmol/L of each deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), and sterile water up to a final volume of 50 μL. The cycle conditions were after an initial denaturation step at 95°C for 5 min; 35 cycles of 95°C for 30 s, 63°C for 30 s and 72°C for 1 min followed by a final extension at 72°C for 7 min. Thereafter, samples were chilled to 4°C. PCR products were run on a 2% agarose gel to confirm the proper length of the product. After PCR, samples were digested with 50 mg of proteinase K (65°C for 15 min and 95°C for 10 min) in order to inactivate Taq polymerase. All DNA used for quantitative analyses in this study was quantified by picogreen measurements (Molecular Probes/Invitrogen).

Synthesis of Mutant Internal Standards

Site-directed mutagenesis was used to introduce a 2 bp substitution to the PCR fragment. The mutagenesis primer used

for this purpose was: 5′-tcc gtg ccc aca ggt cta ccc tcc aat cgc cac tga aca ca**c g**tg gga aaa tgc-3′, which resulted in a mutated PCR fragment with the substitution of a gc for a cg at positions 156 and 157 of the PCR product. This fragment was subsequently used as an internal standard.

Cloning of Wild-type MDM2 Fragment and Internal Standard

Purified PCR fragments were subcloned into a pCR 2.1-TOPO vector and transformed into chemically competent TOP10F′ *Escherichia coli* following the protocol recommended by the company (TOPO TA Cloning kit, Invitrogen). Isolated plasmids were checked for desired inserts both by restriction digest and by sequencing (ABI PRISM 3100 Genetic Analyzer; Applied Biosystems). Plasmid DNA concentrations were determined by picogreen before being diluted in TE and stored at −80°C.

LDR for MDM2 *Gene Amplification Determination*

PCR followed by LDR was originally developed to identify single-base substitutions in genomic DNA (16, 37). The LDR conditions were modified to enable precise detection and quantification of the *MDM2* gene. For each LDR reaction, three primers were used: two upstream fluorescence-labeled primers of which one was specific for the native DNA fragment and one was specific for the mutant fragment (internal standard), plus one common downstream primer. Primer sequences were as follows: wild-type–specific primer, FAM-5′-aaccatgc attttcc-cagc-3′; mutant-specific primer, FAM-5′-**aaat**aaccatgc attttcc-ca**cg**-3′; and common primer, phos-5′-tgtgttcag tggcgattggagg-3′/3AmM/. The resulting LDR products were consequently 41 bp for the wild-type fragment and 45 bp for the mutant fragment. The ligation reaction was carried out in a 25 μL reaction volume consisting of 1 μL (40 units/μL) of Taq DNA Ligase (New England BioLabs), 500 fmol of each discriminating primers and 1,500 fmol of the common primer, plus 2 μL of PCR product in 1× NEBuffer for Taq DNA ligase. The reaction mixtures were incubated at 94°C for 1.5 min, then cycled 10 times at 94°C for 1 min and 65°C for 4 min, whereafter the
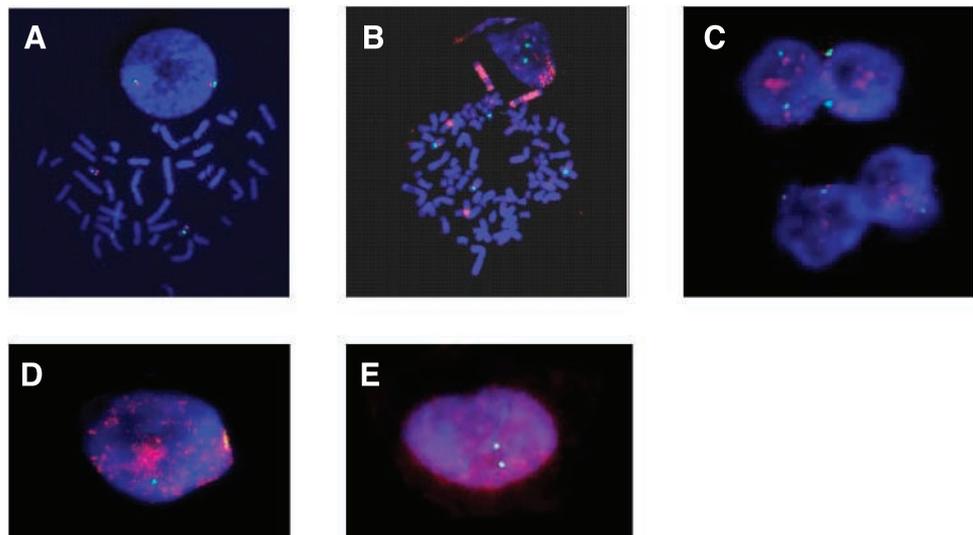

FIGURE 2. FISH analyses in (**A**) normal lymphocytes, (**B**) SJSA-1 16× fold *MDM2*-amplified, (**C**) 203T 21× fold *MDM2* amplification, (**D**) 140T 27× fold *MDM2* amplification, (**E**) 167T 32× fold Mdm2 amplification. Green, chromosome 12–specific centromere probe (CEP-12). Red, *MDM2*-specific probe.

ligation products were put on hold at 4°C until analyzed further. Wild-type and mutant ligation products were separated by capillary electrophoresis on a Genetic Analyzer (ABI PRISM Genetic Analyzer 3100, Applied Biosystems). GeneScan 500 LIZ Size Standard (Applied Biosystems) was used as a molecular weight marker. Fluorescently labeled products were detected and analyzed using GeneScan Analysis Software (version 6.7.1) and ABI PRISM Genotyper Software (version 3.7) All LDR analyzes were repeated at least once on a new PCR reaction.

Positive Control

Genomic DNA was extracted from the human osteosarcoma cell line SJSA-1 (CRL-2098, former OsA-CL from the American Type Culture Collection), which is known to contain gene amplifications of *MDM2* (4, 12). For every new set of PCR/LDR reactions, SJSA-1 was included as a positive control for *MDM2* gene amplification.

TP53 Mutation Analysis

TP53 mutational status was successfully determined in 181 of the primary colon cancers using a combination of multiplex PCR/LDR with Universal DNA microarray analysis and endonuclease V/ligase mutation scanning plus direct sequencing of exons 5 to 8 (38).

MDM2 SNP Analysis

The genotype of SNP309 located in intron 1 of the *MDM2* gene was determined by PCR amplification and subsequent LDR analysis. Primers flanking the SNP309 were used to PCR amplify a 117-bp-long fragment containing the polymorphism. PCR primers used were: 5′-CGG GAG TTC AGG GTA AAG GTC AC-3′ (forward primer) and 5′-CAG ACT ACG CGC AGC GTT CAC-3′ (reverse primer). Fifty nanograms of DNA was amplified as described for *MDM2* gene amplification analyses, with the exception of 2.5 mmol/L of $MgCl_2$ and an annealing temperature of 59°C. After PCR, samples were digested with 50 mg of proteinase K (65°C for 15 min and 95°C for 10 min) before being analyzed further by LDR. The LDR primers used were 5′ Fam-TAA AAA GGA CCT CCC GCG CCG C 3′ for the detection of the G/G variant allele and 5′ Fam-AA GGA CCT CCC GCG CCG A 3′ for the detection of the wild-type SNP309 allele (T/T). The sequence of the common probe used was 5′ phos-AGC GGC CCC GCA GCC CTA AAA TAA AA-3′/3AmM/. Ligation products with the length of 44 bp for the wild-type (T/T) allele and 48 bp for the polymorphic allele (G/G) were separated on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems; Fig. 3).

FISH

PCR/LDR results were validated by FISH analysis. Metaphases from the osteosarcoma cell line with known *MDM2* gene amplification, and touch preparations from 18 frozen tissue samples with various levels of *MDM2* gene copy numbers were analyzed. Human bacterial artificial chromosome clone RP11-1137N1, containing MDM2, was used as a template for the *MDM2* gene–specific FISH probe.
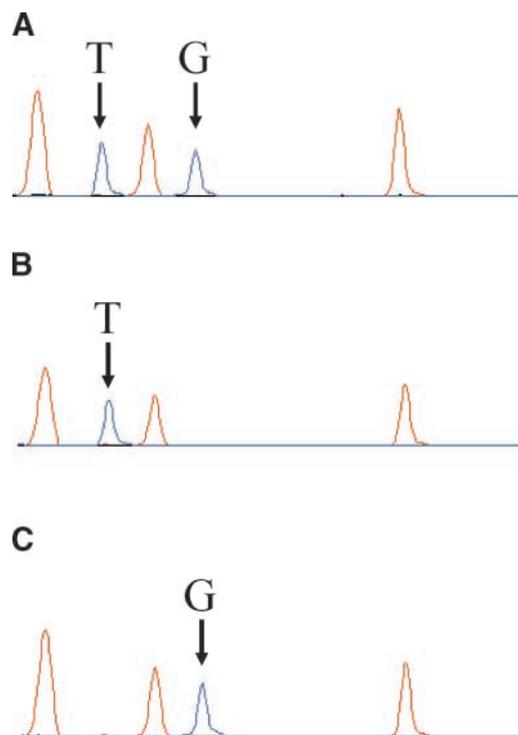

FIGURE 3. Three samples of (**A**) T/G, (**B**) T/T, and (**C**) G/G genotypes for the SNP309 location. Red peaks, size markers.

MSI

MSI was determined, as described previously (39), by a multiplex assay analyzing three of the markers included in the five-marker panel recommended by the National Cancer Institute workshop (40). The markers included were two mononucleotide markers (BAT25 and BAT26) and one dinucleotide marker (D2S123).

Statistical Methods

The association of *MDM2* gene amplification to clinical and molecular variables was examined using Fisher's exact test for categorical variables and Student's *t* test for continuous variables. Multivariate logistic regression, with *Mdm2* gene amplification as the dependent variable, was used to analyze its level of association with stage (or M stage) while adjusting for other clinical and molecular variables, in order to examine the possible presence of confounding factors.

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