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Abstract

As a result of increasing interests in discovering single nucleotide polymorphisms (SNPs) associated complex diseases and high cost of existing sequencing technology, genotype imputation is developed as a statistical method to overcome the limitations of current sequencing technology and to increase the power of the association method of Genome-Wide Association Studies (GWAS). In this paper, genotype imputation is conducted on Tianjin sample with two reference panels; the 1000 Genomes reference panel and the TOPMed reference panel. First, pre-imputation quality control is applied to remove individuals or genetic markers that may induce high error rates when conducting imputation. Principal component analysis was conducted to show the East Asian ancestry of the sample. Using Minimac3, imputation was performed on a sample of 437 individuals with 499,148 genetic variants after haplotype inference with Eagle software from Illumina 660W. Approximately 47 million and 88 million genetic variants were imputed using 1000G and TOPMed reference panels respectively. The estimated squared Pearson's correlation (R^2) was used to determine which of the imputed SNPs passed the post-imputation quality control. Approximately 9.5 million imputed SNPs from the 1000G reference panel and 11 million imputed SNPs from the TOPMed reference panel exceeded the R^2 threshold. To assess imputation quality, imputation was again performed on the original 437 individuals, but with 5% of the directly genotyped genetic variants randomly masked. The imputed variants for chromosomes 1, 11, and 21 were selected to calculate the true squared Pearson's correlation; and the masking results for the two reference panels were compared to determine which reference panel is more suited for this sample. Overall, the imputation provides an accurate set of genetic markers that can be used in the downstream GWAS analysis to explore SNPs associated with lung cancer.

Introduction

The National Human Genome Research Institute (NHGRI) Catalog of Published Genome-Wide Association Studies (GWAS) is a publicly available catalog, which encompasses 3,420 publications of 62,652 unique Single Nucleotide Polymorphisms (SNPs) that are associated with complex diseases or other specific traits (Welter et al. 2014; Burdett et al. 2018). Many of the discoveries were conducted with genotype imputation, a crucial statistical technique that uses haplotype patterns from genotyped reference panels to accurately predict genetic markers of a study sample after sequencing a small amount of genotypes on a commercial array (Li et al. 2009).

One may wonder the intention for using this type of "incomplete" information instead of sequencing the whole genome of every individual in the sample to test for association (Li et al. 2009). The cost and time required to conduct whole genome sequencing of a sample with thousand(s) of individuals is currently unfeasible. The first human genome sequence was conducted with Sanger DNA sequencing, developed in 1977 and costed 0.5-1 billion dollars (Reuter et al. 2015; Sanger et al. 1977). In the last 40 years, much advancement has been made with 'next generation' DNA sequencing (NGS) - supplanted Sanger sequencing with reduction in cost and time required to sequence DNA and RNA (Shendure et al. 2017; Spiliopoulou et al. 2017). Although sequencing technologies have improved speed, cost, and quality over the last several decades, the error rate remains between 0.5%-1.0% errors per raw base, which is considerably high (Li et al. 2011). However, such limitations of DNA sequencing can be resolved with genotype imputation.

Genotype Imputation is a fast, cost-effective, and highly developed technique that can accurately estimate unobserved genotypes, or genotype probabilities from a densely characterized reference panel (Howie et al. 2009; Howie et al, 2011; Howie et al. 2012). This technique can also be used to increase power of GWAS analyses, allowing researchers to conduct meta-analysis of GWAS and analyze the results from multiple studies that depend on different genotyping platforms (Browning et al. 2009; Howie et al. 2011; Li et al. 2010; Welter et al. 2014).

Genotype imputation has led to the discoveries of thousands of SNP-trait associations, which have substantially impacted human health. Researchers and doctors will have a deeper understanding of genetically-associated diseases, thus enabling them to develop better methods to detect, treat, and prevent diseases (Genome-Wide Association Studies 2015). Genetics researchers have claimed that genotype imputation is a necessary tool used in GWAS to accurately impute the unobserved genotypes, and the research goal is to implement genotype imputation on a sample of 437 Tianjin women with 499,257 variants and evaluate the results of the genotype imputation using 1) estimated R-square hat and 2) 5% masking process of the genotyped genetic variants.

Materials and Method

A sample of 445 participants from Tianjin, China is genotyped using Illumina 660 SNP array. A total of 560,525 genetic markers were genotyped and may be used to conduct genotype imputation in order to assess the SNPs-lung cancer association. 1000 Genomes reference panel and TOPMed reference panel were used to complete the imputation process.

M.1 Pre-imputation Quality Control

Pre-imputation quality control is conducted on both sample and marker level to remove individuals or markers that may induce high error rates when performing imputation. It is assumed that the removal of a small sample of genotyped data will not significantly affect the overall power of the study. However, it should be noted that the removal of any SNP could lead to an overlooked SNPs-trait association even though the genotype imputation can recover the removed SNPs.

First, sample-level quality control was performed based on the following three criteria: 1) call rate, 2) gender checking, and 3) relatedness. With regards to call rate, individuals with more than 10% missing genotypes were removed. Then the individuals with discordant sex information using the X chromosome from the genotype data were identified and removed from further analysis. Lastly, individuals in a sample are expected to be unrelated from each other to ensure no genotypes are over-represented and to keep a fair reflection of the allele frequency of the sample. In this case, assessment is conducted on the degree of shared alleles between pairs of individuals. The maximum relatedness between any pair of individuals is the third degree relative; thus, one individual is removed from each pair when pairs are third-degree relative or less.

After the removal of individuals who did not pass the sample level quality control, performance on marker-level quality control was conducted to identify any genetic variants that may induce bias. The criteria for sample level quality control are genotypes 1) with an excessive missing genotype, or 2) show a significant deviation from Hardy-Weinberg equilibrium (HWE), or 3) with a very low minor allele frequency. Markers with a call rate less than 95% are removed from further study. Markers with an extensive deviation from HWE suggest genotype calling error, thus markers with a p-value threshold < 10^{-6} are removed. Lastly, SNPs with minor allele frequency (MAF) <0.5% are removed. Although the goal of quality control is to produce more accurate imputed genotypes, genotyping errors can still persist after pre-imputation quality control. Both sample-level and marker level pre-imputation quality control was conducted using plink program v.190 (Chang et al. 2015).

M.2 Principal Component Analysis (PCA)

After the removal of individuals and markers that may have particularly high error rates, a joint PCA, sample combined with the 1000 Genomes (1000G) reference panel, was conducted to identify the ancestry of the sample and discern outliers using the smartpca program of the EIGENSOFT software package v. 6.1.4 (Patterson et al. 2006). PCA is a mathematical method that reduces large sets of potentially correlated variables to a number of uncorrelated variables, which are called principal components (Reich, David, et al). The first principle component accounts for the greatest variability in the data, while the succeeding principle components account for the remaining variability (Reich, David, et al).

Using plink v.190, a pruned subset of markers based on correlation between genotype allele counts are extracted from the sample that are in approximate linkage equilibrium with each other. When the correlation between any pair of SNPs within a 50 kilobase window is > 0.1, then the SNP is labeled and pruned from the current window. Once no such pairs were retained, the window is shifted across the 5kb at a time. After the production of a subset of markers that are in approximate linkage equilibrium, the alleles of the sample were ensured to be coded on the same strand as the 1000G reference panel by using plink v.190 to flip it. Then, the shared SNPs between the Tianjin sample and 1000G were extracted using vcftools with SNPs from different chromosomes merged into one dataset. SNPs with missing call rate over 10% were filtered from the merged dataset. SMARTpca is used to generate the principle components for each individual, and the first two principal components are plotted to display the ancestry of the sample.

M.3 Strand-Flip

SNPs were genotyped with microarray on either the forward or reverse strand. Strand-flip was conducted to ensure the SNPs from the study sample and the reference panels are coded to the same allele to improve imputation accuracy. Non-ambiguous SNPs, which are SNPs that are not A/T or C/G, are easier to detect. When more than two individuals in the sample study have genotyped on a different strand compared to the reference panel, plink v.1.90 is used to flip the SNPs. The frequencies of the minor alleles for each data are computed to compare to that of the alleles in the reference panels in order to identify the variants that need to be flipped. For ambiguous SNP with MAF >0.55 in sample study but <0.45 in reference, they would be flipped using plink v.1.90. However, if an ambiguous SNP with MAF of 0.45 to 0.55, then the SNP is not flipped due to the uncertainty.

M.4 Pre-phasing and Imputation

This study uses Eagle v.2.3.5 (Loh et al. 2016) for pre-phasing with 1000G as the reference panel. This step is included to discern markers that are located on the same chromosomes. Then Minimac3 v.2.0.1 (Das et al. 2016) is used to perform imputation.

M.5 Post Imputation Quality Control

To assess which of the imputed SNPs can be used for further analysis, the squared Pearson's correlation (R-square) between directly genotyped genotypes and imputed genotypes is estimated and used. Due to lack of availability of true genotypes, R^2 value is estimated by comparing the variance of the imputed allele counts to the theoretical expectation of the variance of allele counts using Hardy-Weinberg equilibrium. For genetic variants that are poorly imputed, the variability will be much less than that of the theoretical expectation, thus leading to a low R^2 value. An R^2 threshold of 0.30 will be used and all variants with R^2 value above 0.30 are then used to calculate the average R^2. If the average is above 0.80, then the threshold used suggests that the majority of the bad imputed SNPs have been removed from the overall imputed sample. However, when the average R^2 value is below 0.80, then the original threshold of 0.30 will be raised by 0.10 each time until the average R^2 value is above 0.80. It is expected that 70% of the badly imputed SNPs will be removed with this method of evaluation.

M.6 Post Imputation Quality Assessment

Since the R^2 value is estimated without having the true genotypes, then it is uncertain how valid the imputations were on this study sample. To validate the quality of the imputation on this Tianjin sample study, R^2 values between imputed genotypes and true genotypes are calculated by randomly removing 5% of the directly genotyped SNPs from the dataset. Then the remaining 95% of the directly genotyped genetic variants are again used to conduct imputation with Minimac3. To ensure at least some genetic variants with low minor allele frequency (MAF) would be included in the evaluation process, masking 5% of the directly genotyped variants was conducted 10 times and evaluated altogether. With this method, the imputation quality is evaluated by calculating the true squared Pearson's correlation of the 5% removed genotypes. Ten samples of imputed genetic variants of three chromosomes (1, 11, and 21) of distinctively different sizes were selected to assess the imputation quality of this sample.

Result

R.1 Pre-imputation Quality Control Results

For sample-level quality control, the three standards are call rate, gender-checking, and relatedness of the sample. All 455 individuals have less than 10% missing rate. In this study, only female participants are of interest; therefore, two male participants and one ambiguous sex participant based on (table 1). Sexuality is inferred with F estimates, which is an indicator of sex call. Female participants should have a F- estimate of < 0.2 and male participants have a F-estimate of > 0.8.

Table 1: Individuals with Ambiguous Sex

Individual ID	Pedigree Sex	SNP Sex	Predicted Sex	Status	F estimate
TGS095013	Female	0	Unknown	Problem	0.4199
TGS024524	Female	1	Male	Problem	0.9997
TGS072771	Female	1	Male	Problem	1.000

Lastly, sample relatedness checking using pairwise IBD (identity by descent) estimate was used to determine individuals who appear to be more related than expected in a random sample. The probability of sharing 0, 1, or 2 alleles IBD for any two individuals is estimated to calculate PI-HAT. A threshold of PI-HAT ≤ 0.125, equate to a 3rd degree or less relatedness. Five pairs of individuals were too closely related, thus the second member of each pair is removed from further analysis (table2). After conducting sample level quality control, 8 participants were removed from the sample, and 437 participants were left in the sample for marker level quality control (table 3).

Table 2: Pair of Individuals who are Closely Related

Individual ID1	Individual ID2	Z0	Z1	Z2	PI_HAT
TGS083386	TGS068937	0.7604	0.1870	0.0526	0.1461
TGS059956	TGS091499	0.6652	0.2958	0.0390	0.1869
TGS046727	TGS002359	0.0026	0.9427	0.0547	0.5260
TGS090327	TGS095445	0.2121	0.4232	0.3647	0.5763
TGS086132	TGS086132	0.0018	0.9506	0.0476	0.5529

Table 3: Results from Sample Level Quality Control

Criteria	# Participants	Percent
Call rate <90%	0	0.00%
Gender Check	3	0.67%
Relatedness	5	1.12%

In regards to marker level quality control, SNPs with a call rate of less than 95%, or an extreme departure from HWE ($p<1X10^{-6}$), or a minor allele frequency of less than 0.005 were excluded from the sample. A total of 1,009 SNPs were found to have a call rate of less than 95%, 283 SNPs were qualified as having an extreme deviation from the HWE, and 60,085 SNPs were excluded due to having a MAF <0.5%. A summary of the number of and the percentage of SNPs removed are shown in table 4. As a result, a total of 61,377 variants were removed from sample, and 499,148 variants passed the quality control test.

Table 4: Results from Marker Level Quality Control

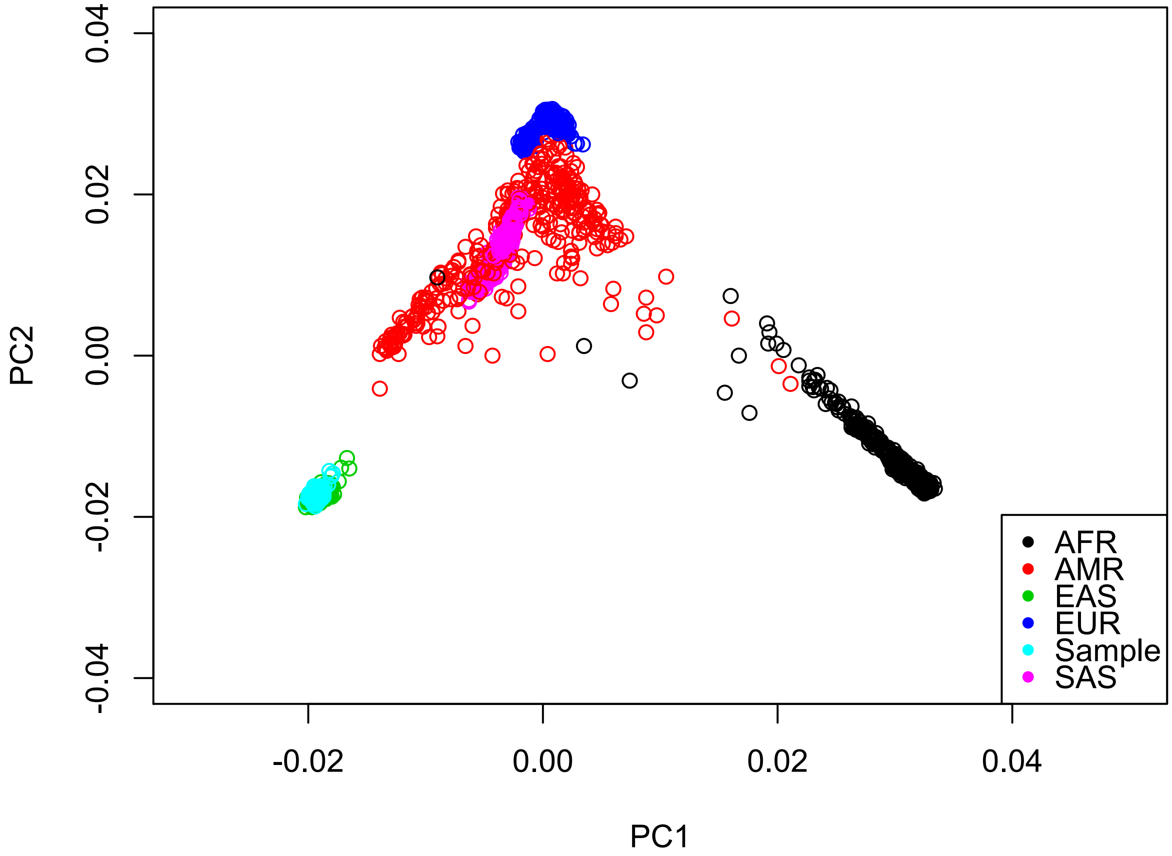
Criteria	SNPs	Percent
Call rate < 95%	1009	0.18
Deviation from HWE	283	0.05
MAF < 0.005	60085	10.72

As a result of quality control on both sample and marker level, the sample is left with 437 Tianjin female individuals with 499,148 genetic variants.

R.2 Principle Component Analysis (PCA)

A total of 60,182 SNPs remained for the principle component analysis after conducting linkage disequilibrium pruning. A joint PCA is conducted with 1000G-reference panel (figure 1).

Figure 1: Joint PCA of Tianjin Individual Sample with 1000G Phase 3 Reference Panel



AFR: African; AMR: American; EAS: East Asian; EUR: European; SAS: South Asian

All the individuals of the Tianjin sample are clustering together with the East Asian cohort of the 1000G-reference panel. Thus the Tianjin sample has individuals of only East Asian descent and it can be noted that there are no outliers in this sample.

R.3 Strand flipping results

By calculating the frequencies of the MAF for each SNP, 503 ambiguous and 94,849 non-ambiguous SNPs were identified that needed to be flipped to ensure alleles from the sample and the 1000G reference panel are labeled consistently. For TOPMed reference panel, 453 ambiguous and 92,834 non-ambiguous SNPs were flipped. The proportions of SNPs flipped and kept for 1000G and TOPMed reference panel are shown in figure 2 and 3 respectively.

Figure 2: Percept of SNPs Flipped or Kept

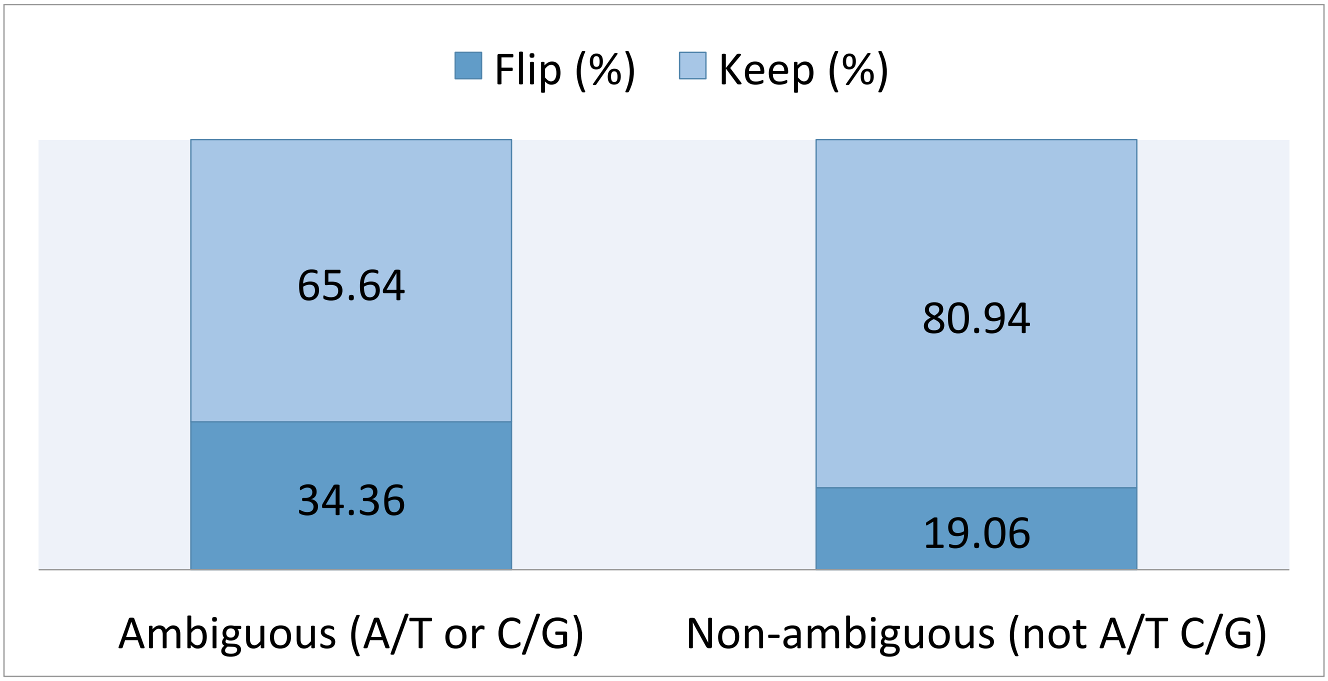
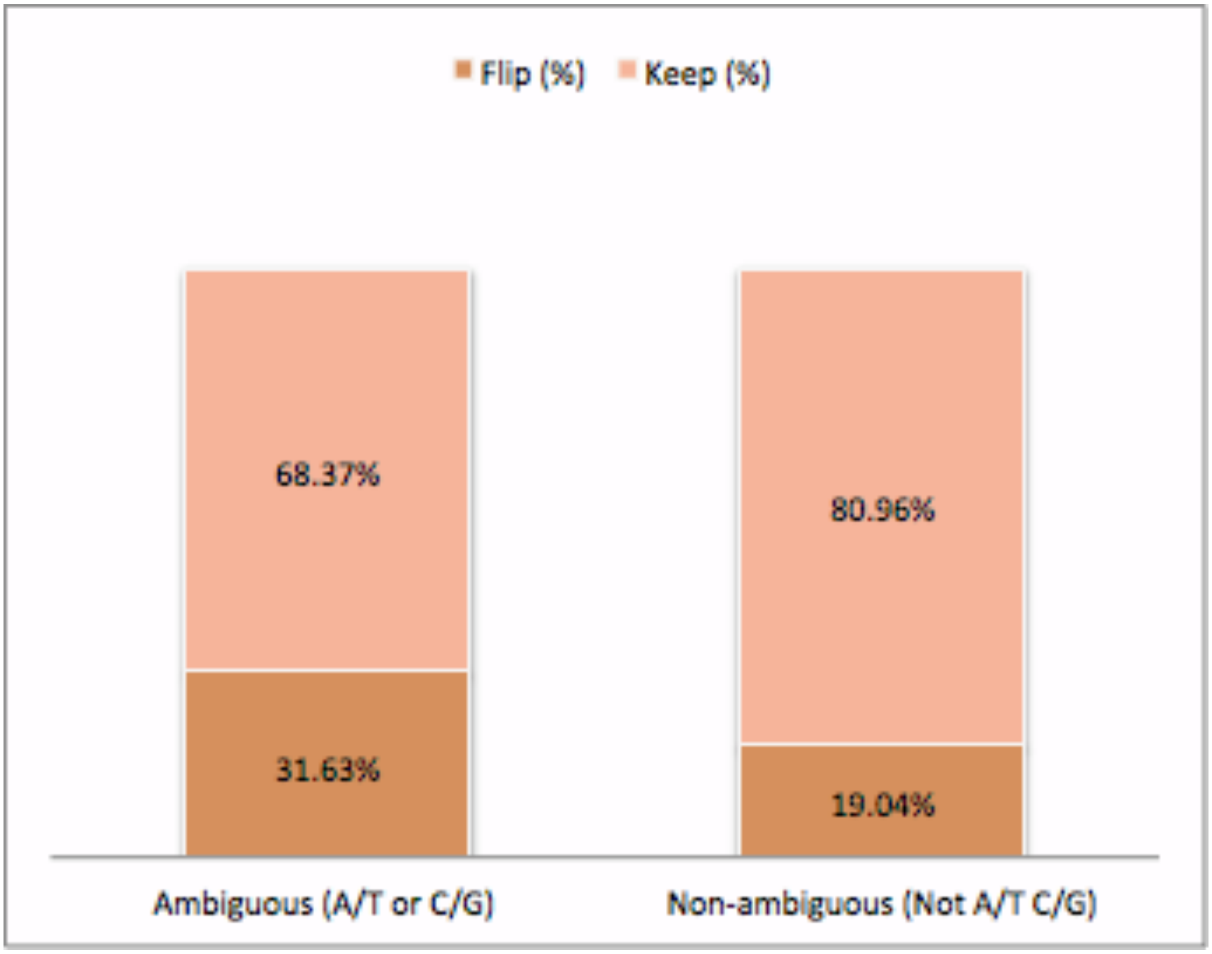


Figure 3: Percent of SNPs Flipped or Kept for TOPMed Reference Panel



R.4 Pre-phasing Imputation Results

Based on the imputation results, approximately 47 million SNPs were imputed using Minimac3. Rare genetics variants (SNPs with MAF of 0.0-1.0%) must surpass an R^2 threshold of 0.7 for their conditional mean of R^2 value to be above 0.80. Even though majority of the imputed SNPs are from genetic variants with MAF of 0.0-0.2%,, only approximately 1.50% of the imputed SNPs passed the post-imputation quality control. Approximately 13.93% of the imputed SNPs with MAF of 0.2-0.5 and 23.94% of the imputed SNPs with MAF of 0.5-1.0% are regarded as “well imputed.” For SNPs with MAF between 1.0-3.0%, 65.74% of the imputed SNPs are well imputed using a threshold of 0.5. SNPs with MAF>3.0%, above 90% of the SNPs, are well imputed using a R^2 threshold of 0.3, which is considerably great. As the MAF increases, the percentage of SNPs that exceed the R^2 threshold also increases. In total, 9.5 million imputed markers passed the post imputation quality control with 1000G reference panel.

Table 5: Summary Statistics of Imputation Quality of Tianjin Sample for 1000G Reference Sample

MAF (%)	**R^2 Threshold**	**Total # of Markers**	**# (%) of Markers Exceed Threshold**	**Conditional Mean of R^2**
0.0-0.2	0.7	33079479	497590 (1.50)	0.83606
0.2-0.5	0.7	3482744	485011 (13.93)	0.84368
0.5-1.0	0.7	1649738	394908 (23.94)	0.86016
1.0-3.0	0.5	1710217	1124244 (65.74)	0.80638
3.0-5.0	0.3	725647	680812 (93.82)	0.83116
5.0-50.0	0.3	6461606	6337304 (98.08)	0.92243
		Total # of QC+ Markers	9,519,869	

Using TOPMed reference panel, approximately 88 million SNPs were successfully imputed with minimac3. Imputed SNPs with MAF of <1.0% have to exceed the R^2 threshold of 0.5 for average R^2 to exceed 0.80. 2.11% of the imputed SNPs with MAF of <0.20%, 49.20% of the imputed SNPs with MAF of 0.20-0.50%, and 53.56% of the imputed SNPs with MAF of 0.50-1.0% surpassed the R^2 threshold. For SNPs with MAF of 1.0-3.0%, a R^2 threshold of 0.50 is necessary for the conditional mean of R^2 to exceed 0.80. Lastly, for SNPs with MAF >3.0%, 88% and 96% of the imputed SNPs in MAF categories 3.0-5.0% and 5.0-50.0%, respectively, passed the post-imputation quality control. Similar pattern is observed using TOPMed reference panel, in which as the MAF increases, higher percentage of the imputed SNPs are regarded as "well-imputed." Overall, almost 11 million imputed markers passed the post imputation quality control with TOPMed reference panel.

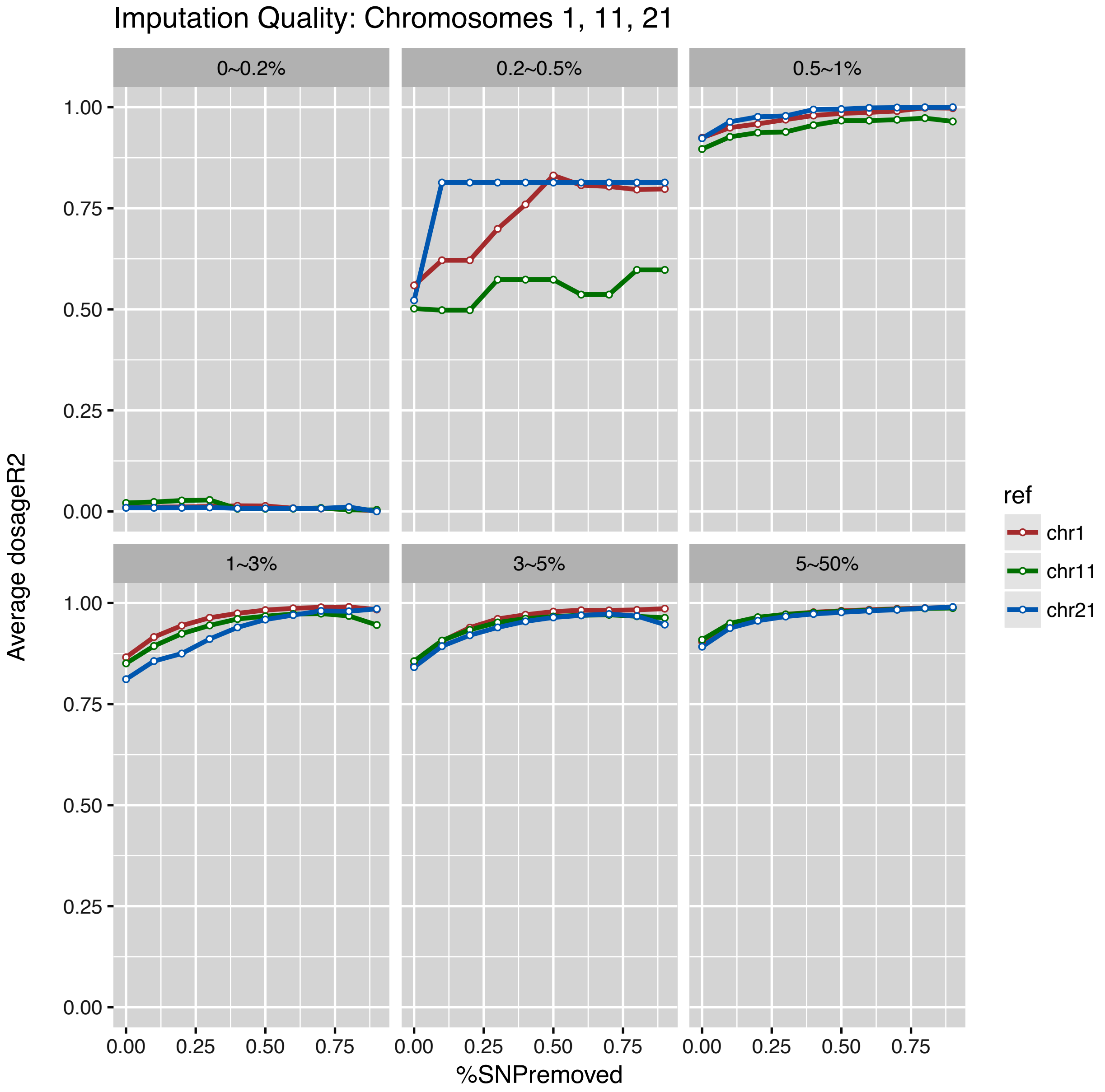
Table 6: Summary Statistics of Imputation Quality of Tianjin Sample for TOPMED reference Panel

MAF (%)	R^2 Threshold	Total # of Markers	# (%) of Markers Exceed Threshold	Conditional Mean of R^2
0.0-0.2	0.6	75,794,901	1,592,902 (2.11)	0.81146
0.2-0.5	0.6	2,796,008	1,375,570 (49.20)	0.80785
0.5-1.0	0.6	1,429,111	765,436 (53.56)	0.81472
1.0-3.0	0.5	1,749,929	1,152,142 (65.84)	0.82849
3.0-5.0	0.3	704,748	624,162 (88.57)	0.82145
5.0-50.0	0.3	5,587,541	5,395,434 (96.56)	0.89315
		Total # of QC+ Markers	10,905,646	

R.5 Evaluation of Imputation with 5% masked

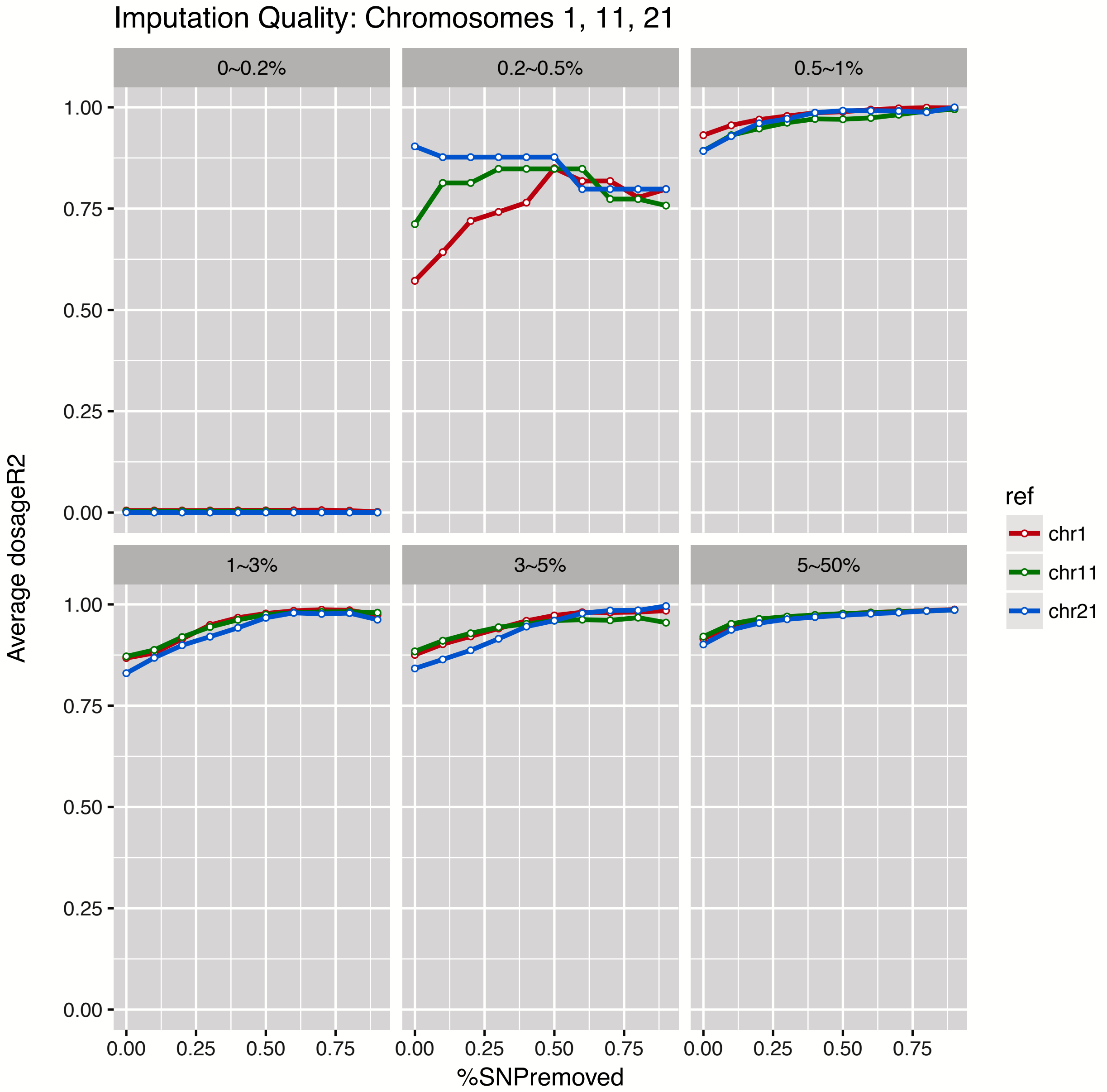
To see how well Minimac3 recovers the 5% masked SNPs from the sample, R was used to create qqplots using two variables -the average dosage R^2 and the percentage of badly imputed SNPs removed.

Figure 4: True Average Dosage R^2 for chromosomes 1, 11, 21 with 1000 Genomes Reference Panel



With 1000G reference panel, figure 4, it can be observed that genetic variants with MAF of <0.2% have an average dosage R^2 less than 0.10 despite removing 90% of all the bad SNPs. Similarly, most SNPs with MAF of 0.2-0.5% did not have the average dosage R^2 above 0.80 after removing a considerable amount of badly imputed SNPs. However, for SNPs with MAF of > 0.5%, almost all imputed SNPs from the three chromosomes have an average dosage R-square of greater than 0.80 without the removal of any SNPs, indicating that approximately 100% of the imputed SNPs are "well-imputed". Figure 4 suggests that genotype imputation with 1000G reference panel can recover a considerable amount of genetic variants, thus imputation quality is good for such study sample.

Figure 5:True Average Dosage R^2 for chromosomes 1, 11, 21 with TOPMED Reference Panel



With TOPMed reference panel, a similar pattern is observed. The average dosage R^2 is very low for genetic variants with MAF of <0.2%. For SNPs with MAF of 0.2-0.5%, chromosomes showed an unusual pattern of decreasing in R^2 value after the removal of 50% of the poorly imputed SNPs, indicating there was some noise in the computation. SNPs with MAF of > 0.5% showed a strong recovery since all the imputed SNPs from the three chromosomes have an average dosage R^2 of greater than 0.80 without the removal of any SNPs, indicating that approximately 100% of the imputed SNPs were recovered through imputation. Figure 5 indicates that TOPMed reference panel can also provide a good prediction as to the genetic variants for this study sample.

Figure 6:Comparing 1000G Reference Panel with TOPMED Reference Panel using 5% Masking Process

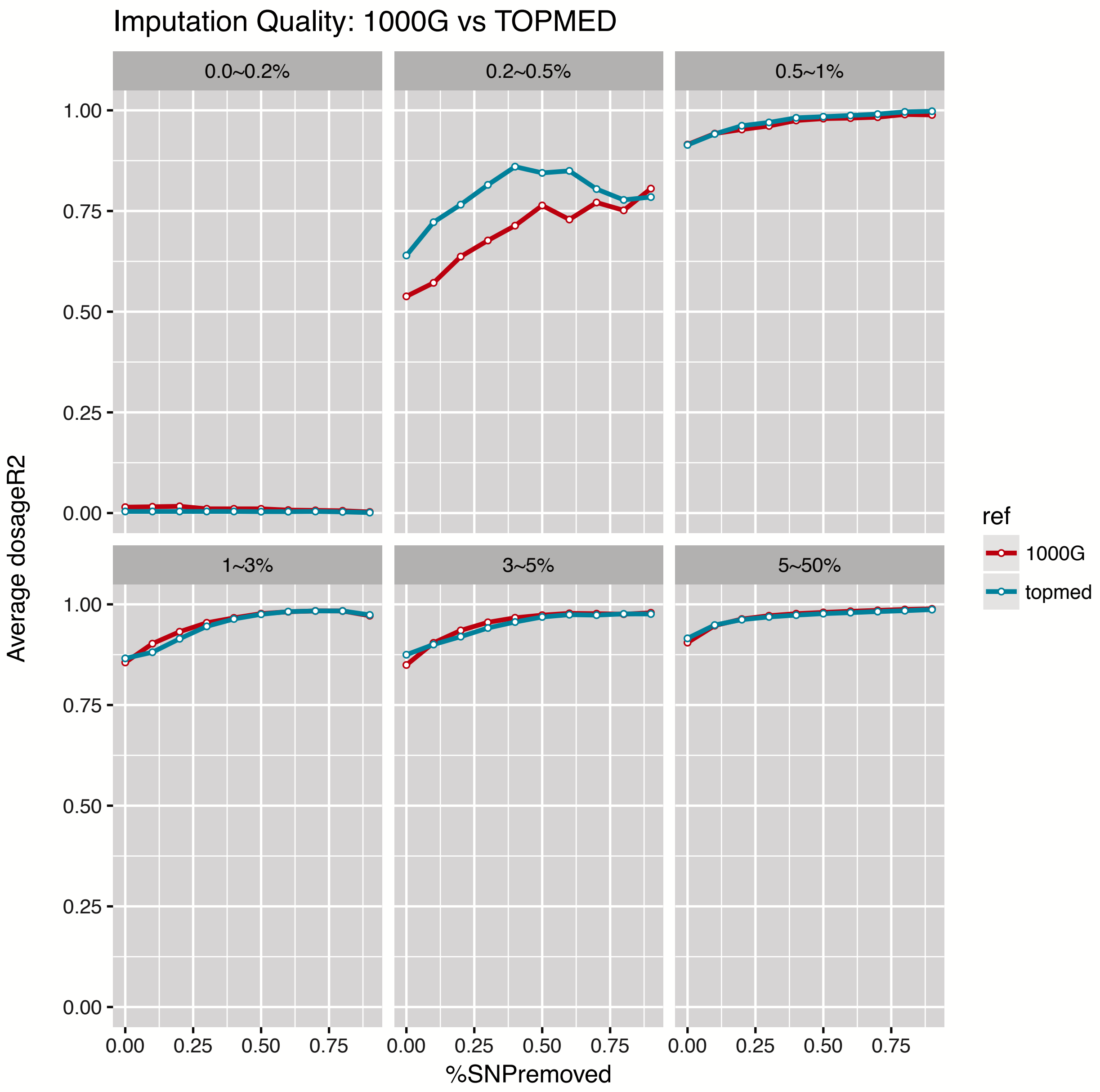


Figure 6 is combining the masking results of the three chromosomes, 1, 11, and 21 using two different reference panels. There is not a considerable difference in using either panels in different MAF categories, although TOPMed performed slightly better in the MAF category of 0.2-0.5%. Overall, the difference between the two reference panels is miniscule and both are useful for conducting imputation.

Discussion

With genotype imputation, approximately 47 million and 88 million genetic variants were imputed with 1000G and TOPMed reference panel, respectively. However, only approximately 9.5 million from 1000G and 11 million from TOPMed of the imputed genetic variants qualify as "well-imputed" and can be used in further analysis. It should be noted that even though the majority of the imputed SNPs were SNPs with MAF<0.2% and only approximately 2% of the imputed SNPs in that category passed the post imputation quality control, the percentage of "well-imputed" SNPs increases with increasing MAF. Thus, imputation is more difficult with rare variants, which is also verified with the 5% masking process. All genetic variants in the category of 0.0-0.2% did not recover using either of the reference panels. While approximately 50% of the genetic variants were recovered in the 0.2-0.5% category, almost all genetic variants with MAF >0.5% were recovered. Thus, even though genotype imputation may not have reached high accuracy for rare genetic variants, imputation is still highly accurate for variants greater than 1%. Despite the large number of imputed SNPs that did not pass quality control, it is still valid to conclude that genotype imputation is a highly accurate statistical tool since it was able to recover most of the masked SNPs using Minimac3. Thus the imputation accuracy for this sample study is still high.

Instead of simply just using the originally ~500,000 directly genotyped genotypes to conduct association between the SNPs and lung cancer, researchers can use at least 10 million SNPs to seek association with imputation. Of the 10 million SNPs, approximately 95% of the genetic variants are imputed with statistical software. Therefore, it is important to conclude the significance and advantages of this statistical method.

One limitation of this study is the study sample size. With only 499,148 genetic variants available to conduct imputation, it was limiting the accuracy of rare SNPs; therefore, limiting the number of imputed genetic variants that could be used to assess association with lung cancer. Additionally, even though TOPMed reference panel imputed more genetic variants that can be used for further analysis, it is still difficult to assess whether TOPMed reference panel outperforms 1000G reference panel. With a larger sample size, the comparison between the two reference panels can be done more vividly.

With genotype imputation for study samples, instead of using only 499,148 SNPs to identify SNPs that are associated with lung cancer, researchers can now use at least 10 million SNPs to detect SNPs associated with lung cancer of non-female nonsmokers. The additional 9.5 million genetic variants can not only facilitate meta-analysis but also increase statistical power in conducting association tests.

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