**Biomarker Insights**

**ORIGINAL RESEARCH**

**Variants in the Signaling Protein TSAd are Associated with Susceptibility to Ovarian Cancer in BRCA1/2 Negative High Risk Families**

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**Abstract:** A substantial fraction of familial ovarian cancer cases cannot be attributed to specific genetic factors. The discovery of additional susceptibility genes will permit a more accurate assessment of hereditary cancer risk and allow for monitoring of predisposed women in order to intervene at the earliest possible stage. We focused on a population with elevated familial breast and ovarian cancer risk. In this study, we identified a SNP rs926103 whose minor allele is associated with predisposition to ovarian but not breast cancer in a Caucasian high-risk population without BRCA1/BRCA2 mutations. We have found that the allelic variation of rs926103, which alters amino acid 52 of the encoded protein SH2D2A/TSAd, results in differences in the activity of this protein involved in multiple signal transduction pathways, including regulation of immune response, tumor vascularization, cell growth, and differentiation. Our observation provides a novel candidate genetic biomarker of elevated ovarian cancer risk in members of high-risk families without BRCA1/2 mutations, as well as a potential therapeutic target, TSAd.

**Keywords:** ovarian cancer, familial cancer predisposition, cancer genetics, single nucleotide polymorphism, signaling pathway

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Introduction
Breast and ovarian cancers are among the leading causes of cancer-related death in the Western female population.\(^1\) Some of these cancer cases are linked to hereditary risk factors, although most are considered sporadic. Despite progress made in this area of research, most familial risk of ovarian cancer still remains unattributed to any particular genetic loci.\(^2\) The chances of long-term patient survival are higher when the cancer is discovered at an early stage of the disease. This is especially true for ovarian cancer, with a more than 90% 5-year survival rate in cases when the disease is detected at an early stage, as opposed to less than 30% chance when detected at stages 3 and 4 due to the absence of definitive symptoms.\(^3,4\)

Screening of asymptomatic populations is an effective way of early detection of different malignant lesions, including breast and ovarian cancer,\(^5\) and as a result persons who have been identified to be at high risk of cancer due to familial factors should be regularly screened to allow early disease detection. Intensive surveillance as well as risk-reduction procedures (such as prophylactic mastectomy or oophorectomy) have been implemented for women at high risk of breast and ovarian cancer as potentially life-saving health-care strategies.\(^6\)

Modifiers of cancer risk can be genetic changes or epigenetic modifications such as DNA methylation at CpG islands, which have been implicated in the development of malignancies because they alter gene expression, usually by promoter silencing.\(^7,8\) In order to identify new biomarkers of elevated cancer risk, we screened genomic DNA from blood samples of cancer patients, as well as samples from unaffected subjects who presented for genetic counseling and testing due to a strong family history of breast and/or ovarian cancer.

Materials and Methods
Human subjects
The study samples were retrieved from the Karmanos Cancer Institute Genetic Registry (KCIGR), a database and specimen repository comprising persons at elevated risk of harboring a \(BRCA1/2\) mutation based on personal and family history of cancer. This risk profile was determined in a clinical cancer genetic counseling session. Samples used for this study were collected between 1999 and 2012. In general, breast and ovarian diagnoses (or “no cancer”) were reported at the time the patient presented for genetic counseling and testing. Inclusion criteria for this retrospective cohort study specified Caucasian female \(BRCA1/2\) positive and negative breast and ovarian cancer patients, unaffected participants that were at high risk (>10%, risk determined by assessment models described below) of carrying \(BRCA1/2\), or were otherwise deemed candidates for clinical genetic testing by a certified genetic counselor. All participants were either confirmed \(BRCA1/2\) mutation carriers or \(BRCA1/2\) negative after full sequencing or testing for the three common Ashkenazi Jewish mutations. Ashkenazi Jews (those with Eastern European ancestry) have a substantially elevated risk of breast and ovarian cancer due to a high frequency of two \(BRCA1\) mutations (187delAG and 5385 insC) and one mutation in \(BRCA2\) 6174delIT. Approximately 1 in 40 Ashkenazi Jews have one of these three founder mutations. It has been shown that these three mutations account for 90% of the mutations found in Ashkenazi Jews.\(^9\) \(BRCA1/2\) mutations detected by either method were included in the “\(BRCA1/2\) positive” category for statistical analysis purposes.

Genetic testing for \(BRCA1/2\) was conducted by Myriad Genetics Laboratories (Salt Lake City, Utah). We excluded from this study KCIGR participants with unknown \(BRCA1/2\) mutation status.

Informed consent was signed and permission was obtained for the collection of blood samples and for access to medical records for all subjects. The protocol was approved following Full Board Review by the Human Investigation Committee at Wayne State University, Detroit, Michigan. The study complies with the Declaration of Helsinki.

Risk assessment was performed using BRACAPRO and Myriad II. BRACAPRO\(^10\) is a computer-based Bayesian probability model that uses breast and/or ovarian cancer family history to determine the probability that a \(BRCA1\) or \(BRCA2\) mutation accounts for the pattern of these cancers in the family. Key attributes include the population prevalence of \(BRCA\) mutations, age-specific penetrance, and Ashkenazi Jewish heritage. Myriad II is a set of prevalence tables categorized by ethnic ancestry (Ashkenazi Jewish or non-Ashkenazi Jewish), the age of onset (age < 50 years or age ≥ 50 years) of breast cancer, and the presence of ovarian cancer, in the patient...
and/or first- or second-degree relatives. Myriad II is based on actual test data from the Myriad Genetic Laboratories clinical testing service.11

SNP sequencing
DNA from a blood sample, drawn specifically for KCIGR use, was isolated by the Karmanos Applied Genomics Technology Center (Detroit, Michigan). DNA was extracted with QIAamp DNA mini kit (Qiagen) and amplified by PCR using primers Fwd-GCTCCAGCTGTTCGGCTTCCA and Rev-CCTCGGAGGCCTTGTG. The PCR DNA fragments were purified on agarose gel prior to sequencing.

Statistical analysis
Hardy-Weinberg Equilibrium was tested in the controls using the R “genetics” package. Logistic regression was used to estimate the association between SNP genotype and the risk of cancer adjusted for age at diagnosis. The genotype was analyzed in two different models: the dominant model—combining the genotypes consisting of the minor allele to form a two-level categorical variable—and the continuous model—counting the number of minor allele to form a one degree of freedom continuous variable with values 0, 1, and 2. A Huber-White sandwich estimator was used to construct confidence intervals because a small percentage of the participants in the sample are relatives. There was no evidence of imbalance or genotype error in the control group from KCIGR. Total genotype frequencies from the examined samples set (A/A = 0.130, A/G = 0.447, G/G = 0.423) were similar to that of the general European population in the 1000 Genome Project database (0.121, 0.446, 0.433 respectively, as assessed from 379 participating individuals of 1000GENOMES:EUR subset).12

Functional studies
Entry clones of TSAd variant 2 and LCK open reading frames (ORFs) in pDONR233 vector were kindly provided by Dr. R. Finley. Using LR Clonase (Invitrogen), the ORFs were recombined as fusion constructs intro pIZ4 attR-2 (with GAL1-promoter induced B42 acid patch activation domain, AD) and pNLEX attR (with LexA DNA binding domain, DBD) plasmids, creating, firstly, AD-TSAd and DBD-LCK fusion constructs for two-hybrid experiments, and secondly, a BDB-TSAd fusion construct for transcription activation experiments. The G to A nucleotide replacement at rs926103 SNP was performed with a QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). The DNA sequence of all constructs was confirmed by sequencing.

Yeast strains Y309 (containing pSH18-34 LacZ reporter plasmid controlled by LexA operator sequences) and RFY231 S. cerevisiae strains were kindly provided by Dr. R. Finley. Yeast were maintained in SD media lacking the appropriate amino acids when necessary, and transformed separately with pJZ4 attR-2- and pNLEX attR-based constructs by PEG/Lithium acetate protocol (as described).13 For two-hybrid experiments, yeast strains were mated overnight in nonselective liquid medium and then selected for diploids on SD plates lacking appropriate amino acids.

For β-galactosidase assays, yeast were grown overnight in SD media lacking the appropriate amino acids to maintain selective pressure. For experiments that included constructs with inductive GAL1 promoter, dextrose in the SD medium was substituted with raffinose (1%) and galactose (2%). Liquid Culture Assay using ONPG as a substrate was performed as described.13 β-Galactosidase activity units were calculated according to Miller’s protocol.14

Results and Discussion
Minor allele of the rs926103 SNP is associated with ovarian cancer predisposition
The original goal of this study was to determine whether epigenetic changes accounted for an increased risk of breast and/or ovarian cancer in those testing negative for BRCA1/2. While looking for cancer-associated changes using a methylation array (manuscript in preparation), we discovered a genetic rather than epigenetic variation associated with increased ovarian cancer frequency risk (Table 1). In this study, we used a Karmanos Cancer Institute Genetics Registry (KCIGR) collection of peripheral blood lymphocyte (PBL) DNA. This collection was gathered from 280 women at high risk of carrying BRCA1/2 mutations, as determined by the BRCAPRO and Myriad risk assessment models.10,11 Fifty nine were cancer free, 208 had breast or ovarian cancer, and 13 had
both breast and ovarian cancer. All participants were either BRCA1/2 deleterious mutation carriers or were confirmed BRCA1/2 negative (Table 1).

Using direct sequencing followed by statistical analysis, we found an association between a minor allele of rs926103 SNP and an elevated frequency of ovarian cancer specifically in the BRCA1/2 negative subgroup. The association was detected both in dominant and continuous models (Table 1: dominant model odds ratio 3.51, \( P = 0.018 \)). No significant association was detected between the frequency of the rs926103 minor allele and ovarian cancer risk in the BRCA1/2 positive subgroup (Table 1, \( P = 0.3 \)).

A similar analysis did not reveal a significant association of breast cancer frequency with rs926103 (Table 1, \( P = 0.1 \)) in either the BRCA1/2 negative or positive subgroup; we therefore concluded that this SNP was associated with hereditary risk of ovarian but not breast cancer. Further investigation of a larger and more diverse sample size will be required for elucidation of the possible association of this SNP with other cancer types and for verification of our findings.

Minor allele of the rs926103 SNP which results in Arg:Ser variation influences TSAd protein activity

The non-synonymous SNP rs926103 is located within the open reading frame of SH2D2A, a gene coding for signal transduction pathways protein TSAd. TSAd functions as a modulator of various cell signaling pathways as well as a transcription activator. Although TSAd does not have any identified catalytic region, it interacts with various tyrosine kinases, gets phosphorylated, and is involved in modulation of their activity and downstream signaling. The participation of this protein has been demonstrated in several signaling cascades originating from such receptors as TCR in the peripheral immune system, VEGFR-2 in vesicular endothelial cells, and KDR in lung epithelial cells. TSAd-deficient mice demonstrated diminished tumor vascularization and growth. In addition, a reduced level of this protein predisposes subjects toward the development of autoimmune diseases.

The G:A variation at the SNP rs926103 results in a serine:asparagine alteration in the amino acid 52 in TSAd protein, S52N, (Fig. 1A). The function of this

![Figure 1. TSAd variants activity in protein-protein interaction. (A) TSAd protein scheme with marked functional domains. Arrow indicates the position of rs926103 SNP. Y = tyrosine. Based on. 24 (B) β-galactosidase activity level was measured by liquid culture ONPG assay in cells carrying the reporter LacZ plasmid and co-expressing fusion proteins as indicated. Notes: rs926103 SNP variants A and G result in TSAd_N52 and TSAd_S52 respectively. Experiment has been performed in triplicate and repeated 3 times with independent transformation clones. Error bars represent standard deviation.](image-url)
region upstream of the SH2 domain is not known, but given that TSAd is involved in phosphorylation-dependent signal transduction pathways, it is plausible that the presence or absence of the serine residue as a potential phosphorylation site can significantly alter the protein’s activity. To evaluate the influence of the rs926103 SNP variants on the activity of TSAd, we used a S. cerevisiae two-hybrid system co-expressing TSAd as a fusion with an activation domain and the TSAd-regulated lymphocyte-specific protein tyrosine kinase, LCK, as a fusion protein with a DNA binding domain. Interaction of TSAd with LCK in that system causes the activation of β-galactosidase expression from the reporter plasmid. We detected an increased interaction when the rs926103 major G allele (serine) is replaced with minor allele A (asparagine) (Fig. 1B). This amino acid difference could therefore affect one of the multiple signal transduction pathways involving TSAd.

A variation of the same reporter expression system, using TSAd as a fusion protein with a DNA binding domain, permitted a comparison of the transcriptional activity of TSAd variants. We detected an approximately 3-fold increase in the transcription activity of TSAd with the A allele (asparagine) of rs926103 SNP compared to G allele (serine) (Fig. 2).

The enhanced protein interactions and/or transcription activation of the TSAd_N52 protein may influence the risk of ovarian cancer. Interestingly, TSAd does not have a nuclear localization sequence and requires the assistance of one of its interacting partners, VCP/TERA, for nuclear import. VCP/TERA also interacts with BRCA1. TSAd is involved in a number of processes with cancer-related mechanisms. Tumor vascularization and growth are inhibited in TSAd-deficient mice. TSAd is also involved in the regulation of the peripheral immunological response, which plays an important role in augmentation of ovarian cancer. We are inclined to believe that the effect of TSAd S52N on ovarian oncogenesis occurs through the immune system or neovascularization, given that we found that TSAd knock-down with siRNA in ovarian cancer cell cultures has no apparent effect on cell proliferation (data not shown). Interestingly, the same minor allele of rs926103 SNP predisposes individuals to the autoimmune disease multiple sclerosis (MS) and the immune suppressant drug treosulfan—which was successfully applied for treatment of MS—was also approved for the treatment of ovarian cancer.

As the rs926103 minor allele occurs in a significant fraction (>25%) of the Caucasian population, it is likely that its effect on ovarian cancer predisposition occurs in a context of other genetic variations through genetic interaction leading to unidirectional or mutual alteration of influence. Additional non-synonymous SNPs or other mutations in TSAd may also influence the risk of ovarian cancer, although we did not find any additional mutations in the TSAd coding region of the 10 BRCA1/2 negative ovarian cancer patients from the KCIGR cohort that contained homozygous major allele genotype at rs926103 (data not shown). There is a possibility that the minor allele of rs926103 alone or in combination with other genetic variations does not lead to cancer on its own accord, but rather modifies through a genetic interaction the penetrance of some major cancer related gene(s), for example, hypothetical members of the BRCA1X (non-BRCA1/BRCA2) genes.

Although we have identified an association between the minor allelic variant of rs926103 and ovarian cancer predisposition, we were not able to evaluate the possibility of a specific influence on the age of the disease onset or on the disease recurrence. This was due to the limited sample size preventing statistical analysis of sub-cohorts. These issues have

Figure 2. TSAd variants activity in transcription.

Notes: β-galactosidase activity level was measured by liquid culture ONPG assay in cells carrying the reporter LacZ plasmid and expressing fusion protein DBD-TSAd and control DBD. The rs926103 SNP variants A and G result in TSAd_N52 and TSAd_S52, respectively. Each experiment was performed in triplicate and repeated 3 times with independent transformation clones. Error bars represent standard deviation.
a great influence on the clinical approaches to ovarian cancer risk evaluation and medical management, and it is therefore essential that they should be addressed in larger BRCA1/2 negative high risk populations.

Our observation of the association between the minor allele of rs926103 and familial ovarian cancer predisposition provides a novel candidate genetic biomarker for the identification of elevated ovarian cancer risk in high-risk families without BRCA1/2 mutations. This SNP may contribute to further differentiation of the risk of ovarian cancer among high-risk subjects without a BRCA1/2 mutation in their family. Once the results of this small pilot study are confirmed by a large scale analysis, women bearing these SNPs and other TSAd cancer associated variants might benefit from enhanced clinical surveillance, as is routinely performed for individuals carrying other known high-risk mutations.

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Author Contributions
Conceived and designed the experiments: MAT, LK, ALF, NKL, SL, SA. Analysed the data: WC, MAT, LK, ALF, NKL. Contributed to the writing of the manuscript: WC, NP, JLB, RG, SL, MSS, RTP, ARM. Agreed with manuscript results and conclusions: MAT, LK, ALF, WC, NKL, NA, NP, JLB, RG, SL, MSS, RTP, ARM. Jointly developed the structure and arguments for the paper: MAT, LK, NKL. Made critical revisions and approved final version: MAT, LK, NKL, WC. Acquired patients’ consents and collected specimens: NKL, MSS, RTM, ARM, RG, NP, JLB. Managed specimens database: NKL. All authors reviewed and approved of the final manuscript.

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Competing Interests
Author(s) disclose no potential conflicts of interest.

Ethical Approval
This study was approved by the Human Investigation Committee at Wayne State University (Detroit, Michigan) in accordance with the Helsinki Declaration. Informed consent was obtained from all study participants.

Disclosures and Ethics
As a requirement of publication author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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