Etiology of Developmental Spinal Stenosis: a Genome-Wide Association Study

ABSTRACT

Background: Lumbar developmental spinal stenosis (DSS) is likely due to genetic influences during both fetal and postnatal development of the lumbar vertebrae. DSS patients with pre-existing narrowing of the spinal canal are at-risk of multi-level compression and recurrence after surgery. Various genetic polymorphisms have been suggested to be associated with the disease entity of DSS but the genetic basis of a narrowed bony spinal canal of a developmental origin have yet to be discussed. Therefore, the aim of this study was to identify possible single nucleotide polymorphisms (SNPs) via a genome-wide association study (GWAS) approach and a candidate gene platform that were associated with developmental narrowing of the lumbar spinal canal.

Methods: Southern Chinese population-based study volunteers were assessed (age range: 18-55 years). DSS was defined as the anteroposterior bony spinal canal diameter on T1-weighted axial MRI of L1 to S1. Genotyping was performed using the Illumina HumanOmniZhongHua-8 BeadChip. Using the canal diameter as the quantitative trait, genomic statistical analyses was performed.

Results: A total of 469 subjects were recruited. The mean axial AP measurements noted were: L1:21.8mm, L2:21.9mm, L3:22.4mm, L4:20.2mm, L5:19.6mm, and S1:17.3mm. Q-Q plots of genome-wide associations found significant differences in L4 and L5 measurements. More significant SNPs were found on chromosomes 8, 11, and 18. Low-density lipoprotein receptor-related protein 5 on chromosome 11 was found to be an important functional gene in canal bony

development via candidate gene approach. We found 2 clusters in the findings with one including the upper levels (L1-L4) and the other the lower levels (L5 and S1).

Conclusion: This is the first GWAS addressing DSS. The presence of multiple SNPs suggests a multi-factorial origin of DSS. Further analyses noted region-specific genetic predisposition, delineating distinct upper to lower lumbar regions of DSS. With better understanding of the DSS phenotype and genetic markers, the at-risk population can be identified early, preventative measures can be initiated, lifestyle/activity modification can be implemented, and more novel and precision-based therapeutics can be developed.

Level of Evidence: Level I Study

1

INTRODUCTION

Lumbar spinal stenosis is one of the most common spine conditions worldwide that often requires surgery.[1] Patients generally have good clinical response after decompression surgery.[2-4] However, reoperation is not an uncommon event. In the Spine Patient Outcomes Research Trial (SPORT) for spinal stenosis, 8% of patients who underwent an operation required another operation within two years of the index operation and 13% had another operation within four years.[3,4] In a similar study, 23% of patients were reported to require a repeat surgery by ten years.[5] In Korea, Kim *et al* showed that reoperation rates were up to 74% at 1 year, 9% at 2 years and 13.4% at 5 years after surgery.[6,7] This raises an important concern in clinical practice as reoperation occurs in both fusion and decompression only surgeries.[8] Developmental spinal stenosis (DSS) is a possible risk factor for reoperation as patients are prone to developing symptoms at multiple levels due to the presence of pre-existing narrowed canals.

Characteristically, patients with DSS have generalized short pedicles suggestive of genetic disturbances during the fetal and postnatal period.[9] A narrowed spinal canal can be due to developmental problems, such as the articular processes in an early embryonic stage, disproportional growth of the lamina and pedicles, and also of the spinal canal and spinal roots.[9] Pathological changes in DSS include narrowing of the dorsal aspect of the spinal canal due to bulging of the inferior articular facets. A paradoxical relationship with the degree of ligamentum flavum fibrosis has also been observed.[10] The lamina is also enlarged with narrowed interlaminar spaces and the pedicles are also shortened leading to decreased interpedicular distances.[11] Vertebral bodies may also be wedged and present with posterior lipping, contributing to the narrow spinal canal.[12] Occasionally, the lower lumbar vertebral levels may produce a trefoil or

three-leaf clover shaped canal.[13] This configuration may also predispose compression of lumbar and sacral nerve roots.[13] Thus, with this distorted canal morphology, patients may develop claudication symptoms more readily than patients with normally developed canals.

The DSS phenomenon has not been well-defined in the past and its prevalence among the general population or impact on lumbar spine disease is unknown. Previous studies are limited by largely generalized measurements of the entire lumbar spine, utilization of variable imaging modalities, lacking control groups, and were based on heterogeneous populations.[14-21] Recent work suggests that the anteroposterior (AP) bony spinal canal diameter is the most relevant measurement associated with DSS and can be assessed on both T1- and T2-weight scans.[22,23] DSS has been defined as level-specific values of developmental narrowing for levels L1-S1 and critical values have been determined as <14 mm at L4, <14 mm at L5 and <12 mm at S1.[22]

Despite the previous work relating to phenotyping, there is a lack of substantial evidence relating to its genetic predisposition. Even for degenerative lumbar spinal stenosis, gene association studies have been focused on its symptoms rather than the actual imaging measurements and are sporadic[24,25] and other studies have often confounded the genetic association with other degenerative findings (e.g. disc degeneration).[26] Furthermore, previous genetic studies were limited to implementing a candidate gene approach rather than the more accepted and robust measure of genome-wide association studies (GWAS),[24-26] whereby hundreds of thousands of single nucleotide polymorphisms (SNPs) can be screened. With better understanding of this phenotype-genotype relationship, there is potential to use genetic profiling to identify the at-risk population. In doing so, preventive measures such as lifestyle and activity modification, and better monitoring for stenosis development may be implemented. Thus, the

aim of our study was to identify genetic associations of SNPs with narrowed lumbar spinal canals via a GWAS approach. The information gathered was used to find where allele frequency differences between spinal canal diameters are greatest and to investigate for any patterns of clustering between spinal canal AP diameter and genotypes.

MATERIALS AND METHODS

Subjects

Based on the Hong Kong Disc Degeneration Population-Based Cohort subjects (approximately 3,000 probands, ranging from 10 to 88 years of age), a total of 469 adult individuals who were not symptomatic for stenosis-related symptoms were randomly selected for analyses. This cohort is a population-based study that openly recruited individuals of Southern Chinese origin, irrespective of their pain status.[27-34] The details of the protocol of this cohort have been reported elsewhere over the past decade. These 469 subjects (age range: 18-55 years) were sex- and age-matched. All 469 individuals underwent magnetic resonance imaging (MRI) examination of the lumbar spine **(see MRI assessment section)** and blood sampling for DNA extraction. This study was approved by the local ethical committee and informed consent was obtained by all subjects.

MRI Assessment

For the current study assessing the spinal canal, all subjects underwent axial T1-weighted 3T MRI (Siemens, Berlin and Munich, Germany; Phillips, Amsterdam, Netherlands) of L1-S1. The axial cut used for measurements included visualization of the bony canal and the thickest pedicle width. All the clinical and additional imaging details were blinded to the imaging assessor before and during the measurements. Intra-observer reliability analysis of the canal was performed on ten subjects. The first and second round of measurements was not performed on the same day. The program used for measurements was the Centricity Enterprise Web V3.0 (GE Medical Systems, 2006).

The imaging protocol used included a field of view of 18x18 cm for axial scans. Slice thickness was 4 mm and slice spacing was 0 mm for axial scans, and the imaging matrix was 288x192.The TR was 700-800 ms and the TE was 8-10 ms. There were 11 slices per vertebral level and parallel slices were made according to the pedicle levels. The quantitative measurement used was the AP bony spinal canal diameter in which we have previously determined it to be the key parameter for diagnosis of DSS.[22] On the axial cut, we measured this phenotype from the posterior midline of the vertebral body to the corresponding midline at the lamina (**Figure 1**).[22]

Genetic Testing and Data Cleaning

Genomic DNA for sequencing was isolated from peripheral blood from all 469 individuals. A similar protocol for isolation has been performed in previous studies.[27,35,36] To extract DNA from blood, we used a blood extraction kit from Qiagen (consumables company). This kit

extracted DNA from white blood cells in the blood samples. First, 10ml venous blood would be collected by ethylene-diamine-tetra-acetic acid (EDTA) tubes to prevent coagulation. The samples were then stored in -20 degrees Celsius, before running into the DNA extraction kit. DNA was extracted using a QIAamp DNA mini-kit (Qiagen, Hilden, Germany). This kit could break blood cells to release their contents, and capture DNA using the provided filter with charge. After washing the enzyme digested samples with buffers and water, blood DNA could then be obtained. Genotyping was carried out using the Illumina HumanOmniZhongHua-8 BeadChip as the genotyping platform. This product could genotype 900,015 SNP markers and was tailored made for studies involving Chinese individuals. This gene chip had coverage of 77% for the SNPs with minor allele frequency (MAF) >5%, and coverage of 73% for the SNPs with MAF >2.5%.

Quality control was performed with PLINK v1.07.[37] For SNPs exclusion quality control, SNPs with call rates of <95% were removed as very low call rate might be caused by the low-quality markers; SNPs that showed deviation from the Hardy-Weinberg equilibrium ($p \leq 1 \times 10^{-6}$), non-autosomal SNPs, monomorphic SNPs, SNPs that were not shared among cases and controls, and SNPs with MAF <5% were removed. The genomic inflation factor λ was calculated to quantify the extent of the bulk inflation and the excess false positive rate. Twenty samples were chosen randomly as duplicates and were genotyped. SNPs with Kappa value less than 0.95 were excluded.

Statistical Analyses

Descriptive and frequency statistics were performed of the measurement data. All values were expressed as mean with ± standard deviation (SD). Reliability assessment was based on Cronbach's alpha analysis. Alpha values of reliability were regarded as follows: excellent (a>0.90), good, (a>0.80), fair (a>70) and poor (a<0.60).[38,39] All MRI measurements were expressed in millimeters (mm). Statistical analyses were performed in PLINK using a linear model. Linear regression was used for continues trait and logistic regression was used for binary trait. Age, body weight (kg), body height (m), body mass index (BMI; kg/m^2), and sex-type were used as covariates to adjust for the influences of these factors. The significance level was adjusted by Bonferroni correction for multiple testing (0.05/SNPs number). Assuming 800,000 SNPs were included after quality control and analysis, the GWAS significance level of the p-value was set at $< 6.2 \times 10^{-8}$. Linear regression analysis and ANOVA testing were utilized if the slope was significantly greater/smaller than 0.

Quantile-quantile (QQ) plots and Manhattan plots were generated to visualize the association results. Quantile-quantile (Q-Q) plots were used to investigate if p-values were more significant (smaller) than that expected by chance. The $-\log^{10}(P)$ was plotted for emphasis for smaller p-values and converted to a positive value. Significant SNPs were indicated by upward deviation from the diagonal line on the Q-Q plot.

Manhattan plots were used to determine position of significant SNPs in relation to their chromosome. The Manhattan plot was conducted by plotting $-\log^{10}(P)$ against chromosomes. The -log was used to emphasize the SNP with the smallest p-value ($-\log^8$ was genome wide significant). Candidate gene analysis of previously utilized and identified SNPs in relevant

lumbar degeneration genes was performed. A list of these candidate genes are listed in **table 1**. Clustering of the SNPs in this study was performed using the correlation study via the R statistical package. This was performed according to each vertebral level to look for patterns of involvement and region specific variation between lumbar levels.

RESULTS

The mean age for subjects was 52.8 (SD:9.9) years old and there were 195 (41.6%) males and 274 (58.4%) females. Excellent intra-observer reliability (a=0.94-0.99) was noted. The mean body weight was 61.8 kg (SD:11.2), mean body height was 1.63 m (SD:0.1) and the mean BMI was 23.2 kg/m^2 (SD:3.3). The bony spinal canal measurement findings are illustrated in **Table ~~1~~2**:

According to Q-Q plot analyses and inspecting significant differences between the observed and expected p-values, **(Figure 2)** we found that L1, L2 and L3 were not significant while S1 was marginally significant. L4 and L5 were more significant and L4 was the most significant. Based on the Manhattan plot analyses, we found the most significant SNP in L4 **(Figure 3A)** to be 4kb from the ZNF704 gene (4.33×10^{-7}) on chromosome 8. For L5 **(Figure 3B),** the most significant SNP was found at the DCC gene ($p= 4.67 \times 10^{-7}$) on chromosome 18.

Implementing a candidate gene approach, the most significant SNP was rs3781579 ($p = 8.21 \times 10^{-4}$; Bonferroni threshold: 1.62×10^{-4}) of the low-density lipoprotein receptor-related protein 5 (LRP5) gene indicating that it was a significant candidate gene responsible for DSS **(Figure 4)**. Other more promising candidate gene results were rs5277 (Prostaglandin-

endoperoxide synthase 2, COX2 at p= 2.63 x 10^{-3} and rs731236 (VDR Taq I) at p= 6.30 x 10^{-3}, but did not reach Bonferroni threshold for significance. Based on clustering analysis using the SNPs discovered in the GWAS analyses, we found that the upper levels of L1 to L4 and the lower levels of L5 and S1 were clustered separately, indicating that there could be a different pattern of region-specific genetic predisposition for upper level and lower level involvement for DSS **(Figure 5)**.

DISCUSSION

In pre-existing narrowed bony spinal canals, even mild changes associated with degeneration or aging, such as intervertebral disc bulging and facet hypertrophy, may readily cause compressive symptoms. The neural structures in an already compromised spinal canal are especially at-risk. By accurately delineating the heterogeneous complex traits involved in DSS, we can detect true genetic associations regarding this condition. This is the first study to attempt to link DSS with genetic markers based on a GWAS approach. Our findings suggest that DSS has a multi-factorial genetic origin with the presence of multiple SNPs. In addition, DSS behaves in two clusters, demonstrating "region-specific" genetic predisposition between the upper (i.e. L1 to L4) to the of the lower lumbar spine (L5 and S1).

Predisposing factors of symptomatic DSS includes degeneration of intervertebral discs, facet joints or the ligamentum flavum.[1,10,19,22,40] Some genetic factors have been implicated in the etiology of lumbar spinal degeneration.[41,42] [35,36,43] There is likely a direct correlation between spinal stenosis and disc degeneration because these two pathologies usually coexist. However,

similar to the situation in spinal stenosis, many studies that discuss lumbar disc degeneration are not consistent in their definitions.[44 35,36,434345464748-5051]

To confirm our hypothesis, genomic DNA from peripheral blood of the subjects were sequenced and the AP diameter of the spinal canal AP was measured as the quantitative trait in the analysis. From our Q-Q and Manhattan plots, several suggestive signals were found in the genetic association analysis. We found the most significant single SNP in L4 to be 4kb from the *ZNF704* gene on chromosome 8. Previously, this SNP has been found to be marginally associated with amyotrophic lateral sclerosis.[52] For L5, the most significant SNP was found at the *DCC* gene on chromosome 18. This gene codes for a protein that functions as a tumor suppressor and is frequently mutated or down-regulated in colorectal and esophageal carcinoma. Although both SNPs have limited relevance regarding bone development, our candidate gene approach yielded an interesting finding. Using candidate gene data, significant SNPs were rs3781579 (Lipoprotein receptor related protein 5; *LRP5* on chromosome 11), rs5277 (Prostaglandin-endoperoxide synthase 2; *COX2)* and rs731236 (*VDR* Taq I). In particular, *LRP5* reached Bonferroni significance and is a key component of the Wnt signalling pathway important for bone development. As such, our preliminary analyses have suggested -- for the first time in the literature -- that specific genetic markers may be associated with the development of the stenosis phenotype.

Our study represents the first GWAS. The study was performed on a homogenous Southern Chinese population based on a reliable MRI phenotype. Although the GWAS did not identify any genetic associations that reached genome-wide significance ($p< 5.0 \times 10^{-8}$), we did identify several important candidate genes that are potential candidates for further study. In

addition, results suggested that the more significant SNPs were found at the L4 and L5 levels, and clustering showed that the upper and lower vertebral levels had different associations. These findings further stress the point that DSS has a multifactorial genetic origin, with variable region-specific genetic predispositions that are not uniformed throughout the lumbar spine. This non-uniformity of region-specific risk factors related to disc degeneration and other MRI phenotypes (e.g. endplate abnormalities, Modic changes, etc) have also been reported elsewhere.[27,34] Although our study represents a homogenous population, further replication studies are needed to validate our findings in other ethnic groups to assess global generalizability. However, due to the homogeneity of our sample, this further limits the potential confounds often associated with heterogenous populations.

Hypothetically, if the patients with DSS can be identified at the index operation, reoperation at adjacent levels can be avoided with prophylactic canal widening surgery. This may lead to an overall better function for the patients since they can avoid repeated operations. However, this is not advocated, is yet premature and the practicality of this approach needs further investigation. Alternatively, in the advent of the omics era whereby blood biomarkers and profiling for more precision or personalized approaches to spine care are being sought, having a refined and better understanding of genetic factors related to various spinal disorders/conditions, such as DSS, may prove essential. This would allow early profiling of individuals that may be at risk, which may necessitate further or follow-up imaging analyses, clinical consult and/or perhaps lifestyle modification. Future investigations into biological pathways and novel therapeutics early-on in an individual's lifespan may be further explored. Ultimately, early identification of DSS may lead to improved patient management, enhanced outcomes and

decreased health-care costs with less reoperations. Future work should aim to assess these findings for replication purposes in other ethnic populations.

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FIGURE LEGENDS

Figure 1: Axial MRI scan noting the measurement of the bony spinal canal anteroposterior diameter (red line).

Figure 2: Q-Q plots of L1 to S1. Note that Q-Q plot compares the observed to that of the expected p-values. Deviation from the diagonal line equates to a suspected significant result with respect to the expressed phenotype in relation to genetic markers.

Figure 3: Manhattan plots for the **(A)** L4 and **(B)** L5 anteroposterior bony spinal canal.

Figure 4: Candidate gene analysis illustrates that chromosome 11 reaches Bonferroni threshold of 10^{-4}, indicating that *LRP5* was a significant candidate gene responsible for developmental spinal stenosis.

Figure 5: Correlation of levels demonstrates genetic clustering of the upper (L1 to L4) to that of the lower spinal levels (L5 and S1).