A SNP in the *flt-1* promoter integrates the VEGF system into the p53 transcriptional network

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The VEGF system is essential for angiogenesis. VEGF overexpression frequently correlates with increased microvasculature and metastasis and decreased spontaneous apoptosis. Although a precise mechanism has not been established, studies suggest that VEGF expression is negatively regulated by p53, a master regulator and tumor suppressor. There are no reports of additional components of the VEGF signal transduction pathway being part of the p53 transcriptional network. A target of VEGF, the VEGF receptor 1/*flt-1*, can regulate growth and migration of endothelial cells and modulate angiogenesis. VEGF appears to be up-regulated in various cancers in which *flt-1* may have a role in tumor progression and metastasis. We identified a C-to-T SNP upstream of the transcriptional start site in ~6% of the people examined. The SNP is located within a putative p53 response element. Only the promoter with the T SNP (FLT1-T) was responsive to p53 when examined with reporter assays or by endogenous gene expression analysis in cell lines with different SNP status. In response to doxorubicin-induced DNA damage, there was clear allele discrimination based on p53 binding at the FLT1-T but not FLT1-C promoters as well as p53-dependent induction of *flt-1* mRNA, which required the presence of FLT1-T. Our results establish that p53 can differentially stimulate transcription at a polymorphic variant of the *flt-1* promoter and directly places the VEGF system in the p53 stress-response network via *flt-1* in a significant fraction of the human population. We suggest that the p53-VEGF-*flt-1* interaction is relevant to risks in angiogenesis-associated diseases, including cancer.

single-nucleotide polymorphism | genotoxic stress | VEGF receptor 1 | cancer cells

VEGF is a multifunctional cytokine that is pivotal to blood vessel formation, including angiogenesis and vasculogenesis (1), and is expressed in a variety of cells (2), including smooth muscle, endothelial, and epithelial cells, monocytes/macrophages, T lymphocytes, and polymorphonuclear neutrophils. Alternative splicing of the human VEGF gene results in at least four isoforms, of which VEGF₁₆₅ is the most prevalent. VEGF exerts its biological effects through two high-affinity receptors: Fms-like tyrosine kinase 1 (*flt-1*) receptor 1 (VEGFR-1) and VEGF receptor 2 (VEGFR-2/FLK-1/KDR) (1). These VEGF receptors are found at the surface of endothelial cells, hematopoietic stem cells, leukocytes, osteoblasts, and many tumor cells (3).

FLK-1 is considered the principal receptor of VEGF-dependent angiogenic signals, with an important role in normal vessel growth (1) and hematopoiesis, as well as other functions (4). The precise function of *flt-1* is still under debate (5). Park *et al.* (6) initially proposed that *flt-1* might not be primarily a receptor transmitting a mitogenic signal, but rather a “decoy” receptor, able to negatively regulate VEGF activity on the vascular endothelium by preventing VEGF binding to VEGFR-2. In addition, *flt-1* is implicated in up-regulation of tissue factor, urokinase-type plasminogen activator, and plasminogen activator inhibitor 1 (7) in endothelial cells. However, direct proliferative, migratory, or cytoskeletal effects

mediated by *flt-1* receptor remain to be demonstrated (9). In other cell types *flt-1* has additional and different roles, such as inducing tissue factor and chemotaxis in monocytes and enhancing matrix metalloproteinase expression in vascular smooth muscle (10, 11). Recent evidence indicates that *flt-1* is not only involved in lung-specific metastasis by matrix metalloproteinase 9 induction (12), but is also involved in ligand-induced pathological angiogenesis in tumor cells (13).

Flt-1^{-/-} mice die at embryonic day 8.5 (14). The endothelial cells form normally but fail to assemble correctly into organized blood vessels, apparently as a consequence of increased commitment of mesenchymal cells to becoming hemangioblasts, the common precursors of both blood and endothelial cells (15).

Levels of *flt-1* expression can vary considerably across tissues (e.g., lung, kidney, heart, liver, and brain) and in response to stimuli (e.g., hypoxia) (16). Given the association of *flt-1* function with cell growth, development, and various pathologies (see *Discussion*), variations in expression are expected to contribute to its cellular impact. In this work we searched for possible genetic sources of variation in *flt-1* expression focusing on 1,196 bp of the *flt-1* proximal promoter. A SNP was found in ~6% of the population sampled. Surprisingly, the SNP occurred in a putative p53 response element (RE) sequence, with the less frequent SNP resulting in an RE predicted to be more p53-responsive (17). We characterized the influence of the SNP on promoter activity by reporter gene assays as well as endogenous *flt-1* gene expression in cell lines heterozygous for the SNP. Analysis of transactivation, promoter occupancy, and responses to genotoxic stress show that only one, the FLT1-T allele, was p53-responsive. Thus, the VEGF system is directly placed in the p53 stress-response transcriptional network via *flt-1* in a significant fraction of the human population. For those individuals with the uncommon polymorphic promoter variant, p53-mediated stress responses could markedly influence the role of *flt-1* in various biological functions.

Results

Identification of a SNP in the *flt-1* Promoter. We used a combination of molecular analysis, custom bioinformatics applications, and *in*

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Abbreviations: ChIP, chromatin immunoprecipitation; HMEC, human microvascular endothelial cell; RE, response element; RFLP, restriction fragment length polymorphism; SSCP, single-strand conformation polymorphism; TSS, transcription start site; wtp53, wild-type p53.

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Fig. 1. Identification of a SNP in the *flt-1* promoter region. (A) SSCP analysis of a 200-bp *flt-1* promoter fragment (positions 465–665; GenBank accession no. D640169) from healthy volunteers (lanes 1–8). An extra band is seen in lane 2. Sequence analysis revealed a single heterozygous C/T nucleotide polymorphism (C519T) located 677 bases upstream of the TSS (position 519, GenBank accession no. D64016). Bioinformatic analysis predicted that this SNP falls within a potential p53 RE. (B) RFLP analysis of HMEC, MCF-7, and HCT116 cells revealed that only HCT116 cells harbor a heterozygous C/T allele, whereas HMEC and MCF-7 cells are homozygous for the C allele. The T allele SNP results in an NSP1 site that enables the T (+) and C (–) to be distinguished. A DNA sample from an individual with heterozygous C/T alleles was used as a positive control. (C) Schematic representation of the putative p53 consensus RE containing the C/T SNP in the promoter sequence of the *FLT-1* gene. The asterisk indicates the location of SNP c519t, and the underlined region corresponds to the spacer sequence. The second half site contains three mismatches from the consensus (lowercase letters).

putative RE for the tumor suppressor gene and transcription factor p53 (p53RE; Fig. 1C). The p53 consensus sequence is RRRCW-WGYYYnRRRCWWGYYY, where R is purine, Y is pyrimidine, W is C or T, and *n* is a spacer of 0–13 nt between the two half sites (18). The SNP is located at the second W (C/T) of the first half site in the sequence GGACA(c/T)GCTccctgGGACcTgagC, where the two half sites are separated by 5 bases (ccctg). The infrequent T variant would result in a good match to the first consensus half site and might increase responsiveness to wild-type p53 (wt-p53). However, the second half site contains three mismatches from consensus (lowercase letters) and would be expected to interact only weakly with p53.

Fig. 4. The endogenous FLT1-T allele in HCT116 (FLT1-C/T) is specifically induced by doxorubicin via p53. (A) Western blots of p53 from HCT116 p53^{+/+} and p53^{-/-} cell lines that are heterozygous for the FLT1-C/T alleles after 24 h of doxorubicin treatment (0.3 μ g/ml). Expression is normalized to β -actin levels. (B) *flt-1* mRNA is regulated by p53 in HCT116 cells harboring a heterozygous C/T allele. Shown are the relative *flt-1* mRNA expression levels as analyzed by real-time PCR analyses after 24 h of doxorubicin treatment (0.3 μ g/ml). Expression is normalized to GAPDH levels. p53-dependent *flt-1* mRNA expression was com-

pared in HCT116 p53^{+/+} and HCT116 p53^{-/-} cells. The mean of the control gene expression level was set as 1. Each bar represents the mean of six independent experiments $\pm$ SD. (C) After exposure to doxorubicin, ChIP was performed on extracts with antibodies against p53. Total lysate was used as a control for PCR amplification (input). PCR was performed with gene-specific primers. p53 binding was shown to be much greater to the T allele than to the C allele in the promoters of the endogenous genes. There was no *flt-1* or p21 DNA recovered by ChIP from HCT116 p53^{-/-} cells. Presented are the averages and standard deviations for three independent experiments. (D) RFLP analysis of the PCR products from the ChIP experiment involving p53^{+/+} cells in C. Presented as controls (first three columns) are restriction analyses of PCR products of the FLT1-T and FLT1-C reporter constructs with and without treatment by NspI as well as PCR products of the endogenous *flt-1* promoter region (HCT116). +, NspI restriction enzyme.

contrast, in p53-positive breast cancer MCF-7 (homozygous FLT1-C/C) cell line even transient p53 overexpression failed to induce *flt-1* mRNA expression (Fig. 5A). To establish that differences between the HCT116 cell lines in terms of ability to transactivate the FLT1-T vs. FLT1-C alleles were strictly due to differences in p53 status, cells were exposed to doxorubicin after transfection with the FLT1-T and FLT1-C luciferase reporter plasmids. Only the FLT1-T plasmid exhibited expression, and this was restricted to the p53^{+/+} cell line (Fig. 6A, which is published as supporting information on the PNAS web site). Furthermore, transfection of p53 plasmid into the p53-null cell line also resulted in strong expression of the FLT1-T, but not the FLT1-C luciferase reporter (Fig. 6B), demonstrating that this cell line has the potential to be induced by p53 from the FLT1-T allele.

We conclude that there is strong endogenous FLT1-C/T allele discrimination by damage-induced as well as overexpressed p53.

Discussion

Variation in targeted p53 RE sequences can alter transcriptional control and may contribute to transactivation specificity in response to stress signals, which in turn could result in biological diversity within a population. Reliable identification of cis-acting promoter genetic variants that can affect gene regulation is still a challenge in genomics because of the plasticity of promoter architecture or the difficulty to predict which regulatory SNP can affect recruitment of transcription factors to REs (17, 19–22). Such regulatory genetic variants can be expected to act as low-penetrance modifiers of disease risk, and their identification is important especially for the molecular characterization of complex traits (19). Ultimately, the transcriptional consequences of the variants need to be assessed.

Here we report a functionally distinct genetic variation in the promoter of the *flt-1* gene in humans. The infrequent FLT1-T variant results in the inclusion of this gene into the network coordinated by the master transcription regulator p53, a tumor suppressor gene that is highly responsive to a variety of stress conditions.

Having observed significant variation in *flt-1* expression between cell types and individuals, we investigated underlying genetic sources as contributors to expression differences. Published information on genetic variants (dbSNP, Human Gene Mutation Database, Human Genic Bi-Allelic Sequences, Ensembl) revealed only one SNP in the promoter region. However, that SNP (rs17086745: G/C, chr 13: 27967272) was not confirmed by subsequent genotyping (23). Therefore, we searched for allelic variants in the *flt-1* promoter and 5' UTR region by SSCP analysis. Subsequent DNA sequence analysis revealed a C $\rightarrow$ T SNP 677 bases upstream of the TSS. The frequency of C/T heterozygous individuals was 6%.

Our results establish that only the *flt-1* promoter with the T-SNP is responsive to p53 and can recruit p53 proteins based on ChIP assays, which was determined by using both transient transfection assays and analysis of the endogenous *flt-1* gene in cell lines that are

heterozygous for the newly identified regulatory SNP. The use of various human cell lines with differences in p53 status, together with the ectopic expression of functionally distinct p53 alleles or with the activation of p53 by using different genotoxic stresses, allowed us to confirm the specific responsiveness of the FLT1-T promoter, but not FLT1-C, to p53.

In particular, we took advantage of the HCT116 cell line derived from colon cancer, which is heterozygous for the FLT1-T SNP. The availability of a p53-null variant obtained by gene targeting (24) provided a close-to-isogenic control cell line to assess the role of p53 on *flt-1* expression under controlled conditions. In HCT116, we compared responses from the endogenous FLT1-C/T alleles and from transfected FLT1-T or FLT1-C promoter fragments. There was allele discrimination in both cases either by p53 stabilized through doxorubicin treatment or by ectopic p53 overexpression. ChIP analysis followed by *flt-1* promoter amplification and RFLP established that p53 bound specifically to the FLT1-T allele.

Our results demonstrate the potential for wtp53 to stimulate transcription at the *flt-1* promoter that depends on an infrequent SNP that generates a consensus half-site RE within an overall weak p53 RE. These results are consistent with our recent genome-wide search of functionally distinct p53 REs that was based on an experimentally derived sequence and structure definition of what constitutes a p53-responsive RE and on predictions of functional changes caused by SNPs in REs (17). We had identified nearly 200 polymorphic p53 REs in novel putative p53 targets that were predicted to be functionally distinct and directly tested six of these. Those six could be induced by genotoxic stress or could be activated directly by transfection with p53 cDNA. The present report broadens the general conclusions from that study on interindividual variation in the p53 transcriptional network. These and other cis-acting polymorphisms in REs (25) represent an important class of genetic variation that has implications for disease such as cancer. Interestingly, sequences like the *flt-1* RE that contain a long spacer between half sites and multiple mismatches were excluded from our previous genome-scale search because of their predicted overall weak activity. The present results with the FLT1-T SNP clearly indicate that this class of REs with a well matched half site can be responsive to p53.

p53 is a prominent tumor suppressor gene that coordinates cellular responses to stress, primarily acting as a sequence-specific transcription factor. Besides genotoxic stresses, other cellular perturbations are known to activate p53, including hypoxia. The long and growing list of established p53 direct transactivation targets include genes involved in angiogenesis such as matrix metalloproteinase 2, PAI-1, and BAI (26–28).

The inclusion of the *flt-1* gene and, therefore, the VEGF pathway into the p53 transcriptional network, but only in a fraction of the human population, adds complexity to the spectrum of possible biological outcomes of p53-mediated responses to genotoxic damage, including chemotherapy. Indeed, *flt-1* overexpression has been associated with pathological angiogenesis, tumor progression, cell survival, proliferation, migration, invasion, and metastasis (1, 29–34). Although we have established a direct interaction between p53 and the FLT1-T allele, an assessment of the biological consequences of the newly identified SNP in the *flt-1* promoter is beyond the scope of our study. Cell culture and animal model studies will be required to determine the impact of the SNP and p53 activation on *flt-1*-induced biological responses. At present, genotype/phenotype relationships in human cells are limited by the lack of isogenic cell line models that allow direct interpretation of the biological consequences SNP alleles (17).

There is a general agreement that FLK-1 is the major mediator of the mitogenic and angiogenic effects of VEGF during transformation and tumorigenesis (4). However, recent reports demonstrate that *flt-1* is present and functional on different human cancer cells and that activation of *flt-1* by VEGF can activate processes involved in tumor progression and metastasis (12, 33, 35). Thus, we

suggest that the FLT1-SNP allele may be a significant risk factor in the development of angiogenesis-driven diseases. The present findings on differences in p53-responsiveness to the FLT1-T and FLT1-C alleles should be considered in epidemiological studies that address related health issues such as cancer susceptibility and therapy, especially given the prominent role of p53 in cancer.

Materials and Methods

Use of Human Materials. This investigation conforms with the principles outlined in the Declaration of Helsinki. DNA was obtained from 150 Caucasian individuals and different endothelial (HMEC), bone-related (SaOS2 and U2OS), colon (HCT15, HCT116, HT29, LoVo, RKO, SW1417, and SW480), breast (BT20, BT474, Cal51, Cama1, H184, HCC1397, TD47D, ZR751, and MCF-7), and lung (CaLU1) tumor cell lines.

DNA Extraction and Screening of the FLT-1 Promoter for Genetic Variations. Information about genetic variations was obtained by database search in the National Center for Biotechnology Information's dbSNP (36), Human Gene Mutation Database, or Human Genic Bi-Allelic Sequences or was experimentally determined by SSCP analysis.

SSCP analysis and RFLP analysis were performed by using standard methods (see *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site).

In Silico Sequence Analysis for Potential cis Elements. Promoter sequences with either C (FLT1-C) or T (FLT1-T) allele at position –912 (i.e., 912 nt upstream of the TSS) in the *FLT-1* promoter were analyzed for potential cis elements by using MATINSPECTOR analysis software and TRANSPLORER with the database TRANSFAC (37) (version 3.5, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany).

Cloning of the Two Polymorphic Variants of the FLT-1 Promoter from Genomic DNA. A 1,196-nt fragment of the *FLT-1* promoter upstream of TSS was PCR-amplified by using the following primers: 5'-GCGCACGCGTGTGGCAACTTTGGGTTACCCAAC-3' and 5'-GCGCAAGCTTTGTGAGAAGCAGACAGCTGAGCA-3'. The two primers contain, respectively, HindIII and MluI restriction sites at their 5' ends. PCR products were purified, digested with HindIII and MluI, and cloned into the luciferase reporter vector pGL3basic (Promega). The identity of the inserts was confirmed by DNA sequencing (ABI PRISM 377, Applied Biosystems).

Transcriptional Regulation of FLT-1. To examine the putative functional significance of the SNP within the *FLT-1* promoter, we compared the activity of promoter fragments of the two SNP alleles cloned in luciferase reporter vectors in different tumor cell lines. The activity of the predicted p53 RE in the FLT1-T promoter was characterized by cotransfections by using the plasmids pCMV-WTp53 or pCMV-(Q331stop)-p53, which express the human wtp53 cDNA or the truncation mutant Q331Stop, respectively, and the control vector pCMV-Neo (38).

Characterization of FLT1 Promoter Activity by Luciferase Assays in Transient Transfection Experiments. For each cell line $1\text{--}2.5 \times 10^5$ cells were seeded in 12-well plates 24 h before transfection. Cells were transfected by using FuGENE 6 (Boehringer Mannheim) according to manufacturer's instructions at $\approx 80\%$ confluence [with 350 ng of FLT-1 promoter-luciferase constructs or the control plasmid pGL3-basic (Promega)]. When appropriate, 0.5 μg of the p53 expression and control plasmids were cotransfected. Cotransfection with 0.5 μg of pSV- β -galactosidase control vector (Promega) was carried out to standardize for transfection efficiency in MCF-7 and HMEC cells, and 150 ng of *Renilla reniformis* luciferase pSRLV40 plasmid (Promega) was used as normalizing control in

SaOS2 and HCT116 cells. Total plasmid DNA per well was adjusted to an equal level by adding the empty vector pCMV-Neo. For doxorubicin treatments, medium was removed 30 h after transfection, and fresh medium was added in the presence of the indicated dose of the drug. In all experiments cells were harvested 48 h after transfection by using the reporter lysis buffer (Promega). We analyzed firefly and *Renilla* luciferase or β -galactosidase activity at room temperature using a Lumat LB 9501 (Berthold Technologies) or a Victor Wallac (PerkinElmer) multilabel plate reader according to the manufacturers' instructions using the Dual-Luciferase Reporter Assay System (Promega) and/or Galacto-Light (Applied Biosystems). For each construct, relative luciferase activity is defined as the mean value of the firefly luciferase/ β -galactosidase or firefly luciferase/*Renilla* luciferase ratios obtained from three independent experiments. A two-tailed *t* test was performed to determine statistical significance.

Transcription Analysis of the Endogenous *flt-1* Gene. TaqMan real-time PCR analysis was used to study induction of endogenous *flt-1* mRNA expression in HCT116 p53^{+/+} and the HCT116 p53^{-/-} cell lines, which are heterozygous for the SNP in the *flt-1* promoter. The MCF-7 p53^{+/+} cells which are homozygous C/C in *flt-1* were used as a negative control for p53-responsiveness. Briefly, the comparative CT method was used to determine the ratio of target to β -actin endogenous control. Total cellular RNA was isolated from transfected cells by using TRIzol reagent (Invitrogen). For RT-PCR analysis, first-strand cDNA was synthesized as described in ref. 39.

TaqMan analysis was carried out according to the manufacturer's instructions, with the use of an Applied Biosystems 7700 system (PerkinElmer). Amplification conditions and primer sequences are described in *Supporting Materials and Methods* and in Table 2, which is published as supporting information on the PNAS web site. Specificity of the *flt-1* RT-PCR products was confirmed by sequencing using an automated sequencing device (ABI PRISM 377, Applied Biosystems). Alternatively, primers on demand (Applied Biosystems) for *flt-1* (Hs00176573), P21 (Hs00355782), and GAPDH (Hs99999908) as endogenous housekeeping gene were also used, and PCR was assessed as described in ref. 17.

Western Blot Analysis. Cell extracts were adjusted to equal protein levels (determined in triplicate according to Bradford with BSA as

a reference standard), resolved by 4–10% BisTris NuPage, and transferred to polyvinylidene difluoride membranes (Invitrogen) with a semidry electroblotter (Owl Separation Systems). Membranes were probed with monoclonal antibodies specific for p53 (pAb1801 and DO-1, Santa Cruz Biotechnology). The quality as well as the equal loading and transfer of protein blots were determined by Ponceau S staining using a monoclonal antibody against β -actin (Sigma). The M_r values of the immunoreactive bands were determined by using molecular weight markers. After washing, blots were incubated with anti-mouse IgG-conjugated peroxidase antibody (Santa Cruz Biotechnology), and immune complexes were visualized by using ECL reagent (Amersham Pharmacia).

ChIP Assay. ChIP assays were performed with an established protocol and minor modifications (40) by using 1 μ g of mouse monoclonal anti-p53 antibody DO7 (Pharmingen). Amplification, primers, and PCR conditions are described in *Supporting Materials and Methods*.

Cell Culture Conditions. The human MCF-7 cells were cultured in DMEM (Biochrom) supplemented with 100 units/ml penicillin plus 100 μ g/ml streptomycin (Biochrom), 10% estrogen-deficient FCS (Biochrom), and 2 mmol/liter L-glutamine (Biochrom). HMEC were cultured according to our recent protocol (8). Human osteosarcoma p53-null cell line SaOS2 was maintained in DMEM with 10% standard FBS and antibiotics. HCT116 p53^{+/+} and its p53^{-/-} derivative (gifts from B. Vogelstein, The Johns Hopkins University, Baltimore), as well as the human osteosarcoma U2OS cell line and lung adenocarcinoma CaLu1 cell lines, were grown in McCoy's A5 medium supplemented with 10% FBS and antibiotics. All cells were incubated at 37°C with 5% CO₂.

Statistical Analysis. Data analysis was performed by using SPSS 11.0 for Windows (SPSS) and SIGMAPLOT 2002 8.0 for Windows (Systat). Values are given as means $\pm$ SD if not otherwise indicated.

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