Allelic imbalance at chromosome 11 in head and neck squamous cell carcinoma in an Indian patient population

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Background: Genetic instability of chromosome 11 is a frequent event in many solid tumours, including head and neck squamous cell carcinoma (HNSCC).

Aims: To perform allelic imbalance analysis of cyogenetically mapped altered regions of human chromosome 11 in patients with HNSCC from eastern India.

Methods: Genomic alterations were investigated using highly polymorphic microsatellite markers in both HNSCC and leukoplakia tissues.

Results: Microsatellite markers D11S1758 from 11p13–15 and D11S925 from 11q23.3–24 had the highest frequency (38% and 32%, respectively) of loss of heterozygosity among all the markers analysed. Allelic loss at the marker D11S925 was seen in both leukoplakia and in all stages of HNSCC tumour tissues suggesting that it is an early event in HNSCC tumorigenesis. Microsatellite size alteration was also found to be high (>20%) in several markers. In leukoplakia samples microsatellite instability was seen at a higher frequency than loss of the allele, indicating such alterations might initiate the process of tumorigenesis in HNSCC.

Conclusions: The high rate of chromosomal alterations at 11q21–24 in HNSCC suggests the presence of a putative tumour suppressor gene in this region.

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common human cancer in all parts of the world, and it has been estimated that there are around 400,000 new cases each year worldwide. It accounts for about 30–40% of all cancer types in India and in the subcontinent. Epidemiological studies have linked chronic exposure to tobacco, betel nut leaf quid, alcohol, and some environmental factors to the occurrence of HNSCC, and the characteristic molecular genetic events associated with the initiation and progression of HNSCC are beginning to be understood. The karyotypes of HNSCC tumours are complex, often near triploid, and there are multiple numerical and structural abnormalities, including deletions, balanced and unbalanced translocations, isochromosomes, dicentric chromosomes, and homogeneously staining regions. Classic cytogenetic analysis of HNSCC tumours has identified a gradual increase in chromosomal aberrations from premalignant to malignant lesions. In other studies, consistent chromosomal deletions and amplifications in several chromosome arms have been reported. In addition, centromeric breakage in several chromosomes has been shown to be important for the development of HNSCC.

Chromosome 11 plays a crucial role in several cancers, and two regions in particular, one in the short arm (11p13–15) and the other in the long arm (11q21–24), have been identified as frequent targets for allelic loss. Allelic deletion at 11p15 has been reported to be a common occurrence in oesophageal cancer. In another report, a microsatellite marker flanking the p57KIP2 gene (a cyclin dependent kinase inhibitor) at 11p15.5 showed 33% LOH and loss of imprinting in HNSCC. A genetic progression model of HNSCC has implicated alteration in chromosomal region 11q13 in the transition from dysplasia to in situ carcinoma. In addition, it has been reported that the 11q23–24 region is frequently deleted in nasopharyngeal carcinoma.

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In our present report, we examine allelic imbalance in HNSCC tumours from the oral cavity, the laryngeal region, and the orofacial region in eastern Indian patient populations at three regions of chromosome 11 (11p15, 11q13, and 11q21–24). We present evidence that the 11q21–24 region may harbour a putative tumour suppressor gene involved in the development of tumours in these three sites of the head and neck. Our result suggests that microsatellite instability is an early event in nasopharyngeal carcinoma.
HNSCC tumorigenesis, and we propose that underlying mechanisms responsible for the generation of microsatellite instability could also be responsible for allelic loss.

**METHODS AND MATERIALS**

**Tumour samples**

Sixty freshly operated primary HNSCC tumours and 13 dysplastic leukoplakia tissue samples, along with their corresponding normal tissues or peripheral blood leucocytes (PBLs), were collected from the patients before treatment. Samples were immediately frozen and stored at −80°C. All tumours were histopathologically diagnosed as squamous cell carcinoma, graded and staged according to the UICC TNM classification. Table 1 shows a detailed history of all 73 patients affected with primary HNSCC tumours and leukoplakia. Among the 60 patients with HNSCC, there were 49 men and 11 women, with a mean age of 49 years. Patients were grouped on the basis of affected primary sites, namely: oral cavity, larynx, and orofacial. Thirty two tumours were from the oral cavity (three alveolus, 16 buccal mucosa, 10 tongue, two tonsil, and one palate), nine tumours were from the larynx, and 14 tumours were from the orofacial region (13 maxilla and one mandible). Histopathologically, the HNSCC tumours were classified as stage I (six tumours), stage II (13 tumours), stage III (17 tumours), or stage IV (19 tumours). Similarly, 13 leukoplakia tissues were classified histologically into three groups, namely: mild (two), moderate (six), and severe (five). Among the 60 tumours, 25 each were lymph node positive and negative. In addition, 34 patients were habitual users of tobacco by different means (bidi, pan, cigarettes, etc) and 17 did not have a tobacco habit. For four patients no history was available.

**Microdissection and DNA isolation**

The normal cells present as contaminants in the primary tumour tissues were removed by a microdissection procedure. More than 50 to 60 serial tumour specimens (10 to 20 µm) were sectioned and placed on glass slides using a cryostat (model CM 1800; Leica, Heidelberg, Germany). Representative 5 μm tumour specimens from different regions of the tumour were stained with haematoxylin and eosin for diagnosis and for marking the tumour rich regions. The normal cells present in the tumour specimens were removed by microdissection. The adjacent normal tissues of the primary tumour were similarly sectioned to identify the presence of tumour cell infiltration. For those cases in which the “normal” tissues were contaminated with tumour cells, PBLs from the corresponding patient were used as the normal sample. Microdissected tumour samples containing <60% tumour cells were not further analysed. Genomic DNA was isolated from tissues by standard proteinase K digestion, followed by phenol/chloroform extraction.

**Microsatellite markers and PCR analysis**

We used 10 highly polymorphic (heterozygosity > 70%) known. No history is available for the patients 4717b, 4717g, 2917, and 2927. Therefore, we used 10 highly polymorphic (heterozygosity > 70% except one) CA repeat markers: two markers from 11p13–15 (D11S1339, D11S2179, D11S1893, D11S924, D11S925, D11S1345, and D11S1328). These microsatellite loci were amplified by the polymerase chain reaction (PCR) in a 10 µl reaction containing 10mM Tris/HCl (pH 8.3), 50mM KCl, 0.2 mM of each dNTP, 2 pmol of each primer, an optimal concentration of MgCl2 (1.5 to 2.0mM; determined separately for each primer pair), and 50–100 ng of template genomic DNA. Reaction mixtures were heated to 94°C for three minutes, cooled to 72°C before the addition of 0.2 units Taq DNA polymerase (Gibco BRL, Gaithersburg, Maryland, USA), and cycled 30 times in a GeneAmp 9700 (Perkin Elmer, Norwalk, Connecticut, USA) PCR machine. Each cycle consisted of 30 seconds at 94°C, 30 seconds at the appropriate annealing temperature (50–58°C), and 30 seconds at 72°C; the final extension was carried at 72°C for seven minutes. Samples without DNA were used as negative controls.
each primer pair. Before the PCR, one of the paired primers was end labelled with \(\gamma^{32}P\) ATP (specific activity, 3000 Ci/mM; Amersham, Buckinghamshire, UK) using T4 polynucleotide kinase (Gibco BRL). The amplified DNA (1 \(\mu\)l) was mixed with formamide dye, denatured, and then electrophoresed on a denaturing 6% polyacrylamide gel at 2300 V for three to four hours. After electrophoresis, gels were dried and exposed to x-ray films (Kodak X-Omat) for autoradiography.

Definition of LOH and MA

LOH was determined by densitometric scanning (CS-900; Simadju, Osaka, Japan) of the autoradiographs. Because the detection of LOH can be compromised by the presence of normal DNA contaminating the tumour samples, we used genomic DNA from microdissected tumour tissues and calculated LOH using the ratios of the band intensities of paired tumour and normal DNA. For informative cases, allelic loss was scored if there was complete loss of one allele or if the relative band intensity of one allele was decreased by at least 50% in the tumours, compared with the same allele in the normal control. Values were calculated as the ratio of band intensities of the larger to the smaller alleles in the tumour DNA divided by the same ratio in the corresponding normal DNA sample. LOH indices of > 1.5 and < 0.67 were considered to be loss of smaller and larger alleles, respectively.

MA was scored if one (MA1) or both (MA2) alleles at a given locus showed size variation—either expansion or contraction—in comparison with the same alleles in normal control DNA. The determination of MA can be erroneous if the samples are not properly paired, so that in our study this was ensured by genotyping with four RFLP markers (see results). To calculate the frequency of LOH, samples showing homozygosity (H) and MA only were not considered; that is, \(\text{LOH} = (\text{LOH} + \text{LMA})/\text{total} - \text{MA} - \text{H}\). For the MA frequency calculation the samples showing LOH only were not considered; that is, \(\text{MA} = (\text{MA} + \text{LMA})/\text{total} - \text{LOH}\). The samples showing both LOH and MA at the same locus was considered for calculating both LOH and MA. MA can sometimes falsely be designated as LOH, and this can be resolved by scanning the autoradiogram. All samples showing LOH and/or MA were subjected to repeat analysis after a second independent amplification for confirmation.

RESULTS

Analysis of LOH and MA in the HNSCC samples

We genotyped 60 primary HNSCC tumours at different stages of development for LOH and MA at 10 different loci on chromosome 11 using highly polymorphic microsatellite markers; two from 11p13–15, one from 11q13, and seven from 11q21–24 (table 1; fig 1). All markers used in our study were informative in more than 70% of the tumours analysed. Each tumour was genotyped with at least eight markers. Broadly, two types of allelic alterations were seen in the matched tumour tissues compared with normal tissues of the same individuals. These were loss of an allele and an increase or decrease in the size of the allele (fig 2). In a few cases, biallelic alterations in the tumour tissues were also seen (data not shown). In those samples, proper matching between the normal and tumour sample was confirmed by genotyping with four RFLP markers, namely: Alu FXIII3B (H = 0.43), Alu D1 (H = 0.49), Alu TPA25 (H = 0.48), and Alu ACE (H = 0.44), respectively (data not shown). It was found that 51 of the 60
HNSCC samples exhibited LOH and/or MA on chromosome 11 for at least one marker (fig 1).

Figure 3 depicts the frequency of LOH and MA in the HNSCC tumours analysed in relation to the markers used. All markers exhibited LOH and MA at a moderate to high frequency. LOH was found in more than 20% of the tumours for three markers (D11S1758, D11S913, and D11S925). Similarly, MA was found in more than 20% of the tumours for four markers (D11S4177, D11S1758, D11S924, and D11S925). Among the markers analysed on the long arm, D11S925 at 11q23.3 displayed the highest frequency of LOH (32%). Interestingly, both D11S925 and another adjacent marker D11S924 within the 11q23.3 region also exhibited a high frequency of MA (22%). Thus, it appears that the 11q23.3 region is prone to genetic instability in HNSCC tumours. It should be noted that two markers from the 11p15 region also exhibited a high frequency of instability (fig 3). Another interesting observation is the moderate degree of instability (16% LOH and 12% MA) of D11S2179, which is intragenic to the ATM gene (fig 3).
Figure 1 shows the allelic imbalance data for the different markers used in relation to the tumour location and stage. Primary site and tumour stage information were available for 55 and 45 tumours, respectively. For five tumours, neither of these pieces of information were available. LOH was seen most frequently for markers D11S925 (13 of 41) and D11S1758 (17 of 45). D11S925 was deleted in eight of 24 (33%) oral cavity tumours and four of 15 (27%) laryngeal/orofacial tumours, whereas D11S1758 was deleted in eight of 17 (47%) laryngeal/orofacial tumours and six of 23 (26%) oral cavity tumours. However, the numbers do not suggest a site specific LOH pattern. Both markers exhibited LOH in all stages of the oral cavity tumours. The group of laryngeal/orofacial tumours was too small to determine the stage specific LOH pattern. Six tumours (numbers 2030, 2539, 5090, 1494, 2323, and 951) exhibited LOH on the short arm of chromosome 11 and none on the long arm. Recently, such chromosome arm specific loss has been documented in colorectal cancer. Further analysis with more markers on the short arm is required to substantiate these data. Four tumours (numbers 4332, 5090, 693, and 2917) suffered biallelic alterations in markers from 11p15, where one allele was lost and the other allele exhibited size alteration (figs 1 and 2). However, biallelic alterations were not seen for the 11q markers.

Furthermore, among the tumours showing changes, 12 of 51 (23%) exhibited only LOH and eight of 51 (16%) displayed MSI exclusively (fig 4). Thus, 31 of 51 (61%) tumours exhibited both LOH and MA (fig 4). Interestingly, 19 of 60 (32%) tumours exhibited MA at two or more loci, indicating a microsatellite instability (MSI) phenotype (fig 1).

Analysis of LOH and MA in leukoplakia
Genotyping of 13 leukoplakia tissues of various stages using a subset (D11S4177, D11S1758, D11S2179, and D11S925) of the above 10 markers revealed several intriguing features (figs 5 and 6). Analysis of the allelic imbalance in leukoplakia samples revealed that dysplastic tissues of mild and moderate stages exhibited both MA and infrequent LOH, whereas severe dysplastic tissues exhibited both types of alteration at equal frequencies (fig 6). Moreover, it was found that three markers had a higher degree of MA than LOH, and one marker (D11S925) exhibited an equal frequency of LOH and MA in these samples (fig 5). These results suggest that MA could be an early event in HNSCC tumorigenesis. Furthermore, the presence of LOH in D11S925 in the leukoplakia tissues suggests that alterations in the 11q23.3–24 region were an early event in the development of HNSCC. In contrast, the absence of LOH in D11S1758 suggests that it is a late event and associated with tumour progression.

DISCUSSION
In our present study, we examined allelic imbalance mainly in two regions (11p15 and 11q21–24) of chromosome 11 in leukoplakia samples. Leukoplakia samples are arranged according to the stage of dysplasia. Markers with their cytogenetic positions are shown in columns at the left hand side of the figure. LOH, loss of heterozygosity; MA, microsatellite size alteration; NI, non-informative; RH, retention of heterozygosity.
patients with HNSCC from three Indian populations. Overall, we found a very high frequency (83%) of allelic alterations in these two regions of chromosome 11 compared with other cancers in different populations. Forty-three of the 60 HNSCC tumours (72%) exhibited LOH in at least one marker. The analysis showed that markers D11S925 at 11q23.3–24 and D11S1758 at 11p15 exhibited the highest frequency of LOH (32% and 38%, respectively). A high frequency of LOH in the 11p15.5 region (D11S988) has also been reported in HNSCC patient populations from the USA. Although frequent allelic loss in the 11q21–24 regions has been reported in many cancers, such alterations in oral cavity, laryngeal, and oro-facial carcinomas of the head and neck region have not been reported so far. The maximum single locus incidence of LOH in the 11q21–24 region varied from 27% in breast cancer to 47% in nasopharyngeal carcinoma, which is comparable to our present data (32% at D11S925) in HNSCC tumours. Several genes involved in the DNA damage response (ATM, MRE11A, and CHEK) and apoptosis (CASP1, CASP4, CASP5, and P53AP1) are located at the 11q21–24 region. Similarly, a few functionally uncharacterised genes (ANC, BRC43, MLL, and ST3), implicated in various cancers, are also located in this region. Another putative tumour suppressor gene (PPP2R1B) found in this region has been excluded from involvement in cervical cancer, but has been predicted to be a possible target in colorectal cancer. It is notable that the two markers flanking D11S925 (D11S924 and D11S1345) had a lower frequency of LOH, so that in our study we have identified a minimal region of 7 cM between D11S924 and D11S1345 that may harbour putative tumour suppressor genes involved in the development of HNSCC. The marker D11S2179, which is intragenic to the tumour suppressor gene ATM, exhibited 16% LOH in HNSCC tumours, indicating that the ATM gene might be involved in the development of HNSCC, and this possibility needs to be investigated in greater detail.

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We found that MA was very frequent (39 of 60; 65%) in HNSCC tumours from Indian populations. Similar results were also reported in a HNSCC patient population from the USA, where 51% of the tumours showed MA in one or more loci. All 10 markers used in our present study exhibited MA in between 12% and 31% of the HNSCC tumours. The markers D11S4177 from 11p15 and D11S924 and D11S925 from the 11q21–24 region exhibited the highest frequency of MA. Interestingly, both markers D11S4177 and D11S924 are adjacent to the markers that exhibited the highest LOH, indicating that these regions are very susceptible to genetic alterations during the development of HNSCC. Recently, it has been suggested that MSI and chromosomal instability phenotypes can coexist in tumours. Previously, we have reported that the MSI phenotype exhibited by HNSCC tumours differs distinctly from that of colorectal tumours. Based on these results, we argue that the underlying mechanisms for MA could also be responsible for LOH.

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REFERENCES

Take home messages
- The high rate of chromosomal alterations at 11q21–24 in head and neck squamous cell carcinoma (HNSCC) suggests the presence of a putative tumour suppressor gene in this region.
- Allelic loss at the marker D11S925 was seen in leukoplakia and in all stages of HNSCC, suggesting that it is an early event in the tumorigenesis of HNSCC.
- Microsatellite size alteration was also found to be high (> 20%) in several markers.
- In leukoplakia samples, microsatellite instability was seen at a higher frequency than loss of the allele, indicating such alterations might initiate the process of tumorigenesis in HNSCC.
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