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Single nucleotide polymorphisms in chemokine receptor and ligand genes contribute to autoimmune thyroid disease (AITD) susceptibility

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ABSTRACT

Chemokine proteins play a role in AITD (Autoimmune Thyroid Disease) immune response, and polymorphisms in chemokine genes have been found to influence AITD susceptibility. The authors of Shin et al., 2023 established that SNPs in chemokine genes are present in individuals with AITD. However, it is not clear if polymorphisms in chemokine receptor genes or chemokine ligand genes contribute more to AITD susceptibility. This study compares the association of polymorphisms in chemokine ligands and chemokine receptors with AITD susceptibility. The goal of the study was to determine if presence of the minor allele in chemokine ligand or chemokine receptor genes was more greatly associated with AITD. The hypothesis of the current study was that polymorphisms in the chemokine ligands will have a greater impact on AITD susceptibility than polymorphisms in chemokine receptors. Using genomic data from Shin et al., 2023 a secondary analysis was performed. The minor allele frequencies (MAF) for each SNP were calculated and Odds Ratio (OR) to determine if the MAF was more likely to be associated with AITD. Fisher's exact test p-values were used to determine significance of associations. This study was unable to reject the null hypothesis, finding no statistically significant difference in the association of the MAF of chemokine receptors and chemokine ligands on AITD. Future studies should expand and diversify the cohort, examine location of SNP (promoter, exon, intron) and its impact on AITD susceptibility, and explore chemokine proteins for potential therapeutic targets for AITD.

INTRODUCTION

Chemokines are signaling proteins which induce chemotaxis in nearby cells. [1] Chemotaxis is a living cell's ability to determine its direction of movement, which can contribute to inflammatory response and manifestations of diseases. [2] There are four families of chemokines based upon their cysteine configurations: XC, CXC, CX3C, and CC. Some chemokines are proinflammatory, meaning they recruit immune cells in inflammation or infection sites.[1] Others are homeostatic, and they are responsible for maintenance and regulation of tissue development and cell migration.[1] Chemokines interact with chemokine receptors which are transmembrane receptors found on the cell membrane of endothelial or

immune cells. [1] Together, chemokines and their receptors recruit various immune cells-T lymphocytes, B lymphocytes, macrophages, and dendritic cells to the impacted area. [1]

AITD (Autoimmune Thyroid Disease) is the result of immune system deregulation which causes an attack on the thyroid gland. AITD has two clinical presentations: Graves' disease (GD) and Hashimoto's thyroiditis (HT). AITD is a T cell mediated organ specific disease, which most often selectively affects the thyroid gland. [5] In AITD disease, T cells; B cells; and Lymphocytes work together in the immune response. AITD affects up to 5 percent of the general population [3] and is overall 10 times more frequent in women than in men (in both HT and GD). [4] Susceptibility to AITD is a combination of genetic and environmental triggers (radiation, smoking, stress, iodine, infection, and drugs) [1] and can be influenced by geographical location.

Several correlations have been found between Chemokine genes, their receptors and AITD diseases. In a study done on GD patients, it was found that in the healthy control group, presence of CXCL10/CXCL9 and their receptor, CXCR3, was poor or absent. However, in patients with Graves' disease, these same chemokines/chemokine receptors were present in most patients. [6] This suggests that CXCL10 and CXCL9 are contributing factors to pathogenesis of Graves' disease. [6] In a similar study performed on HT patients, presence of receptors CXCR3, CCR5, and CRTH2 was assessed in a healthy control group and diagnosed HT patients. CXCR3 and CCR5 levels were higher in patients with HT than those without. [7] CRTH2 levels were higher, but overall low, supporting the idea that Hashimoto's is primarily a Th1 immune response in its initial stages. [7]

The authors of Shin et al., 2023 established that SNPs in chemokine genes are present in individuals with AITD. Genotype frequencies of CCL11 (rs3744508) AA and CCR2 (rs1799864) AA were higher in the total AITD group than in the control; however, CCL17(rs223828) CC was lower in the AITD patient group than in the control. [8] The allele frequency of CCL17 (rs223828) T was higher in the AITD patient group than the control. [8] Overall, this study showed positive correlation between certain chemokine SNPs and AITD disease. [8] However, it is not clear if chemokine ligand genes or chemokine receptor genes are more affected in AITD and if there are differences in SNP presence in chemokine ligand vs receptor genes for AITD versus healthy participants. This project will investigate if SNP prevalence is higher in chemokine receptor genes or chemokine ligand genes among individuals with AITD and in AITD versus healthy individuals using a secondary analysis approach. The raw dataset for this project was provided by the Shin et al., 2023 paper. I hypothesized that polymorphisms in the chemokine ligands would have a greater impact on AITD susceptibility than polymorphisms in chemokine receptors. Chemokines recruit Th1 lymphocytes or other immune cells by binding to their corresponding receptors. These immune cells cause the production of more chemokines and therefore, more immune cells gather at the source which amplifies thyroid inflammation. Since chemokine ligands initiate this process, I believe polymorphisms in the chemokine ligands would impact AITD susceptibility more than receptors.

METHODS

Dataset

This study was a secondary analysis of open source genomic data found on Harvard Dataverse of chemokine polymorphisms in AITD patients and a healthy control group. The genomic data for AITD patients was extracted from 86 Korean pediatric Hashimoto's and Grave's Disease patients at the Pediatric Endocrine Clinics of Seoul St. Mary's Hospital between March 2009 and August 2021. The AITD GD group (50 people) was 76% female, 24% male, and the age at enrollment into the study was 13.2 ± 3.3 years. The age at GD diagnosis was 11.3 ± 3.2 years. In the AITD HT group (36 people), 86.1% of patients were female and 13.9% were male. The HT age at enrollment was 13.1 ± 3.9 years and the age at diagnosis was 10.5 ± 2.7 years. The control group consisted of 183 healthy Korean adults whose age at enrollment was 29.9 ± 3.7 years. The control group was 52.5% female and 47.5% male. This control group was composed of students and staff from the College of Medicine at the Catholic University of Korea.

Individual SNP analysis

Each SNP was classified as belonging to a ligand or receptor gene and the location of each SNP in the genome was noted. Microsoft Excel was utilized to perform a secondary data analysis on the genomic data provided in Shin et al., 2023. Diseased MAF was calculated for each SNP to determine how often the minor allele (present in <50% of the population) was present in an AITD patient. The same test was performed on the healthy population to determine the minor allele presence in the healthy control group. Odds Ratio was calculated for each SNP to determine the likelihood that the MAF was higher in the diseased population versus in the healthy population. The statistical significance of the MAF for individual SNPs was assessed using Fisher's exact test. Fisher's exact test is used for categorical data such as the genomic data being used in this study, and determines whether there is significant association between the minor allele and AITD susceptibility. The p values were adjusted using the Benjamini-Hochberg false discovery rate (FDR) procedure, which reduces the proportion of false-positives among the significant results. The Benjamini-Hochberg FDR procedure was applied because there were many hypothesis tests run simultaneously.

Pooled analysis

Each SNP has a different allele frequency, gene, location in the DNA, and biological function, but in order to answer the question if ligand or receptor SNPs are more responsible for AITD susceptibility, ligand and receptor were treated as categorical data and the total number of minor alleles were counted for all ligand and all receptor genes in healthy and diseased individuals. The chemokine ligand pooled diseased MAF was calculated to determine the MAF in all chemokine ligand genes in AITD patients. The chemokine receptor pooled diseased MAF was calculated to determine the MAF in all chemokine receptor genes in AITD patients. Pooled OR values were calculated to determine if the MAF in ligands or receptors was more strongly associated with AITD. Pooled ligand OR was calculated to determine how likely it was that the minor allele was present in a chemokine ligand gene in AITD patients versus the

healthy control group. Pooled receptor OR was calculated to determine how likely it was that the minor allele was present in a chemokine receptor gene in AITD patients versus the healthy control group. The statistical significance of the MAF for the pooled SNPs was assessed using Fisher's exact test.

RESULTS

Individual SNP analysis

The healthy (MAF= $\frac{healthy\ minor\ allele\ count}{healthy\ alleles\ total\ count}$) and diseased (MAF= $\frac{diseased\ minor\ allele\ count}{diseased\ total\ allele\ count}$) minor allele frequency, Odds Ratio (OR=$\frac{\frac{diseased\ minor\ allele\ count}{diseased\ alleles\ major\ count}}{\frac{healthy\ minor\ allele\ count}{healthy\ alleles\ major\ count}}$), and Fisher P values were calculated for each SNP (Table 2 and Table 3). CCL17 (rs223828) had an OR of 1.735211268. This meant that it was more likely to find the presence of the minor allele was more likely in the diseased versus healthy population. This was statistically significant with the Fisher p exact test (unadjusted p=0.003562997) prior to adjusting for multiple comparisons (Table 2). Statistical significance for FDR-adjusted values was $p<0.05$. The association did not remain statistically significant following FDR-correction (adjusted p= 0.089074918) (Table 3). CCR2 (rs1799864) had an OR of 1.448461954, showing that the minor allele count was higher in the diseased population than in the healthy population. This OR approached statistical significance (unadjusted p= 0.06937035); however, the FDR-adjusted value (adjusted p= 0.721459679), proved no statistical significance.

Pooled analysis

There was no statistically significant difference based on the Pooled Fisher p for the chemokine ligand genes (p = 0.833513851) or the chemokine receptor genes (p = 0.882029597) (Table 4). There is no difference in the pooled MAF for the chemokine ligand genes in the healthy (MAF= $\frac{healthy\ minor\ allele\ count\ in\ ligand\ genes}{healthy\ allele\ total\ \ count\ in\ ligand\ genes}$) (MAF=0.245098039) versus diseased (MAF= $\frac{diseased\ minor\ allele\ count\ in\ ligand\ genes}{diseased\ total\ allele\ count\ in\ ligand\ genes}$) (MAF=0.243006993) populations. There is no difference in the pooled MAF for the chemokine receptor genes in the healthy (MAF= $\frac{healthy\ minor\ allele\ count\ in\ receptor\ genes}{healthy\ allele\ total\ count\ in\ receptor\ genes}$) (MAF=0.2667734973) versus diseased (MAF=$\frac{diseased\ minor\ allele\ count\ in\ receptor\ genes}{diseased\ allele\ total\ count\ in\ receptor\ genes}$)(MAF=0.269174041) population. The pooled odds ratio for ligand genes (OR= $\frac{\frac{diseased\ minor\ allele\ count\ in\ ligand\ genes}{diseased\ major\ allele\ count\ in\ ligand\ genes}}{\frac{healthy\ minor\ allele\ count\ in\ ligand\ genes}{healthy\ major\ allele\ count\ in\ ligand\ genes}}$) was 0.9887298, and the pooled odds ratio for receptor genes (OR= $\frac{\frac{diseased\ minor\ allele\ count\ in\ receptor\ genes}{diseased\ major\ allele\ count\ in\ receptor\ genes}}{\frac{healthy\ minor\ allele\ count\ in\ receptor\ genes}{healthy\ major\ allele\ count\ in\ receptor\ genes}}$) was 1.0125121. Both OR values were close to 1, indicating that the pooled minor alleles were equally likely to occur in diseased and healthy individuals in both chemokine ligand and receptor genes. Therefore, this study failed to reject the null hypothesis.

Table 1
Chemokine ligand and chemokine receptor SNPs investigated in this study.

SNP (rsID)	Gene	Class	Minor Allele	Major Allele
rs1024611	CCL2	Ligand	A	G
rs1024610	CCL2	Ligand	T	A
rs2107538	CCL5	Ligand	A	G
rs2280789	CCL5	Ligand	C	T
rs2280788	CCL5	Ligand	C	G
rs3744508	CCL11	Ligand	A	G
rs223828	CCL17	Ligand	T	C
rs2302004	CCL24	Ligand	T	C
rs2302005	CCL24	Ligand	C	T
rs2302009	CCL24	Ligand	G	T
rs352046	CXCL8	Ligand	G	C
rs4073	CXCL8	Ligand	A	T
rs2227306	CXCL8	Ligand	T	C
rs2276886	CXCL9	Ligand	T	C
rs8878	CXCL10	Ligand	A	G
rs3921	CXCL10	Ligand	C	G
rs1801157	CXCL12	Ligand	A	G
rs1799864	CCR2	Receptor	A	G
rs1799987	CCR2	Receptor	A	G
rs1799988	CCR2	Receptor	C	T
rs2230054	CXCR2	Receptor	T	C
rs1126579	CXCR2	Receptor	C	T
rs3732378	CX3CR1	Receptor	A	G
rs3732379	CX3CR1	Receptor	T	C
rs2671222	CXCR2	Receptor	A	G

Table 2
SNPs and their minor allele frequencies by group. The Odds Ratio and Fisher P values are based on comparisons between diseased versus healthy MAF (from Fisher's exact test)

SNP (rsID)	Gene	Overall MAF	Diseased MAF	Healthy MAF	Allelic OR	Fisher's p
rs1024611	CCL2	0.362453532	0.325581395	0.379781421	0.788389978	0.248794544
rs1024610	CCL2	0.060836502	0.04375	0.068306011	0.624052288	0.326390448
rs2107538	CCL5	0.383895131	0.363095238	0.393442623	0.878894081	0.565467225
rs2280789	CCL5	0.371268657	0.352941176	0.379781421	0.890778286	0.56576942
rs2280788	CCL5	0.170411985	0.148809524	0.180327869	0.794659886	0.388722687
rs3744508	CCL11	0.174157303	0.196428571	0.163934426	1.246666667	0.39009711
rs223828	**CCL17**	**0.43071161**	**0.523809524**	**0.387978142**	**1.735211268**	**0.003562997**
rs2302004	CCL24	0.324626866	0.311764706	0.330601093	0.917214099	0.692647354
rs2302005	CCL24	0.350187266	0.345238095	0.352459016	0.968710359	0.922282966
rs2302009	CCL24	0.097014925	0.117647059	0.087431694	1.391666667	0.275683162
rs352046	CXCL8	0.014981273	0.005952381	0.019125683	0.307100086	0.445429391
rs4073	CXCL8	0.370786517	0.398809524	0.357923497	1.190008314	0.385878374
rs2227306	CXCL8	0.316479401	0.339285714	0.306010929	1.16457529	0.483285343
rs2276886	CXCL9	0.391385768	0.351190476	0.409836066	0.779449541	0.215074825
rs8878	CXCL10	0.037453184	0.017857143	0.046448087	0.373262032	0.141070285
rs3921	CXCL10	0.037313433	0.017647059	0.046448087	0.368791828	0.14083109
rs1801157	CXCL12	0.258426966	0.261904762	0.256830601	1.02676733	0.915464736
rs1799864	CCR2	0.304104478	0.358823529	0.278688525	1.448461954	0.06937035
rs1799987	CCR2	0.473880597	0.494117647	0.464480874	1.126128591	0.577167743
rs1799988	CCR2	0.473880597	0.494117647	0.464480874	1.126128591	0.577167743
rs2230054	CXCR2	0.335205993	0.297619048	0.352459016	0.778478518	0.236569819
rs1126579	CXCR2	0.358208955	0.329411765	0.371584699	0.830753354	0.383905431
rs3732378	CX3CR1	0.048689139	0.047619048	0.049180328	0.966666667	1
rs3732379	CX3CR1	0.048507463	0.047058824	0.049180328	0.95473251	1
rs2671222	CXCR2	0.097014925	0.082352941	0.103825137	0.77462888	0.53098609

Note: Bolded p-values indicate statistical significance (p < .05). (before FDR correction)

Table 3

SNPs and their Fisher's Exact Test p-values and Benjamini-Hochberg FDR-Adjusted p-values.

SNP (rsID)	Gene	Fisher's Exact Test p-value	Benjamini-Hochberg FDR-Adjusted p-value
rs1024611	CCL2	0.248794544	0.721459679
rs1024610	CCL2	0.326390448	0.721459679
rs2107538	CCL5	0.565467225	0.721459679
rs2280789	CCL5	0.56576942	0.721459679
rs2280788	CCL5	0.388722687	0.721459679
rs3744508	CCL11	0.39009711	0.721459679
rs223828	CCL17	0.003562997	0.089074918
rs2302004	CCL24	0.692647354	0.824580183
rs2302005	CCL24	0.922282966	1
rs2302009	CCL24	0.275683162	0.721459679
rs352046	CXCL8	0.445429391	0.721459679
rs4073	CXCL8	0.385878374	0.721459679

rs2227306	CXCL8	0.483285343	0.721459679
rs2276886	CXCL9	0.215074825	0.721459679
rs8878	CXCL10	0.141070285	0.721459679
rs3921	CXCL10	0.14083109	0.721459679
rs1801157	CXCL12	0.915464736	1
rs1799864	CCR2	0.06937035	0.721459679
rs1799987	CCR2	0.577167743	0.721459679
rs1799988	CCR2	0.577167743	0.721459679
rs2230054	CXCR2	0.236569819	0.721459679
rs1126579	CXCR2	0.383905431	0.721459679
rs3732378	CX3CR1	1	1
rs3732379	CX3CR1	1	1
rs2671222	CXCR2	0.53098609	0.721459679

Table 4

Pooled minor allele frequencies and odds ratios by SNP class with Fisher p values based on comparisons between pooled diseased MAF and pooled healthy MAF (adjusted by Bonferroni method).

Class	Number of SNPs	Pooled Disease MAF	Pooled Healthy MAF	Mean SNP Diff.	Pooled OR	Pooled Fisher's p

Ligand	17	0.243006993	0.245098039	-0.002644372	0.988729792	0.833513851
Receptor	8	0.269174041	0.266734973	0.002155083	1.012512097	0.882029597

DISCUSSION

This project showed a statistically significant association between a polymorphism in CCL17 (rs223828) and AITD susceptibility prior to FDR correction. The authors of Shin et al., 2023 likewise found that the frequency of the T allele of CCL17 (rs223828) was higher in the AITD patient group than the control, suggesting that CCL17 (rs223828) is a contributing factor in disease susceptibility. This finding is not an independent replication of the Shin et al., 2023 study, but it is a strength of the paper that analysis of the original dataset in a new way yields the same results.

AITD is a complex disease with a genetic component contributing to susceptibility. However, the genes involved in the AITD immune response are not confined to chemokine receptor and ligand genes. A previous study demonstrated a statistically significant association between the CTLA-4 A/G genotype and AITD disease. [9] CTLA-4 is a Cytotoxic T lymphocyte antigen which regulates immune homeostasis and inhibits T cell activation in order to prevent overactive immune response. [10] Another study confirmed these results (Association between CTLA-4 A/G and HT+GD) and found an association between Allele G of CT60 and GD. [11] CT60 is a SNP within the noncoding region of the CTLA-4 gene. [12] In a different study, CT60 G/G was confirmed to contribute to GD susceptibility in a Taiwanese cohort. [12] Overall, many immune genes work together to contribute to AITD susceptibility.

Many of the polymorphisms in this study did not demonstrate statistical significance to AITD susceptibility. Sample size, effect size, or lack of statistical power could have led to lack of statistical significance. Environmental factors might contribute to AITD susceptibility as well. It is estimated that environmental factors contribute to about 20-30% of AITD pathogenesis. [13] Hypothyroidism has been found to be more common in iodine replete areas. [13] The risk of Graves' disease increases from smoking; however, smoking has been found to lower TPO-Ab antibodies and minimally protect against HT. [13] Selenium deficiency has been attributed to thyroid autoimmunity as well. [13] AITD patients (HT and GD) are more likely to have vitamin D deficiency. [14]

The location in which a polymorphism lies within a gene can influence its functional effect. Promoters are sites on genes where RNA polymerase binds to DNA in order to initiate the process of transcription. [15] SNPs in the promoter region can alter transcription and change gene expression, ultimately affecting the amount of the protein produced. One role of introns is to regulate gene expression, affecting transcription and RNA processing. SNPs in the intron region of chemokine genes could alter gene expression and

affect the amount of chemokine proteins produced. [16] Exons are regions of DNA that are transcribed into mRNA and remain after the introns are removed in RNA splicing. [17] SNPs in the coding exons (not all are protein coding) may adjust the mRNA sequence, and in turn change the structure or function of the protein produced.

CCL17 (rs223828) had the only statistically significant association between the minor allele and AITD susceptibility, but it did not remain statistically significant following the FDR procedure. This polymorphism lies in the intron region [18]. CCR2 (rs1799864) approached statistical significance and lies in the exon, since it is a Missense variant. [19] These findings indicate that both intronic and missense (exon) SNPs can contribute to AITD susceptibility.

Chemokines have been found to contribute to AITD susceptibility and immune cell recruitment; therefore, they have been investigated as AITD therapeutic targets. CXCL10 has emerged as a prominent chemokine in the AITD field, playing an important role in the development of Graves' disease. [20] CXCL10 levels are significantly elevated in patients with Graves' disease; however, after successful treatment with MMI (methimazole) or radioactive iodine, their CXCL10 levels decreased. [20] This suggests that regulation of CXCL10 signaling may be a potential therapeutic strategy for Graves' disease and should be investigated further. Since CCL17 showed statistical significance in this study, it is worth investigating as a potential therapeutic target as well.

LIMITATIONS OF STUDY

The Shin et al., 2023 dataset cohort consisted of 269 participants (183 controls and 86 AITD patients). This is a small sample size for a genotypic study which limited ability to detect associations between SNPs and AITD susceptibility. Additionally, there was no distinction within the dataset between GD and HT patients. Both diseases were grouped together in an AITD category. This may have prevented discovery of disease specific polymorphisms. Another limitation was that the gene on which each SNP was located was not labelled, as well as whether each SNP was on a chemokine ligand or a chemokine receptor.

The lack of age-matching between healthy adults and AITD adolescents was a limitation of the original data set. The average age of the cases was 13.2 ± 3.3 for GD patients and 13.1 ± 3.9 for HT patients. The average age for controls was 29.9 ± 3.7. A study detected allele frequency differences in 8 age groups for 9,817 genotyped variants in 72,635 Taiwanese Han individuals. [21] The results of the study found that 90 percent of all candidate variants had allele frequencies of <0.1% in the population. The magnitude of average allele frequency change in these 151 variants across age groups ranged from 11.5% to 15.46%. [21] This demonstrates that age has a clear impact on allele frequency. Allele frequency differences measured in this study could be impacted due to the differences in mean age group (adolescents vs adults) and therefore not reflect changes due to AITD. However, there is a lack of evidence that MAF in chemokine ligand or chemokine receptor genes changes over time, so it is unclear whether age had a

significant impact on the results of the study. Future genetic studies on AITD should use age matched cases vs controls to eliminate risk of MAF being affected by age group.

Another limitation of this study is that the minor allele counts were pooled for receptor and ligand genes in the pooled analysis. A better study would have been a genome wide association study (GWAS) that used a large sample size to identify SNPs in a diverse population that are associated with AITD. However, that was outside the scope of this project. Future GWAS studies on patients with AITD (cases) and healthy participants (controls) are needed.

Another limitation is that all the genomic data was obtained from patients of Korean ancestry, limiting the applicability of the findings to other ethnic populations, as genetic risk factors vary among different populations. Future directions for this study involve expanding and diversifying the cohort. One way to do this would be to conduct a larger GWAS, as mentioned above. Finally, GD and HT should be analyzed separately.

CONCLUSION

In conclusion, no statistically significant associations were found between any SNP and AITD susceptibility following FDR correction. The pooled MAF for chemokine ligand genes had no statistically significant difference between healthy and diseased populations which means it is equally likely to find a diseased versus a healthy individual with a SNP in a chemokine ligand gene. The same is true for the chemokine receptor genes. No association was found between AITD susceptibility and whether or not a polymorphism is located in a chemokine ligand versus receptor gene. Overall, this means that chemokine ligands and receptors play a role in AITD susceptibility, however, there might be other environmental factors involved. In future studies, study populations should be made larger and more diverse. Additionally, investigating a broader range of immune related genes and chemokine receptor and ligand genes may provide further insight into genetic susceptibility to AITD. The association between CCL17 (rs223828) and AITD susceptibility was found in the Shin et al., 2023 and neared significance in this study, which warrants a further investigation of the CCL17 protein in AITD.

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