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Original Paper

The Patched/Hedgehog/Smoothened Signalling Pathway in Human Breast Cancer: No Evidence for H133Y *SHH*, *PTCH* and *SMO* Mutations

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The patched/hedgehog/smoothened signalling pathway has been implicated in the development of sporadic tumours associated with the naevoid basal cell carcinoma (Gorlin) syndrome (NBCCS). Mutations in sporadic basal cell carcinomas (BCCs) of the skin and medulloblastomas have been found in genes encoding all three proteins of the pathway. A substantial proportion of breast carcinomas has recently been suggested to contain missense mutations in the human *patched* (*PTCH*) and *sonic hedgehog* (*SHH*) homologues. However, an independent study showed that the implicated mutation in *SHH* (H133Y) was absent in a large number of BCCs, medulloblastomas, breast, ovary and colorectal tumours. We searched for the H133Y *SHH* mutation in 84 primary breast carcinomas, but did not detect this change in any sample. In addition, a subset of 45 primary breast tumours was analysed for mutations in the *PTCH* coding region and 48 samples in previously implicated exons of human *smoothened*, but no mutations were found. Although our results do not exclude the presence of clonal alterations of these genes in a small proportion of breast carcinomas, these data do not support the existence of frequent mutations in genes encoding major protein partners of this signalling pathway. The absence of nucleotide changes in *PTCH* may point to another linked gene in the chromosome region 9q22-q23, previously suggested to contain a breast cancer susceptibility gene. © **1999 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

THE patched/hedgehog/smoothened signalling pathway has been shown to control the proliferation of epithelial cells in both *Drosophila* and vertebrates [1]. A human homologue (*PTCH*) of *patched* was found deficient in a cancer-prone dominant disorder known as the naevoid basal cell carcinoma (Gorlin) syndrome (NBCCS), which is characterised by multiple developmental defects and the predisposition to basal cell carcinomas (BCCs) of the skin [2–4]. The *PTCH* gene encodes a transmembrane protein acting as a receptor for the sonic hedgehog (SHH) signal in a receptor complex that involves another membrane protein smoothened (SMO) [5, 6]. Mutations in *PTCH* have been characterised in a number of NBCCS families [2–4] and have also been found in sporadic tumours associated with NBCCS such as BCCs [2, 7] and primitive neuroectodermal tumours [8–11]. In addition to mutations/deletions of the tumour suppressor gene *PTCH*, BCC-like structures have been found to develop in transgenic murine skin overexpressing SHH and BCCs have been reported to contain activating missense *SHH*/*SMO* mutations [12]. Two BCC samples have been found with mutations in both *PTCH* and *SMO* [13]. Missense mutations in signalling partners of PTCH have also been identified in

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primitive neuroectodermal tumours of the central nervous system [13, 14]. These studies suggest a crucial role for this signalling pathway in the development of NBCCS-associated sporadic malignancies.

Recently, a recurrent missense mutation 397C→T in *SHH* was reported in three tumour types [14]. This putative activating change results in the substitution of tyrosine 133 for histidine (H133Y). The authors identified this particular alteration in one of 43 BCCs, one of 14 medulloblastomas and in one of six breast carcinomas analysed. The mutation was neither detected in the germline in any of the 3 patients, nor was it found by screening blood DNA of 100 normal individuals. The observation seemed to be supported by a report of missense mutations in *PTCH* in two of seven analysed breast carcinomas and in other extracutaneous tumours [15]. These data, together with the finding that mice heterozygous for mutated *patched* alleles exhibit a high incidence of rhabdomyosarcomas, the most common soft-tissue sarcoma in children [16], suggested the involvement of the PTCH/SHH/SMO signalling pathway in more common sporadic malignancies, not apparently associated with NBCCS.

Here we analysed tumour DNA extracted from a large number of primary breast carcinomas for mutations in the whole coding sequence of *PTCH* and regions of *SHH* and *SMO* previously reported to contain putative activating mutations.

MATERIALS AND METHODS

Samples

Tumour DNA was extracted from breast cancer tissue by a technique described previously [17]. Tumour tissue for DNA extraction was identified by a histopathologist and was adjacent to that analysed microscopically. Samples with substantial stromal contamination were avoided. Forty-five DNA samples extracted from unselected breast cancer cases were analysed for mutations in 22 *PTCH* coding exons, an additional 39 cases were examined for the *SHH* H133Y mutation ($n = 84$) and 48 primary breast cancer cases for exons 2–6, 9 and 10 of the *SMO* gene.

Mutation analysis

All amplified segments were analysed for mutations using single-strand conformation polymorphism analysis (SSCP) as described previously [18]. This technique has been shown to detect mutations in sporadic BCCs [19], medulloblastomas [9] and in NBCCS patients [19]. The oligonucleotide primers used for SSCP analysis of *PTCH* were as described and can be found at our web site http://www.sics.se/ktm/projects/ptch. The primer pair used for the mutation assay of the H133Y mutation was as follows: 5′-CAG GGC TGG GAC GAA GAT GG-3′ (forward) and 5′-CTG CGG TCG CGG TCA GAC G-3′ (reverse). Oligonucleotide primers for amplifying segments from the human *SMO* gene have been previously reported [12]. The polymerase chain reaction (PCR) contained 100 ng DNA, 0.25 μM of each primer, 100 μM of each dNTP, 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.01% gelatin, 0.2 μCi [α-^{32}P]dCTP (3000 Ci/mmol) and 0.5 units of *Taq* polymerase (Perkin-Elmer) in a volume of 20 μl. Amplification was for 34 cycles at 94°C for 30 sec, annealing at 52–58°C for 45 sec and extension at 72°C for 30 sec in a Perkin-Elmer thermocycler (GeneAmp System 9600). The samples were mixed with 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol, 50 mM NaOH, denatured at 95°C for 10 min and loaded on to 0.4 mm/30 cm/45 cm 5% polyacrylamide/5% glycerol gels (cross-linking 2.5%). The electrophoresis was conducted at 2–5 W at room temperature overnight. The gels were dried and autoradiographed for 1–2 days at −70°C. Amplified segments exhibiting shifts on the SSCP gels were re-amplified together with the negative and positive controls, using both cut fragments exhibiting bandshifts and original DNA samples. DNA was sequenced in both directions using cycle sequencing with AmpliTaqFS (Perkin-Elmer) on the ABI 373A DNA sequencer.

RESULTS

Apart from nucleotide polymorphisms previously found in *PTCH* [2, 9, 18], mutation screening of this gene did not detect any nucleotide changes in any of the amplified segments. This result suggests that, as opposed to BCCs, medulloblastomas and the earlier report [14], primary sporadic breast tumours do not appear to have frequent mutations in genes encoding patched/hedgehog/smoothened proteins.

Similarly, our analysis did not detect the H133Y mutation in any of the cases. This contrasts with a previous report [14], but concurs with another study [20], indicating the absence of the H133Y substitution in the combined sample of 128 samples of breast cancer DNA. No mutations were found in previously implicated exons of the *SMO* gene containing reported amino acid substitutions Y955H and E995G [12].

DISCUSSION

Although this study cannot exclude the presence of point mutations in a small proportion of breast carcinomas, it is unlikely that *PTCH* is inactivated/mutated in a substantial fraction of primary breast cancer, at least at a frequency comparable to BCCs or medulloblastomas. The same technique was able to identify somatic mutations in a number of BCCs and other tumours associated with NBCCS. SSCP is an established and cost-effective scanning method for detecting unknown mutations and is suitable for identifying small insertions/deletions and point mutations [21–23]. This scanning method shows a high sensitivity for such a mutation pattern [21]. The mutation pattern in the germline of NBCCS patients and a large body of evidence for a predominance of point mutations in cancer susceptibility genes make this technique a method of choice for mutation screening in tumour DNA. Although SSCP is unlikely to detect larger deletions or rearrangements in the order of kilobases or more, at present there is no indication that the pattern of putative mutations in breast cancer would mainly involve large deletions or gross rearrangements that would escape detection.

Although contamination of samples by stromal cells may result in a decreased sensitivity of our mutation assay, SSCP was shown to detect point mutations in the *TP53* gene in a mixture of normal and mutated cells containing only 5–10% mutated template [24]. The sensitivity of detection may also reflect the labelling efficiency of DNA fragments, with [α-^{32}P]dCTP giving a highly sensitive option for detecting nucleotide changes in a mixture of normal and mutated cells. However, non-clonal changes present in a small subset of tumour population may go undetected.

Our data concur with a previous report [20], in which no evidence was found for the presence of activating *SHH* H133Y mutation in 36 BCCs, 55 medulloblastomas and in

common cancers, including 44 cases of breast carcinomas, eight breast carcinoma cell lines and 48 ovarian tumours. The absence of the mutation was also documented in our analysis of 37 primitive neuroectodermal tumours and five medulloblastoma cell lines [9], (I. Vořechovský, Karolinska Institute, Sweden).

At least two regions on the long arm of chromosome 9, one of them involving the *PTCH* locus at 9q22-q23, have been implicated as a target of frequent genomic imbalance/loss of heterozygosity in breast [25, 26] and ovarian cancer [27]. If there is a tumour susceptibility gene consistently inactivated in this region in a subset of breast carcinomas, the negative results of *PTCH* mutation screening may rather point to another gene located at 9q22-q23 with a putative role in breast cancer development. This region contains a number of genes expressed in skin and epithelial tissues, such as IR-10 [28], xeroderma pigmentosum complementation group A and Fanconi anaemia complementation group C. Many expressed sequenced tags have now been mapped to this region and it will be interesting to test those involved in the differentiation and proliferation of breast tissue.

In conclusion, in contrast to BCCs, medulloblastomas and an earlier report [14], our study suggests that primary sporadic breast carcinomas do not have frequent mutations in genes encoding major players in the patched/hedgehog/smoothened signalling pathway. A more accurate assessment of the putative involvement of patched/hedgehog/smoothened signalling pathway in human breast cancer would require a more comprehensive analysis in a large series of cases.

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