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**PTCH1 mutation is a frequent event in oesophageal basaloid squamous cell carcinoma**

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Abstract

Basaloid squamous cell carcinoma (BSCC) is a rare and poorly differentiated variant of typical squamous cell carcinoma, and is characterised in part by activation of the Wnt signalling pathway. We previously demonstrated that constitutive activation of the Wnt signalling pathway by epigenetic silencing of secreted frizzled-related protein 4 (SFRP4) is observed in this tumour. Increasing evidence shows that the Wnt signalling pathway cross-talks with other developmental pathways, including the Hedgehog (HH) pathway. The HH pathway is stimulated by inactivating mutations of **PTCH1**, which have a well-described oncogenic role in basal cell carcinoma (BCC) of the skin. We employed polymerase chain reaction followed by direct sequencing to detect inactivating mutations of **PTCH1** using archival tissue samples of 30 oesophageal BSCCs. The frequency of **PTCH1** mutation was compared to that of **Wnt** component genes that we reported previously. We found **PTCH1** mutations in 53.3% (16/30) of cases, revealing T1195S as a hotspot mutation. This frequency is quite high for cancers other than BCC of the skin, and **PTCH1** mutations were almost mutually exclusive with mutations in **APC**, **Axin1** and **Axin2**. Considering the fact that activation of Wnt signalling via down-regulation of **APC** and **SFRP5** due to promoter methylation is observed in BCC of the skin, Wnt signalling activation in oesophageal BSCC might be a secondary effect of the **PTCH1**-inactivating mutations. These findings suggest that the HH and Wnt pathways coordinately contribute to tumourigenesis in oesophageal BSCC. Furthermore, this study provides a potential therapeutic application for HH pathway inhibitors in oesophageal BSCC with highly malignant potential.

Introduction

Basaloid squamous cell carcinoma (BSCC) is a rare and poorly differentiated variant of typical squamous cell carcinoma that is histologically characterised by solid nests with comedo-type necrosis reminiscent of basal cell carcinoma (BCC) of the skin (1). We reported previously that oesophageal BSCC is an invasive carcinoma characterised by poor survival and associated with nuclear accumulation of β-catenin (1) and that constitutive activation of the Wnt signalling pathway by epigenetic silencing of secreted frizzled-related protein 4 (SFRP4) is observed in these tumours (2). Therefore, activation of the Wnt signalling pathway plays an important role in oesophageal BSCC tumourigenesis and progression.

Like the Wnt signalling pathway, the Hedgehog (HH) pathway has well-characterised roles in the developing embryo, including establishment of cell position, body pattern segmentation, polarity and cell fate decisions (3). Driver mutations, which result in the activation of HH signalling, are observed in BCCs, medulloblastomas, and rhabdomyosarcomas associated with Gorlin syndrome (4–6). The most common driver mutations are observed in **PTCH1**, followed by the signal transducer **smoothened** (SMO). In addition, ligand-dependant activation of the HH pathway has been demonstrated in a variety of malignancies, including non-small cell lung cancer, breast cancer and prostate cancer (4, 7–11). Pharmaceutical
therapy targeting the HH pathway has also been used for BCC, some multiple myelomas, and chronic myeloid leukaemia (12, 13).

There is increasing evidence that these developmental pathways cross-talk or intersect with the Notch and bone morphogenic protein pathways (3, 14). The Wnt signalling pathway can also control the Gli3 in the HH pathway (15), and in turn, HH can antagonise Wnt signalling in the colon (16, 17). Cross-talk between the Wnt and HH pathways has also been demonstrated in a gastric cancer cell line (18). The effects of activating mutations of GNAS, the gene encoding the stimulatory G-protein $G_s$ subunit, may have another candidate pathway that integrates with the Wnt signalling pathway. GNAS is a ubiquitously expressed G-protein $G_{s}$-subunit that couples receptors to adenylyl cyclase and is required for receptor-stimulated cyclic adenosine monophosphate generation and protein kinase A (PKA) activation. GNAS-activating mutations have been reported to promote intestinal tumorigenesis in $Apc^{min}$ mice through activation of the Wnt and ERK1/2 mitogen-activated protein kinase (MAPK) pathways (19). In addition, GNAS-activating mutations might inhibit oncogenic sonic HH signalling through PKA activation (20).

Very recently, we reported that activation of the Wnt signalling pathway by epigenetic silencing of $SFRP4$ contributes to oesophageal BSCC tumourigenesis and progression, although mutations in $CTNNB1$, $APC$, $Axin1$ and $Axin2$ are only observed in 20% of cases (2). Because gene silencing due to epigenetic changes is reversible, it is possible that another strong oncogenic switch might exist in oesophageal BSCC. In this study, we searched for $PTCH1$ and GNAS mutations, which have not yet been detected in oesophageal BSCC. Although GNAS mutations were not observed, we found frequent inactivating $PTCH1$ mutations that activate the HH pathway, which has been shown to cross-talk with the Wnt signalling pathway.

Materials and methods

Patients and materials

Informed written consent was obtained from all patients. This study was approved by the Institutional Review Board and ethical committee of our hospital (registration #2011053). A total of 30 BSCC cases from patients treated between 2002 and 2012 were selected from the pathology records at Juntendo University (Tokyo, Japan). All subjects underwent radical surgery to treat oesophageal carcinoma at Juntendo University. In this study, BSCC patients with multiple carcinomas or metastatic disease, as well as those that had received preoperative chemotherapy or radiotherapy, were excluded. The clinicopathological characteristics of these patients were presented in our previous study (2).

Mutational analysis

Mutation analysis of exons 9, 10, 21 and 22 of $PTCH1$ and exons 8 and 9 of GNAS was performed by using polymerase chain reaction (PCR) followed by direct sequencing of genomic DNA derived from 30 formalin-fixed paraffin-embedded (FFPE) BSCC tissue samples (2). The corresponding non-tumour DNA was also extracted to check that the observed mutations were specific to the tumours. We selected these exons based on reports describing $PTCH1$, GNAS mutations in other types of cancers including basaloid carcinoma of skin (21, 22), and in part because the sequencing of the entire region of these genes would be difficult considering the quality of DNA extracted from FFPE. We also hypothesised that the similar histology between basaloid carcinoma of the skin and BSCC of oesophagus might reflect a similar pattern of the mutation spectrum in $PTCH1$. The PCR primer pairs for $PTCH1$ were as follows: Exon9F, 5′-GTGCTGTCCAGGCTTGTG-3′; Exon9R, 5′-AG AGCAGGACGATCATGG-3′; Exon10F, 5′-TCTGGCTTTTTGCG-3′; Exon10R, 5′-CCCCGTGCAATTGTTGCA-3′; Exon21F, 5′-GGAGTCCACCTGTGTCGAC-3′; Exon21R, 5′-AGAACCCACGAAGTGGA-3′; Exon22F1, 5′-GC TCGTGCCAGCGAGTA-3′; Exon22R1, 5′-GTATG GCCGAGCTCTGC-3′; Exon22R2, 5′-TCCGAGTATAGTTCCAGAAG-3′ and Exon22R3, 5′-CTCAGTACCTGATCAGCA-3′. The PCR products were sequenced with deoxyxynucleotides (BigDye Terminator v3.1; Applied Biosystems, Foster City, CA, USA) and specific primers, purified using a BigDye X Terminator Purification Kit (Applied Biosystems) and then analysed with a capillary sequencing machine (3730xl Genetic Analyzer; Applied Biosystems). The sequencing was performed in both directions to confirm the findings. Regarding the possibility of PCR artefact, we performed PCR independently twice for the cases in which a mutation was detected in the first PCR. We considered that when the direct sequencing of independently performed PCR products exhibited the same sequence abnormalities, the sample possessed a mutation.

Results

Mutational analysis

The results are summarised in Table 1 and Figure 1. A total of 20 point mutations that cause amino acid changes were detected in the $PTCH1$ gene, and $PTCH1$ mutations were found in 16 of 30 oesophageal BSCC cases (53.3%). Interestingly, most mutations (15/20) were located in exon 22. Two out of 20 point mutations detected in this study were non-sense mutations, and the others were missense mutations. The $PTCH1$ mutations detected here and the mutations in Wnt component genes ($\beta$-catenin, $APC$, $Axin1$ and $Axin2$), which we reported in our previous study (2), were nearly mutually exclusive, and only two cases harbour one of $PTCH1$ and Wnt component mutations (case 3 and 5) (2). By the comparison with DNA sequences derived from the paired non-tumour tissue, we found that the expression of $PTCH1$ in which the adjacent non-tumour tissue also contained the same mutation in the tumour tissue. All of these four cases harboured the same type of mutation at codon 1195 (T1195S), and we considered these to be germline mutations. Other mutations were confirmed to be tumour-specific mutations. Regarding the type of mutations, the most frequently observed mutations (T1195S) were transversion mutations, and other 12 were transition mutations.

There was no relationship between the presence of a $PTCH1$ mutation and histological ductal differentiation. Furthermore, the presence of a $PTCH1$ mutation did not affect patient prognosis (data not shown). GNAS mutation was not found in any case.

Discussion

Germline loss of function $PTCH1$ mutations in patients with Gorlin syndrome is an autosomal-dominant disease, and somatic mutations in $PTCH1$ have been identified in >90% of sporadic BCC cases (23) and in ~20% of medulloblastomas (24). Our findings for oesophageal BSCC were similar, and $PTCH1$ mutations were observed in 53.3% of cases. Interestingly, it has also been shown that epithelial Hh ligand expression is an early event in mammary carcinogenesis of basal-like breast cancer (BLBC) that is associated with poor outcome
in terms of metastasis and breast cancer-related death, and that ectopic expression of Hh ligand in a mouse model of BLBC led to the development of rapidly growing, high-grade, invasive tumours (11). Considering these findings, activation of the HH pathway might be associated with basal cell morphology and contribute to aggressive behaviour.

In this study, mutation at codon 1195 (T1195S) of PTCH1 seemed to be a potential mutational hotspot site. Mutation at this point has been shown in a French patient with Gorlin syndrome; however, a mutation hotspot was not observed in a series of Gorlin syndrome patients (25). On the other hand, it has been shown that PTCH1 harbours hotspot mutations in the N-terminal region, and that the types of mutations are unique for the different tumour types (26). We have detected T1195S mutations of PTCH1 in eight cases, and four of these were considered to be germline mutations. The relationship between PTCH1 mutation at this position and susceptibility for BSCC of oesophagus is unclear, although it is interesting to note that patients with Gorlin syndrome who harbour germline loss of function PTCH1 mutations have a high risk of BCC of skin, which shows similar histologic features to BSCC of oesophagus. The BSCC patients in this series do not have any history of other malignancies.

It is also interesting to note that mutations in PTCH1 and Wnt component genes were usually mutually exclusive in oesophageal BSCC, except in two cases. Cross-talk between the Wnt and HH pathways has been reported previously. In a gastric cancer cell line, Kim et al. (18) reported that ectopic expression of GLI1 decreased nuclear β-catenin staining, whereas inhibition of GLI1 reversed nuclear β-catenin overexpression, and that ectopic expression of β-catenin also decreased GLI1 expression (18). Furthermore, activation of the HH signalling pathway by PTCH1 mutation may lead to SFRP1 activation in oesophageal BSCC, as was shown in gastric cancer (18). However, as we showed in our previous study (2), in oesophageal BSCC, down-regulation of SFRP4, but not SFRP1, due to promoter methylation continuously activates the Wnt signalling pathway. Activation of Wnt signalling through down-regulation of APC and SFRP5 due to promoter methylation and activation of HH signalling by PTCH1-inactivating mutations has been described previously in BCC of the skin (27). In addition, it has been also shown that ligand-driven, canonical Wnt/β-catenin signalling is required for Hh pathway-driven tumorigenesis (28). The frequent inactivating PTCH1 mutations and Wnt signal activation in oesophageal BSCC suggests that the HH and Wnt pathways coordinately contribute to tumourigenesis. Furthermore, these findings suggest the potential of therapeutic HH pathway inhibitor application in oesophageal BSCC with highly malignant potential.

BSCC of the oesophagus shows diverse histological features, including cribriform pattern, hyaline-like material deposition, microcyt formation and ductal differentiation (1). As we observed in previous studies that ductal (glandular) differentiation in BSCC is related to better survival (1), and that it was also associated with membranous β-catenin expression accompanied by reduced nuclear β-catenin expression (2), we speculated that the mutual exclusivity of mutations in PTCH1 and Wnt component genes might have an impact on histological differentiation. However, there was no relationship between PTCH1 mutation status and histological glandular differentiation in oesophageal BSCC. Furthermore, PTCH1 mutation status did not affect prognosis in oesophageal BSCC. Although PTCH1 mutations were frequently observed in oesophageal BSCC, their impact on the biological behaviour of this cancer needs to be further elucidated.

Somatically acquired activating mutations of GNAS have been identified in a variety of tumours with benign to low-grade malignant

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<td></td>
<td></td>
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<tr>
<td>5</td>
<td>1195</td>
<td>ACA (Thr) to TCA (Ser)</td>
<td>APC and Axin1 mutations (−)</td>
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*Saito et al. (2).  
*Germline mutation.
**Figure 1.** PTCH1 mutations in oesophageal BSCC. (A): A G to A substitution is present at codon 470 in case 1, resulting in an amino acid change from Gly to Arg (left: tumour DNA, right: paired non-tumour DNA). (B): A C to T substitution is present at codon 1159 in case 1, resulting in an amino acid change from Leu to Phe (left: tumour DNA, right: paired non-tumour DNA). (C): A C to T substitution is present at codon 472 in case 3, resulting in an amino acid change from Ala to Val (left: tumour DNA, right: paired non-tumour DNA). (D): A G to A substitution is present at codon 1204 in case 9, resulting in an amino acid change from Val to Met (left: tumour DNA, right: paired non-tumour DNA). (E): An A to T substitution is present at codon 1195 in case 20, resulting in an amino acid change from Thr to Ser. This substitution cannot be seen in the corresponding paired non-tumour DNA (left: tumour DNA, right: paired non-tumour DNA). (F): An A to T substitution is present at codon 1195 in case 25, resulting in an amino acid change from Thr to Ser. This substitution can also be seen in the corresponding paired non-tumour DNA (left: tumour DNA, right: paired non-tumour DNA).
potential, including adrenal hyperplasia, ovarian cysts, thyroid carcinomas (5%), and adrenocortical, pituitary (22–40%), kidney (17%) and Leydig cell tumours (67%) (22, 29–33). We could not find GNAS-activating mutations in any of the BSCC cases in this study. Although GNAS-activating mutations have been reported to activate the Wnt signalling pathway, GNAS mutations seemed less likely to occur in oesophageal BSCC since this tumour is a highly aggressive neoplasm.

In conclusion, we report here for the first time the frequency of PTCH1 mutations (~53.3%) in oesophageal BSCC. This frequency is quite high for cancers other than BCC of the skin.

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References