Developmental Signaling Disorders in Craniofacial Anomalies and Cancers

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Abstract: Normal human development requires the precise functioning and coordination of many complex pathways. Abnormalities in these signaling cascades often result in developmental perturbations, giving rise to congenital anomalies and cancers. There are 21,787 genes in each human nucleus, different gene subsets are expressed in different cell types, and different gene networks make different signal cascades. Among a large number of genes, in this review, we describe signaling disorders of sonic hedgehog and its receptor, patched-1; Tie2; fibroblast growth factor receptor in craniofacial anomalies and oral cancers.

Key words: Sonic Hedgehog, Patched-1, Tie2, Fibroblast growth factor receptor, Gene, Developmental signal, Craniofacial anomaly

Sonic Hedgehog-Patched-1 signaling in craniofacial anomalies and cancers

Sonic hedgehog (SHH) is a mammalian homolog of Drosophila hedgehog (hh), which leads to the activation and repression of target genes, and sets off a chain of events in target cells¹. SHH has a role in establishing left-right body determination, central nervous system development, somite patterning, eye development and limb patterning. The receptor for SHH, Patched-1 (PTCH1), is a human homolog of patched Drosophila segment polarity gene², and it appears to have a tumor suppressor role, at least in some malignancies for which the syndrome predisposes, as has been most convincingly demonstrated for basocellular carcinomas. Its protein product, located in the plasma membrane, is an element of the Shh-PTCH1 signaling pathway. PTCH1 inhibits the seven-transmembrane signaling protein smoothened (SMO). Upon binding of SHH ligand to PTCH1, the PTCH1 inhibition of SMO is relieved, and the transcription factor Gli1 activates target genes, including WNT, BMP, GLI1, Patched2, HIP and Patched-1 itself, resulting in a negative feedback loop⁴. Dysregulation of the SHH-PTCH1 pathway leads to several human diseases, including birth defects and cancers. Loss of SHH signaling in the chick embryonic face causes de-
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Effects similar to the cleft lip/palate and hypotelorism in humans\(^5\), and \(PTCH1\) mutation has been reported to be responsible for nevoid basal cell carcinoma syndrome (NBCCS)/Gorlin syndrome\(^3\). NBCCS is an autosomal dominant disorder, which is characterized by predisposition to basal cell carcinomas and several other tumors, including ovarian fibroma and medulloblastoma. Developmental defects are another prominent feature of the syndrome, including pits of the palms and soles, jaw keratocysts and other dental malformations, midline brain malformations, strabismus, spine and rib abnormalities, ectopic calcifications, and macrocephaly with a characteristic coarse face and generalized over-growth\(^6\). However, the syndrome is particularly noted for its extensive interfamilial as well as intrafamilial variability with respect to the manifestation and severity of the phenotype. The criteria for the diagnosis of NBCCS include presence of at least two major features of the syndrome. Many \(PTCH1\) mutations in Caucasian and African-American NBCCS patients have been reported, however, among the Japanese population, there have been few reports.

Here we describe a novel \(SHH\) missense mutation in a 4-month-old baby with median cleft lip/mandible, and \(PTCH1\) mutations in Japanese NBCCS.

1. \(SHH\) mutation in oral clefts

The boy was born at 40-weeks gestation to healthy non-consanguineous parents. There was no family history of dental or craniofacial anomalies. At birth, agenesis in the mandibular inferior border was recognized (Fig. 1A). The cleft existed in the center of the lower lip, and continued to the gum. The mandibular alveolus part was missing, and the splinter of bone on either side was movable. X-Ray and CT view showed that the mandibular defect was in the central part. Moreover, the boy suffered from congestive heart failure, tetralogy of Fallot (TOF), hepatomegaly, pulmonary hypertension, and valvular disease of the heart.

Genomic DNA was prepared from peripheral blood leukocytes. Each of the 3 exons comprising the \(SHH\) gene was separately amplified. PCR products were analyzed by using PCR-SSCP and direct sequence analysis. PCR-SSCP of exon 1 showed a band shift in patient sample (Fig. 1B). Sequencing analysis revealed that the patient has a 279 C > T mutation in the human \(SHH\) coding region resulting in a Val for Ala substitution (A43V).

Multiple examples of cosegregation with heterozygous \(SHH\) gene deletions or truncations have demonstrated haploinsufficiency of \(SHH\) gene function in holoprosencephaly (HPE). HPE is a complex developmental field defect of the forebrain in which the cerebral hemispheres fail to split into distinct halves. Associated craniofacial anomalies can vary widely, including midline cleft palate, cyclopia, proboscis-like nasal structure and premaxillary agenesis\(^7\). Cleft lip/palate is a typical malformation in the craniofacial region. The frequency of cleft lip with or without cleft palate in Japan is 1 per 500 births. The factors underlying cleft lip/palate are considered to be mutant genes, chromosomal aberrations, environmental teratogens and multifactorial inheritance\(^8\). It has been reported that over 50 syndromes include cleft lip/palate as one manifestation, and gene mutations were identified in 60% of them. Is \(SHH\) a candidate gene for oral clefts? This is a question at issue. Muenke et al. and Odent et al. published that 16% of oral clefts without HPE had \(SHH\) mutations\(^9\).

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Fig. 1 Craniofacial phenotype of a median cleft lip/mandibular patient and PCR-SSCP of sonic hedgehog gene.  
(A): Craniofacial phenotype of the patient. (B): The patient was heterozygous for a PCR-SSCP variant in exon 1 that was not present in the normal control.
Ieda et al. analyzed 220 South American newborns with nonsyndromic cleft lip/palate for \textit{SHH} mutation, however, they concluded that mutations in the coding region and in the exon-intron boundaries of \textit{SHH} are not common in patients with oral clefts. Among oral clefts, median clefts are 0.43–0.73\%, and median cleft mandible is rather rare: there are less than 10 cases in Japan. The case we experienced is very rare; the patient not only has oral clefts but also suffers from circulatory and digestive systemic diseases, such as congestive heart failure, tetralogy of Fallot (TOF), hepatomegalia and pulmonary hypertension. We wonder whether this is a rare syndrome which has not been identified yet. Garavelli’s research group reported a patient with solitary median maxillary central incisor syndrome (SMMCI), who has V332A substitution in \textit{SHH}. SMMCI is a rare dental anomaly which has been reported in cases of HPE with autosomal dominant inheritance and SMMCI syndrome. Interestingly, the \textit{SHH} mutation in our case is A43V, and further studies will be required to determine the role of Ala$\rightarrow$Val substitution in causing oral clefts. In spite of a large amount of literature on the etiology of cleft lip/palate, knowledge of the subject is still insufficient and confused. To date, no report about \textit{SHH} mutation in cleft mandible has been published, and our result indicates that \textit{SHH} might a candidate gene for oral clefts.

2. \textit{Patched-1 (PTCH1)} mutations in nevoid basal cell carcinoma (NBCCS)/Gorlin syndrome

We examined a Japanese NBCCS family (five NBCCS patients and three healthy family members) (Fig. 2) and five NBCCS individuals. Clinical features of familial NBCCS patients are summarized in Table 1. We screened mutations in all PTCH1 exons (except exon 1) and introns. PTCH1 mutations were identified in 7 out of 10 Japanese NBCCS patients and one normal family member examined in this study. In the NBCCS family, three novel PTCH1 mutations were detected, including 3487insAA in exon 19, Gln 853 Lys in exon 15, and 3075 + 21 G$\rightarrow$A in intron 17. 3487insAA was detected in all patients (No. 1, 2, 4, 5, 7) in the family. On the other hand, Gln 853 Lys and 3075 + 21 G$\rightarrow$A were found in a normal family member (No. 8, Table 1). Immunohistochemical study revealed that epithelial cells in NBCCS jaw cysts showed high proliferation activity indicating high risk of recurrence. In fact, all familiar patients suffered local recurrence several times (data not shown).

In addition, one patient showed 2922insTG in exon 17 and a 1875C$\rightarrow$T in exon 12 (silent mutation), and another one showed 1892delC in exon 12. These two deletion/insertion mutations result in a frame shift and introduction of a premature termination codon. Patched-1 is predicted to contain 12 transmembrane domains, sterol sensing domain (SSD) ranging from the second to the sixth transmembrane domain, and two large extracellular loops. SSD is a very important region in patched-1, since (a) SSD has a role in vesicular trafficking of cholesterol and other lipids. (b) The C-terminal region is in close proximity to SSD and together with N-terminus forms a domain involved in SMO inhibition. Extracellular loops of patched-1 are confirmed to be \textit{SHH} binding sites, so mutations in the loops might result in ligand-independent constitutive activation. By reviewing the previously reported PTCH1 mutations in Japanese NBCCS patients, we found that the mutations were mainly concentrated in SSD and the predicted two large extracellular loops, especially the second loop. The two novel PTCH1 mutations we found, Glu853Lys and 2922insTG, were located in the second extracellular loop, 1875C$\rightarrow$T in SSD, and 3487insAA in C-terminus (Fig. 3). Our findings suggest that different domains in patched-1 protein are of special importance for NBCCS development, and PTCH1 mutations in different sites might be correlated with phenotypic variability in NBCCS.
Mutations in Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) play pivotal roles in regulating and coordinating aspects of cell growth, differentiation and apoptosis. Ligand binding to the extracellular domain of RTKs results in receptor dimerization and transphosphorylation of tyrosine residues in the intracellular domain. Tie2 and fibroblast growth factor receptor (FGFR) are typical RTKs. Tie2 is an endothelium-specific receptor tyrosine kinase, which collaborates with vascular endothelial growth factor (VEGF) in regulating angiogenesis and vascular maturation. Tie2 activity is normally regulated by the opposing actions of agonistic and antagonistic ligands. Angiopoietin-1 (Ang1) induces phosphorylation of Tie2 in cultured endothelial cells (ECs), whereas angiopoietin-2 (Ang2) competitively inhibits activation of Tie2 by preventing Ang1 stimulation. Mutational analysis of the Tie2 locus by gene targeting has demonstrated that stringent regulation of Tie2 phosphorylation is crucial to correct vascular assembly and recruitment of pericytes. Patients with inherited venous malformation have been found to have an activating mutation, R849W or Y897S, in the tyrosine kinase domain of the Tie2 gene. These malformed vessels are characterized by a disproportionately large number of ECs versus smooth muscle cells, suggesting that Tie2 plays a critical role in EC-pericyte communication during vascular morphogenesis.

The FGFR family, represented by four major transmembrane receptors, plays an important role in normal angiogenesis and embryonic development. Mutations in FGFRs could produce an altered balance of splice forms, change the balance of homo/hetero-dimers, reducing signaling by dominant negative effect or produce constitutive active dimers. Mutations in FGFR2 on 10q26 are found in Crouzon, Jackson-Weiss, Pfeiffer and Apert syndromes, while mutations in FGFR3 at 4q16 produce achondroplasia, thanatophoric dysplasia hypochondroplasia, Crouzon syndrome with acanthosis nigricans and Muencke's coronal craniosynostosis. Most normal tissues and tumors express multiple FGFRs, including splice variants of each. We have previously reported that all four FGFRs, including FGFR1c, FGFR2b, FGFR3b and FGFR4 were exclusively expressed in cells derived from normal oral epithelia and oral squamous cell carcinomas (OSCC). The growth of normal epithelial cells was stimulated by FGFs while OSCC cell proliferation was not. Mutations in FGFR genes have been reported in human bladder, cervical, gastric and colorectal carcinomas, however no such mutations have been reported in OSCC.

Here we describe Tie2 mutations in intramuscular haemangiomas (IMHs) and FGFR3b mutation in a series of human OSCC.

1. Tie2 signaling in intramuscular haemangiomas (IMHs)

Intramuscular haemangiomas (IMHs) are benign vascular tumors characterized by infiltrating growth in skeletal muscle. IMHs are divided into capillary, cav-
ernous, or mixed type depending on their gross and microscopic appearances. The capillary type is composed of a disordered proliferation of small vessels, whereas the cavernous type is composed of large, thin-walled, dilated vessels by flattened endothelial cells. More than 18% of patients suffered local recurrence after surgical ablation, but the precise pathogenic mechanisms of IMHs are unclear.

We identified two point mutations in the Tie2 kinase domain from 37 haemangioma paraffin-embedded sections: a G2646A change resulting in a glycine to aspartic acid substitution at residue 833 (G833D) in eight unrelated patients and another A2659T change resulting in a glutamine to histidine substitution at residue 837 (Q837H) in one patient. A relative of these patients did not have a history of IMH. All of the patients with the G833D mutation had a large disfiguring lesion affecting one side of the face, and associated early post-operative recurrence (Fig. 4A), whereas the patient with the Q837H mutation had no recurrence during postoperative follow-up after five years (Fig. 4B). Immunohistochemical analysis of G833D IMHs indicated that the expression of VEGF protein was up-regulated when compared to human normal muscular tissue. However, the expression of VEGF was unable to be detected in the ECs of Q837H tumor. Pathological evaluation revealed that the G833D IMHs were capillary type,
whereas the Q837H lesion was an IMH of the cavernous type.

Murine ECs overexpressing G833DTie2 exhibited an increase in cell proliferation at low serum concentrations, high level of VEGF expression and developed angiosarcomas in nude mice, whereas cells overexpressing either wild-type Tie2 or Q837HTie2 failed to elicit these responses. Furthermore, G833DTie2 showed a 2.9-fold increase in ligand-independent tyrosine autophosphorylation relative to wild-type Tie2, whereas Q837HTie2 showed a 20-fold increase relative to wild-type Tie2. Consistent with their ligand-independent phosphorylation, G833DTie2 and Q837HTie2 stimulated activation of STAT3, however, wild-type Tie2 did not exhibit phosphorylation of STAT3 without ligand stimulation (data not shown).

2. FGFR3b mutation in oral squamous cell carcinomas (OSCC)

A G to T mutation at nucleotide position 2128 in the human FGFR3b coding region resulting in a Cys for Gly substitution (G697C) in the tyrosine kinase domain was observed in 62% (44/71) of OSCC paraffin-embedded sections. Immunostained FGFR3b was found in the cytoplasm of prickle cells in normal epithelia, and FGFR3b was localized in the cytoplasm and nucleus in non-FGFR3b mutant OSCC. Overexpressed FGFR3b protein on plasma membranes was noted in OSCC bearing the FGFR3b mutation. The G697C amino acid substitution in the kinase domain of FGFR3 resulted in 23-fold increase in tyrosine kinase activity in comparison with the wild-type (data not shown).

We found three common features between Tie2 mutations and FGFR3 mutation. In the first one, the mutations are located in the second half of tyrosine kinase; in the second one, mutant residues are very important for the molecular structure and function. The G833D mutation in Tie2 occurs at the second glycine residue of the GXGXXG motif (residues 832–835) within the nucleotide-binding loop in the second half of tyrosine kinase, whereas Q837H affects the glutamine adjacent to the third glycine. Structural analysis suggests that these residues around G833, such as E832, N834, and F835, form a self-inhibitory conformation with the neighboring molecule as a safeguard against abnormal ATP binding. Incorporation of the much larger aspartic acid at residue 833 may destabilize the self-inhibitory conformation, thus locking it in the ATP-binding state. In the case of G697C FGFR3b in OSCC, glycine is substituted by cysteine. Because it lacks side chain, glycine is able to participate in extremes of bending and folding of the polypeptide backbone and it is the strongest breaker of α-helices. Glycine provides the flexibility necessary for anchoring the triphosphate moiety of ATP in the correct position for catalysis. Therefore, mutations at these glycines may directly affect the ATP or ADP binding and the hydrolysis of ATP. Cysteine is an important amino acid for maintaining biological activity of proteins since it affects protein conformation through the formation of intra- and inter-molecular disulfide bonds.

The third common feature is that the mutations result in constitutive activation in tyrosine kinase. Our results showed that G833D and Q837H mutations in Tie2 result in ligand-independent hyperphosphorylation of the receptor and promote the activity of STAT3. Tie2 kinase initiates signal transduction pathways downstream of Dok-R and rasGAP. rasGAP is a GTPase-activating protein and functions as a negative regulator of Ras signalling by stimulating the intrinsic rate of Ras GTPase activity. Because Ras signals have been implicated in VEGF expression, as well as in cell growth, differentiation, and tumorigenicity, it is likely that the enhancement of VEGF expression and tumorigenicity by G833DTie2 may be the result of inappropriate signalling through Ras in ECs. On the other hand, on the OSCC bearing the G2128T mutation, the over-expressed G697CFGFR3b protein is similarly localized to the cell surface indicating that the substitution of glycine with cysteine might cause ligand-independent dimerization of FGFR molecules, which results in alterations in receptor function including increased levels of membrane-localized FGFR3b and enhanced autophosphorylation activity.

Human IMHs are benign, nonmetastatic tumors. The high postoperative recurrence of IMHs has previously been attributed to incomplete excision. The data presented here, however, demonstrate that there are significant characteristics of the G833D mutation with respect to transforming activity and VEGF expression. These characteristics could reflect the clinical phenotype of early post-operative recurrence in the human G833D IMHs. Our results and subsequent elucidation of Tie2-mutant-mediated signalling properties may be useful in terms of diagnosis of and possible therapy for
various forms of IMH with Tie2 as a target. In addition, the apparent over-expression of G697C FGFR3b in 62% of the OSCC cases investigated strongly suggests that this particular mutation is involved in the progression of OSCC. FGFR3b may therefore be an important diagnostic and prognostic marker for OSSC and a molecular target for future therapies.

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References


