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TP53 mutations predict for poor survival in *de novo* diffuse large B-cell lymphoma of germinal center subtype

Norafiza Zainuddin [a], Mattias Berglund [a], Alkwin Wanders [b], Zhi-Ping Ren [b], Rose-Marie Amini [b], Monica Lindell [c], Meena Kanduri [b], Göran Roos [d], Richard Rosenquist [b,*,1], Gunilla Enblad [a,1]

[a] *Department of Oncology, Radiology and Clinical Immunology, Uppsala University, Uppsala, Sweden*
[b] *Department of Genetics and Pathology, Uppsala University, Uppsala, Sweden*
[c] *Department of Pathology, Uppsala University Hospital, Uppsala, Sweden*
[d] *Department of Medical Biosciences, Pathology, Umeå University, Umeå, Sweden*

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Abstract

Presence of *TP53* mutations has been associated with poor prognosis in diffuse large B-cell lymphoma (DLBCL), although this has remained controversial. The *TP53* codon 72 polymorphism has shown negative impact on cancer survival, but this has not been analyzed in DLBCL. Furthermore, the *MDM2* SNP309 has been associated with earlier age of onset in DLBCL. Here, we investigated the clinical impact of *TP53* mutations, *MDM2* SNP309 and *TP53* codon 72 polymorphisms on survival in DLBCL of germinal center (GC) and non-GC subtypes. Thirteen of the 102 (12.7%) patients displayed *TP53* mutations. Overall, *TP53* mutations had a significant effect on lymphoma-specific survival (LSS, $P = 0.009$) and progression-free survival (PFS, $P = 0.028$). In particular, inferior survival was observed in *TP53*-mutated DLBCLs of GC subtype (LSS, $P = 0.002$ and PFS, $P = 0.006$). Neither *MDM2* SNP309 nor the *TP53* codon 72 polymorphism had an impact on age of onset or survival. Altogether, our data suggests that *TP53* mutations are associated with poor outcome in GC-DLBCL patients.

Keywords: *TP53* mutation; *MDM2* SNP309; *TP53* codon 72 polymorphism; Diffuse large B-cell lymphoma; Germinal center subtype; Survival

1. Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common type of lymphoma, comprising approximately 30–40% of newly diagnosed lymphomas in western countries [1]. It is an aggressive lymphoma, known to be heterogeneous both regarding morphology and clinical outcome. Based on knowledge obtained from gene expression profiling [2,3] DLBCL is divided into prognostically different subgroups according to cell of origin. The germinal center (GC) subtype is characterized by CD10 and/or BCL6 expression and correlates with a better prognosis [3–6], whereas the activated B-cell like (ABC) group, which has poor clinical outcome, is defined by the absence of GC markers and the presence of the MUM1/IRF4 antigen [3–6]. A small number of cases are unclassifiable, which are usually grouped with the ABC-subtype as non-GC DLBCL.

p53 is known to play a critical role in cellular responses since it responds to damaged DNA by induction of G1 arrest, and, if DNA is not repaired, p53 may induce apoptosis [7]. p53 dysfunction can induce abnormal cell growth and mutant p53 can deregulate apoptosis, resulting in increased cell survival, genetic instability and eventually malignant transformation [8]. Mutated p53 is also associated with resistance to chemotherapy [9]. Alterations in the *TP53* gene are less common in hematological malignancies than in solid tumors, and in B-cell lymphomas, mutations are found in 10–20% of tumors [10,11]. Exons 5–8 of *TP53*, which contain highly

* Corresponding author at: Department of Genetics and Pathology, Rudbeck Laboratory, Uppsala University, SE-751 85 Uppsala, Sweden. Tel.: +46 18 6115947; fax: +46 18 554025.
E-mail address: richard.rosenquist@genpat.uu.se (R. Rosenquist).
[1] RR and GE contributed equally to this work as last authors.

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conserved domains (HCD), have been identified as *TP53* mutational hotspots [12]. Mutations outside hotspot exons account for less than 5% of all mutations and are infrequent in lymphomas [13].

TP53 mutations have been associated with disease progression in follicular lymphomas [14] and poor prognosis in mantle cell lymphoma [15], Burkitt lymphoma [16] and chronic lymphocytic leukemia (CLL) [17]. In DLBCL, the impact of *TP53* mutations on survival remains controversial. Several groups have reported that *TP53* mutations predicted for poor overall in DLBCL [13,18,19]. Leroy et al. [19] have shown that *TP53* mutations were associated with poor outcome in low and low–intermediate risk groups of DLBCL. A more recent study by Young et al. [20] have confirmed that presence of *TP53* mutations, in particular mutations in the core domain of *TP53*, are correlated with poor overall survival. Other studies, however, have failed to demonstrate any correlation between *TP53* mutations and prognosis [21,22].

Two single nucleotide polymorphisms (SNPs) that may impact *TP53* function include a polymorphism in the promoter of the *MDM2* gene and a *TP53* codon 72 polymorphism. The SNP of the *MDM2* gene is referred to as SNP309 (a T → G change) (rs2279744) and it has been shown to promote tumor growth in a p53-independent manner [23]. The G allele of SNP309 increases expression of *MDM2* and accelerates tumor development [24]. Previous studies on various cancers have associated the G/G genotype with an early age onset and increased risk for tumor formation [25–27]. Recently, accelerated age of onset of DLBCL was reported in females carrying G alleles [28].

The pro-apoptotic activity of the p53 protein is dependent on a proline-rich domain containing a common Pro-Arg polymorphism at codon 72 (rs1042522). These two protein variants behave differently; the Arg allele increases mitochondrial localization of p53 in cells and induces cellular death, whereas the Pro allele exhibits lower apoptotic potential and appears to induce a higher level of G1 arrest [29]. There have been contradictory findings on the impact of codon 72 polymorphism on survival in cancer. In several reports, the Pro/Pro genotype was associated with inferior survival [30–32], while in other studies reduced survival was associated with the presence of Arg/Pro genotype [33,34]. A study on CLL patients revealed that the homozygous Arg genotype was associated with an increased susceptibility to CLL, but no impact on survival was found [35].

To further investigate the clinical relevance of *TP53* mutations, *MDM2* SNP309 and *TP53* codon 72 polymorphism, we here investigated a large number of *de novo* DLBCL of GC and non-GC subtypes.

Table 1
Patient characteristics according to immunohistochemically defined GC and non-GC phenotypes

Clinical characteristics	All	GC	Non-GC	*P* value*
No. of patients	102	47	55	
Gender				0.445
Male	53	22	31	
Female	49	25	24	
Median of age (years)	66	62	68	
Age (years)				0.445
≤65	49	25	24	
>65	53	22	31	
Stage				0.950
I to II	47	21	26	
III to IV	55	26	29	
IPI				0.662
Low (0–1)	37	19	18	
Intermediate (2-3)	48	20	28	
High (4–5)	14	6	8	
Missing	3	2	1	
Type of treatment				0.857
Chemotherapy	97	45	52	
Others	5	2	3	
Treatment response				0.069
Complete response	71	37	34	
No complete response	30	9	21	
Missing	1	1	0	

*Chi-square test.

2. Materials and methods

2.1. Study subjects

The study population consisted of 102 *de novo* DLBCL patients treated at Uppsala and Umeå University Hospital, Sweden, between 1984 and 2002. DNA samples from the time of diagnosis and a clinical follow-up were available for all patients. The histological classification of the samples was reviewed by experienced histopathologists and confirmed to be *de novo* DLBCL according to the WHO classification [36]. GC and non-GC subtypes were distinguished based on the algorithm of Hans et al. [6]. The characteristics of the patients with respect to sex, age, stage, International Prognostic Index (IPI) [37] and treatment are listed in Table 1. All patients, except five, were treated with antracycline containing chemotherapy, mainly CHOP or VACOP-B. Five patients received radiotherapy only due to stage I disease and another five patients received Rituximab in combination with chemotherapy.

2.2. TP53 mutation and/or deletion detection

The primer sequences for *TP53* exons 4–8 are listed in Supplementary Table 1 and PCR amplification of exons 4–8 of the gene was performed as detailed in Supplementary information. The PCR products were purified and sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and an ABI 3730 automatic sequencer. The Genome Assembly Program (GAP) software version 1.5, BioEdit Sequence Alignment Editor Version 7.0.5.3 and the GenBank data library (release 160.0) were used to analyze the sequences. The sensitivity of direct sequencing was investigated by diluting DNA from the U-2932 cell line [38], which carries a known *TP53* mutation (TGC to TAC at amino acid 176 of exon 5), with DNA from a healthy control. The mutated and normal DNA were mixed in different ratios ranging from 100% to

10% and exon 5/6 was amplified and sequenced using both forward and reverse primers. The cutoff for detecting the cell line *TP53* mutation was between 20% and 25%.

Cases which did not show any positive PCR products for exons 4–8 were evaluated for *TP53* deletion status using two informative microsatellite markers p53CA (25 kb upstream from the *TP53* gene) and D17S1678 (40 kb telomeric to the *TP53* gene) (Table 2). The oligonucleotide primers were labeled with fluorescent dye FAM™ and the PCR amplification was performed as outlined in Supplementary information. PCR products were submitted for analysis using GeneScan Fragment Analysis (Applied Biosystems, USA). Furthermore, these cases were also analyzed using an independent PCR protocol with *TP53* primers for each of exons 4–8 (see Supplementary information).

2.3. MDM2 SNP309 and TP53 codon 72 genotyping

MDM2 SNP309 and *TP53* codon 72 polymorphisms were determined using PCR-restriction fragment length polymorphism (RFLP). The primer sequences for *MDM2* SNP309 and *TP53* codon 72 are shown in Supplementary Table 1, and the PCR amplification was performed as detailed in Supplementary information. To differ between *MDM2* SNP309 and *TP53* codon 72 genotypes, the resulting PCR products were digested and separated on polyacrylamide gels (see Supplementary information).

2.4. Statistical analysis

Two-tailed P values were generated for two contingency tables, using Fisher's exact test, and for larger contingency tables, using Chi-square analysis. All tests were two-sided and the significance level was set to 0.05. Non-parametric Wilcoxon Mann–Whitney and Kruskal–Wallis tests were used to compare the median age of diagnosis between the different genotypes. Lymphoma-specific survival (LSS) and progression-free survival (PFS) rates were estimated using the Kaplan–Meier method. Survival differences between subgroups were compared by the log-rank test. LSS was calculated from the date of diagnosis to the date of the last follow-up or death caused by lymphoma disease. PFS was calculated from the date of diagnosis to the date of the occurrence of the first relapse. Patients alive and in remission at last follow-up were censored from analysis. Statistica 7.1 software (Statsoft, Tulsa, OK, USA) was applied for all calculations.

3. Results

3.1. TP53 mutations

In total, *TP53* mutations were identified in 13 of 102 (12.7%) DLBCL patients; where 8 of 55 (14.5%) non-GC DLBCL and 5 of 47 (10.6%) GC-DLBCL samples were *TP53* mutated ($P = 0.82$, Table 2). There were no differences in the frequencies of *TP53* mutations between male and female patients or in patients with different IPI (data not presented). Of all *TP53* mutations, eight DLBCL samples showed a single missense mutation, whereas one sample displayed a frameshift mutation (Table 2). Six cases demonstrated *TP53* mutations in the HCD and three of them showed mutations involving two hotspot codons; codon 248 of exon 7 (case 2) and codon 273 of exon 8 (cases 1 and 3). Furthermore, in four DLBCL samples, no PCR product was amplified for exons 4–8, thus indicating homozygous deletions. These deletions were further analyzed using two microsatellite markers (p53CA and D17S1678) as well as an independent TP53 primer set (exons 4–8). No specific PCR product could be amplified for the microsatellite markers or the additional *TP53* primer set in the four cases in contrast to healthy control and DLBCL cases without deletion, thus confirming the presence of homozygous deletions (Table 2). In the following text we have collectively termed point mutations and deletions as *TP53* mutations.

3.2. MDM2 SNP309 and TP53 codon 72 polymorphisms

The wildtype (WT) genotype for *MDM2* SNP309 (T/T) was detected in 33 cases, whereas the heterozygous and homozygous genotypes for SNP309 (T/G and G/G) were present in 48 and 19 cases. Allele frequencies were 57% for T

Table 2
TP53 mutations/deletions identified in 13 of 102 (12.7%) *de novo* DLBCL according to GC and non-GC subtypes

Case	Phenotypes	Mutation/deletion status	Nucleotide change	Amino acid change	Mutation effect
1	GC	Codon 273 exon 8	CGT > CAT	Arg > His	Missense mutation
2	GC	Codon 248 exon 7	CGG > TGG	Arg > Trp	Missense mutation
3	GC	Codon 273 exon 8	CGT > TGT	Arg > Cys	Missense mutation
4	GC	Codon 152 exon 5	CCG > CTG	Pro > Leu	Missense mutation
5	GC	17p deletion[a]	NA	NA	Homozygous deletion
6	Non GC	Codon 281 exon 8	GAC > CAC	Asp > His	Missense mutation
7	Non GC	Codon 305 exon 8	AAG > AAC	Lys > Asp	Missense mutation
8	Non GC	Codon 126 exon 5	TAC > CAC	Tyr > His	Missense mutation
9	Non GC	Codon 109 exon 4	TTC > NA	Phe > NA	Frameshift mutation
10	Non GC	17p deletion[a]	NA	NA	Homozygous deletion
11	Non GC	17p deletion[a]	NA	NA	Homozygous deletion
12	Non GC	Codon 281 exon 8	GAC > GTC	Asp > Val	Missense mutation
13	Non GC	17p deletion[a]	NA	NA	Homozygous deletion

NA = not applicable.

[a] None of *TP53* exons 4–8 amplifications showed PCR products. Homozygous deletions were confirmed using microsatellite markers p53CA (25 kb upstream from *TP53*) and D17S1678 (40 kb telomeric to *TP53*) and an alternative set of *TP53* primers for each exon (4–8).

and 43% for G and the observed genotype frequencies in our series were in Hardy–Weinberg equilibrium. Among DLBCL cases analyzed for *TP53* codon 72 polymorphisms, WT Arg/Arg, heterozygous Arg/Pro and homozygous Pro/Pro genotypes were detected in 60, 29 and 9 cases, respectively. Allele frequencies were 76% for Arg and 24% for Pro. No deviation from the Hardy–Weinberg equilibrium was demonstrated.

3.3. Survival analysis of TP53, MDM2 and TP53 codon 72

101 of 102 patients with *TP53* mutations had survival data available for LSS analysis, whereas PFS analysis was performed according to data available for 100 patients. Overall, there was a significant difference in LSS between DLBCL patients with or without *TP53* mutations (median LSS, 13 *versus* 20 months; $P = 0.009$, Fig. 1A). However, a more pronounced difference in LSS was observed in DLBCL patients of GC subtype ($P = 0.002$, Fig. 1B), where the median LSS was 13 *versus* 49 months in mutant *TP53* compared to WT cases. On the other hand, *TP53* mutations did not predict for poor LSS in the non-GC DLBCL subtype ($P = 0.517$, Fig. 1C). While *TP53* mutations predicted for poor PFS in all patients and patients with GC subtypes ($P = 0.028$, Fig. 2A and $P = 0.006$, Fig. 2B), no significant difference on PFS was found between WT and *TP53*-mutated patients in the non-GC subtype ($P = 0.676$, Fig. 2C).

No significant difference was found in LSS ($P = 0.468$) and PFS ($P = 0.497$) between patients with *MDM2* SNP309 T/T genotype and patients with T/G and G/G genotypes. In addition, SNP309 genotype did not predict for survival in either GC-DLBCL (LSS, $P = 0.957$ and PFS, $P = 0.837$) or non-GC DLBCL (LSS, $P = 0.280$ and PFS, $P = 0.154$). No significant difference was observed in median age of diagnosis, either when the three genotypes were considered independently ($P = 0.494$, Kruskal–Wallis) or when T/G and G/G genotypes were grouped together ($P = 0.545$, Mann–Whitney). In addition, no significant association was found between age of diagnosis according to gender ($P > 0.05$).

TP53 codon 72 genotypes did not predict for LSS ($P = 0.575$) and PFS ($P = 0.216$) in our series of DLBCL. In addition, no significant difference was observed in median age of diagnosis, either when the three genotypes were considered independently ($P = 0.746$, Kruskal–Wallis) or when Arg/Arg and Arg/Pro genotypes were grouped together ($P = 0.559$, Mann–Whitney). Codon 72 genotypes were not significantly associated with survival in either GC-DLBCL (LSS, $P = 0.819$ and PFS, $P = 0.656$) or non-GC DLBCL (LSS, $P = 0.705$ and PFS, $P = 0.274$).

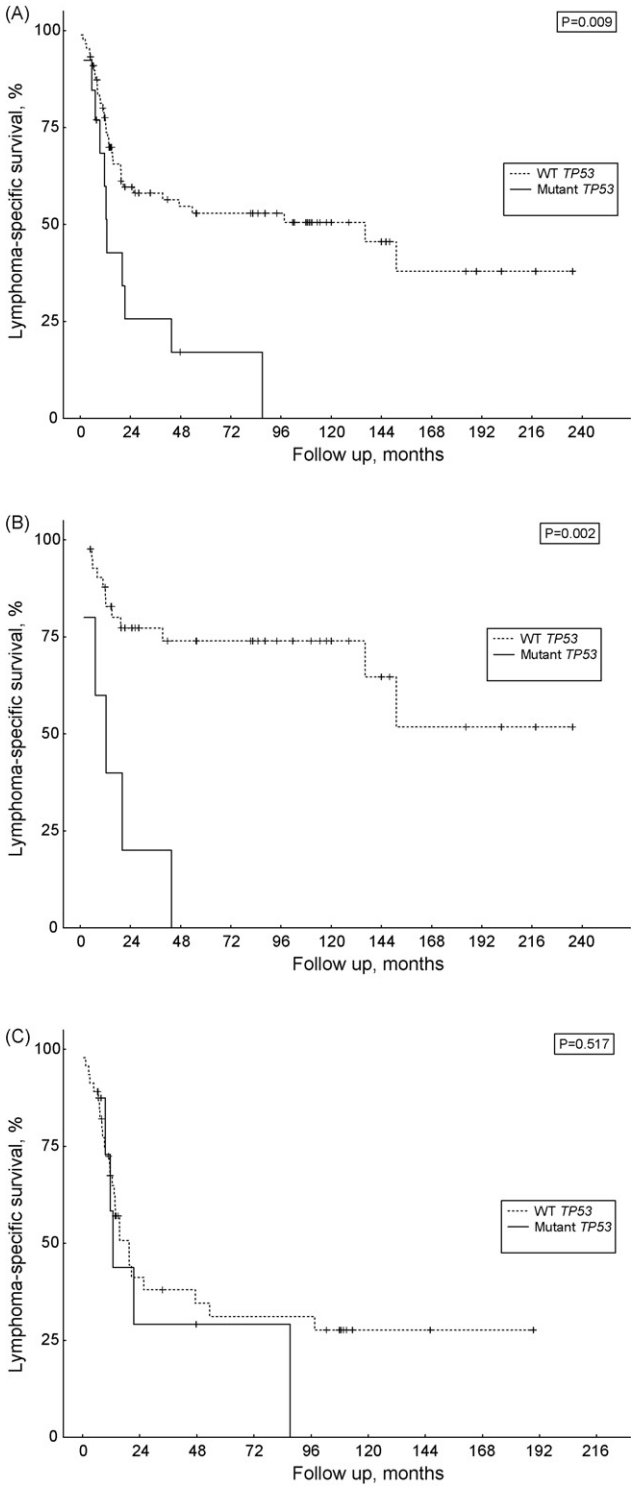

Fig. 1. *TP53* mutations predicts for poor LSS in DLBCL, with significantly inferior survival observed in the GC subtype. (A) Kaplan–Meier curves of LSS in all DLBCL patients. (B) LSS in DLBCL patients with GC subtypes according to *TP53* mutation status. (C) LSS in DLBCL patients with mutant and WT *TP53* within the non-GC subtypes.

4. Discussion

The present study analyzed mutations of *TP53* in exons 4–8, which includes the specific binding domain of the p53

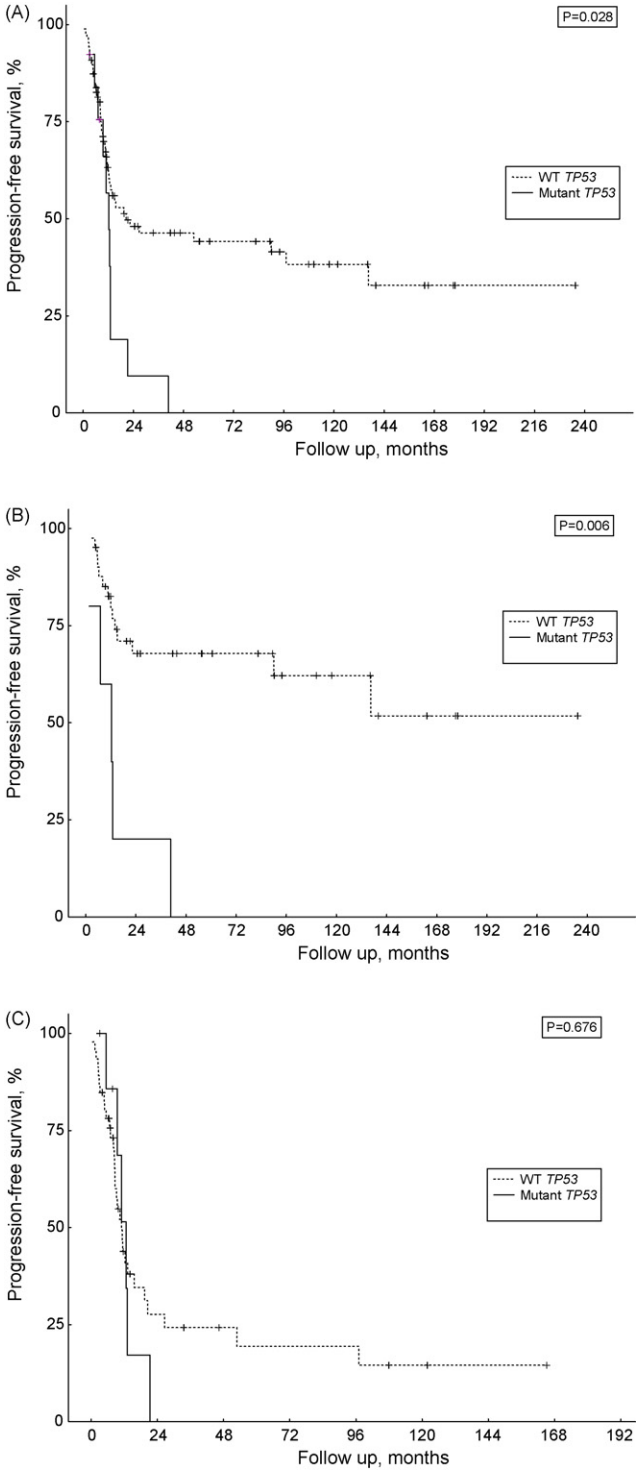

Fig. 2. PFS and *TP53* mutation status in DLBCL. Significant difference was observed in all patients and patients with GC subtypes. (A) PFS in all DLBCL patients with *TP53* mutations *versus* those with WT *TP53*. (B) PFS in DLBCL patients with GC subtypes according to *TP53* mutation status. (C) PFS in DLBCL patients with mutant and WT *TP53* within the non-GC subtypes.

protein, where most human tumor mutations are located. The frequency of *TP53* mutations observed in our series, which is 12.7%, was in range of previously reported frequency of *TP53* mutations in DLBCL (10–23%) [13,18–20,39–41]. The mutations were identical to mutations reported by others in various cancers and collated in the IARC *TP53* Database version R12 [42] and Universal Mutation Database for *TP53* mutations [43,44]. Five of 8 missense mutations in this study were found within exon 8. Previous studies on *TP53* mutations spanning exons 5–8 have reported different common sites for mutation. Kamata et al. [39] found a high frequency of mutations on exon 5 in DLBCL; others reported a high prevalence of mutations on exons 6 and 7 [45,46] in other lymphoma types.

Overall, *TP53* mutations predicted for poor LSS in DLBCL, with a greater prognostic value observed in the GC subtype (LSS, $P = 0.002$). Our findings are thus comparable to several studies in DLBCL which showed that the presence of *TP53* mutations, specifically in exons 5–8, had an adverse impact on overall survival [18,19,40,41]. In addition, our results are in line with a recent study by Young et al. [20], where they showed a tendency towards poor overall survival among *TP53*-mutated patients of GC subtype ($P = 0.057$). Also in parallel with this latter study, no significant difference was observed in survival among patients with non-GC subtypes [20]. We also observed similar frequencies of *TP53* mutations in the GC and non-GC subtypes (10.6% *versus* 14.5%), as in the study by Young et al. [20], suggesting that *TP53* alterations may constitute a common secondary defect in DLBCL [10,47]. No other studies have so far reported the correlation between *TP53* mutations and the molecular subgroups of DLBCL.

What could be the reasons for a more pronounced clinical effect of *TP53* mutations in GC-DLBCL? One explanation to our novel finding might be the involvement of nuclear factor (NF)-κB in p53-mediated apoptosis, where mutant p53 has been reported to activate the anti-apoptotic effect of NF-κB [47], which could render a growth advantage for *TP53*-mutated GC-DLBCL tumors. In contrast, mutant p53 may have limited effect in the non-GC DLBCL subtype, since the NF-κB signaling pathway is already activated in most cases [48].

Various results have been reported regarding *MDM2* SNP309 and its association with survival and age of onset in cancer. SNP309 has been associated with poor survival in certain studies [48–51]; whereas in other studies no impact on survival [52] and age of onset [53] was found. No published data has correlated this polymorphism with DLBCL other than a study by Bond et al. [28] who found an accelerated age of diagnosis in female DLBCL patients with G-allele. In the present study, however, we could not find any association between age of diagnosis and the SNP309, either in female and male, which is in contrast to the findings by Bond et al. [28]. Moreover, our data did not reveal any correlation between the *MDM2* SNP309 and survival.

The *TP53* codon 72 polymorphism has also been evaluated as a predictive marker in human cancer. *TP53* codon 72 genotypes have been shown to have a negative impact on cancer survival, in particular the homozygous Pro genotype [30–32,54]. Other reports have indicated that codon 72 genotypes were not clinically relevant for patient survival [35,55]. From the present study, we conclude that the codon 72 genotypes appear to have no impact on prognosis and survival in DLBCL patients.

In conclusion, the present study supports previous findings that *TP53* mutations are associated with poor outcome in DLBCL patients and our data further suggests that this is most prominent within the GC-DLBCL subtype. This study thus highlights the significance of *TP53* mutation screening as a prognostic marker, although further studies on the predictive capacity of *TP53* mutations in the new era of treatment in DLBCL (R-CHOP) are needed. On the other hand, the *MDM2* SNP309 and *TP53* codon 72 polymorphisms did not show any association with clinical parameters in DLBCL and hence appears to lack clinical relevance in DLBCL.

Conflict of interest

The authors declare no conflict of interest.

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Contributions: Norafiza Zainuddin performed research, analyzed data, and wrote the paper; Mattias Berglund, Alkwin Wanders, Zhi-Ping Ren, Rose-Marie Amini, Monica Lindell and Meena Kanduri performed research and analyzed data; Göran Roos provided samples and associated data; Richard Rosenquist and Gunilla Enblad designed and supervised the research and wrote the paper.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.leukres.2008.06.022.

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