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Zinc transporter genes and urological cancers: Integrated analysis suggests a role for *ZIP11* in bladder cancer

Lang Wu[1,2,3], **Kari G. Chaffee**[1], **Alexander S. Parker**[4], **Hugues Sicotte**[1], and **Gloria M. Petersen**[1]

[1]Department of Health Sciences Research, Mayo Clinic, 200 1st St SW, Rochester, Minnesota, 55905, USA

[2]Center for Clinical and Translational Science, Mayo Clinic, 200 1st St SW, Rochester, Minnesota, 55905, USA

[3]Mayo Graduate School, Mayo Clinic, 200 1st St SW, Rochester, Minnesota, 55905, USA

[4]Department of Health Sciences Research, Mayo Clinic, 4500 San Pablo Road, Jacksonville, Florida, 32224, USA

Abstract

Although zinc transporters were shown to play roles in the development of prostate, bladder and renal cancer, no study has evaluated the genetic variants in zinc transporter genes with risk of urological cancers. A candidate gene association study using genome-wide association study (GWAS) datasets was conducted for variants in 24 zinc transporter genes. Genotypes were analyzed using a logistic regression models adjusted for covariates. The function of identified variants was assessed by using the Encyclopedia of DNA Elements (ENCODE). We further evaluated tumor somatic change of the implicated gene(s) and the associations between identified variants and patient survival from data in The Cancer Genome Atlas (TCGA). A *ZIP11* variant, rs8081059, was significantly associated with increased risk of renal cancer (OR=1.28, 95% CI (1.13–1.45), p=0.049). No zinc transporter variants were associated with prostate cancer risk. Four variants within *ZIP11* were significantly associated with bladder cancer risk: rs11871756 (OR=1.43, 95% CI (1.24–1.63), p=0.0002); rs11077654 (OR=0.76, 95% CI (0.68–0.85), p=0.001), rs9913017 (OR=0.76, 95% CI (0.68–0.85), p=0.002), and rs4969054 (OR=0.78, 95% CI (0.69–0.88), p=0.02); the three protective variants were co-located and highly correlated. These variants were located within predicted transcribed or enhancer regions. Among the 253 bladder cancer patients in TCGA, two had tumors that contained deleterious missense mutations in *ZIP11*. Moreover, rs11077654 was significantly associated with survival of bladder cancer patients (p=0.046). In conclusion, zinc transporter gene, *ZIP11*, may play important role in bladder cancer. Further studies of the gene are warranted.

Corresponding Author: Gloria M. Petersen, Department of Health Sciences Research, Mayo Clinic College of Medicine, 200 First Street SW, Rochester, MN 55905. Phone: 507-538-1563; Fax: 507-266-2478; Petersen.Gloria@mayo.edu.

Conflicts of interest

None

Keywords

Zinc transporter genes; single nucleotide polymorphism; association; risk; urological cancers; survival

Introduction

Zinc is a mineral that is vital for maintaining human health [1]. Zinc ion transporters are critical in sustaining the tightly regulated concentration of zinc in human cells necessary for normal cellular functions [2, 3]. Interestingly, studies have demonstrated that the imbalance of zinc ions and dysfunction of zinc transporters have implications for development of urological cancers [4, 5]. For example, abnormal expression or function of zinc transporters *ZIP1*, *ZIP4*, *ZIP6*, *ZNT4*, and *ZNT7*, as well as the imbalance in zinc ions, play important roles in prostate cancer development *in vitro* and in animal models [6–14]. Moreover, *ZIP10* and *ZNT1* are important in maintaining renal zinc reabsorption, and the zinc imbalance in renal cells is linked to renal cell carcinoma (RCC) development [3, 14–20]. Similarly, zinc imbalance is reported to be associated with the risk of bladder cancer [21–25]. Despite these demonstrated links between zinc and urologic carcinogenesis, to date there have been no systematic studies of the role of genetic variation in zinc transporter genes and risk of urologic cancers. Such a study could suggest a role for identified susceptibility variants in the etiology of the relevant cancer. Moreover, therapeutic strategies based on implicated targets could potentially be developed.

Motivated by this, we hypothesized that people who carry specific genetic variants in zinc transporter genes are at increased risk of developing urologic cancers. To test this hypothesis, we conducted a candidate gene association study [26, 27] of 24 zinc transporter genes (*ZNT1-10*, *ZIP1-14*) to evaluate whether any variants confer susceptibility to three primary urological cancers (prostate adenocarcinoma, transitional cell carcinoma of the bladder and RCC). Additionally, to investigate identified variants/genes, we used available sequencing, genotyping, and survival data from The Cancer Genome Atlas (TCGA)[28] focusing on cancers of interest to evaluate additional evidence.

Materials and Methods

Three genome-wide association study (GWAS) datasets of urological cancers (prostate adenocarcinoma, transitional cell carcinoma of the bladder, and RCC) were downloaded from the database of Genotypes and Phenotypes (dbGaP) in August 2011:

1. Cancer Genetic Markers of Susceptibility (CGEMS) prostate cancer GWAS[29] included approximately 550,000 single nucleotide polymorphisms (SNPs) (Phase 1A with HumanHap300 and Phase 1B HumanHap240, both from Illumina, San Diego, CA) in 1,172 prostate cancer patients and 1,157 controls of European ancestry from the Prostate, Lung, Colon and Ovarian (PLCO, http://www.cancer.gov/prevention/plco/) Cancer Screening Trial (http://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000207.v1.p1). Data on age, stratified into four categories and family history of prostate cancer

were available. Prior to being uploaded to dbGaP, quality control (QC) checks were applied based on SNP and sample call rates, and sample miscalls and duplicates were deleted [29].

2. GWAS for bladder cancer risk included 3,527 cases and 5,119 controls of European descent. There are 591,637 SNPs generated on five Illumina platforms (250, 300, 550, 610 and 1M) (http://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000346.v1.p1&phv=158999&phd=3458&pha=&pht=2183&phvf=&phdf=&phaf=&phtf=&dssp=1&consent=&temp=1). Data on age, stratified into separate categories, and sex were available for this set. QC checks were applied based on SNP call rate and Hardy-Weinberg equilibrium (HWE) threshold. Further QC checks on samples included call rates and heterozygosity; additional sample exclusions included removal of duplicate samples, those with gender discordances, ineligible phenotypes, and non-CEU ancestry [30]. Population structure was assessed, and no notable eigenvectors were found.

3. The National Cancer Institute GWAS of renal cell carcinoma (RCC) (http://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000351.v1.p1) [31] included 1,453 RCC cases and 3,531 controls of European background from 4 studies (3 cohort, 1 case-control), genotyped using the Illumina InfiniumHumanHap 550, 610 and 660W chips. Sex was the only available covariate. SNPs were filtered on call rate and HWE; samples were filtered on completion rate, replications, abnormal heterozygosity, phenotype exclusions, and lack of European ancestry [31].

Within each cancer type, we combined the separate datasets into a single dataset to increase statistical power for each cancer, respectively. We tested for 1,143 variants, 704 variants, and 149 variants in the bladder cancer dataset, RCC dataset, and prostate cancer dataset, respectively. Unconditional multivariable logistic regression models with an additive SNP effect were employed[32]. For the prostate cancer data, we adjusted for age and family history of prostate cancer. For the bladder cancer data, we adjusted for age and sex. Given the well-known gender disparity in RCC (2:1 male to female ratio), in our analysis of the RCC data, we adjusted for sex. In all analyses, odds ratios (OR) and 95% confidence intervals (CI) were computed using Plink (http://pngu.mgh.harvard.edu/~purcell/plink/)[33].

To limit the false-positive results as well as to account for multiple testing and correlations between genetic variants, we computed a family-wise corrected p-value using the maxT permutation tests in plink [34] using 5,000 replicates. To determine whether any of the significant variants were within the same LD block, we used the 1000 Genomes Project [35] browser (http://browser.1000genomes.org/Homo_sapiens/UserData/Haploview). Genetic information of Caucasian population groups (CEU, FIN, GBR, IBS, and TSI) in the most updated data of 1000 Genomes Project (up to April 2014) was used. We further used Haploview[36] to plot the linkage disequilibrium among significant variants.

To investigate the potential functionality of any suggested variants from the association analyses, we performed *in silico* analysis with publicly available data. We used the UCSC

Genome Browser (http://genome.ucsc.edu/cgi-bin/hgTracks?db=hg19) on January 14, 2015, to evaluate the function of both transcribed and non-transcribed variants. The latter was done by comparing the position of significant SNPs to the Encyclopedia of DNA Elements (ENCODE) annotation. In particular, Genome Segmentation by Combined Segway +ChromHMM (GSCSC) [37, 38] track was used to detect whether variants were located in any of the predicted functional regions of the genome. Briefly, computational analysis of chromatin state segmentation at a 200 base pair resolution generated 15 states, ranging from state 1 (active promoter) to state 15 (repetitive/copy number variation) [39]. States 4–7 represent categories of enhancer (from strong to weak/poised [40]), and states 9–11 represent categories of transcription regions (transcriptional transition, elongation, and weak transcribed region, respectively). Publicly available data on several cell lines of human origin were used to explore function prediction, including K562 (erythrocytic leukaemia cells), HepG2 (hepatocellular carcinoma cells), H1-hESC (embryonic stem cells), GM12878 (B-lymphoblastoid cells), HSMM (human skeletal muscle myoblasts), NHLF (normal lung fibroblasts), HUVEC (umbilical vein endothelial cells), HMEC (mammary epithelial cells) and NHEK (normal epidermal keratinocytes). An analogous and productive interrogation approach with ENCODE performed on SNPs in breast cancer has been previously reported [41].

To determine whether any genetic change within identified gene(s) were functional at the somatic/tumor level of patients, we analyzed available data from TCGA. The detailed sequencing procedures and analytic processes have been described previously [42]. The somatic level genetic change of 253 bladder cancer patients and 506 RCC patients were retrieved through the TCGA data portal on November 16, 2014 (https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp). We focused on the GWAS-implicated gene(s) to determine the existence and prevalence of somatic variation in the patients reported by TCGA.

To determine whether any of the identified variants may be associated with survival of cancer patients, we conducted multivariable linear regression analysis employing available data from TCGA. Variables that may influence disease survival (age, gender, race, smoking status, and tumor grade) were adjusted for in the model.

Results

Transitional cell carcinoma of the bladder

Fifteen variants demonstrated association with bladder cancer risk at significance levels of $p \leq 0.002$. Among these, four variants within *ZIP11* were significantly associated with bladder cancer risk after correcting for multiple comparisons (Table 1). One variant, rs11871756, was associated with increased risk of bladder cancer (OR=1.43, 95% CI (1.24–1.63), p=0.0002). Three variants were associated with a reduced risk of bladder cancer: rs11077654 (OR=0.76, 95% CI (0.68–0.85), p=0.001), rs9913017 (OR=0.76, 95% CI (0.68–0.85), p=0.002), and rs4969054 (OR=0.78, 95% CI (0.69–0.88), p=0.02); further linkage disequilibrium (LD) analysis revealed that these three variants are highly correlated (Figure 1) and are within same LD block in chr17:71006408–71021645 (build 37).

Focusing on common SNPs (minor allele frequency, MAF ≥0.10) in the region within which the three protective variants are located, we observed that risk estimates of these variants were mostly protective (OR < 1) among the SNPs closely proximal to the three variants (Table 2 and Figure 1). *In silico* functional analysis found that the three protective variants of interest were located in a block that is a weak/poised enhancer region according to the ENCODE annotation. Specifically, rs11077654 is located within a weak/poised enhancer region by cell lines K562 and HepG2 and a weakly transcribed region by cell lines H1-hESC, GM12878, and HSMM. Variant rs9913017 is located within a weakly transcribed region by cell lines H1-hESC, GM12878, K562, HepG2, HSMM and NHLF. Variant rs4969054 is located within a weak/poised enhancer region by cell lines GM12878 and K562 and a weakly transcribed region by cell lines H1-hESC, HepG2 HSMM and NHLF. The risk variant rs11871756 was predicted to be in a weakly transcribed region by cell lines GM12878 and HepG2 and a weak/poised enhancer region by cell lines K562 and HUVEC.

After evaluating somatic mutations among 253 bladder cancer patients in the TCGA data, we found two patient tumors that contained missense mutations (chr17:70643734_C/A and chr17:71027826_G/A (build 37)). Further *in silico* analysis of functional prediction using both SIFT and PolyPhen-2[43] revealed that these two mutations are predicted to be deleterious.

Two of the suggested variants (rs11871756 and rs11077654) were included in the genotyping platform of the TCGA bladder cancer patients and were tested for their associations with patients' survival using available data on 95 patients, after adjusting for age, gender, race, smoking status, and tumor grade. The SNP rs11871756 did not demonstrate a significant effect on survival. However, rs11077654 was significantly associated with survival by genotype (p=0.046). Specifically, patients with genotype AA had an average survival of 407 days, patients with genotype CA survived 556 days, and patients with genotype CC survived 755 days.

Renal cell carcinoma (RCC)

Seven variants in *ZIP11* were associated with RCC risk at significance level of p≤0.002. Further permutation testing demonstrated that rs8081059 was associated with RCC risk after accounting for multiple comparisons (OR=1.28, 95% CI (1.13–1.45), p=0.049) (Table 1). *In silico* functional analysis suggested it to be within a predicted transcribed region by the GSCSC track and transcriptional transition or elongation by cell line GM12878 and weakly transcribed region by cell lines K562, HepG2, HUVEC, HMEC, HSMM, NHEK and NHLF. Furthermore, this variant was in an open chromatin region according to DNAse I hypersensitivity cluster in 125 cell types and in the intron proximal to the splice site.

Evaluation of *ZIP11* using TCGA data among RCC patients did not reveal any somatic mutations. The identified variant rs8081059 was not captured in the genotyping platform of TCGA patients, thus precluding further exploration of this variant with available data.

Prostate adenocarcinoma

There were no significant associations between any variants and the risk of prostate cancer.

Discussion

Globally, urological cancers represent a major public health burden. For example, in United States, the number of new cases of urological cancers was estimated to be 138,710 in 2015 [44]. Among them, 74,000 were expected to develop urinary bladder cancer and 61,560 were expected to develop kidney cancer [44]. In China, prostate cancer was reported in 2010 to be the 8th most common cancer in urban areas [45]. It is thus critical to better understand the etiology of urological cancers to decrease their health burden. While evidence for a role of zinc in urologic carcinogenesis has been reported at the cellular and animal model levels [21–25], we report the first examination of the role of germline genetic variation in zinc transporter genes as modulators of urologic cancer risk in human subjects. Moreover, this is the first report of an association of *ZIP11* variants with development of RCC and transitional carcinoma of the bladder. Although suggested by basic laboratory research [6–12], variants in several other zinc transporter genes were not associated with urological cancer risk in our study. One explanation is that the variants evaluated in this study were restricted to those SNPs in the genotyping platforms, and those causal mutations/variants affecting gene function were not well captured. Plausible genetic factors beyond SNPs, such as epigenetic factors or possible interactions of *ZIP11* variants with known environmental risk factors for RCC (e.g., smoking, obesity, etc.), could not be assessed because the available data were limited. If our findings are confirmed by independent studies, further work, including targeted sequencing of *ZIP11*, may be warranted to identify additional predisposition variants or mutations for bladder cancer and RCC.

ZIP11 was recently demonstrated to be a zinc importer for cells [46]. Knock-down of this gene can decrease cellular zinc concentrations and metallothionein levels. Though its relationship with development of human cancers is previously unknown, our study suggests that several genetic variants of this gene, which are potentially functional, may modulate risk of bladder cancer and renal cell carcinoma. Further research for clarifying role of *ZIP11* in development of these two cancers, as well as examination of exact roles of identified genetic variants, are warranted.

Non-coding portions of the human genome have long been suspected to be biologically relevant and important, although it was not until recently through the ENCODE project [47] that a comprehensive appreciation and understanding of the potential role of non-coding regions became possible. Located within traditional designated “non-coding” regions, the variants in our study found to be associated with risk of bladder and renal cancers are within predicted functional regions (i.e., transcribed or enhancer regions) from the ENCODE annotation. *In silico* prediction involving knowledge of both exon regions and non-coding regions (using the ENCODE segmentation) tends to be helpful in linking disease associated variants identified through epidemiological studies to their plausible functionality and ultimately to etiology of human diseases [41]. Knowledge generated through this process can be used to guide further research for clarifying the mechanism for disease development.

One limitation of this study is that we could not fully adjust for all known risk factors of studied tumors in the association analyses, due to the unavailability of relevant information. However, evidence from additional analyses, especially the identified somatic mutations in

bladder cancer patients as well as the association with survival of bladder cancer patients, supports the plausibility of *ZIP11*'s important role. Future association studies accounting for all risk factors are warranted to validate our findings.

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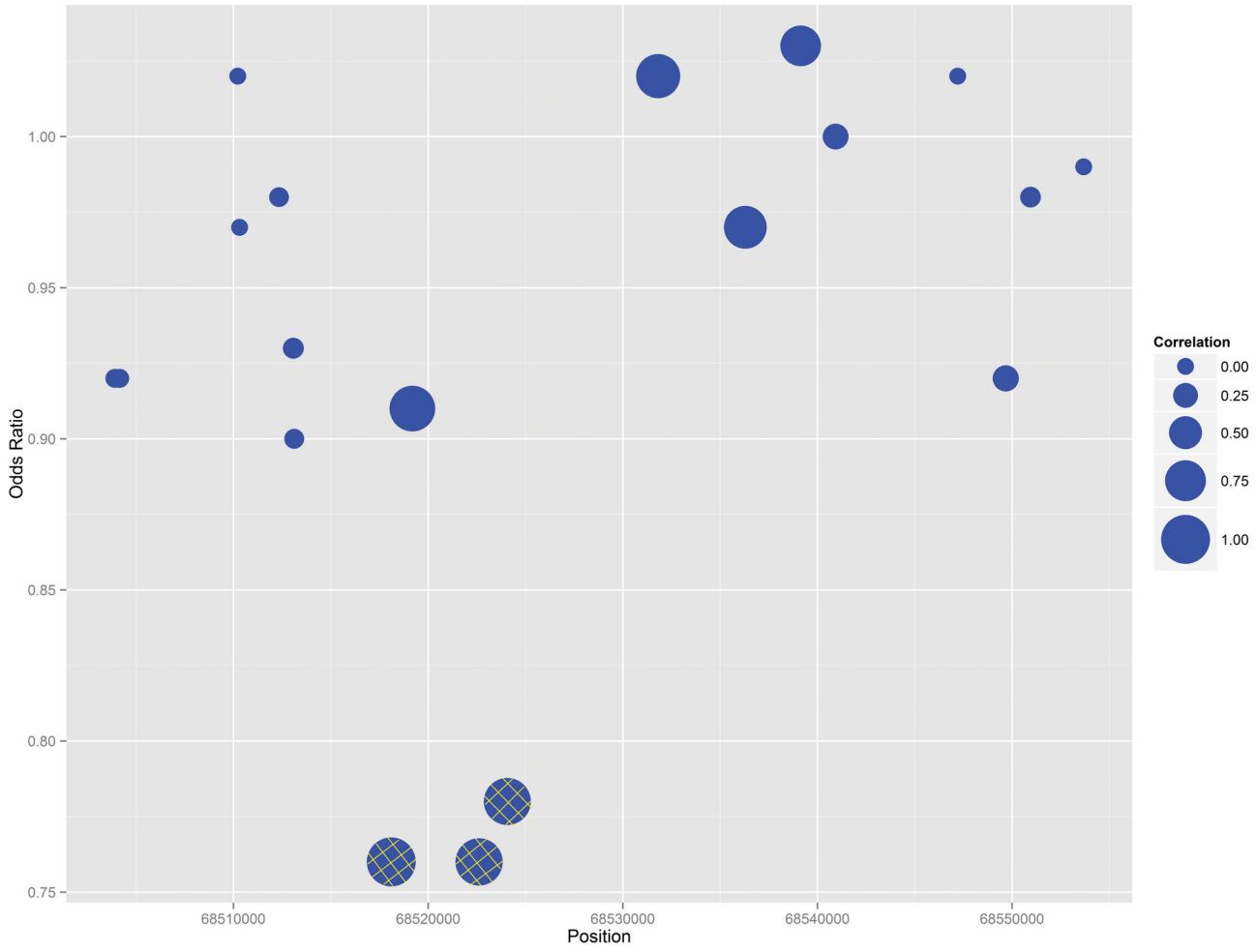

Figure 1.

Odds ratios (OR) and correlation of *ZIP11* common SNPs (MAF ≥0.10) in the region of SNPs observed to be protective for bladder cancer risk. The Y axis denotes ORs with bladder cancer risk. The X axis denotes positions of SNPs (build 36). The size of each SNP's symbol represents its correlation (r^2) with the reference variant rs11077654. The three points that are cross-hatched represent the three SNPs reaching statistical significance ($P<0.05$) after accounting for multiple comparisons.

Table 1

Significant associations between zinc transporter gene variants of *ZIP11* on chromosome 17 and bladder and renal cell cancer risk

SNP	Major/Minor Allele	Position (build 36)	Numbers of cases and controls	MAF*	OR (95% C.I.)	P, permutation**
Bladder Cancer						
rs11871756	C/G	68237839	4590	0.11	1.43 (1.24–1.63)	2×10^{-4}
rs11077654	C/A	68518107	4576	0.17	0.76 (0.68–0.85)	1.4×10^{-3}
rs9913017	C/A	68522615	4548	0.17	0.76 (0.68–0.85)	1.8×10^{-3}
rs4969054	C/G	68524068	4416	0.17	0.78 (0.69–0.88)	0.022
Renal Cell Cancer						
rs8081059	A/C	68580314	4732	0.14	1.28(1.13–1.45)	0.049

* Minor Allele Frequency (MAF) in European population of 1000 Genomes Project [35]

** Multiple comparisons were accounted for by permutation tests using 5,000 replicates

Table 2

Properties of *ZIP11* SNPs with minor allele frequency (MAF) ≥0.10 in the region of SNPs observed to be protective for bladder cancer risk, rs11077654, rs9913017, and rs4969054. Table includes odds ratio (OR) and correlation with reference SNP, rs11077654. Statistically significant SNPs are shown in bold.

SNP (rs ID)	Minor Allele	Major Allele	Position (build 36)	Numbers of cases and controls	OR	95% CI	P, permutation	MAF*	Correlation with rs11077654 (D′)	Correlation with rs11077654 (R^2)
rs11658524	C	A	68503910	7580	0.92	0.86–0.98	1	0.41	0.468	0.064
rs12947636	G	A	68504161	7573	0.92	0.86–0.98	1	0.41	0.468	0.064
rs11077651	G	T	68510222	4585	1.02	0.92–1.12	1	0.22	0.195	0.002
rs4969047	T	C	68510316	7577	0.97	0.90–1.04	1	0.26	0.005	0
rs4969048	C	T	68512342	7565	0.98	0.88–1.08	1	0.1	0.402	0.093
rs9916009	A	G	68513074	7583	0.93	0.87–0.99	1	0.37	0.612	0.129
rs9913553	G	A	68513124	6735	0.9	0.81–1.01	1	0.11	0.39	0.095
rs11077654	**A**	**C**	**68518107**	**4576**	**0.76**	**0.68–0.85**	**0.001**	**0.17**	**reference**	**reference**
rs11658597	C	T	68519196	7575	0.91	0.83–0.99	1	0.16	0.99	0.9
rs9913017	**A**	**C**	**68522615**	**4548**	**0.76**	**0.68–0.85**	**0.002**	**0.17**	**0.98**	**0.95**
rs4969054	**G**	**C**	**68524068**	**4416**	**0.78**	**0.69–0.88**	**0.02**	**0.17**	**0.98**	**0.94**
rs4969005	T	C	68531817	2153	1.02	0.87–1.19	1	0.15	0.97	0.85
rs903104	T	C	68536297	8628	0.97	0.90–1.06	1	0.16	0.94	0.81
rs9912666	G	A	68539138	2152	1.03	0.89–1.19	1	0.19	0.93	0.74
rs9915558	C	T	68540933	8577	1	0.93–1.07	1	0.32	0.81	0.28
rs1552846	T	A	68547208	4587	1.02	0.93–1.12	1	0.27	0.01	0
rs9916389	C	T	68549676	7571	0.92	0.86–0.98	1	0.37	0.91	0.29
rs4969057	T	C	68550945	8635	0.98	0.91–1.04	1	0.31	0.51	0.12
rs8081534	G	A	68553676	8619	0.99	0.92–1.06	1	0.24	0.15	0.001

*Minor Allele Frequency (MAF) in European population of the 1000 Genomes Project [35]