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Novel MDM2 splice variants identified from oral squamous cell carcinoma

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SUMMARY

Introduction: The presence of a variety of MDM2 splice variants has been reported in a range of different tumor types and is associated with poor patient prognosis. Furthermore, several MDM2 variants have been shown to have oncogenic properties. Despite this, MDM2 splice variants have not been comprehensively characterized in oral squamous cell carcinoma (OSCC).

Materials and methods: MDM2 splice variants were identified by polymerase chain reaction (PCR), using cDNA from 35 OSCC and 20 normal oral mucosa (NOM) tissues. MDM2 amplicons from the polymerase chain reactions were cloned and sequenced. The associations between the presence of MDM2 splice variants as well as the types of MDM2 splice variants with OSCC and patient clinicopathological data was examined using Fisher Exact and Chi-square tests.

Results: Thirty-eight MDM2 splice variants were identified from both OSCC and NOM tissues, where the majority (30/38) were exclusively detected in OSCC. Some of these variants were similar to those reported in other cancers whilst 14 novel MDM2 splice variants predicted to code for proteins were also identified. The majority of these variants retained their RING binding domain but had lost the p53 binding site. The presence of MDM2 splice variants was significantly associated with OSCC and increased the risk of OSCC development (OR = 9.98; 95% CI = 2.94–33.90).

Conclusion: MDM2 splice variants were identified in OSCC at a high frequency and were significantly associated with OSCC development. This suggests that MDM2 splice variants may play an important role in oral carcinogenesis and the functional role of these variants in OSCC should be examined further.

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Introduction

Alternative splicing events occur in about 90% of genes in the genome and this phenomenon in the human genome is thought to play an important role in the expansion of protein diversity. However, cancer specific splice variants encoded by particular genes suggest that splice variants could contribute to the pathogenesis of cancers and the identification of these variants not only opens up opportunities to use them as biomarkers for cancer detection and prognostication, but could help us understand the mechanisms by which these splice variants contribute to cancer development. In cancer, one of the most studied genes with numerous reported splice variants is the murine double minute 2 (MDM2).

MDM2, also known as HDM2 in humans, is an oncoprotein that functions as a negative regulator of the p53 tumor suppressor protein. MDM2 antagonizes p53 functions in two ways, firstly, by binding to, and antagonising the transactivation activity of the p53 protein and second, by acting as an E3 ubiquitin ligase that labels the p53 protein for degradation. MDM2 is amplified or over-expressed in one third of the human sarcomas and the over-expression of MDM2 is associated with poor prognosis. MDM2 can also regulate the cell cycle in a p53-independent manner by binding with other partners including the retinoblastoma protein and the transcription factor E2F1.

The occurrence of MDM2 splice variants has been reported in many types of cancers. Since the first report of five alternatively spliced MDM2 variants in ovarian and bladder carcinoma, there are currently more than 40 different splice variants that are associated with diverse types of cancers, including breast, ovarian, bladder, lung, soft tissue sarcoma, liposarcomas, pediatric rhabdomyosarcoma, salivary gland tumors and glioblastoma. Some of

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the MDM2 variants promote tumorigenesis in an extent that is similar to full-length MDM2. Therefore, there is a need to identify and characterize the function of cancer-associated MDM2 splice variants.

MDM2 have been previously implicated in the development of oral squamous cell carcinoma (OSCC). Although a few reports have suggested the presence of MDM2 splice variants in OSCC, none have systematically identified and characterized MDM2 splice variants in OSCC. In this study, we have identified a number of OSCC MDM2 splice variants at the mRNA level and we also demonstrated the correlation between the presence of MDM2 splice variants with p53 mutation, as well as tumor characteristics and patient outcome.

Materials and methods

Patient and tissue samples

Fifty-five OSCC tissues and 20 normal oral mucosa (NOM) specimens were used in this study. OSCC specimens were obtained from patients who had not received any treatment prior to surgery. NOM tissues were obtained from the gingiva of unaffected individuals who had their wisdom tooth removed. Reference slides were made from these tissues to confirm diagnosis and to gauge the percentage of epithelial or tumor cells. The tissue specimens were macrodisected to ensure that there were more than 70% tumor cells (for OSCC specimens) and more than 70% epithelial cells (for NOM specimens), as described previously. RNA was extracted using the RNeasy micro kit (Qiagen, USA) according to the manufacturer’s recommendation. cDNA was synthesized from total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA) in a total volume of 100 l as described in detail previously. Socio-demographic information was obtained from the Malaysian Oral Cancer Database and Tissue Bank System (MOCDTBS). Written consent was obtained before any tissues were collected and this study was approved by the University of Malaya ethical review board (Ethical Approval Code: DF OP 03/06/0018/(L)).

Polymerase chain reaction (PCR)

The open reading frame of MDM2 was amplified as described by others. PCR amplification was performed in a MJ Research PTC-100™ Peltier Thermal Cycler (Bio-Rad, CA, USA) with a total reaction mixture of 25 l consisting of 1.5 mM MgCl₂, 1 μM of each primer sense 5’-TGGGGAGTCTTGAGGGACC-3’ and antisense 5’-CAGGTGTCTAAATTCTCAG-3’, 0.4 mM dNTP, 2.5 units Taq polymerase (Promega, WI, USA) and 1.5 μl cDNA. The PCR conditions were 94 °C (2 min), followed by 30 cycles of 94 °C (1 min), 58 °C (1 min) and 72 °C (2 min) with the final extension at 72 °C for 5 min. Following the first PCR, nested PCR was performed using 2 l from the first PCR reaction as described above using these primers: sense 5’-CTGAGAAGAAGGCTAAAGTA-3’ and antisense 5’-CTCTTAGACAGTCAACTTAG-3’. The PCR products were electrophoresed...
The distribution of the MDM2 in-frame and out-of-frame splice variants found in OSCC and NOM. The majority of the in-frame splice variants were found in OSCC samples, three were found in NOM and only one was found in both OSCC and NOM samples.

Figure 1b The distribution of the MDM2 in-frame and out-of-frame splice variants found in OSCC and NOM. The majority of the in-frame splice variants were found in OSCC samples, three were found in NOM and only one was found in both OSCC and NOM.
on a 1.5% (w/v) agarose gel and stained with ethidium bromide. Visible bands were excised and DNA was purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany).

**Cloning and sequencing analysis**

Purified PCR products were cloned into TA cloning vector pTZ57R/T (Fermentas, USA) according to the manufacturer’s recommendations. Briefly, the purified DNA was ligated to the cloning vector pTZ57R/T in an insert: vector ratio of 3:1 in 20 µl reaction volumes and incubated at 22 °C for 1 h. Five microlitres of the mixture was transformed into *Escherichia coli* DH5α competent cells, and selection was done on LB-ampicillin (50 µg/ml) plates with X-Gal-IPTG for blue white screening. Upon overnight incubation, white colonies were subjected to colony PCR and the selected recombinant plasmids were isolated using the Qiaprep Spin Miniprep Kit (Qiagen, Hilden, Germany). The inserts within the recombinant plasmids were sequenced with the BigDye™ Terminator v3.1 Cycle Sequencing kit. Cycle sequencing was performed in a final volume of 5 µl consisting of 1X sequencing buffer, 5 µM of either M13 forward universal primer or M13 reverse universal primer, 100 ng of recombinant plasmid and 0.5 Unit of BigDye™ Terminator v3.1. The cycle sequencing products were electrophoresed on the ABI 3730XL DNA Analyzer (Applied Biosystems, CA, USA), sequences assembled with the Staden Package22 and compared to the full length MDM2 mRNA sequence (NM_002392.2) for the identification of splice variants, or with sequences of known MDM2 splice variants that were available from the NCBI database to determine the novelty of the splice variants. The nucleotide sequences were then translated to amino acid sequence and analyzed with the ClustalW2 software (EBI, Cambridge, UK) to determine whether the splice variants code for in-frame proteins.

**Figure 2a** Schematic representation of the full length MDM2 protein and the in-frame splice variants which have lost the p53 binding domain.
Statistical analysis was performed using the statistical software package SPSS 16 (SPSS Inc., Chicago, IL, USA). Fisher Exact tests and Chi-square tests were used to investigate the presence of MDM2 splice variants with various clinicopathological parameters. The odds ratios and 95% confidence intervals (CIs) of MDM2 splice variants on OSCC were computed. A \( p \)-value < 0.05 was considered to be statistically significant.

Results

MDM2 splice variants are present in both OSCC and NOM tissue

MDM2 splice variants were detected in 49/55 (89.1%) and 9/20 (45.0%) of OSCC and NOM tissues respectively. All of the cloned PCR products were confirmed to originate from the MDM2 mRNA sequence with specific blocks of the sequences spliced out, generating unique splice variants of MDM2. In total, 38 different splice variants were found in this study, 3 were present in both OSCC and NOM tissues and these were novel variants with the lengths of 313 bp, 544 bp and 660 bp (MYO-6, MYO-19 and MYO-25; Table 1). Thirty of the thirty eight (30/38) splice variants were identified exclusively in OSCC, whilst another 5 were found only in NOM tissues. From the 30 splice variants that were found exclusively in OSCC, 4 were MDM2 splice variants that have been described previously as MDM2B (657 bp), MDM2C (966 bp), MDM-PM2 (393 bp) and MDM2-EU2 (297 bp). The MDM2B splice variant which was detected in 12 OSCC samples was the most frequently detected variant, whilst MDM2C, MDM2-PM2 and MDM2-EU2 were detected in 3, 3 and 1 OSCC samples respectively. None of these variants were detected in the NOM tissues. Amongst the five variants that were present in NOM tissues, one was a previously identified variant named MDM2-KB9 (732 bp). The sequences of the remaining 33 splice variants (26 variants in OSCC, 4 in NOM tissues and 3 in both OSCC and NOM tissues) were novel as these sequences had not been reported in the literature nor found in GenBank and EMBL-EBI. The frequencies of the splice variants identified in this study and their distribution in OSCC and NOM tissues are summarized in Table 1 and Fig. 1a. The full-length transcript of MDM2 is always detectable in both OSCC and NOM specimens. The sequences of novel in-frame splice variants have been submitted to GenBank as indicated in Table 1.

Characteristics of the MDM2 splice variants

As many of the splice variants identified here were novel, we used ClustalW2 to align the amino acid sequences from the novel splice variants against that of the wild type MDM2 to determine if these splice variants code for in-frame proteins. We found that 19/38 (50%) splice variants had sequences which were in-frame and could potentially code for proteins. Of the 19 in-frame splice variants, 1 is common for both OSCC and NOM, 15 and 3 were found exclusively in OSCC and NOM respectively (Table 1, Figs. 1a and 1b).

We further characterized the domains that were present in the MDM2 splice variants identified here. Looking at the 19 splice variants that were predicted to code for in-frame proteins (including

Table 2

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>OSCC</th>
<th>NOM</th>
<th>p-Value</th>
<th>95% Confidence intervals</th>
<th>OR</th>
<th>Lower</th>
<th>Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Splice variants detected</td>
<td>49 (89.1)</td>
<td>9 (45.0)</td>
<td>&lt;0.001*</td>
<td>9.98</td>
<td>2.94</td>
<td>33.90</td>
<td></td>
</tr>
<tr>
<td>No variants detected</td>
<td>6 (10.9)</td>
<td>11 (55.0)</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In-frame variants</td>
<td>33 (60.0)</td>
<td>7 (35.0)</td>
<td>&lt;0.001*</td>
<td>8.64</td>
<td>2.39</td>
<td>31.28</td>
<td></td>
</tr>
<tr>
<td>Out-of-frame variants</td>
<td>16 (29.1)</td>
<td>2 (10.0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No variants detected</td>
<td>6 (10.9)</td>
<td>11 (55.0)</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDM2B present</td>
<td>12 (21.8)</td>
<td>0 (0)</td>
<td>&lt;0.001*</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Other variants detected</td>
<td>37 (67.3)</td>
<td>9 (45.0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>No variants detected</td>
<td>6 (10.9)</td>
<td>11 (55.0)</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

* Odds ratio was not computed due to zero cell size.

* \( p \)-Value < 0.05.
those previously reported), 14/15 (93.3%) OSCC splice variants were found to have lost the p53 binding domain whilst in 1/3 (33.3%) splice variants from the NOM had lost this domain (Figs. 1b, 2a and 2b). The nuclear localization signal (NLS), nuclear export signal (NES) and acidic domains of MDM2 also appeared to be lost in the majority of the variants both in OSCC (14/15; 93.3%) and NOM (3/3; 100%). Interestingly, MDM2 splice variants almost always retained their RING binding domain completely, both in OSCC (13/15; 86.7%) and NOM (3/3; 100%) (Figs. 2a and 2b). The only in-frame splice variant that was found in both OSCC and NOM (MYO-25) retained completely intact p53 binding, NLS and NES domains but had lost the RING binding domain (Fig. 2b).

The presence of MDM2 splice variants is significantly associated with OSCC

We demonstrated that twice as many OSCC samples (89.1%) contained MDM2 splice variants compared to NOM tissues (45.0%), and that the presence of MDM2 is significantly associated with OSCC (p < 0.001; Table 2). Individuals who have MDM2 splice variants have a 9.98 times increase in chance (OR, 95% CI = 2.94–33.90) of developing OSCC. Further, the presence of in-frame splice variants was also significantly associated with OSCC (p < 0.001) and these splice variants increased the risk to OSCC by 8.64 times (OR, 95% CI = 2.39–31.28; Table 2). Notably, the MDM2B splice variant which was the most frequently detected variant in OSCC (MYO-25) retained completely intact p53 binding, NLS and NES domains but was not observed in any of the NOM samples (p < 0.001).

We further determined if the presence of splice variants was associated with specific clinico-pathological characteristics. Only lymph nodes status was significantly associated with the presence of MDM2 splice variants (p = 0.02; Table 3), in which the sensitivity of MDM2 splice variants in predicting lymph nodes metastases was 100% (25/25) and the specificity was 21.7% (5/23). Neither the presence of MDM2 splice variants nor the types of variants (in-frame vs. out-of-frame splice variants; loss of p53 binding domain vs. intact p53 binding domain splice variants) were associated with the presence of p53 mutations (taking into account disruptive and non-disruptive p53 mutations as well as the site of mutation; Table 3). Interestingly, we found that the presence of MDM2B splice variant is significantly associated with Broder’s grading (p = 0.043; Table 3).

Discussion

MDM2 is one of the most commonly spliced genes with more than 40 MDM2 splice variants identified in both normal and tumor tissues.23 Despite the frequent reports of MDM2 splice variants in solid tumors, this is the first study to characterize MDM2 splice variants extensively in OSCC. In this study, we demonstrated that MDM2 splice variants are frequently found in OSCC and that the number of unique MDM2 splice variants is much higher in OSCC compared to NOM. This is consistent with the identification of MDM2 splice variants in a variety of different types of cancers including ovarian, breast cancer, soft tissue sarcoma and pediatric rhabdomyosarcomas with the frequency of detection ranging between 34% and 82%.7,11,24 Although the sample sizes of OSCC (n = 55) and NOM (n = 20) may not be large, a post-hoc analysis yielded a power of 97% to detect the percentage of splice variants found in this study. Notably, normal tissues (including breast, lung and renal) have also been reported to harbor MDM2 splice variants suggesting that these variants may have normal physiological functions that are yet unknown.4,25 However, as demonstrated by our study and others, it is evident that certain splice variants are found exclusively in tumor tissues, suggesting that they could play a significant role in the development of cancer. Indeed, in breast cancer, the presence of MDM2 splice variants has been associated with lymph node metastasis.26 Moreover, MDM2 splice variants were also more frequently observed in glioblastoma multiforme.
compared to lower grade of astrocytic tumors, suggesting that the presence of MDM2 splice variants is frequently linked with more aggressive cancers.

Although many MDM2 splice variants have been discovered in different types of cancer, their functions are however, yet to be fully understood.3 The binding partners of the different domains of MDM2 and their roles have been previously delineated7–25 and therefore, we examined the splice variants to determine which domains were retained. We analyzed only MDM2 splice variants that were in-frame, as out-of-frame variants which have a shift from the original open reading frame that culminates in premature stop codons, are generally targeted by the nonsense-mediated mRNA decay (NMD) mechanisms.36 Consistent with MDM2 splice variants that are found in other cancers, the majority of splice variants in OSCC including the known splice variants detected here have lost the p53 binding domain. By contrast, only a third of the splice variants found in NOD lost the same domain. Furthermore, all except one MDM2 splice variant in OSCC have lost large chunks of mRNA sequence between the NLS and the zinc finger domains, which are both important for protein shuttling between the nucleus and cytoplasm, and the interaction with p14ARF, CBP/p300, RB and L5,6,31–34 However, the RING finger domain which is necessary for the E3 ubiquitin protein ligase and RNA binding activity31,35,36 of MDM2 is retained in all but two of the splice variants found in OSCC. The function of MDM2 splice variants is still unclear and to date, very few splice variants have been characterized for their function. MDM2B which is the most frequently observed splice variant in this study, remains the most studied. MDM2 variants including MDM2A, MDM2B, MDM2C, MDM2D and MDM2E harbor oncogenic properties and are able to transform foci at high frequencies in NIH3T3 cells.11 MDM2 variants (in particular MDM2B, 2C and 2D) have also been shown to drive proliferation and enhance tumorigenesis to the same extent as full length MDM2 independent of its p53 status.15,37 Notably however, despite having lost the p53 binding domain, MDM2 splice variants (MDM2B) can induce p53-dependent growth inhibition through the binding of the full length MDM2,38,39 It appears that MDM2 splice variants can be both tumor suppressing and promoting and this probably occurs under different genetic backgrounds. For example, the MDM2A splice variant appears to have suppressive properties in a p53 wild-type background but enhances transformation when p53 is absent.40 It is perhaps not too surprising that MDM2 splice variants have both growth suppressive and oncogenic functions, as full length MDM2 have been shown to have both these properties as well.41,42 In our study, the majority (13/15) of in-frame MDM2 splice variants found in OSCC lost the p53 binding domain, and retained very similar domains and sequences to those described above, however, given that the same splice variants can have different roles (albeit only a small number of them have been studied), the splice variants identified in this study warrant further investigation with regards to their function, and work in this area is on-going. Although the direct roles of these splice variants remains to be determined their consistent occurrence in OSCC and other cancers strongly suggest that they could contribute to tumorigenesis. In particular, we demonstrated that the presence of splice variants appear to be highly associated with OSCC and the data suggests that the presence of MDM2 splice variants increases risk to OSCC, therefore it would be interesting to determine whether these splice variants are tissue specific and whether they are markers of OSCC susceptibility. The discovery of novel MDM2 splice variants opens up many opportunities to determine if these novel splice variants code for functional proteins, how these putative proteins may modulate tumorigenesis and more importantly, in what genetic context do these variants function, and in parallel, investigations into how they may be useful in the diagnosis and treatment of OSCC.

Conflict of interest statement

None declared.

Role of fund provider

The Ministry of Science, Technology and Innovation, Malaysia provided the funds for this research and monitored the progress of the research project.

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