

MDM2 Splice Variants and Their Therapeutic Implications

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Abstract: *MDM2* splice variants have now been identified in many different tumor types, and their expression has been associated with advanced disease. However, published data concerning their function is contradictory, and therefore their role in tumorigenesis and their potential as a therapeutic target are unclear. Expression of a specific splice variant, *MDM2-B*, in a transgenic mouse model results in tumor development; and expression of several splice variants has been shown to enhance tumor formation in $\text{E}\mu\text{-myc}$ transgenic mice. However, expression of similar variants *in vitro* results in growth inhibition, an observation inconsistent with a transformed phenotype. The observed growth inhibition is p53-dependent, resulting from the binding of splice variants with an intact C-terminal RING finger domain to full-length MDM2 protein. In doing so, p53 can no longer bind MDM2, and p53 activity is elevated. Subsequent inactivation of p53 or p53-mediated apoptosis could contribute to the MDM2 splice variant-mediated tumorigenesis observed *in vivo*. However, MDM2 splice variants, like full-length MDM2, probably display p53-independent activities. Therefore, the potential for MDM2 splice variants as therapeutic targets will be dependent upon their phenotype within specific tumor types.

INTRODUCTION

MDM2 is an oncogene that is amplified in approximately 30% of soft tissue sarcomas, and 7% of all solid tumors [1]. *MDM2* overexpression is a mechanism by which activity of the p53 tumor suppressor protein can be inactivated during tumorigenesis [2]. In the past eight years there has been considerable interest in the alternatively and aberrantly spliced variants of *MDM2* that are expressed in tumors, often at a frequency higher than has been observed for *MDM2* gene amplification [3]. For example, *MDM2* splice variants have been identified in 30% of invasive breast carcinomas, whereas *MDM2* amplification in this tumor type is a rare event [4]. In addition, 82% of pediatric rhabdomyosarcomas express *MDM2* splice variants, but the *MDM2* amplification frequency is only 27% [5]. Similarly, 54% of adult soft tissue sarcomas express *MDM2* splice variants whereas only 28% contain an amplified *MDM2* gene [6]. Even though the presence of *MDM2* splice variants is not associated with *MDM2* gene amplification, full-length MDM2 protein is usually expressed in the same tumors in which the smaller variants have been detected [3-8], suggesting that expression of full-length MDM2 could be important for the function of *MDM2* splice variants

MDM2 AND ITS SPLICE VARIANTS

MDM2 splice variants appear to be generated by either alternative splicing (using alternate splice donor/splice acceptor sites resulting in the loss of complete exons) or aberrant splicing (splicing at sequences that are non-canonical splice sites resulting in fragmented exons). However, the existence of aberrantly spliced variants is controversial. Many of the aberrantly spliced *MDM2* variants have only been identified by RT-PCR, and it cannot be

ruled out that some of these variants could be PCR artifacts. Nevertheless, it is clear that aberrantly spliced products can occur *in vivo* as a result of mutations in splicing regulatory proteins or their binding sites [9]. It has also been suggested that splicing 'errors' might occur from the destruction or creation of splice sites resulting from mutations introduced into the pre-mRNA during transcription [9]. Aberrant splicing is often referred to as being non-functional [10]. However, because splicing errors are likely to occur more frequently in transformed cells when the splicing machinery becomes deregulated [11], the function of these variants once translated cannot be ignored.

Both aberrant and alternative splicing have the potential to generate a C-terminal region that is out-of-frame. However, the majority of *MDM2* splice variants encode a protein in which the C-terminus is in-frame [3]. A common feature of many of the splice variants is a loss of the p53 binding domain, and the nuclear localization and export signals (Fig. (1)); although splice variants containing these domains have been described at a low frequency [3]. There have been over 40 different splice variants described to date. Some of the variants are common to several tumor types, for example *MDM2-A* and *MDM2-B* [5-7]. However, others have only been identified in specific tumors, for example *MDM2-FB25* and *MDM2-FB26* in pediatric rhabdomyosarcoma [5]. The different variants and tumors in which they have been identified have been previously reviewed by Bartel *et al.* in 2002 [3]. Examples of specific *MDM2* splice variants referred to in this review are shown in Fig. (1).

There is limited published information concerning the prognostic impact of *MDM2* splice variant expression in human tumors. Their expression has been shown to be associated with late stage and high grade ovarian and bladder carcinomas [7], and a higher frequency of malignant gliomas express *MDM2* splice variants (69%) compared to lower grade astrocytomas (29%, $p < 0.0003$) [8]. In addition to there being an association between *MDM2* splice variant expression and malignancy, expression of aberrantly spliced *MDM2* mRNAs, (but not those that were alternatively

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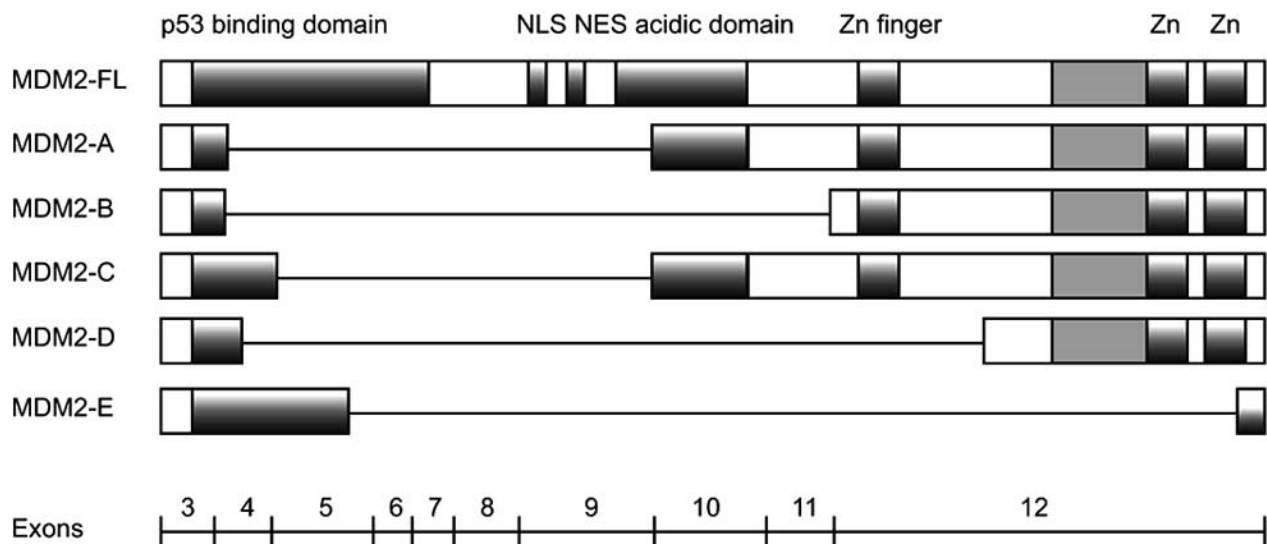


Fig. (1). Schematic representing full-length MDM2 protein and specific MDM2 splice variants discussed in the text. NLS and NES represent the nuclear localization and nuclear export signals, respectively.

spliced) has been shown to correlate with reduced breast cancer patient survival [4]. However, not all published reports are in agreement, since no association was observed between expression of *MDM2* splice variants and prognosis of individuals diagnosed with soft tissue sarcomas [6]. Clearly, additional studies in different tumor histotypes are required to determine whether the expression of *MDM2* splice variants influences tumor prognosis or patient survival. If the consequence of splice variant expression differs between tumor types, as these data suggest, then it will be important to determine their cellular function when expressed in different cell types.

MDM2 SPLICE VARIANTS AND THEIR TUMORIGENIC POTENTIAL

Sigalas and coworkers in 1996 [7] were the first to describe a transforming potential for alternatively spliced *MDM2* isoforms. Their study described five different alternatively spliced mRNAs identified from primary ovarian carcinomas, bladder carcinomas, and human leukemia cell lines. Expression of these variants in NIH3T3 cells resulted in a high frequency of transformed foci. This result was unexpected because four of the five variants had lost the p53-binding domain, and could not bind and inhibit p53 activity [7].

An essential function of full-length *MDM2* is to modulate p53 activity, as demonstrated by the observation that *MDM2* knockout mice are embryonic lethal, and that this phenotype can be rescued by deletion of p53 [12, 13]. Full-length *MDM2* binds p53 protein, inhibits its transactivation potential, and acts as an E3 ubiquitin ligase targeting p53 degradation by the proteasomal pathway [14]. Because most *MDM2* splice variants cannot bind p53, their transforming phenotype must be mediated by a different mechanism. In addition to inactivating p53, full-length *MDM2* has also been shown to exhibit oncogenic properties in the absence of p53. For example *MDM2* transgenic mice develop sarcomas at the same frequency, regardless of whether they express p53 [15]. However, these tumors were found to contain *MDM2*

splice variants that may have played a role in tumor development in these mice.

In order to test the oncogenic potential of one of these splice variants, transgenic mice were developed expressing the variant *MDM2-B* under the control of the GFAP promoter [16]. *MDM2-B* was selected because it was found in all of the full-length *MDM2* transgenic tumors analyzed, but could not be detected in normal mouse tissue. Three transgenic lines were developed, and *MDM2-B* expression was detected in the brain, spleen, liver, kidney, ovary and testis. These mice typically developed tumors between 50 and 104 weeks, with a mean age of 80 weeks. The majority of tumors (70%) were myeloid sarcomas, and 30% were B-cell lymphomas. No brain tumors were observed even though this tissue expressed the highest level of *MDM2-B* protein.

It is of interest that these investigators previously tried to use the chicken β -actin promoter and *CMV* enhancer to drive expression of *MDM2-B* in transgenic mice, but after multiple rounds of injections no founder mice were generated, suggesting that *MDM2-B* is incompatible with normal development. We have also observed similar results when attempting to generate transgenic mice expressing *MDM2-A* (Schuster and Harris, unpublished data). A wild-type p53-dependent growth inhibitory phenotype for these and other similar *MDM2* splice variants has previously been described (see below), suggesting the possibility that the oncogenic potential of *MDM2* splice variants may only be observed once p53, or the p53 pathway, becomes inactivated during tumorigenesis (Fig. (2)). Alternatively, *MDM2* splice variant expression may enhance the selection pressure towards loss of p53 function, as discussed below. Both of these hypotheses are supported by the long tumor latency observed in *MDM2-B* transgenic mice [16] which would allow development of mutations (for example loss of p53) that may be required in order to observe *MDM2-B*-induced tumorigenesis.

The function of *MDM2-B* was also evaluated *in vitro* by the same investigators [16]. Data were consistent with those

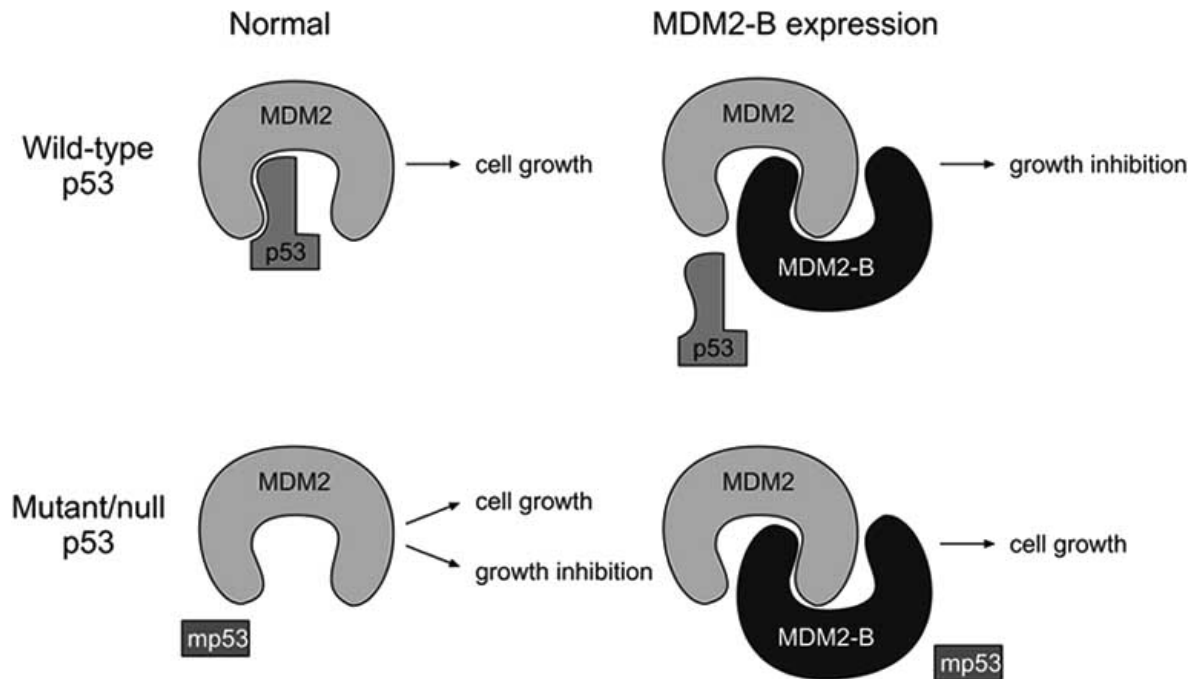


Fig. (2). Model demonstrating the mechanisms of action of MDM2-B, a representative splice variant with an intact C-terminal RING finger domain. mp53 represents mutant p53 protein.

obtained from the transgenic mice, demonstrating that expression of MDM2-B (both human and murine) enhanced the rate of proliferation of NIH3T3 cells, and p53-null, p19(ARF)-null, and Rb-null mouse embryo fibroblasts (MEFs). Consistent with the work of Sigalas [7], *MDM2-B* could also enhance the size and number of foci generated in NIH3T3 transformation assays [16]. However, the levels of p53 protein or its transcriptional activity did not change in the cells expressing MDM2-B, which is in contrast to other published work [17-19], but this observation supports the hypothesis that *MDM2* splice variants may, at least in part, function independently of p53.

A mechanism of MDM2-B-induced tumorigenesis has been proposed to be mediated by induction of RelA expression [16]. RelA is the p65 subunit of NF B, and enhanced NF B activity can suppress apoptosis [20], potentially mediating tumorigenesis [21]. However, this mechanism is not unique to *MDM2* splice variants. Full-length *MDM2* has also been reported to induce RelA expression [22], and therefore this could also be a p53-independent mechanism for full-length MDM2-mediated tumorigenesis.

Additional studies that support a role for *MDM2* splice variants in tumor development has been reported by Fridman *et al.* [23]. These investigators evaluated the activity of *MDM2* splice variants in the E μ -myc transgenic mouse model, a model of human non-Hodgkin's lymphoma [24]. Because *MDM2* splice variants spontaneously arise in both human [25] and murine lymphomas [26], this system was considered appropriate to evaluate the effect of *MDM2* splice variants expression on lymphomagenesis. Four *MDM2* splice variants were evaluated that had previously been identified in human lymphomas [25]. These were equivalent to human variants *MDM2-A*, *-B*, *-D* and *-E* first identified by Sigalas *et al.* [7], and are shown in Fig. (1). Full-length *MDM2* and the splice variants were independently expressed

in hematopoietic stem cells isolated from fetal livers of E μ -myc transgenic embryos, and injected *via* the tail vein into lethally irradiated wild-type recipients. Variants *MDM2-B*, *-D* and *-E* accelerated the rate of lymphomagenesis equivalent to the effect of full-length MDM2. However, expression of *MDM2-A* did not show any effect on lymphoma generation. The difference observed between *MDM2-A* and *-B* was surprising. *MDM2-A* has been detected in tumors [7], and was the most common variant detected in pediatric rhabdomyosarcoma [5]. It encodes 78 additional amino acids compared to *MDM2-B* that contain a portion of the acidic domain and the ARF binding domain, although ARF binding to *MDM2-A* has yet to be confirmed. This region also contains one of the two reported growth inhibitory domains that could potentially inhibit tumorigenic potential [27]. However, if not all *MDM2* variants exhibit a transforming phenotype it is unclear why these variants would be expressed in tumors. Future work comparing the activity of *MDM2* splice variants such as *MDM2-A* and *MDM2-B* will be important in order to determine the mechanism for their oncogenic activity.

Fridman *et al.* observed different activities of the splice variants *in vitro* compared to *in vivo* [23]. None of the splice variants, only full-length MDM2, could induce colony formation in wild-type MEFs, or enhance colony formation when transformed MEFs were grown in soft agar. These data infer that the oncogenic phenotype observed for *MDM2* splice variants B, D and E is limited to their *in vivo* effects. However, this observation is in contrast to the work by Steinman *et al.* [16] and Sigalas *et al.* [7] who demonstrated a growth promoting phenotype for *MDM2-B in vitro* when expressed in NIH3T3 cells and p53-null MEFs. It appears that the phenotype of specific *MDM2* splice variants is highly dependent upon the cell type in which it is expressed. Therefore, in order for *MDM2* splice variants to be considered as a potential target for cancer therapy, their

phenotype in specific tumor histiotypes must first be characterized. This is particularly important considering that *MDM2* splice variants have been detected in normal tissues [4, 28], and can sometimes display growth inhibitory characteristics (see below).

MDM2 SPLICE VARIANTS AND GROWTH INHIBITION

The occurrence of splice variants is associated with mutant p53 in breast cancer [4] and with a stabilized p53 protein in glioblastoma [17]. Therefore, at least in certain tumor histiotypes, *MDM2* splice variants either develop in tumors with inactive p53 protein, or enhance selection against wild-type p53 function.

Evidence supporting the second hypothesis includes work from Evans *et al.* [18] and Dang *et al.* [19]. These investigators demonstrated that *MDM2* splice variants with an intact C-terminal RING finger domain could interact with the RING finger of full-length *MDM2* protein and prevent its interaction with p53. Therefore, expression of specific splice variants can enhance p53 activity [18] and mediate growth inhibition of wild-type MEFs [19]. The growth inhibition was confirmed to be p53-dependent because it was not observed in p53-null MEFs [19]. The *MDM2* splice variants that Dang and coworkers evaluated were murine variants that had been isolated from lymphomas obtained from E μ -myc transgenic mice. These variants did not show any transforming properties, as had previously been reported for similar human variants [7]. However, the variant evaluated by Evans and coworkers, *MDM2-ALTI*, (also called *MDM2-B*), has previously been shown to enhance growth by others [7, 16]. The main differences between these studies are the cell types and epitope tags on the proteins, although whether these factors contribute to the apparently conflicting data is unknown. It is important that the reasons for these contradictory reports be understood so that the phenotype of *MDM2* splice variants might be predicted when expressed within specific tumor types.

The p53-dependent growth-inhibitory activity will be limited only to those *MDM2* splice variants that contain the C-terminal RING finger domain, or potentially the acidic domain, as suggested by Dang *et al.* [19]. Although many of the variants described do have a C-terminus that is in-frame with the N-terminal portion of the protein, many are truncated and would not be expected to bind full-length *MDM2* (reviewed by Bartel *et al.* [3]). Interestingly, variants that contain the RING finger are those most commonly detected in human cancer [3]. *MDM2* splice variants that display a p53-dependent growth inhibitory activity could potentially be tumorigenic by increasing the selection pressure against p53 activity. By inactivating p53, or p53-mediated growth inhibition, tumor progression would likely be enhanced.

Even though full-length *MDM2* is classed as an oncoprotein, under certain conditions it has been shown to display growth inhibitory or apoptotic phenotypes [27, 29, 30]. In addition, there are several examples of *MDM2*-expression in tumors being associated with a favorable prognosis [6, 31, 32]. These data could potentially be explained by enhanced p53 activity mediated by *MDM2*

splice variants in these tumors. However, apoptotic effects of full-length *MDM2* have been observed in cells that do not express wild-type p53 [29 and unpublished data], and if *MDM2* splice variants are expressed in these cells they could potentially bind full-length *MDM2* protein and act in a dominant-negative manner to inhibit its apoptotic phenotype.

However, *MDM2* splice variants have been detected in a wide variety of normal tissues including lymphocytes, lung, spleen, kidney, intestine and breast epithelium [4, 28], suggesting that they may also play a normal physiological role. It is clear that additional work is necessary to evaluate the mechanism of action of alternatively spliced variants of *MDM2* in different cell types, and to determine whether their phenotype is dependent upon the activity of full-length *MDM2* protein.

SPLICE VARIANTS AND DRUG RESISTANCE

Expression of *MDM2* splice variants could potentially have therapeutic implications if their expression were to influence the response of tumor cells to chemotherapeutic agents. Fridman and coworkers observed no effect on cell survival when transformed MEFs expressing *MDM2* splice variants (including *MDM2-B*) were exposed to doxorubicin [23]. However, Steinman *et al.* demonstrated that *MDM2-B* could reduce doxorubicin-induced apoptosis by 60% in NIH3T3 cells [16]. Again, these apparently contradictory data are likely dependent upon the cell type in which the variants are expressed. The Steinman data is consistent with the oncogenic phenotype that these investigators observed in their transgenic mice. However, why they did not observe any changes in p53 activity in the NIH3T3 cells upon *MDM2-B* expression is unclear.

If expression of *MDM2* splice variants in tumor cells results in drug resistance, then *MDM2* splice variants would be an important therapeutic target. As discussed above, activation of the NF κ B pathway could be a mechanism by which drug resistance is mediated [20]. Therefore, targeting this pathway may be an alternate therapeutic strategy other than influencing expression or activity of the splice variants themselves.

MDM2 SPLICE VARIANTS AS THERAPEUTIC TARGETS

Experimental anticancer therapies, for example antisense oligonucleotide or RNAi based therapies designed to target full-length *MDM2* may also affect *MDM2* splice variant expression [33]. Current *MDM2* antisense studies, and the influence of specific oligonucleotides on expression of *MDM2* splice variants have recently been reviewed [33].

If *MDM2* splice variants do in fact exhibit oncogenic or drug-resistant activities independent of the status of full-length *MDM2* or p53, it may be necessary to consider *MDM2* splice variants as specific targets and develop therapies accordingly. In addition to methods to inhibit *MDM2* splice variant expression, small molecule inhibitors of splice variant activity could be developed. Potentially, small molecule screens could be carried out evaluating reversal of *MDM2* splice variant-mediated drug resistance as

an end-point. Alternatively, because it appears that *MDM2* splice variants activate the NF κ B pathway [16], a reduction in NF κ B activity and decreased tumor cell survival might be predicted following inhibition of *MDM2* splice variant activity. If it is determined that activation of the NF κ B pathway is the only mechanism by which *MDM2* splice variants induce tumorigenesis, then specific inhibitors of this pathway downstream from the splice variants should generate similar anti-tumor activity.

However, the majority of published data supports the observation that *MDM2* splice variants, in general, enhance p53 activity, and that tumors expressing these alternatively spliced isoforms would be expected to exhibit a favorable p53-dependent response to therapy. However, enhanced p53 activity will likely result in the eventual selection against p53-mediated apoptosis. If the *p53* gene becomes mutated, or p53 protein inactivated, the rate of either tumorigenesis or tumor progression would probably be enhanced. If this scenario is true, then expression of *MDM2* splice variants would be an early event during carcinogenesis contributing to the loss of p53. Therefore, loss of p53 tumor suppressor activity would ultimately be the factor contributing to carcinogenesis, and loss of *MDM2* splice variants or their activity subsequent to tumor formation would be unlikely to influence tumor growth.

CONCLUSION

The function of *MDM2* splice variants remains an enigma. However, it is clear that under specific conditions they enhance p53 activity and, potentially, increase the rate of carcinogenesis by increasing the selection pressure to inactivate p53. However, tumors do arise in which the p53 pathway appears to be functional. Expression of *MDM2* splice variants under these conditions may predict favorable clinical outcome, by enhancing tumor response to chemotherapeutic agents in a p53-dependent manner. Aside from its p53-dependent activity, full-length *MDM2* has been shown to be oncogenic in a p53-independent manner. This also appears to be true for specific *MDM2* splice variants, suggesting that novel therapies that target these *MDM2* isoforms merits careful investigation. However, before any consideration can be given to inactivation of *MDM2* splice variants as a novel anti-cancer therapy, additional work on their biology is required in order that their activities within specific cell types can be predicted.

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