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Genomic alterations of ribosomal protein genes in diffuse large B cell lymphoma

Ribosomal biogenesis (the process of building new ribosomes for protein synthesis), is frequently altered in cancer. Current evidence suggests that ribosomal biogenesis is involved in different processes other than protein synthesis, acting as a dynamic stress sensor regulating cell cycle and apoptosis through modulation of p53 activity (Bursac *et al*, 2014).

Genomic screening has recently identified copy number alterations and somatic mutations of ribosomal proteins (RPs) in haematological malignancies such as acute lymphoblastic leukaemia, chronic lymphocytic leukaemia (CLL) and multiple myeloma (MM) (reviewed in Sulima *et al*, 2017). In the present study we analysed public datasets containing whole exome sequencing data from multiple studies on mature lymphoid B cell malignancies using the cBioportal website analysis tool (Cerami *et al*, 2012), focusing on genomic alterations of ribosomal protein genes ($n = 80$) and the *TP53* gene (which encodes the p53 protein). Copy number variants (CNV) data were available only for DLBCL [The Cancer Genome Atlas (TCGA) provisional dataset; http://www.cbioportal.org/study?id=dlbc_tcga#summary]. We initially evaluated 7 studies ($n = 1016$ patients) including DLBCL [3 studies: TCGA provisional dataset + (Lohr *et al*, 2012 and Braggio *et al*, 2015) ($n = 116$ patients)], Mantle cell lymphoma (Beà *et al*, 2013; 29 patients), MM (Lohr *et al*, 2014; 205 patients), CLL (Landau *et al*, 2013; Puente *et al*, 2015; 2 studies, 666 patients).

Genomic alterations of RPs occurred at low frequency in 4% of analysed samples ($n = 42$) (Fig 1A). The only RPs found to be mutated in more than one patient were *RPL3* ($n = 3$), *RPL4* ($n = 2$), *RPL10* ($n = 3$), *RPL10A* ($n = 2$), *RPL13* ($n = 4$), *RPSA* ($n = 2$), *RPS2* ($n = 2$), *RPS9* ($n = 2$), *RPS16* ($n = 2$), *FAU* ($n = 3$) (Fig 1A). RP gene mutations were found in DLBCL, CLL and MM and were predominantly missense. Mutations of RPs belonging to the large ribosomal subunit were predominant in DLBCL and MM, whereas CLL was characterized by a higher frequency of mutations of the small ribosomal subunit (Fig 1B). Notably, DLBCL showed the highest frequency of RP genomic alterations (Fig 1C). More precisely, we identified non-recurrent mutations of 13 different RP genes in 14 (12%) of 116 DLBCL patients (Fig 1D). *RPL3, RPL13* and *FAU* were the only RP genes mutated in more than one DLBCL patient sample (2 samples each, 1·7%). Of note, *FAU* mutations affected the ubiquitin-like protein FUBI at the N terminus, sparing the RPS30 protein. Affected genes and mutations are summarized in Table I. Residues involved in *RPL3* and *RPL13* mutations are depicted in Fig 1E. Overall, there was a tendency towards a mutual exclusivity of RP gene mutations,

Fig 1. (A) Heat map showing RP mutations in 7 patient cohorts including 1016 patients affected by lymphoproliferative disorders (116 DLBCL, 205 MM, 29 MCL, 666 CLL patients). Each row represents a gene, each column represents a patient. (B) Charts showing the fraction of samples harbouring mutated RPs in each disease type (left), and the fraction of cases harbouring mutations of the large *versus* small ribosomal subunit RP genes (right). (C) Bar graph showing the incidence of RPs mutations across different B cell lymphoid malignancies, demonstrating significantly higher frequency in DLBCL. $^{**}P$ value <0·01, chi-square test. (D) Heat map showing RP mutations in 3 patient cohorts including 116 DLBCL patients. Each row represents a gene, each column represents a patient. (E) Linear view of the *RPL3* and *RPL13* mutations found in the DLBCL datasets. (F) Heat map showing *TP53*, RP gene mutations and *RPS12* deletions in the TCGA dataset ($n = 48$ patients). (G) Chart showing the proportion of *RPS12*-deleted patients in our validation cohort (DLCL04 study), (upper panel). Mutual exclusivity of *RPS12* deletions and *TP53* mutations in the DLCL04 study (lower panel). (H) Bar graph showing the proportion of patients who had died in the RP mutant *versus* RP wild-type groups. *FAU* mutations sparing the *RPS30* gene were not considered in this analysis. The analysis was restricted to *TP53* wild-type patients. $^{**}P$ value <0·01, chi-square test. (I) Western blot showing the effect of *RPS12* silencing by ShRNAs on P53 stabilization after treatment with doxorubicin in DOHH2 cells. Cells infected with scramble (SCR) and RPS12 ShRNAs (Sh1 and Sh2) were pre-treated with doxycycline 0·1 μmol/l for 48 h in order to induce RPS12 silencing and then incubated with DMSO or doxorubicin 100 nmol/l for 6 h: P53 levels before and after treatment were then detected by Western blot assay. CLL, chronic lymphocytic leukaemia; DLBCL, diffuse large B cell lymphoma; DMSO, dimethyl sulphoxide; DOXO, doxorubicin; MCL, mantel cell lymphoma; MM, multiple myeloma; RP, ribosomal protein.

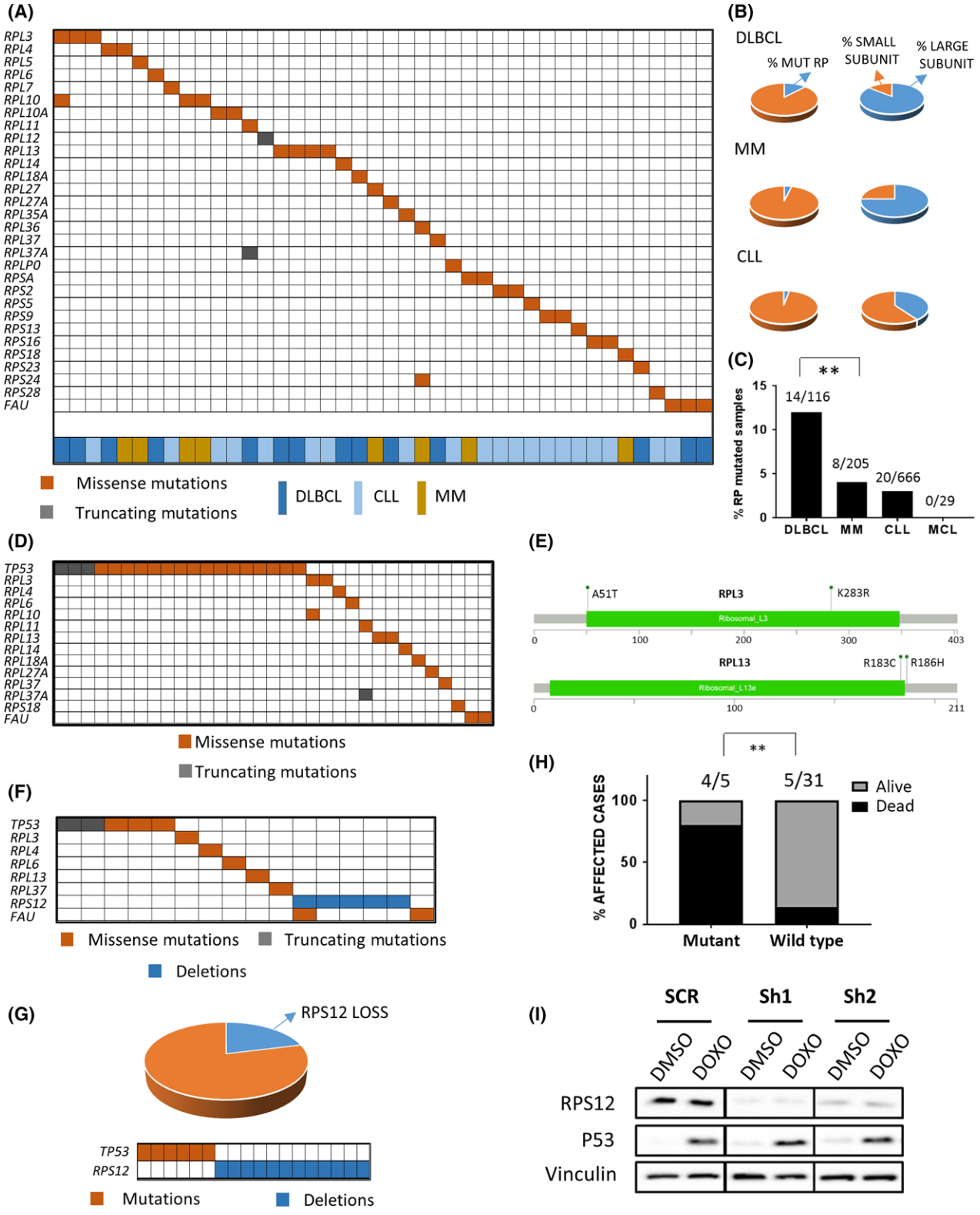

Table I. Genomic alterations of RP genes in DLBCL.

Gene	Chromosome	Mutation type	Amino acid change	Outcome	Study (Reference)
RPL3	22	Missense	A51T	CR 35 months	TCGA
		Missense	K283R	NA	(Lohr *et al*, 2012)
RPL4	15	Missense	A370V	R 209·7 months	TCGA
RPL6	12	Missense	I264V	R 11 months	TCGA
RPL10	X	Missense	A108S	NA	(Lohr *et al*, 2012)
RPL11	1	Missense	A142T	NA	(Lohr *et al*, 2012)
RPL13	16	Missense	R183C	NA	(Braggio *et al*, 2015)
		Missense	R186H	R 9·5 months	TCGA
RPL14	3	Missense	K193E	NA	(Lohr *et al*, 2012)
RPL18A	19	Missense	H146Y	NA	(Braggio *et al*, 2015)
RPL27A	11	Missense	N34Y	NA	(Lohr *et al*, 2012)
RPL37	5	Missense	R79C	R 3·5 months	TCGA
RPL37A	2	Splice	N72N	NA	(Lohr *et al*, 2012)
RPS18	6	Missense	L16F	NA	(Lohr *et al*, 2012)
FAU (also termed *FUBI*)	11	Missense	A26G	CR 32 months	TCGA
		Missense	L3V	CR 22 months	TCGA

Main characteristics of the RP mutations found in the DLBCL cohorts. Involved genes, chromosomes, mutation types, aminoacidic changes and respective study references are shown in the table. Outcome data were available only for the TCGA cohort. CR, complete response; NA, not available; R, relapse; TCGA, The Cancer Genome Atlas.

as only 2 of 14 patients (14%) showed concomitant mutations of multiple RPs (Fig 1D).

At the time of this analysis, genome wide CNV data were available only in the TCGA provisional dataset (48 samples) (Fig 1F). We found losses of the *RPL22* and *RPS12* genes in 4 (8%) and 5 (10%) of 48 cases respectively. Due to the novelty, we focused on CNVs of the *RPS12* gene (Fig 1G–I), which is located on 6q23.2. To validate these findings, we performed genome-wide copy number analysis in an independent cohort of 57 DLBCL patients enrolled in a prospective clinical trial (DLCL04) (Chiappella *et al*, 2017), using the OncoScan(TM) assay. The *RPS12* gene was deleted in 12 (21%) of 57 samples (Fig 1G), being involved in 75% of cases (12/16) with 6q23 deletion. These findings indicate that *RPS12* loss is a common genetic event in DLBCL. Considering the TCGA provisional dataset, the cumulative incidence of RP mutations/RPS12 deletions was 29% (14 of 48 cases) (Fig 1F).

Given that multiple RPs have been shown to regulate p53 stability in response to nucleolar stress by modulating murine double minute 2 (MDM2)-p53 interactions (Bursac *et al*, 2014), we investigated the relationship between RP genomic alterations and the presence of *TP53* mutations in DLBCL. Interestingly RP mutations in all DLBCL cohorts were mutually exclusive with *TP53* mutations (Fig 1D). A similar trend was observed with *RPS12* losses (TCGA provisional cohort) (Fig 1F). To validate these observations, we performed *TP53* DNA Sanger sequencing of the DLCL04 cohort, confirming mutual exclusivity between *RPS12* losses and *TP53* mutations (Fig 1G). Restricting the analysis to patients with available survival data (TCGA cohort), we observed a statistically higher death rate in *TP53* wild-type patients harbouring RP mutations compared to those without RP mutations (Fig 1H). These data suggest that mutations of RPs could provide lymphoma cells with an alternative mechanism to inactivate the p53-mediated response upon nucleolar stress. On the contrary, we did not observe significant differences in outcome between *RPS12* deleted and wild-type patients in the DLCL04 cohort (data not shown). In line with this observation, *RPS12* silencing did not have a significant impact on p53 stabilization after treatment with doxorubicin in a *TP53* wild-type DLBCL cell line (Fig 1I).

This report describes, for the first time, genomic alterations of RP genes in a significant fraction of DLBCL cases. Although the functional and clinical consequences of these RP alterations are yet to be determined, these preliminary observations suggest a possible correlation with adverse outcome, which should be investigated in future studies. This notion could be highly significant as many of the currently used chemotherapeutic drugs (such as doxorubicin) are indeed inhibitors of ribosomal biogenesis (reviewed in Bursac *et al*, 2014), and drugs selectively targeting ribosomal biogenesis are now in clinical development for haematological malignancies. Finally, the observation that both RP mutations and *RPS12* losses were mutually exclusive with *TP53* mutations might imply a role for these RP changes in neoplastic transformation.

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Authors' Contributions

ED designed the study, analysed the data and wrote the manuscript; CA performed the OncoScan assay and helped in manuscript editing; AR, GM, FM and MF analysed the data; AR performed *RPS12* silencing experiments; MR and FS performed the OncoScan assay; FD and AAK performed *TP53* sequencing; AC and UV provided patients samples and helped in manuscript editing, GG provided patients samples, helped in study design and edited the manuscript; CT designed the study, helped with data interpretation and wrote the manuscript; SP designed the study, helped with data interpretation and wrote the manuscript. All authors read and approved the final manuscript.

Disclosures

The authors declare that they have no competing interests.

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Stage I Non-Hodgkin Lymphoma: difference in survival outcome by primary extranodal site of involvement

Lymphoma can develop in any organ or tissue. The percentage of patients presenting with localized disease (Ann Arbor stage I/II) differs by histological subtype may arise in nodal and/or involve other organs or tissues (extranodal). Although certain primary sites have been demonstrated to reflect distinct clinicopathological characteristics and require specific therapy [e.g. primary central nervous system (PCNSL), primary testicular lymphoma and primary mediastinal lymphoma] (Cheah *et al*, 2014; Dunleavy, 2017; Grommes & DeAngelis, 2017), aside from these examples there are limited data to suggest other extranodal sites have prognostic implications. Therefore, we evaluated the survival outcome of patients with stage I non-Hodgkin lymphoma (NHL) among different histological subtypes, focusing on evaluating the differences in survival outcome by primary site of involvement.

The Surveillance, Epidemiology and End Results (SEER) 18 database (https://seer.cancer.gov/) was used to evaluate overall survival (OS) of patients with stage I NHL by different primary site of involvement in patients aged ≥18 years diagnosed between 1998 and 2014. We excluded lymphomas that develop exclusively in extranodal sites, such as PCNSL, primary mediastinal lymphoma, testicular lymphoma, cutaneous T cell lymphoma, primary effusion lymphoma, extranodal Natural Killer cell lymphoma, enteropathy associated T cell lymphoma (EATL) and hepatosplenic T cell lymphoma (HSTL). OS was calculated from diagnosis to death from any cause using the Kaplan-Meier method. Cox proportional hazards models were used to evaluate associations between patient characteristics and survival. All analyses were performed using STATA version 13.1 (StataCorp LP, College Station, TX), with significance set at the 5% level.

After excluding diseases as described above, a total of 58 230 patients were diagnosed with stage I disease during the study period (Table I). With a median follow-up of 68 months (range: 1–203 months), the median OS of patients with diffuse large B cell lymphoma (DLBCL), follicular lymphoma (FL), marginal zone lymphoma (MZL), small lymphocytic lymphoma (SLL), Burkitt lymphoma (BL), mantle cell lymphoma (MCL) and peripheral T cell lymphoma (PTCL, including PTCL-not otherwise specified, angioimmunoblastic T cell lymphoma and anaplastic large cell lymphoma) was 120, 179, 165, 101, not reached, 70 and 109 months, respectively.

Hazard ratios (HR) for OS by multivariate analysis, adjusted for age, sex, race and treatment (classified as none, radiation alone, chemotherapy alone and chemo-radiation), in stage I extranodal disease compared to stage I nodal disease of the same histological subtype are summarized in Table II. The 5-year OS of each site by histological subtypes is summarized in Table SI. Overall, compared to nodal stage I disease, extranodal stage I disease trended towards longer survival in MZL and FL but was associated with shorter survival in PTCL.

The study showed that survival outcomes among patients with stage I NHL are influenced by specific extranodal site of involvement. Compared to nodal disease, cutaneous disease was associated with significantly longer OS in all subtypes except BL and MCL, breast disease was associated with significantly longer OS in FL and SLL, and colonic disease was associated with significantly longer OS in FL, SLL, MZL and MCL. In contrast, gastric and bone disease was associated with inferior OS in FL, BL and PTCL.

Extranodal stage I disease was generally associated with longer OS in FL and MZL but shorter OS in PTCL. Nodal MZL has different biology compared to extranodal MZL and is associated with shorter OS compared to extranodal disease (Swerdlow *et al*, 2008). In contrast, extranodal disease, such as in lung, pleura, stomach, small intestine, liver and bone disease, was associated with shorter OS in PTCL. Interestingly, skin (DLBCL, FL, SLL, MZL and PTCL), colon (FL, SLL, MZL and MCL) and breast (FL and SLL) disease were associated with significantly longer OS across several subtypes. In addition to possible biological difference in lymphomas at those sites, these are the sites routinely screened for other cancers (breast and colon) or can be easily found by patients (skin) and thus may have been detected earlier, resulting in a more favourable clinical condition and prognosis.

The prognostic implications of extranodal disease have been evaluated in other studies, mostly in DLBCL (Moller *et al*, 2004; Lopez-Guillermo *et al*, 2005; Hui *et al*, 2010; Castillo *et al*, 2014). Castillo *et al* (2014) analysed the impact of extranodal involvement in patients with DLBCL using