

## BAK1 Gene Variation and Abdominal Aortic Aneurysms

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**ABSTRACT:** We sought to examine the role of genetics in the multifactorial disease, abdominal aortic aneurysm (AAA), by studying sequence variation in the BAK1 gene (BAK1) that codes for an apoptotic-promoting protein, as chronic apoptosis activation has been linked to AAA development and progression. BAK1 abdominal aorta cDNA from AAA patients and nondiseased individuals were compared with each other, as well as to the BAK1 genomic sequence obtained from matching blood samples. We found specific BAK1 single nucleotide polymorphism (SNP) containing alleles in both aneurysmic (31 cases) and healthy aortic tissue (5 cases) without seeing them in the matching blood samples. These same BAK1 SNPs have been reported, although rarely (average frequency <0.06%), in reference BAK1 DNA sequences. Based on this and other similar observations, we propose a novel hypothesis postulating that multiple variants of genes may preexist in “minority” forms within specific nondiseased tissues and be selected for, when intra- and/or extracellular conditions change. Therefore, the fact that different BAK1 variants can exist in both diseased and nondiseased AA tissues compared to matching blood samples, together with the rare occurrence of these same SNPs in reference sequences, suggests that selection may be a significant factor in AAA ontogeny.

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**KEY WORDS:** intercellular gene variation; tissue specific gene alterations; abdominal aortic aneurysm; BAK1

### Introduction

The sequencing of the human genome, which has resulted in the discovery of hundreds of thousands of single nucleotide polymorphisms (SNPs), has revolutionized the study of multifactorial disease genetics. The HapMap and various SNP databases

[Conrad et al., 2006] have allowed researchers to conduct genome-wide screens of blood from patients suffering from a number of multifactorial diseases, and has resulted in the identification of hundreds of SNPs in hundreds of putative disease-associated genes [Doi et al., 2008; Oksenberg et al., 2008]. Clearly, all of the identified genes are unlikely to be involved in disease phenotypes, but the question of which are significant is hampered by the fact that these disease-associated genes are almost always identified by analyzing nondiseased tissue, that is, blood. One of the basic assumptions underlying many of these studies has been that as the genetic makeup of all an individual's cells is essentially the same, sequencing DNA isolated from blood will reveal the genetic makeup of diseased tissues as well. However, it has already been realized that in some multifactorial diseases such as cancer, this may not be fruitful. The current “two-hit” theory of carcinogenesis considers tissue-specific “loss of heterozygosity” to be a critical event in oncogenesis [Frank, 2005]. In noncancer diseases tissue specific mutational analysis is considerably more problematic, primarily because of the difficulty in obtaining diseased tissues, as opposed to cancer where removal of the diseased tissues, that is, tumors, is often part of the treatment regimen. Nevertheless, a mutation-driven hypothesis of disease ontogeny has remained central to the investigation of virtually all other multifactorial diseases, although like in cancer [Gottlieb et al., 2007] it is still largely unproven.

Abdominal aortic aneurysm (AAA; MIM# 100070) currently affects 6–9% of men over the age of 65, and is the tenth leading cause of death in men over the age of 55 in the United States [Curci et al., 2001]. AAAs are characterized by an inflammatory infiltrate, elastin destruction, and a decrease of medial smooth muscle cells [Lopez-Candales et al., 1997]. Apoptosis of smooth muscle cells is a prominent feature of AAA [Rowe et al., 2000], and is an important pathogenetic event in both atherogenesis [Clarke et al., 2006] and in the formation of aneurysms [Thompson, 2002].

In studies of AAA the vast majority of gene alterations and polymorphisms have been identified using DNA isolated from blood tissues [Pearce and Shively, 2006; Ye, 2000]. Typically, to prove the pathogenicity of these mutations, studies examined gene expression in AAA animal models [Daugherty and Cassis, 2004]. However, doubts have been cast as to exactly how representative of human disease these animal models really are [Calara et al., 2001; Garber, 2006].

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In the present study we decided to investigate if intercellular variation in putative disease-associated genes does occur. We chose to investigate the BAK1 gene (*BAK1*; MIM# 600516) that has been identified as a significant genetic factor in AAA. *BAK1*, a member of the Bcl-2 (MIM# 151430) family, is intimately involved in the activation of caspases that are an intrinsic part of the apoptotic pathway [Ruiz-Vela et al., 2005], and is expressed in atherectomy specimens [Saxena et al., 2002]. We have compared for the first time, *BAK1* alleles from abdominal aortic (AA) tissue from patients suffering from severe aneurismal disease, with *BAK1* alleles from both matching blood as well as normal nondiseased aortic tissue. In undertaking this study we hoped to find answers to a number of critical genetic questions, regarding the possible significance of intercellular genetic differences in *BAK1* alleles and how they contribute to the penetrance, frequency and relative risk of AAA.

## Materials and Methods

### Patient Data

All 31 patients (average age  $70.1 \pm 8.9$  years) provided signed consent. Approval of the research protocol was obtained from the Research and Ethics Review Committee of the Sir Mortimer B. Davis-Jewish General Hospital. Patients' AAA size, estimated by computer-assisted tomography scans and angiography, averaged  $5.7 \pm 0.4$  cm, and they suffered from hypertension ( $n = 22$ ), coronary artery disease ( $n = 12$ ), peripheral vascular disease ( $n = 5$ ), cerebrovascular disease ( $n = 3$ ), and diabetes ( $n = 3$ ). Patients were referred from community hospitals to the Vascular Surgery Service of the Sir Mortimer B. Davis-Jewish General Hospital. AAA samples were immediately frozen and stored at  $-80^\circ\text{C}$  until use.

Human normal abdominal aortic tissues ( $n = 5$ ) were obtained from the Quebec transplant program at the University of Sherbrooke, Sherbrooke, Quebec.

### RNA Isolation, cDNA Preparation, and Sequence Analysis

One hundred milligrams of AAA or normal abdominal aorta vascular tissue was homogenized in RNeasy lysis buffer (Tel-Test Inc., Friendswood, TX) and RNA isolated according to the manufacturer's instructions. SuperScript II (Invitrogen, Carlsbad, CA) RNAaseH-reverse transcriptase and random hexamers were used according to the manufacturer's instructions to create a cDNA. One microliter of the first-strand reaction was used to amplify the *BAK1* cDNA in a 50- $\mu\text{l}$  polymerase chain reaction (PCR) amplification reaction using Hercules DNA polymerase (Stratagene, La Jolla, CA) and *BAK1*-specific primers, according to the manufacturer's instructions.

Forward *BAK1*—CAGGCTGATCCCGTCCTCCACTGAG  
Reverse *BAK1*—GGGCACCCCTGGGAGTCATGATTTG

A portion of the PCR reaction was electrophoresed through acrylamide gels to verify amplification. The remainder was sent to

the McGill Genome Center for automated sequencing. RNA extraction, RT-PCR, and sequence analyses were repeated twice from independent RNA isolates. All samples had both strands sequenced.

### Genomic DNA Isolation, Amplification, and Sequence Analyses

A 5–10-ml blood sample was collected in heparinized tubes. Genomic DNA was isolated from blood samples using the Wizard Genomic DNA Purification Kit (Promega Corp, Madison, WI). Genomic DNA was amplified using Hercules DNA polymerase and the following *BAK1* exon-specific primers, according to the manufacturer's instructions.

**BAK1 exon 2** (covering a.a. 14)

Forward *BAK1*—TGGACATGGCAACCTGTATGGG

Reverse *BAK1*—AGCCTGCCTGAGTCCTGCTCC

**BAK1 exon 3** (covering a.a. 28, 42, 52, and 69)

Forward *BAK1*—CCCTGACTCCCAGCTTTGATC

Reverse *BAK1*—TACTGCCTCCCTGAAGATGTC

**BAK1 exon 4** (covering a.a. 69, 81, and 103)

Forward *BAK1*—ACAGGGCCATGGACAGCTCAG

Reverse *BAK1*—AGCATCATGCAGGCAGGGTATG

Note: a.a. 69 is at the junction between exons 3 and 4.

As above, a portion was used to verify amplification and the remainder sent for automated sequencing. All samples had both strands sequenced.

Samples were also analyzed using restriction fragment length polymorphism. Genomic DNA was amplified using exon 3-specific primers. Fifteen microliters of the PCR reaction was incubated overnight at  $37^\circ\text{C}$  with  $10 \times$  reaction buffer plus or minus *AclI* restriction enzyme. The digests were then electrophoresed through acrylamide and the DNA bands revealed by ethidium bromide staining. The expected fragment pattern of DNA bands based on the reference DNA sequence was 95, 61, 36, and 18 bp. The expected fragment pattern of DNA bands based on the vascular cDNA sequence was 64, 61, 49, and 36. No cutting was ever found to have occurred after incubation without enzyme.

## Results

To establish a reference sequence we examined *BAK1* sequence data from a number of databases (up to five) from different Caucasian populations as listed in the NCBI database (Table 1). Not surprisingly, it was noted that in the coding region of the gene there was a small amount of sporadic sequence variation, naturally assumed to be benign polymorphisms. They consisted of single base substitutions (SNPs) in the codons for seven amino acids, which in four cases resulted in changes to the incorporated amino acid. It should be noted that the occurrence of these SNPs is almost always rare, with a frequency on average of  $<0.06\%$  (Table 1).

**Table 1. SNP Variation in *BAK1* gDNA Reference Sequences from NCBI Databases**

<i>BAK1</i> gDNA	Exon	2	3	3	3	4	4	4
Amino acid		14	28	42	52	69	81	103
bp (cDNA)		253	294	336	366	418	454	520
Sequence change		TGC→TGT	GCC→GTC	CGC→CAC	GTG→GCG	AGC→AGA	ATC→ATT	GCC→GCT
Change in amino acid		synonymous	Ala→Val	Arg→His	Val→Ala	Ser→Arg	synonymous	synonymous
Major allelic frequency	Up to five different databases	0.933–0.792 (C)	1.0–0.994 (C)	1.0–0.5 (G)	1.0 (T)	1.0–0.994 (C)	1.0 (C)	1.0–0.96 (C)
SNP Variant	dbSNP accession #	rs5745585	rs4987115	rs1051911	rs1051912	rs5745592	rs105193	rs561276

**Table 2. Comparison of Non-AAA to AAA Tissue *BAK1* cDNA Sequences and Amino Acids versus Blood and Reference Sequence**

Amino acid #	14	28	42	52	69	81	103
Base #	253	294	336	366	418	454	520
<sup>a</sup> AAA Blood (10) and reference seq.	TGC	GTC	CAG	GCT	AGC	ATC	GCC
Nondiseased AA (5)	TGC	GTC	CAA	GCC	AGC	ATT	GCC
AAA (31)	TGC	GTC	CAA	GCC	AGC	ATT	GCC
AAA Blood (10) and reference aa	Cys	Ala	Arg	Val	Ser	Ile	Thr
Nondiseased AA (5)	Cys	Ala	His	Ala	Ser	Ile	Thr
AAA (31)	Cys	Ala	His	Ala	Ser	Ile	Thr

<sup>a</sup>Note: Although 10 samples were sequenced for all exons, a total of 31 blood samples had exon 3 containing amino acids 42 and 52 sequenced to confirm that SNPs were not present.

We collected tissue samples from a total of 31 AAA patients obtained at the time of surgical repair, together with matching blood samples, as well as five samples of normal nondiseased abdominal aortic tissue, that was obtained from a tissue bank. However, when *BAK1* cDNAs from actual AAA tissue were sequenced gene alterations were found in three of the amino acids (a.a.) (Table 2), which were exactly the same as the rare SNPs that had been identified in the reference databases (Table 1).

In two cases, coding region SNPs resulted in a change in a.a. 42 from arginine to histidine (dbSNP# rs1051911:G>A), and in a.a. 52 from valine to alanine (dbSNP# rs1051912:T>C) respectively (Table 2). In the third case a coding region SNP in a.a. 81 resulted in no change to the amino acid incorporated (dbSNP# 1051913:C>T). This initially suggested that these SNPs could be considered as possible factors in the formation of aneurysms. To confirm these observations, we sequenced *BAK1* cDNAs from nondiseased abdominal aortic tissue. Our results somewhat surprisingly showed that the nondiseased tissue contained the same SNPs as the diseased tissue (Table 2). However, when matching blood from the patients was sequenced it was discovered that none of their *BAK1* alleles contained any of the reported rare SNPs (Table 2).

Thus, the results showed that aortic tissue, whether diseased or not, contained a number of distinct SNPs, that were not present in matching blood samples or in the vast majority (on average >99.4%) of reference sequence DNA (Table 2). However, of interest was that these SNPs had been observed in a very distinct minority of reference sequences (on average <0.06%).

## Discussion

To our knowledge, this is one of the first studies of a multifactorial noncancer disease in which the sequence of a specific gene in a diseased tissue was compared with the same gene in matching blood samples, as well as in nondiseased tissues. For some time genetic differences have been reported between specific tissues and blood in a number of locus specific genetic diseases, that is, diseases in which mutations were identified only in a single gene due to somatic mutations [Erickson, 2003; Gottlieb et al., 2001]. The result has been an ever-increasing number of conditions in which the disease phenotype is the result of a degree of somatic mosaicism [Gottlieb et al., 2001].

In cardiovascular diseases a growing body of literature has started to report the presence of somatic mutations in specific genes associated with disease [De Flora and Izzotti, 2007]. Recently, one

study found somatic mutations in the connexin 40 gene (*GJA5*) to be associated with atrial fibrillation [Gollob et al., 2006], and another found somatic mutations in G protein subunit  $\alpha 2$  associated with tachycardia [Lerman et al., 1998], although in both these cases nondiseased cardiac tissue was not examined.

The present study is the first in which the sequence of a gene isolated from AAA tissue has been compared with the gene sequence from both normal abdominal aortic (AA) tissue and matching blood. We found similar gene alterations (SNPs) in the normal AA and AAA tissues, compared with blood, rather than differences between AAA on the one hand, and blood and normal AA tissues on the other. These are not trivial differences, as it is now widely recognized that nonsynonymous SNPs can affect the functioning of a gene, for example, by altering splice sites and transcriptional regulatory regions [Hull et al., 2007], and even synonymous SNPs can affect gene functioning [Sauna et al., 2007]. Indeed, functional SNPs have already been associated with cardiovascular disease [Anderson et al., 2007; Kullo and Ding, 2007]. Further, SNPs can result in a gain of function [Suzuki et al., 2008], so that it is possible to speculate that these *BAK1* SNP containing alleles could well be significant factors in AAA ontogeny, perhaps by increasing apoptosis. However, to truly assess if apoptosis does play a critical role in AAA we will need to examine AAA tissues for additional apoptotic associated genes, such as BAX and Bcl-2. In addition, to ultimately determine the possible role of apoptosis in AAA, we will also need to examine an additional number of AAA tissues.

As we have noted, traditionally when comparisons between blood and specific diseased tissue were made, nondiseased tissue has not been examined, as it was naturally assumed that somatic mutations were the cause of the disease phenotype. This is understandable, as in the most studied multifactorial disease, that is, cancer; almost all studies have solely involved a genetic comparison between tumor tissue and matched blood samples. The natural conclusion from these cancer studies has been that somatic mutations in genes expressed in tumors are implicated in tumorigenesis.

In a study of prostate cancer, laser capture microdissection (LCM) was used to dissect prostate tumors into cancerous and noncancerous tissue. Initial results [Alvarado et al., 2005] supported the traditional hypothesis of somatic mutations as the causal agents for carcinogenesis, because genetic alterations in the androgen receptor gene (*AR*) were found in cancerous tissues, but not in blood. However, in a follow-up study, *AR* alterations were detected in completely disease-free prostate tissues, remarkably even in prostate tissue from a 1-year-old child [Sircar et al., 2007]. Thus, unlike blood and other tissues, prostate tissue whether diseased or nondiseased and of varying age and maturity, contained variants of the *AR* gene. The results that we are reporting in AAA would appear to be similar to those reported in prostate cancer. These results are interesting, because they question the primacy of the somatic mutation theory as the basis of a common pathogenetic pathway for many multifactorial diseases including atherosclerosis [De Flora and Izzotti, 2007].

This questioning assumes greater significance because reference *BAK1* sequences obtained from blood contain the same SNPs, albeit only on rare occasions (Table 1). The fact that we report that three of these SNPs are present in both the normal and diseased tissue, but not in matched blood, suggests that the genetics of AAA is probably more complicated than a simple case of accumulation of somatic mutations.

It should be noted that transfection of rats with a *BAK1* BH3 peptide containing amino acids 72–87 increased arterial wall hyperpermeability of a *BAK1* BH3<sub>L-to-A</sub> at position 78 mutant

peptide had no effect on arterial wall hyperpermeability following hemorrhagic shock [Childs et al., 2007]. However, although such a mutant BAK peptide is less effective at interfering with the antiapoptotic activity of Bcl-2, it is also possible that the subtle changes of the three identified *BAK1* SNPs could enhance the proapoptotic activity of BAK.

Further, it is important to consider the possibility that the SNPs found in the AA tissues are not directly due to somatic mutational events at all, but rather due to RNA editing [Bass, 2002], in particular because, to ensure that the gene alterations were actually expressed in the AA tissues we examined *BAK1* cDNA. In fact, in a number of disease associated genes, such as the *APOB*, RNA editing has been found to be the cause of several examples of altered disease phenotype [Chen et al., 2007]. Deamination of cytosine in RNA editing has been shown to require a double-stranded RNA structure around the editing site, precedes splicing and usually involves the conversion of C in the mRNA to U, and so leads to T incorporation when amplified in PCR reactions [Scholzova et al., 2007]. A to I editing reactions almost invariably occur within *Alu* repeats [Amariglio and Rechavi, 2007]. *Alu* elements are present in *BAK1* gene introns but not in the coding regions. It is not clear if *BAK1* mRNA forms significant double-stranded character in vivo, allowing it to serve as a substrate for editing enzymes. However, the presence of the same SNPs in germline tissue could be a mitigating factor in considering RNA editing as being the source of the SNPs.

In fact, the occasional presence of the same SNPs in germline tissues, as reported in the NCBI database [Levy et al., 2007], also suggests that these gene alterations may not always be somatic in origin, even though our present data can be most readily interpreted as the SNPs being due to somatic mutations. The presence of SNPs in nondiseased AA tissue suggests that they may be present prior to appearance of disease and may be a consequence of vascular development and maturation. Their presence might be at least partially responsible for the increased susceptibility of AA tissue to aneurysm formation. A possible alternative explanation of their genetic origin is that within nondiseased AA tissues some *BAK1* SNP-containing alleles can exist perhaps as “minority” forms, in a similar manner to what we have observed in prostate tissue [Sircar et al., 2007]. That is, they are only initially present in a very few cells within normal aortic tissue and are then selected for when tissue and cellular conditions change. Thus, known risk factors such as smoking, hypertension and elevated cholesterol levels [Golledge et al., 2006] could then be considered as selection forces that act on susceptible tissues, that is, those that contain *BAK1* with specific SNPs that can cause disease. As aneurysms are a result of a chronic degenerative process [Thompson, 2002] occurring over many years, even a slight increase in apoptosis could contribute to their formation. Further, we also intend to examine both *BAX* and *Bcl-2* genes to fully understand the role of apoptosis in AAA.

Up until now, few researchers have considered the possibility that multiple variants of a gene might preexist within disease-susceptible tissues, but we are beginning to observe this condition in a few cases [Alvarado et al., 2005; Molderings et al., 2007; Sircar et al., 2007]. New sequencing techniques, such as ultradeep pyrosequencing, are able to overcome limitations in current sequencing protocols that effectively only sequence “majority” DNA forms, by being able to “oversequence” individual genes thousands of times. Using such techniques, it is likely that we will be able to much more readily identify such “minority” DNA forms as has already been achieved in the case of HIV [Wang et al., 2007].

In this study we have established for the first time that different *BAK1* variants exist in both diseased and nondiseased AA tissues compared to matching blood samples. Further, the rare occurrence of the same SNPs in reference sequences, suggests that selection may be a significant factor in the ontogeny of AAA.

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