Immunohistochemical Analysis for Mdm2 and p53 Proteins in Methylcholanthrene-Induced Mouse Rhabdomyosarcomas

Haiyan WU and Makoto INOUE

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ABSTRACT. 3-methylcholanthrene (MC)-induced mouse 10 embryonal (ERSs) and 24 pleomorphic rhabdomyosarcomas (PRSs) of the dermis were examined immunohistochemically for nuclear reactivity of Mdm2, p53, and proliferating cell nuclear antigen (PCNA). ERSs were microscopically present in the rhabdium layer of the dermis from 10 to 13 weeks post injection (PI), and PRSs developed from 13 weeks PI. Moderate to marked Mdm2 reactivity was observed in each of the 10 ERSs, and 23 of the 24 PRSs. Moderate to marked p53 reactivity was observed in 5 of the 10 ERSs, and 19 of the 24 PRSs. p53 reactivity increased in PRSs compared with ERSs. The level of Mdm2 expression was significantly higher compared with p53 expression. Discordant Mdm2 overexpression was observed in 5 ERSs and 5 PRSs, and discordant p53 overexpression was observed in 1 PRS, although co-overexpression of Mdm2 and p53 was observed in 5 ERSs and 18 PRSs. PCNA reactivity significantly increased in PRSs compared with ERSs. These results suggest that Mdm2 overexpression is an important pathogenic event in MC-induced mouse rhabdomyosarcomas, and its expression may be induced by p53-independent pathway.

Key words: MC, Mdm2, mouse, p53, rhabdomyosarcoma.


The Mdm2 oncogene was first identified as an amplified gene on a murine double-minute chromosome in the 3T3DM cell line, a spontaneously transformed derivative of BALB/c 3T3 cells [3]. Mdm2 has been shown to interact with a group of cell cycle-related proteins, including the retinoblastoma protein [29], E2F1 and DP1 transcription factors [14], and transforming growth factor-beta [23]. Mdm2 overexpression is frequent in soft tissue and bone tumors in human beings [2, 8, 13, 30] and dogs [16, 22].

The tumor suppressor gene p53 plays a key role in the control of the cell cycle, maintenance of genomic stability, and programmed cell death [12, 27]. p53 mutations are common in methylcholanthrene-induced mouse tumors [4], and have been identified in several types of human neoplasms [18, 21]. The Mdm2 protein directly binds to p53 protein and inhibits the transcriptional function of p53 protein and also targets it for degradation via the ubiquitin-proteasome pathway [15, 27].

Rhabdomyosarcoma is the most common malignant soft tissue sarcoma in childhood and adolescence. Recently, it has been reported that Mdm2 is expressed at low levels and does not show differences between subtypes of rhabdomyosarcomas of human beings [11, 24, 25]. However, little is known about the mechanism of tumorigenesis of rhabdomyosarcoma. Expression of Mdm2 and p53 has not been investigated in methylcholanthrene-induced rhabdomyosarcomas, although expression of some differentiation markers such as vimentin, desmin, and myoglobin has been studied [9, 26]. Thirty-four rhabdomyosarcomas were induced by subcutaneous injection of 3-methylcholanthrene (MC) in C3H/HeJ mice. In this study, we examined nuclear reactivity of Mdm2 and p53 proteins by immunohistochemistry in MC-induced mouse rhabdomyosarcomas, and discussed the relationships between expression of these proteins and tumorigenesis.

MATERIALS AND METHODS

Mice and tumor induction: Endotoxin-resistant and highly tumor susceptible C3H/HeJ mice were purchased from Nippon Clea Co. Ltd. (Tokyo, Japan). Forty-seven four-week-old mice were injected subcutaneously on the back at a single site with 0.2 mg of MC dissolved in corn oil at a concentration of 2 mg/ml, and five normal mice were used as controls. Mice were observed for tumor development and progression over the course of 2–22 weeks. Experiments were performed according to the guidelines for Animal Experimentation of Yamaguchi University.

Histological examination: Tumors were detected in 43 of the 47 MC-treated mice at the site of injection. They were 3 papillomas, 4 squamous cell carcinomas, 34 rhabdomyosarcomas, and 2 complex type tumors (composed of both squamous and rhabdium components). This study was carried out with 34 rhabdomyosarcomas except for papillomas and squamous cell carcinomas and five normal skin tissues obtained from non-MC-injected mice. Mice were euthanized by pentobarbital sodium, and tumor tissues were removed. Tissue samples were initially fixed in buffered formalin prior to embedding in paraffin. Sections from each sample were stained with hematoxylin and eosin and phosphotungstic acid hematoxylin (PTAH), and assigned for histopathologic diagnosis and classification according to the

* Correspondence to: Dr. INOUE, M., Department of Veterinary Pathology, Faculty of Agriculture, Yamaguchi University, 1677–1 Yoshida, Yamaguchi 753–8515, Japan.
criteria established by Parham [17].

**Immunohistochemistry and scoring:** Serial 5-µm sections were mounted on amino-silane-coated slides. The sections were dewaxed and microwave-pretreated in 10 mM citrate buffer, pH 6.0 for 6 min. All the steps of the reaction were conducted at 25°C. Endogenous peroxidase was quenched by immersion in 3% hydrogen peroxide. For Mdm2 and proliferating cell nuclear antigen (PCNA) reaction, an amino acid polymer-peroxidase simple staining method for mouse tissue and mouse primary antibody was used. Endogenous mouse immunoglobulin was blocked with blocking solution (Nichirei Co. Ltd., Tokyo, Japan) for 1 hr, and the sections were incubated for 2 hr with mouse anti-human Mdm2 monoclonal antibody (SMP14), 200 µg/ml, 1:50 dilution (Santa Cruz Biotechnology, Santa Cruz, U.S.A.) or mouse anti-rat PCNA antibody (Novocastra Laboratories Ltd., Newcastle, UK), 200 µg/ml, 1:50 dilution, and then were incubated for 10 min with peroxidase conjugated goat anti-mouse IgG antibody-amino acid polymer (Nichirei Co., Ltd.). For p53 reaction, a biotin-streptavidin-immunoperoxidase method was used. The sections were blocked with normal goat serum (Nichirei Co., Ltd.), 1:10 dilution for 10 min, and incubated with rabbit anti-human p53 polyclonal antibody (FL-393), 200 µg/ml, 1:50 dilution (Santa Cruz Biotechnology) for 30 min. Biotinylated goat anti-rabbit IgG antibody, 1:500 dilution and peroxidase-conjugated streptavidin (Nichirei Co., Ltd.) were applied to the tissues for 10 min, respectively. 3-amino-9-ethylcarbazol (AEC) was used as the substrate chromogen. Sections were counterstained with hematoxylin. Non-immune rabbit or mouse sera, sources of irrelevant primary antibodies, were used as negative controls. To quantify the reaction, positive cells in 100 fields at × 400 magnification were counted; counts were averaged and expressed as percentages. The extent of Mdm2, p53, and PCNA nuclear reactivity were classified as either absent, mild (1 to 10% of tumor cells), moderate (11 to 50%) or marked (51 to 100%).

**Statistical analysis:** The correlation between Mdm2 and p53 reactivity was evaluated by chi-square tests. A p-value less than 0.05 was considered statistically significant.

**RESULTS**

**Histological examination of the rhabdomyosarcomas:** Histological examination revealed 10 embryonal (ERSs) and 24 pleomorphic rhabdomyosarcomas (PRSs). ERSs were microscopically present in the rhabdium layer of the dermis from 10 to 13 weeks post injection (PI), and PRSs developed from 13 weeks PI. In ERSs, small and round undifferentiated primitive cells and short spindle-shaped myoblast-like cells were common. Cross-striations were revealed in a few neoplastic cells with PTAH staining. PRSs were mainly composed of large round, polygonal, or elongated cells with abundant eosionophilic cytoplasm, and multinucleated cells and mitotic cells were common. Cross-striations were revealed in these cells in various degrees with PTAH staining. PRSs showed more aggressive behavior when compared their invasive growth and high frequent mitosis with ERSs. No metastatic lesions were observed in the tumors.

The results of immunohistochemical Mdm2, p53, and PCNA nuclear reactivity are summarized in Table 1. The percentage of Mdm2- or p53-reactive tumor cells in each of the cases is described.

**Analysis of Mdm2 protein:** Normal rhabdium cells in the dermis of 5 controls did not have detectable Mdm2 reactivity. Six (51 to 64% of tumor cells) of the 10 ERSs showed marked reactivity and 4 (25, 26, 42, 46%) showed moderate reactivity. Eleven (51 to 81%) of the 24 PRSs showed marked reactivity (Fig. 1a) and 12 (11 to 47%) showed moderate reactivity; 1 (9%) showed mild reactivity.

**Analysis of p53 protein:** Normal rhabdium cells in the dermis of 5 controls did not have detectable p53 reactivity. Five (17–44% of tumor cells) of the 10 ERSs showed moderate reactivity; and 5 (0–9%) showed mild or no reactivity. Seventeen (15–33%) of the 24 PRSs showed moderate reactivity, and 2 (53, 61%) showed marked reactivity (Fig. 1b); and 5 (0–9%) showed mild or no reactivity. p53 nuclear reactivity increased in PRSs compared with ERSs, although this difference never became statistically significant (P>0.08).

The relationship between Mdm2 and p53 expression in each of the cases is summarized as percentage of Mdm2- and p53-positive tumor cells in Table 2. The cases with moderate to marked Mdm2 and p53 reactivity were regarded as overexpression. The level of Mdm2 reactivity was significantly higher compared with p53 reactivity both in ERSs and PRSs (P<0.01). Discordant Mdm2 overexpression was observed in 5 ERSs and 5 PRSs, and discordant p53 overexpression was observed in 1 PRS, although co-overexpression of Mdm2 and p53 was observed in 5 ERSs and 18 PRSs. There was no correlation between Mdm2 and p53 expression both in ERSs and PRSs.

**Analysis of PCNA:** One (61%) of the 10 ERSs showed marked PCNA reactivity, 6 (22–48%) showed moderate, and 3 (8, 9, 9%) showed mild. Ten (51–67%) of the 24 PRSs showed marked and 14 (18–45%) showed moderate.

<table>
<thead>
<tr>
<th>Tumors</th>
<th>No. Mice</th>
<th>Mdm2</th>
<th>p53</th>
<th>PCNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>−</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>ERSs</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PRSs</td>
<td>24</td>
<td>0</td>
<td>1</td>
<td>12</td>
</tr>
</tbody>
</table>

a) − = absent; + = mild (1–10%); ++ = moderate (11–50%); +++ = marked (51–100%).

b) The number of cases.
PCNA reactivity was significantly higher in PRSs than in ERSs (P<0.01).

**DISCUSSION**

In this study, ERSs were microscopically present in the rhabdium layer of the dermis from 10 to 13 weeks PI, and PRSs developed from 13 weeks PI. Morphologically, ERSs were mainly composed of myoblast-like and undifferentiated primitive cells, and PRSs were mainly composed of large round, polygonal, or elongated cells with abundant eosinophilic cytoplasm, and multinucleated cells and mitotic cells were common. PCNA reactivity was significantly higher in PRSs than in ERSs (P<0.01). A significant difference of malignancy was observed between ERSs and PRSs. Although it has been reported that rhabdomyosarcomas appear 70 days after treatment with MC [5], no serial detection for the tumor progression has been reported. Hirai et al. [6] have reported that epidermal hyperplastic and papillomatous lesions have progressed into squamous cell carcinomas in MC-induced mouse epidermal tumorigenesis. Thus, it is considered that ERS may develop into PRS as time passes in the MC-induced mouse rhabdomyosarcomas.

High levels of Mdm2 overexpression were observed both in MC-induced mouse ERSs and PRSs, but not in normal rhabdium cells in the dermis. PRSs showed more aggressive behavior when compared their invasive growth and high frequent mitosis with ERSs, and PCNA labeling indices were significantly higher in PRSs than in ERSs (P<0.01). These results suggest that Mdm2 overexpression is an important pathogenic event in rhabdomyosarcomas induced by MC, but is not directly associated with tumor malignancy. It has been reported that Mdm2 overexpression is frequent in soft tissue sarcomas of high grade [7, 19, 24, 30]. However, recent studies have suggested that Mdm2 gene amplification and protein overexpression are extremely low in human pediatric rhabdomyosarcomas [11, 25]. Further studies are needed to investigate the role of Mdm2 in myogenic tumorigenesis.

p53 overexpression was observed in five of the 10 ERSs and 19 of the 24 PRSs. This result suggests that p53 overexpression is higher in PRSs than ERSs (P<0.01).

**Table 2. Relationship between Mdm2 and p53 immunoreactivity in MC-induced mouse rhabdomyosarcomas**

<table>
<thead>
<tr>
<th>Mdm2</th>
<th>No. of cases</th>
<th>p53</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>ERSs</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>++</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>+++</td>
<td>11</td>
<td>2</td>
</tr>
</tbody>
</table>

a) = absent; + = mild; ++ = moderate; +++ = marked.

b) The number of cases.

The level of Mdm2 expression was significantly higher compared with p53 expression. Discordant Mdm2 overexpression was observed in 5 of the 10 ERSs and 5 of the 24 PRSs, and discordant p53 overexpression was observed in 1 PRS, although co-overexpression of Mdm2 and p53 was observed in 5 ERSs and 18 PRSs. These results suggest that Mdm2 expression may be induced by p53-independent pathway in MC-induced rhabdomyosarcomas. It has been
reported that Mdm2 protein is overexpressed without p53 mutation in human osteosarcomas [18] and malignant fibrosarcomas and liposarcomas [10]. On the other hand, concomitant expression of Mdm2 and p53 has been demonstrated in human soft tissue sarcomas, and correlated with aggressive tumor behavior and poor prognosis [24, 28].

In conclusion, Mdm2 overexpression is an important pathogenic event in MC-induced rhabdomyosarcomas, and its expression may be induced by p53-independent pathway.

REFERENCES

