Interactions between MDM2 and TP53 Genetic Alterations, and Their Impact on Response to MDM2 Inhibitors and Other Chemotherapeutic Drugs in Cancer Cells

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Abstract  Purpose: MDM2 is a key negative regulator of the p53 signaling pathway. We aimed to evaluate the inter-relationships between MDM2 SNP309, mRNA expression, amplification, and TP53 mutations, as well as their correlations with responsiveness to MDM2 inhibitors and other commonly used cytotoxic drugs tested in the NCI-60 cancer cell panel. Experimental Design: SNP309 was genotyped in the NCI-60 cancer cell lines. MDM2 mRNA levels and gene copy number were measured using real-time PCR. We assessed the inter-relationship between MDM2 genetic alterations, TP53 mutations, and the cytotoxicity of two MDM2 inhibitors (RITA and Nutlin-3) as well as 111 other drugs with known mechanisms of action. Results: In the overall NCI-60 cell panel, MDM2 mRNA levels were not associated with SNP309 but with increased gene copy number. However, SNP309 strongly determined the MDM2 mRNA expression in cancer cells with wild-type TP53. Cancer cells with wild-type TP53 also had significantly higher MDM2 copies. In the overall panel, MDM2 copy number was independently correlated with increased sensitivity to commonly used alkylating agents and topoisomerase I and II inhibitors. SNP309 was significantly associated with increased sensitivity to alkylating agents and topoisomerase I inhibitors in the cells with wild-type TP53. In addition, TP53 mutations were the only factor significantly associated with cellular resistance to the MDM2 inhibitor RITA. Conclusions: Our results suggest that MDM2 copy number and SNP309 may predict for response to alkylating agents and topoisomerase inhibitors. These markers should be tested further, particularly in combination with other putative predictive biomarkers.

It is well established that p53 (the product of the TP53 gene) is one of the most important molecules in human cancers. As a tumor suppressor, p53 is a powerful antiproliferative and pro-apoptotic protein that exerts its effects by coordinating a signal transduction pathway involved in cell cycle arrest, apoptosis, and DNA damage repair (1). Deleted or mutated TP53 has been shown in ~50% of human tumors (2). Many tumors with wild-type (WT) TP53 have attenuated p53 function due to negative regulation by other abnormalities (3). One of these negative regulators is the human homologue of murine double minute 2 (MDM2), which is located on chromosome 12q15 and encodes a 90 kDa protein that negatively modulates p53 by binding directly to and decreasing p53 stability (3). Overexpression of MDM2 in animal models increases tumor formation (3). Somatic mutations in the MDM2 gene have been recently identified in adenocarcinoma of the lung (4), and MDM2 overexpression or amplification has been frequently observed in multiple malignancies (5). In addition, a germline single nucleotide polymorphism (SNP309 T>G; rs2279744) in the promoter region of MDM2 has been identified. The presence of the G allele increases MDM2 gene expression and is associated with an earlier age of onset of human cancers (6).

Due to the importance of the p53-MDM2 interaction, restoration of the p53 activity by inhibition of MDM2 binding represents a novel antineoplastic strategy (2). Small-molecule inhibitors selectively inhibiting the p53-MDM2 interaction have been identified and tested in preclinical studies and early phase clinical trials (7). Understanding the molecular mechanisms underlying sensitivity or resistance to these
drugs is essential to improve therapeutic outcome. Additionally, because the p53-MDM2 signaling pathway has a high impact on cancer development, genetic alterations in MDM2 may play a general role in responsiveness to other chemotherapeutic drugs. Indeed, overexpression of MDM2 was significantly correlated with doxorubicin resistance in treatment of childhood acute lymphoblastic leukemia (8). Transfection of the MDM2 gene into cancer cells also resulted in resistance to topoisomerase II inhibitors (9). The SNP309 G allele has been recently associated with reduced sensitivity to topoisomerase II inhibitors (6) as well. This effect was shown to be due to MDM2-mediated downregulation of the topoisomerase II protein (10).

In this study, we aimed to elucidate the inter-relationship between MDM2 gene expression, copy number changes, and SNP309 and TP53 mutation status. We also aim to evaluate the effect of the genetic alterations mentioned above on RITA and Nutlin-3, two representative MDM2 inhibitors, and other commonly used chemotherapeutic drugs. To this end, we used the NCI-60 cancer cell panel for which extensive cytotoxicity and molecular data have been collected and stored in a publicly accessible database (11).

**Materials and Methods**

RNA, DNA, and complementary DNA preparation from NCI-60 cell lines. Extraction of DNA and RNA and complementary DNA preparation have been described in our previous study (12). Two cell lines, MDA-N and RXF-393, had been discontinued when the study was done.

Genotyping of the SNP309 polymorphism. MDM2 SNP309 was amplified with PCR by generating a 164-bp amplicon. Primer sequences were 5′-GGATTTCGGACGGCTCTCGC-3′ (forward) and 5′-CCGCGCAGCGTTCACACTAGTG-3′ (reverse). PCR were cycled 35 times at 95°C for 20 s, 62°C for 20 min, and 72°C for 30 s after preheating at 95°C for 10 min. PCR products were then purified and directly sequenced from both ends.

Quantification of MDM2 copy number. MDM2 copy number was quantified by real-time PCR as previously described (12) using the Mx3000P system (Stratagene) and IQ SYBR Green Supermix (Bio-Rad Laboratories). Briefly, a 138-bp amplicon spanning exon 8 of MDM2 was amplified with primers (forward: 5′-GCCGCAAGCTGTAATAGCA-3′ and reverse: 5′-GGACCTCCGAGACAGTTG-3′). A 149-bp amplicon in the LINE1 sequence was used as an internal control (13). Real-time PCR for both MDM2 and LINE1 were cycled 45 times at 95°C for 20 s, 55°C for 1 min, and 72°C for 30 s after preheating at 95°C for 10 min. Measurement of both amplicons was repeated thrice. MDM2 copy number was then normalized to that of the LINE1 to obtain a ratio (R1) for each cell line. R1 for each cell line was then normalized to the mean R1 of 10 randomly selected normal DNA samples (R2), and the MDM2 copy number for each cell was calculated by doubling the R2.

Measurement of MDM2 mRNA expression. MDM2 mRNA level was quantified with real-time PCR. Briefly, a 83-bp amplicon was generated by real-time PCR with primers spanning MDM2 exons 8 (5′-GCAATGAATCTACAGGGACGC-3′) and 9 (5′-ATCTGATCCACACAAATCACC-3′). The β-actin gene (ACTB) was used as internal control (12). Reactions were performed with standard curves for MDM2 and β-actin and repeated.
thrice. Real-time PCR was cycled 45 times at 95°C for 30 s, 55°C for 1 min, and 72°C for 30 s after preheating at 95°C for 10 min. The relative expression level of MDM2 was normalized by that of ACTB. The MDM2/ACTB ratios were then normalized to the expression of the cell line with the lowest MDM2 expression (H5778T).

**Extraction of data from the NCI-60 database.** The GI50 (molar concentrations expressed as log10[IC50]) of RITA (NSC652287), Nutlin-3 (NSC732664), and other 111 drugs with known mechanism of action (14) were obtained from the NCI-60 database. These drugs were clustered into six main groups based on their cytotoxic activity: alkylating agents (n = 35), antimitotic agents (n = 15), DNA antimetabolites (n = 15), RNA/DNA antimetabolites (n = 19), topoisomerase I inhibitors (n = 12), and topoisomerase II inhibitors (n = 14).

Additional MDM2 data were also extracted from the NCI-60 database. These include MDM2 mRNA levels measured by microarrays (NCI60 database ID: GC12482, GC28921, GC33920, GC181698, GC33943, and GC34080) and dot blot (MT360) as well as MDM2 copy number data measured by array CGH (Comparative Genome Hybridization, CG2421) and spectral karyotyping (SKY, 12q15, MT1789). Copy number of chromosome 12 measured by SKY (12cen, MT1780) was also extracted. The mutation status of the TP53 gene was obtained from a study recently published (15).

**Statistics.** \(\chi^2\) test was used to test for the Hardy-Weinberg equilibrium of SNP309 and its interaction with TP53 mutation status. Association between p53 mutation status and MDM2 expression, MDM2 copy number, and drug cytotoxicity was done using \(t\) test. For correlations between polymorphisms and MDM2 expression, copy number, or the cytotoxicity data, three univariate genetic models (additive, dominant, and recessive) were tested, as the modes of inheritance were either unknown or not clearly identifiable from the plotted data. For example, for the hypothetical variant A>B, we have genotypes A/A, A/B, and B/B. The additive model would be as follows: A/A = 0, A/B = 1, B/B = 2. The dominant model would be as follows: A/A = 0, A/B = 1, B/B = 1. The recessive model would be as follows: A/A = 0, A/B = 0, B/B = 1. The relationships between MDM2 copy number and cytotoxicity data or MDM2 gene expression were assessed by nonparametric correlation (Spearman). The relative MDM2 mRNA level measured by real-time PCR in each cell line was log transformed (+log10) before analysis. The 111 standard agents were classified into six main groups based on their mechanism of action. In each group, because cytotoxicity data are highly correlated between drugs, the mean of the cytotoxicity data (mean of GI50) for all individual drugs within the group was then used to perform the correlation analysis. All tests were done using GraphPad Prism version 4.0 for Windows (GraphPad Software).

### Results

**Genetic analysis of MDM2.** The SNP309 T/G polymorphism was successfully genotyped in all 58 cancer cell lines. The observed genotype frequency was T/T (46.6%), T/G (36.2%), and G/G (17.2%), similar to what has been previously reported (16). No significant deviation of Hardy-Weinberg equilibrium was observed (\(\chi^2\) test, degrees of freedom = 2; \(P > 0.05\)), indicating minimum possibility of genotyping error and similar genotype frequencies between these cancer cells and germline DNA.

MDM2 copy number was measured by real-time PCR. No high level amplification (defined as copy number of ≥4) of MDM2 was observed (median, 2.05; range, 1.63-2.74). The copy number measured by real-time PCR was highly correlated with that measured previously by array CGH (\(r_s = 0.63, P < 0.0001\)), but weakly with 12cen (\(r_s = 0.38, P = 0.005\)) or 12q15 (\(r_s = 0.26, P = 0.06\)) copies measured by SKY (data not shown), possibly reflecting a differed sensitivity of these methods.

Expression of the MDM2 mRNA was also quantified by real-time PCR. One melanoma cell line (UACC-257) showed significant overexpression of MDM2 when compared with the mean expression in all cell lines (68.41 versus 8.09 ± 10.26). The real-time PCR data were highly correlated with most measurements of MDM2 mRNA expression by microarrays (real-time PCR versus the mean value of the six sets of microarray data, \(r = 0.70, P < 0.0001\); data not shown) but weakly correlated with measurements by dot blot (real-time PCR versus MT360, \(r = 0.28, P = 0.03\); data not shown).

All data obtained in this study have been summarized in the Supplementary Table S1.

**Inter-relationship between SNP309, copy number, gene expression of MDM2, and the p53 mutation status.** In the overall NCI-60 panel, there was no significant allelic or genotypic association between MDM2 SNP309 and TP53 mutations (\(\chi^2\) test, \(P > 0.05\); data not shown). However, there was a significant impact of TP53 mutation status on the interactions between genetic alterations in the MDM2 gene. This was manifested as a strong correlation between MDM2 SNP309, amplification,
and mRNA level in TP53 WT cells, but not in TP53 mutant (MT) cells (Fig. 1). The SNP309 G allele was highly correlated with increased MDM2 mRNA gene expression (dominant model for the G allele; \( r_s = 0.63, P = 0.01 \)) and copy number \( (r_s = 0.79, P = 0.0005) \) in the cells bearing WT TP53 but not in those with MT TP53 (Fig. 1A and B). Similarly, there was also a significant linear correlation between MDM2 mRNA levels and copy number \( (r_s = 0.61, P = 0.02) \) in the cells bearing WT TP53, but not in the cells with MT TP53. It should be noted that UACC-257, the melanoma cell line with significant gene overexpression, also has the highest gene copy number \( (n = 2.75; \text{Fig. 1B}) \).

**Association between MDM2 genetic alterations and drug cytotoxicity.** In the overall NCI-60 panel, MDM2 SNP309 showed a minimum effect on drug response, with no significant correlations found between any genetic model and cytotoxicity data (data not shown). However, when cells were divided into TP53 MT and WT groups, SNP309 was significantly associated with increased sensitivity to alkylating agents (additive model of the G allele; \( r_s = 0.67, P = 0.004 \)) and topoisomerase I inhibitors \( (r_s = 0.60, P = 0.01) \) in the WT group, but not in the cells with MT TP53 (Fig. 2A and B). With regard to the MDM2 amplification and cytotoxicity, although TP53 mutation status had an impact on the correlations, MDM2 copy number was significantly correlated with increased cellular sensitivity to several groups of agents in the overall panel, especially when the apparent outlier UACC-257 was removed from the analyses \( (\text{respective } r_s \text{ and } P \text{ for overall panel, TP53 WT and MT group: alkylating agents } \{r_s = -0.46, P = 0.0004; r_s = -0.66, P = 0.01; r = -0.31, P = 0.05\}; \text{Fig. 3A}), \text{DNA antimetabolites } \{r_s = -0.39, P = 0.003; r_s = -0.48, P = 0.08; r_s = -0.27, P = 0.08; \text{Fig. 3B}), \text{topoisomerase I inhibitors } \{r_s = -0.41, P = 0.002; r_s = -0.47, P = 0.09; r_s = -0.36, P = 0.02; \text{Fig. 3C}), \text{and topoisomerase II inhibitors } \{r_s = -0.46, P = 0.0003; r_s = -0.51, P = 0.06; r_s = -0.33, P = 0.03; \text{Fig. 3D})\} \text{. In contrast to SNP309 and copy number, there was no significant correlation between cytotoxicity data and MDM2 mRNA expression measured by real-time PCR in either the overall panel or the groups stratified by TP53 mutation status (data not shown), which may reflect the stability of DNA markers over the RNA marker. In addition, except for a weak association between MDM2 CGH array data (CG2421) and sensitivity to topoisomerase I inhibitors \( (r_s = -0.27, P = 0.05) \), no other significant association was found between cytotoxicity and MDM2 SNP309 G allele, but not in TP53 mutant (MT) cells (Fig. 1). The SNP309 G allele was highly correlated with increased MDM2 mRNA gene expression (dominant model for the G allele; \( r_s = 0.63, P = 0.01 \)) and copy number \( (r_s = 0.79, P = 0.0005) \) in the cells bearing WT TP53 but not in those with MT TP53 (Fig. 1A and B). Similarly, there was also a significant linear correlation between MDM2 mRNA levels and copy number \( (r_s = 0.61, P = 0.02) \) in the cells bearing WT TP53, but not in the cells with MT TP53. It should be noted that UACC-257, the melanoma cell line with significant gene overexpression, also has the highest gene copy number \( (n = 2.75; \text{Fig. 1B}) \).
data and either 12q15 or 12cen copy numbers measured by array CGH and SKY (data not shown), possibly due to the reduced sensitivity of these methods.

Correlation between TP53 mutation status and drug cytotoxicity data was also tested. The TP53 mutations were significantly associated with resistance to RITA (t test, \( P = 0.005 \); Fig. 4A) but only marginally associated with Nutlin-3 (t test, \( P = 0.07 \); Fig. 4B). Statistically significant correlations were also observed between TP53 mutation and resistance to alkylating agents (t test, \( P = 0.04 \)) and topoisomerase I inhibitors (t test, \( P = 0.04 \); data not shown).

All data used in the analysis mentioned above were also included in the Supplementary Table S1.

**Discussion**

Our study suggests that there is a strong interaction between genetic components of TP53 and MDM2 genes in cancer cells. This interaction further affects the cellular responsiveness to anticancer agents. MDM2 copy number and SNP309 and TP53 mutation status are independently predictive of sensitivity to common cytotoxic drugs. Because these effects are based on analysis of cancer cells of limited number and mixed tissue types, confirmation of these findings in clinical settings would be required before such markers could be considered as a clinical diagnostic.

The significant intercorrelations between SNP309, MDM2 mRNA expression, and gene copy number were only observed in the TP53 WT cells, suggesting that the development of TP53 mutations reduces the regulatory role of MDM2. This is supported by the recent observation that genetic lesions in TP53 were mutually exclusive of those in MDM2 or MDM4 in glioblastoma (17). In contrast, when p53 is normal, MDM2 may regulate p53 activity. The high correlation between the G allele of SNP309 and increased MDM2 gene copy number in TP53 WT cells suggests that there may be an “addiction” to the oncogenic property of MDM2 during carcinogenesis (18).

Our findings highlight the potential importance of MDM2 copy number in regulating gene expression and mediating drug effect in cancer cells. Unlike in TP53, a low rate of somatic mutations and only low-level gene amplification have been observed in MDM2 in cancers (4, 5). This was confirmed in our study as there was only a slight increase in MDM2 copy number in the NCI-60 cancer cells. However, this subtle increase in MDM2 copy number was significantly associated with MDM2 mRNA levels, consistent with a prior study (18). Furthermore, the TP53 mutation status has less impact on the correlation between MDM2 gene copy number and drug response. These findings indicate the potential clinical importance of MDM2 amplification. Given the complexity of the mechanisms underlying drug sensitivity and resistance, it may be worthwhile to evaluate this in clinical settings, especially in combination with other putative predictive biomarkers. This can readily be accomplished using real-time PCR, as shown in this study.

We also found that although mutated TP53 is associated with drug resistance, enhanced MDM2 activity (increased copy number or presence of the SNP309 G alleles) is significantly associated with increased sensitivity to multiple cytotoxic drugs. This suggests that impairment of the p53 function by mutation and by inhibition of MDM2 may have distinct consequences, and additional functions of MDM2 besides down-regulation of p53 may coexist. Indeed, MDM2 has been shown to regulate other proteins such as RB, P73, p14/p19, E2F1-DP1, etc. (19, 20). As a result, this mechanism may lead to differential susceptibility to drug response. This may explain why only p53 mutation affects the response to MDM2 inhibitors whereas the MDM2 genetic alterations have no effect, because the inhibitors specifically target the p53/MDM2 interaction. A recent study has shown that increased MDM2 expression promotes genetic instability and delays DNA double-strand break repair independent of p53 (21). It is therefore possible that impaired DNA damage repair by increased MDM2 activity enhances the cytotoxic effect of DNA-damaging agents. Previous studies observed that either increased MDM2 activity or the SNP309 G allele was associated with cellular resistance to topoisomerase II inhibitors (6, 9, 10). In our study, however, although there is no correlation between SNP309 or MDM2 mRNA level and drug sensitivity, there is a significant correlation between elevated MDM2 copy number and increased sensitivity to topo-II inhibitors. Possible reasons leading to these discrepancies may include differences in cell lines and dose of the drugs tested. Moreover, MDM2 copy number was not tested in previous studies. It is possible that activation of MDM2 by
amplification may be involved in a pathway different from that activated by gene overexpression or SNP309. Nevertheless, the relationship between different genetic alterations in MDM2 and drug sensitivity need to be further investigated.

We recognize the limitations of our study. For example, the NCI-60 panel is derived from nine different tissue types. Because the genetic status of TP53 and MDM2 may vary among different tissue types, our findings may not reflect the gene-drug interactions in each specific tissue type. In addition, as an important molecular marker, the MDM2 protein level has not been measured in this study. We will further address these questions in our future studies. Nevertheless, our study reflects the complexity of genetic interactions in cancer cells and emphasizes the potential importance of germline polymorphisms, as well as somatic mutations in susceptibility to sensitivity and resistance to anticancer agents. Thus, we encourage investigators interested in predictive biomarkers to routinely collect both germline and tumor DNA.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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