Identification of potential pathogenic genes causing occipitalization of the atlas

Shanhang Jia

Xuan Wu Hospital of the Capital Medical University

Wanru Duan

Xuan Wu Hospital of the Capital Medical University

Zong Xin

Xuan Wu Hospital of the Capital Medical University

Boyan Zhang

Xuan Wu Hospital of the Capital Medical University

Qiang Jian

Xuan Wu Hospital of the Capital Medical University

Chen Wei

Beihang University

Zan Chen (✉ chenzan66@163.com)

Xuan Wu Hospital of the Capital Medical University

Research Article

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Abstract

Background

Occipitalization of the atlas (AO) is among the most common congenital or environmental malformations of the craniovertebral region, the rate of incidence of which ranges from 0.08%-2.76% in the general population. The fundamental pathogenic mechanisms and molecular etiology remain largely unknown..

Results

Rare variants were detected in two genes (MEOX1 and MYO18B) with reported association with vertebral malformations, especially in AO. Through the use of a functional prediction tool, Meta-SVM, and genotype-phenotype analysis of 101 candidate genes related to vertebral segmentation abnormalities, TNS1 was identified as having the greatest probability of being associated with AO, followed by FGFR2, AGAP1, TBX2, LRP5, and TONSL. GO-BP and KEGG analyses were then performed on the candidate genes, the results indicating that Fanconi anemia-related genes may drive vertebral malformation in AO patients..

Conclusions

The present study analyzed the WES profile of a cohort of Chinese AO patients and identified 6 novel genes potentially associated with AO, extending the spectrum of known mutants contributing to this disorder. Furthermore, functional gene enrichment analysis provided fresh insight into the Fanconi anemia related pathway and etiology of AO.

Background

Occipitalization of the atlas (AO) is defined as a form of congenital cervical spine dysplasia. AO was first described by Rokitansky in 1844 and demonstrated roentgenographically by Schuller in 1911 (as described in Harcourt & Mitchell, 1990). The reported prevalence of AO in the general population has ranged from 0.08–2.76%, females affected at the same rate as males[1, 2]. The phenotype affects all or part of the anterior arch, lateral mass, or posterior arch of the atlas, forming a bony connection with the occipital bone. AO is considered to be the main cause of bony deformities in the craniocervical junction, including "atlantoaxial dislocation" and "basilar invagination". Congenital atlanto-occipital fusion may affect the physiological development of the brain during growth and development in adolescence, simultaneously limiting the elongation of the slope[3, 4]. Assimilated atlas leads to upward shifting of the axial vertebrae, and further resulting in atlantoaxial instability and compression to the brainstem and cranial nerves. The compression may cause progressive damage to spinal nerve function, blood circulation disorders and cerebrospinal fluid circulation disorders. Symptoms include neck pain, breathing difficulties, quadriplegia, and may even be life-threatening.

The principal etiology of AO is the impaired development of the bone of the cranio-cervical junction, leading to improper segmentation of the vertebrae. Recent studies have suggested that failed development of the craniocervical junction that underlies AO could be caused by defective somitogenesis in the cervical region resulting from gene mutations[5–7]. According to OMIM (Online Mendelian Inheritance in Man), Genecards, and MGI (mouse genome informatics)(MP:0010728), it has been reported that mutations in MEOX1(GCID:GC17M043640, MIM:600147), MYO18B(GCID:GC22P025742, MIM:607295), and the HOX family of genes are associated with AO[7–9]. A study by David (1999) of 30 cases of Klippel-Feil syndrome (KFS) found that a mutation in the Hox gene was the mechanism possibly responsible for atlas assimilation, while reduced or impaired Pax-1 gene expression may result in vertebral fusions[10]. Nevertheless, the etiology of AO remains unclear. To further elucidate the molecular basis for AO at the exome level, we herein present the molecular findings of whole-exome sequencing among a Chinese cohort of 28 AO patients, further investigating rare variants using Meta-SVM, and GO-BP and KEGG analysis.

Results

1. Clinical features and radiographic parameters of the cohort

The study cohort consisted of 33 unrelated Han Chinese patients. There were 28 cases of occipitalization of the atlas and 5 cases, defined as the control group, were cerebellar tonsillar hernia patients without impaired bony development. There were 6 males (21.4%) and 22 females (78.6%) with a mean age at diagnosis of 45.5 ± 14.3 years. Of the 28 AO patients, a mean of 1.6 levels was congenitally fused (range C0-C7), including two different regions of the cervical spine: C0-C1 and C2-C3. 16 cases (57%) were found to present occipitalized atlas and fused C2-C3 vertebrae, diagnosed as KFS type I according to the Samartzis's classification[11]. Twelve cases (43%) presented only fusion of occipital bone and atlas.

The AO patients presented with a variety of extraskelatal and intraspinal anomalies. In the cohort in the present study, atlantoaxial dislocation was the most common clinical feature. There were 21 patients (75%) manifesting atlantoaxial dislocation, followed by cerebellar tonsillar hernia (14 patients, 50%), basilar invagination (17 patients, 53.5%), syringomyelia (10 patients, 35%). Almost all the patients presented with neck pain and numbness in their limbs. The detailed clinical features and radiographic parameters are shown in Table 1.

Table 1
Demographic and clinical characteristics of the cohort

Parameter	AO cohort(n = 28)
Age in years, mean(range)	45,(25–69)
SEX,n(%)	
Men	6(21.4%)
women	22(78.6%)
Level of fusion,n(%)	
C0-C1	12
C0-C1,C2-C3	16
Comorrbidities,n(%)	
atlantoaxial dislocation	21–75%
cerebellar tonsillar hernia	14
basilar invagination	15
syringomyelia	10
scoliosis	2
Clinical manifestations,n(%)	
dizziness	8
Numbness/weakness/paresthesia of extremities	19
limited cervical ROM	10

2. Variants of known KFS-related genes in AO patients

Following the processing of the WES data and its interpretation relating to the variants, filtered rare variants were first checked for their potential to be causative for AO in the reported KFS gene. As a result, 1 variant was found in MYO18B (Table 2) and 1 in MEOX1 (Table 2). In MEOX1, a nonsynonymous SNV (c.G20A:p.S7N) was identified in patient A0751123, a 64 year-old female suffering occipitalization of the atlas (vertebral fusion: C0-C1), tonsillar hernia, basilar invagination, syringomyelia, stiff neck, limited cervical ROM but no rib or other vertebral malformations. The patient underwent posterior atlantoaxial fusion with satisfactory results.

MEOX1 (Mesenchyme Homeobox 1), a protein-coding gene, plays a key role in somitogenesis and is also required for the maintenance of sclerotome polarity and the formation of craniocervical joints. Mohamed et al. found that a frameshift mutation in MEOX1, a 1-bp deletion (94delG) in exon 1, was predicted to result in premature termination in a large consanguineous Saudi family with Klippel-Feil syndrome in 2013[9]. Bayrakli et al. also found Klippel-Feil syndrome resulted from MEOX1 homozygous c.670 G-A transition in a consanguineous Turkish family[12]. Susan et al. reported that the loss of MEOX1 gene function in a mouse model caused defects in the development of the axial skeleton during embryogenesis, especially craniocervical joint malformations, including occipitalization of the atlas[13]. Consistent with the reported phenotype of craniocervical joint malformations in a MEOX1-deficient mouse model, the patient in the present study suffered from occipitalization of the atlas. However, there remain insufficient studies of pathogenic variants of MEOX1 associated with AO due to the limited numbers of samples.

A synonymous SNV (c.T1581G:p.A527A) in MYO18B was identified in patient A0763937, a 33 year-old male with occipitalization of the atlas and vertebral fusion at the C2-C3 level, tonsillar hernia, and syringomyelia. This variant of MYO18B was first reported in a Saudi boy and girl that were unrelated and exhibited KFS with facial dysmorphism and nemaline myopathy[14]. There is still insufficient evidence in terms of its relevance to AO disease.

Table 2
MEOX1 and MYO18B variants and clinical features of patients

Identifier	A0751123	A0763937
Sex/age(years) at diagnosis	F/34	M/33
Mutation	MEOX1	MYO18B
Variant type	nonsynonymous SNV	nonsynonymous SNV
Zygosity	het	het
Chr_Position	17_41738883	22_261166840
Variant nomenclature	c.G20A:p.S7N	c.T1581G:p.A527A
Exac AF	novel	novel
GnomeAD AF	novel	novel
Clinical feature	limited neck ROM, numbness of extremities	dizziness
Fused levels	C0-C1	C0-C1
Clinical manifestations	occipitalization of atlas, tonsillar hernia, basilar invagination, syringomyelia	occipitalization of atlas, tonsillar hernia, syringomyelia

3. Variants identified in potential AO-associated genes

Based on rare variant filtering of the AO-associated list of candidate genes (Table S1), the functional prediction tools SIFT, Polyphen-2, Mutation taster, GERP, and CADD were combined to identify candidate genes which were relevant to the etiology of AO[15–20]. Furthermore, we compared the Meta-SVM score of rare coding variants for each candidate gene in the AO group with controls, finding 6 genes with a high Meta-SVM score that were related to vertebral malformation (Table 3)[21].

A reported nonsynonymous SNV (NM_001308022 c.2191T > A:p.S731T) in TNS1 was identified in patient A0757897, a 21 year-old female complaining of extremity adynamia. Fusion of the craniocervical joint at C0-C1 and C2-C3 was identified by radiological scanning, combined with cerebellar tonsillar hernia, basilar invagination, and syringomyelia. TNS1 codes for a protein localized to focal adhesions, regions of the plasma membrane where the cell attaches to the extracellular matrix. This protein crosslinks actin filaments and contains an Src homology 2 (SH2) domain, often found in molecules involved in signal transduction[22]. Mutations of this gene have been reported in Cowden syndrome and Proteus syndrome[23]. Cowden syndrome is a genetic condition characterized by a large head, hamartomatous lesions affecting derivatives of ectodermal, mesodermal, and endodermal layers, macrocephaly, facial trichilemmomas, and scoliosis[24, 25]. The association of TNS1 with AO or KFS has not been previously reported, thus the present findings may represent a novel disease association[26].

A heterozygous variant of c.1052T > A (p.V351D) in the FGFR2 gene was detected at an ExAC allele frequency of 0.000025. This variant has been predicted as a pathogenic mutation using the functional prediction tools MutationTaster, SIFT, and Polyphen-2, with a CADD score of 33 and GERP score of 6.03. FGFR2 was reported to be associated with Pfeiffer and Crouzon syndromes, related mostly to the premature fusion of particular skull bones and cervical spine abnormalities[27–29].

The variant c.619C > T (p.H207Y) in gene TBX2 was detected in patient A0798026. TBX2 is a member of a phylogenetically conserved family of genes that share a common DNA-binding domain, the T-box. T-box genes encode transcription factors that regulate developmental processes[30, 31]. This gene mutation can cause vertebral fusion and scoliosis[32, 33]. Therefore, a significant association of TBX2 with AO may represent an expansion of diseases known to be associated with this gene mutation.

In AGAP1, LRP5, and TONSL, two other rare heterozygous variants were identified in different patients. It has been reported that these genes associate with vertebral or Chiari malformations[34–39]. The related information is displayed in Table 3.

Table 3
Information on rare variants of 6 novel associated genes

Patient	Gene symbol	Variant type	Zygoty	Chr_Pos	Ref transcription	Variant nomenclature	ExAC	GERP++	CADD	Score
AO757897	TNS1	nonsynonymous SNV	het	2_218712674	NM_001308022	c.T2191A:p.S731T	0.0004	4.45	24.3	0.0004
AO792018	FGFR2	nonsynonymous SNV	het	10_123263355	NM_001144914	c.T1052A:p.V351D	0.00002473	6.03	33	0.00002473
AO751123	AGAP1	nonsynonymous SNV	het	2_236617859	NM_001037131	c.G200A:p.R67Q	0.000008236	3.88	29.1	0.000008236
AO752619	AGAP1	nonsynonymous SNV	het	2_237032686	NM_014914	c.G2335A:p.V779M	0.0007	4.17	22.7	0.0007
AO798026	TBX2	nonsynonymous SNV	het	17_59479268	NM_005994	c.C619T:p.H207Y	/	4.92	27.9	0.0000000001
AO761890	LRP5	nonsynonymous SNV	het	11_68153864	NM_002335	c.G1096A:p.V366M	0.00000829	2.89	23.9	0.00000829
AO749294	LRP5	nonsynonymous SNV	het	11_68125147	NM_002335	c.C518T:p.T173M	0.0004	1.67	18.39	0.0004
AO749294	TONSL	nonsynonymous SNV	het	8_145662165	NM_013432	c.C1865T:p.A622V	/	4.73	31	0.0000000001
AO745185	TONSL	nonsynonymous SNV	het	8_145663874	NM_013432	c.G1633A:p.V545I	0.0003	4.21	26.7	0.0003

4. GO-BP and KEGG enrichment analysis of candidate genes

Table 4 presents the Fanconi anemia-related genes that were identified in 17 patients. Based on the variants of filtered candidate genes, GO-BP and KEGG enrichment analysis was conducted for the AO cohort and controls, respectively. In GO-BP enrichment analysis, the candidate genes of the AO patient cohort were mostly concentrated in terms related to skeletal system development ($p = 1.64 \times 10^{-11}$), epithelial tube morphogenesis ($p = 5.54 \times 10^{-10}$), bone development ($p = 3.42 \times 10^{-11}$), the smoothened signaling pathway ($p = 1.14 \times 10^{-10}$), and epithelial tube formation ($p = 9.03 \times 10^{-9}$). In comparison, the controls were significantly enriched in terms related to skeletal system development ($p = 1.18 \times 10^{-5}$), embryonic organ development ($p = 0.0002$), ear development ($p = 4.12 \times 10^{-5}$), positive regulation of the transmembrane receptor protein serine/threonine kinase signaling pathway ($p = 5.13 \times 10^{-6}$), and chondrocyte differentiation ($p = 1.08 \times 10^{-5}$). Additionally, for KEGG enrichment analysis, the AO patient cohort was significantly enriched in pathways related to Fanconi anemia ($p = 3.53 \times 10^{-12}$), lysine degradation ($p = 7.28 \times 10^{-5}$), and homologous recombination ($p = 0.0006$), while the controls were substantially enriched in pathways related to hepatocellular carcinoma ($p = 6.88 \times 10^{-5}$), proteoglycans in cancer ($p = 0.0002$), and the TGF-beta signaling pathway ($p = 4.50 \times 10^{-5}$) (Fig. 1 and Fig. 2).

Table 4
FA related genes of 17 patients

Patient	Gene symbol	Variant nomenclature
AO748528	FANCF	c.C638T(p.P213L)
AO792018	FANCD2	c.C3335T(p.A1112V)
AO744313	FANCM	c.G2563A(p.D855N)
AO800756	FANCM	c.C1663T(p.R555C)
AO757897	FANCM	c.G1333A(p.E445K)
AO751841	FANCA	c.C2873T(p.A958V)
AO800891	FANCA	c.G2080A(p.D694N)
AO752619	FANCA	c.G3800A(p.G1267D)
AO735654	FANCB	c.C13T(p.Q5X)
AO745185	FANCB	c.T869C(p.M290T)
AO797434	FAN1	c.1985_1994del(p.G663lfs*54)
AO764048	FANCI	c.C3056T(p.T1019M)
AO763937	FANCI	c.A1111G(p.S371G)
AO800895	FANCF	c.C638T(p.P213L)
AO769366	FANCG	c.A55G(p.K19E)
AO800905	FANCE	c.C589A(p.P197T)
AO742782	BRCA2	c.C3883T(p.Q1295X)

Discussion

AO is a relatively rare disorder characterized by congenital synostosis of the occipital bone and cervical vertebrae due to a formation or segmentation defect. However, articles related to the etiology of the mechanism of AO are limited and focused mostly on the type of surgery performed. Thus, this 28 patient cohort is relatively large compared with other reported studies of AO in which to identify the molecular differences by WES.

The clinical manifestations of AO display substantial homogeneity. The typical clinical features of AO are symptoms of spinal cord or nerve root compression and limited neck ROM. These clinical features were recorded in almost all patients in the cohort.

The pathogenesis of the developmental defect in AO was thought to be attributable to the somites at the embryonic stage resulting from a disruption or mutations in genes regulating segmentation or resegmentation, but the underlying etiology of AO remains unclear[40, 41]. Previous studies have suggested that mutations in members of the HOX gene family, MYO18B or MEOX1, are associated with the occipitalization of the atlas in a mouse model[42]. In the present study, we detected rare variants in known AO-related genes. However, these novel nonsynonymous mutations in MEOX1 or MYO18B were still of uncertain significance.

Comprehensive analysis of gene-related phenotypes and functional prediction tools can identify pathogenic genes associated with AO. As a result, we identified 6 genes (TNS1, FGFR2, AGAP1, TBX2, LRP5, and TONSL) with a high Meta-SVM score among candidate genes. TNS1 is involved in cell migration, fibrillar adhesion formation, cartilage development, and linking signal transduction to the cytoskeleton system. Pathogenic mutations in TNS1 are frequently associated with Cowden and Proteus syndromes. Cowden syndrome is a genetic condition characterized by a large head size, facial trichilemmomas, and scoliosis. Wang et al. reported that the TNS1 mutation may result in adolescent scoliosis in 2020[26]. Mutations in FRFR2 have been previously associated with Pfeiffer and Crouzon syndromes. These are rare developmental defect disorders with premature fusion of particular skull bones and cervical spine abnormalities. Meina et al. found three generations of a family with Crouzon syndrome carrying a mutation of FGFR2 in 2019, in which extremely severe scoliosis, heterotopic ossification, and osteoarthritis had manifested. It has been reported that mutations in TBX2 can cause fusion in vertebral anomalies and variable endocrine and T-cell dysfunction[27]. Yinlin et al. demonstrated that TBX2 was important for skeletal development, especially during vertebral segmentation[32]. These reports reinforce the possibility of a key role for TNS1 and FGFR2 in the pathogenesis of congenital occipitalization of the atlas.

In the current study, GO-BP analysis demonstrated that candidate genes of the AO patient cohort were significantly enriched in the skeletal system and bone development. KEGG analysis found that these genes were enriched in the Fanconi anemia (FA) pathway, suggesting that FA pathogenic genes could be associated with AO malformation. FA is a relatively rare genetic disorder associated with a progressive decline in hematopoietic stem cells and bone marrow failure. FA is also characterized by a variety of developmental defects including skeletal malformations and short stature. More than 50% of children affected with FA have radial-ray abnormalities and many patients have early-onset osteopenia/osteoporosis. Although more than 20 FA genes have been identified, the underlying mechanism leading to bone malformations remains elusive.

FA and AO are poorly understood because few reports of these two disorders have been published. Houten et al. described a case of a female with FA and AO in 2013[43]. McGaughan et al. suggested a relationship between FA and vertebral abnormalities[44]. Mazon et al. reported a mouse model with an FA mutant gene that displayed skeletal anomalies such as vertebral fusion and defective bone mineralization[45]. In the present study, 53.6% of patients (15 /28) carried FA-related mutant variants and KEGG analysis indicated that this cohort was affected by FA genetic factors. Furthermore, it has been demonstrated that mice with a mutant FA gene are susceptible to the teratogenic effects of ethanol. Such teratogenicity is largely due to acetaldehyde, a by-product of ethanol catabolism[45].

It is currently unclear how aldehyde and ethanol damage DNA in vivo, but in vitro they can directly modify bases, allowing DNA-DNA and DNA-protein crosslinks. It is likely that acetaldehyde-mediated DNA damage drives FA gene mutant related disorders. In summary, our findings here provide fresh insight into FA in AO.

The study had a number of limitations. The limited sample size could be a cause of false-positive results. Sequencing studies with larger sample sizes are required to reveal the underlying association between phenotype and genotype.

Conclusions

In conclusion, the results represent an AO study in the Chinese population in which we identified a number of candidate genes able to correlate with AO and several FA related mutant genes that correlate with AO etiology. The present research contributes to a more comprehensive understanding of the phenotype-genotype association in AO...

Methods

Recruitment of a cohort of AO patients

From Feb 2019 to Feb 2020, 33 Han Chinese ethnicity patients diagnosed with AO at Xuanwu Hospital were consecutively recruited to this study.

We obtained the demographic information, clinical symptoms, physical examination results, and a detailed medical history.

Radiological evaluation, including anteroposterior, lateral neutral, and flexion-extension plain radiographs, computer tomography (CT) scan, and magnetic resonance imaging (MRI) were performed on all patients in order to determine a robust clinical diagnosis. We also record segmentation of congenitally fused vertebrae and cervical scoliosis. Each radiographic evaluation was performed by a trained neurosurgery surgeon and reviewed by an another observer blinded to the radiographic evaluation.

The study was approved by Department of Scientific Research and the Ethics Committee of Xuanwu Hospital in China.

Genomic DNA preparation and whole-exome sequencing

Genomic DNA was extracted from 33 patients peripheral blood lymphocytes. We use Illumina libraries following whole-exome capture to perform WES. Sequencing was carried out on an Illumina HiSeq 4000 platform by using 150-bp paired-end reads mode (Illumina, San Diego, CA, USA).

Variant annotation and interpretation

Variations in each individual were called using a GATK best practice pipeline. In detail, prior to beginning the aligning process, the quality of raw sequencing reads was evaluated using FastQC v0.11.9[46]. FastQC: A Quality Control Tool for High Throughput Sequence Data [Online]. Available online at: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>. Low-quality reads and contaminated adaptor sequences were removed from further analysis using the paired trimming mode of the cutadapt v3.2 tool, using the parameters: "-q 20 -m 100A [forward_adaptor] a [reverse_adaptor]"[47]. The adaptor sequences were obtained from the sequencing service provider. Remaining sequencing reads were aligned against the human reference genome build 37 (b37) using the mem algorithm of the bwa v0.7.17 tool Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv:1303.3997v2 [q-bio.GN]]. Variants were called using the HaplotypeCaller algorithm of GATK v4.18. Called variants were further filtered using the CNNScoreVariants and FilterVariantTranches modules of GATK v4.18. vcftools v0.1.12b was used to remove variants that did not pass the filter described above[48]. Functional annotation of the remaining variants from the step above was conducted using ANNOVAR and the InterVar pipeline[49]. Disease-causing states were further adjusted according to the clinical information of each patient in accordance with the mutation classification protocol of the American College of Medical Genetics and Genomics (ACMG) [15]. The relationship between mutated genes and human disease and phenotype were obtained from DisGeNET[50].

Statistical analysis

A comparison of the means of relevant features was conducted using a Student's t-test. One-tailed P-values < 0.05 were considered statistically significant.

List Of Abbreviations

AO: occipitalization of atlas; **KFS**: Klippel-Feil syndrome; **WES**: Whole-exome sequencing; **ROM**: Range of motion; **OMIM**: Online Mendelian Inheritance in Man; **ACMG**: American College of Medical Genetics and Genomics; **GERP**: Genomic Evolutionary Rate Profiling; **CADD**: Combined Annotation Dependent Depletion; **SIFT**: Sorting Intolerant Form Tolerant; **ExAC**: Exome Aggregation Consortium

Declarations

Ethics approval and consent to participate

All subjects provided informed consent to take part in the study. Written informed consent was obtained from the parents/guardians of the minors included in this study and all methods were carried out in accordance with relevant guidelines and regulations. The study was approved by the Department of Scientific Research and Ethics Committee of Xuanwu Hospital, Capital Medical University in China.

Consent for publication

Not applicable.

Availability of data and materials

All data analyzed during this study is included in this published article. The raw data is available at the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests.

Author Contributions Statement

SJ, WD,ZX, BZ and QJ performed the study and participated in the experiment including data collection/interpretation. CW and ZC conceived of the study and participated in its design. ZC and CW were responsible for coordination, data collection/interpretation and proofreading of the final manuscript. The final version of the manuscript has been reviewed and approved by all the authors for publication.

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Authors' Information

Co-first authors Shanhang Jia, M.D. ^{1,2}, Wanru Duan, M.D. ^{1,2},

Corresponding authors Chen Wei, Ph.D.,³, Zan Chen, M.D. Ph.D. ^{1,2}

1. Department of Neurosurgery, Xuanwu Hospital, Capital Medical University. Beijing, China
2. Lab of Spinal Cord Injury and Functional Reconstruction, China International Neuroscience Institute (CHINA-INI)
3. Beijing Advanced Innovation Center for Biomedical Engineering, School of Biological Science and Medical Engineering, Beihang University, Beijing 100083, China

Corresponding author

Correspondence to:

Chen Wei, Ph.D.⁴, chenw123@buaa.edu.cn

Zan Chen, M.D. Ph.D.^{1,2}, chenzan66@163.com

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51. Declarations

Figures

Figure 1

GO-BP and KEGG enrichment analysis for the AO cohort and controls, the AO patient cohort were mostly concentrated in terms related to skeletal system development ($p=1.64 \times 10^{-11}$), epithelial tube morphogenesis ($p=5.54 \times 10^{-10}$), bone development ($p=3.42 \times 10^{-11}$);for KEGG enrichment analysis, the AO patient cohort was significantly enriched in pathways related to Fanconi anemia ($p=3.53 \times 10^{-12}$)

Figure 2

GO-BP and KEGG enrichment analysis for the controls. The controls were significantly enriched in terms related to skeletal system development ($p=1.18 \times 10^{-5}$), embryonic organ development ($p=0.0002$), ear development ($p=4.12 \times 10^{-5}$); the controls were substantially enriched in pathways related to hepatocellular carcinoma ($p=6.88 \times 10^{-5}$)

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