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Complement regulatory proteins CFHR1 and CFHR3 and patient response to anti-CD20 monoclonal antibody therapy

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Abstract

Purpose—Anti-CD20 monoclonal antibody (mAb) therapies, including rituximab and obinutuzumab (GA101) are common treatments for follicular lymphoma (FL). In an effort to better understand the role of complement in mAb action, we recently performed germline SNP profiling on 142 FL patients and found rs3766404 genotype correlated with patient response to rituximab. To assess the role of three SNP-associated complement regulatory proteins (CFH, CFHR1 and CFHR3) in clinical response to anti-CD20 mAb, we studied two cohorts of patients treated with anti-CD20 mAb.

Experimental Design—Cohorts included the Iowa/Mayo Lymphoma SPORE observational cohort of subjects with a new diagnosis of FL treated with rituximab, and the GAUSS prospective randomized trial cohort of FL subjects randomized to receive single agent rituximab or obinutuzumab. Circulating protein expression was measured for CFH, CFHR1 and CFHR3 and correlated to clinical outcome.

Results—rs3766404 genotype correlated with expression of the related downstream genes *CFHR1* and *CFHR3*. Loss of CFHR1 expression correlated with inferior patient outcome in the observational cohort, but not in the GAUSS cohort. Loss of CFHR3 correlated with superior event-free survival in GAUSS subjects treated with obinutuzumab, but not rituximab.

Conclusions—We conclude that the relationship between complement regulatory proteins CFHR1 and CFHR3 and response to anti-CD20 mAb therapy varies based on the specific anti-CD20 mAb used. We propose that CFHR3 is a candidate biomarker for obinutuzumab response. Further studies are needed to validate these findings and to better understand how complement pathways and complement regulatory proteins impact on the efficacy of anti-CD20 mAb therapy.

Introduction

Rituximab is an anti-CD20 monoclonal antibody (mAb) that has significantly increased the overall survival of patients with a variety of B cell malignancies, including follicular lymphoma (FL)(1, 2). Obinutuzumab (also known as GA101) is a second-generation anti-CD20 mAb that has recently been approved by the FDA for treatment of chronic lymphocytic leukemia (CLL) and relapsed/refractory FL. The antigen specificity of

obinutuzumab is similar to that of rituximab, but obinutuzuimab and rituximab differ in a number of ways including the glycosylation of the Fc, how they cross-link CD20, and their ability to fix complement(3–7). FL is initially treated with a variety of approaches frequently including combinations of observation, radiation, chemotherapy, and anti-CD20 mAbs. Nevertheless, clinical response to these therapies is variable, and relapse and transformation are common. In order to improve upon current outcomes, we need a better understanding of biological mechanisms contributing to anti-CD20 mAb action and resistance.

Several mechanisms of anti-CD20 action for which the host immune system is important have been identified, including complement dependent cytotoxicity (CDC) and antibody dependent cellular cytotoxicity (ADCC)(8). However, the relative contribution of these mechanisms, and how they interact *in vivo* is unclear. For example, our lab has shown that while complement fixation by rituximab enhances CDC, it can also block ADCC *in vitro*(9). Further evidence that complement activity might be detrimental to rituximab efficacy is our observation that C1qA polymorphisms associated with lower C1q levels correlated with prolonged rituximab response in FL(10). Complement mediated immune responses are tightly regulated by a number of endogenously produced proteins including soluble components such as complement factor H (CFH) and its related family members CFHR1-5(11–13).

In an effort to better understand the role of complement in rituximab action, we recently performed SNP profiling on 142 samples obtained from an observational cohort developed by the Iowa/Mayo Lymphoma SPORE Molecular Epidemiology Resource (henceforth referred to as the “observational cohort”). This included untreated FL patients and those treated with rituximab to determine whether genetic variation influences patient outcome(14). A SNP (rs3766404) in the complement regulatory gene *CFH* was found to be significantly associated with event-free survival in rituximab-treated FL. Patients with the T/T genotype experienced a better outcome than those with T/C or C/C genotypes. Interestingly, rs3766404 genotype was irrelevant for survival of patients who received no therapy, suggesting genotype does not impact the natural history of the disease.

CFH SNP rs3766404 is located in intron 6 and does not have a known function. It is, however, frequently linked to a deletion of two downstream protein-coding genes, *Complement Factor H-Related 3* (*CFHR3*) and *1* (*CFHR1*)(15). CFH itself is a negative regulator of complement activation, acting both to enhance Factor I-mediated degradation of the active complement component C3b, and also to prevent further C3b production by accelerating decay of the alternative pathway C3 convertase(16). Like CFH, CFHR3 and CFHR1 are also considered regulators of complement activation, though the precise mechanisms of regulation—and indeed, whether they have a positive or negative overall impact—are not yet clear(17–19). Because complement activation influences mAb therapy efficacy, it is possible that CFH, CFHR3, and CFHR1 could play a functional role in anti-CD20 action. We therefore examined the relationship between rs3766404 and rituximab in the observational cohort. In addition, we evaluated this relationship in a second cohort of follicular lymphoma patients, namely those enrolled in the GAUSS clinical trial (NCT00576758) that compared rituximab to obinutuzumab, another anti-CD20 mAb approved by the FDA for treatment of B cell malignancies, including relapsed/refractory FL.

Analyses of FL samples from this prospective study are henceforth referred to as the "GAUSS cohort". Obinutuzumab is less effective than rituximab at fixing complement, with almost no C1q bound with antibody concentrations as high as 10ug/ml(3). Thus, evaluating the role of CFH, CFHR3 and CFHR1 in response to both obinutuzumab and rituximab offers the opportunity to gain additional insight into the role of complement regulators in response to anti-CD20 therapy. Further, results could be applied to mAbs targeting other antigens.

Materials and Methods

UI/Mayo Lymphoma SPORE Molecular Epidemiology Resource (Observational Cohort)

All participants provided written informed consent in accordance with the Declaration of Helsinki and this study was approved by the Human Subjects Institutional Review Boards at Mayo Clinic (Rochester, MN) and the University of Iowa (Iowa City, IA). Full details of clinical inclusion criteria have been published previously(20). Additional relevant clinical details are included in Supplemental Table S1.

GAUSS clinical trial study populations (GAUSS cohort)

The GAUSS clinical trial was a randomized phase II clinical trial conducted by F. Hoffmann-LaRoche Ltd. in patients with relapsed CD20+ indolent B-cell non-Hodgkin lymphoma, 85% of which had relapsed FL(21). Only FL patients were included in the present study, and all subjects had received prior rituximab and had responded to therapy before relapsing. In the GAUSS trial, patients were given re-induction therapy with single agent rituximab (n=30) or obinutuzumab (n=31) weekly for 4 weeks, then single doses every 2 months for 2 years. For more demographic information, see Supplemental Table S2.

SNP genotyping

Genotyping for rs3766404 was done using a restriction digest method whereby the SNP and surrounding sequence was PCR amplified using standard cycling conditions for Taq Polymerase (Invitrogen) with a 53°C annealing temperature (primers 5'-TTGCATCTCCATAGCTTTTGACTTC-3' and 5'-ACCATACTTCCACTGTGTCCA-3'), yielding a 397 bp PCR product. Following amplification, PCR products were digested overnight at 37°C with Hinf1, which only cuts if the C allele is present (160 bp and 237 bp). Digested products were run on an agarose gel to determine genotype. This method allows us to identify both homozygous and heterozygous individuals.

CFHR3/1 deletion PCR

Detections of homozygous *CFHR3/1* deletion was performed using the following primer sets and conventional PCR conditions: CFHR1.F 5'-CCCTCCCAAATGCAGGTCCACTG-3', CFHR1.R 5'-TTCAACATCCACTTGGACACA-3', CFHR3.F 5'-CAGTTACATGTACGGAGAAA-3', CFHR3.R 5'-ATAGGTCCGTTGGCAAAACA-3'. No product is amplified in samples with homozygous deletion of *CFHR3* and *CFHR1*. As a template amplification control, beta-2 microglobulin (*B2M*) was amplified in all samples using B2M.F 5'-TTCATCCATCCGACATTGAA-3' and B2M.R 5'-AGAGCTACCCAGCAGGAACA-3'.

Western blotting

Serum samples were diluted 1:100 in PBS, and proteins were denatured under non-reducing conditions and separated using SDS-PAGE. CFH and CFHR1 expression was detected using anti-CFHR1 primary antibody (R&D Systems, #MAB4247, 1:1000), which cross reacts with both proteins. CFHR3 was detected using anti-CFHR3 antibody (Proteintech, #16583-1-AP, 1:500). Blot images were quantified using ImageJ (National Institutes of Health), and band densities were normalized to a reference sample included on each gel that expressed all three proteins.

Statistical analysis

To investigate the associations between protein expression levels and genotype, the Wilcoxon rank sum test was utilized. Event-free survival (EFS) was defined as the time from diagnosis to disease progression, retreatment, or death due to any cause. Patients without an event were censored at time of last known follow-up. Cox proportional hazards models were used to estimate the effects of CFH, CFHR1, and CFHR3 on EFS for patients receiving Rituximab and those who did not. Estimated effects of predictors are reported as hazard ratios (HR) along with 95% confidence intervals. All statistical testing was two-sided and assessed for significance at the 5% level using SAS v9.4 (SAS Institute, Cary, NC).

Results

SNP rs3766404 positively correlates with germline deletion of *CFHR1* in FL patients

Our group recently published SNP analysis of complement regulatory proteins using data from the UI/Mayo Lymphoma SPORE Molecular Epidemiology Resource. This study found that follicular lymphoma (FL) patients treated with rituximab showed differential response based on rs3766404 genotype (n=35, P<0.001)(14). Specifically, patients homozygous for the major T allele had fewer events, including progression or death, after antibody therapy than individuals carrying a C allele. Importantly, rs3766404 genotype did not correlate with event free survival (EFS) in patients not treated with rituximab (observed only), suggesting rs3766404 does not impact natural disease progression. EFS was defined in this study as the time from diagnosis to disease progression, re-treatment, or death due to any cause.

Given the strong correlation between SNP genotype and rituximab response in this cohort, we sought to understand whether rs3766404 was functionally contributing to rituximab efficacy. The polymorphism is located within intron 6 of *Complement Factor H (CFH)*, and is thus not expected to impact the coding sequence of CFH itself. Furthermore, splice prediction algorithms did not predict alterations to *CFH* splice patterns based on SNP genotype(22, 23). Finally, we found that neither plasma levels of CFH protein nor CFH molecular weight significantly differed between T/T individuals and T/C or C/C individuals, suggesting protein levels are unaffected by genotype (Fig. 1A). This led us to conclude that CFH itself is not likely impacted by rs3766404 variation.

Kubista, *et al.* published a correlative study whereby the minor C allele is associated with downstream germline deletion of two CFH family members *CFH Related 3* (*CFHR3)* and *CFHR1*(15). Further, deletion of *CFHR3* and *CFHR1* is associated with a number of

complement-mediated disorders(18, 24). To determine whether SNP genotype correlated with loss of *CFHR3* and *CFHR1* in FL samples from our observational cohort, PCR analysis of genomic DNA was performed (n=9). Individuals with C/C genotypes also lacked all copies of *CFHR3* and *CFHR1* (Fig. 1B). Heterozygous individuals could not be distinguished from homozygous T/T individuals using this approach. Thus, western blot analysis of CFHR3 and CFHR1 proteins in patient plasma was performed and protein levels were quantified (n=109) (Fig 1C). This data confirmed that individuals with the T/T genotype had significantly higher CFHR1 protein expression compared to patients with 1 or 2 minor alleles ($p<0.01$). No such significance was evidenced for CFH ($p=0.54$) or CFHR3 ($p=0.52$) protein expression and rs3766404 genotype (Table 1). This suggests rs3766404 may serve as a marker for germline deletion of *CFHR1*, but cannot be used to predict CFH or CFHR3 expression status.

Serum CFHR1 correlates with rituximab outcome in FL patients in the observational cohort

We next evaluated whether pre-therapy CFHR1 or CFHR3 protein levels correlated with clinical outcome in the observational cohort to investigate the associations between protein levels and event-free survival (EFS) (Table 2). Among FL subjects treated with rituximab as a component of therapy, increased levels of CFHR1 were associated with improved EFS. Specifically, for each 1 unit increase in CFHR1 the risk of an event decreased by a factor of 0.23 (95% CI 0.08–0.67) ($p<0.01$). For those who did not receive rituximab, CFHR1 did not have a significant effect on EFS. The correlation between CFHR1 and EFS was significantly different between subjects who did and did not receive rituximab ($p<0.01$). A trend towards significance between EFS and both CFH and CFHR3 expression was among patients who received rituximab ($p=0.07$ and $p=0.05$, respectively), with increased expression associated with a decline in risk. Given the biologic significance of CFHR1 in complement-mediated disorders and the role complement plays in rituximab action, our data suggest that CFHR1 might directly impact rituximab efficacy and could be therapeutically relevant.

The subjects included in the observational cohort were treated with a variety of regimens including a combination of rituximab and chemotherapy, and there was variability in use of maintenance rituximab. Evaluation of response or relapse was done at the discretion of the treating physician and not based on a rigorous prospective protocol. Therefore, data is limited with respect to overall response rate or duration. In addition, there was no randomization that could be used for comparison. Thus, we sought to confirm the observation that higher CFHR1 levels are associated with superior outcomes in an additional, independent clinical cohort.

Serum CFHR1 does not correlate with rituximab outcome in FL patient in the GAUSS cohort

Samples from the Roche GAUSS clinical trial which compared the efficacy of single-agent rituximab to single-agent obinutuzumab were also analyzed for CFH, CFHR1, and CFHR3 expression status(25). Obinutuzumab differs from rituximab in a number of ways. Unlike rituximab, obinutuzumab causes direct cell killing and enhances ADCC to a greater extent, but it does not induce complement activation as efficiently. The GAUSS trial was a randomized phase II study conducted primarily in Europe in patients with relapsed CD20+

indolent B-cell non-Hodgkin lymphoma, 85% of which had relapsed FL(21). All subjects had received prior rituximab and had responded to therapy before relapsing. In the GAUSS trial, FL patients were given re-induction therapy with single agent rituximab (n=63) or obinutuzumab (n=51) weekly for 4 weeks, then single doses every 2 months for 2 years. Response and progression free survival (PFS) were monitored rigorously and regularly in this controlled prospective trial. As recently reported, preliminary results show improved overall response rate in FL patients treated with obinutuzumab compared to rituximab(21).

Pre-therapy serum and germline DNA (when available) from FL subjects enrolled in the GAUSS study were blindly evaluated for rs3766404 genotype and CFH, CFHR1, and CFHR3 expression. As observed in the observational cohort, the GAUSS cohort data demonstrated that the rs3766404 C allele correlated with a decrease in serum expression of CFHR1, while CFH protein levels remained similar regardless of SNP genotype (Supplemental Fig. S1).

In contrast to results from the observational cohort, we did not observe an association between SNP genotype and EFS in FL patients treated with rituximab in the GAUSS cohort (Fig. 2A). Similarly, the C allele did not correlate with inferior EFS in patients alternately treated with obinutuzumab (Fig. 2B). SNP genotype did not correlate with PFS in either rituximab or obinutuzumab treatment arms (Supplemental Fig. S2). Separate analysis was done for FL subjects defined as responders or non-responders on the GAUSS trial(21). No differences were identified in response based on SNP genotype for either rituximab- or obinutuzumab-treated subjects (Supplemental Table S3).

We also did not observe a significant difference in EFS between FL patients treated with either rituximab or obinutuzumab based on protein expression of CFHR1. In fact, serum protein status, alone or in combination, was not predictive of response to either therapy (Supplemental Fig. S3). Thus, CFH and CFHR1 protein levels were not significantly correlated with either EFS (Table 3) or PFS (Supplemental Table S4) of FL patients in either rituximab or obinutuzumab treatment arms from the GAUSS study. This is in contrast to the observational cohort described above.

Higher serum CFHR3 levels correlated with inferior outcome in patients given obinutuzumab

As noted above, CFHR3 protein expression levels were also measured in both patient populations. For patients in both the observational and GAUSS cohorts, CFHR3 was not predictive of rituximab EFS. However, CFHR3 expression significantly correlated with both EFS (Table 3, P=0.0044) and PFS (Supplemental Table S4, P=0.0045) in obinutuzumab-treated (n=51). CFHR3 serum levels also correlated with the FLIPI index scores of these subjects (Supplemental Table S5, P=0.0101), suggesting that lower CFHR3 serum levels may serve as a positive prognostic biomarker for, or even basis for therapeutic enhancement of, obinutuzumab treatment success. Further validation is required to confirm this, and it would be important to include CFHR3 serum measurements in future clinical trials using obinutuzumab.

Discussion

Complement directly impacts a multitude of processes involved in cancer including angiogenesis, tumor cell signaling and survival, extracellular matrix remodeling and metastasis, and cellular composition of the tumor microenvironment(26). While it is clear that complement is active in a variety of cancers, the role of complement in cancer is complex and is associated with both pro- and anti-cancer effects(27, 28). In patients treated with mAb therapy, complement can enhance tumor cell killing through direct CDC mechanisms, but might do so at the expense of other tumor killing mechanisms, such as ADCC(29–31).

The anti-CD20 antibodies studied here, rituximab and obinutuzumab, differ in their abilities to kill tumor cells directly, fix complement, and enhance ADCC. For example, rituximab has modest direct tumor killing, but efficiently fixes complement and can enhance ADCC. Because obinutuzumab does not fix complement as efficiently as rituximab, its ADCC-promoting activity is also less inhibited by complement activation(3, 5, 7, 30). Obinutuzumab also produces more direct tumor cell killing than rituximab and has been engineered to have greater FcγR affinity so that obinutuzumab is more efficient at inducing ADCC and FcγR-mediated phagocytosis(6, 7). This led us to hypothesize that natural variation in complement and complement regulatory proteins could impact the efficacy of both anti-CD20 antibodies in potentially distinct ways.

Our prior studies of complement regulatory genetic loci identified a number of SNPs that differentially associated with event-free survival (EFS) in FL patients treated with rituximab, including rs3766404(14). In the present study, we found that the minor rs3766404 allele is associated with loss of CFHR1 expression in both the observational and GAUSS study populations (Figures 1 and S1). Further, loss of CFHR1 protein expression correlated with inferior EFS in rituximab-treated patients from the observational cohort. Interestingly, CFHR1 expression did not correlate with EFS in FL patients who were not treated with rituximab, nor does SNP allele frequency differ between treated, observed or the normal population groups. This indicates that CFHR1 may impact rituximab action itself, rather than affecting overall FL incidence or aggressiveness.

The association of CFHR1 with EFS was not observed in rituximab-treated (or obinutuzumab-treated) patients from the prospective GAUSS cohort. Differences between the subject cohorts might explain these contradictory outcomes (data summarized in Table 4). Subjects included in the GAUSS study had relapsed following rituximab or were refractory to rituximab therapy, while those in the observational cohort were previously untreated. Thus, CFHR1 could be important in initial response to anti-CD20 mAb therapy, but not to response at the time of retreatment after relapse or resistance. In addition, the GAUSS study involved subjects treated with single agent anti-CD20, while the subjects from the observational cohort were treated less uniformly with most receiving rituximab in combination with chemotherapy. This difference could indicate that CFHR1 influences response to the combination of mAb therapy and chemotherapy, but does not contribute to response when anti-CD20 mAb is administered alone. Both study cohorts are relatively small (63 and 37 rituximab-treated subjects with protein expression data available in the

GAUSS and UI/Mayo studies, respectively), making it more difficult to draw conclusions that might impact treatment approaches in the clinic.

The role of CFHR1 itself in complement regulation is somewhat disputed, with one study defining it as a negative regulator of terminal complex formation and others finding no effect on complement activation(19, 32–34). Importantly, one group showed no impact of CFHR1 on rituximab-induced complement activation(35). This supports our finding that CFHR1 does not associate with differential outcome in patients treated with obinutuzumab, but may associate with rituximab outcome as observed in the observational cohort. There is some evidence to suggest that macrophages and neutrophils are a major effector population in rituximab-treated follicular lymphoma(36, 37). A recent study shows that CFHR1 can activate neutrophils through direct interaction with complement receptor 3 (CD11b/CD18), also expressed on macrophages(38). Based on this, it is possible that CFHR1 could have long-term advantages on induction of adaptive immune responses to rituximab-treated tumors by enhancing antigen uptake and presentation. This could explain why we observed a therapeutic advantage in rituximab-treated patients expressing higher levels of CFHR1 in the observational cohort (follow-up time 13 years) but not in the GAUSS cohort (follow-up <2 years). Perhaps a more durable immune response is generated in patients expressing more CFHR1, but that the GAUSS data have not matured long enough to see a significant difference in correlation with EFS. More time is needed to adequately assess this in the GAUSS cohort.

CFHR3 expression was associated with EFS of FL patients treated with obinutuzumab in the GAUSS study, with higher protein levels correlating with inferior therapeutic response. This was observed despite the relatively short follow-up time in this study, and suggests CFHR3 is a strong candidate biomarker to predict patient response to obinutuzumab. Further validation is required to confirm this and including CFHR3 serum protein measurements in future clinical trials using obinutuzumab will be necessary.

How CFHR3 might impact obinutuzumab action is not immediately clear. CFHR3 biology remains understudied, and published literature suggests that CFHR3 negatively regulates complement activation early in the complement cascade in a manner that is redundant with CFH(18). If this were important for obinutuzumab efficacy, we might have expected CFH levels to correlate with obinutuzumab therapeutic success, but they did not. Further, obinutuzumab does not activate complement as strongly as rituximab(21). We previously demonstrated that activated complement inhibits rituximab-induced NK cell activation, but not obinutuzumab-induced NK activation(9, 29, 30). Thus, mechanistic differences in rituximab and obinutuzumab actions might explain why CFHR3 was differentially associated with EFS (i.e. only associated with obinutuzumab treatment failure).

Indeed, the roles of the CFHR proteins are highly complex and often overlapping. Population studies have produced conflicting data whereby loss of CFHR1 and CFHR3 is linked with both susceptibility to (autoimmune aHUS(17, 39, 40)) and protection from (AMD(18, 41), IgA nephropathy(42, 43)) complement-mediated pathologies. Further adding to their complexity is the new understanding that some CFHR proteins (CFHR1, CFHR2, and CFHR5) actually exist as homo- and hetero-dimers in serum, and that the relative

abundance of CFHR1 protein in serum can alter dimer abundance, skewing functional output in ways we do not yet understand(33). It may be informative to assess the expression of the remaining CFHR family members (CFHR2, CFHR4 and CFHR5) in a similar manner to the data we presented here, since alterations in this genetic locus frequently involve many family members(44). Indeed, Harris *et al.* have proposed the use of complotyping, a method that incorporates genetic variation across the entire complement system, for predicting disease risk(45, 46). While the effects of individual components, such as CFHR1 and CFHR3, might have small influence on anti-CD20 therapy, the combined effects of a panel of complement components together could give a much clearer picture of anti-CD20 therapeutic success that assessing any one component alone.

It might also be informative to study the effects of CFHR1 and CFHR3 on rituximab and obinutuzumab outcome in other B cell malignancies. Research from our laboratory and others suggests the mechanism of action of either anti-CD20 mAb likely differs depending on the tumor microenvironment, with circulating and solid tumors showing different susceptibilities to complement dependent lysis or ADCC(9, 47). Thus, the impact complement regulators could vary based on the type of B cell malignancy being studied. In addition these studies might lend insight into the basic biological functions of CFHR3 and CFHR1.

We conclude that the role complement plays in determining response to anti-CD20 mAb therapy remains complex. This is particularly true for the CFH family of complement regulatory proteins that have both activating and inhibitory effects at various points in the complement cascade. However, we identified CFHR3 as a candidate biomarker for obinutuzumab treatment success in FL patients. Additional population-based studies, such as that presented here, and laboratory-based studies exploring complement pathways and mechanisms of action, are needed if we are to truly understand the role complement plays in the efficacy of anti-CD20, and other mAb-based therapies, and use this information to develop better mAbs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of translational relevance

Monoclonal antibodies (mAb) are an accepted and vital component of therapy for many patients with cancer. However, our understanding of the factors that determine mAb response, resistance and relapse is incomplete for the anti-CD20 mAbs rituximab and obinutuzumab. Data from our laboratory and others suggest the impact of complement and ADCC on mAb efficacy is complex, and that in some circumstances, complement fixation might reduce the ability of mAb to mediate ADCC. We studied the roles of complement regulators CFH, CFHR1 and CFHR3 in mAb treatment success using two clinical cohorts: the Iowa/Mayo Lymphoma SPORE observational cohort and the GAUSS prospective clinical trial cohort. Our results support CFHR3 serum expression status as a novel biomarker to predict obinutuzumab response in follicular lymphoma. This has further clinical implications for the therapeutic modulation of complement in conjunction with obinutuzumab therapy.

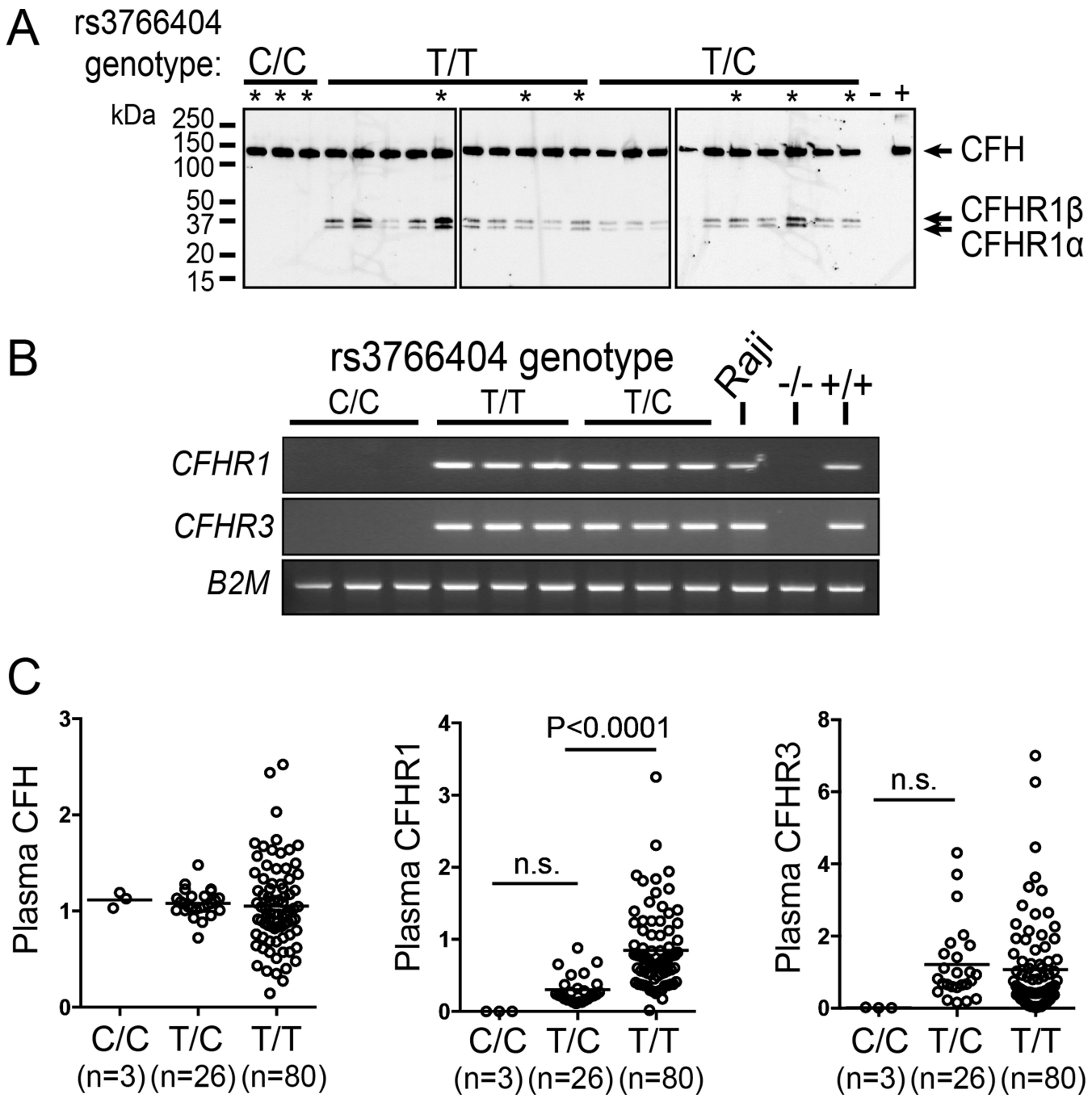

Figure 1. SNP genotype and plasma CFH, CFHR1, and CFHR3 expression

A. Plasma collected from FL subjects in the UI/Mayo Lymphoma SPORE were analyzed by western blot using an anti-CFHR1 antibody that cross reacts with CFH to measure plasma protein levels. Individuals with the C/C genotype retained expression of CFH in their sera, while CFHR1 expression was lost. Asterisks indicate samples genotyped in panel B. Negative control sera, from which CFH and CFHR proteins were depleted, was purchased from CompTech (Tyler, TX). Purified CFH, also purchased from CompTech, was used as the positive control.

B. Genomic DNA was isolated the FL subjects indicated in panel A. PCR amplification of *CFHR1* or *CFHR3* was performed, using Raji cells as a positive control. Individuals with homozygous minor allele (C/C) also exhibited homozygous deletion of *CFHR1* and *CFHR3*. Note this approach cannot detect a heterozygous deletion. The negative control was identified based on lack of plasma CFHR1 and CFHR3 expression.

C. Plasma protein expression in FL subjects was analyzed by western blot as shown in panel A, and expression was quantified using band densitometry. A T/T reference plasma sample from a healthy donor and expressing both CFH and CFHR1 was included on every gel, and protein levels were normalized to these before graphing. While plasma CFH and CFHR3 levels do not vary based on SNP genotype, CFHR1 levels are reduced in individuals with at least one C allele ($P<0.001$, unpaired *t* test). Dots represent individual patients and the horizontal bars represent the mean.

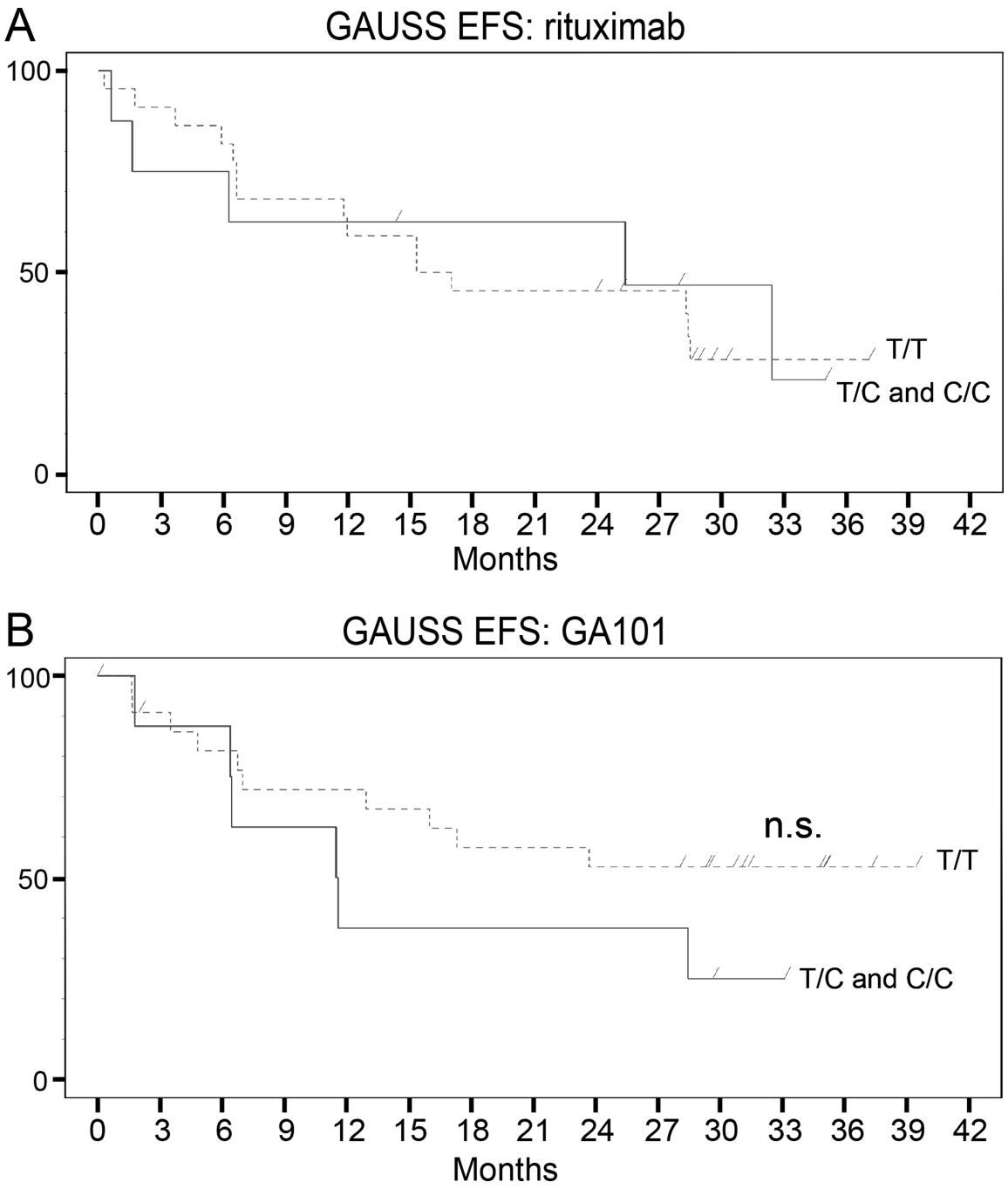

Figure 2. Event free survival by rs3766404 genotype in GAUSS clinical cohort

Kaplan-Meier survival curves of FL subjects participating in the GAUSS clinical trial based on SNP genotype.

A. FL patients treated with rituximab had similar event free survival (EFS) regardless of SNP genotype (P=0.8635, log-rank test). (T/T n=22, T/C and C/C n=8.)

B. FL patients treated with obinutuzumab also had similar EFS regardless of SNP genotype (P=0.1985, log-rank test). (T/T n=23, T/C and C/C n=8.).

Table 1

Plasma protein expression correlated with rs3766404 genotype.

		rs3766404		
Covariate	**Statistics**	**TC or CC N=29**	**TT N=80**	**P-value**
Relative CFH protein expression (normalized to reference CFH)	N	29	80	0.54
	Mean	1.08	1.05	
	Median	1.08	0.97	
Relative CFHR1 protein expression (normalized to reference CFHR1)	N	29	80	**<0.01**
	Mean	0.27	0.85	
	Median	0.22	0.72	
Relative CFHR3 protein expression (normalized to reference CFHR3)	N	28	80	0.52
	Mean	1.08	1.07	
	Median	0.72	0.56	

UI/Mayo SPORE FL patients with the T/T genotype had significantly higher CFHR1 protein expression compared to patients with 1 or 2 minor alleles ($p<0.01$). By contrast, there were no significant differences evidenced for CFH ($p=0.54$) or CFHR3 ($p=0.52$). P-values were calculated by Wilcoxon rank sum test.

Table 2

UI/Mayo plasma protein expression association with FL EFS

	Any Rituximab	No. of Patients	Hazard Ratio	95% Confidence Limits for Hazard Ratio		HR P-value	P-value
CFH	**No**	71	1.09	0.58	2.05	0.80	**<0.01**
	Yes	37	0.45	0.19	1.07	0.07	
CFHR1	**No**	71	1.05	0.62	1.76	0.86	**<0.01**
	Yes	37	0.23	0.08	0.67	**<0.01**	
CFHR3	**No**	71	0.93	0.73	1.19	0.57	0.09
	Yes	36	0.61	0.37	1.00	0.05	

UI/Mayo SPORE FL patients with higher plasma CFHR1 expression experienced superior EFS. Specifically, for each 1-unit increase in CFHR1 the risk of an event decreases by a factor of 0.23 (95% CI 0.08–0.67) for patients who received rituximab ($p<0.01$). For those who did not receive rituximab, CFHR1 did not have a significant effect on EFS but the effect was significantly different from those who received rituximab ($p<0.01$). Regardless of whether or not patients received rituximab, CFH did not have a significant effect on EFS but the direction of the effect was different between the two groups ($p<0.01$). CFHR3 was not found to be a predictor of EFS. A trend towards significance was evidenced for CFH and CFHR3 among patients who received rituximab on EFS ($p=0.07$ and $p=0.05$, respectively) with increased expression associated with a decline in risk.

Table 3

GAUSS serum protein expression association with FL EFS

Covariate	Treatment Arm	No. of Patients	Hazard Ratio	95% Confidence Limits for Hazard Ratio		HR P-value
CFH	**rituximab**	63	1.07	0.94	1.22	0.29
	obinutuzumab	51	1.03	0.88	1.20	0.73
CFHR1	**rituximab**	63	1.09	0.97	1.23	0.14
	obinutuzumab	51	1.01	0.81	1.26	0.95
CFHR3	**rituximab**	63	1.04	0.98	1.10	0.16
	obinutuzumab	51	1.15	1.04	1.26	**<0.01**

GAUSS clinical trial patient serum expression of CFH or CFHR1 did not correlate with EFS in either rituximab or obinutuzumab treatment groups. Interestingly, CFHR3 serum expression did correlate with EFS in the obinutuzumab, but not rituximab, treatment arm, with increased CFHR3 corresponding to inferior EFS with obinutuzumab treatment.

Table 4

Summary of associations observed in this study

	Observational Cohort	GAUSS Cohort	
	Initial therapy with most receiving rituximab combined with chemotherapy	Rituximab relapsed or refractory, treated with single agent anti-CD20 on a randomized study	
	rituximab	rituximab	obinutuzumab
rs3766404 genotype correlates with expression of CFHR1	Yes	Yes	Yes
CFHR1 expression correlates with outcome	Yes	No	No
CFHR3 expression correlates with outcome	No	No	Yes

A significant association between rituximab-treated patient outcome and expression of complement regulatory protein CFHR1 was observed in the observational cohort, but not in the GAUSS cohort. This could be due to longer follow-up time of the patients included in the observational cohort (>10 years) compared to the GAUSS study (2 years). Interestingly, CFHR3 expression was associated with obinutuzumab outcome, but not rituximab outcome. These results indicate CFHR3 may be a useful biomarker for obinutuzumab success in FL patients, and further population-based studies examining CFHR1 and CFHR3 in FL, or other malignancies where antibody therapy is standard, are recommended.