MDM4 expression as an indicator of TP53 reactivation by combined targeting of MDM2 and MDM4 in cancer cells without TP53 mutation

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ABSTRACT

MDM2 and MDM4, a structurally related MDM2 homolog, negatively regulates expression and functions of TP53 tumor suppressor gene. To explore the precise expression patterns and function of MDM2 and MDM4 in wild-type (wt) TP53 cancer cells, we analyzed 11 various cancer cell lines with wt TP53. All cell lines exhibited deregulated expression of MDM2 and MDM4, and were divided into two distinct types; the one expressing high levels of MDM4 and another expressing low levels of MDM4. The low MDM4 type expressed higher MDM2 levels than the high MDM4 type. In cells with high MDM4 expression, knockdown of MDM4 or MDM2 reactivated TP53, and simultaneous knockdown of MDM2 and MDM4 synergistically reactivated TP53. In contrast, in cells with low MDM4 expression, knockdown of only MDM2 reactivated TP53. These results suggest that both MDM2 and MDM4 are closely involved in TP53 inactivation in cancer cells with high MDM4 expression, whereas only MDM2, and not MDM4, is a regulator of TP53 in cells with low MDM4 expression. MDM4 expression in wt TP53-tumors is a potential indicator for TP53 reactivation cancer therapy by simultaneous targeting of MDM4 and MDM2. Specific knockdown of MDM2 and MDM4 might be applicable for TP53 restoration therapy.

INTRODUCTION

The tumor suppressor protein p53 is a transcriptional factor that controls multiple genes to regulate the cell cycle, apoptosis, DNA repair, and senescence [1-4]. Approximately half of human cancers have mutations in the TP53 gene [5], indicating that TP53 inactivation is pivotal in cancer development. The remaining cancers retain the wild-type (wt) status of TP53, which is inhibited by deregulated upstream modulators and/or inactivation of downstream effectors [1, 6].

The human homolog of murine double minute 2 (MDM2) is a major negative regulator of p53 through binding to its transactivation domain, thereby resulting in subsequent suppression of transcriptional activity [7, 8]. In addition, the RING (Really Interesting New Gene) finger domain of MDM2 functions as an E3 ubiquitin ligase that mediates ubiquitin-dependent degradation of p53 [9-11]. MDM2 is a transcriptional target of p53, forming an autoregulatory feedback loop [12, 13]. TP53 is also negatively regulated by MDM4, an MDM2 homologue [14, 15]. Like MDM2, MDM4 represses p53 transcriptional activity by direct binding of its binding domain, which is located in the N-terminal region, to the transactivation domain of p53 [14]. Although MDM4 possesses a RING finger domain, it lacks E3 ligase activity and is unable to directly decrease p53 stability [14], but rather enhances the E3 ligase activity toward p53 by forming a heterodimer with MDM2 via the RING domains of both molecules [16, 17]. MDM2 also destabilizes the structure of MDM4 via ubiquitination [18]. Both MDM2 and MDM4 function as oncogenes and their deregulated expression has been reported in various types of human cancers, including soft tissue sarcoma, breast cancer,
retinoblastoma, and melanoma [19-23]. However, to date, the expression patterns and functional roles of MDM2 and MDM4 in cancer cells with or without TP53 mutations remain uncertain.

Restoration of wt TP53 function in tumors leads to rapid tumor regression by induction of apoptosis or senescence and can be applicable to cancer treatment [19]. Several small molecular inhibitors of the interactions between MDM2 and p53 have been shown to restore TP53 activity in tumors expressing high MDM2 levels [24-26]. Similarly, MDM4 antagonists have been reported. Among them, SAH-p53-8 binds and inhibits more efficiently to MDM4 than to MDM2 and exerts antitumor effects in cancer cells expressing high MDM4 levels [21, 27].

Synthetic small interfering RNAs (siRNAs) are not only a powerful tool for functional gene analysis [28, 29], but has been intensively explored for application to therapy of human cancer and other diseases with some promising results [30-32]. siRNAs often silence the expression of untargeted genes with partial sequence complementarities (off-target effects) [33, 34]. However, such nonspecific effects can be avoided by DNA replacement in the seed region of the guide strand (first 6–8 bases from the 5′ end) and the complementary sequences of the passenger strand, which has been designated as a double-stranded RNA–DNA chimera (dsRDC) [35]. Considering the recent progress in RNAi technology, synthetic siRNAs targeting MDM2 and MDM4 may present an alternative mechanism to induce TP53 restoration.

In the present study, we carefully analyzed MDM2 and MDM4 expression levels in various cancer cell lines with and without TP53 mutations and found that MDM2 and MDM4 were deregulated in all wt TP53 cancer cells. To probe the roles of MDM2 and MDM4 in TP53 regulation in cancer cells, we selected efficient and specific dsRDC-modified siRNAs targeting MDM2 and MDM4. Individual and combined knockdown of MDM2 and MDM4 revealed their roles in TP53 inactivation in wt TP53 cancer cells with different patterns of MDM2 and MDM4 expression, which provided us with a rationale for the selection of MDM2 and MDM4 as targets in TP53 restoration therapy of cancers.

RESULTS

Expression levels of MDM2 and MDM4 in cancer cell lines

We examined the expression levels of MDM2 and MDM4 in 14 cancer cell lines including 11 wt TP53 and three mutant (mt) TP53 cell lines by immunoblotting (Figure 1). wt TP53 cell lines were divided into two groups according to levels of MDM4: seven cell lines (MCF-7, A375, SNU-1, HCT116, NUGC-4, LoVo, and A549) expressed high levels of MDM4, whereas the remaining four cell lines (SJSA-1, HepG2, HuH-6, and C32TG) expressed low levels of MDM4. Interestingly, all cell lines expressing low MDM4 levels accumulated higher levels of MDM2 than those expressing high MDM4 levels. Cell lines carrying mt TP53 (KATOIII, NUGC-3, and DLD-1) expressed various levels of MDM4 and MDM2. p53 was not detected in KATOIII cells, which harbored gross deletions of both TP53 alleles.

Efficient siRNAs targeting MDM2 and their DNA-modified forms

Seventeen new siRNAs targeting human MDM2 transcript variant 1 (NM_002392.4) were selected using siDirect software (Supplementary Table 1) [36]. These siRNAs contained at least three mismatched base pairs in both the guide and passenger strands with a non-redundant sequence set of human genes to minimize off-target effects [37]. siRNA sequences containing single-nucleotide polymorphisms were also excluded to avoid individual differences in response. These new siRNAs targeting MDM2 (siMDM2) and nine previously reported siMDM2s were synthesized and tested for knockdown efficiency by transfection into SJSA-1 cells and subsequent immunoblot analysis (Figure 2a) (Supplementary Figure 1). Six new (1068, 830, 480, 691, 1489, and 2381) and two previously reported siMDM2s (396 and 851) strongly suppressed MDM2 expression. These siMDM2s were converted to dsRDCs with the aim to further reduce off-target effects by decreasing the free energy of pairing stability between the seed region and off-target mRNAs [35]. As shown in Figure 2b, all dsRDC-modified siMDM2s (chiMDM2) were able to silence MDM2 expression with the most efficient silencing achieved by chiMDM2-1489. Quantitative reverse transcription (qRT)-PCR analysis demonstrated the ability of these chiMDM2s to knockdown mRNA to the same or a slightly reduced

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**Figure 1: Expression levels of p53, MDM2, and MDM4 in cancer cell lines.** Expression levels of p53, MDM2, and MDM4 were examined in 14 cancer cell lines (11 wt TP53 cell lines and 3 mt TP53 cell lines) by immunoblotting. SE, short exposure; LE, long exposure.
extent as compared with cognate siRNAs (Supplementary Figure 2).

The effect of chiMDM2s on the growth of cancer cells with high MDM2 expression was examined. SJSAtlanta cells were transfected with chiMDM2s at 1 nM for 5 days and then subjected to the WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenoxy)-5-(2,4-disulfophenyl)-2H-tetrazolium) cell proliferation assay. As shown in Figure 2c, most of the chiMDM2s suppressed the growth of SJSAtlanta cells in proportion to the individual MDM2 knockdown efficiency, with the exception of chiMDM2-1068, which suppressed cell growth to a greater extent than chiMDM2-1489, although the MDM2 knockdown efficiency was inversed, suggesting that chiMDM2-1068 partially exerted MDM2–p53-independent growth suppression. Therefore, these two chiMDM2s were further analyzed for growth suppression of cancer cells carrying mt TP53 (KATO III, NUGC-3, and DLD-1) (Figure 2d). chiMDM2-1489 and chiMDM2-1068 exhibited negligible effects on these cells, with the exception of chiMDM2-1068-mediated suppression of NUGC-3 cell growth.

Selection of siRNAs targeting MDM4 and their DNA-modified forms

siRNAs targeting the coding region of human MDM4 transcript variant 1 (NM_002393.4) were similarly selected as those targeting MDM2. Ten new

Figure 2: Effects of siRNAs targeting MDM2 and their dsRDC forms on MDM2 expression and cell growth. (a) Two previously reported (396 and 851) and 17 new siMDM2s were analyzed for their effects on MDM2 expression in SJSAtlanta cells by immunoblotting. SJSAtlanta cells were transfected with mock, control siRNA (siCtrl), control-R siRNA (siCtrl-R), and siMDM2s at 1 nM for 48 h and then examined for MDM2 expression by immunoblotting. (b) Control siRNA, control-R siRNA, and eight effective siMDM2s, including two previously reported and six new were converted to dsRDC forms (chiCtrl, chiCtrl-R, and chiMDM2s), and examined for MDM2 knockdown activity in SJSAtlanta cells 48 h after transfection at 1 nM. (c) Effect of chiMDM2s on growth of SJSAtlanta cells were examined. The cells were transfected with control dsRDC (chiCtrl) or eight chiMDM2s at 1 nM for 5 days and then assayed for relative viable cell number using the WST-8 assay (mean ± SD; n = 3). (d) The effects of two highly effective chiMDM2s (1068 and 1489) on growth of mt TP53-cancer cells (KATOIII, NUGC-3, and DLD-1) after transfection at 1 nM for 5 days were examined using the WST-8 assay. Viable cell numbers relative to those transfected with control dsRDC (chiCtrl) are shown (mean ± SD; n = 3; *p < 0.05; Dunnett’s test).
MDM4 siRNAs (siMDM4) (Supplementary Table 1) were examined for MDM4 knockdown efficiency in MCF-7 cells, which exhibit high levels of MDM4 expression, by immunoblot analysis (Figure 3a). Seven siMDM4s (317, 347, 452, 582, 788, 861, and 1036) showed strong suppression of MDM4 expression and were converted to dsRDCs (chiMDM4). Six chiMDM4s (317, 347, 452, 788, 861, and 1036) knocked down MDM4 expression in MCF-7 cells as efficiently as their cognate siRNAs (Figure 3b). Among these six chiMDM4s, chiMDM4-452 exhibited the highest silencing activity. qRT-PCR analysis confirmed efficient MDM4 knockdown by each of these chiMDM4s (Supplementary Figure 3). The effect on viability of MCF-7 cells by these chiMDM4s was also tested using the WST-8 assay (Figure 3c). All chiMDM4s induced growth suppression in parallel to the MDM4 knockdown efficiency of each. In fact, potent growth suppression was observed with most chiMDM4s (317, 347, 452, 788, 861, and 1036). chiMDM4-582 exhibited less efficient MDM4 silencing and growth inhibitory activities of MCF-7 cells than other chiMDM4s.

Next, the effect of each chiMDM4 on the growth of mt TP53 cancer cells was evaluated. Six effective chiMDM4s were introduced into three mt TP53 cancer cell lines (KATO III, NUGC-3, and DLD-1) and examined for effects on cell growth suppression using the WST-8 assay. As shown in Figure 3d, chiMDM4-452 and -1036 showed negligible growth suppression, whereas other chiMDM4s exhibited mild growth suppression, but without any statistical differences.

Figure 3: Effects of siRNAs targeting MDM4 and their dsRDC forms on MDM4 expression and cell growth. (a) The effects of siMDM4s on MDM4 expression were analyzed in MCF-7 cells by immunoblotting 48 h after transfection with mock, control siRNA (siCtrl), or 10 siMDM4s at 1 nM. SE, short exposure; LE, long exposure. (b) Seven effective siMDM4s and a control siRNA were converted to dsRDC forms (chiMDM4s and chiCtrl), and analyzed for MDM4 knockdown in MCF-7 cells 48 h after transfection at 1 nM. (c) The effect of chiMDM4s on the growth of MCF-7 cells was examined. The cells were transfected with control dsRDC (chiCtrl) or seven chiMDM4s at 1 nM for 5 days and then assayed for relative viable cell number using the WST-8 assay (mean ± SD; n = 3). (d) The effects of six highly effective chiMDM4s on the growth of mt TP53-cancer cells (KATOIII, NUGC-3, and DLD-1) after transfecting at 1 nM for 5 days were examined using the WST-8 assay. Viable cell numbers relative to those transfected with control dsRDC (chiCtrl) are shown (mean ± SD.; n = 3; *p < 0.05; Dunnett’s test).
Effects of MDM4 and MDM2 knockdown on growth of wt TP53 cancer cells

To examine the effect of MDM4 and MDM2 knockdown on the growth of wt TP53 cancer cells, we tested the 11 previously mentioned wt TP53 cancer cell lines, which included seven with high levels of MDM4 expression (MCF-7, A375, SNU-1, HCT116, NUGC-4, LoVo, and A549) and four with low levels of MDM4 expression (SJSA-1, HepG2, HuH-6, and C32TG). To knock down MDM2 and MDM4, two dsRDCs were chosen for each target (chiMDM2-1068/-1489 and chiMDM4-452/-1036). As shown in Figure 4, each chiMDM2 inhibited growth of all wt TP53 cancer cells regardless of the expression levels of MDM2 or MDM4, whereas chiMDM4 only suppressed the growth of cells with high MDM4 expression and not of those with low MDM4 expression (Figure 4).

Next, we examined the effects of MDM2 and MDM4 knockdown on expression levels of p53 and p21Waf1/Cip1 (p21), a TP53 responsive gene product [38], by immunoblotting. As shown in Figure 5, MDM2 suppression increased levels of p53 and p21 in all wt TP53 cells. MDM4 slightly accumulated in most of the wt TP53 cells after MDM2 knockdown, with the exception of SJSA-1 and HepG2 cells. As shown in Figure 6, in all cells with high MDM4 expression, MDM4 knockdown slightly increased p53 levels in association with the induction of p21 and MDM2, which are known TP53-responsive genes, but had negligible effects on p53, p21, and MDM2 in cells with low MDM4 expression.

The effects of MDM2 and MDM4 knockdown on
p53 and MDM4 varied among three mt TP53 cell lines. As shown in Figures 5, MDM2 knockdown induced mild MDM4 accumulation in NUGC-3 and DLD-1 cells, but not KATOIII cells. As shown in Figure 6, MDM4 knockdown reduced MDM2 levels in all three mt TP53 cell lines. Accumulation of p53 occurred in DLD-1 cells, which expressed low levels of mt p53, but not in NUGC-3 cells, which expressed high levels of mt p53. The induction of p21 did not occur in any of these mt TP53 cell lines in response to MDM2 or MDM4 knockdown.

**Effects of MDM2/MDM4 double knockdown on growth of wt TP53 cancer cells**

We examined the effect of MDM2/MDM4 double knockdown on growth of wt TP53 cells, which had high MDM4 expression, using chiMDM4-452 and chiMDM2-1489, which were the most potent and specific inhibitors of each respective target. Cells were transfected with various chiMDM4 concentrations along with control dsRDC-modified siRNA (chiCtrl) at a total dosage of 2 nM, as indicated. As shown in Figure 7a, chiMDM4 and chiMDM2 suppressed the growth of MCF-7 and A375 cells in a dose-dependent manner. When chiMDM2...
and chiMDM4 were simultaneously transfected at three different ratios, more profound growth suppression was observed in these cells than transfection of either chiMDM2 or chiMDM4 alone at the same dosage, or even at a maximal dosage of 2 nM. Similar enhancement in growth suppression was observed in all cells with high MDM4 expression, including five other cell lines (SNU-1, HCT116, NUGC-4, LoVo, and A549) (Supplementary Figure 4). Combination index values at three different ratios of chiMDM2 and chiMDM4 were calculated in cells with high MDM4 expression with values ranging between 0.20 and 0.72, which showed that these dsRDCs promoted synergistic growth inhibition of tumor cells with high MDM4 expression (Table 1).

chiMDM2, but not chiMDM4, alone dose-dependently suppressed the growth of cells with low MDM4 expression (SJSA-1 and C32TG). Further, chiMDM2-mediated growth inhibition was not enhanced by co-transfection with chiMDM4 in these cells (Figure 7a). Similar results were observed in two other cell lines with low MDM4 expression (Supplementary Figure 5). In mt TP53 cells, chiMDM2, chiMDM4, and a combination of both failed to demonstrate any detectable growth suppression (Supplementary Figure 5).

Effect of *MDM2/MDM4* double knockdown on p53 expression

To explore the mechanism by which *MDM2/MDM4* double knockdown synergistically inhibited the growth of cells with high MDM4 expression, the effects of individual and simultaneous knockdown of *MDM2*

![Figure 6: Effect of MDM4 knockdown on expression levels of MDM4, MDM2, p53, and p21.](image-url)

Mock, control dsRDC (Ctrl), and two dsRDCs targeting *MDM4* (chiMDM4-452 and -1036) were transfected into seven cell lines with high MDM4 expression, four cell lines with high MDM2 expression and three mt TP53 cell lines at 1 nM. Expression levels of MDM2, MDM4, p53, and p21 were analyzed by immunoblotting 2 days after transfection.
and MDM4 on p53 expression was examined in MCF-7 and A375 cells (Figure 7b). MDM2 knockdown was associated with mild accumulation of MDM4 and p53, as well as the product of the downstream gene p21. MDM4 knockdown slightly increased levels of MDM2 and p21 in these cells. A subtle increase in p53 expression was observed in chiMDM4-transfected A375 cells, but not in chiMDM4-transfected MCF-7 cells. Simultaneous knockdown by chiMDM2 (1 nM) and chiMDM4 (1 nM) induced expression of p53 and the downstream p21 gene product more than either chiMDM2 or chiMDM4 alone at 1 or 2 nM in cell lines with high MDM4 expression (MCF-7, A375). In cells with low MDM4 expression (SJSA-1 and C32TG), chiMDM2 alone knocked down MDM2, which resulted in accumulation of both p53 and p21 (Figure 7b). However, chiMDM4 did not induce either p53 or p21 upregulation even though MDM4 was efficiently suppressed. Co-transfection of chiMDM2 and chiMDM4 induced accumulation of p53 and p21 to the same extent as chiMDM2.

**DISCUSSION**

A fraction of wt TP53 tumors expresses oncogenes, such as MDM2 and MDM4, to inactivate TP53 [19]. Precise expression patterns and the functional significance of MDM2 and MDM4 in wt TP53 cancer cells remain to be clarified. In the present study, a careful analysis of cancer cell lines harboring wt and mt TP53 demonstrated that all wt TP53 cancer cell lines included in this study exhibited deregulated expression of MDM2 and MDM4. These cell lines were divided into just two distinct types, according to MDM4 expression levels; the one expressing MDM4 at high levels and another expressing MDM4 at low levels. MDM4 expression occurs when tumor cells have acquired MDM4 amplification [19], activated KRAS mutations [39], or loss of miR-34a-mediated suppression [40]. Among seven wt TP53 cancer cell lines with high MDM4 expression, one cell line (MCF-7) has MDM4 amplification [41]. Four cell lines (SNU-1, HCT116, LoVo, A549) harbor KRAS mutation [42, 43], suggesting that the deregulated expression of MDM4 may be caused by KRAS activation or along with miR-34a abnormality in these cell lines.

It is well established that MDM2 and MDM4 are ideal therapeutic targets for wt TP53 tumors. However, to date, there is no biological rationale of whether MDM2 or MDM4 should be targeted in such tumors. Using wt TP53 cell lines and DNA-modified siRNAs specific to MDM2 and MDM4, we demonstrated here for the first time that knockdown of either MDM4 or MDM2 alone can reactivate the TP53 pathway in cancer cells with high MDM4 expression, whereas knockdown of MDM2, but not MDM4, can reactivate wt TP53 in the low MDM4 cancer cells. Furthermore, simultaneous knockdown of MDM2 and MDM4 synergistically activated TP53 and suppressed cell growth in the cancer cells with high MDM4 expression. Based on these results, we propose that both MDM4 and MDM2 are efficient therapeutic targets in wt TP53 tumors cells with high MDM4 expression, whereas MDM2, but not necessarily MDM4, presents a possible therapeutic target in wt TP53 cancer cells with low MDM4 expression.

We explored the mechanisms by which MDM2/MDM4 double knockdown exhibited synergistic effects on TP53 activation in tumor cells with high MDM4 expression. MDM4 is devoid of a nuclear transport signal and requires MDM2 to translocate from the cytoplasm to nucleus [44, 45]. Therefore, we assessed whether the synergistic effect of MDM2/MDM4 double knockdown on TP53 activation was mediated through the inhibition of nuclear transport of MDM4 by MDM2 (Supplementary Figure 6). We found that MDM2 knockdown had no effect on nuclear localization of MDM4 in cells with high MDM4 expression. MDM4 silencing alone increased p53 expression. Because MDM4 has no intrinsic ubiquitin ligase function, but can enhance MDM2 ubiquitin ligase activity by forming a heterodimer with MDM2 [14, 17], both MDM2 and MDM4 may be involved in p53 degradation through the formation of heterodimers. Alternatively, MDM4 knockdown alone enhances MDM2 expression by releasing p53 transcriptional activity, which subsequently suppresses p53 [12, 14, 46]. Therefore, simultaneous knockdown of

**Table 1: Combination index of chiMDM2 and chiMDM4 in MDM4 overexpressed cancer cell lines**

| chiMDM2 (nM) | chiMDM4 (nM) | Combination index |
|--------------|--------------|------------------|
|              |              |                  |
| 0.4          | 1.6          | 0.57  0.20  0.36 0.17  0.26  0.55  0.55 |
| 1.0          | 1.0          | 0.51  0.28  0.39 0.43  0.39  0.45  0.65 |
| 1.6          | 0.4          | 0.47  0.20  0.56 0.44  0.63  0.31  0.72 |

CI>1.1, antagonistic effect; CI = 0.9–1.1, additive; CI<0.9, synergistic effects and the lower value means the stronger synergistic effect.
MDM4 and MDM2 may result in more potent activation of p53 by blocking this p53-MDM2 negative feedback than silencing MDM4 alone.

With the aim to employ synthetic siRNAs in TP53-restoration therapy, we carefully designed and screened siRNAs with high specificities and potencies to target either MDM2 or MDM4. A series of siRNAs targeting MDM2 and MDM4 was designed using siDirect software, which enabled the selection of siRNA sequences with structural features compatible for the efficient loading of the guide strand into the RNA-induced silencing complex as well as a minimal number of off-target candidates from human genes [36, 47]. Among them, effective siRNAs with high knockdown efficiency were chosen by cell-transfection experiments and then converted to DNA-modified siRNAs with 6-base pair double-stranded DNA substitutions [35]. This modification offers a great advantage by lowering off-target activity by decreasing the free energy between the seed regions and off-target mRNAs and avoiding passenger strand-mediated RNAi [35, 48]. Three dsRDCs targeting MDM2 (chiMDM2-1489, 1068, and 2381) and six targeting MDM4 (chiMDM4-861, 452, 1036, 317, 347, and 788) showed potent silencing activity at a concentration as

![Figure 7: Combined knockdown of MDM2 and MDM4 in wt TP53 cell lines with high and low MDM4 expression.](image)

Effects of individual and simultaneous knockdown of MDM2 and MDM4 on cell growth (a) and expression of p53 and p21 (b) were examined in two cell lines with high MDM4 expression (MCF-7 and A375) and two cell lines with low MDM4 expression (SJSA-1 and C32TG). Cells were transfected with MDM4 dsRDC (chiMDM4-452) alone, MDM2 dsRDC (chiMDM2-1489) alone, or both. The total amount of dsRDCs was adjusted to 2 nM by adding control dsRDC (chiCtrl). Cell viability was determined 5 days after transfection using the WST-8 assay. Viable cell numbers of chiCtrl (2 nM) transfected cells was defined as 100% (mean ± SD; n = 3). Levels of MDM2, MDM4, p53, and p21 were analyzed by immunoblotting 2 days after transfection. In panel b, + and ++ indicates 1 nM and 2 nM of dsRDCs, respectively.
suggested the presence of nonspecific effects, such as inhibition of miRNA generation, even though the siRNA concentration was very low.

In conclusion, we showed that most wt TP53 cancer cells exhibited deregulation of MDM2 and MDM4. Specific knockdown of MDM2 and MDM4 with DNA-modified siRNAs clearly revealed the ability of MDM2 and MDM4 to inactivate wt TP53 in cancer cells. The results of this study provide rationale for the selection of MDM2 and MDM4 as therapeutic targets in cancer cells expressing wt TP53. MDM4 expression in wt TP53-tumors is a potential indicator for TP53 reactivation by combined MDM4 and MDM2-targeted cancer therapy. Our specific and potent DNA-modified siRNAs targeting MDM2 and MDM4 might be applicable to TP53 restoration therapy for human cancers.

MATERIALS AND METHODS

Cell lines

Fourteen tumor cell lines were used: eleven cell lines with wt TP53 (MCF-7 breast cancer, A375 melanoma, HCT116 colon cancer, NUGC-4 gastric cancer, LoVo colon cancer, SJSA-1 osteosarcoma, HepG2 hepatocellular carcinoma, HuH-6 hepatocellular carcinoma, A549 lung cancer, and C32TG melanoma) [42, 64-66], and three cell lines with mt TP53 (KATOIII gastric cancer, NUGC-3 gastric cancer, and DLD-1 colon cancer) [64]. The MCF-7, A375, SNU-1, HCT116, LoVo, SJSA-1, and DLD-1 cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA). The NUGC-4, HepG2, and KATOIII cell lines were obtained from the Riken BioResource Center Cell Bank (Tsukuba, Japan). The NUGC-3, A549, HuH-6 and C32TG cell lines were obtained from the Japanese Collection of Research Biorepositories Cell Bank (Osaka, Japan). MCF-7, SNU-1, NUGC-4, SJSA-1, KATOIII, NUGC-3, and DLD-1 cell lines were cultured in RPMI 1640 medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 5% fetal bovine serum (FBS; Nichirei Biosciences, Tokyo, Japan). A375, HepG2, and HuH-6 cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Nichirei Biosciences, Tokyo, Japan). A375, HepG2, and HuH-6 cells were cultured in Ham’s F12 medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Nichirei Biosciences, Tokyo, Japan).

siRNAs and transfection

Sequences of siRNAs used in this study are summarized in Supplementary Table 1. All siRNAs targeting MDM2 and MDM4 were designed using
siDirect software (http://sidirect2.rnai.jp), as reported previously [36]. The control siRNA was an artificial sequence designed to have all features of siRNAs inducing potent RNAi and the least homology to human and mouse genes. Control-R siRNA consisted of randomized sequences of the control siRNA. Control siRNA and complementary dsRDC-modified forms were included in all experiments [07]. siRNAs were converted to dsRDCs by substituting six ribonucleotides from the 5′ end of the guide strand and eight from the 3′ end of the passenger strand with corresponding deoxynucleotides [35, 67]. siRNA transfection was performed using Lipofectamine RNAiMAX (Invitrogen) as reported previously [67].

**Immunoblot analysis**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis were performed as previously described [68]. The primary and secondary antibodies used in this study were as follows: mouse monoclonal antibody against MDM2 (2A10) (Abcam, Cambridge, UK); rabbit polyclonal antibody against MDM4 (Bethyl Laboratories, Montogomery, TX, USA); mouse monoclonal antibodies against p21/Waf1/Cip1 (DCS60) and β-actin (8H10D10) (Cell Signaling Technology, Danvers, MA, USA); and anti-TP53 mouse monoclonal antibody (BP53-12; Cell Sciences, Canton, MA, USA). Both horseradish peroxidase-conjugated anti-mouse IgG sheep and anti-rabbit IgG donkey sera were purchased from GE Healthcare (Buckinghamshire, UK). Chemiluminescent detection was performed using ECL Prime Western Blotting Detection Reagent (GE Healthare) and the Ez-Capture II Imaging System (Atto Corp., Tokyo, Japan).

**Quantitative reverse transcription (qRT)-PCR**

RNA samples were extracted from cell lysate using 40 µL per well of RealTime ready Cell Lysis reagent (Roch Diagnostics, Mannheim, Germany), according to the manufacturer’s instructions. cDNA was synthesized using 2 µL of RNA and 8 µL of Transcriptor Universal cDNA Master (Roch Diagnostics) in 20 µL reactions. qRT-PCR assays were performed using Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in 96-well plates. Primers and TaqMan probes for MDM2, MDM4, and 18S ribosomal RNA (18S rRNA) were obtained from Applied Biosystems (Assay ID: Hs00234753, Hs00967238, and Hs03928990_g1, respectively). Reactions were performed in duplicate under standard thermocycling conditions in a 20-µL volume containing 0.8 µL of cDNA, 900 nM of primers, 250 nM of the probe, and 10 µL of TaqMan Gene Expression Master Mix (Applied Biosystems), according to the manufacturer’s protocol. The amount of target mRNA was examined and normalized to that of 18S rRNA.

**Cell viability**

WST-8 colorimetric assays were performed using a Cell Counting kit-8 (Dojin Laboratories, Kumamoto, Japan) according to the manufacturer’s protocol. Cells were incubated for 5 days after transfection and then analyzed using an iMark microplate reader (Bio-Rad, Hercules, CA, USA). The absorbance of the plates was read at wavelengths of 450 nm and 620 nm.

**Combination index.**

Quantification of chiMDM2 and chiMDM4 synergy was determined by the Chou–Talalay method for drug combination using CalcuSyn software (Biosoft, Cambridge, UK) [69]. A combination index (CI) < 0.9 indicates synergism, 0.9–1.1 indicates additivity, and >1.1 indicates antagonism.

**Immunofluorescence**

Cells were fixed for 15 min in 4% paraformaldehyde at room temperature, and aldehydes were neutralized by soaking coverslips in phosphate-buffered saline (PBS) with 0.1% tween 20 (PBS-T) containing 50 mM glycine at room temperature. Then, the cells were permeabilized in PBS-T with 0.1% Triton X-100 solution for 15 min on ice, blocked for 60 min in PBS-T solution containing 5% normal goat serum (Vector Laboratories, Burlingame, CA, USA) (blocking solution) at room temperature, and then reacted with rabbit polyclonal antibody against MDM4 (Bethyl Laboratories) diluted with blocking solution. After overnight incubation at 4°C, the cells were reacted with fluorescein isothiocyanate isothiocyanate-conjugated goat anti-rabbit IgG antibody (Bethyl Laboratories) diluted with washing buffer for 60 min at room temperature. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Confocal fluorescence images were obtained using Leica TCS SP5 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany).

**Statistical analysis**

All data are expressed as the mean ± standard deviation (SD). Statistical significance of differences between various groups was evaluated using the Dunnett’s test. P-values < 0.05 were considered significant.

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