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Insights and future directions of potential genetic therapy for Apert syndrome: A Systematic review

Running title: Gene therapy in Apert syndrome

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ABSTRACT

Apert syndrome is a genetic disorder characterised by craniosynostosis and structural discrepancy of the craniofacial region as well as the hands and feet. This condition is closely linked with fibroblast growth factor receptor-2 (FGFR2) gene mutations. Gene therapies are progressively being tested in advanced clinical trials, leading to a rise of its potential clinical indications. In recent years, research has made great progress in the gene therapy of craniosynostosis syndromes and several studies have investigated its influences in preventing/diminishing the complications of Apert syndrome. This article reviewed and exhibited different techniques of gene therapy and their influences in Apert syndrome progression. A systematic search was executed using electronic bibliographic databases including PubMed, EMBASE, ScienceDirect, SciFinder and Web of Science for all studies of gene therapy for Apert syndrome. The primary outcomes measurements vary from protein to gene expressions. According to the findings of included studies, we conclude that the gene therapy using FGF in Apert syndrome was critical in the regulation of suture fusion and patency, occurred via alterations in cellular proliferation. The superior outcome could be brought by biological therapies targeting the FGF/FGFR signalling. More studies in molecular genetics in Apert syndrome are recommended. This study reviews the current literature and provides insights to future possibilities of genetic therapy as intervention in Apert syndrome.

Keywords: Apert syndrome; Craniosynostosis, Gene therapy; Fibroblast Growth Factors; FGF; Non-surgical therapy

INTRODUCTION

Throughout this review, we clarified and discussed the potential benefits of gene/protein regulations in the treatment or prevention of craniofacial and coronal suture discrepancy in Apert syndrome (AS).

Description of the condition

The membranous bones of which the neurocranium is made, are held together by the direct ossification of their sutures. These sutures enclose undifferentiated mesenchymal cells, separating the osteogenic fronts (1). The entire process is coordinated by the interplay of several signaling molecules such as the fibroblast growth factors (FGFs), Bone morphogenetic protein (BMP), hedgehog, and transforming growth factor beta (TGF- β). The calvarial sutures are documented to appear between the 15th day of embryonic development in mice and the 18th week of gestation in humans, and to allow continuous brain growth, they stay open at fetus birth. Craniosynostosis is a result of improper bone formation in the developing skull. It is considered a congenital disorder where the calvarial sutures prematurely seal together, resulting in constraint of the skull's normal growth, and consequently of its brain development and face. It is estimated that 1 in 2,500 live births are born with craniofacial anomalies, making it one of the most common disorders recurring in this field (2). Craniosynostosis can be a localized, non-syndromic, phenomenon or it may be part of a larger syndrome involving the malformations of the digits, the development of defects in the skeleton, in the heart, or other organ anomalies.

Apert syndrome is categorized by premature fusion of sutures of the vault and/or cranial base, accompanied with mid-face hypoplasia, symmetric syndactyly of digits and other general malformations involving craniofacial, visceral features, skeletal, oral, respiratory and cutaneous

(3). Central nervous system abnormalities and intracranial hypertension are frequently identified in these patients. The most distinguishing aspect of an affected child is bilateral coronal synostosis which results in a head that is short in the anteroposterior direction but wide, tall, and towering at the top. Since the characteristic features of the Apert syndrome are acrocephaly and syndactyly, it is referred to as acrocephalosyndactyly. Oral and craniofacial clinical signs being the most prominent features in Apert syndrome patients. Longer soft palate and shorter hard palate than normal with soft cleft palate and bifid uvula were seen in 76 %, (4-6). It has a natal incidence valued between 9.9 and 15.5/million and accounts for about 4.5 % of all craniosynostosis (3).

FGFs are the family of at least 22 known signaling molecules that regulate cell proliferation, differentiation, and migration through a range of complex pathways. They work through the fibroblast growth factor receptors (FGFRs) - a transmembrane receptor tyrosine kinase- a family of 4 tyrosine kinase receptors. FGFRs each contain 3 immunoglobulin-like domains (Ig I-III), a single transmembrane domain (TM), and 2 tyrosine kinase domains (TK1-2) (7). The ligand binds to extracellular immunoglobulin-like domains (IgI, IgII, and IgIII). Ligand-induced dimerisation leads to autophosphorylation of the split tyrosine kinase (TK1 and TK2). The rate of recurrence of mutations in Ig II-III leading to craniosynostosis syndromes (8). Mutation in the FGF signaling pathway plays a crucial role in the development of craniosynostosis syndromes. Apert disorder is linked with a mutation in the FGFR2 gene that maps to chromosome 10q25-10q26 and follows an autosomal dominant inheritance form (9). It includes specific mutations at two adjacent amino acids, Ser252 and Pro253 of FGFR2, anticipated to lie in the linker zone between Ig II and Ig III of the FGFR2 ligand-binding domain (10). This gene is accountable for the development and union of the skull sutures during growth and bone development.

Description of the intervention

In this review, the gene therapy as an intervention for Apert Syndrome compared to surgical intervention is critically appraised and the therapeutic applicability of related genes that might have an important role in AP via gene-protein regulation was highlighted.

Initiated FGFs/FGFR2 signaling disturbs the stability of differentiation, cell proliferation, and apoptosis by its downstream signal pathways. These mutations enhance ligand affinity for the receptor and consequently prompt excessive activation. The mutated FGFR2 causes AS drives cells osteodifferentiation via ERK1/2, AKT, PKC, p38, PLC γ , JNK, EGFR, PDGFR and other signaling pathways. ERK1/2 demonstrates an essential role in osteoblast differentiation. AKT, a downstream target of phosphatidylinositol-3-kinase (PI3-K), is a crucial mediator of cell proliferation and survival by phosphorylation of various targets which lead to initiation or suppression of their functions. Protein kinase C (PKC) is a serine/threonine protein kinase and mediates several cellular functions such as cell proliferation and differentiation. Furthermore, PKC signaling regulates osteoblast differentiation and is vital for basic fibroblast growth factor (bFGF) - induced bone formation. The p38 MAPKs belong to the MAPK superfamily and have been revealed to be involved in proliferation, differentiation, apoptosis, senescence and cytokine production. Phospholipase C (PLC) transforms phosphatidylinositol 4,5-bisphosphate (PIP₂) to inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG) and regulates the creation of vital second messengers which defines cell behavior (3). The signaling pathways that lead to AP is summarized in Figure 1.

Review purpose

In recent years, the understanding of biochemical and molecular signaling, specifically of the FGFRs, plays an significant role in investigating possible pharmacologic and gene therapy that precisely repress the initiation of these pathways which in turn could be useful for the treatment / diminish the complications of AS patients. Genetic study in the field of craniosynostosis will keep on disclosing new targets for non-surgical therapy and lead to the discovery of new technologies. This may ultimately provide a possible alternative to the invasive surgical interventions currently in use.

Understanding the molecular and pathogenic mechanisms involved in Apert syndrome conditions and the role of gene therapy is undoubtedly helpful in designing and implementing new treatment strategies providing insights into the future direction. Hence, we aim to present a systematic review for management of Apert syndrome by gene-protein therapy which may have the potential to diminish the AS complications thus becoming a useful medical alternative to high-risk surgical intervention.

METHODS

Objective

To demonstrate the effectiveness of gene therapy/ pharmacological inhibitors in diminishing the clinical manifestation of the AS with emphasis on their intervention, protocols, and outcomes.

Focused research question

Does gene therapy regulate gene-protein expressions that have a role in craniofacial growth and diminish/prevent AS development?

132 **Condition or domain being studied**

133 The effect of the gene-protein regulations on craniosynostosis prevention or underlie the AS.

134 **Selection criteria**

135 There are no restrictions on the types of study design. Inclusion criteria are any studies on
136 syndromic craniosynostosis with AS which was treated with gene or pharmacological inhibitors
137 therapy, *in vitro*, and *in vivo* (Animals genetically induced AS and cell lines).

138 **Exclusion criteria**

139 Studies on other craniosynostosis syndromes (not relevant to AS), surgical treatment and
140 pharmacological treatment (not relevant to gene therapy). In addition, reviews, abstracts, author
141 debates, summary articles and studies with significant incomplete data were excluded.

142 **Search protocol**

143 The following electronic bibliographic databases: PubMed, EMBASE, ScienceDirect, SciFinder
144 and Web of Science (Science and Social Science Citation Index) were searched for relevant articles
145 until November 2020. The keywords and Boolean terms used for the electronic databases search
146 were: (Apert syndrome OR acrocephalosyndactyly OR Maroteaux Fonfria syndrome) AND
147 (gene* mutation OR DNA OR fibroblast growth factor receptor) AND (therapy* OR treatment
148 OR repair OR inhibitor OR suppressor OR pharmacology) AND (mutation OR damage OR
149 variation OR imbalance OR instability). Study selection eligibility and exclusion criteria were pre-
150 specified. Both *in vivo* studies, which contain animals induced for AS, and *in vitro* studies, which
151 performed in the cell line, were considered for this review. The primary outcome of interest was

gene-protein expressions and regulations that have a role in the craniofacial growth and coronal suture, while the secondary outcome was how gene therapy could prevent or diminish AS progression. With respect to effective outcomes from gene therapy compared to the non-exposed considering untreated control group of AS, pre- and post-test data were required for inclusion.

We manually searched bibliographies of extracted articles to identify relevant references that were potentially missing. The search was not restricted by the year of publication; however, it was limited to studies on the database published in the English language. The searches were re-run just before the final synthesis and any further studies were retrieved and included. Two of the authors performed the search through virtual libraries, screening the titles and abstracts and for final inclusion based on full-text assessments and made decisions on inclusion, data extraction, and quality assessment of included studies independently. A third reviewer served as an arbiter if consensus couldn't be reached. Disagreements between reviewers were resolved by discussion and consensus. The literature search was performed according to the guidelines of the PRISMA statement and the summary of the literature search and screening process is presented using PRISMA flow chart (Figure 2) (11). This systematic review was registered at PROSPERO in March 2018 (registration number: CRD42018089955).

Risk of bias and quality assessment

Two authors individually evaluated the risk of bias among the involved studies by considering the following characteristics using SYRCLE's risk of bias tool for animals studies (12) and OHAT tool (Oral Health Assessment Tool) for cell line (13): adequate randomization, baseline characteristics specified, evidence of adequate concealment of groups, evidence of random housing/experimental conditions, evidence of caregivers blinded to intervention, evidence of

random selection for assessment, evidence of assessor blinded, explanation of missing data, free of selective reporting based on methods/results and free of other high bias risk. A grade (low, high, or unclear) for risk of bias was assigned for each domain in SYRCLE's tool and a grade (Definitely Low, Probably Low, Probably High or Definitely High) for each domain in OHAT tool. Conflicts between the evaluators over the risk of bias in particular studies were solved by debate with a third reviewer.

Data extraction and analysis

Due to the insufficient homogeneity of the primary outcomes among the included studies, a meta-analysis was not performed. The qualitative synthesis was carried out to summaries the extracted data obtained from all the included studies. A total of 20 studies were identified and full-text articles were retrieved. Data extraction and interpretation were conducted for all included studies. Excel worksheets were designed for listing the specific parameters for reporting. The data were collected from the articles included according to the items in the data extraction form and approaches as follows: "Author / Year" - author's reference and year of publication, "Study model" - describes the type of research method carried out, "Type of participants" - specifies the type of animals, cell lines and follow-up time, type of intervention, "Evaluation method" - methods that were used to evaluate a study quality and "Risk of bias" - characterization of risk of bias in each study. As a primary outcome the data of protein and gene analysis, and image analysis pre- and post- gene therapy were extracted from the tables, graphs, and text. Both data (continuous/dichotomous) were collected and compared. Furthermore, other data such as secondary outcome(s): Image analysis (%), CT scan for bone formation analysis quantity and quality (%), Microarray- gene number expression (n), Immunohistochemistry (%), Number of

cells present at follow-up (continuous; total number of cells or cells per mm² OR (%), Animal model (Species, Sex), Method of mutation were extracted and analyzed.

RESULTS

Study selection

A total of 897 titles were obtained from the electronic search until November 2020. The first screening of headlines and abstracts based on the relevance and eligibility criteria led to include only 51 studies. Out of these papers, 31 articles were excluded based on the inclusion and exclusion criteria. Finally, after full text analysis, 20 manuscripts remained to be reviewed (Figure 2).

Risk of bias and quality assessment of included studies

Figures 3 and 4 show the overall results of the risk of bias assessment of the 20 studies, included in this systematic review, using SYRCLE's tool for *in vivo* and OHAT tool for *in vitro* studies. The included studies were generally considered to be of moderate quality. The baseline characteristic, random housing, blinding of participants, incomplete outcome data and selective reporting were adequate in all studies according to SYRCLE's tool. Random sequence generation, allocation concealment and experimental condition were satisfactory in all studies according to OHAT tool. There were 10 studies with low risk of bias and no study had high risk of bias (out of the 20 studied 50 % have low risk and 0 % with high risk).

Characteristics of included studies

All the included studies were cell line and animal based with variations of experimental characteristics. Among the included studies, five were *in vitro* studies and four were *in vivo* studies, while eleven studies were performed using both *in vivo* and *in vitro* models. In *in vivo* studies,

three of them were performed in rats while twelve in mice. In view of gender for *in vivo* experiments, two studies used only males, one study used only females and ten studies did not mention the gender of the animals. Five studies used neonatal animals and others used animals with age ranged from 3 to 12 weeks old. All studies used untreated groups as a control group except Wheldon et al. who considered the standard treatment as a positive control (14). Regarding *in vitro* studies, ten studies used osteoblast cells while four studies used other types of cells including fibroblast, BaF3 cells, HEK-293T cells, Cos7 cells, primary mouse calvarial cells and bone marrow stem cells (BMSCs). The type of cell lines was not clear in two studies. The passage of cells used ranged from 1 to 4 (Table 1).

Intervention design and types of protocol

Different techniques were used as methods of mutation. Mainly the methods were either via genetic interventions or induction. The route of administration varied greatly between the studies. Among the included studies, six studies used FGF2 as an intervention, three studies used U0126 (MEK inhibitor), two studies used juglone (5-hydroxy-1,4 naphthoquinone; 420120, Calbiochem) and other studies used different types of treatments. Eleven studies presented overall protein and gene measurements after treatments, while the others presented their measurements as either protein or gene expressions. The duration of the treatment for *in vivo* studies was ranged from 24 hours to one month (Table 2).

The outcomes of different studies are summarized in Table 3. The morphological appearance in untreated animals (control-mutant animal) revealed overgrowth of calvarial plates, a dome-shaped skull, widely spaced eyes, underdeveloped midface and other features which appeared in AS. However, four studies showed no major difference detected between treated mice and normal mice (15-21). Histological results showed evidence of new bone formation and increasing in the

expression of bone formation markers e.g. osteopontin (OPN), increasing in the number of osteoblasts, decreasing in osteoclast, and secondary ossification centres in mutant mice, proliferating cell nuclear antigen- positive cells and expression of collagen type II alpha 1 and type X (14-26). These results were confirmed radiologically by many studies, which showed premature closure of coronal suture (CS) and delayed fusion of sutures in mutant mice (15, 17, 26, 27). Alterations of FGF biological activity resulted in changes in cellular expression of different proteins e.g. Bax expression and collagen I and TGF- β 1 expression, Runx2 and OPN, Bcl-2, FGF2, and ERK (18, 19, 22, 28, 29).

Miraoui et al, 2020 reported that activation of FGFR2 and upregulation of EGFR led to reduced EGFR ubiquitination expression (30). Furthermore, FGF signalling inhibits expression of ALP and blocks mineralization resulting in inhibition of suture fusion (17, 27, 30).

DISCUSSION

Overall, after screening the literature based on our inclusion and exclusion criteria, only 20 studies were involved in this systematic review within the span of 20 years. The earliest being a study from Mansukhani et al. in 2000 and the latest by Kim et al. in 2020. Although this number can be considered small, these studies provide breakthrough and evidence of progression in the exploration of the potential of gene therapy in syndromic craniosynostosis.

As most of the studies were not homogenous and reported different outcomes, meta-analysis was not conducted. Nevertheless, SYRCLE and OHAT tools allowed us to assess the degree of bias for all included *in vivo* and *in vitro* studies, respectively. In general, most of the articles carry moderate quality with more than half of the studies having a low risk of bias.

Knowledge of the normal and pathological process of cranial development is essential for addressing the abnormality in the complex molecular signalling pathways, and its consequences towards tissue development. Similarities in craniofacial development and its molecular pathway have been found between mouse and human, making mouse as the ideal model for craniosynostosis study because of the genetic and physiological similarities between the species (31-33). Therefore, many *in vivo* studies used mice models to allow the induction of cranial suture fusion and imitating the actual human craniosynostosis in AS.

Apert syndrome molecular pathogenesis

The substantial majority of AS patients have one of two missense mutations in adjacent amino acids; S252W or P253R in FGFR2 gene. The S252W mutation, found in two-thirds of AS patients, has been correlated to the most severe craniofacial abnormalities (34, 35). Gene therapies have great potential to treat diseases that were formerly untreatable. Manufacturers were investing more in this field to revolutionize treatments of an array of diseases. This approach may prevent, alleviate or cure underlying disorders and diminish the needing of the surgical approach.

According to the findings of the included studies in this systematic review, AS presented distinctive morphological and radiological features, histological anomalies as a result of loss-of-function and gain-of-function of FGFR2c mutants. The morphological and radiographical appearance in AS revealed overgrowth of calvarial plates, a dome-shaped skull, widely spaced eyes, underdeveloped midface, and other features as results of mutation of FGF (15-18). Mutations in FGF led to increased cellularity and dysregulated differentiation of osteoblasts in the suture which in turn led to premature fusion. Thus, inhibition of FGF signaling resulted in reduced

cellularity and crew of osteogenic fronts and delayed fusion of the suture (15, 16, 20, 21, 23, 24, 28, 36-38). Similar futures were confirmed by the reported changes in cellular expression of different proteins, for example, increased Bax expression and collagen I and TGF- β 1 expression, Runx2 and Opn, decreased Bcl-2, FGF2 and ERK in mutant animals (15, 18, 21, 25, 39), while treated animals displayed opposite patterns in regulation of these proteins (17, 36-38, 40).

Nakamura et al. (2009) demonstrated that ERK-MAPK signaling was a key mediator of hyperactivated FGF signaling and might contribute to both craniosynostosis and craniofacial growth aberrations; presumably via an osteoblast-dependent mechanism (41). These findings are consistent in some of included studies of mutant mice with AP (16, 17, 21, 25, 28, 38, 42). Thus, sutural patency or fusion secondary to alterations in FGF biological activity was likely because of changes in cellular proliferation. This evidence appeared in histological results as new bone formation and increasing in the expression of bone formation markers such as; Opn, type 1 collagen, and osteocalcin (15, 21, 25, 28, 39, 40) . According to these findings, we concluded that the gene therapy using FGF in AS was critical in the regulation of suture fusion and patency, occurred via alterations in cellular proliferation.

Progress in gene therapy in craniofacial surgery in human

Twenty-five years ago , insertion of a marker gene into lymphocytes from cancer patients was the first clinical gene delivery. Potentially, an improvement in effectiveness, safety and production of gene delivery paths and introduction of gene-editing technologies to the clinic will be seen in the futuer. Gene delivery might demonstrate a cost-effective method for the delivery of biological interventions as reported by Collins in 2015 (43). Promising progress of gene delivery in human

309 trials includes the application of viral vectors of engineered bone marrow stem cells, therapy for
310 inherited retinopathies and haemophilia B as well as in certain malignancy and infectious disease
311 (43).

312
313 Broad and concentrated genetic database are required to allow better insight in the genomic field
314 and the epigenetic influences on this condition (44). Crucially, shortage of molecular genetics data
315 of craniosynostosis published in the literature has become one of the main constraints for the
316 development in genomic approach towards precision medicine. As such, the authors recommended
317 that more studies of molecular genetic in the field of syndromic craniosynostosis is needed and
318 crucial before gene therapy can be part of interventional strategy in AS. In addition, clinical trials
319 are needed to assess gene therapy in patients with Apert syndrome.

321 **Potential difficulties, side effect and complications of gene therapy in Apert syndrome**

322 In general, challenges in gene therapy in syndromic craniosynostosis include comprehensive
323 understanding of AS molecular pathogenesis as the condition involves various phenotyping
324 characteristics. Additionally, effective gene delivery is not predictable in the actual clinical setting
325 since therapy would necessitate intervention during ante-natal phase that may cause an actual risk
326 on mother and fetus.

327
328 In terms of stability and integration, some gene treatment approaches aim to accomplish a long-
329 term effect. Wherever such durability is required, the therapeutic material requires to stay
330 functional for the intended period. As reported there are two possible ways of achieving this, either
331 utilize multiple rounds of gene therapy or combine the therapeutic genes, remained them active

for a longer time (45). On other hand, scientists are investigating ways of accomplishing long-term therapeutic effects without integration, for example by using stable non-integrating vectors. Additionally, safety of vectors are mandatory as viruses; the carrier of choice in most gene-therapy studies, presents several potential difficulties. Thus, improving the immune system to vectors in recipients may make it hard to give reproducible applications of gene therapy.

Viral delivery of tissue nonspecific alkaline phosphatase diminishes craniosynostosis in one of two FGFR2C342Y/+ of Crouzon syndrome in mouse model (46, 47). The authors suggested a new perspective on creating a conducive atmosphere, such as viral vectors with genetic engineering technologies, and personalizing all characteristics of gene therapy treatments to the patient is necessary to transform the lives of patients with genetic disorders in Apert syndrome preceding the clinical trial.

Gene therapy and precision medicine in Apert syndrome

Disorders that evolve from mutations in a single gene were the best candidates for gene therapy. Various routes of gene therapy have been demonstrated from the selected studies involved in this systematic review. In general, for correcting faulty genes, the commonest approach involved in normal gene insertion into a non-specific location within the genome to replace a non-functional gene. Other alternatives were through genome editing for example; swapping an abnormal gene with a normal gene through homologous recombination; abnormal gene repair via selective reverse mutation and through the regulation or alteration of a particular gene (48, 49). Superior outcome could be brought by biological therapies targeting the FGF/FGFR signalling. PD98059, an MEK1 inhibitor, was found to reduce coronal suture fusion in cultured calvarias of FGFR2+/P253R mice

in Yin study (15). In addition, Yokota *et al.* and Morita *et al.* discovered a soluble form of FGFR2 with Ser252Trp mutation that could partially relieve the phenotype of Apert mouse model by alleviating the premature closure of coronal suture in cultured calvarias and transgenic mice (25, 50). Moreover, inhibiting the MEK1/2 by U0126 alleviates the craniosynostosis phenotypes in Fgfr2+/S252W mice was found in Shukla study (16). Pharmacologic inhibitor [juglone (5-hydroxy-1,4 naphthoquinone; 420120, Calbiochem)] reduced RUNX2, inhibited the downstream Dusp6, Spry2, and decreased the expression of Cyclin D1, Cdk2, Cdk4 and PCNA, which resulted in normal closure of coronal sutures (36). Furthermore, it led to expression of collagen type II alpha 1 and type X which in turn inhibited the sutures fusion in mice (20). Adeno-Associated Virus-Mediated RNAi (S2) downregulated the protein levels of FGFR2, phosphorylated ERK1/2 and P38, and decreased the mutant FGFR2, Runx2, Collagen 1, osteocalcin, and osteopontin expression that followed by decreasing in the sutures fusion (38). Tamoxifen increased p-ERK1/2 MAPK and the expression of Runx2, Opn, ALP, Col1A1, osteocalcin and Rankl/Opg ratio, which resulted in normal closure of coronal sutures (21). These results confirmed the impacts of biological inhibition on the activated molecular pathways in AS on skull phenotypes.

As summarized in this systematic review, the primary outcomes measurements varies from protein to gene expressions. These findings are promising evidence towards the progress on precision medicine in AS management. Corresponding to the US Precision Medicine Initiative, precision medicine is defined as an emerging approach for disease treatment and prevention that takes into account individual variability in genes, environment, and lifestyle for each person (51). Nevertheless, other than proteomic and genetic modification, identification of characterization for phenotypic variants is also important as it will allow specific therapies involving either gene or

pharmaceutical approach to target these variants as well as potential lifestyle changes that may modify the outcome.

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534 Figure 1: FGFR2 mutation and signaling pathways of Apert Syndrome.

535 ERK1/2; extracellular signal-regulated protein kinase, AKT; Protein kinase B (PKB), PKC;
 536 Protein kinase C, p38; mitogen-activated protein kinases, PLCγ; Phosphoinositide phospholipase
 537 C, JNK; c-Jun N-terminal Kinas, EGFR; epidermal growth factor receptor, PDGFR; Platelet-
 538 derived growth factor receptor.

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Figure 2: Literature search and screening according to PRISMA statement (Moher et al. 2009)

	Random sequence generation (selection bias)	Baseline characteristics (Selection bias)	Allocation concealment (selection bias)	Random housing (Performance bias)	Blinding of participants and personnel (performance bias)	Random outcome assessment (Detection bias)	Blinding of outcome assessment (detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)	Other bias
Greenwald 2001	+	+	+	+	+		+	+	+	+
Kim 2020		+	+	+	+			+	+	+
Li Zhang 2015		+	+	+	+			+	+	+
Luo 2018		+	+	+	+			+	+	+
Morita 2004		+	+	+	+		+	+	+	+
Pfaff 2016		+	+	+	+			+	+	+
Shen 2009		+		+	+			+	+	+
Shin 2018		+	+	+	+			+	+	+
Shukla 2007		+	+	+	+	+	+	+	+	+
Suzuki 2001		+	+	+	+	+	+	+	+	+
Warren 2003		+	+	+	+			+	+	+
Xu 2017		+	+	+	+			+	+	+
Yeh 2012	+	+	+	+	+			+	+	+
Yin 2008		+	+	+	+	+	+	+	+	+
Yokota 2014		+	+	+	+			+	+	+

Figure 3: Summary of risk of bias for in vivo studies using SYRCLE's tool

	Random sequence generation (selection bias)	Allocation concealment (selection bias)	Experimental conditions (Performance bias)	Blinding of participants and personnel (performance bias)	Exposure characterization (performance bias)	Random outcome assessment (Detection bias)	Incomplete outcome data (attrition bias)	Selective reporting (reporting bias)	Other bias
Greenwald 2001	+	+	+		+	+	+	+	+
Li Zhang 2015	+	+	+	+	+	+	+	+	+
Luo 2018	+	+	+	+	+		+	+	+
Mansukhani 2000	+	+	+	+	+	+	+	+	
McDowel 2006	+	+	+	+	+	+	+	+	
Miraoui 2010	+	+	+		+		+	+	+
Pfaff 2016	+	+	+	+	+		+	+	+
Shen 2009	+	+	+	+	+		+	+	+
Shin 2018	+	+	+	+	+		+	+	+
Suzuki 2001	+	+	+	+			+	+	+
Tanimoto 2004	+	+	+	+		+	+	+	+
Warren 2003	+	+	+	+		+	+	+	+
Wheldon 2011	+	+	+				+	+	+
Yeh 2012	+	+	+	+	+	+	+	+	+
Yin 2008	+	+	+	+	+	+	+	+	+
Yokota 2014	+	+	+	+	+	+	+	+	+

558 Figure 4: Summary of risk of bias for in vitro studies using OHAT tool

559 **Table 1. Study characteristics of included studies**

Study	Study design (<i>in vitro</i> / <i>in vivo</i>)	Species	Sex	Age	Sample size (n)	Control (n)	Cell line	Sample size	Group (n)	Passage (n)
Mansukhani et al., 2000	<i>In vitro</i>	-	-	-	-	Untreated	Osteoblasts	3	4	1
Greenwald et al. 2001	<i>In vivo</i> and <i>in vitro</i>	Sprague-Dawley rats & CD-1 mice	-	Neonatal	10	Untreated	Osteoblasts	3	3	1
Warren et al. 2003	<i>In vivo</i> and <i>in vitro</i>	CD-1 mice	-	18-22 days	6-9	Untreated	Osteoblasts	3	2	1
Tanimoto et al. 2004	<i>In vitro</i>	-	-	-	-	-	Osteoblasts cell	3	2	-
McDowel et al. 2006	<i>In vitro</i>	-	-	-	-	-	BaF3 cells	3	3	-
Shukla et al. 2007	<i>In vivo</i>	U6-Fgfr2S252W shRNA mice & shRNA rescue mice	Female	Neonatal	2	Untreated	-	-	-	-
Yin et al. 2008	<i>In vivo</i> and <i>in vitro</i>	-	Male/female	-	2	Untreated	Osteoblasts & Bone marrow cells	3	4	-
Shen et al. 2009	<i>In vivo</i> and <i>in vitro</i>	Weanling nude rats	-	6-8 week	6	Untreated	Osteoblasts cell	3	2	-
Miraoui et al. 2010	<i>In vitro</i>	-	-	-	-	Untreated cell	Osteoblasts cells	3	3	0
Wheldon et al. 2011	<i>In vitro</i>	-	-	-	-	Chick embryos (treated with electroporated with 2 mg/ml of purified plasmids encoding IIIa-TM or GFP)	HEK-293T cells & Cos7 cells	3	3	0
Suzuki et al. 2012	<i>In vivo</i> and <i>in vitro</i>	CB-17 SCID/SCID mice	Male	5-week old	3	Untreated	Osteoblasts cells	3	3	1 or 2
Yeh et al. 2012	<i>In vivo</i> and <i>in vitro</i>	Nonimmunosuppressed (NIS) Wistar rats	Male	8 weeks	2	-	Primary periosteal fibroblasts	3	12	3-4
Yokota et al. 2014	<i>In vivo</i> and <i>in vitro</i>	Apert Syndrome mice	-	-	2	Untreated	Cos-7 cells, MC3T3-E1 & MC3T3-Ap cells, osteoblast	3	2	-
Morita et al. 2014	<i>In vivo</i>	Fgfr21/Neo-S252W, sFGFR2IIIc S252W, and EIIa-Cre mice	Male/female	Neonatal	-	Untreated	-	-	-	-
Zhang et al., 2015	<i>In vivo</i> and <i>in vitro</i>	Fgfr2 ^{S252W} mice	-	8 weeks	2	Untreated	BMSCs	3	2	-
Pfaff et al. 2016	<i>In vivo</i> and <i>in vitro</i>	Wild-type mice (WT), Fgfr2c-null mice & Crouzon-like mice	-	12 weeks	3	Untreated	Osteoblasts	3	2	-

Xu et al. 2017	<i>In vivo</i>	CMVCreER T2;Fgfr2 ^{+/P25} 3R-neo mice	-	2 months	4	Untreated	-	-	-	-
Shin et al. 2018	<i>In vivo and in vitro</i>	Fgfr2S252W /+ and Pin1- deficient mice	-	Newborn mice	5	Untreated	Primary mouse calvarial cells	3	-	1
Luo et al. 2018	<i>In vivo and in vitro</i>	Fgfr2+/P253 R-neo mice	-	-	3	Untreated	Primary calvarial cells	-	-	-
Kim et al. 2020	<i>In vivo</i>	Fgfr2neo252 W/+; Pin1+/- mouse	-	Newborn mice	3	Untreated	-	-	-	-

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579 **Table2. Interventions and protocols used in included studies**

Study	Method of mutation		Route of administration	Treatment type	Duration of treatment	Measurements	
	Genetic intervention	Induction				Protein	Gene
Mansukhani et al., 2000	FGFR2 cDNA	-	Culture media	FGF1 expression	3 weeks	FGF	-
Greenwald et al. 2001	-	Increased FGF biological activity	1- Culture media. 2- Injected into the region of PF dura	FGF-2alteration (simulation)	Postnatal day 30	FGF1, ERK-1 and FGF-2	TGF- β 1 and Col1A1
Warren et al. 2003	FGF-R1 gene	-	Injected with adenovirus	ADGFP and AdCasfgf2 noggin misexpression	30 days	BMP4 and FGF2	-
Tanimoto et al. 2004	germ line mutation of S252W in the FGFR2 gene	-	Culture media	Cells transfected FGF2 with S252W mutation	2 weeks	FGFR2	Runx2 and Opn
McDowel et al. 2006	FGFR2 (S252W) Mutation	-	-Culture media	FGF2, 7 or 9	40 hours	FGFR2	-
Shukla et al. 2007	Fgfr2Neo-S252W/+EIIa-Cre/+U6shRNA /+ mice	-	Crossing between male and female to generate mutated mice	U0126 (inhibition FGFR2)	Pregnant (p10-p18)	ERK1/2	FGFR2
Yin et al. 2008	Microinjected into blastocysts	-	Microinjected of blastocysts and implanted into the uterus of pseudopregnant mice.	PD98059 (Erk1/2 inhibitor)	9 days	Erk1/2	FGFR2, CBFa1, Opn and osteocalcin
Shen et al. 2009	FGFR2 mutant human osteoblasts	-	Surgical approach	rhNoggin protein	11 days	Alkaline Phosphatase sialoprotein, GP130, LIF receptor, LIF, and IGF I expression	FGFR1 and 2
Miraoui et al. 2010	S252W FGFR2mutant osteoblasts	-	-	1-PKCa inhibitor 2-PDGFR inhibitor	24 hours	ERK	EGFR, PDGFRa, Runx2, ALP, Col1A1 and osteocalcin
Wheldon et al. 2011	Protein was transfected to cells	-	Protein was extracted from transfected HEK-293T cells	FGF2 inhibition	5–7 hours	FGFR2	FGFR1 and 2
Suzuki et al 2012	FGFR2IIIc-S252W; FGFR2IIIc-Ap		1-Clonning 2-Subcutaneously	1-U0126 2-U73122(PLCg inhibitor) 3-SB203580(p38 inhibitor)	1- 3 hrs (<i>in vitro</i>) 2- 3 weeks (<i>in vivo</i>)	ERK, MEK, PLCg, p38, Shc, JNK, and Akt,	
Yeh et al 2012	Cells from AS patients	-	-	JNK inhibitor SP600125	4 weeks and 21 days	ALP and JNK	-
Yokota et al 2014	Fgfr2+/S252W mice	-	Crossing between male and female	Delivery FGFR2with S252W Mutation	3 weeks	Fgf10, Esrp1, ERK,	Runx2, Opn and FGFR2

			to generate mutated mice			MEK, SAPK/JNK, p38	
Morita et al. 2014	EIIa-Cre sFGFR2IIIcS25 W mice	-	Crossing between male and female to generate mutated mice	Delivery FGFR2 With S252W	-	sFGFR2IIIc S252W protein	-
Zhang et al., 2015	Heterozygous mutant	-	Crossing between male and female to generate mutated mice	Wnt3a	21 days	ALP, SRRP1, SFRP2, and SFRP4	PPARc, LPL, SRRP1, 2 & 4, osteocalcin, Opn, Runx2 and osteorix
Pfaff et al. 2016	Heterozygous mutant	-	Crossing between male and female to generate mutated mice	U1026	3 days	ERK ALP	
Xu et al. 2017	FGFR2+/P253R neo mice were crossed with CMV-CreERT2 transgenic mice	-	Bred FGFR2+/P253R neo mice with CMV-CreERT2 transgenic mice	Tamoxifen	One month	P-ERK1/2 MAPK	Runx2, OPN, ALP, Col1A1, and Osteocalcin, Rankl and Opg
Shin et al. 2018	Pin1+/-FGFR2 ^{2Neo-S252W/+} mice with Pin1+/-EIIa-Cre/+ mice	-	Mating Pin1+/-FGFR2Neo-S252W/+ mice with Pin1+/-EIIa-Cre/+ mice	Juglone (5-hydroxy-1,4 naphthoquinone; 420120, Calbiochem)	Pregnant mice	RUNX2,	Dusp6, Spry2, Spry3, CyclinD1, Cdk2, Cdk4 and PCNA
Luo et al. 2018	Transfected 11 siRNAs into primary osteoblasts from Apert mice	-	Culture	Adeno-Associated Virus-Mediated RNAi (S2)	7 days	FGFR2, ERK1/2 and P38	FGFR2, mutant Fgfr2, Runx2, Col1A1, Osteocalcin, and Opn
Kim et al. 2020	Blocks expression of the mutant FGFR2 allele and bred with the Pin1+/- mouse	-	Bred mutant FGFR2 allele and the Pin1+/- mouse	Pharmacologic inhibitor, juglone (5-hydroxy-1,4 naphthoquinone; 420120, Calbiochem)	Pregnant mice	Collagen type I and II and collagen type X	-

FGFR2 cDNA; fibroblast growth factor receptor 2 complementary DNA, FGF; fibroblast growth factor, ERK; Extracellular Receptor Kinase, TGF- β 1; Transforming growth factor beta 1, Adgfp; adenovirus type 5 expresses enhanced green fluorescent protein, AdCAsfgf2; a replication-defective adenovirus (AdCAsFGF-2) coding for human basic fibroblast growth factor (FGF)-2, BMP, Bone morphogenetic protein 4, Runx2; Runt-related transcription factor-2, Opn; Osteopontin, CBFa 1; core binding factor 1, U0126; 1,4-diamino-2,3-dicyano-1,4-bis (2-aminophenylthio) butadiene (selective inhibitor of MEK1 and MEK2 kinases), PD98059; a potent and selective inhibitor of MAP kinase kinases, LIF; Leukemia Inhibitory Factor, GP130; founding member of the “IL-6/IL-12”, PDGFRA; platelet-derived growth factor receptor A, RUNX2; runt-related transcription factor 2, ALP; alkaline phosphatase, COL1A1; Collagen, type I, alpha 1, SB203580; pyridinyl imidazole inhibitor, HEK-293T; human embryonic kidney 293, PLCg; phospholipase Cg, MEK; Mitogen-activated protein kinase, JNK; c-Jun N-terminal kinase, AKT; protein kinase B, Shc; a docking protein involved in the coupling of several receptors, SAPK; stress-activated protein kinase, ESRP1; Epithelial Splicing Regulatory Protein 1, P38; mitogen-activated protein kinases, SFRP; Secreted Frizzled-Related Protein, PPARc; Peroxisome proliferator-activated receptor c, LPL; Lipoprotein lipase, receptor activator of nuclear factor kappa-B ligand; Rankl, osteoprotegerin; Opg, P253R; Substitution - Missense, position 253, P $\rightarrow$ R, CMV-CreERT2; Cytomegalovirus-Tamoxifen inducible Cre; OPN; Osteopontin, COL1A1; Collagen Type I Alpha 1 Chain,

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584 **Table 3. The outcomes of included studies**

Study	Clinical/ Morphological evaluation	Histological	Radiological	Primary Outcome		Secondary Outcome
				Protein expression	Gene expression	Suture closure
Mansukhani et al. 2000	-	Apoptosis, and inhibition the expression of ALP in treated group	-	Grb2/FRS2/Shp2 complex formation, activation of MAP kinase, Increased Bax, decreased Bcl2	-	Inhibition the suture fusion
Greenwald et al. 2001	Overgrowth calvarial plates, fused suture in mutant animal	High-level expression of LacZ transgene in treated mice	-	Over expression of FGFR1, ERK-1 and FGFR-2	Upregulation Collagen I and TGF-b1 expression	
Warren et al. 2003	Increase distance between eyes, increased frontal bone growth in mutant animal	Induced noggin expression in the treated mice	-	Overexpression of BMP4 and FGFR2	-	Inhibition the suture fusion
Tanimoto et al. 2004	-	-	-	Overexpression of FGFR2	Increased expression of RUNX2 and Opn	Inhibitory activity for osteoblastic differentiation
McDowel et al. 2006	-	-	-	2-O- and N-sulfated heparan sulfate, antagonized the over-activated FGFR2b	Increased ligand-dependent FGFR signaling	
Shukla et al. 2007	Dome-shaped skull, widely spaced eyes, premature CS closure and underdeveloped midface in mutant mice	Growing fronts of frontal and parietal bones in apert mice	-	Inhibition phosphorylation and activation of ERK	Change FGFR2 with the S252W substitution	
Yin et al. 2008	Dome shaped skulls and smaller body size in mutant mice	Osteopontin expression in the mutant coronal sutures, decreased in mesenchymal cells, secondary ossification centres in mutant mice	Premature closure of sutures and significant shortened anterior–posterior axis	Increased FGFR2 expression	Increased cbfa1, Opn and OC in mutant	Potential application of Erk1/2 modulator in the treatment of craniosynostosis syndrome.
Shen et al. 2009	-	Lack of bridging, synostosis seen in treated animal.	-	Increased bone sialoprotein, GP130, LIF receptor, LIF, and IGF I expression.	Noggin can counteract the effect of constitutive FGFR2 signaling	New bone formation

Miraoui et al. 2010	-	-	-	EGFR inhibitor reduced 40–60% Runx2, ALP and type I collagen and oc.	Pharmacological inhibition of FGFR	Reduces osteoblastic dysfunction in Apert syndrome
Wheldon et al. 2011	-	Increased Lamp2 expression in treated cells	-	FGF2 induced ERK activation.	Loss-of-FGFR2 function	
Suzuki et al. 2012	-	-	-	Increased Runx2 and Opn expression. Activated ERK, MEK, and p38		Change the phenotypes in AS
Yeh et al. 2012	-	Mutation increased osteogenic	-	Inhibition of JNK, ALP activity treated model		Osteogenic differentiation was reversed through inhibition of JNK.
Yokota et al. 2014	-	Synostosis of the CS	-	- FGF2 stimulated Erk1/2, MEK, SAPK/JNK, and p38 - U0126 inhibited Erk1/2, MEK, p38, and Akt. - SB203580 inhibited p38 and Akt	Increased Runx2, Opn and FGFR2 expressions	Appropriate delivery of purified sFGFR2IIIcS252W could be effective for treating AS.
Morita et al. 2014	-	Ectopic bone formation and greatly thickened calvarial cartilage in AS mice	Premature closure of suture	Increased sFGFR2IIIcS252W protein	-	Prevent AS-like phenotypes <i>in vivo</i>
Zhang et al., 2015	-	-	Reduced trabecular and cortical bone	- Decreased expression levels of Wnt3a, PPARc and LPL, but increased ALP. - The levels of OC, OP, Runx2 and osteorix Col1 were lower on day 4, but got higher on days after.	- Upregulated PPAR, SRRP1, 2 & 4, downregulated of LPL. OC, , Runx2 and osteorix - The levels of OC, OP, Runx2 and osteorix mRNA expression in markedly lower on days 4, 7, and 14, but markedly higher on day 21	Mutant BMSCs and osteoblasts differentiation can be ameliorated by Wnt3a treatment
Pfaff et al. 2016	Dome-shaped skulls, truded midface. ocular proptosis, dysmorphia, frontal height: skull height ratio in mutant mice	- Reduced cellular proliferation within the suture mesenchyme in treated mice. - Coronal sutures are completely fused in mutant mice	Premature closure of sutures	Decreased ERK and ALP activity	-	FGFR2c- mediated ERK– MAPK signaling as a key mediator of craniofacial growth and coronal suture development.
Xu et al. 2017	No significant difference in the body size and	-Structure of growth plates in treated mice	Increased trabecular bone	- increased p-ERK1/2 MAPK	Increased the expression of Runx2, Opn,	Normal closure of sutures

	skull shape between treated and untreated mice	resembled that in normal mice - significantly increased the number of osteoblasts in mutant - The peak osteoclast surface was significantly lower in mutant mice			ALP, Col1A1, Osteocalcin and Rankl/Opg ratio	
Shin et al. 2018	-	-Normal closure of coronal sutures -Decreased RUNX2 in treated mice	-Longer skull and nasal region - Rescued of early fusion of sutures	Reduced RUNX2	-Inhibited the downstream Dusp6, Spry2 -Decreased the expression of CyclinD1, Cdk2, Cdk4 and PCNA	Normal closure of coronal sutures
Luo et al. 2018	-	Decreased overlapping region between parietal bone and frontal bone in Apert calvarias after treatment	Decreased bone volume of Apert calvarias	Downregulated the protein levels of FGFR2, the phosphorylated ERK1/2 and P38	-Decreased FGFR2 and mutant FGFR2 expression Decreased the expressions of Runx2, Collagen 1, Osteocalcin, and Osteopontin	Decrease sutures fusion
Kim et al. 2020	Improved the midface Shortening and Cranial Base Flexion in treated mice	- Proliferating cell nuclear antigen-positive cells -Expression of collagen type II alpha 1 and type X in mutant mice	Complete or partial fusion of premaxillary suture, patent or widened interpremaxillary suture, and partial fusion of maxilla and palatal bone	Expression of collagen type II alpha 1 and type X	-	Inhibition the sutures fusion

ALP: Alkaline phosphatase; Grb2: Growth Factor Receptor Bound Protein 2; FRS: Fibroblast Growth Factor Receptor Substrate; ERK: Extracellular Receptor Kinase; TGF- β 1; Transforming growth factor beta 1; BMP: Bone morphogenetic protein; Opn: osteopontin; OC: osteocalcin; GP: Glycoprotein; LIF: Leukemia inhibitory factor; IGF: Insulin-like growth factor 1; AP: Apert syndrome; Runx2, Runt-related transcription factor 2; Col1A1, Collagen Type I Alpha 1 Chain; Cdk, Cyclin dependent kinase; PCNA, Proliferating cell nuclear antigen;

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