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The important role of MDM2, RPL5, and TP53 in mycophenolic acid-induced cleft lip and palate

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Abstract

Mycophenolate embryopathy (MPE) is a mycophenolic acid (MPA)-induced congenital malformation with distinctive symptoms. Cleft lip/palate (CLP) is one of the most common symptoms of MPE. The aim of this study was to screen and verify hub genes involved in MPA-induced CLP and to explore the potential molecular mechanisms underlying MPE.

Overlapping genes related to MPA and CLP were obtained from the GeneCards database. These genes were further analyzed via bioinformatics. The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis results were visualized with the Cytoscape ClueGO plug-in. Gene protein-protein interaction (PPI) networks were constructed based on data obtained from the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database.

Overall, 58 genes related to MPA and CLP were identified. The genes most relevant to MPA-induced CLP included ABCB1, COL1A1, Rac1, TGFβ1, EDN1, and TP53, as well as the TP53-associated genes MDM2 and RPL5. GO analysis demonstrated gene enrichment regarding such terms as ear, mesenchymal, striated muscle, and ureteric development. KEGG analysis demonstrated gene enrichment in such pathways as the HIF-1 signaling pathway, glycosylphosphatidylinositol-anchor biosynthesis, the TNF signaling pathway, and hematopoietic stem cell development.

Bioinformatic analysis was performed on the genes currently known to be associated with MPA-induced CLP pathogenesis. MPA-induced CLP is mediated by multiple ribosome stress related genes and pathways. MDM2, RPL5 and TP53 could be the main contributor in this pathogenesis, along with several other genes. ABCB1 polymorphism could be related to the probability of MPA-induced CLP.

Abbreviations: ATP = adenosine triphosphate, BP = biological process, CLP = Cleft lip/palate, CsA = Cyclosporine, DBA = Diamond-Blackfan anemia, GO = Gene Ontology, IMPDH = inosine-5'-monophosphate dehydrogenase, KEGG = Kyoto Encyclopedia of Genes and Genomes, MMF = Mycophenolate mofetil, MPA = Mycophenolic acid, MPE = Mycophenolate embryopathy, PPI = Protein-protein interaction, STRING = Search Tool for the Retrieval of Interacting Genes/Proteins, TCS = Treacher Collins Syndrome, TGFβ = Transforming growth factor-beta.

Keywords: bioinformatics, cleft lip and palate, mycophenolic acid

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Highlights

1. The combination of MDM2, RPL5 and TP53 plays a major role in the ribosome stress in the mycophenolic acid -induced cleft lip and palate
2. COL1A1, RAC1, TGFB1 and EDN1 may also contribute in the mycophenolic acid -induced cleft lip and palate.
3. Polymorphism of ABCB1 could be related to the probability of mycophenolic acid -induced cleft lip and palate.

1. Introduction

Mycophenolate mofetil (MMF) is an inactivated precursor of mycophenolic acid (MPA). In 1995, MMF (CellCept) was approved for clinical use and quickly became a widely prescribed immunosuppressant.[1] With the widespread application of MMF in the clinic, its embryonic toxicity has been observed and reported. Relevant clinical observational reports showed that intrauterine exposure to MMF was associated with fetal malformation.[2] The company manufacturing MMF reviewed

post marketing adverse event surveillance data from 1995 to 2007. Of 77 women exposed to MMF during pregnancy, 25 had a spontaneous abortion, and 14 had infants or fetuses with abnormalities, including 6 auricle abnormalities.[3] Notably, the reported MMF-related birth defects exhibit a relatively constant pattern, with major symptoms including microtia, CLP and other related malformations.[2,4–6] Regarding MMF, the occurrence of a rare malformation is associated with rare cases of prenatal drug exposure, and since the incidence of both conditions occurring simultaneously is low, this phenomenon suggests a particular embryopathy.[3,7]

Although most researchers believe that the teratogenic effect of MMF is related to its pharmacological action of inhibiting inosine-5'-monophosphate dehydrogenase (IMPDH),[8,9] the pathogenesis of MMF and its association with CLP have not been fully elucidated. Bioinformatic analysis can play an important role in distinguishing gene expression.[10] Numerous gene expression profiling studies based on microarray technology have been reported.[6] Therefore, analyzing the currently known genes that are related to both MMF and CLP with integrated bioinformatic methods might represent a means of filling this gap in the literature.

The present study aimed to identify hub genes and pathways involved in the pathogenesis of CLP resulting from MPA by assessing gene data collected from the GeneCards database and performing bioinformatic analysis. Based on the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) results, these hub genes and pathways involved in CLP resulting from MPA could help to elucidate the pathogenesis of CLP and possibly contribute to its prevention.

2. Methods

2.1. Selection of the intersection of the MPA genes and CLP genes

As a bioinformatics study, the data were all from the GeneCards, an open access internet database, so the Ethical review in Methods section is not necessary. The GeneCards database was employed to identify the genes that are related to MPA and CLP. GeneCards is the largest international database for *Homo sapiens*, providing integrated genetic, genomic, and biological data to facilitate the study of human health and disease.[11] After acquisition of the genes, the gene sets were overlapped to obtain common genes. The results were visualized in a Venn diagram. A total of 226 common genes were identified (Fig. 1). GeneCards gives confidence scores to every correlation between a candidate gene and disease. We used a score of 1 as a screening criterion to ensure the reliability of the data in this study and to narrow the sphere of exploration. However, some of the genes are not correlated with CLP and MPA. In fact, previous studies indicated that some of the common genes are not related to CLP or MPA. Other studies have mentioned MPA because of its curative effect on dermatomyositis. Some of the genes were reported to be related to CLP or MPA simply because they were mentioned in the same articles, while they have virtually no relationship with CLP or MPA. These genes were identified and deleted, and 58 of the 226 common genes were screened for further analysis ([Fig. 2).

2.2. Construction of a PPI network for the common genes

Functional links between proteins are usually inferred from genomic associations between the genes that encode them: groups of genes that are required for the same function tend to exhibit similar species coverage and are often located in close proximity on the genome. The search tool for the retrieval of interacting genes (STRING) (http://www.string-db.org/), which is a web biological database for the prediction of known and unknown protein interaction relationships, was employed to construct the protein-protein interaction (PPI) networks.[12] In the present study, PPIs with medium confidence scores were selected to balance the possibility and accuracy of the mining data. The PPI results were visualized by Cytoscape (Fig. 3). The node degrees of the common genes were rated and visualized by R language bar plots (Fig. 4).

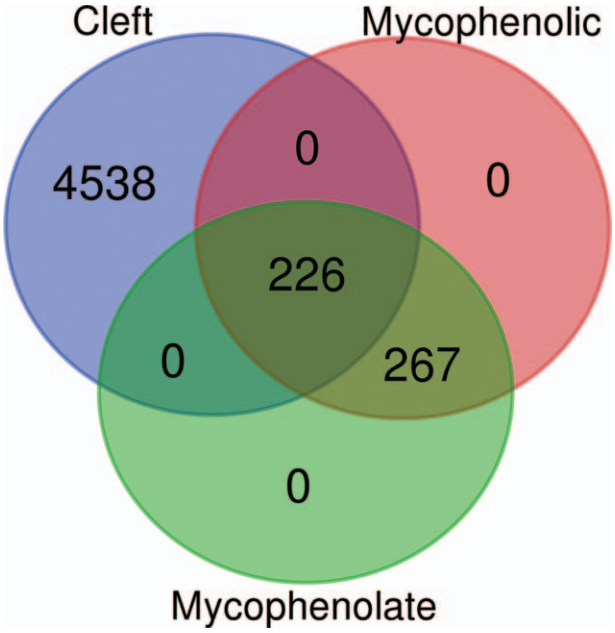

Figure 1. Venn diagram containing genes related both to MPA, MMF and CLP. All genes in the Mycophenolate set belong to the Mycophenolic set. 226 common genes have been found to both related to MPA and CLP.

2.3. Functional and pathway enrichment analyses

The GO biological process (BP) category contains concepts or classes used to describe gene functions and relationships. KEGG is a database used to understand the high-level functions and utilities of biological systems. The ClueGo plug-in of Cytoscape was used to classify and visualize common genes by their BPs through GO analysis.[13] This plug-in was also used to perform KEGG pathway enrichment analysis. In both the GO and KEGG analyses, an adjusted *P*-value (*P*. adjust) $< .01$ was considered to be the cutoff point.

3. Results

3.1. Screening of common genes for MMF, MPA and CLP

Although MMF is an inactive form of MPA, we investigated it to rule out the possibility that certain genes may be directly linked to the MMF, instead of MPA, by an unknown mechanism. The results showed that the MMF set is included in the MPA set, indicating that all currently known genes related to MMF are also

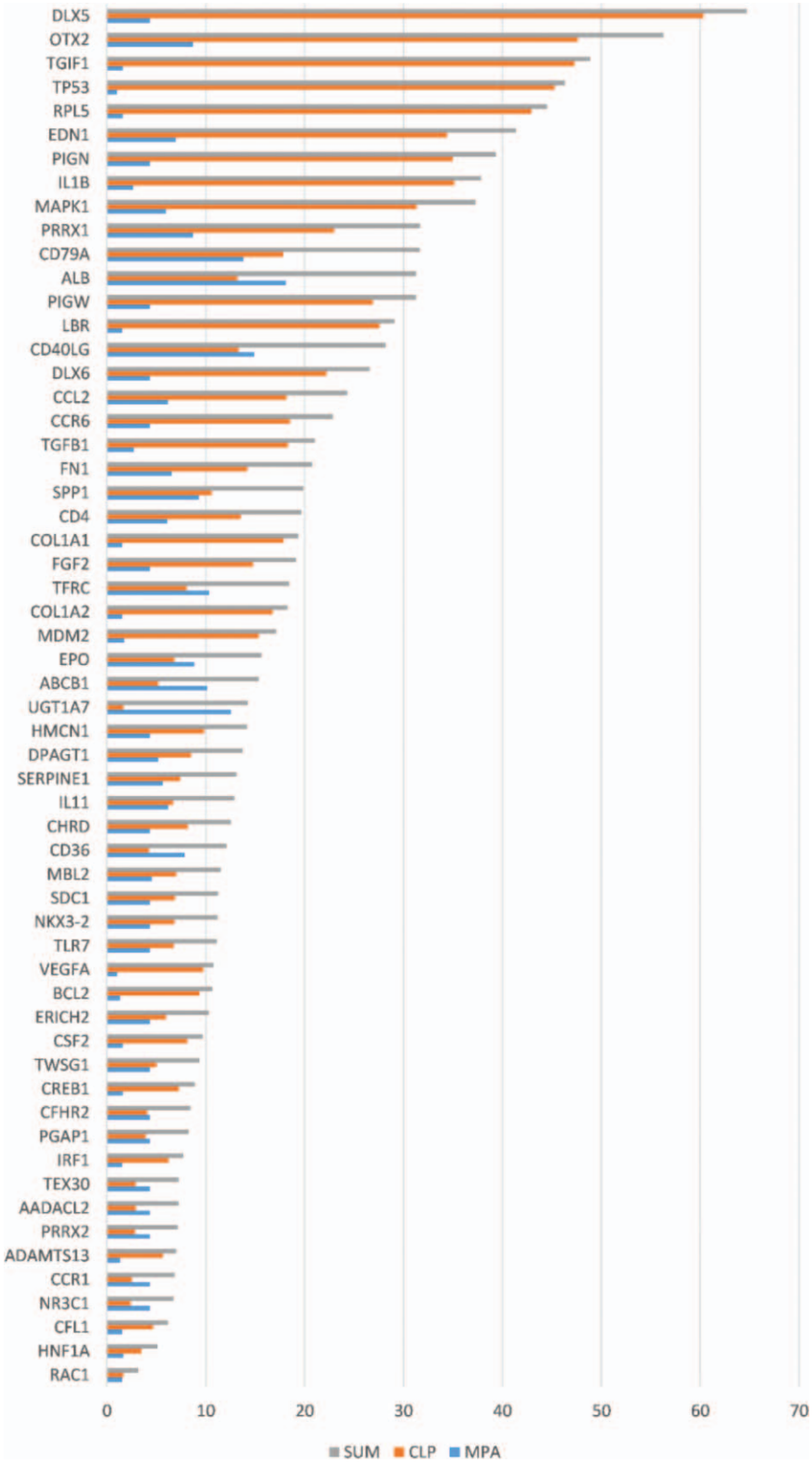

Figure 2. Confidence bar plot ranks common genes according to their confidence score combined. Blue bars represent confidence of correlation between genes and MPA. Orange bars represent confidence of correlation between genes and CLP. Gray bars are confidence scores of each gene combines.

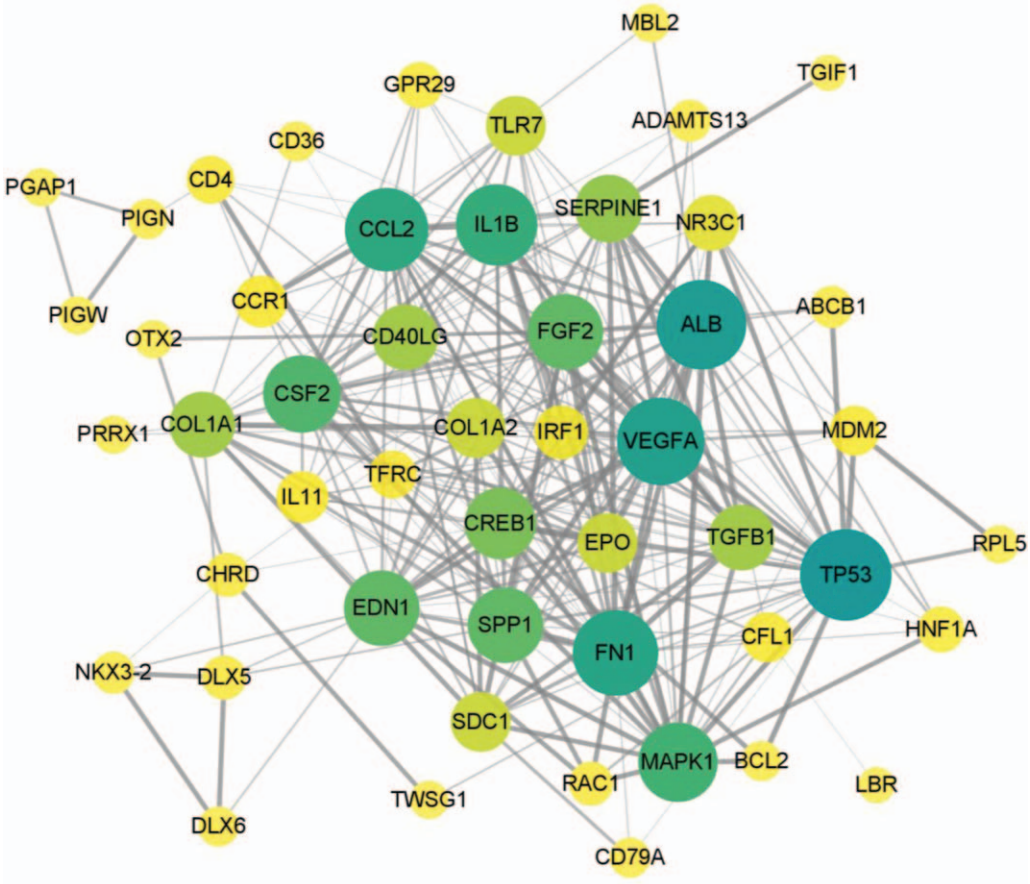

Figure 3. PPI net work of genes both related to MPA and CLP. Visualized by Cytoscape. The color and the size of dotes represent the edgecount of each gene. With the blue and the larger size goes to the larger edgecount, and the yellow and smaller size goes to the smaller edgecount. The thickness and transparency represent the confidence of possible interaction between each gene, with the thicker and less transparent goes to more confident interactions, and the thinner and more transparent goes to less confident interactions.

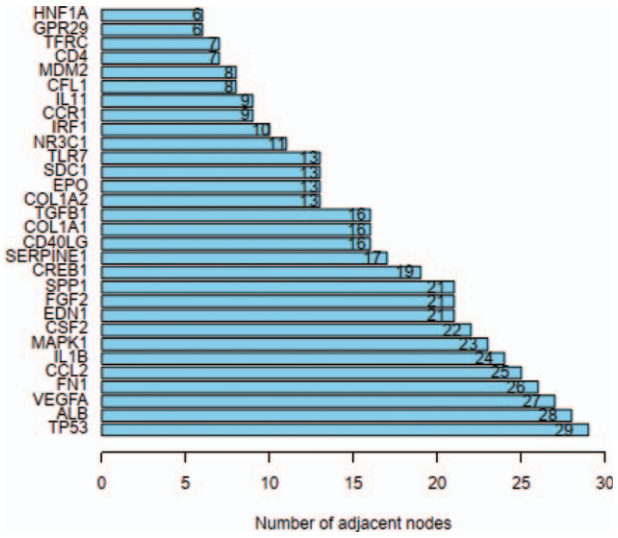

Figure 4. Gene node ranking bar plot. Top 30 common genes ranked according to their node degree from PPI network.

related to MPA. The common genes of MMF, MPA and CLP are all included in the intersection of MPA. A total of 226 genes related to both MPA and cleft were selected (Fig. 1). These genes are considered to be pathogenic genes due to their correlation to both MPA and cleft. Having filtered out the genes with confidence scores lower than 1 and the genes that are not correlated with the pathogenesis of CLP or MPA, 58 genes were screened and arranged by their confidence scores in Figure 2.

3.2. PPI network construction

According to the information in the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) and Cytoscape databases, the PPI relationships of the 58 genes were determined and visualized with Cytoscape (Fig. 3). In the network, TP53 is the most closely correlated with other proteins. In particular, TP53 directly correlated with MDM2 and RPL5. A bar plot ranking common genes by their node degrees depicts the TP53 gene with the highest node degree (Fig. 3).

3.3. Functional annotation of common genes

The pathways and associated BPs of common genes related to both CLP and MPA were detected via GO and KEGG pathway

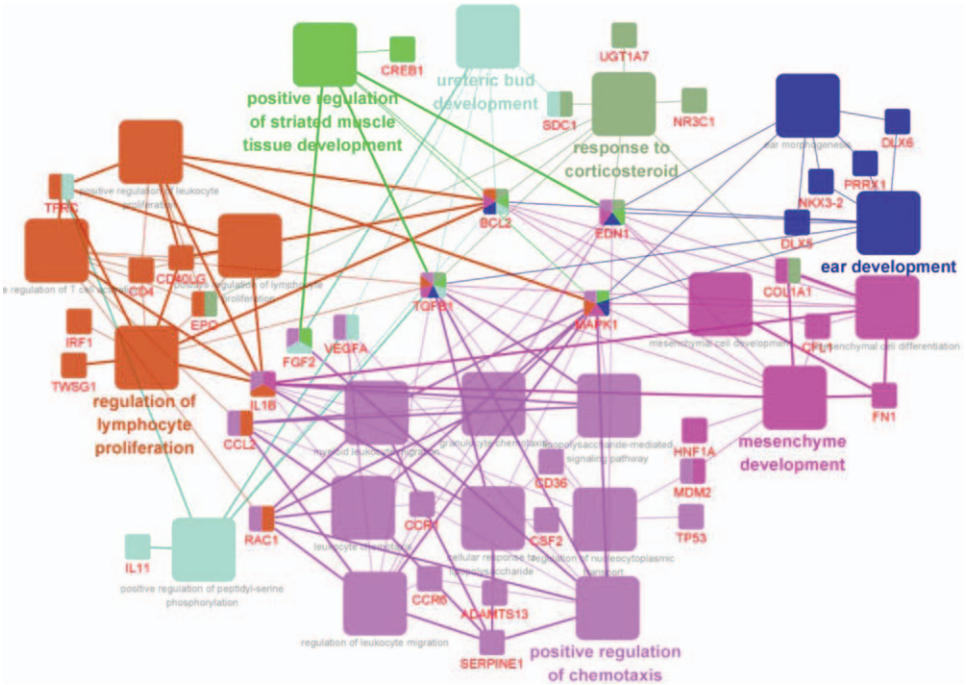

Figure 5. GO BP analysis of genes both related to MPA and CLP.

analyses. The GO terms and the genes belonging to each of them are presented in Figure 5. The GO analysis identified genes enriched for the development of multiple organs and tissues, including ear, mesenchymal cells, striated muscle, and ureteric tissue. Moreover, the KEGG pathways most relevant to the pathogenesis of CLP are presented in Figure 6. The majority of genes were strongly enriched for the HIF-1 signaling pathway, glycosylphosphatidylinositol-anchor biosynthesis, the TNF signaling pathway, and hematopoietic stem cell development.

4. Discussion

MPE is an MPA-induced congenital malformation with a characteristic pattern of symptoms, of which cleft palate/lip is one of the most common.[3] Le Ray et al reported a female renal transplant recipient who was maintained on MMF 250 mg bid during the first trimester of pregnancy. The pregnancy was terminated at 22 weeks due to the prenatal diagnosis of multiple fetal malformations, including cleft lip and palate, microtia and external auditory duct atresia.[2] Nicole M. Sifontis et al reported two female renal transplant recipients receiving MMF during pregnancy. Both cases presented syndromes comprised of CLP and microtia.[14] In 2007, in another case report of MMF exposure, patients suffered from syndromes comprised of bilateral upper cleft lip with complete cleft palate, bilateral microtia, hypertelorism, micrognathia, and mild left ptosis. Perez-Aytes et al proposed the existence of mycophenolate-associated embryopathy, with CLP, microtia, micrognathia and hypertelorism serving as syndrome features.[3] Not all patients suffering from MMF exposure exhibit the same pattern of syndromes. The effect and severity may be correlated with the dose, timing, and duration of MMF exposure.[15] To date, of all MMF-exposure patients, 44% have been reported to have CLP.[16]

A variety of animal experiments have been conducted to elucidate the mechanism underlying MMF embryopathy. Mice with the IMPDH gene knocked out in the neural crest exhibited malformations, including osmotic enteric ganglion disease, craniofacial bone hypoplasia, cardiac outflow tract and great vasculature, suggesting that IMPDH2-mediated guanine nucleotide synthesis is essential for normal development of the ENS and other neural crest derivatives.[17] Jiang et al studied the direct effect of MPA exposure on zebrafish embryos and found that the tail curvature of zebrafish embryos and morphological defects, such as severe pericardial edema caused by MPA, were dose-dependent and that MPA reduced the expression levels of IMDPH1 and IMDPH2. Therefore, it was believed that MPA damaged the development of zebrafish embryos by inhibiting the activity of IMDPH.[18] In *in vitro* studies on rats and rabbits, Tendron et al showed that MMF could cause anacreontic deformity and anophthalmia.[19] Schmidt et al used whole embryo culture methods and found that rat embryos exposed to MPA produced adverse reactions, including missing branchial arches, optic nerves and ear capsules; the deformity observed in rat embryos after exposure to MMF is similar to that of human embryos in the womb. The activity of IMDPH has already been observed in several tissues, such as the heart and brain. Therefore, Schmidt et al proposed a teratogenic mechanism by which MPA inhibits IMDPH. However, the mechanism of MMF embryopathy has not been fully elucidated to date. Bioinformatic analysis is a powerful tool for analyzing the mechanism of pathogenesis. In this study, the GeneCards database and bioinformatic analysis were combined to explore the genes potentially involved in the pathogenesis of CLP resulting from MPA exposure.

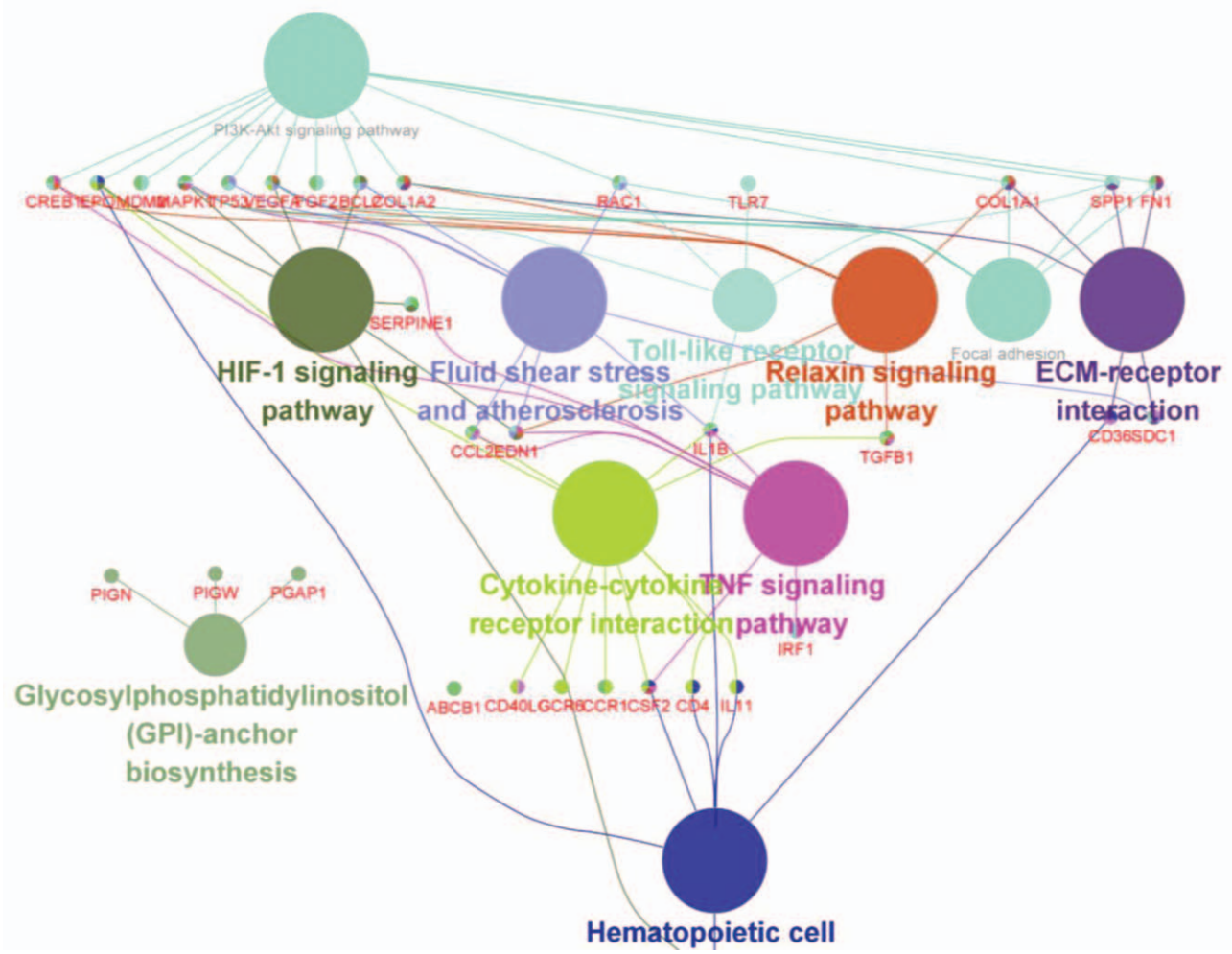

Figure 6. KEGG analysis of genes both related to MPA and CLP.

In the first step, we used a Venn diagram to obtain the intersection of the gene symbols related to MPA and CLP. All genes related to MMF were also determined to be within the range of MPA. This finding is in keeping with the fact that MMF is an inactivated form of MPA. All pharmacological action of mycophenolate can only be achieved by MPA, an activated form of MMF. Subsequently, according to the results of the Venn diagram, there are 226 genes related to both MPA and CLP. We deleted genes with confidence scores lower than 1, and the genes do not correlate with the pathogenesis of CLP or MPA, obtaining 58 genes that are related to both MPA and CLP. These genes are the ones that may be involved in the pathogenesis of CLP resulting from MPA and are to be enriched and annotated in the steps to follow.

4.1. TP53 and the associated MDM2 and RPL5 genes may be involved in MPA-induced CLP

The TP53 gene, also known as the P53 gene, encodes a protein that suppresses tumor proliferation through transcriptional activation and DNA binding.[20,21] MDM2 and p53 are enriched in the pre-migratory neural crest.[22,23] MPA can be used as a reagent to cause ribosomal or nucleolar stress, thereby causing RPS14 to bind to the central acidic domain of MDM2 and restricting p53 levels in the MDM2-p53 feedback loop in response to elevated TP53 expression.[24] In addition, MPA may also trigger a nucleolar stress response that induces p53 activation by inhibiting MDM2 via the ribosomal protein L5 (RPL5).[25] In response to alcoholic cellular stress, alcohol was observed to disrupt the Snai2-p53 relationship, and MDM2 and p53 silencing was sustained, leading to apoptosis of NCCs.[26] Under the regulation of the NEIL1 and NEIL2 DNA glycosylases, MDM2 serves as a target gene of TP53, possibly protecting NCCs from mitochondrial oxidative stress and TCS.[27]

A growing body of research has shown that increased expression of TP53 could underlie a wide range of developmental defects, including Treacher Collins Syndrome (TCS).[28] TCS is a congenital abnormality that presents with microtia, mandibular hypoplasia, retrognathia and CLP (in 40% of cases).[29] The major mutation in the TCS is the Tcof1 (Treacle) gene. Haploinsufficiency of Tcof1 leads to nuclear stabilization of the p53 protein and activation of p53-dependent transcriptional targets, such as Ccng1, in the neuroepithelium. This activation directly correlates with cell cycle arrest and caspase-3-mediated apoptosis of NCC progenitors in the neuroepithelium of Tcof1 +/− embryos; these events account for the NCC deficiencies

underlying TCS.[30] The inhibition of the TP53 gene can also alleviate the apoptosis in neuroepithelial cells and the cranial-maxillofacial deformity of zebrafish with mutant POLR1C and POLR1D. These mutated genes encode the subunits of RNA polymerase I and RNA polymerase III in human TCS.[31–33] Considering the close occurrence of CLP (44% in the MPE, 40% in the TCS) and the important role played by TP53 in the PPI network, we surmised that MPE and TCS may have an identical pathogenesis regarding ribosome stress resulting from TP53 activity. This hypothesis merits further research.

Diamond-Blackfan anemia (DBA) is a rare congenital pure red cell aplasia with a variety of physical malformations, including craniofacial, genitourinary, orthopedic and cardiac malformations.[34] Cleft palate represents approximately 10% of the DBA clinical presentations.[35] TP53 also plays an important role in DBA pathophysiology. Studies of various model systems have shown that haploinsufficiency of the RPS19, RPL5, or RPL11 genes leads to p53 stabilization, increased p53 phosphorylation, and activation of transcriptional targets, including P21, Bax, and NoXA.[36–41] Some ribosomal proteins (RPS3, RPS7, RPS27, RPS27a, RPL5, RPL11, and RPL23) directly bind to the MDM2 gene, releasing p53.[42] p53 may also independently exert its effects on the embryonic stem cells of mice, resulting in DBA without the involvement of RPS19 and RPL5.[43] Downregulating RPL5 is a means of developing a mouse model of DBA syndrome.[44] Mutations in ribosomal protein-coding genes are found in approximately 50% of DBA patients, and the RPL5 mutation is one of the most common.[45] RPL5 can also directly contact the central acidic domain of MDM2 and act as an inhibitor of this gene.[24,46] Patients with mutations in RPL5 display a high frequency of developmental anomalies, especially cleft palates and triphalangeal thumbs.[47] In KEGG analysis, the term hematopoietic cell also demonstrated a possible correlation between DBA, a hematopoietic disease, and MPA-induced CLP, a cranial malformation.

4.2. ABCB1 may be related to the susceptibility of MPA-induced cleft lip/palate

ABCB1 encodes P-glycoprotein, an adenosine triphosphate (ATP)-dependent efflux pump expressed in multiple human tissues, such as the small intestine, liver, kidney, and blood-brain barrier, as well as in lymphocytes. P-glycoprotein is a membrane protein that determines the plasma concentration of MPA by altering its absorption and deposition.[48] The haplotype of ABCB1 in conjunction with sex is correlated with the risk of extrarenal adverse effects resulting from MPA.[49] Mothers who carry the ABCB1 3435C > T polymorphism are at significantly increased risk for having offspring with CLP, especially mothers using medication in the periconceptional period;[50] this risk is presumably attributable to suboptimal exclusion of xenobiotics at the fetal–maternal interface.[51]

4.3. COL1A1 could be involved in Stickler syndrome, similar to MPA-induced CLP

COL1A1 encodes the alpha 1 chain of collagen type I, which can be inhibited by MPA.[52] Clinical exome sequencing indicated that COL11A1, in addition to COL1A1, is involved in the pathogenesis of Stickler syndrome,[53] a genetic disorder of connective tissue presented with submucous cleft or a complete cleft of the hard palate.[54]

4.4. RAC1 could be involved in MPA-induced CLP

MPA decreased the GTP-bound (active) form of RAC1 and specifically altered the expression level of RAC1, inhibiting the proliferation of renal mesangial cells.[55] Trans-interaction of nectin-1 and nectin-4 induces the activation of RAC1 and regulates E-cadherin-mediated cell-cell adhesion. Nectin-1 mutations cause CLP ectodermal dysplasia.[56]

4.5. TGFB1 exhibits the potential of mediating the teratogenic effect of MPA-induced CLP

Transforming growth factor-beta (TGFβ) represents a group of 25-kDa proteins that are actively involved in the development and differentiation of various tissues. Cyclosporine (CsA) is thought to enhance transforming growth factor beta 1 (TGFβ1) production *in vitro* and *in vivo*, which may have a negative effect on long-term graft survival. BJ van der Mast et al converted postoperative therapy from CsA to MMF in renal allograft recipients and observed no significant downregulation of TGFβ1 levels in the plasma.[57] This finding attracted our attention because TGFβ1 is involved in the pathogenesis of CLP.[58] TGFBβ1 and TGFβ3 are differentially expressed and are correlated with the CLP phenotype.[59] However, Ginila T Raju reported that TGFβ1 gene polymorphisms do not confer risk for nonsyndromic oral clefts but instead affect the stability and the activation process of TGFβ1.[60] Elucidating this controversial role of TGFβ1 in the pathogenesis of CLP would enhance our understanding of the mechanism underlying MPA-induced CLP.

4.6. Suppression of EDN1 by MPA could be related to the pathogenesis of cleft lip/palate

EDN1 encodes endothelin 1, a vasoconstrictive peptide that results in ischemic renal failure and drug-induced renal injury during treatment with CsA. This side effect can be reversed after conversion to MPA, as MPA significantly inhibits endothelin 1 mRNA in renal artery endothelial cells,[61] while EDN1 mutation has been found to be involved in CLP pathogenesis.[58,62–64]

4.7. Various genes are involved in the development of multiple organs and tissues

The results of GO analysis demonstrated the involvement of GO terms for multiple organs and tissues. This finding is in keeping with the fact that phenotypes resulting from MPA are comprised of malformation of different organs, with ear development being the most compromised (microtia).[16] Therefore, the genes participating in MPA-induced CLP are also likely to be involved in the malformation of other organs and tissues. This phenomenon reflects the syndromic characteristics of mycophenolic acid embryopathy. MPA may result in a spectrum of malformations, including CLP, by affecting a variety of target genes and pathways.

As a bioinformatics research, however, there are still some limitations. The conclusion that ribosome stress-related genes in the TP53-mediated signaling pathway play an important role in the MPA-induced pathogenesis of CLP remains to be further validated. In addition, although we obtained the core genes by setting the threshold on the correlation score, that doesn't mean that the genes that are filtered out have nothing to do with the disease at all. This strategy only provides a more reliable research

direction for further study. With the advancement of more valuable research, it will bring more abundant data to the bioinformatics database, and some new genes may also be found.

5. Conclusion

The present study provides significant results that may help to elucidate the molecular mechanisms underlying MPA-induced CLP pathogenesis. The common genes associated with both MPA and CLP were identified in this study. The combination of MDM2, RPL5 and TP53 plays a major role in the pathogenesis in the MPA-induced CLP. This pathogenetic process is very resembling to that of TCS, as the TP53-induced TCS share a common clinical feature and symptom occurrence. Other genes such as COL1A1, RAC1, TGFB1 and EDN1 may also contribute in the MPA-induced CLP, a syndromic pathogenesis combined with multiple organ malformations. The polymorphism of ABCB1 could be related to the probability of MPA-induced CLP by altering the absorption and determining the ureteric concentration of MPA. It is suggested that the teratogenic effect of MPA resulting in CLP is mediated by multiple genes and pathways.

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Visualization: Yangyang Lin.
Writing – original draft: Yangyang Lin, Elsa M. Ronde, Gang Ma, Huiqin Cui.
Writing – review & editing: Yangyang Lin, Elsa M. Ronde, Gang Ma, Huiqin Cui.

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