

Functional *FAS* Promoter Polymorphisms Are Associated with Increased Risk of Acute Myeloid Leukemia¹

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Abstract

The *FAS* (*TNFRSF6/CD95/APO-1*) gene is silenced in many tumor types, resulting in an inability to respond to proapoptotic signals. The *FAS* promoter is polymorphic, including a G to A substitution at –1377 bp and an A to G substitution at –670 bp, which occur within SP1 and signal transducers and activators of transcription 1 transcription factor binding sites, respectively. In a case-control study of adult acute myeloid leukemia (AML), we show a significantly increased risk of AML associated with heterozygotes (GA) and homozygote variants (AA) at position –1377 bp (32.3% in cases versus 22.0% in controls; odds ratio, 1.69; 95% confidence interval, 1.32–2.16). Extended haplotype analysis revealed that the –1377A/–670A haplotype was significantly associated with disease (3% versus 0.5%; odds ratio, 6.72; 95% confidence interval, 3.13–14.51). These data suggest that variation in the *FAS* gene promoter may affect *FAS* gene expression and modulate apoptotic signaling, contributing to an increased risk of AML.

Introduction

FAS (*TNFRSF6/CD95/APO-1*) is a cell surface receptor involved in apoptotic signal transmission in many cell types, including cells of the immune system (1). *FAS* belongs to the family of tumor necrosis factor receptors, and binding to the receptor by the *FAS* ligand (*CD95L*) triggers receptor trimerization and subsequent assembly of the death-inducing signaling complex (2). This mechanism of *FAS*-mediated apoptosis is believed to be involved in the removal of autoreactive lymphocytes during normal development of the immune system, and perturbation of this pathway occurs in a number of autoimmune disorders including systemic lupus erythematosus (3). The *FAS*-mediated apoptotic pathway is also dysregulated in several immune system malignancies, resulting in down-regulation of apoptosis and subsequent persistence of the malignant clone (2). Germ-line mutations or deletions within *FAS*, resulting in a loss or a reduction in receptor function, have been shown to cause autoimmune lymphoproliferative syndrome, a condition associated with systemic autoimmunity and generalized lymphoproliferation, as well as an overall increased risk of hematological malignancies (4). Aberrant apoptosis is known to be important in the pathogenesis of AML⁴ because up-regulation of the antiapoptotic protein *BCL2* is a common feature of leukemic blasts and may be a critical event in myeloid

transformation (5). Down-regulation of apoptosis can prolong the normal cellular life span, allowing cells to acquire mutations and facilitating tumor progression. *BCL2* transgenic mice with targeted expression in myeloid cells develop a myeloproliferative condition analogous to human chronic myelomonocytic leukemia; however, they rarely go on to develop acute leukemia. Interestingly, when mice constitutively expressing *BCL2* are crossed onto a *FAS*^{–/–} background, approximately 15% develop AML, implicating *FAS*-mediated apoptosis in the pathogenesis of AML (6). Myeloblasts are known to express high levels of *FAS*, and functional deficiencies of *FAS* signaling have been shown to be important in several subtypes of AML, providing further evidence for *FAS*-mediated apoptosis in the etiology of AML (7). *FAS* expression levels may be affected by mutations or polymorphisms in the promoter region of *FAS*, particularly when they affect transcription factor binding sites. We have developed a TaqMan-based assay and used an established RFLP assay (8) to determine the frequency of the polymorphisms at –1377 and –670 bp of the *FAS* promoter in a series of AML cases and controls. We have examined haplotype frequencies using maximum likelihood methods and log-linear modeling to determine the risk of AML. Our data indicate that haplotypes that include –1377A have a significantly increased risk of AML compared with the control population. An examination of the functional consequences of these polymorphisms revealed that both –1377 and –670 bp affect transcription factor binding sites within the *FAS* gene promoter.

Materials and Methods

Study Design. All samples included in this study were derived from the Leukaemia Research Fund population-based case-control study of adult acute leukemia conducted in the United Kingdom from April 1991 to December 1996. Further details of study design are given elsewhere (9). Participation rate, sample collection, and sample processing have been reported previously (10).

TaqMan-based Allelic Discrimination. A total of 471 of 479 AML cases and 931 of 952 controls were successfully genotyped for the –1377 bp *FAS* promoter polymorphism. An additional 21 control samples (2.2%) and 8 cases (1.6%) could not be amplified. Oligonucleotide primers and probes were designed using Primer Express software (Applied Biosystems, Warrington, United Kingdom). Allele-specific probes were labeled with either FAM (AA-GACCCTGGGTGTGCCAGCCT) or VIC (CCCTGGGCGTGCCAGCCT). PCR oligonucleotide primers spanning the –1377 polymorphism were as follows: *FAS*-F, CTATTAGATGCTCAGAGTGTGTGCA; and *FAS*-R, GCT-TGTCTCTGTTCACCTTTCA. Standard TaqMan cycling conditions were used, incorporating 40 cycles with an annealing temperature of 61°C.

RFLP-based Genotyping. A total of 454 of 479 cases and 934 of 952 controls were successfully genotyped for the –670 bp *FAS* promoter polymorphism as described elsewhere (8). *Scr*FI-digested PCR products were separated on 2.5% agarose gels, stained with ethidium bromide, and visualized under UV light.

Genotype Verification. TaqMan-based genotyping was verified in 10% of randomly chosen samples by allele-specific PCR. Oligonucleotide primers specific for the –1377G allele (ATGAGGAAGACCCTGGGC) or –1377A allele (ATGAGGAAGACCCTGGGT) were used with a common oligonucleo-

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⁴ The abbreviations used are: AML, acute myeloid leukemia; STAT, signal transducers and activators of transcription; OR, odds ratio; CI, confidence interval; EMSA, electrophoretic mobility shift assay.

tide primer (CTATTAGATGCTCAGAGTGTGTGCA) in an allele-specific PCR reaction (95°C for 10 min followed by 45 cycles of 95°C for 20 s, 58°C for 20 s) using Stoffel Gold enzyme (a kind gift from Roche Molecular Systems; Ref. 11). Final confirmation of both the -1377 and -670 bp genotyping assays was obtained in a random selection of 47 samples using DNA sequencing (Big Dye Terminator; Applied Biosystems Inc.) and analyzed using the Sequence Analysis program (Applied Biosystems Inc.).

Statistical Analysis. ORs and 95% CIs were derived using unconditional logistic regression with all available controls adjusting for age, sex, and region of residence. Maximum likelihood regression of haplotype frequencies was conducted using the Haploglm function in Stata (12), and ORs were derived using log-linear modeling (version 7; Stata Corp.).

EMSA. Oligonucleotide probes representing the -1377A allele (GGCTGGCACACCCAGGGTCTTC), -1377G allele (GGCTGGCACGCCA-GGGTCTTC), -670G allele (TGTCATTCCAGGAACGTCTG), or -670A allele (TGTCATTCCAGAAACGTCTG) were end-labeled using [γ -³²P]ATP (ICN, Basingstoke, United Kingdom) and polynucleotide kinase (Promega, Southampton, United Kingdom). A consensus SP1 site (GGCAGCCCA) was contained within the -1377G oligonucleotide probe, whereas a consensus STAT1 binding site was contained within the -670G oligonucleotide probe (TTCCAGGAA). Oligonucleotides were annealed to their complementary sequence by heating to 95°C for 10 min followed by cooling overnight. For -1377 bp EMSAs, K562 cells were grown to 10⁶ cells/ml in RPMI 1640 containing 2 mM L-glutamine and 10% fetal bovine serum. Nuclear protein extracts were made as described elsewhere (13) using 10⁷ cells. Determination of protein concentration was carried out using the Bradford reagent according to the manufacturer's instructions (Bio-Rad, Hemel Hempstead, United Kingdom). For -670 bp EMSAs, phorbol ester-treated HeLa nuclear extract was used (Autogen Bioclear, United Kingdom Ltd., Wiltshire, United Kingdom).

EMSAs were carried out using the Gel Shift Assay System (Promega). For each lane, 10 μ g of nuclear lysate were incubated with 4 ng of labeled double-stranded oligonucleotide primers for 30 min at room temperature followed by the addition of 20 μ l of 80% glycerol. Samples containing antibodies were preincubated for 30 min with anti-SP1 or anti-STAT1 antibody (Autogen Bioclear United Kingdom Ltd.) before the addition of labeled oligonucleotide. DNA-protein complexes were resolved on a 6% polyacrylamide gel (Sigma, Dorset, United Kingdom) and run at 140 V for 3 h in 0.5 \times Tris-borate EDTA (Invitrogen Ltd., Paisley, United Kingdom).

Results

FAS Genotype at -1377 and -670 bp. Allele and genotype frequencies for the -1377 and -670 bp polymorphisms are shown in Table 1. Both alleles within either the case or control group are in Hardy-Weinberg equilibrium at -1377 bp and -670 bp; however, at -1377 bp, the allele frequencies are significantly different in cases

Table 2 FAS -1377/-670 haplotype frequency distribution of controls and AML cases

FAS promoter polymorphism		Haplotype frequency ^a			
-1377	-670	Controls	AML cases	OR	95% CI
G	G	638.9	283.8	1	
G	A	978.0	456.2	1.05	0.88-1.26
A	G	208.0	133.2	1.44	1.11-1.87
A	A	9.0	26.8	6.72	3.12-14.51
χ^2 (P) ^b =		164.52 (<0.001)	64.78 (<0.001)		

^a Haplotype frequency estimated by EH.

^b Likelihood ratio test comparing interaction model with additive model (1 degree of freedom).

compared with controls ($\chi^2 = 17.1$; $P = 0.0002$), suggesting a link with risk of AML. There was an increased risk of AML associated with heterozygotes at -1377 bp (28.9%) compared with controls (20.0%; OR, 1.66; 95% CI, 1.29-2.15). The risk associated with AA homozygotes at -1377 bp in the case population (3.4%) is also higher than that in the control population (2.0%; OR, 1.92; 95% CI, 0.97-3.78). The narrow CIs indicate that these results are both accurate and precise. These data suggest that an increased risk of developing AML is associated with inheritance of the -1377A allele in a dose-dependent manner. The associated risk of AML when carrying an adenine residue at -1377 bp was shown to be independent of age, sex, and region (data not shown). In contrast, no significant differences in genotype distribution between AML cases and controls were observed at -670 bp (Table 1). Previous studies have shown that GSTT1-null and GSTM1-null genotypes and tobacco smoking were each associated with risk of AML (9, 10). However, our findings remained unchanged when these additional factors were adjusted for (data not shown).

Haplotype Analysis. The distribution of -1377 bp/-670 bp haplotypes (Table 2) was significantly different in cases compared with controls for both the -1377A/670G haplotype (OR, 1.44; 95% CI, 1.11-1.87) and the -1377A/-670A haplotype (OR, 6.72; 95% CI, 3.12-14.51). Although the genotype at -670 bp is not independently associated with risk, haplotype analysis reveals that in the presence of -1377A, -670A increases the risk of AML much more than -670G.

DNA Binding at -1377 and -670 bp of the FAS Promoter. Having demonstrated an association of the FAS promoter polymorphisms with risk of developing AML, we sought to define the functionality of the different alleles. Using EMSAs, we have shown that -1377A has a greatly reduced ability to bind the transcription factor

Table 1 FAS -1377 bp and -670 bp genotype distributions of controls and AML cases, with ORs and 95% CIs

FAS promoter polymorphism	Controls (n = 952)		AML cases (n = 479)	
	No. (%)	No. (%)	OR ^a	95% CI ^b
-1377				
GG	726 (78.0)	319 (67.7)	1	
GA	186 (20.0)	136 (28.9)	1.66	1.29-2.15
AA	19 (2.0)	16 (3.4)	1.92	0.97-3.78
SNA ^c	21	8		
GG	726 (78.0)	319 (67.7)	1	
GA + AA	205 (22.0)	152 (32.3)	1.69	1.32-2.16
Hardy-Weinberg equilibrium test Pearson χ^2 (P)	2.93 (0.087)	0.10 (0.749)		
-670				
GG	205 (21.9)	97 (21.4)	1	
GA	449 (48.1)	228 (50.2)	1.07	0.80-1.43
AA	280 (30.0)	129 (28.4)	0.97	0.71-1.34
SNA	18	25		
GG	205 (21.9)	97 (21.4)	1	
GA + AA	729 (78.1)	357 (78.6)	1.03	0.79-1.36
Hardy-Weinberg equilibrium test Pearson χ^2 (P)	0.975 (0.32)	0.04 (0.841)		

^a ORs and 95% CIs estimated by unconditional logistic regression adjusting for age, sex, and region.

^b Using GG as the reference population.

^c SNA, sample not amplified.

SP1 compared with -1377G (Fig. 1A). The signal produced by -1377G can be supershifted by addition of anti-SP1 antibody (Lane 6). The -1377G signal can be completely eliminated when the extract is incubated with a 250-fold excess of unlabeled -1377G oligonucleotide (Lane 7) but is only reduced in the presence of a 250-fold excess of unlabeled -1377A oligonucleotide (Lane 8), providing further evidence that the A variant is less efficient at binding SP1.

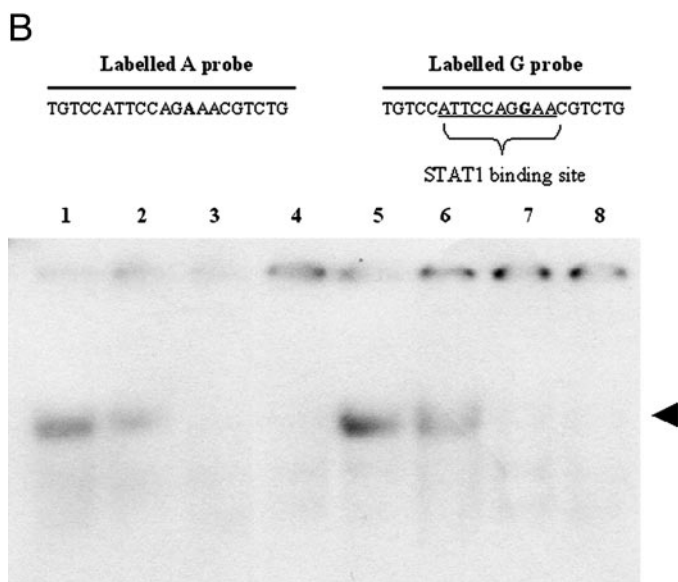
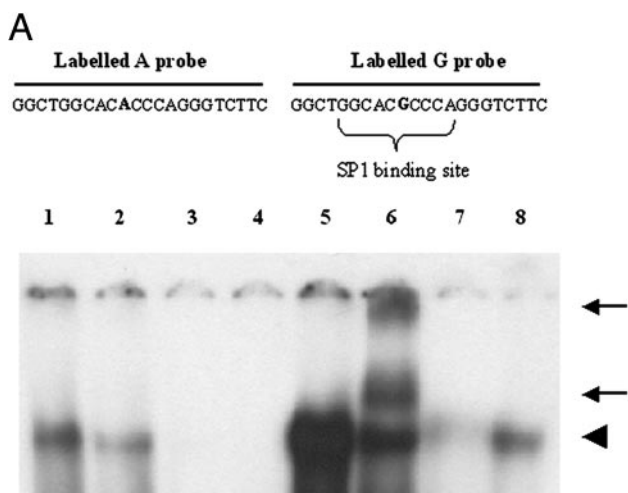


Fig. 1. A, DNA binding activity at -1377 of the *FAS* promoter. Extracts of K562 (20 μ g/lane) were incubated with radioactively labeled oligonucleotide probes containing either the A (Lanes 1-4) or G variant (Lanes 5-8) at position 10. Lane 1, extract incubated with labeled A probe; Lane 2, extract incubated with labeled A probe and anti-SP1; Lane 3, extract incubated with labeled A probe and 250-fold excess of unlabeled A probe; Lane 4, extract incubated with 250-fold excess of unlabeled G probe; Lane 5, extract incubated with labeled G probe; Lane 6, extract incubated with labeled G probe and anti-SP1; Lane 7, extract incubated with labeled G probe and 250-fold excess of unlabeled G probe; Lane 8, extract incubated with labeled G probe and 250-fold excess of unlabeled A probe. Arrowhead shows bandshift when SP1 binds to the probe. Arrows indicate supershifted bands with anti-SP1. B, DNA binding activity at -670 of the *FAS* promoter. Extracts of phorbol ester-treated HeLa nuclear extract (20 μ g/lane) were incubated with radioactively labeled oligonucleotide probes containing either the A (Lanes 1-4) or G variant (Lanes 5-8) at position 10. Lane 1, extract incubated with labeled A probe; Lane 2, extract incubated with labeled A probe and anti-STAT1; Lane 3, extract incubated with labeled A probe and 125-fold excess of unlabeled A probe; Lane 4, extract incubated with 125-fold excess of unlabeled G probe; Lane 5, extract incubated with labeled G probe; Lane 6, extract incubated with labeled G probe and anti-STAT1; Lane 7, extract incubated with labeled G probe and 125-fold excess of unlabeled G probe; Lane 8, extract incubated with labeled G probe and 125-fold excess of unlabeled A probe. Arrowhead shows bandshift when STAT1 binds to the probe.

The -670 bp polymorphism is located within a STAT1 binding site (Fig. 1B). Both -670A and -670G alleles can bind STAT1 (Lanes 1 and 5), producing similar intensity bands. This experiment was repeated several times with no detectable difference in band intensity between -670A and -670G. Competition using either a 125-fold excess of unlabeled -670A or a 125-fold excess of -670G completely eliminated the signal produced by -670A or -670G, suggesting that both variants can bind STAT1 equally well. Addition of anti-STAT1 antibody disrupts the signal and reduces the intensity of the band (Lanes 2 and 6).

Discussion

We have demonstrated that the risk of developing AML is associated with the -1377 bp polymorphism in the promoter region of the *FAS* gene, which affects a SP1 transcription factor binding site. Individuals homozygous for adenine at -1377 bp are almost twice as likely to develop AML (OR, 1.92; 95% CI, 0.97-3.78), with heterozygotes having an intermediate risk (OR, 1.66; 95% CI, 1.29-2.15) compared with individuals homozygous for guanine. However, there was no evidence of an increased risk of AML associated with the -670 bp polymorphism (Table 1). Work from other laboratories has shown that the genotype at -670 bp is associated with cervical squamous cell carcinoma (14) and Alzheimer's disease (15), whereas the -1377 bp genotype showed no association with disease (16, 17). Haplotype analysis (Table 2) reveals that the risk of AML is driven by the presence of the -1377A allele, with the -1377A/670A haplotype conferring the highest risk (OR, 6.72; 95% CI, 3.12-14.51).

Previous studies have suggested that cytogenetics can be used to define distinct subgroups of AML with different prognoses and age distributions (18). Using cytogenetic data from this study, we separated the data set into five cytogenetic subgroups: normal karyotype; translocation; trisomy 8; deletions of 5q/7q; or other. Comparison of the *FAS* -1377 bp and -670 bp genotypes across these groups revealed no significant associations of genotype with karyotype (data not shown), suggesting that the risk associated with polymorphisms in the *FAS* promoter is not restricted to a particular AML subtype and that the downstream effects of genetic variation in the promoter are likely to be exerted via mechanisms common to all subtypes.

The genotype frequencies in our control population at both -1377 bp and -670 bp are in good agreement with previous studies (16, 19). To our knowledge, haplotype frequencies have only been reported in one other study examining systemic lupus erythematosus cases in a Japanese population (17), but, as would be expected, allele and genotype frequencies in the control population were different from those obtained in this study; hence direct comparisons cannot be made.

Detection bias, in which one allele is more efficiently amplified or detected than another, in large genotyping studies has been reported in the literature, as have false associations in small studies (20). The large size of this study, combined with a rigorous verification of TaqMan and RFLP-based genotyping using allele-specific PCR and direct sequencing, has ensured that accurate genotype frequencies have been calculated. For studies claiming association of specific polymorphisms with a biological effect, it is also important to be able to demonstrate a potential mechanism of action. In this study, we have confirmed that the -1377 and -670 bp polymorphisms occur within transcription factor binding sites and bind SP1 and STAT1, respectively. Moreover, we have shown that the presence of an adenine residue at position -1377 bp of the *FAS* promoter significantly reduces SP1 binding compared with a guanine residue. The SP1 site at -1377 bp lies between two putative silencer regions, although the site itself is not within a known silencer consensus sequence (21). SP1

binding is associated with transcriptional activation (22), and we postulate that reduced SP1 binding at -1377 bp in the *FAS* promoter results in a decrease in *FAS* expression. Although the allele and genotype frequencies at -670 bp in the case and control populations are not significantly different, haplotype analysis reveals that the -1377A/-670A haplotype increases the risk of AML six times above those with -1377G/1377G. Hence the -670A allele appears to modulate risk of AML when associated with -1377A, but not when associated with -1377G. Previous work has suggested that STAT1 binding alone cannot be equated with a biological function and that concomitant SP1 binding may be required to achieve transcriptional activation (23). This complex association at the protein level may explain why the -1377A/-670A haplotype shows such an increased risk of AML.

The importance of *FAS* in hematopoietic tissues is illustrated by the mouse *lpr* model. Mutations in *FAS* lead to lymphadenopathy, systemic lupus erythematosus, and an increase in the incidence of B-cell lymphoma (2). Autoimmunity and related conditions are believed to be due to a failure of T cells to undergo negative selection during development, where efficient apoptosis is crucial in the removal of autoreactive lymphocytes (3). Loss of function mutations in *FAS* and components of the *FAS* apoptotic pathway have also been implicated in myeloid malignancies (24). *lpr* mice that constitutively express the *Bcl-2* proto-oncogene develop murine AML, whereas constitutive *Bcl-2* expression alone does not result in malignancy (6). These studies suggest that *FAS* is one of several genes critical to normal myeloid differentiation, potentially regulating myeloid expansion. Our study has shown that the -1377 bp *FAS* promoter polymorphism is associated with an increased risk of AML. Furthermore, we have shown that the presence of an adenine residue rather than a guanine residue at -1377 bp significantly attenuates transcription factor SP1 binding and may contribute to a reduction in *FAS* expression and ultimately to the enrichment of apoptosis-resistant clones in AML.

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