Subject Review

# *MDM2* and Its Splice Variant Messenger RNAs: Expression in Tumors and Down-Regulation Using Antisense Oligonucleotides

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## Abstract

**Alternative splicing has an important role in expanding protein diversity. An example of a gene with more than one transcript is the *MDM2* oncogene. To date, more than 40 different splice variants have been isolated from both tumor and normal tissues. Here, we review what is known about the alteration of *MDM2* mRNA expression, focusing on alternative splicing and potential functions of different MDM2 isoforms. We also discuss the progress that has been made in the development of antisense oligonucleotides targeted to MDM2 for use as a potential cancer therapy.**

## Introduction

MDM2[1] appears to play a role in many normal physiological and pathological pathways. It has been shown that MDM2 not only acts as an oncogene (1–3) but also displays growth-inhibitory functions (4, 5). MDM2 can also function independently of p53; for example, MDM2 interacts with transcription factors of the E2F family and the human TATA binding protein (6, 7) and inhibits retinoblastoma growth-regulatory function (8). A question remains whether the same MDM2 protein is responsible for the diverse functions reported or whether different isoforms or post-translationally modified MDM2 proteins could be involved. We examine here the different MDM2 variants that are likely translated from alternatively spliced *MDM2* pre-mRNAs and how antisense oligonucleotide (AS-ODN) therapies might be useful for down-regulating the expression of both full-length and alternatively spliced variants of *MDM2*. Down-regulation of full-length MDM2 expression in tumors that overexpress MDM2 would be predicted to result in p53 protein stability and sensitization to DNA-damaging agents (chemotherapeutic drugs and radiation) that act via p53-dependent pathways (Fig. 1). In addition, p53-independent functions of MDM2, such as in regulating the cell cycle, would also be abrogated, thereby restoring growth control in the tumor (Fig. 1). Progress has been made in the *in vitro* and *in vivo* uses of MDM2 antisense to down-regulate MDM2 expression.

## Splicing of the *MDM2* Messenger RNA

Three human *MDM2* mRNA transcripts of 6.7, 4.7, and 1.9 kb long were reported by Pinkas *et al.* (9) in breast carcinoma cells, with the 1.9-kb mRNA having lost exon 12. In addition, several truncated MDM2 isoforms of 85, 76, and 57 kDa have been described in a panel of human breast carcinomas, together with the full-length 90-kDa protein (10). During the last few years, detailed expression analyses of the *MDM2* mRNA in various cancer types and in normal tissue have revealed alternative as well as aberrant splicing (Fig. 2; reviewed in Ref. 11). The tumor types investigated to date include ovarian and bladder cancer (12), glioblastomas (13), glioblastoma cell lines (14), breast carcinomas (15, 16), soft tissue sarcomas (17–19), giant cell tumors of the bone (20), and Hodgkin's lymphoma (21). The majority of the greater than 40 splice variants that have been detected to date lack sequences that encode at least part of the p53 binding domain, the nuclear localization and export sequences, the p300 binding domain, and the acidic domain. *In vitro* expression studies confirmed that the protein isoforms encoded by at least four of these splice variants (*MDM2*-A, -B, -C, and -D; 12) are unable to bind p53. The splice form *MDM2*-B, which is the most frequently detected transcript variant, has been described in various tumor types as well as in normal tissue, whereas many of the other variants have only been found in one particular cancer type.

A problem that has developed, however, is that some investigators have chosen to give different names to previously published isoforms, although most of the sequences of published splice variants are in Genbank. For example, the recently detected splice form *MDM2*-HD1 (accession no. AJ5505169) corresponds to the PM2 form (accession no. AJ278977), and another splice variant, *MDM2*-Del.G (15), which lacks the sequence between nucleotides 182 and 1432 of the coding region of the *MDM2* mRNA, exactly corresponds to the 219bp form

Received 10/1/03; revised 11/11/03; accepted 11/13/03.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
**Grant support:** NIH grants CA92401 and CA21765, American Lebanese Syrian Associated Charities (L. C. H.), Deutsche Krebshilfe e.V. grant 2130-Ta2, Land Sachsen-Anhalt grant 3347A/0021B, and GSGT e.V. (F. B. and H. T.).
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[1]The abbreviations used are *MDM2*, human gene and oncogene; MDM2, human protein and isoform; *mdm2*, mouse gene; Mdm2, mouse protein.

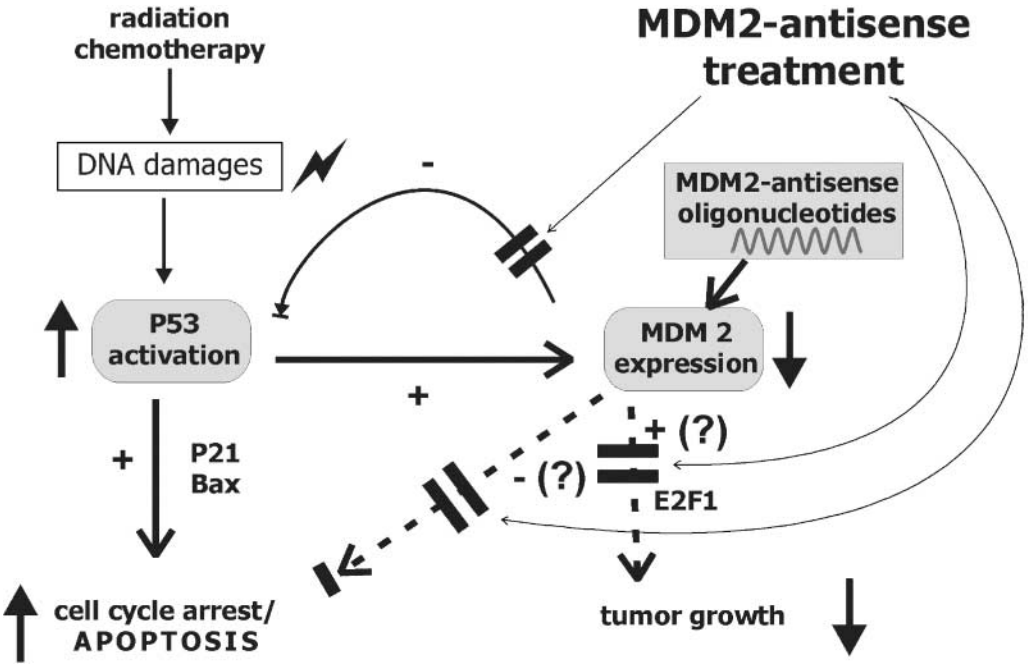


**FIGURE 1.** Proposed mechanism of action of MDM2-AS-ODNs (modified from Ref. 58). MDM2 and p53 form an autoregulatory feedback loop in which the MDM2 expression is up-regulated by p53 and MDM2 binds to p53, thereby promoting its degradation. In many tumors, however, MDM2 is overexpressed and can limit the effect of p53 activation by DNA-damaging drugs or radiation. By inhibiting the expression of MDM2 by targeting MDM2 mRNA with AS-ODNs, p53 should be activated to induce cell cycle arrest and/or apoptosis. In addition, it is predicted that MDM2-AS-ODN treatment will also inhibit p53-independent functions of MDM2.

described by Lukas *et al.* (16). This makes it difficult to determine the frequency of occurrence of specific isoforms and to compare the variants expressed within different tumor types.

A large proportion of the known *MDM2* transcripts is aberrantly spliced (*e.g.*, MDM2-PM2, -EU2, -KB3, and -219bp). These examples have a common splicing pattern that is illustrated in Fig. 3. Splicing occurs at cryptic splice donor and acceptor sites in regions with high sequence homology and that are present as many as four times within the coding region of the *MDM2* mRNA. For example, a 9-bp repeat sequence (AAGAGACCC in exon 3 and AAGAAACCC of exon 12) is involved in the splicing of the EU2 variant (a splice variant detected in soft tissue sarcomas; 17). Similar findings have been described by Hong and Li (22) for the retinitis pigmentosa GTPase regulator. Here, various portions of a purine-rich region were removed as introns. When analyzing the purine-rich sequences within the *RPGR* gene, several exonic splice enhancers that promote splicing through interaction with splicing factors were found, and Western blot analysis revealed many different sizes of RPGR proteins (22). Similar observations have also been made when MDM2 protein expression is evaluated in primary tumor samples (Bartel, unpublished observations) and in several normal tissues (23).

Given the fact that alternative splicing of the *Drosophila* Dscam pre-mRNA gives rise to over 38,000 transcripts (24), the splicing pattern of *MDM2* mRNA is still somewhat concise. However, the question remains as to why there are so many splice variants. We have described a mechanism by which a diverse collection of mRNAs can be generated from a single gene (Fig. 3), but it is unknown how many of the more than 40 splice variants described for *MDM2* are real and functionally relevant. It has been shown that many of the documented splicing errors can be caused by mutations in the genomic DNA, thereby creating new or destroying normal splice sites (25). Furthermore, mutations within binding sites of splicing regulatory proteins can cause "missplicing" (26, 27). However, whether this is also true for *MDM2* is not known. There are only a few reports describing analysis of mutations within the *MDM2* cDNA, but it is unknown whether these contribute to the diversity of the *MDM2* mRNA transcripts. Point mutations have been described in the zinc finger-encoding region of *MDM2* cDNA isolated from non-Hodgkin's lymphomas, leukemias, and hepatocellular carcinomas (28) and in other domains in liposarcomas (19). However, the mutation frequency of the *MDM2* gene in general is rather low, as other reports detected no mutations in the *MDM2* cDNA isolated from other tumor types (29–31).

The fact that some splice variants have been detected only in particular tumor types suggests that they might contribute to the transformed phenotype of these tumors, whereas others (*e.g.*, *MDM2*-A) may be associated with tumorigenesis in general. However, it is important to note that a considerable number of *MDM2* splice variants have been detected in normal tissues (23), demonstrating that MDM2 isoforms do not always possess oncogenic properties.

## Functions of MDM2 Isoforms

There are several variants that share great structural homology (Fig. 2), suggesting that they may perform similar cellular functions. In addition, because multiple isoforms with

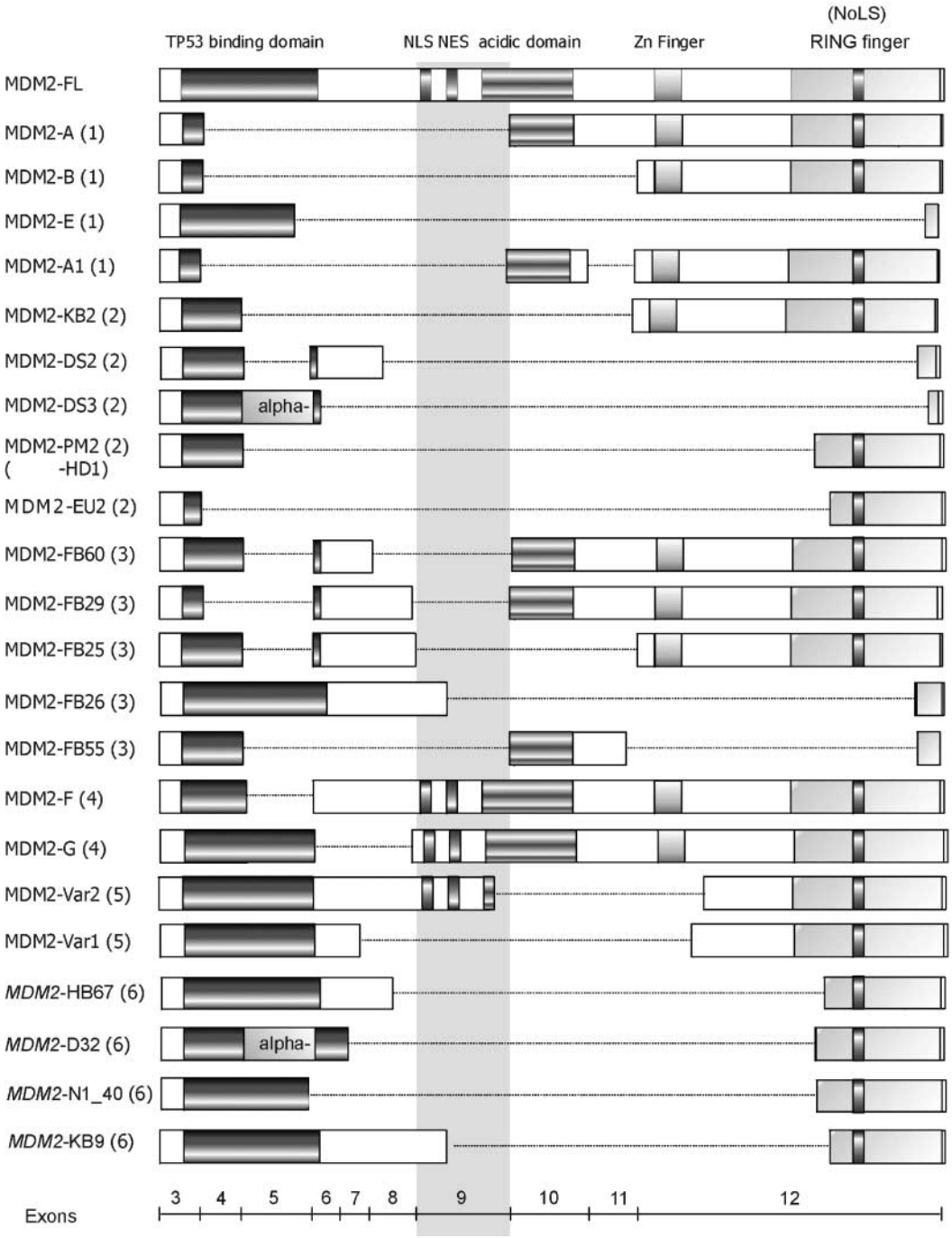


**FIGURE 2.** Summary of the most frequently expressed MDM2 splice variants and the domains that they encode. The sequence that is spliced out in most of the variants is highlighted with a *light gray box*. *Numbers in parentheses,* (*1*) Sigalas *et al.* (12), (*2*) Bartel *et al.* (17), (*3*) Bartel *et al.* (18), (*4*) Tamborini *et al*. (19), (*5*) Schlott *et al*. (59), and (*6*) Bartel *et al.* (unpublished observations).

similar sequences are often expressed within the same tumor (11), some isoforms are functionally redundant. Many examples of alternative splicing result in the region encoding the COOH terminus to be out of frame. This frame shift leads to the generation of novel amino acid sequences; however, because these new sequences differ in every case and are very short (only eight or nine amino acids), they are unlikely to contribute to a common novel function. However, it is not without precedent that these unique sequences could contribute to a gain of function as has been shown for MDMX-S, an isoform of the MDMX protein (32, 33). The MDMX-S isoform is characterized by a short unique amino acid sequence, which increases 12-fold the affinity of MDMX-S binding to p53 compared with MDMX (33).

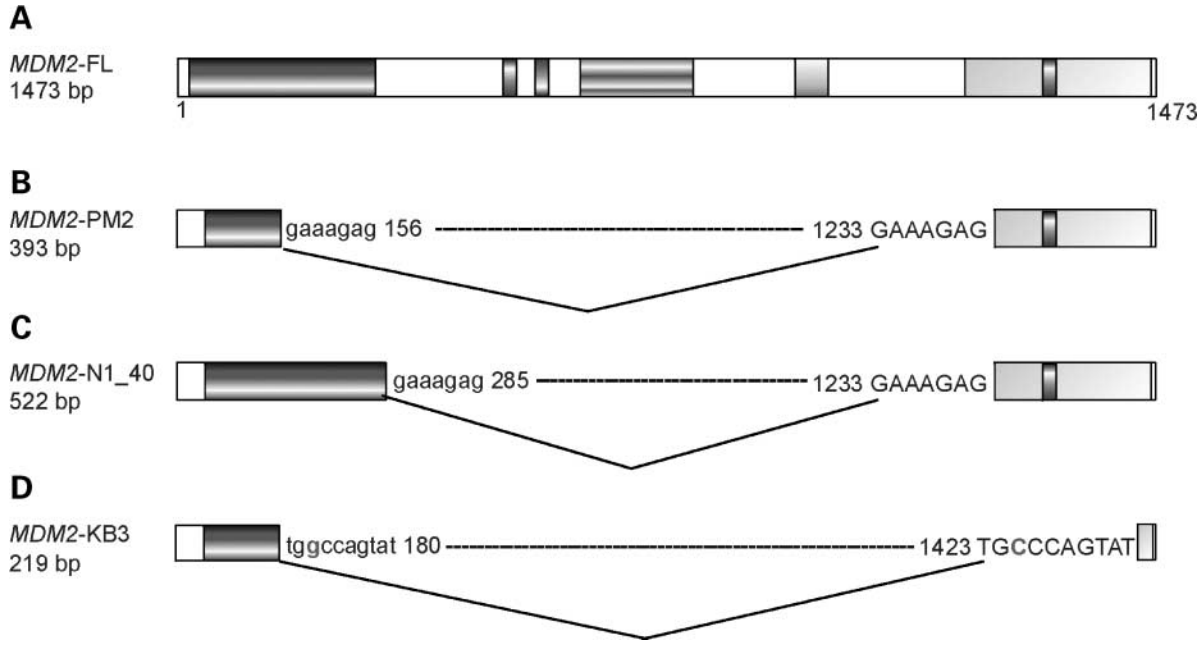


**FIGURE 3.** Mechanism of aberrant splicing at repetitive sequences. *MDM2-FL,* full-length MDM2; *capital letters,* sequence that is present in the splice variant. A detailed description of the splicing mechanism is given in the text.

Evans *et al.* (34) have shown that at least one splice variant (*MDM2*-B or *ALT1*) encodes a protein that binds full-length *MDM2*, resulting in sequestration of both proteins in the cytoplasm. This finding is important because alternatively and aberrantly spliced *MDM2* mRNAs are usually expressed together with full-length *MDM2* transcripts. *MDM2*-B is the most frequently expressed *MDM2* splice variant. It has been observed in numerous types of cancer, including ovarian and bladder cancers (12), breast cancer (13, 16), soft tissue sarcomas (17, 19), and giant cell tumors of the bone (20), but it also occurs in normal breast tissue (16).

Evans *et al.* (34) demonstrated that binding of *MDM2*-B to full-length MDM2 increased wild-type p53 activity, an observation that was also described by Dang *et al.* (35) with some of the murine variants. On binding of *MDM2* splice variants with an intact COOH-terminal RING finger domain to full-length MDM2 protein, p53 protein becomes stabilized, resulting in a growth-inhibitory phenotype.

A correlation between expression of *MDM2* splice variants and stabilization of wild-type p53 has also been described in glioblastoma cell lines despite an amplified *MDM2* gene (14). In soft tissue sarcomas, the expression of *MDM2* splice forms was also associated with an overexpression of mutant and wild-type p53 (17). These findings suggest that p53 accumulation arises as a consequence of alternative and aberrant *MDM2* splicing independent of the mutational status of p53. Although wild-type p53 overexpression induced by MDM2 isoforms is inconsistent with tumor progression, it is conceivable that the stabilization of mutant p53 might contribute to transformation and tumor growth. It is also possible that *MDM2* splice variant-mediated p53 activation could result in enhanced selective pressure to inactivate the p53 apoptotic pathway, thereby increasing accumulation of other genetic defects and promoting tumorigenesis. However, the association between *MDM2* splice variants and malignancy is controversial. Initial studies by Sigalas *et al.* (12) demonstrated that the expression of *MDM2* splice forms in transfected NIH3T3 cells could grow as colonies in soft agar. These findings have been supported by data from Steinman *et al.* (36) who demonstrated that *MDM2*-B, which is the most prevalent isoform identified in tumors, can cause tumors in a transgenic mouse model. Recent data from Fridman *et al.* (37) show that the murine equivalents of the human *MDM2*-B, -D, and -E splice forms, but not *MDM2*-A, significantly accelerated lymphomagenesis in an Eμ-*myc* transgenic mouse model. The lymphomas produced by the splice variants were aggressive and displayed a similar pathology to lymphomas expressing full-length *MDM2*. These data provide evidence that at least some MDM2 isoforms can contribute to tumor development in an *in vivo* mouse model. However, in contrast to a transforming function, *MDM2*-B is expressed in both normal and malignant mammary tissues (16).

Another aspect to be considered regarding a potential transforming function of the MDM2 isoforms is the amount and/or the translation efficiency of a given mRNA as well as the ratio of two or more splice variants. Because it is unknown under what conditions p53-independent functions of full-length *MDM2* may be active, it is impossible to predict the function of each splice variant in all the model systems evaluated. In many cases, MDM2 isoforms may not be functional and, therefore, would not affect the cell negatively. However, many of the proteins synthesized from alternatively and/or aberrantly spliced mRNAs can play either a transforming role or a "normal" physiological role dependent on the cell type. In addition, recent data from Bartl *et al.* (38) suggest a new RNA-based function of MDM2. The authors describe a 365-bp,

**Table 1. Sequence of the Anti-MDM2 Oligonucleotides**

| Oligonucleotide | Sequence[a] | Target Sequence in Exon | Characteristic | References |
|---|---|---|---|---|
| Kondo | GACATGTTGGTATTGCACAT | Exon 3 | Phosphorothioate oligonucleotide | (39–41) |
| HDMAS5 | GATCACTCCCACCTTCAAGG | Exon 7 | Phosphorothioate oligonucleotide | (42, 43) |
| Anti-MDM2-MBO | UGACACCTGTTCTCACUCAC | Exon 7 | Mixed backbone oligonucleotide | (44–46, 48, 51, 55–57) |

[a]Underlined sequences, 2′-*O*-methyl-RNA linkages between nucleotides.

alternatively spliced transcript that is composed of the first five *MDM2* exons and is highly stress inducible. It appears to be the major processing product of the *MDM2* mRNA both in normal and in cancer cells (38). Therefore, it seems likely that even transcript variants that are not translated fulfill a function as small non-mRNAs. Future studies are required to clearly define the functions of the many *MDM2* transcript variants and how their expression is controlled.

## Down-Regulation of the *MDM2* Messenger RNA by AS-ODNs

The use of AS-ODNs targeted to different *MDM2* mRNAs may be a useful approach to evaluate the functions of alternatively spliced isoforms. In addition, MDM2 antisense designed to down-regulate splice variant and/or full-length *MDM2* mRNAs may be a useful anticancer therapeutic approach (Fig. 1). However, many of the MDM2-AS-ODNs reported to date were designed to target sequences not present within the majority of splice variants and therefore would only down-regulate full-length MDM2 expression. Here, we summarize current progress on the development of MDM2-AS-ODNs as a potential anticancer therapy.

The first study to use MDM2-AS-ODNs (Table 1) to down-regulate *MDM2* expression was published by Kondo *et al.* (39). It was shown that glioblastoma U87-MG cells were more susceptible to cisplatin-induced apoptosis when the cells were cotreated with cisplatin and MDM2-AS-ODNs that targeted the first 20 nucleotides of the open reading frame. A decrease of the MDM2 protein level could be observed, although p53 protein levels remained constant. These findings (39) suggest an important role for MDM2 in the development of resistance to cisplatin. Teoh *et al.* (40) studied the effect of the same oligonucleotide in a series of multiple myeloma cell lines. Treatment with MDM2-AS-ODNs resulted in decreased DNA synthesis and cell viability as well as a $G_1$ cell cycle arrest associated with pRb-E2F1 binding. Furthermore, apoptosis was induced in the AS-ODN-treated cells (40). In another study using the same MDM2-AS-ODNs described by Kondo *et al.* (39), we observed decreased MDM2 protein expression 24 h after antisense treatment and an 80% decrease in the colony-forming ability of MDM2-AS-ODN–treated undifferentiated sarcoma cells (US8/93) compared with control cells that received a scrambled control oligonucleotide (41). Although *MDM2* splice variants were not evaluated during these studies, the MDM2-AS-ODN used had the potential to down-regulate many of the common splice forms

**Table 2. Summary of Published *in Vivo* Applications of Anti-MDM2 Oligonucleotides**

| Tumor Type | Cell Line | Animal Model | p53 Gene Status | Oligonucleotide, Amount, Schedule | Drugs | Effects of Cotreatment | References |
|---|---|---|---|---|---|---|---|
| Prostate cancer | DU-145, PC-3 | Mice | ND[a] | Anti-MDM2-MBO, 25 mg/kg/day, 4 × 5 days/wk | Irinotecan, paclitaxel, rituxan | Synergistic effect (+ irinotecan) slightly increased activity (+ paclitaxel, rituxan) | (56) |
| Colon cancer | LS174T | Mice | Wild-type | Anti-MDM2-MBO, 20 mg/kg/day, 5 days/wk | 10-Hydroxy-camphothecin, 5-fluorouracil | Synergistically or additive therapeutic effects | (46, 57) |
| | DLD-1 | | Mutant | | | | |
| Glioblastoma multiforme | U87-MG | Mice | Wild-type | Anti-MDM2-MBO, 25 mg/kg/day, 5 days/wk | Irinotecan, paclitaxel | Inhibition of tumor growth, 39- and 63-fold activity of irinotecan and paclitaxel | (48) |
| Rhabdomyosarcoma | RD | Rat | Mutant | Anti-MDM2-MBO, 100 μg continuously over 1 wk | – | Significantly reduced tumor growth, decreased mutant p53 levels | (51) |
| Breast cancer | MCF-7 | Mice | Wild-type | Anti-MDM2-MBO, 25 mg/kg/day, 3 × 5 days/wk | Irinotecan, paclitaxel, 5-fluorouracil | Synergistically or additive therapeutic effects | (47) |
| | MDA-MB-468 | | Mutant | | | | |
| Colon cancer | GEO | Mice | ND | Anti-MDM2-MBO, 10 mg/kg/day, 2 × 5 days/wk | Cisplatin, topotecan | Potentiation of effects of cisplatin, topotecan | (45) |
| Osteosarcoma | SJSA | Mice | ND | Anti-MDM2-MBO, 25 mg/kg/day | 10-Hydroxy-camphothecin, Adriamycin | Synergistic effect of cotreatmant | (44) |
| Choriocarcinoma | JAR | Mice | ND | Anti-MDM2-MBO, 25 mg/kg/day | 10-Hydroxy-camphothecin, Adriamycin | Synergistic effect of cotreatmant | (44) |

[a]ND, not determined

(Fig. 2) in addition to full-length MDM2. However, because splice variant expression was not analyzed during these studies, it is not possible evaluate how potential changes in splice variant expression may have influenced the observed results.

Chen *et al.* (42) investigated the effects of improved phosphorothioate MDM2-AS-ODNs in JAR, SJSA, and MCF-7 cells. The MDM2 protein level was reduced up to 5-fold by using an oligonucleotide termed HDMAS5-ODN (Table 1), resulting in activation of p53 (42). This oligonucleotide was directed against a sequence within exon 7 of the MDM2 mRNA, a region that is omitted in many splice variants. Similar results were observed by Sato *et al.* (43) in osteosarcoma U2-OS cells treated with HDMAS5-ODN in combination with DNA-damaging drugs such as mitomycin C and cisplatin. The drug-mediated cell killing were even more pronounced when the expression of both MDM2 and p21 was blocked by antisense transfection (43). Encouraging *in vitro* results of MDM2-AS-ODN treatment led to the development of xenograft models of different tumors. These xenograft models include osteosarcoma SJSA cells and choriocarcinoma JAR cells (44), colon cancer GEO cells (45), LS174T and DLD-1 cells (46), breast cancer MCF-7 and MDA-MB-468 cells (47), several glioblastoma multiforme cell lines (48), and prostate cancer cell lines (49). In these studies, the human tumor cells were injected into the inguinal area, grown to xenografts, and subsequently treated with either an anti-MDM2 mixed backbone oligonucleotide (anti-MDM2-MBO; Table 1; 44) alone or in combination with chemotherapeutic drugs such as irinotecan, paclitaxel, 5-fluorouracil, or radiation (Table 2; 47–51). MDM2-MBO is another oligonucleotide targeted to exon 7 that is deleted in most splice variants (Fig. 2). In most studies, the MDM2-AS-ODNs were given by i.p. injection at 5 consecutive days. Compared with untreated animals, treatment of the respective xenografts with MDM2-AS-ODNs led to significant antitumor activity in terms of slowed tumor growth and prolonged survival that was attributed to inhibition of MDM2 expression. In cell lines with wild-type p53 such as MCF-7 (47) and U87-MG (48), these antitumor effects were accompanied by elevated levels and increased activity of wild-type p53, whereas in cell lines with mutant p53 such as MDA-MB-468 (47) and T98G (48), the p53 protein levels remained unaffected. In contrast, treatment of an orthotopic xenograft model of rhabdomyosarcoma RD cells (p53mt/−) in the peritoneum of nude rats with MDM2-AS-ODNs resulted in decreased levels of both MDM2 and mutant p53 (51). In general, synergistic effects were observed when cells or xenografts were treated with a combination of MDM2-AS-ODNs and the respective drug or radiation compared with the treatment with either of these agents alone (47–50). However, combination of irinotecan and a MDM2 mismatch control oligonucleotide had the similar effect as the combination of irinotecan and MDM2-AS-ODNs (47–49). The authors speculated that treatment with oligonucleotides, in general, increases the uptake of the active metabolite of irinotecan, SN-38 (46).

## Conclusions

It has been shown that MDM2 not only acts as an oncogene but also displays growth-inhibitory functions. However, it is currently unclear how these different functions are controlled or influenced by expression of alternatively spliced isoforms of MDM2. The use of AS-ODNs to inhibit the expression of genes of the p53-MDM2 pathway has the potential to become an exciting new cancer therapy (52, 53). MDM2-AS-ODNs specifically inhibit MDM2 expression, and they employ their antitumor activity via different mechanisms, regardless of p53 status. This is particularly noteworthy given the high mutation frequency of the p53 gene in human cancer (54). The activity of conventional drugs or radiation can be synergistically enhanced if given in combination with MDM2-AS-ODNs, and the effects appear to be independent of p53 gene status or the different MDM2 isoforms that might be expressed. In light of the current findings, effective cancer therapy may involve inhibiting MDM2 expression, in addition to blocking binding of MDM2 to p53, to inhibit all the growth-promoting activities of MDM2. However, whether the AS-ODNs influence expression of any MDM2 splice variants is currently unknown. As specific splice variants have now been shown to play a role in tumorigenesis, knowing whether they are down-regulated together with full-length MDM2 will be important for interpretation of future data.

## Acknowledgments

We apologize to colleagues whose excellent work in the field of mRNA splicing was not cited due to space limitations.

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