Developmental Signaling Disorders in Craniofacial Anomalies and Cancer

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1. Sonic Hedgehog-Patched in craniofacial anomalies and cancer

1) Sonic Hedgehog (SHH) in craniofacial anomalies

Multiple examples of cosegregation with heterozygous sonic hedgehog gene deletions or truncations have demonstrated haploinsufficiency of SHH gene function in horoprosencphary (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 cyclopia, proboscis-like nasal structure, midline cleft palate, and premaxillary agenesis.

We describe a patient with median cleft lip/madibular and identified a novel *SHH* missense mutation. Sequencing analysis revealed that the patient has a C to T mutation at nucleotide position 279 in the human *SHH* coding region resulting in a Val for Ala substitution (A43V) (Fig 1).

2) *Patched (PTCH)* mutation in nevoid basal cell carcinoma/Gorlin syndrome

Nevoid basal cell carcinoma syndrome (NBCCS)/Gorlin syndrome is a rare autosomal dominant disorder 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 and include pits of the palms and soles, jaw keratocysts and other dental malformations, midline brain malformations, strabismus,

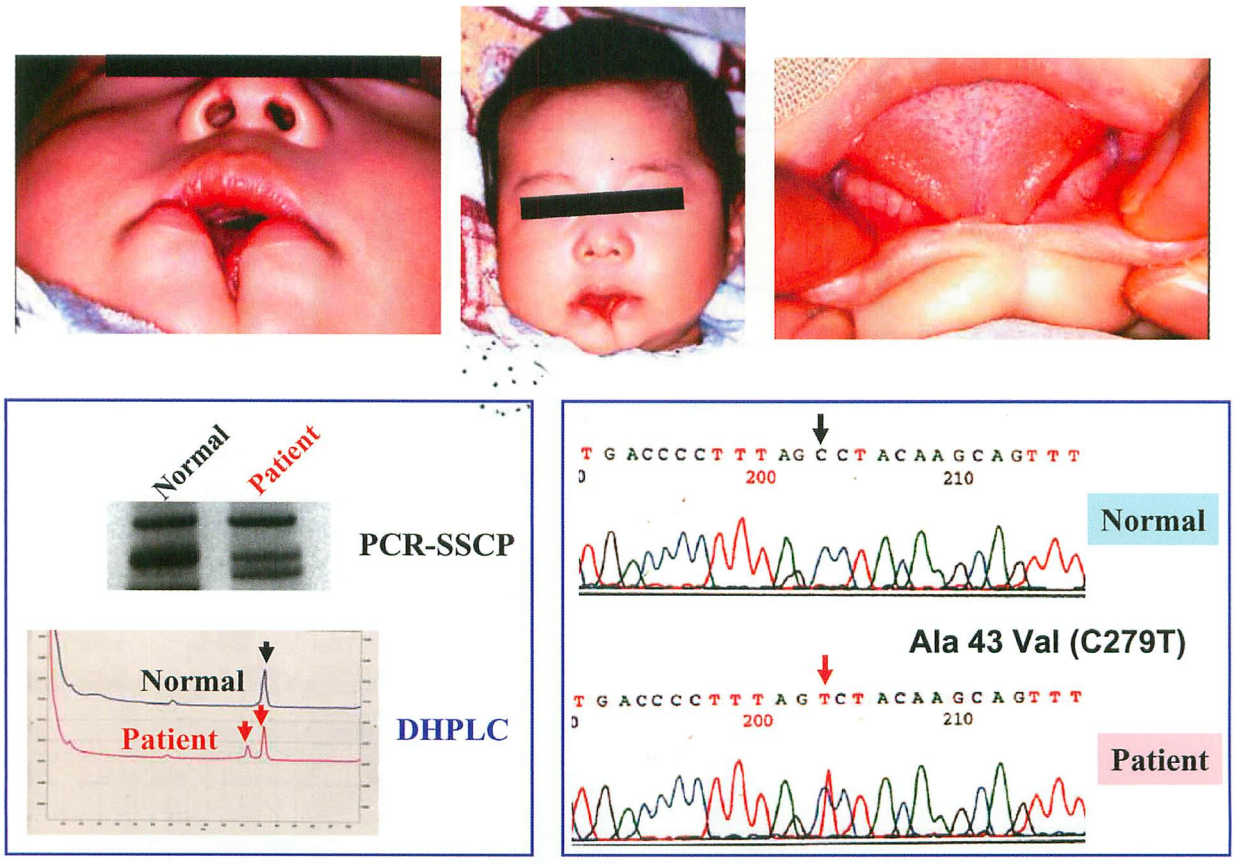

Fig. 1 Craniofacial and intra-oral phenotypes of the median cleft lip/madibular patient, PCR-SSCP, DHPLC and sequence analysis of *sonic hedgehog gene*.
Sequencing analysis revealed a C to T mutation at nucleotide position 279 in the human *SHH* coding region resulting in a Val for Ala substitution (A43V).

spine and rib abnormalities, ectopic calcifications, and macrocephaly with a characteristic coarse facies and generalized over growth. 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, such as multiple basal cell carcinomas, or onset of basocellular carcinomas before the age of 20, or pits of palms and soles, jaw cysts, calcification of falx cerebri, and having a first degree relative with NBCCS.

This heritable condition has been associated with constitutional hemizygous in activation of the *PTCH* gene, which is a human homologue of ptch, Drosophila segment polarity gene. The gene 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 a part of the Shh/ PTCH signalling pathway. It has a receptor role for the Shh ligand through close connection to smoothened (smo), another membrane protein component. PTCH suppresses smo's continuous signalling into cytoplasm by binding to it. That prevents *smo* from inducing expression of several downstream genes.

We examined one Japanese NBCCS family (Fig 2) for mutations in all *PTCH* exons and introns by PCR-SSCP analysis and direct sequencing of the PCR products. As a result, we identified 3 novel *PTCH* mutations in the family. Patients (number 2, 4, 7) exhibited the three muatation including 3487insAA in exon 19, Gln 853 Lys in exon 15, and 3075+21 G→A in intron 17. Patients (number 1, 5) posessed 3487insAA in exon 19, and 3075+21 G→A in intron 17. On the other hand, Gln 853 Lys in exon 15, and 3075+21 G→A in intron 17 has been found in normal family member (number 8). It has been suggested that 3487insAA in exon 19 resulting in protein truncation due to the insertion of a couple of adenine might be a responsible cause for this Gorlin syndrome family (Table 1).

3) PTCH mutation in squamous cell carcinoma (SCC) cells

In the present study, we have analyzed tumor deoxyribonucleic acid from oral squamous cell carcinoma (OSCC) cells for *PTCH* mutations using an exon-by-exon single strand conformation polymorphism assay and direct sequencing. We found two missense mutations which affected the conserved residue in the transmembrane domains of the gene product and in the intracellular loop at the C-terminal residue implicated in regulating the smoothened molecule (Table 2). In addition, we

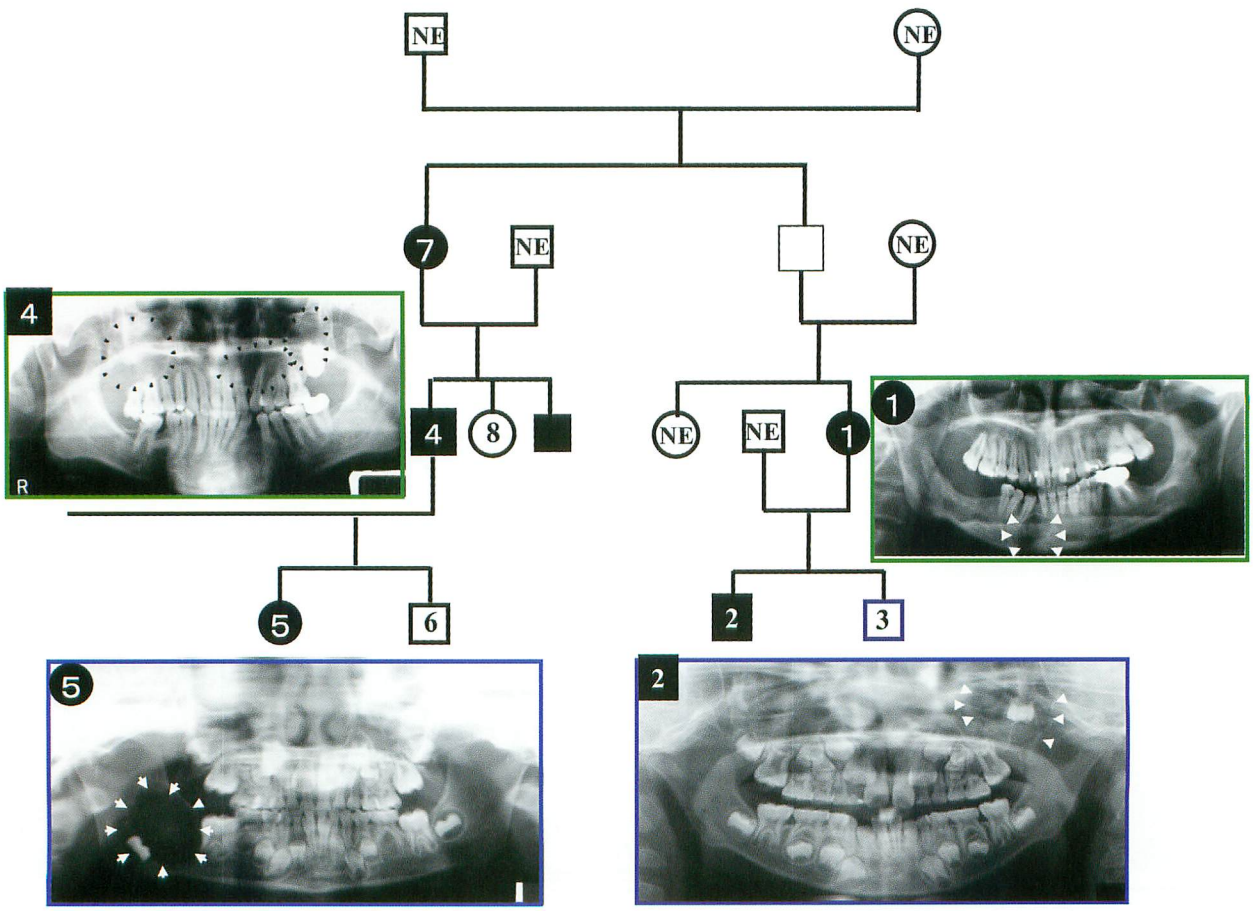

Fig. 2 Pedigree of a Gorlin syndrome family with panoramic roentogenograph exhibiting multiple jaw cysts. Number 1, 2, 4, 5, 7 were diagnosed as a Gorlin syndrome. Affected members are shown as closed symbols and nonaffected as opened symbols.

Table 1 Summary of the symptoms and *PTCH* mutations in a NBCCS/Gorlin syndrome family.

Patients	JC	P	HS	BS	CFC	AK	3487insAA (Exon 19)	Gln 853 Lys (Exon 15)	3075+21 G→A (Intron 17)
1 ●	+	+				+	+		+
2 ■	+	+					+	+	+
3 □									
4 ■	+	+	+	+	+	+	+	+	+
5 ●	+	+					+		+
6 □									
7 ●	+	+	+		+		+	+	+
8 ○								+	+

JC: Jaw Cysts
HS: Hyposeoliosis
CFC: Calcification of falx cerebri
P: Pits
BS : Bony bridging of the sella turcica
AK: Abnormal Keryotype

Table 2 Summary of PTCH mutations in SCC cells and their growth response to recombinant SHH.

Cell line	PTCH exon	Nucleotide change	Protein change	Codon	LOH	Growth Stimulation by SHH
A431	exon 23	T3944C	Leu to Pro	1315	No	No
KA	exon 12	T1682G	Met to Arg	561	No	No
KO	exon 12	T1682G	Met to Arg	561	No	No
NA		N.D	N.D			Yes
NI	exon 12	T1682G	Met to Arg	561	No	No
UE	exon 12	T1682G	Met to Arg	561	No	No

demonstrated that the N-terminal fragment of sonic hedgehog (Shh-N) stimulates the growth of normal epithelial cells, the OSCC cell line, NA, and the salivary gland adenocarcinoma cell lines, HSG and HSY, which have no detectable mutation in patched. On the other hand, Shh has no effect on human SCC cells (UE, KA, KO, NI, A431 cells) that have mutations in patched. These results strongly suggest that a Shh-patched signaling is involved in the cell growth of oral epithelial cells and in the tumorigenesis of OSCCs[1].

Furthermore, to evaluate the biological significance of patched mutations in human OSCC cells and A431 epidermoid carcinoma cells, we constructed a VSV-G pseudotyped retrovirus vector carrying the wild-type patched gene and transduced it into two human squamous cell carcinoma (SCC) cell lines, A431 and KA, that express only mutant patched mRNA. When SCC cells were transduced with Ptc virus, colony forming activity in soft agar was drastically reduced and these cells recovered anchorage independent growth when Sonic hedgehog (Shh), the ligand of Patched (Ptc), was added into the soft agar culture. Expression of exogenous patched, however, had no effect on anchorage independent growth of Ras-transformed NIH3T3 cells or SCC cell line, NA, which expresses wild-type patched mRNA (Table 3). Cyclopamine, a specific inhibitor of the Shh/Ptc/Smo signaling pathway, efficiently suppressed anchorage independent growth of A431 and KA cells. These results indicate that loss of patched function plays a major role in the acquisition of oncogenic potential in these SCCs and further that Ptc virus would be an effective reagent for suppressing tumorigenicity of such SCCs[2].

2. Angiopoietin-Tie-2 in haemangiomas

Human IMHs are benign, nonmetastatic tumours. The high postoperative recurrence of IMHs has previously been attributed to incomplete excision. This was based on their localization in deep muscle and their infiltrative growth, which make tumour resection difficult.

Table 3 Effects of PTCH virus-transfection on anchorage independent growth and growth in monolayer of SCC cells.

cells	colony formation in soft agar (%) *		growth in monolayer			
			doubling time (%) *		saturation density (%) *	
	*MOI 0.1	MOI 3	MOI 0.1	MOI 3	MOI 0.1	MOI 3
A431	35 ± 10	21 ± 3	105 ± 1	107 ± 2	95 ± 5	74 ± 8
KA	30 ± 5	25 ± 2	107 ± 3	114 ± 2	96 ± 20	88 ± 11
NA	97 ± 10	74 ± 13	101 ± 2	92 ± 2	104 ± 11	108 ± 20
NIH-ras	82 ± 7	97 ± 9	96 ± 3	110 ± 3	97 ± 19	105 ± 11

* percentage to those of control virus-transduced cells
* MOI (multiplicities of infection)

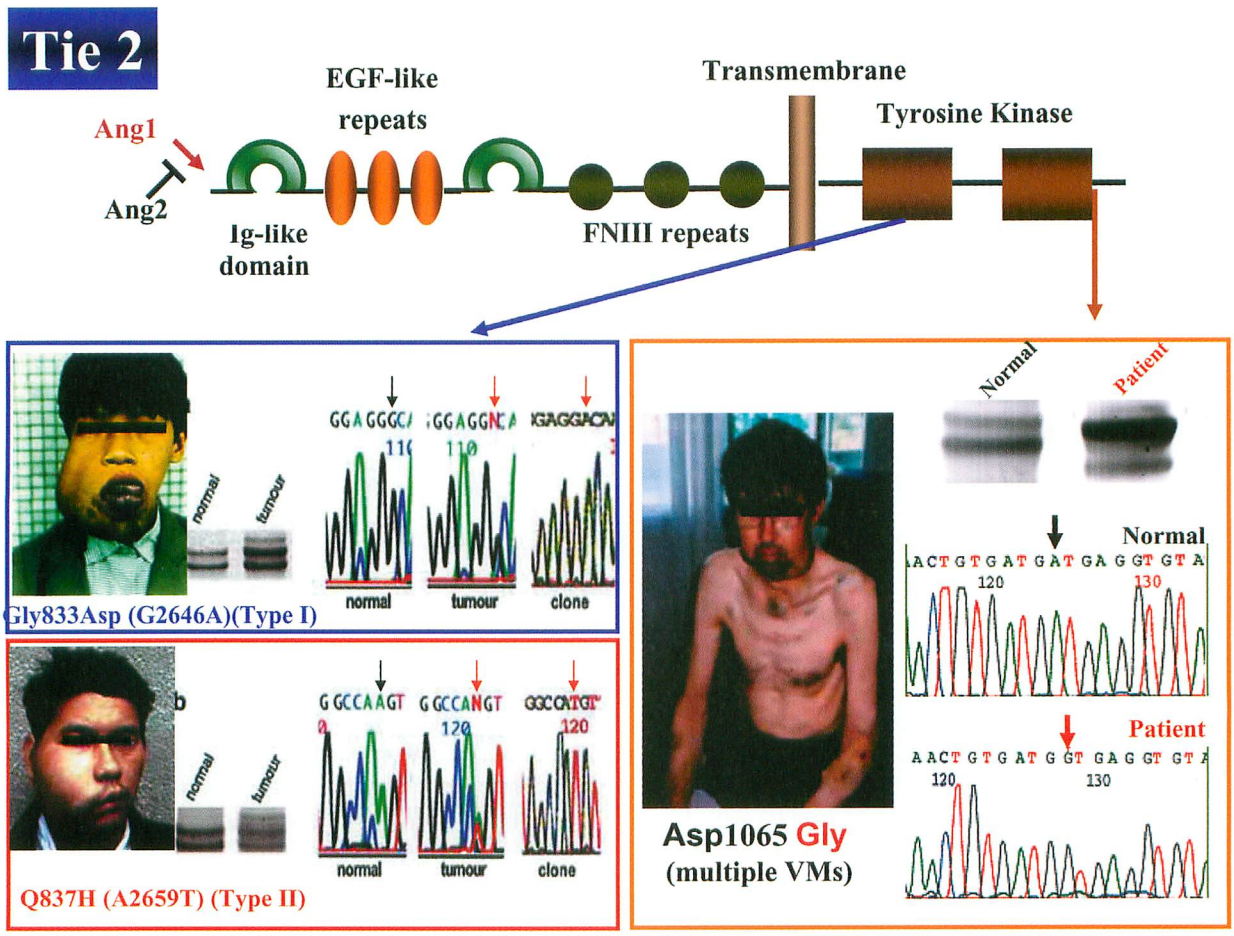

Fig. 3 Tie 2 mutations in human intramuscular haemangiomas.
The patients (left upper) with the G833D (G2646A) mutation had a large disfiguring lesion affecting one side of the face, and associated early post-operative recurrence, whereas the patient (left lower) with the Q837H (A2659T) mutation had no recurrence during postoperative follow-up after five years. The patient with multiple haemangiomas (right) has D1065G mutation in c-terminal tail of Tie 2.

Tie2, an endothelial-cell-specific receptor tyrosine kinase, collaborates with vascular endothelial growth factor (VEGF) in regulating angiogenesis and vascular maturation. Here, we report a mutation of glycine to aspartic acid at the second glycine of the GXGXXG motif of Tie2 (G833DTie2) in human intramuscular haemangiomas (IMHs) of the capillary type (Fig 3). Murine endothelial cells (ECs) overexpressing this G833DTie2 receptor

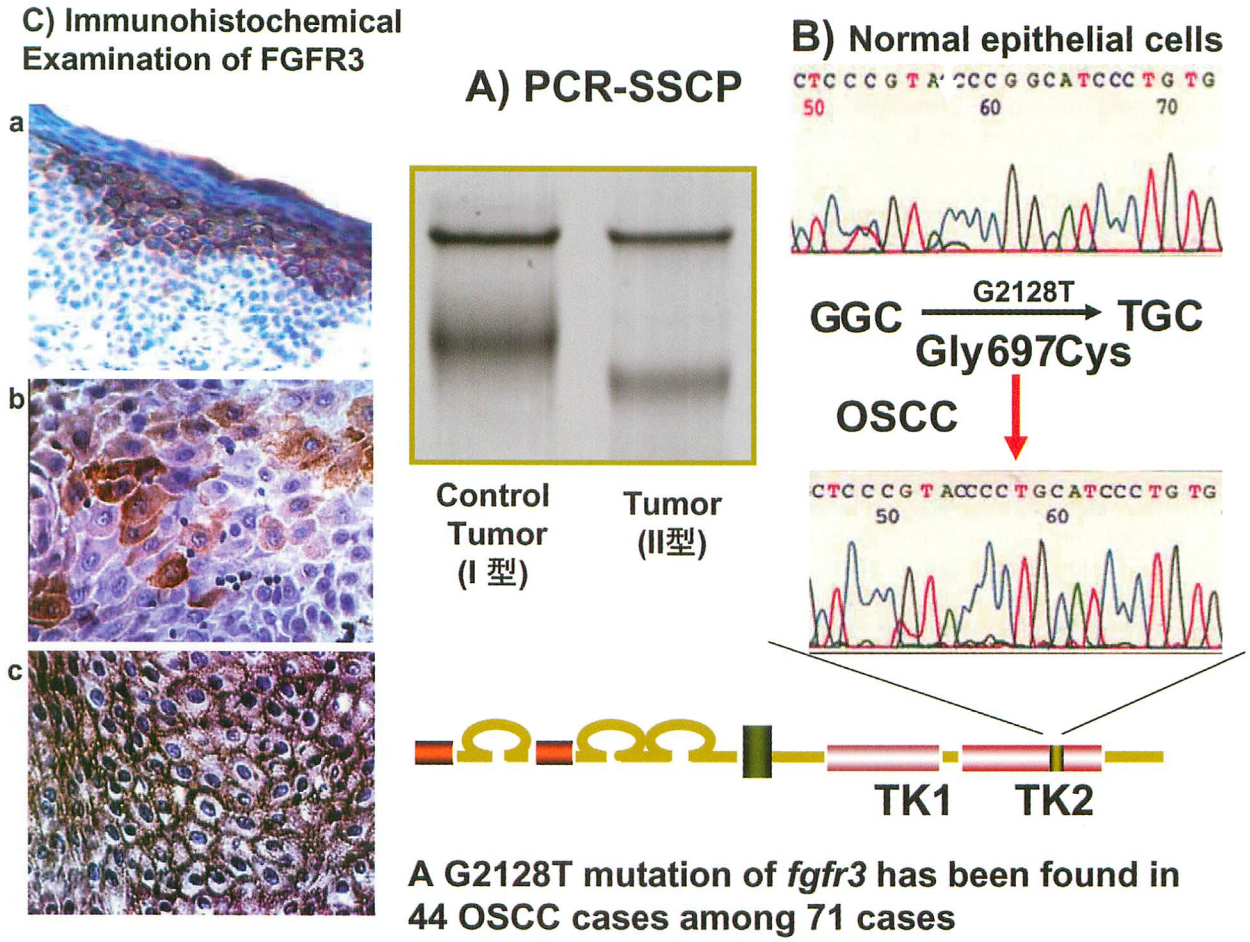

Fig. 4 Detection of mutation in exon 17 of FGFR3b gene and an immunostaining of FGFR3 protein in normal epithelia and OSCC tissues.
A) Representative PCR-SSCP analysis. PCR product from OSCC DNA (lane 2) was run alongside product from normal DNA (lane 1) isolated from non-neoplastic gingiva. *Arrow* indicates mobility shift band. B) Representative sequence analysis of exon 17 from OSCC specimen and normal control. Histogram of sequencing in the forward direction are shown. *Arrow* indicates sequence alteration. C) a, The expression of FGFR3 was observed in prickle cells in normal epithelia (X200). b, In OSCC having no G2128T mutation, the expression of FGFR3 was observed in cytoplasm and nucleus of cancer cells (X400). c, In OSCC having G2128T mutation, the strong expression of FGFR3 was seen in the cell membrane (X400).

exhibited an increase in cell proliferation at low serum concentrations and angiosarcomas developed in nude mice, whereas cells overexpressing either wild-type Tie2 or Q837H Tie2 failed to elicit these responses. Furthermore, the G833D Tie2 receptor increased VEGF expression in ECs. These characteristics could reflect clinical phenotype of early post-operative recurrence in the human G833D IMHs. These findings provide molecular mechanisms for pathogenesis of IMH. 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. They may also extend our understanding of receptor kinase function in vascular development. The data presented here, however, demonstrate that there are significant characteristics of the G833D mutation with respect to transforming activity and VEGF expression[3].

3. FGF-FGFR in Salivary gland Adenocarcinomas and Oral Squamous cell Carcinomas

1) Growth inhibition by keratinocyte growth factor receptor/FGFR2b of human salivary adenocarcinoma cells through induction of differentiation and apoptosis

We have previously reported that normal human salivary gland-derived epithelial cells exclusively express keratinocyte growth factor receptor (KGFR). In the process of malignant transformation of human salivary gland tumors, KGFR gene expression disappeared concomitantly with the de novo expression of the fibroblast growth factor receptor 1 (FGFR1) and FGFR4 genes.

In the present study, we introduced wild-type KGFR cDNA or chimeric KGFR/FGFR1 cDNA, which encoded the extracellular domain of KGFR and the intracellular domain of FGFR1, into the HSY human salivary adenocarcinoma cell line. The KGFR tyrosine kinase suppressed the activity of FGF receptor substrate 2 (FRS2)

and inhibited the growth of HSY by inducing differentiation and apoptosis in vitro and in vivo (data not shown)[4]. Our results provided significant insight into the mechanism of KGFR tumor suppression and suggest that KGFR gene therapy might be a viable method of inhibiting human salivary adenocarcinoma growth.

2) *FGFR3b* mutation in oral squamous cell carcinomas

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 oral squamous cell carcinomas (OSCC) examined. Immunostained FGFR3-IIIb was found in the cytoplasm of prickle cells in normal epithelia, and FGFR3b was localized in the cytoplasm and nucleus in non-FGFR3-IIIb mutant OSCC. Overexpressed FGFR3-IIIb protein on plasma membranes was noted in OSCC bearing the FGFR3-IIIb mutation. Enhanced tyrosine kinase activity of G697CFGFR3-IIIb was confirmed[5] (Fig 4). Our results indicate that G697C is an activating mutation causing constitutive ligand-independent FGFR3-IIIb signaling. This mutation may be involved in the progression of OSCC and thus the FGFR3b coding sequence may have diagnostic or prognostic value for OSCC.

ACKNOWLEDGEMENTS

The author thank Drs. Yan Zhang, Hua Wang, and Shigeaki Toratani for their contribution and collaboration to this work and also thank Dr. Kamegai at Kyoto Second Red-Cross Hospital, Dr. Hosoda at Kawasaki Medical Collage, and Dr. Iba at Tokyo University for their collaboration and helpful discussion, and Dr. Yasufumi Sato of Tohoku University for the murine EC line. This work was supported in part by grants from the Japan Society for the Promotion of Science, and by a grant from the Smoking Research Foundation to T. O.

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