Genomewide Single Nucleotide Polymorphism Microarray Mapping in Basal Cell Carcinomas Unveils Uniparental Disomy as a Key Somatic Event

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Abstract
Basal cell carcinoma is the most common human cancer with increasing incidence reported worldwide. Despite the aberrant signaling role of the Hedgehog pathway, little is known about the genetic mechanisms underlying basal cell carcinomas. Towards a better understanding of global genetic events, we have employed the Affymetrix Mapping 10K single nucleotide polymorphism (SNP) microarray technique for “fingerprinting” genomewide allelic imbalance in 14 basal cell carcinoma–blood pair samples. This rapid high-resolution SNP genotyping technique has revealed a somatic recombination event–uniparental disomy, leading to a loss of heterozygosity (LOH), as a key alternative genetic mechanism to allelic imbalances in basal cell carcinomas. A highly conserved LOH region at 9q21-q31 was found in 13 of 14 (93%) basal cell carcinomas. Further statistical and fluorescence in situ hybridization analyses confirmed that the 9q LOH was a result of uniparental disomy in 5 of 13 (38%) basal cell carcinomas. De novo mutations in the Patched 1 gene (PTCH) were found in 9 of 13 (69%) basal cell carcinomas with 9q LOH. A second important locus, containing LOH at 6q23-q27 was found in 5 of 14 (36%) basal cell carcinomas, suggesting that the presence of an additional putative tumor suppressor gene may be contributing to basal cell carcinoma development. This study shows that the rate of 9q LOH in basal cell carcinomas has been previously underestimated. Furthermore, we provide the first evidence that uniparental disomy due to somatic recombination constitutes one of the mechanisms of LOH in basal cell carcinoma tumorigenesis.

Introduction
The increasing incidence of basal cell carcinoma worldwide is a cause of significant morbidity and has a major impact on public health care resources (1). There has been a wealth of experimental data confirming the role of aberrant Sonic Hedgehog (Shh) pathway, via Patched (PTCH) gene mutations (2, 3), as a key cellular signaling event in basal cell carcinoma.

tumorigenesis. However, relatively little is known about the global genetic mechanisms that may occur in basal cell carcinomas. The availability of microarray-based high-resolution single nucleotide polymorphism (SNP) analysis allows a reproducible and rapid determination of genomewide allelic changes such as genomic instability and loss of heterozygosity (LOH) from a single DNA sample (4, 5). This technique has been employed for SNP genotyping in various human cancers (5–8), and in this report, we present the first data using the SNP microarray mapping technique to characterize basal cell carcinoma DNA samples from 14 patients with sporadic nodular basal cell carcinomas, the most common subtype of basal cell carcinoma.

Materials and Methods
Clinical samples. All tumor and blood samples were obtained with patients’ consent and ethical approval was obtained from the local research ethics committees. All 14 basal cell carcinoma samples were confirmed to be sporadic nodular basal cell carcinomas based on clinicohistopathologic classification. Tumor purity was estimated to be no less than 90% according to both histology and the number of informative SNP calls (average call rate for basal cell carcinomas, 94.8 ± 1.7%; see Table 1). Freshly collected basal cell carcinoma tumors were snap-frozen in liquid nitrogen and stored at −70°C. The age range was 49 to 98 years with a mean ± SD age of 76.2 ± 13.5 years and a male-to-female ratio of 1:1.

DNA extraction and quantification. DNA was extracted from blood and basal cell carcinoma samples using the Nucleon Genomic DNA Extraction kit BACC3 (RPN8512, Amersham Biosciences, Bucks, United Kingdom) according to the manufacturer’s instruction. DNA concentrations and purity were determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop, Rockland, DE).

Single nucleotide polymorphism microarray mapping assay. Patient DNA samples were processed according to the standard GeneChip Mapping 10K (V2.0) Xba Assay protocol (Affymetrix Inc., Santa Clara, CA) as described previously (9, 10). Briefly, 350 ng of DNA was digested with XbaI and ligation to the XbaI adaptor prior to PCR amplification (35 cycles) using AmpliTaq Gold with Buffer II (Applied Biosystems, Foster City, CA). Hybridized arrays were processed with an Affymetrix Fluidics Station 450 and fluorescence signals were detected using the Affymetrix GeneChip Scanner 3000. Signal intensity data was analyzed by the GeneChip DNA analysis software based on a model algorithm to generate SNP calls (4). Statistical analysis was done with the R statistical package (11).

Fluorescence in situ hybridization. Fluorescence in situ hybridization (FISH) was done on touch imprints made from snap-frozen basal cell carcinoma tumor samples using 1 μg of BAC DNA containing the PTCH gene locus (12) at 9q22.3 (RP11-34D4, Wellcome Trust Sanger Institute,
Cambridge, United Kingdom) labeled with Spectrum Orange dUTP (Vysis, Downers Grove, IL). A green chromosome 9 centromere probe (Vysis) was used as a control. Cells were counterstained with 4',6-diamidino-2-phenylindole and the fluorescence images analyzed with MacProbe software (Perceptive Scientific Instruments, League City, TX). A minimum of 50 interphase cells were analyzed for each tumor sample.

Mutation analysis of PTCH. Coding exons 3 to 22 of the PTCH gene were screened for mutations by heteroduplex analysis on the Transgenomic WAVE system and subsequent verification of mutations by direct sequencing. Primers were designed to amplify PTCH exons and the adjacent intron boundaries. PCRs were carried out in 25 µL reaction volume using Promega (Southampton, England, United Kingdom) Taq DNA polymerase and touchdown cycling. For heteroduplex analysis, tumor PCR products were combined with an equal volume of equivalent PCR product amplified from a normal control DNA sample with no history of basal cell carcinomas, denatured at 95°C and slowly cooled to 25°C using a ramp rate of 0.03°C/s. Five microliters of heteroduplexed DNA was analyzed on the Transgenomic WAVE DNA analysis system according to the manufacturers' instructions, using optimum conditions predetermined for each PTCH fragment using WaveMaker software. Sequencing was done using ABI Prism BigDye Terminator cycle sequencing reagent v.1.1 (Applied Biosystems) with 3.2 pmol primer in 10 µL reaction volumes. Sequencing products were ethanol-precipitated and run on an ABI 3700 sequencer. De novo mutations found in this study were identified firstly by denaturing high-pressure liquid chromatography separation and then characterized by sequencing, and also by sequencing the blood DNA of the same patient to cross-verify each mutation found. Restriction digest was also used to confirm some of the de novo mutations.

### Table 1. Clinical, chromosomal LOH, and mutational information of the 14 basal cell carcinomas

<table>
<thead>
<tr>
<th>ID</th>
<th>Sex</th>
<th>Age</th>
<th>Location</th>
<th>PTCH Mutation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>76</td>
<td>nose</td>
<td>P504Q, TM4 (1699C&gt;A, ex11)</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>83</td>
<td>abdomen</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>79</td>
<td>arm</td>
<td>G1167W, TM12 (3687G&gt;T, ex20)</td>
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<tr>
<td>4</td>
<td>F</td>
<td>81</td>
<td>shin</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>83</td>
<td>ear</td>
<td>Y831X, ECL4 (2680-2681insAGTA, ex14)</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>97</td>
<td>scapula</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>82</td>
<td>knee</td>
<td>Q466X, ICL1 (1584C&gt;T, ex10)</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>60</td>
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<td>A558fsX618, TM5 (1861-1883del23, ex12a)</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>82</td>
<td>arm</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
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<td>48</td>
<td>temple</td>
<td>ND</td>
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<td>11</td>
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<td>M</td>
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<td>ear</td>
<td>C727fsX737, ICL3 (2366-2367insC, ex13)</td>
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<td>F</td>
<td>79</td>
<td>ear</td>
<td>W399X, ECL1 (1384G&gt;A, ex13)</td>
</tr>
</tbody>
</table>

NOTE: The mean ± SE of SNP call rates for the 14 basal cell carcinoma and blood samples are 94.8 ± 1.7% and 97.4 ± 1.4%, respectively. The overall mean ± SE of SNP call rate for all samples was 96.1 ± 1.1% (n = 28). Values in boldface indicate LOH regions encompassing the PTCH gene locus at 9q22.3.

*Indicates the amino acid change (with corresponding location in the PTCH protein; fs, frame shift; TM, transmembrane; ECL, extracellular loop; ICL, intracellular loop; see Fig. 2B) and the position of nucleotide mutation (numbering based on Genbank accession NM_000264/GI:25121959) within the PTCH gene. ND, mutation not detected.

*Contains mutation within the PTCH gene.
Figure 1. Genomewide LOH and allelic imbalance profiles in basal cell carcinomas. A, a total of 14 sporadic nodular basal cell carcinomas with corresponding lymphocytic DNA samples taken from the same patients were analyzed using the 10K SNP microarray. LOH regions were identified by analyzing the somatic differences in SNP genotypes between germ line (blood) and basal cell carcinoma tumor DNA of each patient. The LOH regions were further characterized into uniparental disomy (pink), allelic loss (gray), or allelic gain (green) according to signal intensity data analyzed using the CopyNumber tool within the Affymetrix GeneChip DNA analysis software. B, a summary of LOH data obtained in (A) showing the minimal regions of recurring LOH (blue) within the 14 basal cell carcinomas. The LOH region at 9q21.11-q31.1 was detected in 13 of 14 (93%) basal cell carcinomas analyzed. Of the 9q LOH regions, 5 of 13 (38%) were a result of uniparental disomy. *, presence of PTCH gene mutation (see Table 1 and Fig. 2) within the 9q22.3 locus.
Results and Discussion

We have employed a high-resolution Affymetrix 10K SNP microarray mapping technique (7, 9) to obtain genomewide SNP genotype “fingerprints” in 14 sporadic nodular basal cell carcinomas samples. By comparing the SNP genotype fingerprints in basal cell carcinoma tumor DNA with lymphocytic DNA from the same patient, we investigated de novo global somatic events such as allelic instability and LOH profiles that may underlie basal cell carcinoma tumorigenesis. We have shown that 13 of 14 (93%) sporadic basal cell carcinomas analyzed in this study contain chromosome 9q LOH (Fig. 1A). A highly conserved LOH region at 9q21.1-q31.1 (Fig. 1B, shaded blue) was found in all 13 basal cell carcinomas containing 9q LOH. The tumor suppressor PTCH gene, responsible for the autosomal dominant condition, Gorlin, or nevoid basal cell carcinoma syndrome (MIM 109400; refs. 2, 3), is located within

![Figure 1: Mutational analysis of the PTCH gene in basal cell carcinoma. A, electropherograms of PTCH gene mutations: patients #1 and #7 with point mutations, patient #5 with 4 bp insertion, and patient #9 with 2 bp deletion. B, a schematic diagram summarizing the approximate locations of the PTCH mutations found in this study.](image-url)
Figure 3. Confirmation of uniparental disomy in basal cell carcinoma using statistical and FISH analyses. A, a FISH on metaphase chromosomes illustrating the specificity of a probe (RP11-34D4, red; ref. 12) for the PTCH gene on 9q22.3. A centromere probe (green) was used as a chromosome 9 control. B and C, statistical analysis was done on chromosome 9 of basal cell carcinoma–blood pair samples from patients #4 and #7 with their corresponding interphase FISH images showing uniparental disomy and deletion, respectively. The ratio of LOH calls was calculated in a running window of 20 Mbp. The LOH score (black) is calculated based on the SNP allelic ratios between the tumor and blood samples from the same patient. A score of >1.0 indicates LOH. The DNA intensity signal score (blue) is based on the mean SNP signal ratios between the tumor and blood sample pair. Any reduction in signal ratio score implies a chromosomal loss. The chromosome 9 ideogram (not to scale) and SNP call map (AA or BB homozygous calls in green and AB heterozygous calls in red) above each SNP score graphs illustrates the corresponding regions of LOH with either uniparental disomy (shaded pink) or loss/deletion (gray), respectively.
this minimal region of LOH at 9q22.3. A significant number of basal cell carcinomas with 9q LOH 9/13 (69%) was found to contain de novo mutations including missense, nonsense and frameshift in the PTCH gene (Table 1; Fig. 2A). These PTCH gene mutations were not found in the lymphocyte DNA of the corresponding patients. No PTCH gene mutations were found in 4 of 13 (31%) of the basal cell carcinomas with 9q LOH. This could be due to confounding limitations of the nucleotide sequencing technique in detecting mutations in samples containing large intragenic deletions or the presence of other gene-inactivation mechanisms such as mutations within the promoter region or gene silencing by methylation. Nevertheless, the rate of PTCH gene mutation found in the present study, despite a smaller cohort of basal cell carcinoma used, is comparable to a recent study (13) and strengthens the published role of somatic PTCH inactivation in basal cell carcinoma (3).

Interestingly, the second most common LOH locus at 6q23.2-q27 was found in 5 of 14 (36%) basal cell carcinomas indicating that the presence of an additional putative tumor suppressor gene may be contributing to basal cell carcinoma development. To our knowledge, the LOH at 6q has not been previously reported in basal cell carcinomas. Except for 9q21.1-31.1 and 6q23.2-q27, very few other conserved regions of chromosomal LOH have been identified, suggesting that basal cell carcinomas are a genetically homogenous cancer with the key locus being 9q21-31 containing the PTCH gene which we found to contain various types of de novo mutations (Fig. 2A).

Further analysis of SNP call signal intensity data for quantification of allelic copy number, we found that about half of the chromosomal LOH regions were due to allelic loss/deletion (Fig. 1A, shaded gray) and 13% were due to allelic gain (green). Interestingly, 42% (19 of 45) of all the detected LOH regions do not exhibit a change in copy number. The SNP signal scores calculated (7) within these LOH regions were indicative of two copies (Fig. 1A, shaded pink) which were confirmed by FISH analysis (Fig. 3 for chromosome 9q). This indicates that a genetic event, other than allelic imbalance is responsible for producing LOH in these chromosomal regions. We concluded that uniparental disomy, a somatic recombination event, is responsible for the LOH regions bearing two copies. In 10 of the 12 patients with uniparental disomy as a cause of chromosomal LOH (Fig. 1A), over half of the regions with uniparental disomy show segmental LOH extending to the telomeres, indicating that segmental uniparental disomy was a result of somatic recombination. Whereas in three patients (#9, #12, and #13; Fig. 1A), there was entire chromosomal uniparental disomy in 9, 22, and 19, respectively, indicating that the uniparental disomy had arisen from either a somatic recombination close to the centromere or a nondisjunction event with subsequent chromosomal duplication.

Further investigations in the 13 basal cell carcinomas containing 9q LOH found that 5 of 13 (38%) of the 9q LOH were a result of uniparental disomy (Fig. 1A, shaded pink), whereas 8 of 13 (62%) were a result of allelic loss/deletion (gray). No copy number gain was found within the 9q LOH detected in this study. The 9q copy number was confirmed by both statistical and FISH analyses (Fig. 3). The FISH analysis using a BAC probe containing the PTCH gene locus at 9q22.3 (12) showed that basal cell carcinomas with 9q uniparental disomy (Fig. 3A, patient 4) contained two copies of the PTCH gene, whereas basal cell carcinomas with 9q allelic loss (Fig. 3B, patient 7) contained only one copy. This suggests that the SNP signal intensity information provides an accurate determination of allelic copy number. Conventional genotyping methods using cytogenetic and microsatellite analyses on basal cell carcinomas found, in agreement with our data, a consistent pattern of allelic loss in 9q LOH (14, 15). However, previous studies have failed to detect the presence of uniparental disomy as a mechanism which we now find responsible for 38% of 9q LOH regions detected in the basal cell carcinomas. Our finding that 93% of basal cell carcinoma possess 9q LOH shows that the reported rate of 9q LOH in basal cell carcinomas has been underestimated, possibly due to the limitation of microsatellite and cytogenetic analyses (14, 15). Uniparental disomy as a somatic recombination event in tumorigenesis has been reported in breast cancer (16) and more recently in acute myeloid leukemia (7), suggesting that uniparental disomy may be a rather common genetic mechanism important in tumorigenesis. The high-resolution genomewide SNP genotyping technique, capable of producing detailed fingerprints of LOH and allelic copy number, has helped to reveal uniparental disomy in cancers.

This study establishes sporadic basal cell carcinomas as a genetically homogenous cancer as proposed in a study using a less dense microsatellite marker set (17). We have previously shown that aberrant Hedgehog signaling, via activation of glioma (Gli) transcription factor, led to the activation of FOXM1, a cell cycle transcription factor, in basal cell carcinomas (18). A recent study established that FOXMI has a major role in maintaining chromosomal stability (19). Our finding that nodular basal cell carcinomas show significant genetic homogeneity and a conserved genomic pattern of chromosomal LOH profile is in agreement with such molecular signaling events in basal cell carcinomas. The intrinsic genomic stability in basal cell carcinomas may account in part for the lack of biological aggression in most basal cell carcinomas (20), but the molecular mechanisms responsible remain to be defined.

In summary, the use of a high-resolution SNP mapping technique has revealed, for the first time, the presence of uniparental disomy, a result of somatic recombination, in sporadic basal cell carcinomas. We hypothesize that uniparental disomy, in addition to allelic imbalance, constitutes a novel genetic mechanism involved in tumorigenesis. Furthermore, the highly conserved pattern of 9q LOH found in this study hallmarks an early somatic event in basal cell carcinoma.

Acknowledgments


Grant support: Cancer Research UK, Association for International Cancer Research, and Barts and the Royal London Charitable Foundation.

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