Mdm2 Gene Amplification in Gastric Cancer Correlation with Expression of Mdm2 Protein and *p53* Alterations

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***Mdm2*, localized on chromosome 12, is considered a negative regulator of *p53* function and seems to play a role in the pathogenesis of a variety of tumors. The *mdm2* amplification in advanced-stage gastric carcinoma has not yet been investigated.**

***Mdm2* amplification was determined in 43 gastric carcinomas, and the genetic results were correlated with mdm2 protein expression, *p53* alterations, and clinicopathologic data. The tumors were classified according to Lauren: 20 intestinal-type tumors, 19 tumors of diffuse growth inclusive of a primary small cell carcinoma, and 4 carcinomas with mixed differentiation. Staging was based on the pTNM classification system. *Mdm2* and *p53* were demonstrated by immunohistology on formalin-fixed and paraffin-embedded tumor tissue. The *mdm2* oncogene was amplified by nonradioactive hybridization of tumor DNA with an *mdm2* cDNA probe. The Southern blots were evaluated densitometrically. For *p53* mutation screening, we analyzed the highly conservative regions of the *p53* gene (exons 4 to 8) with the use of the polymerase chain reaction–single-strand conformation polymorphism technique. Polymerase chain reaction products with band shifting were directly sequenced.**

***Mdm2* amplification was demonstrated in 18 tumors (41.8%). The *mdm2* gene was amplified more frequently in carcinomas with a diffuse growth pattern. Gastric carcinomas of the intestinal type, however, showed a higher frequency of *p53* alterations. There was no statistical significance of the molecular genetic and immunohistologic results of the *mdm2*/*p53* status to staging as well as to age and sex of the patients.**

The *mdm2*/*p53* pathway is a part of the carcinogenesis of gastric carcinoma. Only approximately 20% of gastric carcinomas failed to show *mdm2* and/or *p53* alterations. The upregulation of the *mdm2* oncogene and the accompanying inactivation of the tumor suppressor gene 53 seem to play a role above all in carcinomas of the diffuse type.

KEY WORDS: Gastric cancer, *mdm2*, *p53*.

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The *mdm2* gene has been mapped to chromosome 12q13–14 and encodes a 90 kDa cellular oncoprotein. The gene structure on the human chromosome was identified in 1992 (1). Like viral proteins, mdm2 protein is also capable of binding to *p53* (2–4). The complex that develops in this process inactivates the tumor suppressive function of p53 and prevents p53 from intervening into the cell cycle. The functional effect of inactivation through the formation of a p53-mdm2-complex thus corresponds to a complex formation between wild-type p53 and mutated p53 (5). Moreover, mdm2 is capable of forming complexes with both wild-type p53 and mutated p53. The interaction and relative ratios of p53 and mdm2 protein seem to play an important role in regulating cell division (3). Simultaneously, mdm2 inhibits the p53 function by binding to its transcriptional activation domain (6).

Furthermore, it was shown that *p53* activates the expression of the *mdm2* gene in an autoregulatory feedback loop (7, 8). Otherwise, the overexpression of *mdm2* can reduce the amount of endogenous *p53* through enhanced proteasome-dependent degradation (9). Recent research has suggested that *mdm2* also promotes the rapid degradation of *p53* under conditions in which *p53* is otherwise stabilized (10).

At the level of amplification or protein overexpression, mdm2 plays a role in a variety of tumors such as sarcomas (11, 12) and epithelial neoplasms, such as breast and lung tumors (13–15). In the early gastric carcinogenesis, however, mdm2 protein

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overexpression seems to play no role (16). To the best of our knowledge, this study is the first to investigate mdm2 expression and amplification in advanced-stage gastric cancer.

MATERIALS AND METHODS

Tissue

We investigated a series of 43 gastric carcinomas received in the Department of General Surgery, Magdeburg University, between 1995 and 1996. The resected stomachs were sent to the Department of Pathology for rapid section.

In addition to routine preparation, further representative tumor material was prepared, snap-frozen in liquid nitrogen, and stored at −70° C. All tumors showed a deep invasion of the gastric wall and were classified as advanced-stage cancers according to the pTNM system (pT 2–4). The number, origin, and involvement of the regional lymph nodes excised were also determined according to the International Union Against Cancer TNM system (17). Clinical data on the presence of metastases were included in this classification. For the histologic tumor typing, Lauren's classification system was used (18). There were 18 carcinomas of the diffuse type (mean age of patients, 60.9; range, 33 to 76; sex: f/m, 9/9), 20 intestinal cases (mean age of patients, 66.2; range, 34 to 83; sex: f/m, 5/16), and four mixed-type tumors (mean age of patients, 67.5; range, 64 to 71; sex: f/m, 2/2). One tumor was classified as a primary small cell carcinoma of the stomach.

Immunohistology

Mdm2 and p53 protein

Immunohistochemical analyses of p53 and mdm2 protein were performed on formalin-fixed, paraffin-embedded material. For the reactions, standardized and automated immunohistochemistry were applied (Nexes; Ventana Medical Systems, Tucson, AZ). Sections (4 μm thick) were deparaffinized in a series of graded alcohols and microwaved in EDTA buffer for 2 × 10 min at 450 W.

After the sections were cooled to room temperature and rinsed with Tris buffer, incubation was performed with anti-p53 (Do-1; Calbiochem, Cambridge, MA) or anti-mdm2 (SMP-14; DAKO, Hamburg, Germany) in an immunostainer. Before incubation of the primary antibodies, endogenous peroxidase was blocked by preincubation of the slides with 0.3% H_2O_2. The p53 antibody reacted specifically with both the wild type of protein p53 and its mutant form, recognizing an epitope between the amino acids 37 and 45 (19). Anti-p53 was diluted 1:30 and incubated at 37° C for 26 min; anti-mdm2 was diluted 1:50 and incubated at 37° C for 30 min. Bound primary antibodies were detected by the avidin-biotin-complex method (Ventana Medical Systems) labeled with horseradish peroxidase and diaminobenzidine as substrates. All detection steps were performed at 37° C in the immunostainer.

Assessment of both antigens was made by counting 1,000 cells per section at high magnification (high-power field, 40×), and the percentage of positive tumor cells was determined. According to Kawai *et al.* (20), a tumor was considered positive for p53 when more than 10% of the tumor cells were immunopositive. With regard to positive mdm2 antibody reaction, the cutoff point was defined as 10%. For both antibodies p53 and mdm2, only the positive nuclear staining was evaluated. Sections from a breast carcinoma were used as a positive control for mdm2 and p53, respectively. The immunohistochemistry slides were evaluated independently by two pathologists (TG and CH).

Molecular Genetic Methods

DNA preparation

DNA was prepared from 2- to 10-mg fresh tissue through a phenol-chloroform extraction (21).

To estimate the percentage of tumor cells in hematoxylin and eosin slides, tumor tissue corresponding to all snap-frozen tumor samples was obtained. We used only those cases in which at least 70% tumor cells were seen. A dilutional effect caused by nontumor cells on the DNA analysis should thus be excluded.

Mdm2 amplification

Southern blot analysis was performed by 10 μg of DNA with *Eco*RI (AGS, Heidelberg, Germany), separating the fragments by electrophoresis on 0.8% agarose gels, and by transferring them to nylon filters (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany). The DNA on the filters was then sequentially hybridized with probes for *mdm2* (a cDNA fragment containing −300 to 1600 nt of the *mdm2* gene [*i.e.*, almost the entire coding region, provided by Dr. B. Vogelstein]) and for β-actin used as a control (1) for unequal amounts of DNA loaded. Probe labeling was done with the use of random oligonucleotide primers (Gene Images; CDP Star; Amersham Pharmacia Biotech Europe).

Hybridization signals were quantified by densitometry (VDS Amersham Pharmacia Biotech Europe; Dorval, Quebec, Canada; Biotech, Bethesda, MD). Signals that had an intensity at least three times higher than those from samples with a normal copy number of the gene (placenta) were scored as amplification. The osteosarcoma cell line

OsA-CL (ATCC, Rockville, MD), in which *mdm2* is amplified, was used as a positive control (1).

Polymerase chain reaction–single-strand conformation polymorphism technique

The conserved regions of the *p53* gene (exons 4 to 8) were amplified and sequenced. Primers have recently been described by Günther *et al.* (13).

A total volume of 50 μl polymerase chain reaction (PCR) mixture contained 100 ng DNA, 250 μM dNTPs, 25 pmol primer each, 1.5 mM $MgCl_2$, 1 × PCR buffer: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 2.5 u *Taq*-DNA-polymerase (Life Technologies, Karlsruhe, Germany). PCR consisted of 35 cycles (92° C for 1 min, 52 to 62° C for 5 min, 72° C for 1 min) and was performed in an automated thermocycler (PTC, Watertown, MA).

Routine evidence of the PCR products was given on ultrathin polyacrylamide gels (PAA, 0.3- to 0.45-mm thick, 8 to 15%, depending on the fragment length) at 15° C for approximately 2.5 h in a horizontal electrophoresis system (Multiphor, Pharmacia/Biotech). DNA fragments were demonstrated using a modified silver staining protocol according to Budowle *et al.* (22). The single-strand conformation polymorphism (SSCP) technique (23) served as a prescreening method for the detection of mutations. Each PCR product that showed aberrantly migrating single strands was always amplified twice to exclude amplification artifacts. In brief, 4.5 μl of the PCR product and 4.5 μl of 100% formamide buffer (0.05% bromphenol, 0.05% xylencyanol) were denatured at 98° C for 5 min, subsequently chilled on ice, and applied to a 0.5 × mutation-detection-enhancement gel (AT Biochem). Gels were stained according to a protocol described by Goldman and Merril (24).

PCR products that showed mobility shifts of their single strands were directly sequenced on an automated fluorescence sequencer (ALF-Express, Pharmacia, Biotech).

Statistical Evaluation

The significance of associations between genetic and immunohistologic findings and clinicopathologic factors was evaluated statistically by Fisher's exact test (two tail) using SAS statistical software system (SAS Institute, Inc., Cary, NC). A probability of $P < .05$ was considered significant.

RESULTS

Mdm2

Of 43 gastric carcinomas investigated, 18 (41.8%) showed an amplification of the *mdm2* oncogene (Fig. 1). The gene was upregulated in 9 of 18 (50%) diffuse-type carcinomas, in the small cell carcinoma, and in 3 of the 4 mixed-type tumors. Only 5 of the 20 (25%) intestinal-type carcinomas were found to show *mdm2* gene amplification.

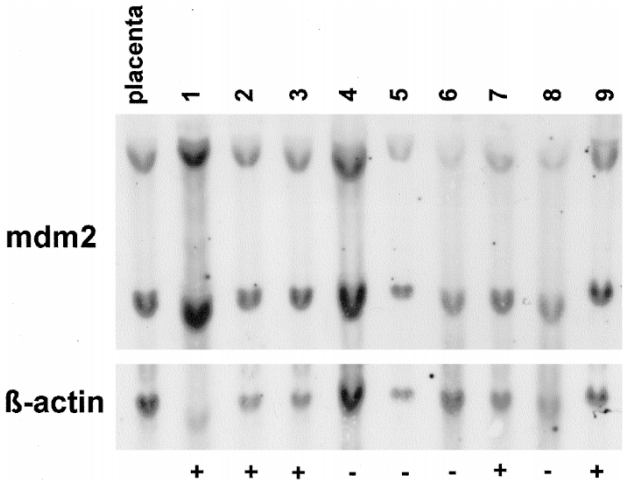

FIGURE 1. Representative Southern blot of Eco-RI-digested genomic DNA demonstrating the level of *mdm2* amplification in gastric carcinomas. Signal ratios of *mdm2*: β-actin (estimated densitometrically), which were three times higher than those of placenta, were scored as amplification (+). Samples without *mdm2* amplification are scored (−).

The expression of the mdm2 protein could also be demonstrated in 18 tumors. In 9 of 23 (39.1%) carcinomas with a diffuse growth pattern (Fig. 2), including 2 of the mixed type, the reaction was positive in more than 10% of the tumor cells. Nine of 20 carcinomas of the intestinal type (45%) were mdm2 positive.

Nontumor cells, such as connective tissue cells and smooth muscle cells of the stomach wall or epithelial cells of the mucosa, reacted only occasionally to the mdm2 antibody. Only the parietal cells of the corpus mucosa showed a nonspecific positive immunoreaction.

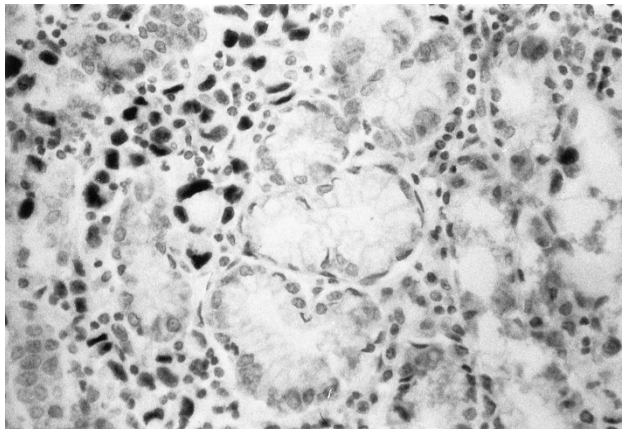

FIGURE 2. *Mdm2*-positive immunostaining in a diffuse-type gastric carcinoma, localized in the corpus (×400).

p53

The screening of carcinomas for genetic *p53* alterations by SSCP analysis and direct sequencing revealed mutations in 9 of 43 (20.9%) tumors (Figs. 3 and 4). Only one carcinoma of the diffuse type showed a *p53* mutation (5.5%); a second mutation was verified in the small cell carcinoma. Mutations were also seen in 7 of 20 intestinal-type tumors (35%). The four carcinomas with mixed differentiation showed no *p53* mutations. The mutation spectrum is presented in Table 1.

p53 overexpression was demonstrated in 14 of 20 (70%) tumors with a glandular growth pattern (Fig. 5). Twelve of them were of the intestinal type, and 2 were carcinomas of the mixed type, which expressed *p53* in their glandular component. In contrast, the p53 protein was found by immunohistology in only four carcinomas with a diffuse growth pattern. No p53 overexpression was seen in the small cell tumor.

As summarized in Table 2, tumors with a diffuse growth pattern, including those classified as mixed type and the small cell carcinoma, tended to show a higher frequency of *mdm2* amplification than carcinomas of the intestinal type ($P = .062$). There was also a tendency for *p53* gene mutations to occur more frequently in the intestinal type ($P = .059$).

The mdm2 protein expression showed no differences concerning the tumor type ($P = .763$), whereas a significantly higher frequency of the p53 protein overexpression could be found in carcinomas of the intestinal type ($P = .001$).

Altogether, approximately 80% of the gastric carcinomas investigated showed mdm2 and/or p53 alterations (Table 3), either at the level of genetic changes or at the protein level.

DISCUSSION

Numerous investigations have addressed alterations of the *p53* tumor suppressor gene as well as the accumulation of the p53-coded protein in the genesis of different tumors (25). Several studies have demonstrated that p53 alterations, including protein overexpression, also play a role in the pathogenesis of gastric carcinoma (25, 26). On the one hand, the functions of the *p53* gene, regulating the cell cycle (27) and apoptosis, are disturbed by gene mutations and deletions. On the other hand, both viral proteins and oncoproteins, such as mdm2, may manipulate the *p53* function and thus, alternatively, exert an influence on the cell cycle via this pathway (2–4). The frequency of the *mdm2* amplification in gastric carcinoma has not yet been investigated. Although in our study the *mdm2* gene

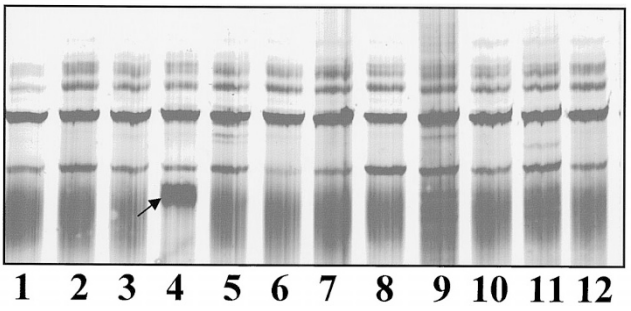

FIGURE 4. Single-strand conformation polymorphism analysis of exon 8 of the *p53* gene in gastric tumor samples. In lane 4, an aberrantly migrating single strand can be seen, which was confirmed as a heterozygous missense mutation in codon 272 (tumor 6 in Table 1) in the sequence analysis.

TABLE 1. *p53* Mutation Spectrum in Gastric Cancer

Tumor	Exon/Codon	Base Substitution	Type	Consequence
1	8 bp splice-donor exon 6	G to A		Splicing?
2	5/185	Insertion TA	Frame shift	Stop in exon 7
3	4/52	Insertion CAT	In frame	+ His
4	5/175	CGC to CAC	Missense	Arg to His
5	8/295	CGG to TGG	Missense	Arg to Trp
6	8/272	GTG to GCG	Missense	Val to Ala
7	8/280	AGA to AAA	Missense	Arg to Lys
8	7 bp splice-donor exon 5	C to T		Splicing?
9	6/192	CAG to TAG	Missense	Stop

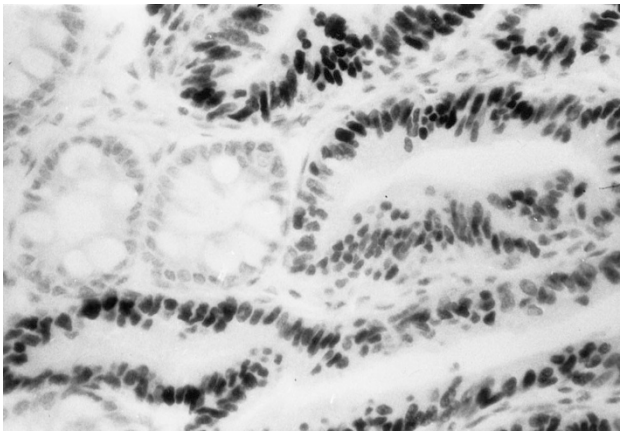

FIGURE 5. *p53*-positive immunoreaction in an intestinal-type gastric carcinoma. Intestinal metaplasia on the left (×400).

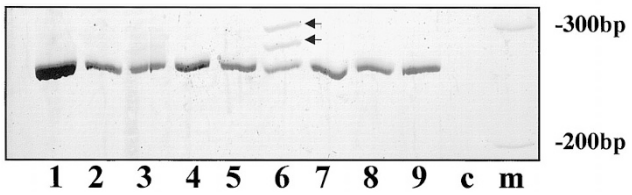

FIGURE 3. Polyacrylamide control gel of polymerase chain reaction of exon 5 of the *p53* gene. c, negative control without DNA; m, DNA ladder; 1–9, gastric carcinomas. In lane 6 (tumor 2 in Table 1), a heteroduplex formation (***arrows***) can be seen, reflecting the heterozygous TA insertion confirmed by sequence analysis.

TABLE 2. Distribution of *mdm2* and *p53* Alterations in Intestinal-Type Carcinomas and Tumors with a Diffuse Growth Pattern, Including Mixed-Type Carcinomas and Small Cell Carcinoma

mdm2/*p53* Alteration	Carcinoma with Diffuse Growth Pattern (n = 23)	Carcinoma of the Intestinal Type (n = 20)	p Value
mdm2 amplification	13 (56.5%)	5 (25%)	0.062
mdm2 protein expression	9 (39.1%)	9 (45%)	0.763
p53 gene mutation	2 (8.7%)	7 (35%)	0.059
p53 protein expression	4 (17.4%)	14 (70%)	0.001

TABLE 3. *mdm2*/*p53* Alterations in Gastric Cancer (n = 43)

p53 Alterations	*mdm2* Alterations	Gastric Cancer n (%)
+	+	16 (37.2)
+	−	8 (18.6)
−	+	10 (23.2)
−	−	9 (20.9)

+, observed alterations; −, no gene alterations.

was amplified in 9 of 18 diffuse-type carcinomas, in the primary gastric small cell carcinoma and in 3 of 4 cases classified as mixed-type tumor, only 5 of the 20 intestinal-type tumors showed an amplification. Of the total of 43 gastric carcinomas investigated, the *mdm2* amplification rate amounted to 41.8%. Marchetti *et al.* (15) determined an amplification rate of only 6% in non–small cell carcinomas of the lung. Investigating 52 breast carcinomas, the same group demonstrated *mdm2* amplification in 23% (14). Higher amplification rates were reported in some soft tissue tumors. Leach *et al.* (11) observed *mdm2* amplification in approximately 33% of the liposarcomas and malignant fibrous histiocytomas investigated. Another study of leiomyosarcomas found *mdm2* amplification in only 2 of 29 tumors (6.9%) (12). In osteosarcomas, *mdm2* upregulation was found in 14% (28). In contrast, as in Ewing's sarcoma (29), *mdm2* seems to play no role in the pathogenesis of carcinomas of the cervix uteri (30). Blok and co-workers (16, 31) investigated the mdm2 protein expression in 45 early gastric cancers. At this stage of tumorigenesis, there was no evidence of an mdm2-linked dysfunction of *p53*. In our study, an immunohistologic proof of the *mdm2* gene product could be given in 18 advanced gastric carcinomas. We found no significant difference between the carcinomas of the diffuse type (38.9%) and the intestinal type (45%). A statistically significant association between *mdm2* gene amplification and mdm2 protein expression could not be demonstrated. Mdm2 protein was expressed in eight tumors, without a gene amplification being present. Such a constellation was also partly observed in a series of breast carcinomas investigated by Marchetti *et al.* (14). Cordon-Cardo *et al.* (32) also described similar phenomena in soft tissue tumors. This could be ascribed to an autoregulative activation of *mdm2* expression by p53 (7). In seven cases, the *mdm2* gene was amplified, but there was no expression of the mdm2 protein. Cordon-Cardo and co-workers (32) found no protein in 6 of 11 soft tissue tumors with *mdm2* amplification. This could be explained by an alteration in the amplified gene that prevents transcription. In this connection, Marchetti *et al.* (14) reported that the antibodies used may recognize only different epitopes so that immunohistologic evidence is not always possible.

In nine tumors (20.9%), *p53* gene mutations were detected by the SSCP technique, followed by direct sequencing. Concerning the tumor type, the distribution of *p53* alterations is in contrast to that of *mdm2* amplification. Only one diffuse-type carcinoma (5.5%) and the small cell carcinoma showed *p53* gene mutations. In contrast, 7 of 20 (35%) carcinomas of the intestinal type showed gene alterations. Ranzani *et al.* (33) also found considerably higher mutation rates in intestinal-type tumors.

p53 protein overexpression was seen in 12 of the 20 intestinal-type tumors. In two carcinomas of the mixed type, p53 was demonstrated in their glandular component. Only 22.2% of the diffusely growing carcinoma showed protein expression. The markedly higher expression of the p53 protein in the intestinal type is in accordance with the results obtained by Fléjou *et al.* (26).

Because only approximately 20% of the gastric carcinomas show *mdm2*/*p53* alterations neither at the genetic nor at the posttranslational level, we can draw the following conclusions: (1) Alterations in the *mdm2*/*p53* pathway are a part of the pathogenesis of gastric carcinoma. (2) Tumors with a diffuse growth pattern in particular are frequently accompanied by an amplification of the *mdm2* oncogene during genesis, whereas *p53* alterations are expected to occur in intestinal-type carcinomas. The question as to which stage of tumorigenesis allows *mdm2* alterations to gain importance remains to be answered in further investigations.

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