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Title

THE KRUPPEL LIKE FACTOR 6 (KLF6) GENOTYPE IS ASSOCIATED WITH FIBROSIS IN NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD).

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Short Title

A Role for KLF6 in NAFLD

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List of abbreviations:

NAFLD: Nonalcoholic Fatty Liver Disease

KLF6: Kruppel-like Factor 6

NIDDM: Non-Insulin Dependant Diabetes Mellitus

HCC: Hepatocellular Cancer

SNP: Single Nucleotide Polymorphism

TDT: Transmission Disequilibrium Test

αSMA: alpha smooth muscle actin

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Abstract

Background & Aims: Although NAFLD is increasingly common, only a minority of affected individuals develop fibrotic liver disease. Based on its role in liver growth and repair, we explored whether Kruppel-like factor 6 (KLF6) plays a role in NAFLD progression.

Methods: KLF6 expression in 31 Brunt scored NAFLD liver biopsies was assessed by real-time PCR. Transfected minigene constructs were used to study the effect of a polymorphism, KLF6-IVS1-27G>A, that promotes KLF6 alternative splicing *in vitro*. We genotyped KLF6-IVS1-27G>A in 3 groups of patients (UK Group 1: n=306; Italian Group 2: n=109; Trio Group 3: n=61 children and parents).

Results: KLF6 expression was increased in association with increased steatosis, inflammation and fibrosis in NAFLD livers. KLF6-IVS1-27G>A promoted alternative splicing of KLF6 and abrogated the up-regulation of both αSMA and collagen 1 in LX2 cells. Group 1 genotyping identified KLF6-IVS1-27G>A in 44 of 306 (14.4%) patients. Notably, KLF6-IVS1-27G>A was significantly associated with milder NAFLD, with only 25% having more advanced fibrosis compared to 45% of wild type (wt) individuals. This trend was confirmed in Group 2. A linear regression analysis including all 415 patients, adjusted for age, sex, body mass index and blood glucose, confirmed that presence of the wt KLF6 allele was an independent predictor of fibrotic NAFLD. Furthermore, we have shown preferential transmission of the wt allele to children with fibrotic NAFLD.

Conclusions: We report a functional polymorphism in the KLF6 gene associated with advanced NAFLD and believe further study of KLF6 may enhance our understanding of this disease process.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the liver manifestation of the metabolic syndrome, characterised by central obesity, atherogenic dyslipidaemia, hypertension and insulin resistance. NAFLD encompasses a spectrum of liver disease from simple fatty liver to cirrhosis. The prevalence of NAFLD is growing rapidly and it is now the most common cause of chronic liver disease in Western countries.[1] The cardiovascular health implications of the metabolic syndrome are significant, with the risk of progressive liver disease, cirrhosis and liver cancer set to have a further major impact.[2] Despite its high prevalence, however, less than a quarter of subjects with NAFLD ever progress beyond steatosis to significant fibrosis and liver cancer. [3-5] The reasons for these differences in individual susceptibility to progressive disease are unclear, but family/ethnic studies suggest that genetic factors play a significant role.[6,7] While several candidate genes have been studied, as yet, no genetic associations with advanced NAFLD have been replicated in large studies.[8]

Kruppel-like factor 6 (KLF6) belongs to the Kruppel-like family of transcription factors known to play diverse roles in differentiation, development, cell growth, apoptosis, and angiogenesis.[9] Similar to other members of the family, KLF6 has 3 contiguous C_2H_2 zinc fingers at the carboxyl-terminal domain and recognizes GC box motifs or CACCC motifs in responsive promoters.[10] While expression of some KLF transcription factors is tissue specific, KLF6 is ubiquitously expressed.[11] It was identified as an early gene expressed in activated hepatic stellate cells (HSC) after liver injury,[12,13] raising the possibility it may be involved in the process of liver fibrogenesis. Furthermore, KLF6 transactivates several genes critical for the development of liver fibrosis, including collagen 1, TGFβ1, and types I and II TGFβ receptors in HSC.[13, 14] The recent demonstration of increased expression of KLF6 in

response to oxidative stress in a methionine-choline deficient diet model of non-alcoholic steatohepatitis (NASH), linked to increased TGFβ1 expression, provides support for a role of KLF6 in NAFLD.[15] KLF6 has also been proposed as a tumour suppressor gene, being located on chromosome 10p15 and frequently deleted in a number of cancers including prostate [16] and hepatocellular cancer (HCC).[17] Its expression is reduced in the majority of hepatitis B and C virus associated HCCs.[18] Moreover, retrovirally mediated over-expression of KLF6 in HCC HepG2 cells is associated with reduced proliferation and increased differentiation,[18] suggesting a key role in the regulation of hepatocyte growth.

A functional single nucleotide polymorphism (SNP) in the KLF6 gene has recently been identified.[19] *KLF6–IVS1-27G>A* (rs3750861; NCBI Entrez SNP database) is a *KLF6* SNP located within the first intron of the gene and is associated with an increased incidence of familial prostate cancer.[19] Further characterisation of the SNP demonstrated that it created a novel binding site for the splicing factor, SRp40.[20] Furthermore, the presence of the SNP *in vitro* in prostate cancer cells promoted alternative splicing of the KLF6 gene into dominant negative alternative isoforms that fail to upregulate p21, facilitating enhanced proliferation. Alternative splicing of KLF6 has now been accepted as an additional means of inactivation of its wt tumour suppressor function in prostate[20] and ovarian cancers [21], as well as in glioblastomas. [22]

Based on the suspected roles of KLF6 in hepatic fibrosis associated with oxidative stress, as well as in hepatocyte proliferation and differentiation, we have investigated and demonstrated increased levels of KLF6 in liver biopsies of patients with advanced versus earlier stages of NAFLD. We have also shown that the *KLF6-IVS1-27G>A* SNP promotes splicing of KLF6 to its alternative forms in both HCC

and HSC cell lines. In the latter, we have confirmed an increase in both αSMA and collagen I production stimulated by KLF6 WT, attenuated in the presence of SNP associated alternative splice forms. We have identified wt *KLF6* as an independent predictor of the presence of fibrosis in two independent cohorts of NAFLD patients, with those with the variant allele having less advanced fibrosis. Finally, we have also shown preferential transmission of the wild type allele to children with fibrotic NAFLD using transmission disequilibrium testing in a family study. We believe KLF6 to be a strong candidate for further studies in this increasingly common disease.

Patients and methods

Patients

We enrolled 415 patients with biopsy proven NAFLD at different stages of disease from Newcastle and Turin hospitals according the regulations and ethical requirements of the participating centres. All patients had clinical features and liver biopsies diagnostic of NAFLD. Females and males consuming greater than 14 or 21 units of alcohol per week respectively were excluded, as were any individuals with viral or autoimmune liver diseases. Clinical and laboratory data were collected on the date a diagnostic liver biopsy was performed. Body mass index (BMI) was calculated using the formula: weight(kilograms)/height(m)^2. The presence of diabetes mellitus (fasting glucose ≥ 7.1 mmol/L or treatment with anti-diabetic drugs) and hypertension (blood pressure ≥130/85mmHG or on treatment for hypertension) was recorded. Laboratory evaluation included liver biochemistry; blood count; total- and HDL-cholesterol and total triglycerides; fasting glucose; fasting insulin; viral serology and autoantibodies. The degree of insulin resistance was determined by the homeostatic model assessment (HOMA).[23] Patients included 306 North East UK patients (Group

1), and 109 Italian patients (Group 2). The confirmation of results in two independent European NAFLD populations reduces the risk that any association observed in the first group was a "chance" finding subject to a type I error.

In addition we assessed 71 Italian family "trios" with both parents alive and an index child with biopsy-proven NAFLD. Our particular interest was in those with fibrotic disease, of which there were 61 (stage 1 n=59; stage 2 n=2). We used transmission disequlibrium testing (TDT)[24] to look for preferential transmission of either KLF6 alleles to the affected children. This approach is not subject to the potential confounding effects inherent in case-control studies and is significantly more powerful at detecting true associations.

Liver biopsy

Ultrasound guided liver biopsy was performed and read by a single liver pathologist in each participating center. The severity of steatosis, necroinflammatory grade and stage of fibrosis were scored according to modified Brunt criteria.[25] Liver tissue from 31 patients undergoing liver biopsy for suspected NAFLD also had a 0.5-1cm core of tissue stored in RNA later solution (Ambion).

RNA extraction from Liver tissues

mRNA was isolated from liver tissue using TRIzol reagent (Invitrogen) according to manufacturer's instructions with minor modification in the precipitation step: the aqueous phase with isopropanol, 5 mg/ml glycogen (Ambion) and a high salt solution (1.2M NaCl, 0.8M Na-citrate) was incubated at -20ºC for 1h prior to washing and resuspension in water. 1µg cDNA was synthesised with Reverse Transcription System (Promega).

Real-time PCR analyses

Semi-quantitative RT-PCR assays were using either Taqman (KLF6 quantification) or SYBR Green (KLF6, αSMA; collagen 1) technology on a 7900HT sequence detection system (Applied Biosystems). The SYBR Green primers used to compare the wt KLF6 mRNA (KLF6 WT) with all isoforms of KLF6 (KLF6 Total) were as previously described.[19] Taqman semi-quantitative PCR was similarly performed using the following primer and probe sets: KLF6 Total forward – 5' CGGACGCACACAGGAGAAAA 3', reverse – 5' GGTTAACTCATCACTTCTTGCAAA 3', probe – 5' AGGGTGTGAGTGGCGT 3'; KLF6 WT forward – 5' AATTTGACAGCCAGGAAGATCTG 3', reverse – 5' CAGTTCGGATTCCTCCTTTTTC 3', probe – 5' ACCAAAATCATTCTGGCTCGGG 3', GAPDH forward – 5' GCACCGTCAAGGCTGAGAA 3'; reverse 5' AGCATCGCCCCACTTGATT 3'; and probe 5' CATCTTCCAGGAGCGAGAT 3'. The TaqMan master mix was used according to manufacturer's instructions (Applied Biosystems). 1µl of cDNA was used per well in a total volume of 10µl with standard cycling parameters (95ºC for 2min, 95ºC for 10min then 40 cycles of; 95ºC for 15sec, 60ºC for 1min). Data generated was analysed using SDS 2.2 (Applied Biosystems). The Relative Quantity (RQ) of each target gene was determined from replicate samples anaysed on three separate occasions using the formula $2^{-\Delta\Delta C_T}$. All data was presented as RQ values±min/max for the relative amounts of target gene, normalized to GAPDH, and relative to expression values in a chosen comparator sample. In the liver biopsy tissue analyses, the comparator sample served only as a reference for standardisation of all other samples and was not included in any subsequent analyses. It was created from

equal quantities of cDNA from 4 samples with previously determined low expression of KLF6, including one histologically normal sample and three samples with simple steatosis.

Cell Culture

HCC cell lines were obtained from the American Tissue Culture Collection, while the LX2 cells were created and characterised as previously described.[26] Cells were cultured in Dulbecco's modified Eagle medium containing 10% Fetal Bovine Serum with 100U/ml penicillin, 0.1mg/ml streptomycin and 2mM L-glutamine (Sigma). Media was changed every 48h. Cells were passaged 1-2 times per week. Cells were seeded 24h before transient transfection experiments. LX2 cells were similarly maintained, but in 1% serum.

Transient transfections of HCC cell lines were performed using 4ng DNA and Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen). For analyses in LX2 cells, which have a very low transient transfection efficiency, cell lines stably expressing minigene constructs were created and harvested at 1-2 weeks. $2x10^5$ LX2 cells were seeded into each well of a 6-well plate and grown overnight. Transfection was with Effectene reagent (Qiagen). 0.5µg of each minigene construct was combined with 0.5µg of pCI-Neo and incubated in enhancer solution for 5min at room temperature. The effectene reagent was added for a further 10min before adding to the cells in 1ml of normal growth medium. The medium was replaced the following day and after 48h G418 was added at a concentration of 600µg/ml to select for transfected cells.

Genotyping

Blood samples (5mL) were collected from each subject, and DNA was extracted by Tissue DNeasyTM (Quiagen) according manufacturer instructions. The intron 1/exon 2 region of *KLF6* was amplified as previously described.[19] Successful DNA amplification was determined by the visualisation of a 290bp fragment on 1% agarose gel electrophoresis. The presence of the *KLF6-IVS1-27G>A* SNP (rs37508611) was determined by BsaAI (New England BioLabs Inc.) digestion, which cuts wild type DNA at recognition sequence 'CGCG' at position 84 of the amplicon. The detection of 3 bands indicated heterozygosity. Positive and negative controls to confirm complete digestion were used on every gel. Every positive sample was confirmed by an independent PCR and restriction analysis. Each of these independent PCR reactions included random wild type samples, similary re-analysed, in order to confirm a lack of cross contamination.

Statistical analysis

The data was analysed using SPSS 14 (Statistical Package for Social Sciences) licensed to Newcastle University. Comparisons of parametric data were analysed using a students t-test or ANOVA, while non parametric data analyses were performed by a Mann-Whitney test for quantitative and Pearson Chi-square test for categorical variables. Comparisons of KLF6 expression associated with different stages of histologically scored disease was performed with using a Kruskal-Wallis test. A p value < 0.05 was considered significant.

The main aim of this study was to evaluate the possible role of the polymorphism of KLF6 in predicting patients with progressive NAFLD. Using the web based calculator available at www.tufts.edu/, we confirmed that the KLF6 polymorphism was in Hardy-Weinberg (HW) equilibrium in each of the British and

Italian groups studied. The minimum sample size required to analyse wild type KLF6 being a risk factor for fibrosis in NAFLD was calculated by QUANTO Software as detailed in supplementary information. We considered the presence of stage 2 fibrosis as indicative of progressive disease as stage 1 fibrosis in adults often does not, or can take many years, to progress. Logistic regression was used to determine the independent nature of a number variables, including age, BMI, bood glucose, platelet count, AST/ALT ratio and KLF6 genotype, on the presence of stage 2 or more of fibrosis. The Forward Stepwise Wald method was used. The goodness of fit of the model was assessed by the Hosmer-Lemeshow test for significance. Results are shown as odds ratio (OR) and 95% confidence interval (95% CI).

The transmission disequilibrium test (TDT) test[24] was used to look for intra-familial allelic association in the family study. The Chi-square statistics (using R and UNPHASED, freeware software) to compare the observed number if parent-offspring transmission of *KLF6-IVSI-27G>A* allele with the number of transmission expected by chance. A p value <0.05 was considered statistically significant.

Results

KLF6 expression is increased in association with more advanced stages of NAFLD.

KLF6 is a ubiquitous transcription factor and immediate early gene induced in response to stressful stimuli. We have compared its expression at the mRNA level using two different sets of Taqman real-time primers and probe, one of which quantifies all isoforms of KLF6 ('KLF6 Total') and one of which quantifies the full length wild type version only ('KLF6 WT'). Both are expressed as a Relative Quantity (RQ) value, relative to the same control gene (GAPDH) and the same comparator sample. The difference between the two primer sets can be attributed to

alternatively spliced KLF6 mRNA. Relative levels of Total KLF6, WT KLF6 and alternatively spliced KLF6 are summarized in Table 1. The fat, necroinflammatory and fibrosis scores for each sample are also included. The levels of KLF6 expression were subsequently analyzed with respect to histological scoring and there was clearly a significant increase in KLF6 expression in association with more advanced histological disease, as shown in Figure 1. The expression of the full length isoform, KLF6 WT, was significantly increased with more advanced stages of steatosis, inflammation and fibrosis. The data for 'Total KLF6 isoforms' was similar, but with some notable differences. While a significant increase was seen in association with increasing inflammatory score, there was no significant differences in Total KLF6 isoforms relative to different stages of steatosis or fibrosis. These data suggest that while all isoforms of KLF6 are expressed in response to liver injury and ongoing inflammation, it is the predominance of the full length WT isoform that is associated with more advanced fibrotic stages of NAFLD.

KLF6 IVS1-27G>A enhances splicing in liver cell lines and abrogates the KLF6wt induced transcriptional increase in αSMA and type 1 collagen mRNA

Using transfected control LacZ expressing and KLF6 expressing minigene constructs, the latter either with or without the KLF6 SNP, we demonstrated that KLF6 alternative splicing to its smaller isoforms occurs readily in the presence of the SNP in a liver cell environment. Cell lines studied included the HepG2, Huh7 and Hep3B liver cancer cell lines, as shown in Figure 2A, and the human HSC cell line, LX-2, shown in Figure 2B. Both αSMA and type 1 collagen are markers of HSC activation, the latter being a known transcriptional target of wt KLF6. As shown in Figure 2C, both were increased at the mRNA level in LX-2 cells transformed with the

KLF6 wt minigene. The increases in αSMA and collagen 1 expression were attenuated in the LX-2 cells expressing the *KLF6 IVS1-27G>A* minigene, producing increased levels of KLF6 splice forms.

KLF6-IVS1-27G>A is associated with reduced fibrosis in NAFLD

In order to determine whether the *KLF6-IVS1-27G>A* SNP plays a role in susceptibility to NAFLD-related liver fibrosis, we examined whether the presence of the SNP was associated with histological parameters in a large cohort of patients with NAFLD. The combined study populations comprised a total of 415 Caucasian European patients (Group 1: 306 from Newcastle, UK and Group 2: 109 from Turin, Italy). Clinical details are reported in Table 2. While there were some biologically relevant differences (eg. age, BMI, diabetes), and others which may reflect minor differences attributable to variation in laboratory, age and sex normal ranges (Alkaline Phosphatase, bilirubin, cholesterol), there was no difference in the prevalence of liver fibrosis, the HOMA score reflecting insulin resistance, nor in the frequency of the *KLF6 IVS1-27G>A* SNP between UK (14.4%) and Italian (13.8%) cohorts. The latter was similar to the KLF6 IVS1-27G>A SNP frequency in an independent group of Europeans as reported in the HAP-MAP-Project (NCBI Entrez SNP database: http://www.ncbi.nlm.nih.gov/entrez; rs3750861; heterozygous genotype incidence 15%, 2N=120;).

Both groups of patient data were subsequently analysed in relation to the presence or absence of *KLF6 IVS1-27G>A.* No significant differences were identified in a range of clinical and laboratory variables between those with and without the SNP. Data for the combined group are shown in Table 3(i). This was not the case, however, for the histological fibrosis score, where significant differences between the

wt and heterozygous individuals were present. In Group 1 (UK NAFLD patients), the *KLF6 IVS1-27G>A* heterozygotes had significantly less fibrosis (75% having fibrosis stage 0 or 1) compared to wt individuals (only 55% staged 0 or 1), as shown in Table 3(ii). Those with fibrosis of stage 2 or more accounted for 45% wt KLF6 individuals and only 25% of heterozygous patients. This difference was statistically significant (p=0.015, Pearson Chi Square Test). The findings in Group 2 (Italian NAFLD patients) were very similar, with 67 % of heterozygous patients having stage 0 or 1 fibrosis compared to only 46% of wild type individuals. This difference did not reach statistical significance (p=0.134) owing to the smaller number of patients. In combination with Group 1, however, the statistical significance for the protective effect of *KLF6-IVS1-27G>A* (73% having stage 0 or 1 fibrosis, compared to only 53% of wt KLF6 individuals) was enhanced (p=0.004).

Univariate analyses of differences between the defined control group (fibrosis 0+1) and the case group (fibrosis 2-4) are shown in Table 4(i). Variables analysed include both reported risk factors for progressive disease (Age, BMI and serum glucose) as well as two reported associations with progressive disease (Platelet count and AST/ALT ratio).[27] Logistic regression analysis was used on the combined dataset to determine whether the association between *KLF6* and advanced stage 2-4 fibrosis was independent of these factors. As presented in Table 4(ii), wt KLF6 remained a highly significant and independent predictor of the presence of fibrosis, with an odds ratio of 2.76 [1.295-5.908], p=0.009.

Wild type KLF6 IVS1-27 is preferentially transmitted to children with fibrotic NAFLD

We analysed 71 families with two surviving parents and an index child with NAFLD and genotyped parents and children for the *KLF6 IVS1-27G>A* SNP. The

majority of these family trios formed part of a previously reported and well characterised series.[28,29] Our particular interest was in those with fibrotic disease, of which there were 61 (stage 1 n=59; stage 2 n=2). All of these cases had NASH, with a necroinflammatory score of either stage 1 or 2. Forty were male (mean age 11.47±2.89; BMI 25.46±3.1) and 21 were female (mean age 9.9±2.31; BMI 25.17±4.75). Twenty-four families were "informative" in that 1 parent was heterozygous for the SNP. TDT testing using these families revealed preferential transmission of the wt allele to the children (17 wt alleles transmitted versus 7 not transmitted, TDT χ^2 4.16667; p = 0.041; odds ratio (OR) of the wt allele being transmitted to affected children being 2.42).

Discussion

NAFLD is increasingly common in western societies. Established predictors of those likely to develop progressive disease characterized by advanced fibrosis include age, increased BMI and a raised blood glucose.[27] Why some individuals with these risk factors progress and others do not is unclear, but twin and ethnic studies suggest that genetic factors play a role.[5,6] To date, SNPs in a number of genes have been associated with NAFLD severity, including microsomal triglyceride transfer protein, manganese superoxide dismutase,[30] phosphatidylethanolamine *N*-methyltransferase,[31] and tumor necrosis factor alpha.[32] However, none of these studies were sufficiently large to exclude a type 1 error (number of patients <110) and none have been replicated.

The identification of a functional alternative splicing promoting *KLF6* SNP as a susceptibility factor for familial prostate cancer has lead to further studies establishing alternative splicing as a means of KLF6 deregulation in cancers.[20, 21, 33]

The suspected role of KLF6 in the regulation of both hepatocyte growth and HSC activation, as well as its induction in an established animal model of NASH,[13] provides justification for examining both the role of KLF6 in human NAFLD and the SNP as a susceptibility factor affecting disease stage. In this paper, we have presented KLF6 mRNA expression data from liver biopsy samples of patients with NAFLD and demonstrated increased total and wild type isoforms in association with increased levels of inflammation. From these data alone we were unable to draw firm conclusions on the role of wt KLF6 or on the contribution of alternatively spliced KLF6 to the development of fibrotic disease. It was interesting, however, that wt KLF6 rather than the Total KLF6 isforms was associated with the more advanced stages of fibrotic NAFLD. We hypothesized that, through promotion of fibrosis and inhibition of hepatocyte proliferation, wt KLF6 was involved in the pathogenesis of more advanced fibrotic liver disease. To test this hypothesis we investigated whether possession of the SNP promoting alternative splicing of *KLF6* to its dominant negative isoforms protected against the development of fibrotic disease in a large cohort of well characterized patients with NAFLD.

Functionality of the *KLF6 IVS1-27G>A SNP* was confirmed by demonstrating increased expression of alternatively spliced products in a liver cell environment and the subsequent attenuated up-regulation αSMA and collagen 1 in human HSC. Subsequently we performed both a classical allelic association study to determine if the frequency of the SNP differed according to the presence of more advanced fibrosis and an intra-familial allelic association study to look for preferential transmission of the wt allele to children with fibrotic NAFLD. In our first group of 306 biopsy–proven UK NAFLD patients, we demonstrated that presence of the *KLF6 IVS1-27G>A* SNP was associated with less fibrosis. Not only was this trend

confirmed in an independent group of Italian patients, analysis of the two groups combined identified the presence of wt KLF6 as a predictor of level 2 or more fibrosis independently of all other established risk factors of progressive disease. Clearly, using case control methodology to study disease progression is subject to the potential bias that some "controls" (fibrosis stage <2) could, with time, "cross-over" to become "cases". Given the small age difference between the cases and controls (6 yrs) relative to the slow and infrequent progression of early NAFLD[5] (5-25% at 14 years depending on presence of NASH), and the fact that KLF6 remained significantly associated with more advanced fibrosis in the logistic regression analysis that included age, we feel this is unlikely to have significantly affected our results. With longer follow-up of our cohort conducting a time-to-progression ("survival") type analysis should be possible in the future. In the family study we have shown preferential transmission of the wt allele to affected children. The size of Group 1 (n=306 is three times larger than any previous study of NAFLD genetic susceptibility), the replication in an independent cohort, the disproportionate transmission to affected children and the confirmed functionality of the SNP make it extremely unlikely that the association is a result of type 1 error or that the SNP is a marker of some other disease-causing SNP in the same haplotype block. We therefore conclude that the wt *KLF6* genotype is a significant susceptibility factor for fibrotic NAFLD, while *KLF6 IVS1-27G>A* protects against the development of fibrosis.

In the presence of inflammation and ongoing oxidative stress, we believe that increased wt KLF6 promotes HSC activation and fibrogenesis, as well as inhibiting hepatocyte growth. However, in the presence of *KLF6 IVS1 -27G>A*, the production of dominant negative splice variants of KLF6 in addition to the wt KLF6 isoform will

render wtKLF6 less active, delaying fibrogenesis, but also facilitating hepatocyte regeneration in the face of ongoing inflammation. It is interesting that a SNP in a tumour suppressor gene associated with rapid tumour growth and an increase in the incidence of familial prostate cancers is so common in the populations studied. Our observation that this SNP has beneficial effects in promoting cell survival in a non-cancer, chronic disease situation may offer an explanation. Whether individuals with *KLF6 IVS1-27G>A* who do get cirrhosis are at a greater risk of developing hepatocellular cancer remains to be determined. Further study of this interesting gene in other chronic hepatic and non-hepatic diseases and cancers will help to determine its increasingly recognised role in regulation of these processes.

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Figure 1. Increased wt KLF6 expression with advanced stages of NAFLD.

Either Total KLF6 mRNA isoforms or the wt isoform only have been quantified by semi-quantiative Taqman RT-PCR. The individual Brunt histology scores and KLF6 Relative Quantity (RQ) values are shown in Table 1, while mean expression levels (RQ log) +/- standard error within histologically defined grades of NAFLD are shown in Figure 1 (n=31; p values are shown). Panel 1 shows a stepwise increase in both Total KLF6 isoforms and wt KLF6 in association with increasing levels of inflammation. While wt KLF6 was also significantly elevated in association with advanced steatosis and fibrosis (panels 2 and 3), Total KLF6 isoforms were not.

Figure 2. *KLF6 IVS1-27G>A* promotes alternative splicing of KLF6 and abrogates the upregulation of αSMA and collagen 1. Cells were transfected with either transfection reagent alone (control), a control minigene expressing LacZ (LacZ), a KLF6 minigene with the IVS1-27G>A SNP (G>A), or a *KLF6* wt minigene construct (WT). Agarose gel electrophoresis of cDNA products amplified using 5' and 3' flanking primers detecting all KLF6 isoforms identified is shown (2A). KLF6 in the WT minigene transfected cells was predominently the full length wt isoform, while in the cells transfected with the KLF6 IVS1-27G>A minigene there were additional smaller bands representing alternative splice forms. Relative quantities of these isoforms were confirmed by semi-quantitative RT-PCR (n=3, data not shown). Figure 2B shows semi-quantitative RT-PCR data from RNA extracted from stably transfected LX2 cells harvested at 1 week. Total KLF6 was increased in cells transfected with either of the KLF6 minigene constructs, while only a relatively modest amount of this total KLF6 was the wt isoform in the G>A transfected cells. The marked increase in wt KLF6 in the LX2 cells transfected with the WT KLF6

minigene was associated with significant increases in both αSMA and Collagen I mRNA, as shown in 2C, which was not present in those cells transfected with the G>A SNP containing minigene expressing dominant negative KLF6 isoforms in addition to the wt isoform.

Table 1. KLF6 in NAFLD Liver biopsies

	Bunt score			RQ	RQ
	Fat	N/I	Fib	Total KLF6	WT KLF6
1	1	1	2	3.33	3.49
2	1	2	2	2.76	4.68
3	1	1	0	2.34	3.16
4	1	2	0	1.85	1.98
5	1	1	0	3.89	4.82
6	1	2	1	2.68	3.44
7	1	2	0	3.19	5.43
8*	1	2	0	3.04	3.38
9	1	1	0	2.53	4.33
10	1	3	3	3.38	2.23
11	1	0	2	2.42	2.83
12	1	0	0	0.87	1.66
13	1	0	0	0.87	1.36
14	1	0	0	4.38	1.20
15*	3	3	2	14.65	7.80
16*	3	1	1	1.31	1.25
17	2	0	0	1.55	2.02
18	3	0	0	5.68	8.15
19	2	1	0	6.78	1.52
20	1	0	0	1.11	1.61
21	1	1	1	5.74	2.57
22	3	0	0	1.90	3.15
23	1	1	2	1.07	2.72
24	3	1	2	1.56	3.60
25	3	1	2	1.76	5.53
26	2	1	1	0.92	3.14
27	2	1	1	11.53	2.40
28	2	1	3	2.25	4.35
29	2	1	1	38.67	1.10
30	2	1	2	0.98	2.14
31	1	1	1	1.08	2.35

** denotes individuals heterozygous for KLF6 IVS1-27G>A.* Fib - fibrosis; N/I - necroinflammation; RQ - relative quantity; WT - wild type.

Table 2. Clinical features of patients with NAFLD.

	ALL	Group 1	Group 2	p
Number (n).	415	306	109	
Country / Ethnicity	Caucasian	UK Caucasian	Italy Caucasian	
KLF6 IVS 27G>A	59(14.2%)	44(14.4%)	15(13.8%)	0.874
Sex (Male)	290 (66.8%)	193 (63%)	87 (81%)	0.001
Age	48.14±12.66	49.56±13.14	44.16±10.27	0.000
BMI	32.21±6.25	34.10±5.45	26.97±5.29	0.000
Hypertension	165/379 (43%)	134/272 (49%)	31/107 (29%)	0.000
Diabetes	124/406 (31%)	116/299(39%)	8/107(7.5%)	0.000
Platelets ($x10^9$/L)	235±72	239±79	217±63	0.010
Total Bilirubin (µmol/L)	13.3±10.1	12.44±10.2	16.01±8.90	0.000
AST (IU/L)	49±33	51±35	42±26	0.001
ALT(IU/L)	78±60	78±63	77±49	0.208
AST/ALT	0.73±0.36	0.78±0.4	0.59±0.19	0.000
gGT(IU/L)	116±167	125±188	91±88	0.016
Alkaline Phosph. (IU/L)	96±44	99±45	85±41	0.000
Fasting Gluc. (mmol/L)	6.3±2.9	6.67±3.26	5.35±1.19	0.000
Fasting Insulin (mU/L)	22.8±28.9	24.12±20.89	20.67±14.27	0.731
HOMA Score	6.32±2.92	6.51±6.3	5.00±3.99	0.259
Total Chol. (mmol/L)	5.51±1.32	5.61±1.37	5.24±1.15	0.018
HDL Chol. (mmol/L)	1.24±0.36	1.21±0.35	1.32±0.38	0.018
Triglycerides (mmol/L)	2.41±1.61	2.71±1.71	1.59±0.91	0.000
Fibrosis Score				0.263
0	156 (38%)	119 (39%)	37 (34%)	
1	75 (18%)	59(19%)	16 (15%)	
2	71 (17%)	49 (16%)	22 (20%)	
3	68 (16%)	43(14%)	25 (23%)	
4	45 (11%)	36 (12%)	9 (8%)	

The table shows the mean ± SD for continuous variables, number (%) for binary variables, and number (%) per group for categorical variables.

Table 3. The KLF6 genotype is associated with the fibrosis score in NAFLD patients

(i). Clinical and demographic variables in the combined groups

	KLF6 IVS1- 27G>A	**KLF6 WT**	***p***
Number	59	356	
Sex (M)	41(69.5%)	239 (67%)	0.721
Age	47.76 ± 12.04	48.21 ± 12.78	0.729
BMI (Kg/m^2)	32.28 ± 5.45	32.20 ± 6.38	0.718
Glucose (mmol/L)	5.95± 2.16	6.38±3.03	0.719
Insulin	27.5± 21.2	22.0± 18.2	0.127
Triglyceride (TG) (mmol/L)	2.37± 1.47	2.42± 1.64	0.719
Cholesterol (mmol/L)	5.63±1.54	5.49±1.28	0.804
HDL (mmol/L)	1.21±0.36	1.24±0.36	0.702
Total Bilirubin (µmol/L)	12.79± 8.07	13.36±10.38	0.545
Alanine transaminase (ALT)	77± 49	78± 61	0.520
Aspartate transaminase (AST)	53± 40	48± 32	0.656
AST/ALT ratio	0.71±0.33	0.73±0.37	0.624
Gamma glutamyl transferase (GGT)	96± 94	120± 177	0.668
Platelets x 10^9 L	239± 55	234± 74	0.680
Hyperten sion	27/53 (51%)	138/326 (42%)	0.241
Diabetes	19/58 (33%)	105/348 (30%)	0.693
Fat Score			0.432
1	20 (34%)	131 (38%)	
2	30 (52%)	119 (34%)	
3	8 (14%)	97 (28%)	
Inflammation	38/58 (65%)	232/348 (67%)	0.864
Fibrosis			**0.055**
0	24 (41%)	132 (37%)	
1	19 (32%)	56 (16%)	
2	7 (12%)	64 (18%)	
3	5 (8%)	63 (18%)	
4	4 (7%)	41 (11%)	
Fibrosis >2	16 (27%)	168 (47%)	**0.004**
HCC	1 (1.7%)	3 (0.84%)	0.535

(ii) The KLF6 genotype association in individual and combined groups

KLF6 IVS1-27 genotype	**Group 1 (n=306) Fibrosis score**		***p***	**Group 2 (n=109) Fibrosis score**		***p***	**Combined (n=415) Fibrosis score**		***p***
	0+1	**2+3+4**		**0+1**	**2+3+4**		**0+1**	**2+3+4**	
GA or AA	33 (75)	11 (25)	0.015	10 (67)	5 (33)	0.134	43 (73)	16 (27)	0.004
GG (WT)	145 (55)	117 (45)		43 (46)	51 (54)		188 (53)	168 (47)	

Table 4. KLF6 genotype is a predictor of fibrosis stage in patients with NAFLD

(i) Univariate Analyses of controls (fibrosis 0+1) versus cases (fibrosis 2+3+4)

Fibrosis	**0-1**	**2-3-4**	***p***
number	231	184	
Sex (M)	164(71.0%)	116 (63.0%)	0.086
Age	45.39 ± 12.25	51.61 ± 12.35	0.000
BMI	31.50 ± 6.46	33.15 ± 5.89	0.009
Blood glucose	5.85 ± 2.39	6.93 ± 3.40	0.000
Platelets x 10^9 L	253.45 ± 60.74	209 ± 77.95	0.000
AST/ALT Ratio	0.654 ± 0.25	0.813 ± 0.45	0.000
KLF-IVS1-27G>A	43 (18.60%)	16 (8.69%)	0.004

(ii) Binary Logistic Regression analysis presenting factors independently associated with advanced fibrosis.

Groups 1 & 2 (n=415)	**Odds Ratio (OR)**	**95% C.I.**	***p***
Age at biopsy	1.025	1.001-1.049	0.042
BMI	1.070	1.026-1.117	0.002
Blood glucose	1.119	1.022-1.224	0.015
Platelets x 10^9 L	0.992	0.988-0.996	0.000
AST/ALT Ratio	2.884	1.124-7.402	0.028
KLF wt	2.766	1.295-5.908	0.009

Figure 1

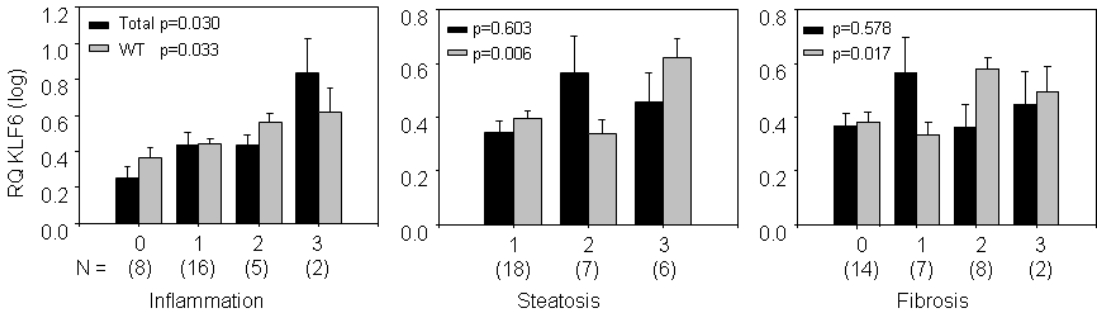

FIGURE 2