Lower frequency of TLR9 variant associated with protection from breast cancer among African Americans

Madison R. Chandler, Kimberly S. Keene, Johanna M. Tuomela, Andres Forero-Torres, Renee Desmond, Katri S. Vuopala, Kevin W. Harris, Nancy D. Merner, Katri S. Selander

Abstract

Introduction

Toll-like receptor 9 (TLR9) is an innate immune system DNA-receptor that regulates tumor invasion and immunity in vitro. Low tumor TLR9 expression has been associated with poor survival in Caucasian patients with triple negative breast cancer (TNBC). African American (AA) patients with TNBC have worse prognosis than Caucasians but whether this is due to differences in tumor biology remains controversial. We studied the prognostic significance of tumor Toll like receptor-9 (TLR9) protein expression among African American (AA) triple negative breast cancer (TNBC) patients. Germline TLR9 variants in European Americans (EAs) and AAs were investigated, to determine their contribution to AA breast cancer risk.

Methods

TLR9 expression was studied with immunohistochemistry in archival tumors. Exome Variant Server and The Cancer Genome Atlas were used to determine the genetic variation in the general EA and AA populations, and AA breast cancer cases. Minor allele frequencies (MAFs) were compared between EAs (n = 4300), AAs (n = 2203), and/or AA breast cancer cases (n = 131).

Results

Thirty-two TLR9 variants had a statistically significant MAF difference between general EAs and AAs. Twenty-one of them affect a CpG site. Rs352140, a variant previously associated...
with protection from breast cancer, is more common in EAs than AAs ($p = 2.20E-16$). EAs had more synonymous alleles, while AAs had more rare coding alleles. Similar analyses comparing AA breast cancer cases with AA controls did not reveal any variant class differences; however, three previously unreported TLR9 variants were associated with late onset breast cancer. Although not statistically significant, rs352140 was observed less frequently in AA cases compared to controls. Tumor TLR9 protein expression was not associated with prognosis.

Conclusions

Tumor TLR9 expression is not associated with prognosis in AA TNBC. Significant differences were detected in TLR9 variant MAFs between EAs and AAs. They may affect TLR9 expression and function. Rs352140, which may protect from breast cancer, is $1.6 \times$ more common among EAs. These findings call for a detailed analysis of the contribution of TLR9 to breast cancer pathophysiology and health disparities.

Introduction

Toll Like Receptor-9 (TLR9) is an endosomal DNA receptor that belongs to the innate immune system. It recognizes and reacts to both microbial and vertebrate (self) DNA that has entered cells, either during microbial infections or during cell death.[1–3] Activation of TLR9 by DNA stimulation results in a rapid and a robust inflammatory reaction, with increased production of Th1-biased inflammatory mediators that activate the adaptive immune system.[4–6] The outcome of this reaction is an immunological elimination of the invading microbe and the infected cells.[4, 7] A similar inflammatory response occurs during sterile tissue damage, such as infarction or trauma.[8, 9]

TLR9 is also widely expressed in various human cancer cell lines and clinical tumors, including breast, brain, lung, ovarian, prostate, kidney and GI-tract cancers.[10–19] Stimulation of TLR9 with synthetic DNA-ligands (CpG-oligonucleotides) or cell-derived DNA has been shown to induce cytokine expression in TLR9-expressing cancer cells in vitro.[20, 21] In addition, both synthetic TLR9-ligands as well as cell-derived DNA induce cancer invasion in vitro.[3, 10, 12, 22, 23] TLR9 can also regulate cancer cell invasion ligand-independently.[15, 24] The clinical relevance of these findings and the significance of TLR9 in cancer pathophysiology has however, remained unclear. A recent meta-analysis by Wan et al. suggested that certain TLR9 variants (such as rs187084) might be associated with an elevated cancer risk, especially in cervical cancer.[25] Other TLR9 variants (such as rs352140 and rs5743836, respectively) were suggested to be protective of breast and digestive system cancers.[25] Currently, little is known about the functional effects of the various TLR9 genetic variants on cancer or other cells.[26–29] When studied with immunohistochemistry, tumor TLR9 expression can indicate cancer-specific prognoses; on one hand, high TLR9 expression was associated with decreased survival in brain, prostate and esophageal cancers.[11, 18, 30] On the other hand, in triple negative breast cancer (TNBC), renal cell carcinoma, mucopidermoid salivary gland carcinoma, and most recently in pancreatic cancer, low tumor TLR9 protein expression is associated with poor survival.[13, 15, 31–33]. This suggests that high tumor TLR9 expression protects from relapses in these malignancies. The mechanisms for such protection are unclear.

Of all breast cancer patients, those with TNBC have the worst prognosis.[34–36] These tumors are generally aggressive and lack the expression of drug targets, such as estrogen...
receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2).\[34–36\] TNBC is especially troubling in African American (AA) women. Firstly, large population-based studies have identified a higher proportion of TNBC among premenopausal AA women.[37] Secondly, AA women have higher mortality rate from breast cancer than Caucasian or European Americans (EA) even when socioeconomic factors are taken into account. [37] Although the results of a recent study suggested that TNBC in AA women is not a unique disease compared to TNBC in Caucasian women, biological differences have been indeed detected.\[37–39\] TLR9 has not been studied in breast or other cancer disparities previously. The aim of this study was to evaluate tumor TLR9 expression among AA TNBC patients and to assess its relationship to survival and recurrence in this patient population. We also aimed to identify the landscape of germline TLR9 variants in both EAs and AAs, and determine if such variants contribute to AA breast cancer risk.

Materials and methods
Breast cancer specimens
AA females with TNBC treated between 2000 and 2008 at The University of Alabama at Birmingham (UAB; Birmingham, Alabama, U.S.A) and with tissue available for analysis were identified in a pathology database. The working data was for 51 patients, but the follow-up data was available only for 43–49 subjects. The associated paraffin-embedded tumor blocks were processed for immunohistochemical staining and evaluation of TLR9 expression using standard techniques and as previously explained.[15, 40] Specifically, we used the following definition of TNBC to select the tumors for the immunohistochemical stainings: Tumors exhibiting any nuclear estrogen/progesterone receptor expression in invasive tumor cells were considered as steroid receptor-positive and excluded. Membranous HER2 expression was also studied by means of immunohistochemistry (IHC) and if a specimen exhibited a HER2-positive result in IHC, the HER2 gene amplification status was determined by means of chromogenic in situ hybridization. HER2-positive tumors were excluded. All patients got standard treatment and care, consisting of surgery, radiation and chemotherapy (adjuvant or neo-adjuvant). The research was approved by the UAB Institutional Review Board and by the Ethics Council of The Northern Ostrobothnia Hospital District (Oulu, Finland).

Tumor TLR9 staining and scoring
Immunohistochemical staining for TLR9 and scoring of the staining intensities were performed as previously described.[40] Briefly, tissue sections of 5 μm in thickness were cut from the formalin-fixed, archived, paraffin-embedded tissue blocks. Immunohistochemical staining was performed with a LabVision Autostainer™ (LabVision, Fremont, CA, USA), using the Envision™ Detection System (K500711; Dako Denmark A/S, Glostrup, Denmark). The antibody used was the anti-TLR9 (Img-305A, diluted 1:200, Imgenex, San Diego, CA, U.S.A). TLR9-staining intensity scores (0–16) were divided into low (<8) and high (≥8), according to the previously used criteria.[15, 25, 40] Clinical information was obtained from patient records. TLR9 expression scores were compared with retrospective outcome data, including ipsilateral breast cancer, disease-free and overall survival. TLR9 scores and the associated survival data are shown in S1 Table.

Statistical analysis of TLR9 protein expression data
Clinical and pathologic characteristic were summarized as means (sd) for continuous variables and frequencies for categorical variables. The relationship between TLR9 and survival, disease-
free survival and ipsilateral breast tumor recurrence were evaluated using the log-rank test and Kaplan Meier survival curves with TLR9 dichotomized at the approximate median value.

Genetic analyses

Firstly, TLR9 variants reported in the Exome Variant Server (EVS; http://evs.gs.washington.edu/EVS/; release ESP6500SI-V2) of the National Heart Lung and Blood Institute (NHLBI) GO Exome Sequencing Project (ESP) (https://esp.gs.washington.edu/drupal/) were recorded to determine variant calls and frequencies in both AAs and EAs, which represented the general population for each ethnicity. Overall, all called variants were compared between the two ethnicities to identify variants with statistically significant different allele frequencies. The statistical analyses involved Fisher Exact tests using the program R (R 3.3.2 GUI 1.68 Mavericks build (7288)). To determine if TLR9 variants play a role in AA breast cancer susceptibility, a request (#44682–1) for The Cancer Genome Atlas (TCGA) data access was submitted and project (#10805) was approved. Sequencing data from select breast cancer-affected individuals was collected from the Genomic Data Commons (GDC) Data Portal.[41, 42] Cases were filtered for ‘Project:’ TCGA-BRCA, ‘Disease Type:’ Breast Invasive Carcinoma, ‘Race:’ black or AA, and ‘Samples Sample Type:’ Blood Derived Normal. Files were filtered for ‘Experimental Strategy:’ WXS (whole exome sequencing), and ‘Data Format:’ BAM (binary sequence alignment-mapping). BAM files were produced by mapping/aligning sequencing data to the GRCh38.p0 reference genome using Burrows-Wheeler Aligner with Mark Duplicates and Cocleaning.[41] After filtering, 170 whole-exome BAM files remained. The manifest of the selected BAM files was downloaded from the GDC Data Portal; 131 of the 170 whole-exome BAM files were downloaded using the GDC Data Transfer Tool (version 1.2.0) [43]. HaplotypeCaller from the Genome Analysis Toolkit (GATK; version 3.6) was used to call variants from the downloaded BAM files and to generate Variant Calling Format (VCF) files for the whole-exome [44]. Variants located on chromosome 3 between base pair 52221079 and 52226163 were extracted from the whole-exome VCF files using VCFTools (version 0.1.12a).[45] The extracted VCF files that contain variants located in TLR9 were compressed and indexed using Tabix (version 0.2.6). Ultimately, extracted VCF files were merged for all 131 AA breast cancer cases and allele frequencies were calculated and compared to the frequencies reported in AAs in EVS. Clinical data for the breast cancer-affected individuals was collected from the GDC Data Portal (S2 Table).[41]

Results

No association between tumor TLR9 expression status and survival among AA TNBC patients

The baseline and clinico-pathologic characteristics of the studied breast cancer patient population are shown in S3 Table. Fifty-one patients had tissue evaluable for review. Median age at the time of diagnosis was 51 years. Most patients (n = 31, 72%) were early stage (I–II), and the majority of the patients were also node-negative (n = 30, 67%). Most patients had ductal infiltrating carcinoma (n = 43, 90%). All patients received standard of care, consisting of surgery, radiation and chemotherapy. Most patients had mastectomy (n = 28, 61%), with the minority undergoing lumpectomy. All patients received some form of chemotherapy (26% in the neo-adjuvant setting, 74% adjuvant systemic therapy). Radiation therapy was used in 66% of patients in the post-operative setting. At a median follow-up of 3.5 years, 25 patients (58%) experienced some type of recurrence. Eleven patients (25%) had ipsilateral breast tumor recurrence. TLR9 staining in the paraffin-embedded tumor sections was performed as previously
Examples of high and low TLR9 staining patterns in the studied AA TNBC specimens are shown in Fig 1. The TLR9 staining range was similar as previously detected in the EA cohort (data not shown). However, unlike previously shown for Caucasian TNBC patients,[15] tumor TLR9 protein expression score was not significantly associated with breast cancer recurrence, ipsilateral breast cancer or breast cancer-specific survival, Fig 2.

**TLR9 variant differences in general AA and EA populations**

A total of 147 different variants were called in EVS between both ethnicities; this included 31 variants that were detected in both EAs and AAs, as well as 65 variants that were unique to EAs and 51 variants that were unique to AAs. Of the 31 overlapping variants, 15 had no statistically significant difference in the minor allele frequencies (MAFs) between ethnicities; nine variants had statistically significant higher MAFs in AAs compared to EAs (Table 1), and six variants had statistically significant higher MAFs in EAs (Table 2). Of the 65 variants that were unique to EAs, 62 were extremely rare with MAFs between 0.01 and 0.06% with no significant MAF difference between ethnicities, and three variants had a statistically significant higher MAF compared to AAs (Table 2). Regarding the 51 unique AA variants, 37 were extremely rare with MAFs between 0.02 and 0.04% with no significant MAF difference between ethnicities, and 14 variants had a statistically significant higher MAFs compared to EAs (Table 1).

Overall, EAs have a total of nine variants with a statistically significant higher MAF compared to AAs (Table 2). This included two noncoding and seven coding variants. The coding variants included six synonymous variants and a rare frameshift mutation. Interestingly, the frameshift mutation, p.(A59Qfs*54), results in a truncated protein of 111 amino acids, which is likely pathogenic, but currently not associated with any disease (Table 2). Furthermore, despite that the synonymous variants do not have an effect on the protein sequence, 83% (five out of the six) disrupt a CpG site (Table 2). Considering that CpG sites are known to be sites of DNA methylation that reduce gene expression when methylated, these variants could result in EA-specific TLR9 expression patterns.[47] Noteworthy, one of the synonymous variants is p.
Differences in TLR9 variants associated with breast cancer protection between European and African Americans

**Fig 2. Patient outcomes stratified by tumor TLR9 expression status.** a) Breast cancer recurrence probability stratified by median tumor TLR9 expression status, b) survival probability stratified by ipsilateral breast cancer (IBTR) and tumor TLR9 expression status and c) breast cancer specific-survival probability stratified by tumor TLR9 expression status \( (n = 43) \).

https://doi.org/10.1371/journal.pone.0183832.g002

\( (P545 =) \), which is also known as rs352140 (Table 2); despite not being recognized as having a clinical link in EVS, rs352140 has been previously reported to have a protective effect against breast cancer.\[25\] Considering that rs352140 is significantly more common in EAs than AAs (Table 2), EAs likely benefit more from such protective effects than AAs. This variant disrupts a CpG site, thus its effect on expression could play a role in disease susceptibility.

AAs have a total of 23 variants with a statistically significant higher MAF compared to EAs, including 12 non-synonymous (missense) and 11 synonymous variants (Table 1). Seven of the missense variants are located in the extracellular domain, and five are in the cytoplasmic domain. Four are predicted to have strong pathogenic effects through PolyPhen (Table 1) \[48\]; these are all extremely rare variants that could have detrimental effects on protein function. Of the 11 synonymous variants, 64% disrupt a CpG site. In fact, 70% of all the statistically significant AA variants affect a CpG site (Table 1). Overall, AAs had a significantly higher percentage of variants that had statistically significant higher MAFs compared to EAs \( (P = 6.43 \times 10^{-4}) \).

Furthermore, there was a significant difference in the total number of allele counts for all variant types between ethnicities (Table 3). Overall, EAs had more synonymous alleles compared to AAs but AAs had significantly more rare coding alleles (Table 3).

**TLR9 germline variants detected in TCGA AA breast cancer cases**

A total of 21 different germline TLR9 variants were detected in the 131 TCGA AA breast cancer cases (Table 4). This included eight missense and 13 synonymous variants. Only one of the missense variants (rs115440379) was predicted to be pathogenic, and it did not appear to be associated with breast cancer (Table 4 and S4 Table). Of the 13 synonymous variants, 8 disrupted a CpG site (Table 4); however, none appeared to be associated with breast cancer. Despite not being statistically significant, p.(P545 =) (rs352140) was observed even less frequently in the AA breast cancer cohort (Table 4 and S4 Table). Of the 13 synonymous variants, 8 disrupted a CpG site (Table 4); however, none appeared to be associated with breast cancer. Despite not being statistically significant, p.(P545 =) (rs352140) was observed even less frequently in the AA breast cancer cohort (Table 4 and S4 Table), which supports the previously reported protective effect.\[25\] Six of the breast cancer variants are common (MAF > 1%) in the general AA population with no statistical differences, and 15 are rare (MAF < 1%; Table 4 and S4 Table). Three of the latter were previously unreported and, individually, appeared to be associated with a later onset of breast cancer (diagnosed over the age of 45 years; Table 4 and S4 Table). Despite that aggregation analyses did not indicate a breast cancer association with particular TLR9 variant classes, there were more rare coding variants reported in individuals diagnosed over the age of 45 compared to under 45 years of age (Table 5).

**Discussion**

We described recently a novel, poor prognosis subtype in TNBC, as characterized by low tumor TLR9 expression upon diagnosis.\[15\] Specifically, patients whose TNBC tumors had low TLR9 expression levels upon diagnosis had significantly shorter breast cancer specific survival, as compared with those patients whose tumors had higher TLR9 protein expression levels.\[15\] This finding has since been independently verified by a group of French scientists.\[32\] Furthermore, these findings are not limited to TNBC as low TLR9 expression predicts poor survival also in renal cell carcinoma, pancreatic cancer and possibly also in mucoepidermoid salivary gland carcinoma.\[13, 31, 33\] Whether low tumor TLR9 expression is only a biomarker for poor survival, or whether it actually contributes to cancer pathophysiology in these tumors,
Table 1. Variants with a statistically significant MAF in AAs.

| Variant Status | rs ID          | Alleles | GVS Function | GMA Change | Protein Change | PolyPhen2 (Class: Clinical) | Link | EVS MAF (%) | EA, AA | Odds Ratio | 95% CI | P value |
|----------------|---------------|---------|--------------|------------|----------------|-----------------------------|------|-------------|--------|------------|--------|---------|
| A4 and EA     | 3:52223630    | G>A     | missense     | c.13C>T    | p.(R5C)       | benign:0.002                 |      | 0.12 CI     |        | 0.01       | 0.002  | 3.79    |
|               | 3:52223612    | G>A     | missense     | c.504C>T   | p.(A168 =)    | unknown unknown no effect    |      | 0.01 CI     |        | 0.01       | 0.001  | 2.20E-07|
|               | 3:52223591    | G>A     | coding-synonymous | c.1149G>A | p.(T383 =)    | unknown no effect            |      | 0.03 CI     |        | 0.001      | 0.000  | 9.56E-06|
|               | 3:52223419    | G>A     | missense     | c.696C>T   | p.(I232 =)    | unknown no effect            |      | 0.06 CI     |        | 0.01       | 0.006  | 5.46E-05|
|               | 3:52223397    | G>A     | missense     | c.2585G>A  | p.(G862E)     | unknown no effect            |      | 0.07 CI     |        | 0.01       | 0.009  | 2.10E-04|
|               | 3:52223295    | G>A     | missense     | c.2588G>A  | p.(R962H)     | probably-damaging:1.0        |      | 0.08 CI     |        | 0.01       | 0.018  | 4.07E-04|
|               | 3:52223204    | G>A     | missense     | c.2667C>T  | p.(L889 =)    | unknown unknown disrupt      |      | 0.09 CI     |        | 0.01       | 0.014  | 6.07E-04|
|               | 3:52222902    | G>A     | missense     | c.2885G>A  | p.(L204 =)    | unknown no effect            |      | 0.10 CI     |        | 0.01       | 0.016  | 1.48E-03|
|               | 3:52222686    | G>A     | missense     | c.3565G>A  | p.(N889 =)    | unknown unknown disrupt      |      | 0.11 CI     |        | 0.01       | 0.020  | 5.98E-05|
|               | 3:52222585    | G>A     | missense     | c.441C>A   | p.(H40 =)     | unknown no effect            |      | 0.12 CI     |        | 0.01       | 0.020  | 5.98E-05|
|               | 3:52221431    | G>A     | missense     | c.70C>T    | p.(L24 =)     | unknown unknown no effect    |      | 0.13 CI     |        | 0.01       | 0.020  | 5.98E-05|
|               | 3:52221321    | G>A     | missense     | c.120C>T   | p.(H40 =)     | unknown no effect            |      | 0.14 CI     |        | 0.01       | 0.020  | 5.98E-05|
|               | 3:52221246    | G>A     | missense     | c.1093G>A  | p.(A365T)     | possibly-damaging:0.62      |      | 0.15 CI     |        | 0.01       | 0.03   | 3.24E-02|
|               | 3:52221141    | G>A     | missense     | c.1715G>A  | p.(L571M)     | probably-damaging:0.992     |      | 0.16 CI     |        | 0.01       | 0.03   | 3.24E-02|
|               | 3:52220940    | G>A     | missense     | c.1885G>A  | p.(G629S)     | benign:0.022                 |      | 0.17 CI     |        | 0.01       | 0.03   | 3.24E-02|
|               | 3:52220735    | G>A     | missense     | c.2175G>C  | p.(E652K)     | probably-damaging:0.999     |      | 0.18 CI     |        | 0.01       | 0.03   | 3.24E-02|
|               | 3:52220234    | G>A     | missense     | c.2398G>A  | p.(Q799R)     | probably-damaging:0.987     |      | 0.19 CI     |        | 0.01       | 0.03   | 3.24E-02|

Differences in TLR9 variants associated with breast cancer protection between European and African Americans.
Table 2. Variants with a statistically significant MAF in EAs.

| Variant Status       | GRCh38 Position | rs ID | Alleles | GVS Function | cDNA Change | Protein Change | PolyPhen2 (Class:Score) | Clinical Link | CpG site | EVS MAF (%) | p values         | Odds Ratio | CI 95%          |
|----------------------|-----------------|-------|---------|--------------|-------------|----------------|-------------------------|---------------|-----------|--------------|----------------|------------|----------------|
| EA and AA            | 3:52224140      | unknown | R>A1    | frameshift   | c.175del1   | p. (A59Qfs*54) | unknown               | unknown       | no effect | 0.67         | 0.33            | 1.52E-02   | 0.49 [0.3–0.9] |
| Unique EA variants   | 3:5222789       | rs35342983 | C>T     | coding-     | c.1527G>A   | p.(S509 =)     | unknown               | unknown       | disrupt   | 0.40         | 0.07            | 6.51E-04   | 5.69 [1.8–29.0] |
|                      | 3:52222681      | rs352140  | C>T     | coding-     | c.1635G>A   | p.(P545 =)     | unknown               | unknown       | disrupt   | 55.17*       | 34.52*          | 2.20E-16   | 2.33 [2.2–2.5]  |
|                      | 3:52221802      | rs138032346 | G>A     | coding-     | c.2514C>T   | p.(L838 =)     | unknown               | unknown       | no effect | 0.33         | 0.07            | 3.60E-03   | 2.79 [1.5–24.7] |
|                      | 3:52221376      | rs445676  | G>A     | coding-     | c.2940C>T   | p.(Y980 =)     | unknown               | unknown       | disrupt   | 1.73         | 0.41            | 2.20E-16   | 25.74 [8.6–126.0] |
|                      | 3:52221199      | rs5743848 | C>G     | utr-3       | c.*18G>C    | NA             | unknown               | unknown       | no effect | 1.14         | 0.07            | 2.94E-14   | 16.9 [5.6–83.4]  |
|                      | 3:52225443      | rs373979034 | T>C     | intron      | c.3+84A>G   | NA             | unknown               | unknown       | no effect | 0.57         | 0              | 1.56E-07   | Inf [6.1-Inf]   |
|                      | 3:52222519      | rs143900156 | C>T     | coding-     | c.1797G>A   | p.(S599 =)     | unknown               | unknown       | disrupt   | 0.16         | 0              | 3.85E-03   | Inf [1.7-Inf]   |
|                      | 3:52222270      | rs143703479 | G>A     | coding-     | c.2046C>T   | p.(L682 =)     | unknown               | unknown       | disrupt   | 0.10         | 0              | 3.33E-02   | Inf [1.0-Inf]   |

Accession #: NM_017442
* used T allele

https://doi.org/10.1371/journal.pone.0183832.t002
is not currently known. Our studies in pre-clinical cancer models however demonstrated that despite slower in vitro growth, TNBC cells with low TLR9 expression formed in vivo significantly larger tumors than those with high TLR9 expression.[15] Notably, all our previous (Caucasian) cohorts consisted of patients of European ethnicity, from Northern and Eastern Finland.[15, 33] Ethnicity of the patients with results similar to ours was not disclosed in the French cohort.[32] Despite these promising results, tumor TLR9 expression is not currently used as a prognostic clinical tool. To be used as such will require further clinical studies, and preferably a demonstration that use of the TLR9 biomarker improves patient outcomes.

AA women with TNBC have a worse outcome than Caucasian women. The reason for this health discrepancy has remained unclear and controversial as both socioeconomic factors and tumor biology have been suggested as etiologic factors.[39, 49, 50] To investigate the possible prognostic significance of tumor TLR9 in AA TNBC, we compared tumor TLR9 staining intensity upon diagnosis with disease outcomes in this patient group. Surprisingly, unlike among Caucasian TNBC patient population,[15, 32] high tumor TLR9 protein expression did not protect from relapses among AA TNBC patients. In our previously published EA breast cancer cohort, ~ 90% of TNBC patients with high tumor TLR9 expression survived over 10 years. Of patients in the low TLR9 group, ~ 40% died within the first 5 years.[15] The survival gap between the high and low tumor TLR9 TNBC groups was even greater in the French study.[32] It is currently unclear how high tumor TLR9 expression protects from relapses in various cancers and especially in TNBC. The mechanisms involved could include effects on tumor immunity, tumor invasion, or autophagy. [3, 10, 15, 51] There may however be, SNP-based modifications of the effect of TLR9 on these cellular processes among AA TNBC patients. This could explain the lack of protection that tumor TLR9 provides in other ethnic groups. These issues will, however, require further detailed experimentation at the cellular and molecular level.

In 2003, Lazarus et al. reported differences in TLR9 genetic variation between various ethnic groups.[52] Such differences might result in ethnic-specific TLR9 function or expression patterns that could, ultimately, also contribute towards disease susceptibility, progression, or

---

**Table 3. Comparison of allele counts between ethnicities for different variant classes.**

| Variant class                  | Cohort       | Number of minor alleles reported | p value   | Odds ratio |
|-------------------------------|--------------|---------------------------------|-----------|------------|
|                               | Ethnicity    | Size                            |           |            |
| Total coding                  | EA           | 8600                            | 5242      | 2.35E-05   | 1.13 Cl_{95}[1.1–1.2] |
|                               | AA           | 4406                            | 2356      |            |            |
| Rare coding                   | EA           | 8600                            | 348       | 1.01E-11   | 0.58 Cl_{95}[0.5–0.7] |
|                               | AA           | 4406                            | 310       |            |            |
| Missense                      | EA           | 8600                            | 95        | 2.20E-16   | 0.11 Cl_{95}[0.1–0.1] |
|                               | AA           | 4406                            | 463       |            |            |
| Rare missense*                | EA           | 8600                            | 95        | 4.54E-16   | 0.34 Cl_{95}[0.3–0.4] |
|                               | AA           | 4406                            | 143       |            |            |
| Probably damaging missense    | EA           | 8600                            | 16        | 2.20E-16   | 0.12 Cl_{95}[0.1–0.2] |
|                               | AA           | 4406                            | 65        |            |            |
| Synonymous                    | EA           | 8600                            | 5070      | 2.20E-16   | 1.38 Cl_{95}[1.3–1.5] |
|                               | AA           | 4406                            | 1874      |            |            |
| Rare Synonymous*              | EA           | 8600                            | 176       | 1.41E-05   | 0.61 Cl_{95}[0.5–0.8] |
|                               | AA           | 4406                            | 148       |            |            |

*MAF less than or equal to 1%

https://doi.org/10.1371/journal.pone.0183832.t003
| Position | Accession #: NM_017442 | GRCh38 cDNA | Protein Change | PolyPhen2 change site | Position | TCGA BC (n = 131) | EVS AA—45 years at diagnosis (n = 21) | TCGA BC—45 years at diagnosis (n = 110) | EVS AA—45 years at diagnosis (n = 2203) | Odds ratio | p value | Odds ratio | p value | Odds ratio | p value | Odds ratio | p value | Odds ratio | p value |
|----------|-------------------------|-------------|----------------|-----------------------|----------|------------------|----------------------------------------|------------------------------------------|------------------------------------------|------------|----------|------------|----------|------------|---------|------------|---------|------------|---------|
| c.13C>T  | GRch38:c.13C>T          | missense    | R5C            | benign:0.002          | disrupt  | 2.67             | 4.76                                   | 2.27                                      | 0.673                                     | 0.47      | 0.35    | 1.5        | 0.15     | 0.673                      |
| c.70C>T  | GRch38:c.70C>T          | missense    | I114 =) unknown | affect                | disrupt  | 0.38             | 0.00                                   | 0.45                                      | 0.32                                     | 0.357     | 0.59    | 0.45        | 0.32     | 0.357                      |
| c.342C>T | GRch38:c.342C>T         | missense    | D175 =) unknown | affect                | disrupt  | 0.38             | 0.00                                   | 0.45                                      | 0.32                                     | 0.357     | 0.59    | 0.45        | 0.32     | 0.357                      |
| c.504C>T | GRch38:c.504C>T         | missense    | D175 =) unknown | affect                | disrupt  | 0.38             | 0.00                                   | 0.45                                      | 0.32                                     | 0.357     | 0.59    | 0.45        | 0.32     | 0.357                      |
| c.747C>T | GRch38:c.747C>T         | missense    | V233I          | benign:0.010          | disrupt  | 0.38             | 0.00                                   | 0.45                                      | 0.32                                     | 0.357     | 0.59    | 0.45        | 0.32     | 0.357                      |
| c.1635G>A| GRch38:c.1635G>A        | missense    | L830 =) unknown | affect                | disrupt  | 0.38             | 0.00                                   | 0.45                                      | 0.32                                     | 0.357     | 0.59    | 0.45        | 0.32     | 0.357                      |
| c.2490G>C| GRch38:c.2490G>C        | missense    | F873 =) unknown | affect                | disrupt  | 0.38             | 0.00                                   | 0.45                                      | 0.32                                     | 0.357     | 0.59    | 0.45        | 0.32     | 0.357                      |
| c.2593G>A| GRch38:c.2593G>A        | missense    | E865K          | benign:0.040          | disrupt  | 4.20             | 2.38                                   | 4.55                                      | 3.52                                     | 0.495     | 1.20   | 3.55        | 2.38     | 0.495                      |
| c.2698G>A| GRch38:c.2698G>A        | missense    | R704W          | probably-damaging:0.990 | disrupt  | 0.38             | 0.00                                   | 0.45                                      | 0.32                                     | 0.357     | 0.59    | 0.45        | 0.32     | 0.357                      |

Note: Differences in TLR9 variants associated with breast cancer protection between European and African Americans.
Table 5. Comparison of allele counts between AA cases and controls for different variant classes.

| Variant class | Number of minor alleles reported | TCGA BC cohort (n = 131) | TCGA BC cohort > 45 years at diagnosis (n = 21) | EVS AA - Population controls (n = 2203) | Comparing population controls (n = 2203) to TCGA BC cohorts | Comparing TCGA cohorts ≤ 45 years at diagnosis to > 45 years at diagnosis |
|---------------|---------------------------------|--------------------------|-----------------------------------------------|----------------------------------------|----------------------------------------------------------|-------------------------------------------------------------------------|
|               |                                 | TCGA BC cohort ≤ 45 years at diagnosis (n = 110) | TCGA BC cohort > 45 years at diagnosis (n = 110) | EVS AA - Population controls (n = 2203) | Comparing population controls (n = 2203) to TCGA BC cohorts | Comparing TCGA cohorts ≤ 45 years at diagnosis to > 45 years at diagnosis |
|               |                                 | p value                   | Odds ratio                                    | p value                                 | Odds ratio                                                  | p value                                 | Odds ratio                                    |
| Total         | Coding                          | 0.411                     | 0.90 CI[0.7–1.1]                             | 0.592                                  | 0.85 CI[0.5–1.2]                                           | 0.514                                  | 0.92 CI[0.7–1.2]                             | 0.882                                  | 1.08 CI[0.8–Inf]                             |
|               | Missense                        | 0.755                     | 0.81 CI[0.6–1.4]                             | 0.796                                  | 0.84 CI[0.5–1.5]                                           | 0.911                                  | 0.95 CI[0.6–1.5]                             | 0.777                                  | 1.40 CI[0.4–7.6]                             |
|               | Synonymous                      | 0.480                     | 0.91 CI[0.7–1.2]                             | 0.775                                  | 0.90 CI[0.5–1.6]                                           | 0.565                                  | 0.92 CI[0.7–1.2]                             | 1.000                                  | 1.02 CI[0.5–2.1]                             |
| Rare*         | Coding                          | 1.000                     | 0.98 CI[0.6–1.6]                             | 0.111                                  | 0.00 CI[0.3–1.3]                                           | 0.505                                  | 1.16 CI[0.7–1.9]                             | 0.085                                  | Inf CI[0.8–Inf]                              |
|               | Missense                        | 0.856                     | 0.82 CI[0.3–1.8]                             | 0.641                                  | 0.98 CI[0.2–3.1]                                           | 1.000                                  | 0.98 CI[0.4–2.1]                             | 0.601                                  | Inf CI[0.5–Inf]                              |
|               | Synonymous                      | 0.482                     | 1.25 CI[0.8–2.3]                             | 0.646                                  | 0.00 CI[0.0–2.8]                                           | 0.191                                  | 1.49 CI[0.7–2.8]                             | 0.224                                  | 1.08 CI[0.8–Inf]                             |
| Common**      | Coding                          | 0.359                     | 0.90 CI[0.7–1.1]                             | 1.000                                  | 0.97 CI[0.5–1.7]                                           | 0.348                                  | 0.88 CI[0.7–1.2]                             | 0.760                                  | 0.90 CI[0.5–1.7]                             |
|               | Missense                        | 0.903                     | 0.95 CI[0.5–1.6]                             | 1.000                                  | 0.98 CI[0.2–3.1]                                           | 1.000                                  | 0.94 CI[0.5–1.6]                             | 1.000                                  | 0.95 CI[0.3–5.4]                             |
|               | Synonymous                      | 0.361                     | 0.89 CI[0.7–1.1]                             | 1.000                                  | 0.97 CI[0.5–1.8]                                           | 0.320                                  | 0.87 CI[0.7–1.1]                             | 0.744                                  | 0.90 CI[0.5–1.8]                             |
| Pathogenic    | Missense                        | 0.269                     | 0.25 CI[0.0–0.5]                             | 1.000                                  | 0.00 CI[0.0–0.6]                                           | 0.373                                  | 0.31 CI[0.0–1.8]                             | 1.000                                  | Inf CI[0.0–Inf]                              |

*MAF less than or equal to 1%
**MAF greater than 1%

https://doi.org/10.1371/journal.pone.0183832.t005
survival. We therefore carried out a similar but larger effort to understand TLR9 genetic differences between EAs and AAs. We indeed detected differences. Thirty-two TLR9 variants had a statistically significant MAF difference between AAs and EAs, most of which have a higher MAF in AAs. Most of the detected differences are predicted to affect CpG-methylation, and thereby, possibly gene expression.[47] Some of the missense variants with statistically significant MAF differences between the ethnicities are predicted to have profound effects on the TLR9 protein and function. Overall, rare missense alleles are more frequently present in AAs compared to EAs; these rare variants could be related to ethnic-specific disease risk. The most notable finding is the MAF distribution of rs352140. This TLR9 variant has been previously associated with a decreased breast cancer risk.[25] Specifically, the T allele was suggested to be protective of breast cancer in a recent meta-analysis consisting of 12,197 cancer cases and 13,488 controls. [25] We identified a significantly higher T allele frequency in EAs compared to AAs (55.17 vs. 34.52, p = 2.20E-16), suggesting EAs benefit more from the T allele’s protective effects than AAs. This synonymous variant disrupts a CpG site; thus its effect on expression could play a role in disease susceptibility. The mechanism of how the T allele of rs352140 might protect from breast cancer has been investigated, but it is currently debatable.[29, 52–55] In fact, rs352140 has even been associated with an increased risk of certain cancers.[56] Further investigation is required to more clearly understand rs352140 and cancer-specific risk. Noteworthy, through our case/control analysis, we detected even less T alleles in AA breast cancer cases compared to AA controls, which is expected if the allele is protective against breast cancer; however, our results were not statistically significant.

Our efforts to test an association of germline TLR9 variants with AA breast cancer risk did not reveal any variant class differences between cases and controls; however, three previously unreported TLR9 variants appears to be slightly associated with late (>45 years) onset breast cancer. These included two missense (p.(D864N) and p.(E865K)) and one synonymous (p. (F873 =)) variants and are predicted to be benign. Furthermore, only the synonymous variant affects a CpG site. Each variant was detected in one breast cancer case in the cohort; thus, these findings need to be replicated. Furthermore, our AA breast cancer cohort did not specifically address TNBC cases, due to limited clinical information available for TCGA data. Therefore, genetic associations specifically with TNBC are warranted. In addition to assessing breast cancer risk, also the contribution of these SNPs to breast cancer specific-survival requires further studies.

It is currently unclear what molecular events drive the expression of the TLR9 variants. Especially the rs352140 variant has been, however, associated with infections such as malaria and meningitis. [53, 57] Specifically, the minor T allele has been associated with increased inflammation and thereby increased symptoms of these infectious diseases, possibly indicating a stronger immune reaction, as also noted in placental inflammation.[29, 58] Whether or not such increased inflammatory response also protects from the development of breast cancer remains to be characterized. Conversely, the weaker immune response, and thereby lesser symptoms may have initially provided an evolutionary survival advantage, possibly explaining the enrichment of such variant in people with African ancestry. Taken together, these hypotheses require further studies.

In conclusion, unlike among Caucasian TNBC patients, high tumor TLR9 protein expression is not associated with improved survival among AA TNBC patients. This may be due to differences in TLR9 function or expression, caused by variants in the TLR9 gene. Our results show that EAs more frequently carry the T allele of rs352140 when compared to AAs, which has been associated with protection from breast cancer. Thus, this TLR9 variant may be a previously unknown source of health disparity in breast cancer. Our results need further
confirmation, especially in TNBC cohorts. They also call for more in depth studies on the molecular mechanisms of how TLR9 could affect breast cancer development and treatment responses.

Supporting information

S1 Table. Immunohistochemical TLR9 staining scores and the associated clinical data for AA TNBC patients.
(XLSX)

S2 Table. Clinical and sequencing file information of the 131 AA TCGA breast cancer cases.
(DOCX)

S3 Table. Descriptive and clinico-pathological parameters of triple-negative breast cancer patients.
(DOCX)

S4 Table. Genotypes and MAFs of TLR9 germline coding variants detected in AA BC-affected individuals in the TCGA and population controls.
(DOCX)

Acknowledgments

This study was supported by grants from Breast Cancer Research Foundation of Alabama (K. S.S.), Maud Kuistila memorial foundation (J.T), Orion-Farmos Research foundation (J.T), Turku University foundation (J.T), Finnish Cultural foundation (J.T), Jane and Aatos Erkko Foundation (J.T.) and K. Albin Johansson foundation (J.T). The results shown here are in part based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov/.

Author Contributions

Conceptualization: Katri S. Selander.

Data curation: Kimberly S. Keene, Nancy D. Merner, Katri S. Selander.

Formal analysis: Madison R. Chandler, Kimberly S. Keene, Johanna M. Tuomela, Renee Desmond, Nancy D. Merner, Katri S. Selander.

Funding acquisition: Kimberly S. Keene, Johanna M. Tuomela, Andres Forero-Torres, Katri S. Selander.

Investigation: Madison R. Chandler, Kimberly S. Keene, Johanna M. Tuomela, Andres Forero-Torres, Kevin W. Harris, Katri S. Selander.

Methodology: Kimberly S. Keene, Johanna M. Tuomela, Renee Desmond, Katri S. Vuopala, Katri S. Selander.

Resources: Katri S. Selander.

Supervision: Kimberly S. Keene, Katri S. Vuopala, Katri S. Selander.

Writing – original draft: Renee Desmond, Katri S. Vuopala, Kevin W. Harris, Nancy D. Merner, Katri S. Selander.

Writing – review & editing: Kevin W. Harris, Nancy D. Merner, Katri S. Selander.
Differences in TLR9 variants associated with breast cancer protection between European and African Americans

References

1. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A Toll-like receptor recognizes bacterial DNA. Nature. 2000; 408(6813):740–5. Epub 2000/12/29. https://doi.org/10.1038/35047123 PMID: 11130078.

2. Lamphier MS, Siros CM, Verma A, Golenbock DT, Latz E. TLR9 and the recognition of self and non-self nucleic acids. Ann N Y Acad Sci. 2006; 1082:31–43. https://doi.org/10.1196/annals.1348.005 PMID: 17145922.

3. Tuomela J, Sandholm J, Kaakinen M, Patel A, Kauppila JH, Ilvesaro J, et al. DNA from dead cancer cells induces TLR9-mediated invasion and inflammation in living cancer cells. Breast Cancer Res Treat. 2013; 142(3):477–87. https://doi.org/10.1007/s10549-013-2762-0 PMID: 24212717; PubMed Central PMCID: PMC4238912.

4. Kaisho T, Akira S. Toll-like receptor function and signaling. The Journal of allergy and clinical immunology. 2006; 117(5):979–87; quiz 88. Epub 2006/05/06. doi: S0091-6749(06)00439-8 [pii] https://doi.org/10.1016/j.jaci.2006.02.023 PMID: 16675322.

5. Marshak-Rothstein A, Rifkin IR. Immunologically active autoantigens: the role of toll-like receptors in the development of chronic inflammatory disease. Annu Rev Immunol. 2007; 25:419–41. https://doi.org/10.1146/annurev.immunol.25.022806.094514 PMID: 17378763.

6. Wagner H. Interactions between bacterial CpG-DNA and TLR9 bridge innate and adaptive immunity. Current opinion in microbiology. 2002; 5(1):62–9. Epub 2002/02/09. PMID: 11834371.

7. Paul WE. Bridging innate and adaptive immunity. Cell. 2011; 147(6):1212–5. Epub 2011/12/14. https://doi.org/10.1016/j.cell.2011.11.036 PMID: 22153065.

8. Chen GY, Nunez G. Sterile inflammation: sensing and reacting to damage. Nature reviews Immunology. 2010; 10(12):826–37. Epub 2010/11/23. https://doi.org/10.1038/nri2873 PMID: 21088693; PubMed Central PMCID: PMC3114424.

9. Hoque R, Malik AF, Gorelick F, Mehdi WZ. Sterile inflammatory response in acute pancreatitis. Pancreas. 2012; 41(3):353–7. Epub 2012/03/15. https://doi.org/10.1097/MPA.0b013e3182321500 PMID: 22415665; PubMed Central PMCID: PMC3306133.

10. Ilvesaro JM, Merrell MA, Swain TM, Davidson J, Zayzafoon M, Harris KW, et al. Toll like receptor-9 agonists stimulate prostate cancer invasion in vitro. Prostate. 2012; 41(3):353–7. Epub 2012/03/15. https://doi.org/10.1002/pros.20562 PMID: 17373717.

11. Kauppila JH, Takala H, Selander KS, Lehenkari PP, Saamio J, Karttunen TJ. Increased Toll-like receptor 9 expression indicates adverse prognosis in oesophageal adenocarcinoma. Histopathology. 2011; 59(4):643–9. Epub 2011/10/22. https://doi.org/10.1111/j.1365-2559.2011.03991.x PMID: 22014045.

12. Merrell MA, Ilvesaro JM, Lehtonen N, Sorsa T, Gehrs B, Rosenthal E, et al. Toll-like receptor 9 agonists promote cellular invasion by increasing matrix metalloproteinase activity. Mol Cancer Res. 2006; 4(7):437–47. https://doi.org/10.1159/000329115 PMID: 16849519.

13. Ronkainen H, Hirviko斯基 P, Kauppila S, Vuopala KS, Paavonen TK, Selander KS, et al. Absent Toll-like receptor-9 expression predicts poor prognosis in renal cell carcinoma. J Exp Clin Cancer Res. 2011; 30:84. https://doi.org/10.1186/1756-9966-30-84 PMID: 21929816; PubMed Central PMCID: PMCPMC3182949.

14. Takala H, Kauppila JH, Soin Y, Selander KS, Vuopala KS, Lehenkari PP, et al. Toll-like receptor 9 is a novel biomarker for esophageal squamous cell dysplasia and squamous cell carcinoma progression. J Innate Immun. 2011; 3(6):631–8. Epub 2011/08/31. https://doi.org/10.1159/000329115 PMID: 21876325.

15. Tuomela J, Sandholm J, Karihtala P, Ilvesaro J, Vuopala KS, Kauppila JH, et al. Low TLR9 expression defines an aggressive subtype of triple-negative breast cancer. Breast Cancer Res Treat. 2012; 135 (2):481–93. https://doi.org/10.1007/s10549-012-2181-7 PMID: 22847512.

16. Vaisanen MR, Vaisanen T, Jukkola-Vuorinen A, Vuopala KS, Desmond R, Selander KS, et al. Expression of toll-like receptor-9 is increased in poorly differentiated prostate tumors. Cancer Sci. 2010; 101 (4):1059–66. Epub 2010/02/17. https://doi.org/10.1111/j.1349-7006.2010.01491.x PMID: 20156214; PubMed Central PMCID: PMC3188854.

17. Berger R, Fieg H, Goebel G, Obexer P, Ausserlechner M, Doppler W, et al. Toll-like receptor 9 expression in breast and ovarian cancer is associated with poorly differentiated tumors. Cancer Sci, 2010; 101 (4):1059–66. Epub 2010/02/17. https://doi.org/10.1111/j.1349-7006.2010.01491.x PMID: 20156214; PubMed Central PMCID: PMC3188854.
Differences in TLR9 variants associated with breast cancer protection between European and African Americans

Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y, et al. Identification of human TLR9 variants associated with breast cancer protection between European and African Americans

Zhang YB, He FL, Fang M, Hua TF, Hu BD, Zhang ZH, et al. Increased expression of Toll-like receptors 4 and 9 in human lung cancer. Mol Biol Rep. 2009; 36(6):1475–81. Epub 2008/09/03. https://doi.org/10.1007/s11033-008-9338-9 PMID: 18763053.

Di JM, Pang J, Pu XY, Zhang Y, Liu XP, Fang YQ, et al. Toll-like receptor 9 agonists promote IL-8 and TGF-beta1 production via activation of nuclear factor kappB in PC-3 cells. Cancer Genet Cytoenet. 2009; 192(2):60–7. Epub 2009/07/15. https://doi.org/10.1016/j.cancergeny.2009.03.006 PMID: 19596255.

Mukherjee S, Siddiqui MA, Dayal S, Ayoub YZ, Malathi K. Epigallocatechin-3-gallate suppresses proinflammatory cytokines and chemokines induced by Toll-like receptor 9 agonists in prostate cancer cells. Journal of inflammation research. 2014; 7:89–101. Epub 2014/06/28. https://doi.org/10.2147/JIR.S61395 PMID: 24971028; PubMed Central PMCID: PMC4070858.

Ilvesaro JM, Merrell MA, Li L, Wackchoure S, Graves D, Brooks S, et al. Toll-like receptor 9 mediates CpG oligonucleotide-induced cellular invasion. Mol Cancer Res. 2008; 6(10):1534–43. https://doi.org/10.1186/1475-2875-11-168 PMID: 22594374; PubMed Central PMCID: PMC3426492.

Nurmenniemi S, Kuvaja P, Lehtonen S, Tiuraniemä S, Alahuhta I, Mattila RK, et al. Toll-like receptor 9 ligands enhance mesenchymal stem cell invasion and expression of matrix metalloproteinase-13. Exp Cell Res. 316(16):2676–82. Epub 2010/06/18. doi: S0014-4827(10)00287-9 [pii] https://doi.org/10.1016/j.yexcr.2010.05.021 PMID: 20563713.

Sandholm J, Tuomela J, Kauppila JH, Harris KW, Graves D, Selander KS. Hypoxia regulates Toll-like receptor-9 expression and invasive function in human brain cancer cells in vitro. Oncology letters. 2014; 8(1):266–74. Epub 2014/06/25. https://doi.org/10.3892/ol.2014.2095 PMID: 24959258; PubMed Central PMCID: PMC4063648.

Wan GX, Cao YW, Li WQ, Li YC, Zhang WJ, Li F. Associations between TLR9 polymorphisms and cancer risk: evidence from an updated meta-analysis of 25,685 subjects. Asian Pac J Cancer Prev. 2014; 15(19):8279–85. PMID: 25339018.

Lange NE, Zhou X, Lasky-Su J, Himes BE, Lazarus R, Soto-Quirós M, et al. Comprehensive genetic assessment of a functional TLR9 promoter polymorphism: no replicable association with asthma or asthma-related phenotypes. BMC medical genetics. 2011; 12:26. Epub 2011/02/18. https://doi.org/10.1186/1471-2350-12-26 PMID: 21324137; PubMed Central PMCID: PMC3048492.

Kubarenko AV, Ranjan S, Rautanen A, Mills TC, Wong S, Vannberg F, et al. A naturally occurring variant in human TLR9, P99L, is associated with loss of Cpg oligonucleotide responsiveness. J Biol Chem. 2010; 285(47):36486–94. Epub 2010/09/17. https://doi.org/10.1074/jbc.M110.117200 PMID: 20843814; PubMed Central PMCID: PMC2978577.

Carvalho A, Osorio NS, Saraiva M, Cunha C, Almeida AJ, Teixeira-Coelho M, et al. The C allele of rs5743836 polymorphism in the human TLR9 promoter links IL-6 and TLR9 up-regulation and confers increased B-cell proliferation. PLoS One. 2011; 6(11):e28256. Epub 2011/12/02. https://doi.org/10.1371/journal.pone.0028256 PMID: 22132241; PubMed Central PMCID: PMC3223238.

Omar AH, Yasunami M, Yamazaki A, Shibata H, Ofori MF, Akanmori BD, et al. Toll-like receptor 9 (TLR9) polymorphism associated with symptomatic malaria: a cohort study. Malar J. 2012; 11:168.

Vaisanen MR, Jukkola-Vuorinen A, Vuopala KS, Selander KS, Vaarala MH. Expression of Toll-like receptor-9 is associated with poor progression-free survival in prostate cancer. Oncol Lett. 2013; 5(5):1659–63. https://doi.org/10.3892/ol.2013.1204 PMID: 23761830; PubMed Central PMCID: PMCPMC3678868.

Korvala J, Harjula T, Siirila K, Almanghash A, Aro K, Maktilie AA, et al. Toll-like receptor 9 expression in mucoepidermoid salivary gland carcinoma may associate with good prognosis. J Oral Pathol Med. 2014; 43(19):827–9. PMID: 25339018.

Meseure D, Vacher S, Drak Alsiabi K, Trassard M, Nicolas A, Leclere R, et al. Biopathological Significance of TLR9 Expression in Cancer Cells and Tumor Microenvironment Across Invasive Breast Carcinomas Subtypes. Cancer Microenviron. 2016; 9(2–3):107–18. https://doi.org/10.1007/s12307-016-0186-1 PMID: 27392414.

Leppanen J, Helminen O, Huhta H, Kauppila JH, Isohookana J, Haapasaari KM, et al. High toll-like receptor (TLR) 9 expression is associated with better prognosis in surgically treated pancreatic cancer patients. Virchows Arch. 2017. https://doi.org/10.1007/s00428-017-2087-1 PMID: 28191612.

Carotenuto P, Roma C, Raffigliò AM, Botti G, D’Alessio A, Normanno N. Triple negative breast cancer: from molecular portrait to therapeutic intervention. Critical reviews in eukaryotic gene expression. 2010; 20(1):17–34. Epub 2010/06/10. PMID: 20528735.

Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. J Clin...
Invest. 121(7):2750–67. Epub 2011/06/03. doi: 10.1172/JCI45014 PMID: 21633166; PubMed Central PMCID: PMC3127435.

36. Anders CK, Carey LA. Biology, metastatic patterns, and treatment of patients with triple-negative breast cancer. Clin Breast Cancer. 2009; 9 Suppl 2:S73–81. https://doi.org/10.3816/CBC.2009.s.008 PMID: 19596646; PubMed Central PMCID: PMCPMC2919761.

37. Sturtz LA, Melley J, Mamula K, Shriver CD, Ellsworth RE. Outcome disparities in African American women with triple negative breast cancer: a comparison of epidemiological and molecular factors between African American and Caucasian women with triple negative breast cancer. BMC Cancer. 2014; 14:62. https://doi.org/10.1186/1471-2407-14-62 PMID: 24495414; PubMed Central PMCID: PMCPMC3916697.

38. Loo LW, Wang Y, Flynn EM, Lund MJ, Bowles EJ, Buist DS, et al. Genome-wide copy number alterations in subtypes of invasive breast cancers in young white and African American women. Breast Cancer Res Treat. 2011; 127(1):297–308. Epub 2011/01/26. https://doi.org/10.1007/s10549-010-1297-x PMID: 21264507; PubMed Central PMCID: PMC3224104.

39. Stewart PA, Luks J, Roycik MD, Sang QX, Zhang J. Differentially expressed transcripts and dysregulated signaling pathways and networks in African American breast cancer. PLoS One. 2013; 8(12):e82460. https://doi.org/10.1371/journal.pone.0082460 PMID: 24324792; PubMed Central PMCID: PMCPMC3853650.

40. Jukkola-Vuorinen A, Rahko E, Vuopala KS, Desmond R, Lehenkari PP, Harris KW, et al. Toll-like receptor-9 expression is inversely correlated with estrogen receptor status in breast cancer. J Innate Immun. 2009; 1(1):59–68. https://doi.org/10.1159/000151602 PMID: 20375966.

41. U.S. Department of Health and Human Services NIH, National Cancer Institute, USA.gov. Genomic Data Commons Data Portal

42. Grossman RL, Heath AP, Ferretti V, Varmus HE, Lowy DR, Kibbe WA, et al. Toward a Shared Vision for Cancer Genomic Data. N Engl J Med. 2016; 375(12):1109–12. https://doi.org/10.1056/NEJMp1607591 PMID: 27653561.

43. U.S. Department of Health and Human Services NIH, National Cancer Institute, USA.gov. GDC Data Transfer Tool.

44. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 2010; 20(9):1297–303. https://doi.org/10.1101/gr.107524.110 PMID: 20644199; PubMed Central PMCID: PMCPMC2928508.

45. Danecek P, Auton A, Abecasis G, Albers CA, Banks E, DePristo MA, et al. The variant call format and VCFtools. Bioinformatics. 2011; 27(15):2156–8. https://doi.org/10.1093/bioinformatics/btr330 PMID: 21653522; PubMed Central PMCID: PMCPMC3137218.

46. Li H. Tabix: fast retrieval of sequence features from generic TAB-delimited files. Bioinformatics. 2011; 27(5):718–9. https://doi.org/10.1093/bioinformatics/btq671 PMID: 21208982; PubMed Central PMCID: PMCPMC3042176.

47. Long MD, Smiraglia DJ, Campbell MJ. The Genomic Impact of DNA CpG Methylation on Gene Expression: Relationships in Prostate Cancer. Biomolecules. 2017; 7(1). https://doi.org/10.3390/biom7010015 PMID: 28216563.

48. Adzhubei I, Jordan DM, Sunyaev SR. Predicting functional effect of human missense mutations using PolyPhen-2. Curr Protoc Hum Genet. 2013;Chapter 7:Unit7.20. https://doi.org/10.1002/0471142905.hg0720s76 PMID: 23319928; PubMed Central PMCID: PMCPMC4486030.

49. Hyslop T, Michael Y, Avery T, Fui H. Population and target considerations for triple-negative breast cancer clinical trials. Biomarkers in medicine. 2013; 7(1):11–21. Epub 2013/02/08. https://doi.org/10.2217/bmm.12.114 PMID: 23387481; PubMed Central PMCID: PMCPMC3670335.

50. Ogden A, Garlapi C, Li XB, Turaga RC, Oprea-Ilies G, Wright N, et al. Multi-institutional study of nuclear KIFC1 as a biomarker of poor prognosis in African American women with triple-negative breast cancer. Sci Rep. 2017; 7:42289. https://doi.org/10.1038/srep42289 PMID: 28216233; PubMed Central PMCID: PMCPMC5316996.

51. Lim JS, Kim HS, Nguyen KC, Cho KA. The role of TLR9 in stress-dependent autophagy formation. Biochem Biophys Res Commun. 2016; 468(3–4):219–26. https://doi.org/10.1016/j.bbrc.2016.10.105 PMID: 27793667.

52. Lazarus R, Klimecki WT, Raby BA, Vercelli D, Palmer LJ, Kwiatkowski DJ, et al. Single-nucleotide polymorphisms in the Toll-like receptor 9 gene (TLR9): frequencies, pairwise linkage disequilibrium, and haplotypes in three U.S. ethnic groups and exploratory case-control disease association studies. Genomics. 2003; 81(1):85–91. PMID: 12573264.
53. Wang XH, Shi HP, Li FJ. Association between Toll-like receptor 9 gene polymorphisms and risk of bacterial meningitis in a Chinese population. Genet Mol Res. 2016; 15(3). https://doi.org/10.4238/gmr.15037641 PMID: 27525854.

54. Chen KH, Zeng L, Gu W, Zhou J, Du DY, Jiang JX. Polymorphisms in the toll-like receptor 9 gene associated with sepsis and multiple organ dysfunction after major blunt trauma. Br J Surg. 2011; 98 (9):1252–9. https://doi.org/10.1002/bjs.7532 PMID: 21633947.

55. Kwiatkowski DP. How malaria has affected the human genome and what human genetics can teach us about malaria. Am J Hum Genet. 2005; 77(2):171–92. https://doi.org/10.1086/432519 PMID: 16001361; PubMed Central PMCID: PMCPMC1224522.

56. Zhang L, Qin H, Guan X, Zhang K, Liu Z. The TLR9 gene polymorphisms and the risk of cancer: evidence from a meta-analysis. PLoS One. 2013; 8(8):e71785. https://doi.org/10.1371/journal.pone.0071785 PMID: 23990988; PubMed Central PMCID: PMCPMC3747197.

57. Zhang P, Zhang N, Liu L, Zheng K, Zhu L, Zhu J, et al. Polymorphisms of toll-like receptors 2 and 9 and severity and prognosis of bacterial meningitis in Chinese children. Sci Rep. 2017; 7:42796. https://doi.org/10.1038/srep42796; PubMed Central PMCID: PMCPMC5311876.

58. Karody V, Reese S, Kumar N, Liedel J, Jarzembowski J, Sampath V. A toll-like receptor 9 (rs352140) variant is associated with placental inflammation in newborn infants. J Matern Fetal Neonatal Med. 2016; 29(13):2210–6. https://doi.org/10.3109/14767058.2015.1081590 PMID: 26371589; PubMed Central PMCID: PMCPMC5238957.