Activation of innate anti-viral immune response genes in symptomatic benign prostatic hyperplasia

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INTRODUCTION

Benign prostatic hyperplasia (BPH) is a condition that affects millions of men in the United States every year and costs the US $4 billion annually. The prevalence of BPH increases with age: roughly 50% of men in their 50s exhibit symptoms of BPH, and 70% of men age 70 years old having symptomatic BPH. BPH is a condition in which growth of the prostate tissue is abnormally impeded by factors such as fibroblast growth factor 2 and insulin growth factor, demonstrating that androgens have a role in this disease, as well as various growth factors such as fibroblast growth factor 2 and insulin growth factor. Additionally, inflammation is frequently associated with BPH. Several cytokines and inflammatory mediators are upregulated in BPH including interleukin 1 alpha (IL1α), IL2, IL8, IL15, IL17, nuclear factor κ B, chemokine (C-X-C motif) ligand 5 (CXCL5) and CXCL12. In a study that examined gene expression differences between cultured normal prostate transitional zone stromal cells, genes involved in the innate immune response, including complement factor I (CFI) and AP0BEC3G, were found to be upregulated in the BPH-affected cells when compared with the normal transitional zone stromal cells.

The goal of this current study is to determine molecular differences between symptomatic and asymptomatic BPH in terms of the innate anti-viral immune response. To accomplish this, we compare gene expression differences in symptomatic BPH patient tissue samples from TURP and asymptomatic BPH patient tissue samples from patients undergoing prostatectomy for prostate cancer that had accompanying asymptomatic BPH. BPH typically arises in the transitional zone of the prostate, whereas prostate cancer most often occurs in the peripheral zone. Since these two diseases arise in different zones of the prostate,
molecular alterations due to prostate cancer will likely not be present in BPH-affected tissue, although this cannot be completely ruled out. Because a previous study had found upregulation of genes involved in the innate immune response in cells isolated from BPH-affected tissues and cultured in vitro, we wanted to determine if these findings were applicable to patient tissue samples. Our results indicate that an innate anti-viral immune response is activated in symptomatic BPH, which distinguishes patients with BPH requiring surgery from those who are either asymptomatic or able to endure the symptoms.

RESULTS

Activation of innate anti-viral immune response genes in symptomatic BPH

Relative CFI mRNA expression was determined by real-time PCR analysis using symptomatic BPH \((n = 17)\) or asymptomatic BPH samples \((n = 9)\). The patients with asymptomatic BPH reported AUA (American Urological Association) scores of \(\leq 12\). Specifications regarding patient samples can be found in Supplementary Table 1. CFI inactivates C3b and C4b; two key players in the innate immune systems complement pathway activation. Analysis of the CFI real-time PCR data using the BPH1 cell line as a calibrator and glyceraldehyde 2-phosphate dehydrogenase (GAPDH) as a reference gene reveals that the symptomatic BPH samples exhibit a fivefold increase in CFI expression compared with the asymptomatic BPH samples (Figure 2a, \(P = 0.0012\), t-test).

The expression profile of OAS2 was then examined. OAS2 recognizes virally produced double-stranded RNA and produces 2’-5’ oligoadenylates to initiate RNA destabilization through activation of RNaseL, thus contributing to the anti-viral response. Quantitative PCR for OAS2 reveals a greater than twofold increase in OAS2 gene expression in symptomatic BPH \((n = 16)\) when compared with the asymptomatic BPH \((n = 9)\) (Figure 1b; \(P = 0.0043\), Mann–Whitney).

Long interspersed nuclear element 1 (LINE-1) retroelements are methylated in normal prostate tissue but frequently become demethylated in cancer. We discovered that marked demethylation of LINE-1 also occurs in BPH tissue from TURPs when we used BPH-affected tissue as a ‘normal’ prostate control for our studies of LINE-1 methylation in prostate cancer (Figure 2a, compare symptomatic BPH lanes 3–5 with donor lanes 9–11). Methylation is measured by combined bisulfite restriction analysis involving isolation of genomic DNA, bisulfite conversion of the DNA that converts unmethylated cytosines to uracil and conserves methylated cytosines, and LINE-1 PCR followed by restriction digestion of the PCR product by an enzyme that preferentially cuts based on the presence of a cytosine that was conserved during bisulfite conversion due to methylation. Therefore, digested product indicates the presence of methylation. A statistically significant difference exists between symptomatic BPH samples \((n = 11)\) and histologically normal prostate tissue from organ donors \((n = 9)\) (Figure 2b; \(P = 0.040\), Mann–Whitney). Next, we examined LINE-1 methylation status in asymptomatic BPH tissues. LINE-1 is demethylated and expressed in prostate cancer and a field defect exists in prostate cancer in which normal prostate tissue adjacent to the tumor also demonstrates demethylation of LINE-1 (unpublished data). Since the asymptomatic BPH samples come from patients undergoing prostatectomy due to prostate cancer, any demethylation of LINE-1 observed in the asymptomatic BPH samples may be due to the field defect from the prostate cancer. Although the average relative methylation of the asymptomatic BPH samples was higher than symptomatic BPH samples, it did not reach statistical significance (Figure 2b).

Since we observed a statistically significant difference in LINE-1 methylation between symptomatic BPH and donors, we were interested in pursuing a potential cause for this demethylation. It is not known what mechanism(s) lead to demethylation of DNA, however, recent evidence suggests that apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like proteins (APOBECs) may target cellular genomic DNA leading to the demethylation of cytosines. APOBEC3G is a cytidine deaminase that can mutate uridines. APOBEC3G typically performs this function on the viral genomes of viruses that have infected the cell. Since APOBEC3G might be involved in both DNA demethylation and the innate anti-viral response, we examined its expression in our BPH tissue samples. Real-time PCR for APOBEC3G was performed on symptomatic BPH \((n = 16)\), asymptomatic \((n = 13)\) BPH, and donor \((n = 6)\) samples with the BPH1 cell line used as the calibrator. There is over a twofold increase in APOBEC3G expression in symptomatic BPH when compared with asymptomatic BPH (Figure 2c; \(P = 0.011\), Mann–Whitney) and donors \((P = 0.043\), t-test).
In an attempt to distinguish activation of these genes in response to virus versus other pathogens, we examined the expression profile of interferon-induced tetratricopeptide 1 (IFIT1). IFIT1 expression is activated in response to viruses as well as interferon (IFN)α/β, which are induced in response to virus. The function of IFIT1 is to inhibit cellular and viral processes such as translation, migration, proliferation, signaling, and viral replication.13 Real-time PCR for IFIT1 revealed no statistically significant difference between the symptomatic (n = 16) and asymptomatic BPH (n = 13) groups (Figure 3a). However, we observed that the asymptomatic BPH samples with the largest mass had the highest fold change in IFIT1. Large prostate size is oftentimes associated with LUTS. When we remove the four samples that came from asymptomatic BPH patients with a prostate mass of >60 g, there is a statistically significant twofold increase in IFIT1 expression in symptomatic BPH samples (n = 17) when compared with asymptomatic BPH (n = 9) (Figure 3b; P = 0.012, Mann–Whitney).

Correlations observed in gene expression data and patient parameters
When analyzing our IFIT1 data we observed that the asymptomatic BPH samples with large masses had the highest fold changes in IFIT1 expression. Therefore, we wanted to determine if there was a significant correlation between IFIT1 fold change and the mass in grams of the asymptomatic BPH prostates. We found that there is a strong positive correlation between IFIT1 expression and the mass of the asymptomatic BPH prostates (Pearson’s r = 0.65), and this was found to be statistically significant (Figure 4a; P = 0.017).

Additionally, we observed that the symptomatic BPH samples demonstrating a strong induction of IFIT1 expression also showed strong induction of APOBEC3G. Therefore, we examined whether there was a strong correlation between expression of these two genes in the symptomatic BPH samples. Indeed, we found that there is a strong correlation between IFIT1 fold change and APOBEC3G fold change in the symptomatic BPH samples (Figure 4b; Pearson’s r = 0.65, P = 0.0066). We were unable to correlate IFIT1 expression with prostate size for symptomatic BPH samples, as only the affected tissue and not the entire prostate is removed.

LINE-1 retrotransposon expression is not elevated in symptomatic BPH samples
After observing the activation of an innate anti-viral immune response in symptomatic BPH, we wanted to determine the mechanism potentially causing this response. Since we know that LINE-1 is demethylated in symptomatic BPH samples, we hypothesized that LINE-1 retroelements were being expressed and activating the anti-viral immune response in symptomatic BPH. Examination of LINE-1ORF2 (LINE-1 open reading frame 2) expression in symptomatic BPH samples compared with donors demonstrates that there is a statistically significant difference in LINE-1 expression between symptomatic BPH (n = 19) and donor tissue (n = 8) (Figure 5; P = 0.015, Mann–Whitney). Examination of
asymptomatic BPH samples \( (n = 14) \) indicates that the mean was lower than the symptomatic BPH mean but did not reach statistical significance (Figure 5). Again, LINE-1 expression in asymptomatic BPH may be due to the field defect observed in prostate cancer.

**DISCUSSION**

In the current study examining molecular differences between symptomatic BPH and asymptomatic BPH, we found four genes involved in the innate anti-viral immune response to be upregulated in symptomatic BPH: CFI, OAS2, APOBEC3G and IFIT1. CFI is a major regulator of the complement system in the innate immune response. Activation of the complement pathway leads to various signaling cascades, all of which converge at the point of C3a and C4a production. CFI is a protease that cleaves C3b and C4b yielding C3 and C4 fragments resulting in inhibition of complement signaling.\(^9\) Interestingly, some viruses such as vaccinia, mumps and Epstein-Barr virus utilize CFI during infection to prevent complement activation and allow for successful infection.\(^{14-16}\) Additionally, some cancer cells such as glioma cells and non-small cell lung cancer cells express increased levels of CFI.\(^{17,18}\) Expression of CFI by cancer cells may be contributing to protection of these altered cells from complement attack. In the current study, we observe a statistically significant increase in CFI expression in symptomatic BPH samples when compared with asymptomatic BPH. In fact, the average fold change of the symptomatic BPH samples is five times higher than the average fold change of the asymptomatic BPH samples.
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It is interesting that a potent inhibitor of one arm of the immune system, the complement cascade, is activated in symptomatic BPH since BPH is a disease currently characterized by a strong immune/inflammatory response. Importantly, factors involved in inflammation, namely IL6 and IFN gamma (IFN-γ), are known activators of CFI.9,20 It is possible that CFI is playing a similar role in symptomatic BPH as it is in glioma and non-small cell lung cancer cells: CFI may be contributing to the protection of altered symptomatic BPH cells from complement attack. Protection from the complement system in symptomatic BPH may allow for tissue remodeling and growth, thus leading to LUTS. This finding warrants further investigation into the role CFI is playing in symptomatic BPH and provides insight into a potential pathway to target in symptomatic BPH.

OA2, APOBEC3G and IFIT1 are all induced by Type I IFNs and all are involved in the innate anti-viral response. OA2 is an anti-viral enzyme that recognizes virally produced double-stranded RNA and initiates RNA degradation through activation of RNaseL within infected cells. APOBEC3G is critical in the innate anti-viral response, because it recognizes and subsequently mutates the viral genome, and with the exception of HIV, usually renders the virus non-functional.15 Finally, IFIT1 is strongly induced by Type I IFNs and viral infection and can lead to inhibition of cellular and viral protein synthesis and replication.18 Recently, IFIT1 has also been shown to be involved in binding viral 5′ triphosphate-containing RNA.21 In the present study, we demonstrate a statistically significant upregulation of APOBEC3G, OA2 and IFIT1 in symptomatic BPH when compared with asymptomatic BPH. Additionally, IFIT1 expression correlates with the size of the prostate in the asymptomatic BPH group, with the largest prostates resulting in the biggest induction of IFIT1. Larger prostates are more likely to be associated with LUTS, therefore, it is consistent with our results that IFIT1 expression would correlate with prostate size. Another correlation that we observe is between IFIT1 and APOBEC3G. It is not surprising to observe a correlation in the expression of two genes involved in response to the same/similar stimuli.

It is interesting that an anti-viral response is observed in symptomatic BPH, since this condition has not been linked to viral infection of the prostate. Studies have, however, demonstrated a link between sexually transmitted viral infections and LUTS.22,23 The significance of this correlation is currently unknown. The anatomical location of the prostate in the urogenital tract makes it possible that sexually transmitted viral infection may contribute to the innate anti-viral immune response in symptomatic BPH. In addition, it is possible that inflammation associated with BPH results in the production of endogenous ligands (‘Sterile Inflammation’), which activate the innate immune receptors to induce Type I IFN and/or these anti-viral innate immunity-associated genes.

Activation of the innate anti-viral immune response in symptomatic BPH could also be due to activation of endogenous retroelements. Methylation of LINE-1 suppresses expression of these elements. There was a statistically significant demethylation of LINE-1 in symptomatic BPH samples when compared with histologically normal prostate tissue from organ donors (Figure 2a). We also found that there was a significant upregulation of LINE-1 expression in the symptomatic BPH samples when compared with donors (Figure 5), indicating that demethylation of LINE-1 may be having an important role in symptomatic BPH. Asymptomatic BPH samples did show a higher level of LINE-1 methylation and lower LINE-1 expression than symptomatic BPH; however, these did not reach statistical significance. This may be due to the field defect observed in prostate cancer in regards to methylation of LINE-1. Future studies examining the role of LINE-1 in symptomatic BPH could utilize samples from patients with prostate cancer and accompanying symptomatic BPH versus asymptomatic BPH. It will be important to tease out the role of LINE-1 expression in symptomatic BPH, as expression of LINE-1 retroelements may be triggering activation of the innate anti-viral immune response.

Two examples of diseases in which LINE-1 is demethylated, expressed and believed to be responsible for an innate anti-viral immune response are the autoimmune disorders Sjogren syndrome (SS) and systemic lupus erythematosus.24 Inflammation is present in SS and systemic lupus erythematosus, as well as in BPH. The anti-viral immune response in SS and systemic lupus erythematosus is likely to be responsible for the inflammation present in these diseases; therefore, the anti-viral immune response may be responsible for the inflammation observed in BPH.24 Previously, it has been suggested that BPH does have characteristics of an autoimmune disease, because significantly more IgC antibodies to prostate-specific antigen were present in BPH samples when compared with controls.25 As we see in BPH, part of the anti-viral immune response in SS is the expression of APOBEC3 family members. APOBECs may be having multiple roles in regard to LINE-1 hypomethylation and expression. Some APOBEC family members are able to target RNA of cellular retroelements, providing a line of defense against aberrantly expressed retrotransposons.12 Recently, however, it has been suggested that some APOBECs may be able to demethylate cellular DNA.11 Upregulation of APOBECs could then be contributing to demethylation of LINE-1. Determination of the effect that APOBECs have on LINE-1 demethylation and expression in BPH, SS and systemic lupus erythematosus is critical. It will be important to answer the question of whether APOBECs are being upregulated and demethylating LINE-1 or is LINE-1 being demethylated by some other cause, which is leading to the upregulation of APOBECs, allowing for subsequent targeting of LINE-1 RNAs.

Taken together, we observe an innate anti-viral immune response involving the upregulation of CFI, OA2, APOBEC3G and IFIT1 in symptomatic BPH when compared with asymptomatic BPH. Additionally, LINE-1 retroelements are demethylated and expressed in symptomatic BPH tissue compared with donors. This study gives insight into the molecular changes in symptomatic BPH, a disease in which knowledge at the molecular level is
lacking. Further investigation into the cause of this anti-viral immune response and how this response contributes to LUTS is necessary. Targeting a factor involved in this anti-viral immune response may be therapeutically beneficial in the prevention and/or treatment of symptomatic BPH.

MATERIALS AND METHODS

Tissue acquisition

Symptomatic BPH samples were collected from patients undergoing TURP to alleviate symptoms. These samples were de-identified and supplied to us by the University of Pittsburgh HSTB (Health Sciences Tissue Bank) after Institutional Review Board approval. Asymptomatic BPH samples were collected from patients undergoing prostatectomy due to prostate cancer. These patients also had accompanying histologic BPH. Tissues utilized as asymptomatic BPH were from patients with an AUA score of <12. A University of Pittsburgh HSTB genito-urinary pathologist isolated the transitional zone containing histologic BPH from the asymptomatic BPH samples and gave us the de-identified tissue. Organ donor prostate tissue was also provided by the University of Pittsburgh HSTB and a genito-urinary pathologist confirmed that they were histologically normal. The zone of origin of the donor prostate tissue is unknown. All samples were stored at –70°C.

The same symptomatic and asymptomatic BPH samples were used for the CFI, OA2, APOBEC3G and IFIT1 real-time PCR. Additional asymptomatic BPH samples were prepared and used for the APOBEC3G and IFIT1 real-time PCR to increase statistical power. A large overlap in samples exist in the LINE-1 real-time PCR and LINE-1 methylation assays, however, there are some additional samples used because samples for these assays were prepared differently (RNA was DNase treated for the LINE-1 real-time PCR).

The AUA symptom score is determined by a questionnaire that patients fill out regarding the severity of their LUTS. Questions range from how many times the patient urinates at night to how often the patient has to strain to urinate. The patients answer each question on a scale of 0-5, with 0 being not at all and 5 representing almost always. Scores for all of the questions are then added up and the total is the patients AUA symptom score, with a score of 35 being the maximum possible. Scores between 0 and 7 indicate mild symptoms; 8-19 are moderate and 20-35 severe.

RNA isolation

RNA was isolated from TURP or asymptomatic BPH tissue using the Trizol method. Briefly, ~50 mg of tissue was placed in 500 μl of Trizol (Invitrogen, Grand Island, NY, USA) and was homogenized. The homogenized tissue was allowed to incubate in Trizol for 5 min at room temperature. In all, 100 μl of chloroform was then added to the tissue/Trizol. Samples were shaken vigorously for 15 s and then allowed to incubate at room temperature for 3 min. Samples were then centrifuged at 12000 g for 15 min at 4°C. The supernatant was again removed and the RNA pellet was allowed to air dry for ~30 min. The RNA pellet was then resuspended in diethylpyrocarbo-
nate-treated water and the concentration was read on a Nanodrop 2000C spectrophotometer (Thermo Scientific, Asheville, NC, USA). In all, 500 ng of RNA was then run on a 0.8% agarose/tris-borate-EDTA gel and visualized to ensure intact 28S and 18S ribosomal RNA.

DNase treatment of RNA

RNA utilized to make complementary DNA (cDNA) for real-time PCR for CFI, OA2, APOBEC3G and IFIT1 was not DNase treated whereas RNA used to make cDNA for LINE-1ORF2 real-time PCR was DNase treated. This is because the primers designed for the LINE-1ORF2 real-time PCR recognize genomic DNA as well as cDNA, so it is essential to remove all contaminating genomic DNA for this particular assay. All other real-time PCR primers were designed not to recognize genomic DNA. In all, 4 μg of RNA was treated with 1 μl of Ambion/Life Technologies (Grand Island, NY, USA) TURBO DNase at 37°C for 30 min. The RNA was extracted from the enzyme via Phenol-Chloroform extraction. RNA concentration was then read using the nanodrop and 500 μg of RNA was run on a 0.8% agarose/ TBE gel and visualized to confirm RNA integrity.

cDNA production

For the non-DNase-treated RNA samples, 1 μg of RNA was utilized to make cDNA using random hexamers and the SuperScript III First-Strand Synthesis System for Reverse Transcription-PCR (RT–PCR) (Invitrogen) as per manufacturer’s instructions. The DNase-treated RNA samples produced cDNA starting with 1 μg of DNase-treated RNA and utilization of random primers and the Promega (Madison, WI, USA) M-MLV RT First-Strand Synthesis of cDNA kit.

Real-time PCR

Quantitative real-time PCR for APOBEC3G, IFIT1, LINE-1ORF2 and GAPDH was performed on a Bio-Rad (Hercules, CA, USA) iQ5 Multicolor Real Time PCR detection system real time PCR machine whereas real-time PCR for CFI and OA2 was performed on the Applied Biosystems StepOnePlus Real time PCR system/machine (Carlsbad, CA, USA). The APOBEC3G primer/probe set from Solaris/Thermo Scientific (Catalog number AK-013072-00-0100; Lafayette, CO, USA) was used at a 1× concentration along with a 1× concentration of the Solari’s QPCR Master Mix (Catalog number AB-4350) in a total volume of 20 μl. The real-time PCR was performed using the manufacturer-recommended thermocycling conditions, which yielded a satisfactory efficiency (103.7%). SYBR green real-time PCR was performed for IFIT1. In all, 0.625 μM of each primer (F1—5’-caagcaacactagtgactaagg-3’ and R1—5’-ctgctcatctagtacttc-3’) was used with a 1× concentration of Bio-Rad’s iQ SYBR Green Supermix in a 20 μl total volume. The following thermocycling conditions resulted in a satisfactory efficiency (94.3%): 94°C for 1 min and 45 cycles × (94°C for 30 s, 60°C for 45 s, 76.5°C for 15 s). SYBR green real-time PCR was also performed for LINE-1ORF2 using 0.625 μM final concentration of each primer (F1—5’-cagccatactaa-
taattgcaggg-3’ and R1—5’-tatccagtctgttgtcgct-3’) and a 1× final concentration of the Bio-Rad’s iQ SYBR Green Supermix in a total volume of 20 μl. Thermocycling conditions consisted of 94°C for 30 s and 45 cycles × (94°C for 15 s, 52°C for 60 s), resulting in an acceptable efficiency (92%). A primer/probe set designed by Sigma-Aldrich (St Louis, MO, USA) was utilized for the GAPDH real-time PCR. In all, 0.75 μM of each primer (F2—5’-ccagccattgtctct-3’ and R2—5’-gatgatgtctgtgagag-3’) and 0.1 μM of the probe (5’-FAM-aaggctgcccctgatgctcatt-black hole quencher 1) were used along with a final 1× concentration of the iQ Supermix (170-8860) from Bio-Rad in a total volume of 20 μl. In all, 94°C for 30 s and 45 cycles × (94°C for 15 s, 52°C for 60 s) was the thermocycling conditions allowing for an acceptable efficiency (102%). CFI and OA2 real-time PCR were performed using primer/probe sets from Applied Biosystems (Catalog numbers Hs00989715_m1 and Hs00942643_m1, respectively). The ABI Master Mix was used at a final 1× concentration and manufacturer-recommended thermocycling conditions were used. These conditions resulted in a satisfactory efficiency for CFI (93.6%), however, the OA2 efficiency was only 84.2%.

Real-time PCR analysis

The deltadelta Ct method was used to analyze the APOBEC3G, IFIT1, LINE-1ORF2 and CFI real-time PCR results using BPH1 or LNCaP cell lines as the calibrator. GAPDH was used as a reference gene. The Pfaffi method was used to analyze the OA2 real-time PCR results to account for the reduced efficiency. The BPH1 cell line was used as a calibrator and GAPDH as the reference gene. Statistical analysis included the Student’s t-test which was used to analyze the data that was normally distributed, or the Mann–Whitney rank-sum test that was utilized in the case of a non-normal distribution. Pearson’s correlation coefficient was calculated for the correlations performed. Statistical tests were performed using Systat software version 3.5 (Systat Software, San Jose, CA, USA).

Global DNA methylation combined bisulfite restriction analysis

LINE-1 elements are found so frequently in the human genome that its methylation status is used as a surrogate marker for global DNA methylation. Genomic DNA was prepared from patients using the phenol-chloroform extraction method. Briefly, samples digested with proteinase K were mixed in equal volume with phenol/chloroform/soybean alcohol reagent at a 25:24:1 dilution. Samples were then shaken vigorously for 15 s and centrifuged at maximum speed for 10 min at 4°C. The aqueous (top) phase was then put in a new tube. Half of the original volume of 7.5 mM ammonium acetate and two volumes of 100% ethanol were added. The
samples were then centrifuged at maximum speed for 20 min at 4 °C. The supernatant was removed and the pellet washed with 70% ethanol followed by another centrifugation at maximum speed for 10 min at 4 °C. The wash step and centrifugation was repeated, the pellet air dried and then resuspended in water. Genomic DNA and controls were then bisulfite converted using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA). Bisulfite treatment of DNA converts non-methylated cytosines into uracil via deamination, which is replicated as a thymidine during PCR. 5-Methylcytosines are protected and identified as cytosines in the PCR product. The LINE-1 PCR products were then subjected to restriction digestion with HinfI (New England Biolabs, Ipswich, MA, USA). Digestion products were then run on a 1.8% agarose/TBE (Tris base, EDTA) gel and stained with ethidium bromide. Digestion with HinfI indicates methylation of LINE-1, as only methylated and thus conserved (methyl)cytosine during bisulfite conversion creates a HinfI digestion site in the LINE-1 PCR product. The ratio of the top digestion product to the uncut band was determined using Image J and this was utilized to calculate the percent methylation. This was then calculated relative to the calibrator.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Genes and Immunity website (http://www.nature.com/gene)