Homozygous *FCGR3A-158V* alleles predispose to late onset neutropenia after CHOP-R for Diffuse Large B-cell Lymphoma

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Abstract word count: 209

Main text word count: 2463

Short title: Impact of LON and FCGR3A-V158F in DLBCL

Keywords: **Diffuse Large B-cell Lymphoma; Late onset neutropenia; Rituximab; Genetic polymorphisms**

Background

Recent reports suggest genetic polymorphisms influence susceptibility to rituximab induced late-onset neutropenia (LON), which in turn may be a predictor of good outcome in B-cell lymphoma.

Aims

We report the largest study to date assessing *FCGR3A-V158F* polymorphisms in Diffuse Large B-cell Lymphoma (DLBCL) treated with CHOP-R. The influence of *C1qA-A276G* polymorphisms in DLBCL, and the impact of both polymorphisms on susceptibility to LON and outcome were also examined.

Methods

115 DLBCL patients treated with CHOP-R were compared with 105 healthy Caucasian controls with regards to *FCGR3A-V158F* and *C1qA-A276G* polymorphisms. LON incidence and event free and overall survival (EFS and OS) were analysed for linkage to either polymorphism.

Results

The *FCGR3A-V158F* but not the *C1qA-A276G* polymorphism influenced the risk of developing LON. 50% of *FCGR3A-158V/V* patients experienced LON. In contrast, only 7% *V/F* and 2% *F/F* experienced LON. The *FCGR3A-158V/V* genotype was associated with LON compared to *V/F* ($p=0.028$) and *F/F* genotypes ($p=0.005$). Although no patients with either LON or *FCGR3A-158V* homozygosity relapsed compared to 33% *FCGR3A-158F/F* and 21% non-LON, this did not translate into improved EFS or OS.

Conclusions

Polymorphic analysis may be a predictive tool to identify those at high-risk of LON. Prospective studies are required to definitively establish if LON or *FCGR3A-158V/V* genotype influences outcome.

Introduction

Diffuse Large B-cell Lymphoma (DLBCL) is the commonest aggressive B-cell lymphoma. Rituximab is a routine component of DLBCL therapy, typically as part of ‘CHOP-R’ (cyclophosphamide / hydroxydaunorubicin / Oncovin [vincristine] / prednisone / rituximab). It has resulted in a marked improvement in response and survival.¹ The importance of host genetics on the mode of action of rituximab in DLBCL is unclear.

Two principle mechanisms of action are postulated. The first is antibody dependent cellular cytotoxicity (ADCC) whereby rituximab binds to FCGammaReceptor (*FCGR*) bearing Natural Killer (NK) cells, resulting in destruction of CD20⁺ normal and malignant B-cells by the reticulo-endothelial system.²⁻⁵ The other is direct lysis via complement dependent cytotoxicity (CDC).⁶⁻¹⁰

FCGR3A is a low-affinity receptor capable of binding the FC portion of complexed but not monomeric IgG. A polymorphism at amino acid 158, alternatively encoding for a valine (V) or phenylalanine (F), has been identified. *FCGR3A-V158* has a higher binding affinity for IgG₁ than *FCGR3A-F158*.¹¹ There is evidence that this polymorphism is important in the treatment of colon and breast cancers with cetuximab and transtuzumab respectively.^{12, 13} Data on the impact of the *FCGR3A-V158F* polymorphism in DLBCL treated with CHOP-R is conflicting.^{14, 15} There are also reports that the polymorphism may contribute to the development of late-onset neutropenia (LON).^{16, 17} This is a relatively rare but well-recognized late complication of rituximab containing therapy, defined as grade 3-4 neutropenia occurring after neutrophil recovery from last therapy in the absence of other causes. Although not designed for survival analysis, it is notable that in previous case series lymphoma patients with LON have a strikingly low incidence of disease progression. It has

therefore been proposed that development of LON is a measure of good outcome, perhaps reflecting enhanced potency of rituximab.¹⁸

Binding of C1q to the Fc portion of immune complexes activates CDC through initiation of the complement cascade. C1q is encoded by *C1qA*, whose sole coding polymorphism is at position 276, coding for adenine (*C1qA-A276*) or guanine (*C1qA-G276*). *C1qA-A276* results in lower C1q protein levels than the *C1qA-G276* polymorphism. Breast cancer patients heterozygous or homozygous for the *C1qA-G276* genotype have a higher rate of metastasis.¹⁹ In a study of 133 patients with follicular lymphoma treated with single agent rituximab, possession of the *C1qA-A276* allele was associated with increased response rates and prolonged response duration, even after adjusting for *FCGR3A-V158F* polymorphisms.²⁰ The role of C1qA polymorphisms in DLBCL has yet to be evaluated.

Our study is to our knowledge the first examining the influence of *C1qA-A276G* polymorphism in DLBCL. It is also the largest to date to assess *FCGR3A-V158F* polymorphisms in DLBCL treated with CHOP-R, and the first to assess the influence of either polymorphism on both outcome to chemo-immunotherapy and susceptibility to LON.

Methods

Analysis was restricted to DLBCL patients treated with CHOP-R between 1st January 2003 – 1st January 2010 at the Princess Alexandra Hospital, Brisbane. Since 2003 all lymphoma cases at PAH have been recorded on a prospectively maintained database. Cases were chosen solely on the availability of tissue for PCR but were otherwise unselected. One hundred and fifteen patients were identified. DNA extracted from formalin fixed paraffin embedded tissue (FFPE) was of sufficient quality to perform PCR analysis in 90 patients for *FCGR3A-V158F* polymorphisms and 81 patients for *C1qA-A276G* polymorphism. One hundred and five consenting healthy adult Caucasian volunteers served as controls. Controls specifically denied haematological or autoimmune disorders of any kind. The study was approved by the Hospital / Research Institute Ethics Committees and was performed in accordance with the Declaration of Helsinki.

CHOP-R consisted of an intravenous infusion of cyclophosphamide 750 mg/m², adriamycin 50 mg/m², vincristine 1.4 mg/m² (capped at 2mg), oral administration of 100 mg prednisone on days 1 to 5 (CHOP), and Rituximab 375 mg/m² at day 1 before CHOP chemotherapy began. Patients with stage I/II disease typically received 4 courses of chemo-immunotherapy followed by involved-field radiotherapy (30-40 Gy), while patients with advanced stage disease received 6 to 8 cycles of chemo-immunotherapy followed by radiotherapy to bulky sites. In 2006 our therapeutic practice shifted from a 21 day chemo-immunotherapy cycle (CHOP-R-21) without G-CSF support, to a 14 day cycle (CHOP-R-14) with pegylated G-CSF administration. In patients receiving < 8 cycles of combination therapy, further rituximab was administered as monotherapy so that in total patients received 8 doses. Maintenance rituximab after completion of CHOP-R therapy was not administered. The response to CHOP-R therapy was radiologically evaluated after completion of the third and sixth cycle of CHOP-R

chemotherapy and 1 month after completion of all therapy, as per standard criteria.²¹ In those with documented bone marrow lymphoma, restaging bone marrow biopsies were performed.

Inclusion criteria for LON was neutrophils <1000/ μ l occurring greater than six weeks after receiving last rituximab, with no other identifiable causes of neutropenia. Patients with human immunodeficiency virus infection, pelvic irradiation or marrow involvement at restaging were excluded. Follow-up evaluation (included full blood counts) was performed at least every three months for the first two years.

DNA was extracted from FFPE tissue (patients) or buccal scrapes (controls) using standard procedures and analysis performed in batches. All samples were analyzed in the same laboratory. *FCGR3A-V/F158* and *C1qA-G/A276* genotyping were performed using allele specific PCR based on protocols previously described.^{11, 22}. In selected samples results were confirmed by directed sequencing.

Event-free and overall survival (EFS and OS) were estimated using the Kaplan-Meier product-limit method. EFS was measured from the date of diagnosis to first documented progression, change to alternate therapy, death, or the last follow-up visit. OS was calculated from the date of diagnosis to death from any cause or the last follow up. Survival rates were compared for statistical differences by using log-rank analysis. P values less than 0.05 were considered statistically significant and all correspond to 2-sided significance tests. The chi-squared and Fishers exact tests were used to compare clinical and laboratory parameters between the different polymorphisms. All statistics were performed on the Graphpad Prism platform (version 5).

Results

Patient characteristics by *FCGR3A-V158F* and *CIqA-A276G* polymorphism status.

Of 115 patients mean age was 62 years (range 18-90) and 52 were female. Sixty four (56%) patients had stages III/IV disease, 38 (33%) had two or more extranodal sites, 34 (30%) had an ECOG>2 and 70 (61%) had a raised lactate dehydrogenase (LDH). There were 39 (34%) with low (0/1) international prognostic index (IPI) scores, 46 (40%) with intermediate (2/3) IPI and 30 (26%) with high (4/5) IPI scores. After a median follow-up of 31 months (range 1-76 months), the actuarial 3 year EFS and 3 year OS rates were 71% and 77%, respectively. The response to treatment with CHOP-R is comparable to published results.²³ As expected, the international prognostic index (IPI) differentiated patients into three prognostic groupings (3 year OS: 0/1 89%, 2/3 85%, 4/5 44%). *FCGR3A-V158F* and *CIqA-A276G* polymorphic groupings (V/V, V/F, F/F and A/A, A/G, G/G respectively) were evenly distributed across all clinical or laboratory parameters.

PCR amplification was successful for *FCGR3A-V158F* polymorphisms in all controls and in all but three for *CIqA-A276G*. The distributions observed in control participants were in agreement with those previously reported (Table I). The polymorphism distributions in the overall cohort of DLBCL patients were similar to healthy controls. Within DLBCL patients there were no significant differences regarding age, gender, stage, performance status, LDH, extranodal involvement, or international prognostic index (IPI) for *FCGR3A-V158F* and *CIqA-A276G* respectively.

Late onset neutropenia according to *FCGR3A-V158F* and *CIqA-A276G* polymorphism status.

Seven (6%) of 115 DLBCL patients developed LON, with a mean neutrophil count of 300 μ l/l (range 40-860 μ l/l). None of the seven had a truncated course of CHOP-R chemotherapy due to neutropenia, although four did receive pegylated G-CSF (as per the CHOP-R-14 schedule) which may have masked the onset of early neutropenia. Two patients had bone marrow biopsies, with both showing myeloid maturation arrest. The onset of LON occurred at a mean of 90 days (range 48-150) after last treatment. There was no difference between the occurrence of LON in CHOP-R-21 (three patients) and CHOP-R-14 (four patients). No LON patients had any infective sequelae. One patient received a brief course of G-CSF, whilst others were simply observed and their neutropenias resolved spontaneously. No patients required in-patient admission. The mean duration of LON was 12 days (range 6-35). Interestingly, none of the seven patients who developed LON have had any survival events to date, compared to 21% of patients with non-LON. However this did not reach significance compared to those without LON (EFS $p=0.09$; OS $p=0.11$).

50% of *FCGR3A-158V/V* patients experienced LON. In contrast, just 7% *FCGR3A-158V/F* and 2% *FCGR3A-158F/F* had LON. The *FCGR3A-158V/V* genotype was associated with LON compared to *FCGR3A-158V/F* ($p=0.028$) and *F/F* genotypes ($p=0.005$). The *FCGR3A-158V/V* genotype occurred more frequently in LON patients than in normal controls ($p=0.026$) or non-LON DLBCL patients ($p=0.005$). (Table II) There was no significant association between LON and the *CIqA-A276G* polymorphisms. The combination of *FCGR3a-158V* allele with the *CIqA-276A* allele was not associated with LON.

Patient Outcome by *FCGR3A-V158F* and *CIqA-A276G* polymorphism status.

The *FCGR3A-158V/V* genotype was combined with *FCGR3A-158V/F* to assess outcome against patients with the *FCGR3A-158F/F* genotype. The distribution of *FCGR3A-V158F* polymorphisms had no impact on EFS and OS (p=0.16 and p=0.37 respectively) (Figure 1). However none of the six patients homozygous for the *FCGR3A-158V* polymorphism have had any event to date, against 13 of 40 patients with *FCGR3A-158F/F*. *CIqA-A276G* polymorphisms had no impact upon either EFS or OS. Of the 5 *CIqA-276G/G* patients, one has had an event compared to 7 of 40 DLBCL patients homozygous for the *CIqA-276A* polymorphism. The combination of *FCGR3A-158V* allele with the *CIqA-276A* allele was not associated with altered survival.

Discussion

To our knowledge this is the largest series assessing the impact of *FCGR3A-V158F* polymorphisms on susceptibility to LON in DLBCL after CHOP-R. It is also the first study examining the influence of *CIQA-A276G* polymorphisms in any aggressive B-cell lymphoma. We show that homozygosity for the high-affinity *FCGR3A-158V* allele but not *CIQA-A276G* polymorphisms are strongly associated with LON.

Previous studies examining the role of *FCGR3A-V158F* polymorphisms on LON are limited by heterogeneous lymphoma populations and treatment regimens. The Stanford group analysed 33 relapsed / refractory / high-risk lymphoma patients with miscellaneous indolent and aggressive histologies.¹⁷ Rituximab monotherapy was administered following high-dose chemotherapy and/or total-body radiation with CD34⁺ enriched, B-cell depleted hematopoietic stem cell rescue. In agreement with our findings, they found that the *FCGR3A-158V* allele correlated with the incidence of LON. The influence of *FCGR3A-V158F* polymorphisms on LON in a more frequent clinical scenario, namely B-cell lymphomas treated with (various) chemo-immunotherapies regimens as initial therapy was analysed within a Taiwanese population.¹⁶ Fifty six patients with either indolent or aggressive B-cell lymphomas were analysed. The *FCGR3A-V158F* polymorphism was associated with development of LON.

Whilst the occurrence of LON is not infrequent, it is rarely associated with adverse outcomes such as serious infection and seems to resolve spontaneously. Indeed there is some suggestion that patients who develop LON have improved lymphoma outcomes. A recent review of nine LON studies totalling 92 patients, commented that only one patient (with Follicular lymphoma) experienced lymphoma relapse after LON.¹⁸ Testing for *FCGR3A-V158F* was not performed in any of these studies. In our

cohort of patients with a median follow-up of over 2.5 years, no patients with LON and none with homozygosity for the *FCGR3A-158V* allele have relapsed. By contrast, 33% of patients with *FCGR3A-158F/F* and 21% of non-LON patients have relapsed lymphoma. Our findings regarding the impact of LON and *FCGR3A-V158F* polymorphisms on outcome should be interpreted with caution. In line with the observations of Mitrovic (58 DLBCL patients) and Kim (113 DLBCL patients) in which subjects were treated exclusively with CHOP-R, we found *FCGR3A-V158F* polymorphisms did not significantly impact EFS or OS.^{14, 15} Homozygosity for the *FCGR3A-V158* allele leads to stronger binding of effector cells to rituximab which appears to translate to improved response rates in indolent lymphomas. Thus the absence of relapse in LON patients may relate to enhanced rituximab efficacy in association with the *FCGR3A-V158F* polymorphism may and not LON explain *per se*. In addition, because LON is not diagnosed until six weeks after chemo-immunotherapy, this may indicate that these patients do not have refractory disease which would skew towards their having a more favourable prognosis.

We observed a 6% incidence of LON in DLBCL patients treated with CHOP-R chemo-immunotherapy. Previous reports of LON after induction chemo-immunotherapy for B-cell lymphoma have not been restricted to a single histological sub-type or treatment regimen. In addition the neutrophil cut-off used to identify LON has varied between grade 2 to grade 4.²⁴⁻²⁶ Once studies are restricted to those using a definition of grade 3/4 neutropenia, then irrespective of ethnicity the incidence of LON (range 7-13%) is of a similar order of magnitude to our series.^{16, 27, 28} In these as with our study, incidence may be underestimated as patients are frequently asymptomatic. The incidence of LON appears to rise markedly in studies that include patients

undergoing high-dose therapy with autologous stem cell rescue.^{17, 29} Put together, it may be that intensity of treatment regimen (use of high-dose over conventional dose chemotherapy) is the principal determinant of LON incidence rather than lymphoma histology.

The mechanism behind LON is yet to be adequately defined. Notably bone marrow histology has been variously reported as maturation arrest (most cases) or myeloid hypoplasia (some cases), perhaps indicating that several mechanisms are responsible. Investigators have hypothesized anti-neutrophil antibodies or increased large granular lymphocytes in the absence of B-cells leading to FAS ligand mediated destruction of neutrophils.^{30, 31} However this has not been consistently observed. A report of six cases of aggressive lymphoma (including two with AIDS related lymphoma) treated with DA-EPOCH-R (dose-adjusted etoposide / prednisone / Oncovin [vincristine] / cyclophosphamide, hydroxyduranubicin / rituximab), implicated perturbations of stromal derived factor-1 (SDF-1) / CXCL12 during B-cell recovery as a potential aetiology.²⁴ The SDF1 chemokine is important for granulocyte egress from the bone marrow and also in B-cell development. A shift of SDF-1 towards B-cell recovery over granulocyte homeostasis may result in LON due to the characteristic maturation. Cytokine kinetics were evaluated in detail in a case of LON associated with granulocytic hypoplasia following fludarabine, cyclophosphamide and rituximab for Waldenstroms macroglobulinaemia. In this patient LON was associated with markedly raised levels of serum B-cell activating factor (BAFF).³²

It may be that the pharmacokinetics of rituximab are different in patients homozygous for the *FCGR3A-V158* polymorphism. *FCGR3A-V158* has a higher binding affinity for IgG₁ antibodies (such as rituximab) than *FCGR3A-F158*.¹¹ Thus *FCGR3A-V158* may result in enhanced clearing of CD20 expressing cells by ADCC by

NK cells. *FCGR3A* transcripts are higher in NK cells from subjects with *FCGR3A-V/V158* versus the *V/F* or *F/F* genotype and *V/V* homozygotes have enhanced *in-vitro* ADCC activity. *In-vivo* this may result in more profound B-cell depletion.³³ Upon B-cell recovery, heightened stimulation of lymphopoiesis may result in temporary imbalance of cytokines and transient ineffective granulopoiesis.

In contrast with our findings regarding *FCGR3A-V158F*, there was no significant association between LON and the *C1qA-A276G* polymorphisms. Furthermore, the combination of *C1qA-A276G* and *FCGR3A-V158F* did not appear to expose any polymorphic linkage and did not appear to influence outcome or development of LON. These findings suggest that *C1qA* is not implicated in the pathogenesis of LON. We did not observe any difference in EFS or OS related to the *C1qA-A276G* polymorphism.

Similarly to *FCGR3A-V158F* polymorphism findings, other groups have looked at *FCGR2A-H131R* and response to rituximab in lymphoma. (34) In a small series looking at late onset neutropenia, *FCGR2A-H131R* was not found to contribute to the development of neutropenia post stem cell transplant.(35) In contrast to *FCGR3A-V158F* and *FCGR2A-H131R*, an *FCGR2B-I232T* polymorphism inhibits ADCC when being co-engaged by antibody. Therefore, it is possible that this polymorphism may influence the effector cells' ability to perform ADCC and their anti-tumour effect. One small study does not show the *FCGR2B-I232T* polymorphism influencing outcome in rituximab treated follicular lymphoma, however there is currently no data on the incidence of LON in association with this polymorphism.(36)

In conclusion in our uniformly treated group of DLBCL patients who received rituximab, 6% of patients developed LON. The *FCGR3A-I58 V/V* genotype was significantly associated with development of LON. Polymorphic analysis may be a

predictive tool to identify those at high-risk of LON. Although no patients with either LON or *FCGR3A-158V* homozygosity relapsed, neither were associated with improved EFS or OS after CHOP-R. Large prospective studies are required to establish if *FCGR3A-V158F* polymorphisms have a bearing on response rates in DLBCL, and whether either LON or *FCGR3A-V158F* influences outcome.

Acknowledgments

C.K. is supported by a Leukaemia Foundation Clinical Fellowship. M.K.G.'s research salary is provided by the NHMRC (Australia). This study was supported by the Cancer Council of Queensland and the Queensland Office of Health and Medical Research.

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Figure Legends

Figure 1. Kaplan-Meier survival curves. (A) event and (B) overall survival by FCGR3a-V158F polymorphisms status; (C) event and (D) overall survival by C1qA-276A polymorphisms; (E) event and (F) overall survival according to development of LON post rituximab therapy.

Table 1 . Frequency of FCGR3a-V158F and C1q-A276G genotypes and alleles in healthy controls and DLBCL patients.

Genotype	Controls (%)	Patients (%)	p value
<i>FCGR3A-158F/F</i>	46 (44%)	40 (44%)	0.97
<i>FCGR3A-158V/F</i>	50 (48%)	44 (49%)	
<i>FCGR3A-158V/V</i>	9 (8%)	6 (7%)	
<i>C1qA-276G/G</i>	13 (13%)	5 (6%)	0.12
<i>C1qA-276G/A</i>	52 (51%)	36 (45%)	
<i>C1qA-276A/A</i>	37 (36%)	40 (49%)	
Allele Frequency			
<i>FCGR3A-158F</i>	96 (92%)	84(93%)	0.9
<i>FCGR3A-158V</i>	59 (56%)	50(56%)	
<i>C1qA-276G</i>	65 (64%)	41(51%)	0.26
<i>C1qA-276A</i>	89 (87%)	76(94%)	

Table 2. Distribution of FCGR3a-V158F genotypes in healthy controls and DLBCL patients with and without LON.

Genotype	LON	Non LON	p value	LON	Controls	p value
<i>VV</i>	3 (43%)	3 (4%)	0.005	3 (43%)	9 (8%)	0.026
<i>VF/FF</i>	4 (57%)	80 (96%)		4 (57%)	96 (92%)

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