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Functional *FLT1* genetic variation is a prognostic factor for recurrence in stage I-III non-small cell lung cancer

Dylan M. Glubb, PhD[1,2], **Laia Paré-Brunet, PhD**[3], **Eloisa Jantus-Lewintre, PhD**[4], **Chen Jiang, PhD**[5], **Daniel Crona, PharmD**[1], **Amy S. Etheridge, BS**[1], **Osman Mirza, MS**[6], **Wei Zhang, PhD**[7], **Eric L. Seiser, PhD**[1], **Witold Rzyman, MD**[8], **Jacek Jassem, MD, PhD**[9], **Todd Auman, PhD**[1], **Fred R. Hirsch, MD, PhD**[10], **Kouros Owzar, PhD**[5], **Carlos Camps, MD, PhD**[11], **Rafal Dziadziuszko, MD, PhD**[9], and **Federico Innocenti, MD, PhD**[1,12]

[1] Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina, USA [2] Department of Genetics and Computational Biology, QIMR Berghofer Medical Research Institute, Brisbane, Australia [3]Department of Genetics, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain [4]Molecular Oncology Laboratory, General University Hospital Research Foundation, Valencia, Spain [5]Department of Biostatistics and Bioinformatics, Duke University Medical Center, Durham, North Carolina, USA [6]Department of Medicine, University of Chicago, Chicago, Illinois, USA [7]Department of Pediatrics, University of Illinois at Chicago, Chicago, Illinois, USA [8]Department of Thoracic Surgery, Medical University of Gdansk, Gdansk, Poland [9]Department of Oncology and Radiotherapy, Medical University of Gdansk, Gdansk, Poland [10]Department of Medicine, University of Colorado Cancer Center, Aurora, Colorado, USA [11]Department of Medicine, University of Valencia, Department of Oncology, General University Hospital of Valencia, Valencia, Spain [12]Lineberger Comprehensive Cancer Center, School of Medicine, University of North Carolina, Chapel Hill, North Carolina, USA

Abstract

Hypothesis—We propose that single-nucleotide polymorphisms (SNPs) in genes of the VEGF-pathway of angiogenesis will associate with survival in non-small cell lung cancer (NSCLC) patients.

Methods—Fifty-three SNPs in VEGF-pathway genes were genotyped in 150 European stage I-III NSCLC patients and tested for associations with patient survival. Replication was performed in an independent cohort of 142 European stage I-III patients. Reporter gene assays were used to assess the effects of SNPs on transcriptional activity.

Results—In the initial cohort, five SNPs associated ($q<0.05$) with relapse-free survival (RFS). The minor alleles of intronic *FLT1* SNPs, rs7996030 and rs9582036, associated with reduced RFS (HR=1.67 [95% CI, 1.22 to 2.29] and HR=1.51 [95% CI, 1.14 to 2.01], respectively) and reduced transcriptional activity. The minor alleles of intronic *KRAS* SNPs, rs12813551 and rs10505980, associated with increased RFS (HR=0.64 [0.46 to 0.87] and HR=0.64 [0.47 to 0.87], respectively)

Corresponding author: Federico Innocenti, MD, PhD University of North Carolina at Chapel Hill CB 7361, 120 Mason Farm Rd. Chapel Hill, NC 27599-7361 Tel: 919-966-9422 Fax: 919-966-5863 innocent@unc.edu.

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and the minor allelic variant of rs12813551 also reduced transcriptional activity. Lastly, the minor allele of the intronic *KRAS* SNP rs10842513 associated with reduced RFS (HR=1.65 [95% CI, 1.16 to 2.37]). Analysis of the functional variants suggests they are located in transcriptional enhancer elements. The negative effect of rs9582036 on RFS was confirmed in the replication cohort (HR=1.69 [0.99 to 2.89], p=0.028) and the association was significant in pooled analysis of both cohorts (HR=1.67 [1.21-2.30], p=0.0001).

Conclusions—The functional *FLT1* variant rs9582036 is a prognostic determinant of recurrence in stage I-III NSCLC. Its predictive value should be tested in the adjuvant setting of stage I-III NSCLC.

Keywords

non-small cell lung cancer; SNPs; VEGF-pathway; *FLT1*; enhancer

INTRODUCTION

Disease stage appears to be the most important prognostic factor in NSCLC patients, but disease recurrence is common even for patients with stage I-III disease[1]. Molecular biomarkers that identify patients who will develop recurrence and might benefit from adjuvant therapy are sorely needed[2]. Currently, there are no prognostic molecular markers or expression signatures in clinical use in resectable NSCLC[2].

The angiogenic potential of many cancers, including NSCLC, impacts their clinical course. Angiogenesis is an essential event in tumor growth, progression and metastasis formation, and is regulated by several angiogenic cytokines, mainly those of the VEGF-pathway[3-5]. In NSCLC, tumor vascularization and levels of VEGF-pathway proteins have been found to be associated with patient outcomes[6]. Trials testing bevacizumab in the adjuvant setting have been negative and an ongoing randomized study (ECOG 1505) might provide the definitive answer to the role of adjuvant bevacizumab.

As angiogenesis is a host-mediated process, germline genetic variation in the VEGF-pathway is likely to affect the angiogenic potential of a tumor[7, 8]. A small number of single-nucleotide polymorphisms (SNPs) in VEGF-pathway genes have been tested for association with outcomes in NSCLC and other solid tumors (reviewed in[9-11]), but there is little information about the functional significance of germline variation in genes of the VEGF-pathway. Thus, there is no mechanistic basis to support many of these associations, in part explaining the inconsistent results from these studies[11]. Without prospective validation of findings already built in at the time of the initial discovery, the demonstration of the clinical validity of biomarkers is a lengthy and difficult process.

The aim of this study was to identify germline variants in the VEGF-pathway genes that associate with NSCLC survival and may act as markers of angiogenic-dependent tumor recurrence. NSCLC patients were genotyped for candidate VEGF-pathway SNPs to test associations with patient survival. To aid the interpretation of these associations, the molecular function of SNPs was characterized by examining their effects on transcriptional activity in reporter gene assays. Finally, the SNPs found to associate with survival were also

prospectively tested in a validation cohort of European stage I-III NSCLC patients to provide an independent assessment of our findings.

METHODS

Ethics statement

Tissue banking and related research was approved by institutional review boards at the Medical University of Gdansk, Poland and the General University Hospital of Valencia, Spain. All patients signed an informed consent.

Initial patient cohort

The initial cohort consisted of 150 White European stage I-III NSCLC patients who underwent pulmonary resection. They belonged to a cohort of unselected patients systematically diagnosed with resectable NSCLC with tumor samples collected at the Medical University of Gdansk, Poland. Median follow-up of the study group was 63.4 (range, 13.1 to 82.3) months. Relapse-free survival (RFS) was defined from the date of surgery to the date of local or distant relapse, death of any cause, or last follow-up. Overall survival (OS) was defined from the date of surgery to the date of death of any cause or last follow-up. Relapse of disease was assessed by chest radiograms or CT scans every 3 months for the first two years and every 6-12 months thereafter. The patient characteristics are described in Table 1. Adjuvant therapy was given to only 4.6% of patients[12]. Postoperative chemotherapy was not routinely administered in the analyzed period per institutional guidelines. Primary tumors were fresh frozen at the time of surgery. All patients signed an informed consent. Further details of the cohort were described previously[12].

SNP selection and genotyping in the initial cohort

In the initial patient cohort, 53 SNPs (minor allele frequency (MAF) >5%) in 13 candidate genes (identified from the PharmGKB VEGF signaling pathway web resource: www.pharmgkb.org/pathway/PA2032#tabview=tab1&subtab=) were selected using several approaches: SNPs associated with mRNA expression in lymphoblastoid cell lines (LCL); SNPs identified from our prior study[13]; SNPs in *HIF1A* and *FLT1* (not expressed in LCLs) predicted to be functional by FastSNP and FuncPred; non-synonymous SNPs predicted to change protein structure according to FastSNP; SNPs identified from previous association studies. Detailed information on the SNPs according to these criteria is provided (see table, Supplemental Digital Content 1, which identifies VEGF-pathway gene SNPs genotyped in the initial cohort).

Genomic DNA prepared from fresh-frozen patient tumor samples (AllPrep DNA/RNA kit, Qiagen, Germantown, MD) was used for genotyping. Five *KDR* SNPs had been genotyped as described previously[14]. The remaining SNPs were genotyped by TaqMan® (Applied Biosystems, Foster City, CA) per the manufacturer's instructions using a CFX384 Real-Time System (Bio-Rad, Hercules, CA), and Sanger-based DNA sequencing (Mammalian Genotyping Core at UNC) was used to validate representative samples and determine thresholds for allelic discrimination. SNP allele frequencies were comparable to those previously reported from the HapMap and 1,000 Genomes projects (see table, Supplemental

Digital Content 1, which identifies VEGF-pathway gene SNPs genotyped in the initial cohort). For rs9582036 in *FLT1* and rs10505980 in *KRAS*, additional quality control of the genotyping was performed using the genotype calls in the tumor DNA and matching germline DNA in squamous NSCLCs from The Cancer Genome Atlas (see table, Supplemental Digital Content 2, which illustrates *FLT1* rs9582036 and *KRAS* rs10505980 genotype calls from squamous NSCLC genotyped samples from The Cancer Genome Atlas). Linkage disequilibrium (LD) was analyzed using the SHEsis application. No SNPs deviated from Hardy-Weinberg equilibrium (HWE) after controlling for multiple testing using a false discovery rate (FDR) at $q<0.05$ (see table, Supplemental Digital Content 1, which identifies VEGF-pathway gene SNPs genotyped in the initial cohort).

Reporter gene assays

To support the interpretation of the associations between SNPs and survival, we analyzed the molecular effects of five SNPs which passed a FDR threshold ($q<0.05$) for association with RFS in the initial cohort. Luciferase reporter assays can test the transcriptional effects of genetic variants in potential regulatory genetic regions. When the functional effects of SNPs are unknown (as for the five SNPs associated with RFS in this study), these assays are critical to provide the mechanistic basis of the clinical associations[15]. The pGL4.26 (Promega, Madison, WI) plasmid with minimal promoter and *Firefly* luciferase gene was used and the cloning approach is described in Supplemental Digital Content 3. Three DNA clones of each reporter gene construct were prepared for transfection into murine endothelial (SVEC4-10, kind gift from Mark Lingen at the University of Chicago) and human embryonic kidney (HEK-293) cells. Cell culture conditions are described in Supplemental Digital Content 3. Cells were transfected using lipofectamine 2000 (Invitrogen, Carlsbad, CA), the reporter gene construct of interest and *Renilla* TK plasmid (Promega, Madison, WI). Each construct was transfected in three independent experiments, using triplicate wells. The Dual-Luciferase Reporter Assay kit (Promega, Madison, WI) was used to measure luciferase activity as per the manufacturer's instructions. Luciferase activity was defined as a ratio of *Firefly* to *Renilla* luciferase and was normalized to the luciferase activity of the wild-type construct in each experiment.

Validation patient cohort

We tested prospectively whether any of the five SNPs passing the FDR for association with RFS in the initial cohort (Table 2) would associate with RFS in an independent, external validation cohort. Hence, these five SNPs were genotyped in DNA extracted from fresh frozen tumor samples of 142 White European stage I-III NSCLC patients from Spain[16]. A CONSORT chart is provided (see figure, Supplemental Digital Content 4, which shows the CONSORT chart). Clinical and demographic variables of the patients are provided in Table 1. Patients were systematically diagnosed with operable, histologically confirmed NSCLC at the General University Hospital of Valencia, Spain. Median follow-up of the study group was 37.3 months (95% CI, 29.3 to 43.5). Recurrence of disease was assessed by chest CT scan every 3 months for the first two years and every 6-12 months thereafter, using the same criteria of the initial cohort. Further details of the cohort were described previously[16].

SNP genotyping methods in the validation cohort

Patient tumor specimens were obtained at the time of the surgery and preserved in RNAlater® (Applied Biosystems, Carlsbad, CA) at −80°C until the analysis. Nucleic acid isolation and genotyping using TaqMan SNP assays are described in Supplemental Digital Content 3.

Statistical analyses

Survival was estimated according to the Kaplan-Meier method. Patient characteristics were tested for association with survival using a Mantel-Cox log-rank test to identify potential prognostic factors. The genetic associations with survival in each cohort were then adjusted for these factors, and the independence of the genetic associations was tested using additive Cox proportional hazards models. Since we propose that functional VEGF-pathway SNPs impact tumor angiogenesis and growth, we have chosen RFS as the primary endpoint as it relates to tumor growth or the appearance of new lesions[17] and, compared to OS, is less likely to be affected by events (including treatment) occurring after recurrence. In the initial cohort, FDR was applied to correct for multiple testing (a $q<0.05$ was considered significant), genotypes were coded additively, and associations were adjusted for stage, the only variable associated with RFS in this cohort (Table 1). In the validation cohort, identical statistical methods to those of the initial cohort were used; however, a one-sided test was used because we hypothesized we would observe the same directionality of association in the validation cohort. Moreover, because the mode of heritance for these SNPs in the initial cohort was always dominant, a dominant model was used to test the associations in the validation cohort. Moreover, in the validation cohort, the associations were adjusted for histology, the only variable associated with RFS in this cohort (Table 1). In the combined cohort test, a one-sided test and a dominant model were used, and the associations were not adjusted.

Differences in reporter assays were analyzed by Student's t-test or ANOVA followed by Dunnett's multiple comparisons test in GraphPad Prism ($p<0.05$ for significance). All other statistical analyses were carried out using the R Statistical environment along with extension packages.

RESULTS

Five SNPs associate with RFS in the initial NSCLC cohort

Out of the 53 SNPs genotyped in 150 European stage I-III NSCLC patients, five SNPs (two in *FLT1* and three in *KRAS*) associated with RFS and passed the FDR threshold for significance ($q<0.05$, Table 2). Disease stage was incorporated into these models because it significantly associated with RFS and OS ($p<0.0001$, Table 1).

The minor alleles of the intronic *FLT1* SNPs rs7996030 and rs9582036 associated with reduced RFS (HR=1.67 [95% CI 1.22 to 2.29] and HR=1.51 [95% CI 1.14 to 2.01], respectively); for rs7996030 in a dominant model, the median RFS of patients with GA+AA genotypes was 2.1 fold shorter than that of GG patients (HR=2.13 [1.38 to 3.28], p=0.0006, Fig. 1A); for rs9582036 in a dominant model (in modest LD with rs79906030, $r^2=0.44$), the

median RFS of patients with CA+CC genotypes was 2.9 fold shorter than that of AA patients (HR=1.77 [1.18 to 2.67], p=0.006, Fig. 1B).

The minor alleles of the intronic *KRAS* SNPs rs12813551 and rs10505980 associated with increased RFS (HR=0.64 [0.46 to 0.87] and HR=0.64 [0.47 to 0.87], respectively); for rs12813551 in a dominant model, the median RFS of patients with TC+CC genotypes was 4.3 fold longer than that of TT patients (HR=0.49 [0.32 to 0.74], p=0.0008, Fig. 1C); for rs10505980 in a dominant model (in high LD with rs12813551, r^2=0.83), the median RFS of patients with GA+AA genotypes was 3.7 fold longer than that of GG patients (HR=0.49 [0.33 to 0.75], p=0.0008).

The minor allele of the intronic *KRAS* SNP rs1084251was associated with reduced RFS (HR=1.65 [1.16 to 2.37]), and in a dominant model the median RFS of patients with CT+TT genotypes was 3.8 fold shorter than that of CC patients (HR=1.97 [1.22 to 3.18], p=0.006, Fig. 1D). An additional seven SNPs nominally associated with RFS (p<0.05) but did not pass the FDR threshold (Table 3).

As a secondary analysis, associations with OS were similarly tested. Nine SNPs nominally associated with OS (p<0.05, see table, Supplemental Digital Content 5, which identifies initial cohort nominal associations of SNPs with OS (p<0.05) in Cox regression models adjusted for stage), but none passed the FDR threshold. However, all five SNPs passing FDR for RFS (Table 2) had an effect on OS concordant with that on RFS (p<0.02 for these SNPs).

Three SNPs demonstrate functional effects on reporter gene expression

To provide a mechanistic explanation for the genetic associations with RFS, SNPs were assessed for regulatory activity using luciferase reporter gene assays. Genomic regions containing the SNPs were cloned downstream of the luciferase gene in a construct containing a minimal promoter and assays were performed to determine the effects of the SNPs on the transcriptional activity of the minimal promoter. An intronic region containing the minor allele of the *FLT1* SNP rs9582036 or rs7996030 (associated with reduced RFS) reduced transcriptional activity in an endothelial cell line (SVEC) by 33% (p<0.0001) and 23% (p<0.0001), respectively, when compared to a reference construct containing the major allelic variants (Fig. 2A). The effect on transcriptional activity was even more pronounced when both minor alleles were tested in the same construct: activity was reduced up to 45% (p<0.0001; Fig. 2A). Neither SNP had a significant effect on transcriptional activity in HEK-293 cells (Fig. 2B), suggesting that the effects of the variants may be cell or tissue type specific, as is characteristic of transcriptional enhancers. Moreover, analysis of ENCODE functional genomic data indicated that the *FLT1* region examined contains a putative enhancer element, coincident with rs7996030 and possibly extending to rs9582036 (see Supplemental Digital Content 6A, which illustrates evidence of putative transcriptional enhancer elements at *FLT1* loci).

For the *KRAS* SNPs rs12813551 and rs10505980, an intronic region containing the minor alleles of both variants (associated with increased RFS) reduced transcriptional activity in SVEC cells by 18% (p=0.014; Fig. 2C). Neither SNP had a significant effect by itself in

SVEC cells, though the minor allele of rs12813551 reduced transcriptional activity by 15% (p=0.051). In HEK-293 cells, the minor allele of rs12813551 reduced transcriptional activity by 19% (p<0.001), but neither the minor allele of rs10505980 nor the construct containing the minor alleles of both SNPs had a significant effect (Fig. 2D). Analysis of ENCODE data suggests that rs12813551 is coincident with a putative transcriptional enhancer while rs10505980 is ~1.5 kb downstream of this element (see Supplemental Digital Content 6B, which illustrates evidence of putative transcriptional enhancer elements at *KRAS* loci).

An intronic region containing the minor allele of the *KRAS* SNP rs10842513 (associated with reduced RFS) demonstrated no effect in SVEC cells (Fig. 2E) and only a marginal increase (9%, p=0.054) in transcriptional activity in HEK-293 cells (Fig. 2F). ENCODE data did not provide evidence for substantive transcriptional enhancer activity at this SNP locus (see Supplemental Digital Content 6C, which illustrates lack of evidence of putative transcriptional enhancer elements at *KRAS* rs10842513 locus).

The association of rs9582036 and RFS replicates in validation cohort

We prospectively tested whether any of the five SNPs passing the FDR for the association with RFS in the initial cohort (Table 2) associated with RFS in an independent, external validation cohort of 142 European stage I-III NSCLC patients (Table 1)[16]. Out of the five SNPs, only rs9582036 in *FLT1* had a significant and concordant effect (Table 2): the RFS of patients with the AC+CC genotypes was shorter than that of AA patients (HR=1.57 [0.93 to 2.66], p=0.045, Fig. 3A). When this association was adjusted for histology (the only parameter associated with RFS, Table 1), the HR was 1.69 [0.99 to 2.89], p=0.028. The adjustment for stage (as in the initial cohort) did not improve this association (HR=1.57 [95% CI 0.92 to 2.66], p=0.049).

In a pooled analysis of both cohorts (n=292), the HR of rs9582036 for RFS (adjusted for stage, using a dominant model) was 1.67 (95%CI 1.21 to 2.30), with a marked separation between the two survival curves (p=0.0001, Fig. 3B); without adjusting for stage, the HR was 1.81 [1.32 to 2.49], p=0.0001).

DISCUSSION

The VEGF-pathway genetic studies of NSCLC outcome have focused so far on a small number of genes and genetic variants, in particular *VEGFA*[9]. We rationally selected 53 SNPs from 13 VEGF-pathway genes and identified five SNPs in *FLT1* and *KRAS* that associated with RFS after correction for multiple testing. We subsequently validated the association of the *FLT1* SNP rs9582036 with RFS in an independent cohort of stage I-III European NSCLC patients. Molecular studies of germline SNPs associated with patient outcome are essential to guide the assessment of their clinical utility[15] and we found functional evidence to support the clinical associations of the two *FLT1* SNPs (rs7996030 and rs9582036) and one of the *KRAS* SNPs (rs12813551). Furthermore, all three functional variants were found to be located in or adjacent to putative transcriptional enhancer elements. The finding that the *FLT1* SNPs had an additive effect on gene expression is consistent with a recent proposal that multiple enhancer variants cooperatively act to modestly alter gene expression and account for genetic associations with disease[18].

The most important result of this study is that the variant allele (C) of the *FLT1* SNP rs9582036 is associated with shorter RFS in both the initial and the validation cohorts (Table 2, Fig. 1 and Fig. 3). rs9582036 is a common germline variant (frequency of about 30% in Caucasians) and this association is supported by the finding that this variant is functional. A putative transcriptional enhancer element is located immediately adjacent to rs9582036 and possibly extends into the locus of this SNP in lung tissue (see Supplemental Digital Content 6A, which illustrates evidence of putative transcriptional enhancer elements at *FLT1* loci). The minor allele of rs9582036 reduces transcriptional activity and, thus, suggests that this variant may act through this element to reduce *FLT1* expression. Intriguingly, the effect of rs9582036 was only observed in the SVEC cell line, indicating that the effect may be specific to endothelial cells and that this variant may have effects on angiogenesis in vivo.

There is evidence to suggest that the clinical association and the molecular effect of rs9582036 reconcile with the biology of *FLT1*. *FLT1* encodes the VEGF receptor 1 (VEGFR-1), a mediator of tumor endothelial function[19]. Its level of phosphorylation in response to VEGFA is low[20, 21], and its soluble form is expressed by endothelial tumor cells, sequestering VEGFA through the formation of a complex[22]. Soluble VEGFR-1 also inhibits VEGFA in a dominant manner by heterodimerizing with the ligand binding region of VEGFR-2[23], the most potent receptor of VEGFA which mediates most of the pro-angiogenic effects of VEGFA. Enhanced in vivo expression of soluble VEGFR-1 by tumor cells inhibits solid tumor growth, impedes metastatic nodule development, and extends host survival[19]. In addition to its soluble form, upregulation of membrane-anchored VEGFR-1 in endothelial cells contributes to the readjustment of the tumor endothelial phenotype in response to increased oxygen supply and vessel normalization[24]. These lines of evidence are strongly suggestive of *FLT1* being a negative regulator of neovascularization (see review[19]). Hence, collectively, these findings fit a mechanistic model where reduced endothelial expression of VEGFR-1, mediated by rs9582036, could accelerate NSCLC recurrence through increased angiogenesis.

Based upon the results of this study, we hypothesize that carriers of the minor allele of rs9582036 *FLT1* variant may be less responsive to angiogenesis inhibition, as a result of a potentiated angiogenic tumor phenotype. Indeed, rs9582036 is predictive of shorter survival in bevacizumab-treated patients with metastatic pancreatic and renal-cell carcinoma[25]. The negative predictive effect is also observed in other nonrandomized studies of anti-VEGF therapies: bevacizumab (colorectal[26]) and sunitinib (renal cell[27]). Because results from different tumor types might not be necessarily extended to the setting of stage I-III NSCLC, more molecular and cellular studies in ex vivo angiogenesis models are needed to dissect the resulting effect of *FLT1* genetic variation on both the basal NSCLC endothelial phenotypes and response to VEGF blockade.

A major question is whether there is now sufficient evidence to regard rs9582036 as a validated prognostic marker of recurrence in stage I-III NSCLC. RFS was the primary endpoint in our study, and for a genetic association that relates to the biology of the tumor, RFS should be robust to confounders which could not be accounted for. We acknowledge that the demonstration of a prognostic role of rs9582036 in *FLT1* for cancer-related OS is probably warranted before this novel marker could be used to inform treatment decisions in

stage I-III NSCLC patients. In this setting, interventions after recurrence should be taken into account to avoid the confounding related to imbalances in active therapies. The lack of recommendations on the use of adjuvant chemotherapy in higher-risk NSCLC following surgery in the cancer institute treating study patients may also represent a limitation of the present study.

Our study did not replicate the effect of three *KRAS* SNPs on RFS (Table 2). These findings may have been false positives or the effects of the SNPs could have been confounded by hidden heterogeneity between the two cohorts. The patients in the validation cohort were not enrolled to match the characteristics of the initial cohort; therefore intrinsic patient differences across the two cohorts might hamper the detection of true positive associations. Furthermore, only rs12813551 demonstrated functionality and its effects were modest in the reporter gene assays. It may be that these SNPs are in linkage disequilibrium with the causal variants underlying the clinical associations and, thus, fine mapping studies may help clarify these associations. Nonetheless, the *KRAS* SNP rs12813551 should still be tested in other studies as its molecular effect is consistent with the clinical association and the oncogenic biology of *KRAS* in NSCLC. Moreover, a negative prognostic impact of increased *KRAS* expression in operable NSCLC has been previously demonstrated[28] and the minor allele of rs12813551, associated with increased RFS, had a negative effect on transcriptional activity.

In summary, we identified several associations between VEGF-pathway SNPs and NSCLC outcome, and provided biological interpretations through molecular studies. A prospective validation study has selected rs9582036 in *FLT1* as a germline variant associated with poor prognosis in stage I-III NSCLC. These results provide the foundation for testing the prognostic and predictive value of functional VEGF-pathway SNPs in NSCLC patients administered chemotherapy and anti-VEGF therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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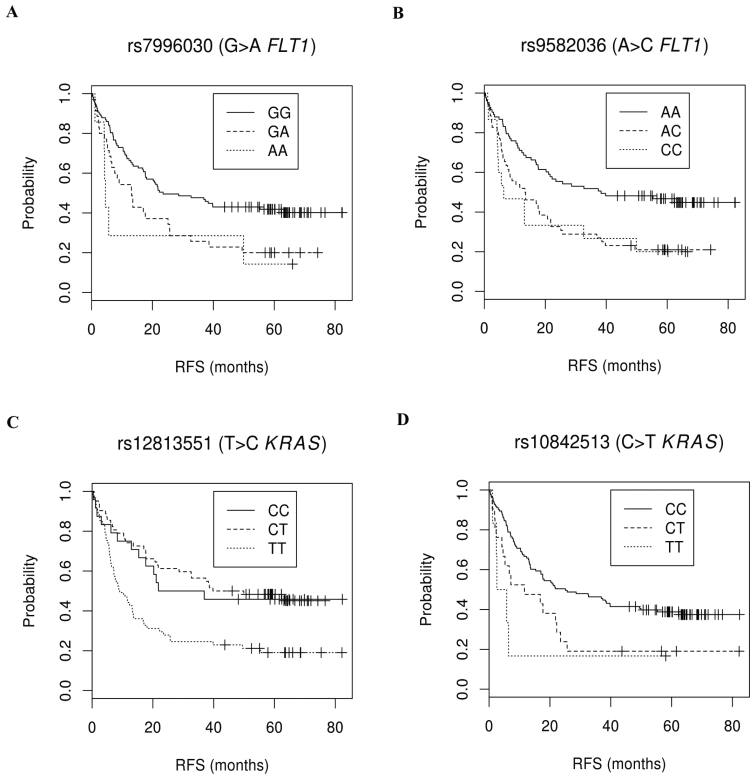

Fig. 1. Kaplan-Meier plots of significant SNP-RFS associations after false discovery rate (FDR) correction (q<0.05) in initial cohort

Symbols denote censored points. (A) rs7996030: median RFS is 11.1 (95% CI, 5.8 to 25.7) and 23.5 (17.7-NR) months for patients with GA+AA and GG genotypes. (B) rs9582036: median RFS is 13.1 (7.2 to 20.7) and 38.3 (21.1 to NR) months for patients with CC+AC and AA genotypes, respectively. (C) rs12813551: this plot is also representative of rs10505980 due to the high LD with rs12813551. Median RFS is 39.2 (21.8 to NR) and 9.1 (7.0 to 16.8) for patients with CC+TC and TT genotypes, respectively. (D) rs10842513: median RFS is 6.6 (4.6 to 22.2) and 25.1 (17.1 to 49.9) months for patients with CC+TC and TT genotypes, respectively. While these results are for associations adjusted for stage, the plots are for unadjusted data.

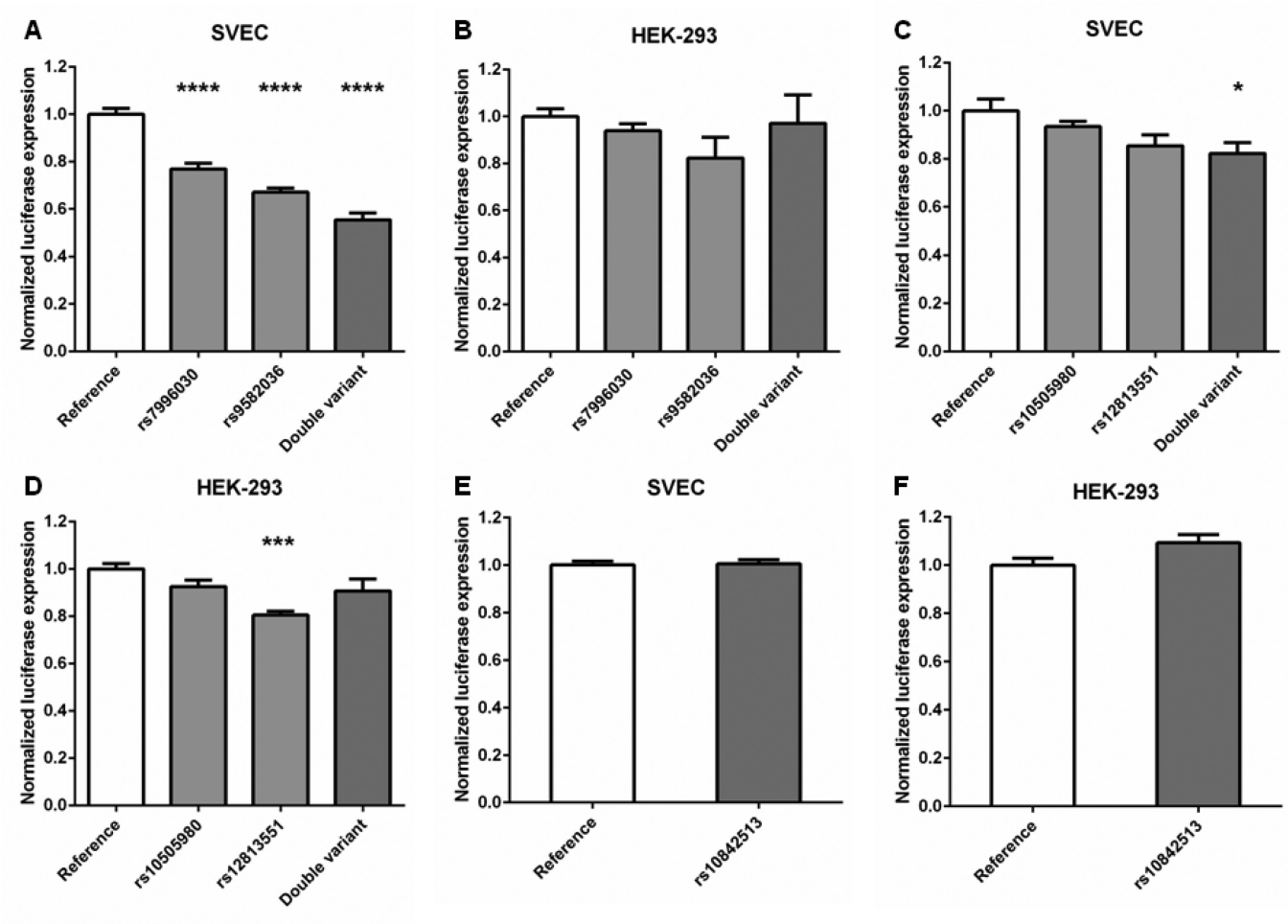

Fig. 2. *FLT1* and *KRAS* SNPs effect transcriptional activity in reporter gene assays

Relative luciferase activity of *FLT1* SNPs in SVEC (A) and HEK-293 (B) cell lines, and *KRAS* SNPs in SVEC (C, E) and HEK-293 (D, F) cell lines. Values were normalized to those of the reference sequences containing the major allelic variants of the SNPs of interest. Significance was tested by Student's t-test or ANOVA followed by Dunnett's multiple comparisons test in GraphPad Prism (for significance: *p<0.05; ***p<0.001; ****p<0.0001)

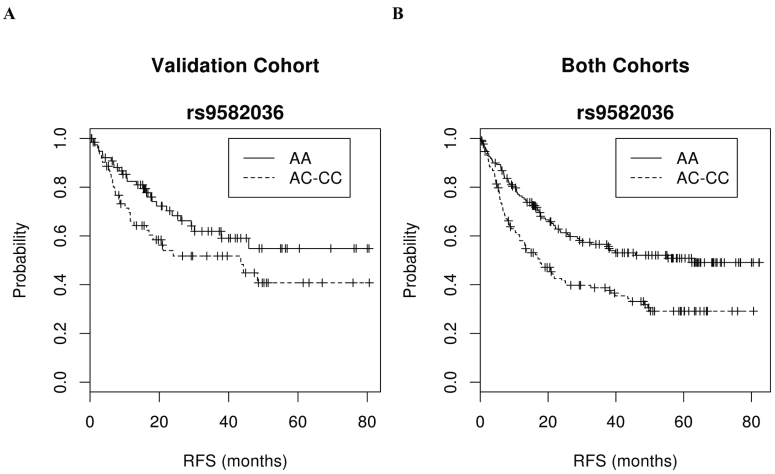

Fig 3. Kaplan-Meier plots of the association between the rs9582036 SNP in *FLT1* and RFS in the validation cohort (A) and in the combined initial and validation cohorts (B)

Symbols denote censored points. The plots are for unadjusted data.

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Table 1

Initial and validation cohorts: patient characteristics, relaspe-free survival (RFS) and overall survival (OS) (log-rank test).

	Initial cohort					Validation cohort				
	n (%)	**Median OS months (95% CI)**	**p value**	**Median RFS months (95% CI)**	**p value**	**n (%)**	**Median OS months (95% CI)**	**p value**	**Median RFS months (95% CI)**	**p value**
Sex			0.08		0.14			0.26		0.26
Female	37 (25)	62 (15.4-NR[#])		49 (13.7-NR)		20 (14)	NR (NR-NR)		NR (NR-NR)	
Male	113 (75)	23 (17.5-39.8)		18 (13.1-25.7)		122 (86)	67 (30.9-NR)		44.3 (26.2-NR)	
Disease stage			<0.0001		<0.0001			0.64		0.30
I	64 (43)	NR (48.6-NR)		62 (36.9-NR)		78 (55)	NR (32.3-NR)		48.3 (43.5-NR)	
II	33 (22)	37 (20.3-NR)		22 (16.8-NR)		35 (25)	42.9 (29.8-NR)		26.2 (16.3-NR)	
III	53 (35)	10 (7.8-13.3)		8 (6.0-11.0)		29 (20)	NR (17.2-NR)		NR (11.2-NR)	
Histology			0.31		0.42			0.03		0.04
Squamous	88 (59)	25 (16.2-41.6)		17 (11.6-36.9)		67 (47)	NR (42.6-NR)		48.3 (26.2-NR)	
Adenocarcinoma	42 (28)	61 (20.9-NR)		36 (17.7-NR)		55 (39)	67 (42.9-NR)		NR (37.9-NR)	
Others[*]	20 (13)	15 (9.1-NR)		15 (7.2-NR)		20 (14)	17.1 (12.9-NR)		11.2 (7.3-NR)	
Ever Smokers			0.96		0.69			0.40		0.61
No	7 (5)	21 (7.16-NR)		18 (3.4-NR)		19 (13)	NR (NR-NR)		NR (17.9-NR)	
Yes	143 (95)	28 (17.8-48.6)		21(13.5-36.9)		123 (87)	67 (32.3-NR)		45.8(29.2-NR)	
Age (years)			0.13		0.33			0.30		0.30
<65	78 (52)	36 (21.3-NR)		22 (13.7-62.4)		74 (52)	NR (42.6-NR)		NR (43.5-NR)	
>65 Median (range)	72 (48)	21 (12.6-48.6) 64 (37-85)		18 (8.4-38.6)		68 (48)	53.3 (27.9-NR) 64 (26-82)		31.9 (21.1-NR)	

In the validation cohort, adjuvant therapy was administered to 56 patients and was not associated with either RFS or OS (results not shown).

[#]NR, Not reached

[*]Initial cohort = Not otherwise specified, Validation cohort = 6 LCC, 4 ADL-SCC, 2 carcinoid, 8 not otherwise specified.

Table 2

Initial and validation cohorts: associations of SNPs with relapse-free survival (RFS) (Cox regression).

			Initial Cohort				Validation Cohort		
SNP	**Alleles**	**Gene**	**HR**	**95% CI**	**P value**	**Q value**	**HR**	**95% CI**	**P value**
rs7996030	G>A	*FLT1*	1.67	1.22 to 2.29	0.0014	0.0490	1.37	0.78 to 2.42	0.1382
rs9582036	A>C	*FLT1*	1.51	1.14 to 2.01	0.0044	0.0490	1.69	0.99 to 2.89	0.0275
rs10505980	G>A	*KRAS*	0.64	0.47 to 0.87	0.0049	0.0490	1.17	0.68 to 2.01	0.2890
rs12813551	T>C	*KRAS*	0.64	0.46 to 0.87	0.0052	0.0490	0.91	0.53 to 1.58	0.3698
rs10842513	C>T	*KRAS*	1.65	1.16 to 2.37	0.0060	0.0490	1.09	0.49 to 2.44	0.4136

In the initial cohort, data are adjusted for stage and pass the false discovery rate (FDR) correction ($q<0.05$) for multiple testing in additive genetic models. In the validation cohort, data are adjusted for histology, associations are for dominant genetic models (because all five associations in the initial cohort had a dominant model), and the p values are for a one-sided test, as the associations were tested prospectively on the basis of the results obtained from the initial cohort. SNPs are ranked by p value of SNP-RFS associations observed in the initial cohort.

Table 3

Initial cohort: nominal associations of SNPs with relapse-free survival (RFS, $p<0.05$) in Cox regression models adjusted for stage.

SNP	Alleles	Gene	HR	95% CI	P value	Q value
rs4246229	A>G	*KRAS*	0.71	0.54 to 0.94	0.0168	0.1120
rs34176876	A>-	*KRAS*	0.72	0.55 to 0.95	0.0191	0.1120
rs1570360	G>A	*VEGFA*	1.39	1.03 to 1.87	0.0290	0.1428
rs1951795	C>A	*HIF1A*	1.47	1.03 to 2.11	0.0341	0.1428
rs11549465	C>T	*HIF1A*	1.62	1.02 to 2.57	0.0392	0.1428
rs542403	A>G	*FRS2*	0.66	0.44 to 0.98	0.0404	0.1428
rs2076139	C>T	*MAPK11*	0.70	0.49 to 0.99	0.0417	0.1428

SNPs are ranked by significance of SNP-RFS associations. The associations with $p>0.05$ are available upon request.