Deletion of Chromosome 9p21 Noncoding Cardiovascular Risk Interval in Mice Alters Smad2 Signaling and Promotes Vascular Aneurysm

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Background—Vascular aneurysm is an abnormal local dilatation of an artery that can lead to vessel rupture and sudden death. The only treatment involves surgical or endovascular repair or exclusion. There is currently no approved medical therapy for this condition. Recent data established a strong association between genetic variants in the 9p21 chromosomal region in humans and the presence of cardiovascular diseases, including aneurysms. However, the mechanisms linking this 9p21 DNA variant to cardiovascular risk are still unknown.

Methods and Results—Here, we show that deletion of the orthologous 70-kb noncoding interval on mouse chromosome 4 (chr4\(\Delta 70kb/\Delta 70kb\)) mice is associated with reduced aortic expression of cyclin-dependent kinase inhibitor genes p19Arf and p15Inkb. Vascular smooth muscle cells from chr4\(\Delta 70kb/\Delta 70kb\) mice show reduced transforming growth factor-β–dependent canonical Smad2 signaling but increased cyclin-dependent kinase–dependent Smad2 phosphorylation at linker sites, a phenotype previously associated with tumor growth and consistent with the mechanistic link between reduced canonical transforming growth factor-β signaling and susceptibility to vascular diseases. We also show that targeted deletion of the 9p21 risk interval promotes susceptibility to aneurysm development and rupture when mice are subjected to a validated model of aneurysm formation. The vascular disease of chr4\(\Delta 70kb/\Delta 70kb\) mice is prevented by treatment with a cyclin-dependent kinase inhibitor.

Conclusions—The results establish a direct mechanistic link between 9p21 noncoding risk interval and susceptibility to aneurysm and may have important implications for the understanding and treatment of vascular diseases.


Key Words: aneurysm ■ genetics ■ models, animal ■ myocytes, smooth muscle

Abdominal aortic aneurysm is the most common form of vascular aneurysm in humans. It is an age-associated disease that affects ≈8% of men aged >65 years and is responsible for a significant number of deaths in Western countries. Other important risk factors include smoking and low high-density lipoprotein cholesterol levels. Although abdominal aortic aneurysm is frequently associated with atherosclerosis, the bulk of evidence suggests distinct pathophysiological determinants. The major features include alterations in vascular smooth muscle cell (VSMC) proliferation, survival, migration and phenotype, extracellular matrix degradation, neoangiogenesis, and the presence of inflammatory infiltrates within the vascular media and adventitia. Despite this knowledge, the only treatment involves surgical or endovascular repair or exclusion. There is currently no approved specific medical therapy for this condition, which highlights the urgent need for a better understanding of the determinants of the disease.

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Intriguingly, several recent reports identified strong association between genetic variants, particularly 9p21 DNA variants and the occurrence of vascular aneurysms in humans (independent of the reported associations with coronary heart disease). Interestingly, this association holds true for different forms of aneurysms, including abdominal aortic aneurysm and intracranial aneurysms. These observations highly suggest common pathogenic mechanisms for apparently distinct forms of aneurysms.

Here, we took advantage of the recent availability of a mouse with a targeted deletion of the 9p21 noncoding cardiovascular risk interval on mouse chromosome 4 (chr4\(\Delta 70kb/\Delta 70kb\)) mice is associated with reduced aortic expression of cyclin-dependent kinase inhibitor genes p19Arf and p15Inkb. Vascular smooth muscle cells from chr4\(\Delta 70kb/\Delta 70kb\) mice show reduced transforming growth factor-β–dependent canonical Smad2 signaling but increased cyclin-dependent kinase–dependent Smad2 phosphorylation at linker sites, a phenotype previously associated with tumor growth and consistent with the mechanistic link between reduced canonical transforming growth factor-β signaling and susceptibility to vascular diseases. We also show that targeted deletion of the 9p21 risk interval promotes susceptibility to aneurysm development and rupture when mice are subjected to a validated model of aneurysm formation. The vascular disease of chr4\(\Delta 70kb/\Delta 70kb\) mice is prevented by treatment with a cyclin-dependent kinase inhibitor.

Conclusions—The results establish a direct mechanistic link between 9p21 noncoding risk interval and susceptibility to aneurysm and may have important implications for the understanding and treatment of vascular diseases.


Key Words: aneurysm ■ genetics ■ models, animal ■ myocytes, smooth muscle

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Methods

Aneurysm Model

chr4Δ70kb+ mice were obtained from Jackson Laboratories. We used littermate mice between 8 and 12 weeks of age. Angiotensin (Ang)-II was infused via subcutaneous osmotic pumps at 1000 ng/kg per minute for a maximum of 28 days.8 Anti–transforming growth factor (TGF)-β antibody (clone 2G7) was administered every 3 days at 10 mg/kg as previously described.10,11 Systolic blood pressure was measured weekly in all mice using a computerized tail-cuff system (BP2000 Visitech Systems). Aneurysm severity (stages I–IV) was evaluated as previously described.10,12 Stage IV was attributed to rupture. In some experiments, a group of mice was treated intraperitoneally with the cyclin-dependent kinase (CDK) inhibitor flavopiridol (5 mg/kg per day; 5 days/week) for 28 days.

For bone marrow transplantation experiments, C57Bl/6 mice, which bear the same major histocompatibility class-II as 129S6 mice, were lethally irradiated (9.5 Gy) and repopulated with bone marrow (107 cells) from chr4Δ70kb/Δ70kb or chr4+/- littermate mice. After 4 weeks of recovery, mice were subjected to AngII and anti–TGF-β treatment.

Experiments were performed under French Ministry of Agriculture permit No. 02934. The study was also approved by the Home Office, PPL 80/2426, United Kingdom.

Characterization of Aneurysmal Lesions

Paraffin-embedded aortic sections were used. Elastin staining was visualized using orcein, and the number of degraded elastin lamellae was quantified by a researcher blinded to the experimental protocol. VSMCs were stained using anti–α-smooth muscle actin (clone 1A4; Sigma-Aldrich), and T lymphocytes using anti-CD3 antibody (Dako). The percentage of cellular area with positive staining was quantified using Image J. VSMCs were stained using anti–transforming growth factor–β antibody (clone 2G7) was administered every 3 days at 10 mg/kg for 28 days.300 nmol/L with 3H-thymidine. 3H-thymidine incorporation was quantified by a researcher blinded to the experimental protocol.

Proliferation Assay

Smooth muscle cells were isolated from the aorta (ascending arch, descending thoracic, and suprarenal) of chr4Δ70kb or chr4+/- littermate mice as previously described.15 Cells were plated at 2.5×104 cells per well in Roswell Park Memorial Institute medium, 1% antibiotics, and 20% fetal bovine serum at passage 3. Cells were incubated with or without flavopiridol for 24 hours at 300 nmol/L, with 3H-thymidine. 3H-thymidine incorporation was quantified as previously described.16

Western Blotting

VSMCs were isolated from the aorta of chr4Δ70kb/Δ70kb or chr4+/- littermate mice as previously described15 and cultured in RPMI, 1% antibiotics, and 20% FBS. Experiments were performed on cells up to passage 5. Subconfluent cells were incubated overnight in serum-free media containing 2% BSA (Sigma-Aldrich) before treatment with or without flavopiridol (300 nmol/L for 60 minutes; Sigma-Aldrich) followed by TGF-β1 (10 ng/mL for 15 minutes; R&D Systems). Cells were washed in PBS and lysed in lysis buffer (20 mmol/L Tris-HCL, pH 8.0, 5 mmol/L magnesium chloride, 10 mmol/L EGTA, 1% TritonX100, 10 mmol/L sodium orthovanadate, 250 mmol/L sodium fluoride, and protease inhibitor cocktail [Roche]). Lysates containing equal amounts of protein were fractionated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. After blocking with Odyssey blocking buffer (Licor), membranes were incubated with antibodies against Smad2 S465/467 or Smad1 S245/250/255 (Cell Signaling) and GAPDH (Millipore) at 4°C overnight. After washing, membranes were incubated with secondary antibodies (Licor) for 1 hour at room temperature. Bands were visualized and quantified using the Odyssey infrared imaging system (Licor).

Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction was performed on a Roche Light Cycler 480 II in triplicate. CT for Gapdh (R: 5′-CGTCCCGTAGACAAATGTGAA-3′; F: 5′-GCCGTGAGTGTAGTCATACTGGAACA-3′) was used to normalize gene expression. The following primer sequences were used for p19 (R: 5′-CGGGGAGAAGTTAGTGGG; F: 5′-GCCGCCGCTTCGGCCAGTTC TTGTA-3′), p15 (R: 5′-GGTTGGCCAGGTGCGCCAGT-3′; F: 5′-ACGGTGGCGAGCAGCTCCTGGA-3′), Cmm1 (R: CCGGTGACGTGACCCTCATC; F: GCACATCTTTGAGGCCAACGA), Myocd (R: GCCCATTCCTACTGCTGTCACT; F: CACGGCAAAAGGTCTCTC), Acta2 (R: GCCCGTAGACATGACGTT; F: TCGGACCTACATGTGACTCTGTAG), and Mmp12 (R: GGGTTTCACTGGGGCTCCATA; F: GCCATGCTTTTTATCCTGACCT).

Statistics

Values are expressed as percentage or mean±SEM when appropriate. Statistical tests included ANOVA, unpaired t test, χ2 test for a trend, and Fisher exact test where appropriate. Kaplan–Meier survival curves were constructed and analyzed using log-rank (Mantel–Cox) test. P values of <0.05 were considered significant.

Results

Increased Susceptibility of chr4Δ70kb/Δ70kb Mice to Aneurysm

We subjected chr4Δ70kb/Δ70kb mice8 to a validated model of aortic aneurysm formation and rupture that involves the administration of AngII with a neutralizing anti–TGF-β antibody for a duration of 28 days.10 chr4Δ70kb/Δ70kb mice are on 129S6 genetic background that is highly resistant to AngII-induced abdominal aortic aneurysm. However, despite the resistant genetic background and the development of an appropriate blood pressure response to AngII infusion (Figure 1A), we found that chr4Δ70kb/Δ70kb mice were highly susceptible to aneurysm formation and more frequently developed severe aneurysms (increasing severity from stage 0 indicating no aneurysm to stage IV, indicating fatal aortic rupture) compared with control mice (Figure 1B). Interestingly, there seemed to be a genetic–dose effect as chr4Δ70kb+ mice showed intermediate susceptibility compared with chr4Δ70kb/Δ70kb and chr4+/- littermates (Figure 1B). We next examined the impact of selective deletion of the noncoding risk interval in bone marrow–derived cells on aneurysm formation and rupture using bone marrow transplantation (see Methods section of this article). We found that deletion of the noncoding risk interval in bone marrow cells did not aggravate the development/severity of aortic aneurysm (Figure 1C and 1D), suggesting a predominant role for chr4Δ70kb/Δ70kb deletion in vascular cells on disease susceptibility.

Reduced Canonical Smad2 Signaling in Aortic SMCs of chr4Δ70kb/Δ70kb Mice

We then addressed potential molecular mechanisms linking the noncoding risk interval to aneurysm formation. We reasoned that an abnormal response to TGF-β in aortic SMCs of chr4Δ70kb/Δ70kb mice might predispose them to aneurysm.10,17 We therefore assessed the induction of SMC differentiation markers by TGF-β.18 Interestingly, induction of α-actin (Acta2) and calponin (Cmm1) gene expression by TGF-β was
significantly blunted in aortic SMCs of chr4Δ70kb/Δ70kb mice compared with controls (Figure 2A and 2B). Interestingly however, myocardin (Myocd) expression tended to follow a different pattern (Figure 2C). Given the differential involvement of canonical Smad2/3 signaling in the induction of SMC markers, the results strongly suggested abnormal Smad2 signaling in aortic SMC of chr4Δ70kb/Δ70kb mice in response to TGF-β. This was confirmed by the marked reduction of canonical TGF-β–dependent Smad2 tail S465/467 phosphorylation in VSMCs recovered from aortas of chr4Δ70kb/Δ70kb mice compared with controls (Figure 2D).

### Increased Noncanonical CDK-Dependent Linker Smad2 Phosphorylation in Aortic SMCs of chr4Δ70kb/Δ70kb Mice

In contrast to canonical tail phosphorylation, noncanonical Smad2/3 phosphorylation has been associated with reduced Smad transcriptional activity and antiproliferative function. This occurs through CDK-dependent phosphorylation of Smad2/3 at linker sites, which represses Smad transcriptional activity, limits canonical TGF-β–dependent Smad2/3 phosphorylation, and promotes resistance to TGF-β–mediated growth inhibition in cancer.

The 9p21 risk variants have been associated with reduced vascular expression of CDK inhibitors, which has been linked, at least in part, to modulation by ANRIL (antisense noncoding RNA in the INK4 locus). Although Anril is absent in mice, the orthologous region on mouse chromosome 4 includes the exon structure of an equivalent noncoding transcript of unknown function, AK148321, and synteny with neighboring genes Cdkn2a, Cdkn2b, Mtap, and Dmrta1 is preserved. Therefore, we examined potential alterations in gene expression of Cdkn2a (encoding p16Ink4a and p19Arf in mice) and Cdkn2b (encoding p15Inkb) at the various vascular...
We were not able to detect p16Ink4a expression in the aorta even in wild-type animals (data not shown). Interestingly, the expression of p19Arf and p15Inkb was significantly reduced in aortic arches and suprarenal aortas of chr4\(^{Δ70kb/Δ70kb}\) and chr4\(^{Δ70kb/+}\) mice compared with chr4\(^{+/+}\) littermate controls (Figure 3A). Reduced vascular expression of CDK inhibitors in chr4\(^{Δ70kb/Δ70kb}\) mice suggests that increased CDK activity might alter Smad phosphorylation at linker sites. Remarkably, we found increased TGF-β–independent phosphorylation of Smad2 at linker S245/250/255 sites (Figure 3B) in VSMCs recovered from aortas of chr4\(^{Δ70kb/Δ70kb}\) mice (KO) compared with chr4\(^{+/+}\) controls (WT), with or without pre-incubation with flavopiridol (Flavo). Representative blots are given for 2 experiments performed on 2 separate cultures of VSMCs. In B the graph shows the average normalized band intensities (phosphorylated Smad normalized to GAPDH) from 4 separate experiments, for which \(P=0.002\) (2-way ANOVA). C Proliferation of smooth muscle cells (SMC) recovered from the aortas of chr4\(^{+/+}\) (WT, \(n=3\)) and chr4\(^{Δ70kb/Δ70kb}\) (KO, \(n=3\)) mice and incubated with or without flavopiridol (Flavo). \(P<0.05\) vs Flavo, Student t test.

Finally, to test the hypothesis that increased CDK activity is involved in the susceptibility of chr4\(^{Δ70kb/Δ70kb}\) mice to aneurysm formation, we treated a group of chr4\(^{Δ70kb/Δ70kb}\) mice with flavopiridol. Remarkably, flavopiridol treatment significantly reduced the incidence and severity of aortic aneurysms in chr4\(^{Δ70kb/Δ70kb}\) mice infused with AngII and anti–TGF-β (Figure 4A), which was associated with
shown to promote aneurysm formation in this model,\(^\text{10}\) not shown, suggesting a modifying role for lineage-specific\(^{34}\) parison with VSMCs of the descending thoracic aorta (data previously associated with a TGF-\(\beta\)-resistant phenotype,\(^{21,22}\) signaling, noncanonical Smad2 phosphorylation at linker sites, and microenvironment-related cues. Further studies will be elucidated. Interestingly, however, we found that reduced CDK inhibition was associated with reduced aortic expression of \(Mmp12\), a matrix metalloproteinase known to be regulated by TGF-\(\beta\),\(^{44}\) and shown to promote vascular pathology in the setting of aneurysm.\(^{10}\) Additional studies are required to more precisely define the mechanisms of vascular protection after CDK inhibition and to validate these hypotheses in various models of aortic aneurysms. It is interesting to note in this regard the study by Leeper et al\(^{45}\) showing increased aneurysm formation in mice deficient for \(Cdkn2b\) using a model of elastase infusion.

In conclusion, deletion of the orthologous noncoding cardiovascular risk interval on mouse chromosome 4 leads to reduced aortic expression of \(p19\)Arf and \(p15\)Inkb, reduced canonical TGF-\(\beta\)-dependent Smad2 phosphorylation, but increased CDK-dependent Smad2 linker phosphorylation. These abnormalities are associated with increased susceptibility to aneurysm development and rupture and are reversed, at least in part, by treatment with a CDK inhibitor. The results validate the use of this mouse model to study the molecular links between the noncoding risk region and aneurysmal disease. They also shed new light on the pathophysiology of vascular aneurysms and identify new therapeutic targets for this severe disease.

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**Disclosures**

None

**References**


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**Discussion**

There are 3 major findings in this study. Recent data established a strong association between genetic variants in the 9p21 chromosomal region in humans and the presence of cardiovascular diseases, including aneurysms. However, the causality of the association has not been tested directly in an animal model. This is the first study to show that deletion of the 9p21 orthologous region on mouse chromosome-4 affects the susceptibility to aortic aneurysm and vascular rupture and establishes a direct link between the 9p21 noncoding risk interval and susceptibility to a vascular disease. The increased risk of aneurysms is not dependent on manipulation of the risk interval in bone marrow-derived cells and seems to be related to intrinsic abnormalities of vascular cells. chr4\(^{\Delta70kb}\) mice seem to be a suitable model to further address the mechanistic links between 9p21 risk variants and cardiovascular diseases.

The second important finding is that deletion of the orthologous 9p21 interval in mice is associated with reduced TGF-\(\beta\)-dependent canonical Smad2 signaling in VSMCs. The mechanisms responsible for such alterations are still to be elucidated. Interestingly, however, we found that reduced TGF-\(\beta\)-dependent Smad2 phosphorylation tended to be more prevalent in VSMCs retrieved from the aortic arch and abdominal (suprarenal) aorta of chr4\(^{\Delta70kb}\) mice in comparison with VSMCs of the descending thoracic aorta (data not shown), suggesting a modifying role for lineage-specific\(^{14}\) and microenvironment-related cues. Further studies will be required to address these important issues in more detail.

We then found that, in contrast to canonical Smad2 signaling, noncanonical Smad2 phosphorylation at linker sites, previously associated with a TGF-\(\beta\)-resistant phenotype,\(^{21,22}\) was increased in VSMCs of chr4\(^{\Delta70kb}\) mice and was driven, at least in part, by CDK activation. The results further implicate abnormal TGF-\(\beta\) signaling in disease pathophysiology and reinforce previous findings linking alterations in TGF-\(\beta\) signaling to the progression of vascular diseases, including atherosclerosis\(^{35–40}\) and various forms of vascular aneurysms.\(^{10,17,41–43}\)

Finally, the study identifies a target for therapeutic modulation and shows that inhibition of CDK activity significantly reduces the susceptibility of chr4\(^{\Delta70kb}\) mice to AngII-induced aneurysm formation. The protective effect of CDK inhibition was associated with reduced aortic expression of \(Mmp12\), a matrix metalloproteinase previously shown to promote aneurysm formation in mice deficient for \(Cdkn2b\) (Figure 5A), a matrix metalloproteinase previously shown to promote aneurysm formation in this model,\(^{10}\) and with a significant prevention of elastin degradation (Figure 5B).

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**Disclosures**

None

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**CLINICAL PERSPECTIVE**

Recent data established a strong association between genetic variants in the 9p21 chromosomal region and the presence of aneurysms in humans. However, the causality of the association has not been tested directly. Here, we took advantage of the recent availability of a mouse with a targeted deletion of the 9p21 noncoding cardiovascular disease risk interval to examine the link between 9p21 DNA variants and vascular aneurysm. We show that this deletion is associated with reduced aortic expression of cyclin-dependent kinase inhibitors, reduced transforming growth factor-β–dependent canonical signaling but increased cyclin-dependent kinase–dependent Smad2 phosphorylation. We also show that these abnormalities are associated with increased susceptibility to aneurysm development and rupture in 9p21 mice. Interestingly, the disease phenotype is reversed by treatment with a cyclin-dependent kinase inhibitor. The results validate the use of this mouse model to study the molecular links between the noncoding risk region and aneurysmal disease. They also shed new light on the pathophysiology of vascular aneurysms and identify new therapeutic targets for this severe disease.
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