MDM2 phenotypic and genotypic profiling, respective to TP53 genetic status, in diffuse large B-cell lymphoma patients treated with rituximab-CHOP immunochemotherapy: a report from the International DLBCL Rituximab-CHOP Consortium Program

Zijun Y. Xu-Monette, 1 Michael B. Møller, 2 Alexander Tzankov, 3 Santiago Montes-Moreno, 4 Wenwei Hu, 5 Ganiraju C. Manyam, 6 Lei Fan, 7 Carlo Visco, 8 Karen Dybkær, 9 April Chiu, 10 Wayne Tam, 11 Youli Zu, 12 Govind Bhagat, 13 Kristy L. Richards, 14 Eric D. Hsi, 15 William W.L. Choi, 16 J. Han van Krieken, 17 Qin Huang, 18 Jooryung Huh, 19 Weiyun Ai, 20 Maurilio Ponzoni, 21 Andrés J.M. Ferreri, 21 Jane N. Winter, 22 Lin Wu, 23 Xiaoying Zhao, 24 Ronald S. Go, 25 Sa A. Wang, 1 Carlos E. Bueso-Ramos, 1 Yong Li, 26 Miguel A. Piris, 9 L. Jeffrey Medeiros, 1 and Ken H. Young 1¶

1Department of Hematopathology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA; 2Odense University Hospital, Odense, Denmark; 3University Hospital, Basel, Switzerland; 4Hospital Universitario Marques de Valdecilla, Santander, Spain; 5Cancer Institute of New Jersey, University of Medicine and Dentistry of New Jersey, New Brunswick, NJ, USA; 6Department of Biostatistics and Bioinformatics, The University of Texas MD Anderson Cancer Center, Houston, TX, USA; 7The First Affiliated Hospital of Nanjing Medical University, Jiangsu Province Hospital, Nanjing, China; 8San Bortolo Hospital, Vicenza, Italy; 9Aalborg Hospital, Aarhus University Hospital, Aalborg, Denmark; 10Memorial Sloan-Kettering Cancer Center, New York, NY, USA; 11Weill Medical College of Cornell University, New York, NY, USA; 12The Methodist Hospital, Houston, TX, USA; 13Columbia University Medical Center and New York Presbyterian Hospital, New York, NY, USA; 14University of North Carolina School of Medicine, Chapel Hill, NC, USA; 15Cleveland Clinic, Cleveland, OH, USA; 16University of Hong Kong Li Ka Shing Faculty of Medicine, Hong Kong, China; 17Radboud University Nijmegen Medical Centre, Nijmegen, Netherlands; 18City of Hope National Medical Center, Duarte, CA, USA; 19Asan Medical Center, Ulsan University College of Medicine, Seoul, Korea; 20University of California San Francisco School of Medicine, San Francisco, CA, USA; 21San Raffaele H. Scientific Institute, Milan, Italy; 22Feinberg School of Medicine, Northwestern University, Chicago, IL, USA; 23Roche Molecular Systems, Pleasanton, CA, USA; 24Zhejiang University School of Medicine, Second University Hospital, Hangzhou, China; 25Mayo Clinic, Rochester, MN, USA; 26University of Louisville, Louisville, KY, USA

Key Words: MDM2, p53, p53 mutations, Diffuse large B-cell lymphoma, SNP309, Immunohistochemistry, FISH, Oscillation, Gene expression profiling

Running Heads: MDM2 phenotypic and genotypic profiling in DLBCL
¶ Correspondence:
Ken H. Young, MD, PhD
The University of Texas MD Anderson Cancer Center
Department of Hematopathology
1515 Holcombe Boulevard
Houston, Texas 77030-4009
Phone: 1-713-745-2598
Fax: 1-713-792-7273
Email: khyoung@mdanderson.org

Miguel A. Piris, MD, PhD
Servicio de Anatomia Patologica
Hospital Universitario Marques de Valdecilla Fundacion IFIMAV
39008 Santander, Spain
Phone: 34-942203492
Email: mapiris@humv.es
Key Points

- Phenotypic and genotypic profiling of MDM2 in DLBCL
- MDM2 as a negative regulator of p53 tumor suppressor function

Abstract

MDM2 is a key negative regulator of the tumor suppressor p53, however, the prognostic significance of MDM2 overexpression in diffuse large B-cell lymphoma (DLBCL) has not been defined convincingly. In a p53-genetically defined large cohort of de novo DLBCL patients treated with R-CHOP chemotherapy, we assessed MDM2 and p53 expression by immunohistochemistry (n=478), MDM2 gene amplification by fluorescence in situ hybridization (n=364), and a single nucleotide polymorphism in the MDM2 promoter, SNP309, by SNP genotyping assay (n=108). Our results show that MDM2 overexpression, unlike p53 overexpression, is not a significant prognostic factor in overall DLBCL. Both MDM2 and p53 overexpression does not predict for an adverse clinical outcome in patients with wild-type p53, but predicts for significantly poorer survival in patients with mutated p53. Variable p53 activities may ultimately determine the survival differences as suggested by the gene expression profiling analysis. MDM2 amplification was observed in 3 of 364 (0.8%) patients with high MDM2 expression. Presence of SNP309 did not correlate with MDM2 expression and survival. This study indicates that evaluation of MDM2 and p53 expression correlating with TP53 genetic status is essential to assess their prognostic significance, and important for designing therapeutic strategies that target the MDM2-p53 interaction.
Introduction

MDM2/Hdm2, the human homolog of murine double minute 2 (Mdm2) or p53 E3 ubiquitin protein ligase homolog (mouse) [Homo sapiens], is a key negative regulator of the tumor suppressor p53 and has other p53-independent functions. The MDM2 gene is transactivated by p53 and thus p53 degradation by MDM2 forms the other direction of a negative feedback loop. MDM2 is frequently overexpressed in cancers, but its prognostic importance has been elusive in many disease entities. MDM2 overexpression has been shown to facilitate B-cell lymphomagenesis in vivo, and to inactivate the tumor suppressor function of wild-type p53 (WT-p53) in vitro. MDM2 overexpression, however, has correlated inconsistently with adverse clinical outcomes in patients with hematological malignancies.

Several factors could account for the inconsistent results: (1) small study sizes; (2) different cutoffs for MDM2 expression; (3) unclear expression and function of MDM2 isoforms; and (4) post-translational modifications or subcellular localization of MDM2. In stress conditions, both p53 and MDM2 are modified (for example, phosphorylation by ATM), resulting in reduced affinity and increased degradation of MDM2. In addition, MDM2 nuclear entry is inhibited via induction of p53-responsive PTEN, and p53-inducible p21 maintains another positive feedback loop. Another of fifth possible factor is oscillation of the p53-MDM2 autoregulatory feedback loop (Figure 1C), which has not been recognized by the previous prognostic studies. Elegant models and laboratory observations have shown that cellular levels of WT-p53 and MDM2 fluctuate in an oscillatory fashion in response to stress, such as DNA damage, hypoxia, or oncogene activation, and that the numbers of pulses and the fraction of cells with oscillatory pulses increase with the strength of DNA damage. The oscillatory kinetics and the variable amplitude of p53/MDM2 pulses over cell population may affect the measurement of MDM2 expression using immunohistochemistry (IHC), a commonly used method in clinical diagnostic and prognostic studies. If the study cohort is too small, or the cutoff is inappropriately established, the survival difference between two groups may not be truly reflected. To obtain evaluable results beyond the “noise” caused by oscillation, a large cohort of cases becomes essential.

In comparison, MDM2 function towards mutant p53 (MUT-p53), and the kinetics of MDM2 and MUT-p53 levels under stress are not well defined. In a mouse model, MDM2 and...
DNA damage regulate MUT-p53 levels in a manner similar to WT-p53. However, most MUT-p53s have lost the ability to transactivate \textit{MDM2}, so basal MDM2 levels cannot compensate for increased MUT-p53 due to sustained stress. Conversely, in a panel of cell lines, mutual stabilization of MDM2 (increased half-life time from 30 minutes to 2 hours) and MUT-p53 was observed.

The prognostic significance of MDM2 expression is further complicated by other non-oncogenic functions of MDM2. MDM2 monoubiquitinates p53 leading to only nuclear export and not p53 degradation. MDM2 promotes p53 translation by binding to p53 mRNA, simultaneously impairing its E3 ligase activity. After exposure to the p53-activating drug RITA, MDM2 can enhance the apoptotic response induced by p53 through down-regulation of p21 (cell cycle inhibitor) at both the transcriptional and posttranscriptional levels. In normal human or murine cells, MDM2 induces G0/G1 cell cycle arrest. These MDM2 functions suggest that MDM2 expression can be a positive prognostic factor in certain cellular contexts. In mouse models, Mdm2 appeared to be both a tumor suppressor and an oncprotein depending on its cellular levels.

Mdm2 exerts p53-independent tumorigenesis in mouse models, but the mechanisms are unclear although several proteins (such as p73, p63, RB, Sp1, E2F1, L5) have been identified to have interaction with MDM2. However, the principal role of Mdm2 as a negative p53 regulator is supported by the similar survival curves and tumor spectrum of \textit{p53–/– Mdm2–/– Mdm4–/–} and \textit{p53–/–} mice, or the similar tumor incidence in \textit{p53–/– Mdm2–/–} and \textit{p53–/–} mice. On the other hand, few p53-independent mechanisms upregulating MDM2 are known. In many tumors, MDM2 overexpression is not caused by gene amplification. The single nucleotide polymorphism (SNP) 309 T→G in the \textit{MDM2} promoter is associated with increased affinity with the transcriptional activator Sp1 resulting in elevated MDM2 expression, in a gender-specific (females) and hormonal-dependent manner. On the other hand, in Burkitt lymphoma cells, MDM2 overexpression was caused by enhanced translation.

To explore the prognostic significance of MDM2 expression correlating with p53 expression that MDM2 functions towards, and recognize the possible factors that could complicate the analysis of the results, we assessed for MDM2 expression, \textit{MDM2} amplification
and polymorphism, p53 expression and $TP53$ genetic status in a large cohort of patients with diffuse large B-cell lymphoma (DLBCL).
Materials and Methods

Patients

The initial study cohort consisted of 478 de novo DLBCL patients treated with rituximab, cyclophosphamide, hydroxydaunorubicin, vincristine, and prednisone (R-CHOP) therapy in 29 medical centers. The diagnostic criteria, review process, treatment regimens, treatment response criteria, and eligibility/exclusion criteria has been described previously. At last follow-up 36% (173/478) of the patients had died. The median follow-up interval for the 305 censored patients was 56.5 months (range, 12.0 to 180.3 months). The validation set consisted of another 227 DLBCL patients with similar clinical feature and treatment regimen. This study was approved as being of minimal to no risk or as exempt by the Institutional Review Boards of all participating centers, and at The University of Texas MD Anderson Cancer Center. This study was conducted in accordance with the Declaration of Helsinki.

p53 and MDM2 Immunohistochemical Staining

Biopsy samples obtained at diagnosis were fixed in formalin and embedded in paraffin, and tissue microarrays (TMAs) were constructed using a tissue arrayer (Beecher Instruments, Silver Spring, MD). The GEO accession number is GSE#31312. IHC was performed on TMAs using antibodies DO-7 for p53 from Dako, and IF2 for MDM2 (N-terminal) from Calbiochem (Billerica, MA) as described previously. Expression levels of p53 and MDM2 for each sample were determined by counting the percentage of positive cells in TMA cores, in combination with assessment of intensity. Each TMA core contained approximately 500 tumor cells. Six pathologists in addition to the pathologist at each contributing medical center independently evaluated the tissue cores by light microscopy for p53 and MDM2 expression without knowledge of the clinical outcomes. Discrepancies were resolved by joint review on a multi-head microscope.
TP53 Mutations and SNP309 Genotyping of MDM2

p53 mutation status was determined by resequencing microarray using p53 AmpliChip® (Roche Molecular Systems, Pleasanton, CA) as described previously.34

The SNP309 (rs2279744) status of the MDM2 promoter, was determined by using a Taqman SNP genotyping assay as previously described.32 Primers were purchased from Applied Biosystems (Foster City, CA).

Gene Expression Profiling (GEP)

GEP was performed using Affymetrix GeneChip Human Genome HG-U133 Plus 2.0 chips.36 Normalized microarray data was analyzed for differential expression between MDM2+ and MDM2– patients, or p53+ and p53– patients with WT-p53 or MUT-p53. Univariate analysis was performed to identify differentially expressed genes using t-test. The P values obtained by multiple t-tests were corrected for false discovery rate (FDR) by BUM method. Differentially expressed genes (DEGs) were identified with P value cutoffs .0012-.0035 for respective comparisons at a FDR of .30.

Fluorescence in situ Hybridization (FISH) for MDM2 Gene Amplification Analysis

A bacterial artificial chromosome clone (RP11-1064P9, obtained from the BACPAC Resource Center of the Children’s Hospital Oakland Research Institute, http://bacpac.chori.org/home.htm) localized on chromosomal region 12q13-15 was used as a probe for MDM2 FISH and labeled with Spectrum Orange. A control region from the centromere of chromosome 12 was used as ploidy reference and labeled with Spectrum Green. Dual-color FISH was performed in 4 micron sections of the TMAs. Images were captured and archived using Cytovision software (Applied Imaging, Santa Clara, CA). Fluorescence signals were scored by two independent investigators by counting the number of MDM2 and reference probe signals in 150–250 well-defined nuclei. A sample was scored as amplified when the ratio between the test and control signals was above 2.
Statistical Analysis

Clinical and laboratory features at time of presentation between different DLBCL subgroups were compared using the $\chi^2$ test. Overall survival (OS) was calculated from time of diagnosis to time of death from any cause. Progression-free survival (PFS) was calculated from time of diagnosis to time of progression or death from any cause. Patients who remained alive or progression-free were censored at last follow-up. Univariate and multivariate analysis for survival of the study cohort was performed with IBM SPSS statistics 19.0 using the Cox proportional hazards regression model and forward-stepwise method. OS and PFS curves of different groups were analyzed by GraphPad Prism 5 using the Kaplan–Meier method and differences were compared using the log-rank (Mantel-Cox) test. All differences with $P \leq .05$ were considered to be statistically significant.
Results

MDM2 and p53 Expression

Our results showed variable p53/MDM2 levels (0 - 100%) among 478 patients and all combinations of IHC staining patterns (+/+, +/−, −/+, −/−) (Table 1A-B), which could possibly be explained by oscillations of p53/MDM2 levels in patients with WT-p53 (Figure 1C), and other regulatory factors that affect p53 and MDM2 levels. The mean percentage of MDM2-positive cells in 478 patients was 22%. The mean percentage of p53-positive cells was 23% in 474 patients with p53 expression data available. Based on occurrence distribution of different MDM2 or p53 expression levels (Figures 1A-B), both MDM2 and p53 overexpression (MDM2+, p53+) were defined as >10% of cells positive for IHC staining, which is similar to cutoffs used by others in earlier lymphoma studies of MDM2 and p53 expression. Using the >10% cutoff, 193 of 478 (40.4%) DLBCLs had MDM2 overexpression and 182 of 474 (38.4%) DLBCLs had p53 overexpression. The frequency of MDM2 overexpression was higher (but not significant) in DLBCLs with WT-p53 (41.9%, 156 out of 372) than in DLBCLs with MUT-p53 (34.9%, 37 out of 106). Correspondingly, there is a higher mean level of MDM2 expression in DLBCL patients with WT-p53 than DLBCLs with MUT-p53 (24% vs 17%, Table 1A). Comparatively, p53 overexpression is significantly more common in DLBCLs with MUT-p53 than with WT-p53 (71.4% vs 29.1%) (P< .0001, see case numbers in Table 1A), and higher mean level of p53 expression in DLBCL with MUT-p53 (54% vs 14%). MUT-p53 was overexpressed compared to WT-p53 in both MDM2– and MDM2+ DLBCLs (Table 1B), suggesting that overexpression was not readily explained by reduction in MDM2-mediated degradation alone.

Also notable, all patients (n=16) with non-sense, splice-site or frame-shift p53 mutations had a p53-negative immunophenotype; all but 4 patients were also MDM2-negative. In contrast, 84% of patients with missense mutations had a p53+ phenotype and 72% of patients have a high IHC score ≥50% of tumor cells (mean level: 64%). MDM2 expression had no good correlation with missense-MUT-p53+ phenotype: p53+/MDM2−: 52.7%; p53+/MDM2+: 30.8%; p53−/MDM2−: 11.0%; p53−/MDM2+: 5.5%.
Clinical Features of the Cohort, Univariate and Multivariate Analysis

Clinical features of the patients were compared between MDM2– and MDM2+ DLBCL, in the entire cohort and in subcohorts of patients with WT- or MUT-p53 (Table 2A).

Univariate analysis regarding to survival indicated that an International Prognostic Index (IPI) score of >2 and the five risk factors composing the IPI (age, stage, serum lactate dehydrogenase level, performance status, and extranodal sites), as well as B-symptoms, tumor size, TP53 mutation and p53 overexpression were significantly associated with poorer survival. MDM2 overexpression was not associated with poorer survival.

The prognostic factors according to univariate analysis, and MDM2 >10% and gender that are significantly different between MDM2+ and MDM2– DLBCLs (Table 2A) were then entered a multivariate survival analysis. The IPI >2, TP53 mutations and B-symptoms remained as the independent prognostic factors, whereas p53 >10% and MDM2 >10%, tumor size >5cm and gender were not associated with significantly poorer survival (Table 2B).

However, in the MUT-p53 subcohort, the IPI >2, p53 >10% and MDM2 >10% remained prognostically significant in the multivariate analysis, whereas B-symptoms and tumor size >5cm did not independently predict a poorer prognosis (Table 2B).

Prognostic Significance of MDM2 and p53 Overexpression

MDM2 overexpression was not significantly associated with poorer survival for the entire cohort (P values: .18 for OS and .41 for PFS. Figures 2A, 3A), or within patients with WT-p53 (P values: .71 for OS and .99 for PFS. Figures 2C, 3C). However, MDM2 overexpression conferred an inferior OS for patients with MUT-p53 (median OS: 30.0 versus 87.3 months; hazard ratio [HR] = 2.92; 95% confidence interval [CI], 2.33-3.50; P= .0072) (Figure 2E). Similar trend was observed for PFS curves (Figure 3E).

In contrast, p53 overexpression correlated with poorer survival for the entire cohort (P=. 017 for OS, and P=. 019 for PFS). However, the prognostic significance of p53 overexpression was restricted to DLBCL patients with MUT-p53 (median OS: 33.3 versus 82.4 months; HR =
2.48; 95% CI, 2.0-2.96; \( P = .016 \); median PFS: 25.8 versus 82.4 months; HR = 2.16; 95% CI, 1.23-3.79; \( P = .0073 \)) (Figures 2-3B, D, F).

The prognostic significance of MDM2 or p53 overexpression is confirmed in the validation set (Supplemental Figure 1).

**Concurrent Evaluation of p53 and MDM2 Expression**

Not only the survival curves for p53 and MDM2 overexpression showed similar patterns in the WT-p53 and MUT-p53 subgroups (Figures 2-3C-F), patients with p53+/MDM2, p53–/MDM2+, or p53+/MDM2+ had comparable survival (Figures 4A-D), reinforcing the idea that MDM2+ and p53+ patients are prognostic equivalents in the WT- or MUT-p53 subgroups. If stratify patients with either MDM2 or p53 overexpression as a single group, the impact of their overexpression on survival was similar with that of p53 overexpression (shown in Figures 4E-F for the MUT-p53 subgroup).

**Differentially Expressed Genes**

DEGs were identified by comparing GEPs of different groups: MDM2+ vs MDM2–DLBCLs with WT-p53 (DEGs1: 157) or MUT-p53 (DEGs2: 302), as well as p53+ vs p53–DLBCLs with WT-p53 (DEGs3: 547) or MUT-p53 (DEGs4: 0) (Figures 5A-C). Although DEGs1 and DEGs2 shared 11 genes (Supplemental Table 1), they appeared to be p53- and MDM2- independent suggesting the different and heterogeneous transcription programs in the MUT-p53 subgroup. Common 49 genes (Supplemental Table 2) between DEGs1 and DEGs3 include CDKN1A/p21, MDM2, MDM4 and ATM. Albeit the stress transmitter and p53 activator, ATM was downregulated both in MDM2+ and p53+ DLBCLs with WT-p53, probably by p53-inducible Wip1.12 Only one gene (probable ATP-dependent RNA helicase DDX59) overlaps DEGs2 and DEGs3.

In order to identify genes and pathways responsible for the different clinical outcomes of MDM2+ versus MDM2– DLBCLs patients with MUT-p53, DEGs2 with fold change >2 are examined (Supplemental Table 3). In MDM2+ DLBCL patients, 8 genes with known function (http://www.uniprot.org) were expressed at significantly higher levels (1.66-2.64 fold change, \( P < \))
.0023), whereas 21 genes were expressed at significantly lower levels (1.66-2.46 fold change, \( P < .0023 \)). None of these genes have been reported to interact with MDM2, but \( CXCL5,^{40} MBD4,^{41} PAK2, ATG7,^{42} \) and \( DCUN1D1^{43} \) have been reported to interact with the TP53 pathway, and many genes downregulated in MDM2+ patients are related to DNA repair or cell death. Upregulation of \( CXCL5 \) suggests gain-of-function of MUT-p53s in MDM2+ DLBCL,\(^40\) whereas downregulated \( MBD4, PAK2, ATG7, \) and \( DCUN1D1 \) in MDM2+ DLBCL suggest loss-of-function of MUT-p53s. Interestingly, not all upregulated genes in patients with MDM2+ DLBCL are oncogenic (such as \( MAD/MXD1 \) antagonizing MYC function), and there are several oncogenes downregulated in MDM2+ DLBCLs (such as \( ATAD2 \) and \( DCUN1D1 \)). When we further compared GEPs of MDM2+ DLBCLs with WT-p53 vs MDM2+ DLBCLs with MUT-p53, only 14 transcripts were significantly differentially expressed (\( P \) cutoff: .000113; FDR: .30), which appears to indicate the simultaneous presence of WT-p53 transcription activities in some MDM2+ patients with MUT-p53.

**MDM2 Amplification**

\( MDM2 \) amplification was identified by FISH (Figure 5D) in 3 of 364 patients (0.8%). All three patients had strongly MDM2+ tumors (100%, 95% and 60%). The two patients with 100% or 95% tumor cells expressing MDM2 had WT-p53, and the patient with 60% MDM2+ cells had MUT-p53. These tumors had 90%, 70% and 0% p53-positive cells, respectively. All the three patients were alive and censored at last follow-up.

**MDM2 SNP309 Polymorphism and Survival**

SNP309 genotyping was analyzed in 108 patients treated with R-CHOP. Clinical features of patients with homogenous T/T genotype vs with T→G polymorphism were compared (Table 2A). The only significantly different variant is the number of extranodal sites. Most female patients were elderly. In this cohort, no significant difference in the mean MDM2 expression levels was detected in patients with different SNP309 genotypes (Table 1C).
OS and PFS were compared between patients with G/G (n=10), G/T (n=57), and T/T (n=41) genotypes. The SNP309 T→G change did not correlate with poorer survival in DLBCL patients (Figure 5E-F).

**Discussion**

In contrast with several reports in the literature for other types of human cancer, we show in DLBCL patients that MDM2 overexpression has no significant (P > .05) adverse impact on survival in the entire cohort (Figures 2-3A) and in the WT-p53 subcohort (Figures 2C-3C) with a >10% cutoff or with other cutoffs of 20-70%, suggesting that suppression of p53 by MDM2 in tumor cells did not significantly affect WT-p53 function under stress condition. Possible explanations include: (1), posttranslational modifications of p53 and MDM2 resulted in reduced repression by MDM2, and more degradation of tumorigenic MDM2 rather than degradation of p53; (2), interaction of p53/MDM2 can be regulated by many other factors (such as ARF, L11, MDM4); (3), during the time delay of MDM2 repression, the tumor suppressor function of WT-p53 is already exerted. (4), other MDM2 functions positive to the p53 function may also exist. Correspondingly, MDM2 overexpression did not significantly impact the clinical outcomes of DLBCL patients with WT-p53 in our study, which may explain why clinical trials of MDM2 inhibitors have not shown impressive efficacy.

Similarly, p53 overexpression by IHC did not predict poorer survival in patients with WT-p53 (Figures 2-3D). This is true for cutoffs of 10-70%. Overexpression of WT-p53 in tumor cells could be caused by high endogenous cellular stress (DNA damage or oncogene activation), which increases the fraction of cells with oscillatory p53/MDM2 pulses (only part of which were detected by IHC). The correlation between levels of p53 reflecting pre-treatment cellular stress level and clinical outcomes was not significant, probably because after chemotherapy treatment p53 would be activated to high levels due to the widespread DNA damage rendered by chemotherapy or radiation. What determined the tumor cell fate and therapeutic response was whether p53 functions properly after the treatment, not the p53 expression level before treatment.

In contrast, in DLBCL patients with MUT-p53, p53 overexpression correlated with significantly worse survival (Figures 2-3F), which stayed significant with cutoffs of 10-60% (P values are marginal for 40-60%). Since all non-sense, splice-site and frame-shift p53 mutants...
had a p53-negative phenotype (accounting for 53% of MUT-p53-negative cases) and MUT-p53+
patients carried exclusively missense mutants with probable dominant oncogenic gain-of-
function, the different prognosis of MUT-p53+ and MUT-p53-negative arms may attribute to the
variable loss-of-function and gain-of-function of MUT-p53s.47 However, the survival difference
between patients with missense p53 mutations and other patients is not significant. Moreover,
after excluding other types of p53 mutants, patients with missense p53 mutants are still stratified
into two groups with significant different survival according to p53 overexpression or not before
chemotherapy. In a mouse model, MUT-p53 was stabilized by oncogene activation.44 Higher
MUT-p53 expression levels at diagnosis might indicate higher DNA damage, sustained
oncogene activation, loss-of-function (including MDM2 activation) and gain-of-function of
MUT-p53 at presentation; chemotherapy probably further stabilized and increased levels of
MUT-p53,44 and added more DNA damage to tumor cells due to the absence of WT-p53
function. Therefore, pre-treatment MUT-p53 expression level correlated with patient survival
even after chemotherapy.

Since MUT-p53s have oncogenic gain-of-function, the MDM2-mediated degradation of
MUT-p53 should alleviate oncogenesis. Unexpectedly, overexpression of MDM2, which was
thought to regulate oncoprotein MUT-p53 in the same way as WT-p53 (MDM2 \rightarrow p53),24 also
correlated with poor survival in our MUT-p53 subcohort mirroring p53 overexpression (Figures
2-3E). To explain this observation, perhaps the impact of MDM2 \rightarrow MUT-p53 on p53 function
was just as not significant as MDM2 \rightarrow WT-p53, or MDM2 \rightarrow MUT-p53 was lost in MUT-p53
DLBCL.45 However, none of the MUT-p53s in our study had mutations in the MDM2-binding
domain (N-terminus);45 most MUT-p53s had mutations in the DNA-binding domain (DBD),34
which might have altered conformation and interact with MDM2 by other sites alternative to the
N-terminus yet could still be efficiently degraded by MDM2 through a pathway different than
WT-p53.46 Conversely, it has been reported in cell lines that MDM2 and MUT-p53 stabilize each
other (p53\rightarrow MDM2 and MDM2\rightarrow p53).25 Supporting this theory, coexistence of MDM2 and
MUT-p53 was observed in lung carcinomas and the authors speculated this resulted in a gain-of-
function phenotype, stabilizing MDM2 which otherwise has a short half-life. Moreover, MDM2
mRNA levels were not significantly different between MDM2+ and MDM2– DLBCLs with
MUT-p53 according to our GEP analysis (Figure 5B, Supplemental Table 3), suggesting that
MDM2 overexpression is due to increased stability at the protein level.37.
Another explanation is the p53-independent oncogenic function of MDM2.\textsuperscript{2,12} However, this idea is weakened by the fact that the same effect was not shown in the WT-p53 subgroup. In addition, none of the differentially expressed genes between MDM2+ and MDM2– patients with MUT-p53 have been found to relate to MDM2 (Supplemental Table 3). Instead, 5 genes are related to WT- or MUT-p53. Therefore, it seems likely that the survival difference between MDM2+ and MDM2– patients can be ultimately attributed to the variable function and activities of different MUT-p53s.\textsuperscript{47} In this setting, MDM2 expression should be evaluated concurrently with MUT-p53 expression for prognostic studies.

Stratification by concomitant evaluation of p53 and MDM2 expression showed MDM2+ and p53+ patients are prognostic equivalents in WT- or MUT-p53 subgroups (Figure 4), supporting that MDM2+ and p53+ evaluation can be impacted by the oscillatory characteristics of p53/MDM2 levels, and that MDM2 overexpression may largely reflect p53 activity, an indication of cellular stress in tumor cells, or the oncogenic activity of MUT-p53.

MDM2 overexpression potentially could be caused by \textit{MDM2} gene amplification, enhanced transcription and translation, or reduced degradation. MDM2 SNP309 T→G causes enhanced \textit{MDM2} transcription, resulting in attenuated p53 function.\textsuperscript{32,33} In our study, \textit{MDM2} was rarely amplified. Similarly, increased MDM2 expression was not shown in the homozygous (G/G) or heterozygous (G/T) for SNP309 tumor cells among 108 patients in this study, and SNP309 T→G did not correlate with significantly poorer survival. Although similar results have been shown by some other reports,\textsuperscript{48,49} the lack of significance might be attributable to a small number of patients, similar estrogen level in the SNP309 groups, oscillation of MDM2 pulses, and possibility that endogenous MDM2 level could not be distinguished by IHC (which scores fraction of positive cells but not the intensity).

In our large DLBCL patient cohort, high MDM2 was not necessarily associated with low p53 (64 of 157 MDM2+ patients with WT-p53 had p53+ phenotype), p53 level could be high in patients with WT-p53 (only ~44% of p53+ patients had MUT-p53), and expression of MDM2 and p53 mutations were not mutually exclusive abnormalities of the TP53 pathway (Figure 2E and Table 1). These observations may reflect the existence of oscillatory p53/MDM2 pulses in tumor cells, or other regulatory factors that affect p53 and MDM2 levels (such as ARF, L11, MDM4, ATM, Akt, p300/CBP, p73).\textsuperscript{1,2} Nonetheless, several trends were observed by evaluating
the mean p53/MDM2 expression levels (Table 1). These trends suggest that MDM2 was induced by WT-p53 whereas WT-p53 level was low due to the reduced cellular stress by WT-p53 function and increased degradation by MDM2. In contrast, in patients with MUT-p53, MDM2 level was not increased due to the loss-of-function of MUT-p53 (according to the yeast p53-functional assay, only 4 MUT-p53s in MDM2– cases and 5 MUT-p53s in MDM2+ cases of this study preserved function to transactivate MDM2); MDM2+ phenotype in 35% of MUT-p53 cases is likely due to enhanced stabilization (otherwise short half-life of MDM2) by oncogenic activities of MUT-p53. MUT-p53 levels maintained high due to the sustained cellular stress, which activated and stabilized MUT-p53 similarly to WT-p53, and reduced degradation by lower levels of MDM2 (Table 1B).

In conclusion, our results in R-CHOP treated DLBCL patients show that p53 or MDM2 overexpression predicts a significantly poor survival in patients with MUT-p53, but not in patients with WT-p53. The prognostic value of p53 overexpression is attributable to the poor prognosis of MUT-p53+ but not WT-p53+, and that MUT-p53-negative patients were not necessary to have a poor survival. Stratification by p53 overexpression can be further improved by concomitant evaluation of MDM2 expression. These findings may explain the inconsistent prognostic value of p53 and MDM2 by several previous reports in the literature and provide direction to the future therapeutic drug designs targeting the p53-MDM2 pathway.
**Acknowledgements**

Z.Y.X.M. is a recipient of Harold C. and Mary L. Daily Endowment Fellowships at The University of Texas MD Anderson Cancer Center. A.T. is a recipient of the Stiftung zur Krebsbekaempfung Zurich Grant 269 award. M.A.P is funded by Ministerio de Ciencia e Innovación, Spain (RETICC, SAF2008-03871) and the Spanish Association against Cancer (AECC). K.H.Y is supported by The University of Texas MD Anderson Cancer Center Institutional Research Grant Award, an MD Anderson Lymphoma Specialized Programs of Research Excellence (SPORE) Research Development Program Award, an MD Anderson Myeloma SPORE Research Development Program Award, and MD Anderson Collaborative Research Funds with High-Throughput Molecular Diagnostics and Roche Molecular Systems. This work was also partially supported by National Cancer Institute and National Institutes of Health grants (R01CA138688, 1RC1CA146299, P50CA136411 and P50CA142509), and by the MD Anderson Cancer Center Support Grant CA016672.

**Authorship Contributions**

Designed research: Z.Y.X.-M. and K.H.Y.

Performed research: Z.Y.X.-M. and K.H.Y.


Analyzed data: Z.Y.X.-M., and K.H.Y.
Performed and supported statistical analysis: Z.Y.X.-M., and K.H.Y.

Conflicts of Interest
The authors declare no conflicts of interest.
REFERENCES


Tables

**Table 1.** MDM2 and p53 expression in DLBCL patients treated with R-CHOP.

**Table 2.** (A) Clinical characteristics of 478 *de novo* DLBCL treated with R-CHOP: comparison between patients with or without MDM2 overexpression or with different SNP309 genotypes. (B) Multivariate survival analysis.
Table 1. MDM2 and p53 expression in DLBCL patients treated with R-CHOP

(A) MDM2 and p53 expression in DLBCL patients with WT-p53 or MUT-p53

<table>
<thead>
<tr>
<th></th>
<th>Overall</th>
<th>WT-p53</th>
<th>MUT-p53</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of MDM2– patients</td>
<td>285</td>
<td>216</td>
<td>69</td>
</tr>
<tr>
<td>Number of MDM2+ patients</td>
<td>193</td>
<td>156</td>
<td>37</td>
</tr>
<tr>
<td>Mean MDM2 expression (% cells)</td>
<td><strong>22%</strong></td>
<td><strong>24%</strong></td>
<td><strong>17%</strong></td>
</tr>
<tr>
<td>Number of p53– patients</td>
<td>292</td>
<td>262</td>
<td>30</td>
</tr>
<tr>
<td>Number of p53+ patients</td>
<td>182</td>
<td>107</td>
<td>75</td>
</tr>
<tr>
<td>Mean p53 expression (% cells)</td>
<td><strong>23%</strong></td>
<td><strong>14%</strong></td>
<td><strong>54%</strong></td>
</tr>
</tbody>
</table>

(B) MDM2 and p53 expression in DLBCL patients with or without p53 overexpression

<table>
<thead>
<tr>
<th></th>
<th>Number of p53+</th>
<th>p53–</th>
<th>Mean p53 expression (% cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDM2– patients</td>
<td>79</td>
<td>204</td>
<td>19%</td>
</tr>
<tr>
<td>WT-p53/MDM2–</td>
<td>32</td>
<td>183</td>
<td>9%</td>
</tr>
<tr>
<td>MUT-p53/MDM2–</td>
<td>47</td>
<td>21</td>
<td>39%</td>
</tr>
<tr>
<td>MDM2+ patients</td>
<td>103</td>
<td>88</td>
<td>28%</td>
</tr>
<tr>
<td>WT-p53/MDM2+</td>
<td>75</td>
<td>79</td>
<td>17%</td>
</tr>
<tr>
<td>MUT-p53/MDM2+</td>
<td>28</td>
<td>9</td>
<td>50%</td>
</tr>
</tbody>
</table>

Note: WT-p53 indicates wild-type p53 protein; MUT-p53, mutated p53 or p53 mutants.

(C) Numbers of DLBCL patients with different SNP309 genotypes and the mean MDM2 expression level

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of patients</th>
<th>Mean MDM2 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>G/G</td>
<td>10</td>
<td>33%</td>
</tr>
<tr>
<td>G/T</td>
<td>57</td>
<td>42%</td>
</tr>
<tr>
<td>T/T</td>
<td>41</td>
<td>42%</td>
</tr>
</tbody>
</table>
**Table 2. (A) Clinical characteristics of patients with de novo DLBCL**

<table>
<thead>
<tr>
<th></th>
<th>Overall (n=478)</th>
<th>WT-p53 (n=372)</th>
<th>MUT-p53 (n=106)</th>
<th>SNP (n=108)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MDM2−</td>
<td>MDM2+</td>
<td>P</td>
<td>MDM2−</td>
</tr>
<tr>
<td><strong>n</strong></td>
<td>285</td>
<td>193</td>
<td></td>
<td>216</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 60</td>
<td>124</td>
<td>68</td>
<td></td>
<td>95</td>
</tr>
<tr>
<td>≥ 60</td>
<td>161</td>
<td>125</td>
<td>0.07</td>
<td>121</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>128</td>
<td>69</td>
<td></td>
<td>97</td>
</tr>
<tr>
<td>M</td>
<td>157</td>
<td>124</td>
<td>0.046</td>
<td>119</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I - II</td>
<td>136</td>
<td>79</td>
<td></td>
<td>101</td>
</tr>
<tr>
<td>III - IV</td>
<td>137</td>
<td>108</td>
<td>0.11</td>
<td>103</td>
</tr>
<tr>
<td><strong>B-symptoms</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>158</td>
<td>123</td>
<td></td>
<td>118</td>
</tr>
<tr>
<td>Yes</td>
<td>80</td>
<td>51</td>
<td>0.35</td>
<td>63</td>
</tr>
<tr>
<td><strong>LDH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>83</td>
<td>64</td>
<td></td>
<td>69</td>
</tr>
<tr>
<td>Elevated</td>
<td>180</td>
<td>103</td>
<td>0.17</td>
<td>130</td>
</tr>
<tr>
<td><strong>No. of extranodal sites</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 - 1</td>
<td>217</td>
<td>138</td>
<td></td>
<td>164</td>
</tr>
<tr>
<td>≥ 2</td>
<td>55</td>
<td>45</td>
<td>0.27</td>
<td>41</td>
</tr>
<tr>
<td><strong>Performance status</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 - 1</td>
<td>218</td>
<td>137</td>
<td></td>
<td>163</td>
</tr>
<tr>
<td>≥ 2</td>
<td>30</td>
<td>27</td>
<td>0.21</td>
<td>23</td>
</tr>
<tr>
<td><strong>Size of largest tumor</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 5cm</td>
<td>131</td>
<td>87</td>
<td></td>
<td>101</td>
</tr>
<tr>
<td>≥ 5cm</td>
<td>88</td>
<td>62</td>
<td>0.78</td>
<td>63</td>
</tr>
<tr>
<td><strong>IPI risk group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 - 2</td>
<td>170</td>
<td>99</td>
<td></td>
<td>130</td>
</tr>
<tr>
<td>3 - 5</td>
<td>83</td>
<td>71</td>
<td>0.064</td>
<td>61</td>
</tr>
<tr>
<td><strong>Therapy response</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td>225</td>
<td>139</td>
<td>0.081</td>
<td>176</td>
</tr>
<tr>
<td>PR</td>
<td>33</td>
<td>30</td>
<td></td>
<td>22</td>
</tr>
<tr>
<td>SD</td>
<td>13</td>
<td>4</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>PD</td>
<td>14</td>
<td>20</td>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

- 26 -
(B) Multivariate analysis

<table>
<thead>
<tr>
<th></th>
<th>OS</th>
<th>PFS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hazard Ratio</td>
<td>95% CI</td>
</tr>
<tr>
<td>Overall cohort</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IPI &gt; 2</td>
<td>3.04</td>
<td>1.96-3.96</td>
</tr>
<tr>
<td>B-symptoms</td>
<td>1.43</td>
<td>1.00-2.04</td>
</tr>
<tr>
<td>TP53 mutation</td>
<td>1.59</td>
<td>1.06-2.26</td>
</tr>
<tr>
<td>MUT-p53 subcohort</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IPI &gt; 2</td>
<td>2.03</td>
<td>1.05-3.93</td>
</tr>
<tr>
<td>p53 &gt; 10%</td>
<td>2.70</td>
<td>1.04-7.04</td>
</tr>
<tr>
<td>MDM2 &gt; 10%</td>
<td>2.24</td>
<td>1.17-4.27</td>
</tr>
</tbody>
</table>

Note: WT-p53, wild-type p53; MUT-p53, mutated p53 or p53 mutants; LDH, lactate dehydrogenase; IPI, International Prognostic Index; CR, complete remission; PR, partial response; SD, stable disease; PD, progressive disease. For therapy response, we calculated P values as CR vs other responses. Some clinical features of certain cases were not available.
Figures

**Figure 1.** (A-B) Histogram showing the distribution of MDM2 and p53 expression levels in the DLBCL cohort. X-axis, percentage of immunopositive cells in tumors; Y-axis, numbers of DLBCL patients. (C) Illustration of p53 and MDM2 kinetic pulses in tumor cells under stress conditions. In a single cell, due to the fluctuating p53/MDM2 levels in oscillatory pulses the IHC pattern is 1) p53–/MDM2– at zero time point A or E after cellular stress is removed; 2) p53+/MDM2+ at time point B; 3) p53–/MDM2+ at time point C; and 4) p53+/MDM2– at time point D. Same patterns over cell population may be generated by evaluation of the percentage of MDM2 positive cells and defining overexpression by certain cutoffs. (D) Representative MDM2 immunohistochemical staining patterns.

**Figure 2.** Impact of MDM2 and p53 expression on overall survival in *de novo* DLBCL patients with WT- or MUT-p53.

**Figure 3.** Impact of MDM2 and p53 expression on progression-free survival in *de novo* DLBCL patients with WT- or MUT-p53.

**Figure 4.** Concurrent evaluation of p53 and MDM2 overexpression in DLBCL.

**Figure 5.** (A) Differentially expressed genes in patients with MDM2+ and MDM2– DLBCL with WT-p53. (B) Differentially expressed genes in patients with MDM2+ and MDM2– DLBCL with MUT-p53. (C) Differentially expressed genes in patients with p53+ and p53– DLBCL with WT-p53. (D) *MDM2* amplification shown by FISH. (E-F) Impact of MDM2 SNP309 on overall survival and progression-free survival in DLBCL patients.
Figure 1

A. Bar chart showing the number of patients across different percentage ranges of MDM2 expression.

B. Bar chart showing the number of patients across different percentage ranges of p53 expression.

C. Graph showing the levels of p53 and MDM2 over time.

D. Images showing MDM2 expression levels: 0%, 10%, 40%, and 100%.
Figure 2

(A) MDM2

- MDM2 ≤10% (n=285)
- MDM2 >10% (n=193)

P=0.18

(B) p53

- p53 ≤10% (n=292)
- p53 >10% (n=182)

P=0.017

(C) WT-p53, MDM2 ≤10% (n=216)

P=0.71

(D) WT-p53, p53 ≤10% (n=262)

P=0.88

(E) MUT-p53, MDM2 ≤10% (n=69)

P=0.0072

(F) MUT-p53, p53 ≤10% (n=30)

P=0.016
Figure 4

A

WT-p53, p53+/MDM2- (n=183)

WT-p53, p53+/MDM2+ (n=75)

WT-p53, p53-/MDM2- (n=79)

WT-p53, p53+/MDM2- (n=32)

P = 0.93

B

WT-p53, p53+/MDM2- (n=183)

WT-p53, p53+/MDM2+ (n=75)

WT-p53, p53-/MDM2+ (n=9)

WT-p53, p53+/MDM2- (n=47)

P = 0.99

C

MUT-p53, p53-/MDM2- (n=21)

MUT-p53, p53-/MDM2+ (n=9)

MUT-p53, p53+/MDM2- (n=47)

MUT-p53, p53+/MDM2+ (n=28)

P = 0.0088

D

MUT-p53, p53-/MDM2- (n=21)

MUT-p53, p53+/MDM2+ (n=9)

MUT-p53, p53+/MDM2- (n=47)

MUT-p53, p53+/MDM2+ (n=28)

P = 0.0088

E

MUT-p53, p53 & MDM2 ≤10% (n=21)

MUT-p53, p53 or MDM2 >10% (n=84)

P = 0.0029

F

MUT-p53, p53 & MDM2 ≤10% (n=21)

MUT-p53, p53 or MDM2 >10% (n=84)

P = 0.0027
Figure 5

A, B, C, D, E, F: Various images and graphs illustrating gene expression and survival analysis.
MDM2 phenotypic and genotypic profiling, respective to TP53 genetic status, in diffuse large B-cell lymphoma patients treated with rituximab-CHOP immunochemotherapy

Zijun Y. Xu-Monette, Michael B. Møller, Alexander Tzankov, Santiago Montes-Moreno, Wenwei Hu, Ganiraju C. Manyam, Lei Fan, Carlo Visco, Karen Dybkær, April Chiu, Wayne Tam, Youli Zu, Govind Bhagat, Kristy L. Richards, Eric D. Hsi, William W.L. Choi, J. Han van Krieken, Qin Huang, Jooryung Huh, Weiyun Ai, Maurilio Ponzoni, Andrés J.M. Ferreri, Jane N. Winter, Lin Wu, Xiaoying Zhao, Ronald S. Go, Sa A. Wang, Carlos E. Bueso-Ramos, Yong Li, Miguel A. Piris, L. Jeffrey Medeiros and Ken H. Young