Association of deletion in the chromosomal 8p21.3-23 region with the development of invasive head & neck squamous cell carcinoma in Indian patients


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**Background & objectives:** Deletions in chromosome 8 (chr.8) have been shown to be necessary for the development of head and neck squamous cell carcinoma (HNSCC). Attempts have been made in this study to detect the minimal deleted region in chr.8 associated with the development of HNSCC in Indian patients and to study the association of clinicopathological features with the progression of the disease.

**Methods:** The deletion mapping of chr.8 was done in samples from 10 primary dysplastic lesions and 43 invasive squamous cell carcinomas from the head and neck region of Indian patients to detect allelic alterations (deletion or size alteration) using 12 highly polymorphic microsatellite markers. The association of the highly deleted region was correlated with the tumour node metastasis (TNM) stages, nodal involvement, tobacco habit and human papilloma virus (HPV) infection of the samples.

**Results:** High frequency (49%) of loss of heterozygosity (LOH) was seen within 13.12 megabase (Mb) region of chromosomal 8p21.3-23 region in the HNSCC samples, whereas the dysplastic samples did not show any allelic alterations in this region. The highest frequency (17%) of microsatellite size alterations (MA) was observed in the chr.8p22 region. The loss of short arm or normal copy of chr.8 and rare bi-allelic alterations were seen in the stage II-IV tumours (939, 5184, 2772, 1319 and 598) irrespective of their primary sites. The highly deleted region did not show any significant association with any of the clinical parameters. However, HPV infection was significantly associated (P < 0.05) with the differentiation grades and overall allelic alterations (LOH/MA) of the samples.

**Interpretation & conclusion:** Our data indicate that the 13.12 Mb deleted region in the chromosomal 8p21.3-23 region could harbour candidate tumour suppressor gene(s) (TSGs) associated with the progression and invasion of HNSCC tumours in Indian patients.

**Key words** Chromosome - head and neck cancer - squamous cell carcinoma - microsatellite - tumour suppressor genes

Head and neck squamous cell carcinoma (HNSCC) is a common cancer world-wide with around 616,000 cases being diagnosed in 2000. It accounts for 30-40 per cent of all cancer types in India. Epidemiological
studies have linked tobacco, betelnut leaf quid, alcohol, some environmental factors and infection with oncogenic types of HPV to the development of HNSCC. Among HPVs, the high risk HPV types 16 and 18 are most prevalent in the HNSCC tumour.

The karyotypes of the HNSCC tumours are complex often near triploid and are composed of multiple numerical and structural abnormalities of the chromosomes. Cytogenetic studies have identified deletions in several chromosomal arms e.g., 3p, 4p, 8p21-pter, 9p13-p23, 18q22-qter etc., and also centromeric breakage in several chromosomes necessary for HNSCC development.

Allelotyping studies (using microsatellite markers) of HNSCC have identified >25 per cent LOH in several chromosomal regions e.g., 3p, 4q, 6p, 8p, 9p, 11q etc. Among these regions, the deletions in 4q, 6p, 8p/q are proposed to be associated with the development of invasive HNSCC tumours. The deletion mapping of HNSCC tumours by different investigators have identified several regions in chr.8 e.g., 8p23, 8p22, 8p21 and 8p12, which might harbour candidate TSGs. But there is ambiguity in the localization of the minimum common deleted region among the different studies. Also there is uncertainty about the timing of these deletions during the progression of the tumour. El-Naggar et al. have shown that deletions in 8p21 and 8p22 regions were associated with early tumourigenesis of oral and laryngeal squamous cell carcinomas, whereas according to Califano et al. deletions in the 8p22 and 8q21.1-21.2 regions might be necessary for the development of invasive HNSCC tumours. Besides LOH, MA is detected in different cancers, and it may represent a form of genomic instability. In HNSCC tumours, the occurrence of MAs in the Indian patient population has been shown to be high compared to the Western patient population. Recently, we have detected high frequencies (16-28%) of MA in and around the two high LOH regions of chr.3 in primary HNSCC, which indicate the importance of MA in the development of this tumour.

Thus to find out the importance of chr.8 alterations in the development of HNSCC, attempts have been made to map the highly deleted regions on chr.8 in head and neck lesions of Indian patients and to study the association of some clinicopathological features with the identified region(s).

### Material & Methods

#### Sample collection and clinical data:
Freshly operated tumour specimens (n=53) from the head and neck regions of 52 patients were collected along with normal tissue or peripheral blood leukocytes (PBL) from patients admitted to the Chittaranjan National Cancer Institute, Kolkata and Cancer Center & Welfare Home, Kolkata during 1995 to 2000. The secondary recurrent tumour (B) of one patient (5090) on the tooth gum was collected six months after collection of the primary tumour (A) on the inner side of the cheek of the same patient. All the samples were collected prior to treatment of the patients (except tumour 5090B) and after obtaining prior consent from the patients. The samples were frozen immediately after collection and stored at -80°C until use. The study had clearance from the hospital’s ethics committee.

The detailed clinical features of the patients was recorded (Table). To study the association of the allelic alterations of chr.8 with the clinicopathological features, 20 samples from stage I + II HNSCC and 23 samples from stage III + IV HNSCC were collected. These included samples from 31 lesions of the oral cavity (including 10 dysplastic lesions), 13 tumours from the larynx and 9 tumours from the orofacial region. The tumours were graded and staged according to the Tumour Node Metastasis (TNM) classification of Union Internationale Contre le Cancer (UICC). The patients were considered as tobacco habituated if they smoked at least 10-15 cigarettes/bidis or chewed the equivalent amount of chewable tobacco per day for at least 10 years.

#### Microdissection and DNA isolation:
The normal cells present as contaminant in the specimens were removed by microdissection procedure as described by Nawroz et al. The specimens were microdissected to 10-20µm serial sections using a cryostat (Leica CM 1800, Germany). The representative sections of 5µm thickness were taken from different regions of the samples and were stained with haematoxyline and eosin for diagnosis as well as marking of the dysplastic epithelium or tumour rich regions. The normal cells were removed from the marked regions of the sections by the microdissection procedure. The samples containing > 60 per cent dysplastic epithelium/tumour cells were taken for DNA extraction.
DNA was extracted from the microdissected tissue sections and their corresponding normal tissue or PBL by proteinase-K (Sigma, USA) digestion followed by phenol: chloroform extraction. When the adjoining normal tissue of the lesion was contaminated with infiltrating tumour cells, then PBL was taken for normal DNA extraction.

**Microsatellite markers:** To evaluate LOHs and MAs, the following 12 highly polymorphic microsatellite markers were used, from chr.8: D8S277 (forward primer – GATTTGTCCCTCATGCAGTGT, reverse primer- ACATGTTATGTTTGAGAGGTCTG); D8S265 (forward primer - ACCTCTTTCCAGATAAGCCC, reverse primer- CCAATGGTTTCGGTTACTGT); D8S261 (forward primer- TGCCACTGTCTTGGAAAAATCC, reverse primer- TATGGCCCAAGCAATGTGTAT); LPL (forward primer- CAGGTGATGTCCCCAGAGG reverse primer: ACATGTTATGTTTGAGAGGTCTG).
CGAACATGAATTAGAAATCCAGTG); D8S137 (forward primer- GCTAATCAGGGAATCACCCAA reverse primer: AAATACCGAGACTCACACTTCT), D8S87 (forward primer- GGTTGTTGTGTAATTTAAC, reverse primer- TGCTAATACATTAAGCAGA); D8S285 (forward primer- GCTAATCAGGGAATCACCCAA reverse primer: AAATACCGAGACTCACACTTCT), D8S283 (forward primer- TTGGGTGTTATGCTACACTTCT, reverse primer: AGATACAGATGTAGATCTCTCCG); D8S87 (forward primer- GGTTGTTGTGTAATTTAAC, reverse primer- TGCTAATACATTAAGCAGA); D8S260 (forward primer- AGGCTTGCCAGATAAGGTTG, reverse primer- GCTGAAGGCTGTTCTAGGA), D8S257 (forward primer- TTGGGTGTTATGCTACACTTCT, reverse primer: AGATACAGATGTAGATCTCTCCG); D8S283 (forward primer- TTGGGTGTTATGCTACACTTCT, reverse primer: AGATACAGATGTAGATCTCTCCG); D8S557 (forward primer- CAGGGTACAGACATGCTTG, reverse primer- CCTGGGGTCCTAGAGATTT) and D8S256 (forward primer- GTTCAAGGGCTCAGGGTTCT, reverse primer- CTTCCACCTTTAGCCAAGGA).

The information and primer sequence of the microsatellite markers were obtained from [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov). All the markers showed at least 60 per cent informativeness in our samples (data not shown).

**Microsatellite analysis:** The PCR was carried out in a 20 µl reaction volume containing 67mM Tris-HCl (pH 8.7), 16.6mM (NH4)2SO4, 1-2 mM MgCl2, 0.01 per cent Tween-20 (Sigma, USA), 4 pmol of each primer, 0.2 mM of each dNTPs, 50-100ng of template DNA and 0.5 units of Taq-DNA polymerase (Gibco-BRL, USA) as described by Nawroz *et al* and Dasgupta *et al*13. One of the paired primers in the reaction mixture was end labeled with [γ32p] ATP (specific activity 3000ci/mmol, Amersham, UK) using T4-polynucleotide kinase (Gibco-BRL, USA). The PCR conditions were 95°C for 5 min, next 30 cycles at 95°C for 1 min, annealing at appropriate temperature (50-60°C) for 1 min and extension at 72°C for 2 min followed by final extension at 72°C for 7 min.

The samples showing LOH and MA were confirmed by a second independent amplification. To verify the proper pairing of the samples, additional typing was done with different RFLP markers (NAT-KpnI, Hb-HinfI, Hb-HincIII, CYPIAI-MspI, ALAD-RsaI) as reported by Dasgupta *et al*13. In all cases the results were in concordance with the earlier findings (data not shown).

**Interpretation of LOH and MA:** The LOH and MA were interpreted in our samples by densitometric scanning (Shimidazu, CS-9000, Japan) of the autoradiographs16. The allelic loss was recorded if there was a complete absence of one allele or if the relative band intensity of one allele was reduced at least 50 per cent in the tumour in comparison to the homologous allele in the corresponding normal DNA sample. An LOH index > 1.5 (loss of the smaller allele) or <0.67 (loss of the larger allele) corresponded to at least 50 per cent reduction in relative band intensities16. The MAs were detected by a shift in the mobility of one or both alleles in comparison to the same alleles in the corresponding normal DNA17. For calculation of LOH at a locus, samples showing homozygosity and MA at that locus were not considered, whereas for calculation of MAs the samples showing LOHs only were not considered18. A sample showing loss of one allele and size alteration of the other allele at the same locus was considered for calculating both LOH and MA.

**Detection of HPV types 16 and 18:** The presence of HPV in the head and neck lesions was detected by PCR using primers (MY09 and MY011) from the consensus L1 region2. The typing of HPV type 16/18 in the L1 positive samples was done by PCR using specific primers from the HPV type 16 E6 and HPV type 18 E7 region19. The PCR products were electrophoresed in 2 per cent agarose gel and stained with ethidium bromide for visualization under UV-light and photographing. For final confirmation of the HPV types, the PCR products after gel electrophoresis were transferred to the nylon containing 8M urea for electrophoresis at 50W for 2-5 h. After electrophoresis the gels were exposed to X-ray film (Kodak, France) and kept at -80°C for 4-36 h.

This microsatellite analysis procedure used for allelotyping could detect LOH and MA in the presence of 50 per cent and 10-30 per cent tumour DNA respectively13.
membrane for Southern hybridization with $^{32}$P-labelled HPV type specific probes. As a positive control for HPVs, the DNAs from SiHa (for HPV type 16) and HeLa (for HPV type 18) cell lines (obtained from American Type Culture Collection, Manassas, VA, USA) and the HPV type specific plasmids were used.

**Statistical analysis of the clinical data:** To determine the association between the highly deleted region and different clinicopathological features (tobacco habit, nodes at pathology, tumour stages and HPV infection) of the tumours the Chi square analysis was performed. Probability values of $P<0.05$ was considered as statistically significant.

**Results**

**Analysis of allelic alterations (LOH/MA) in the HNSCC samples:** Thirty four (79%) of the 43 HNSCC tumours, showed LOH and/or MA on chr.8p for at least one marker (Figs 1,2). However, only 10 samples showed LOH and/or MA in both arms of chr8 for at least one marker. This indicated that the LOH and/or MA in chr.8p were necessary for the development of HNSCC. The preference for deletions in larger and smaller alleles was seen in 53 and 47 per cent of the affected alleles respectively (data not shown) which indicated that the deletion phenomenon was independent of the size of the allele. Overall highest frequencies (27-33%) of LOHs were observed within 13.12 Mb of chromosomal 8p21.3-23 region (D8S277-LPL) (Figs 1,3). In case of MA, the expansion and contraction of alleles were observed in 41 per cent and 59 per cent of the affected alleles respectively (data not shown). Overall highest frequency (17%) of MA was seen in the D8S261 locus (Fig. 3). The locations of the high LOHs and MAs were the same among the different anatomical sites (orofacial, oral cavity and larynx), though the frequency of alterations of the LOHs and MAs varied differentially (Fig.1).

The tumour 5184 showed either homozygosity or allelic alterations (LOH/MA) in all the markers studied, whereas the tumour D102 showed similar changes only in the chr.8p markers (Fig. 2). This might be due to the loss of either wild type chr.8 or short arm of chr.8 in the tumours 5184 and D102 respectively. In the analysis of primary (A) and secondary (B) tumour of the patient 5090, it was seen that there was a common allelic deletion of the marker D8S277 (Figs 1 and 2) indicating their common clonal origin. However the differences in allelic alterations in the markers D8S265, D8S261 and D8S257 between the primary (A) and secondary (B) sites of the tumour indicated that there might be a subclonal evolution in the secondary site due to field cancerization.

In this study 5 tumours (939, 5184, 2772, 1319 and 598) showed either loss of one allele and MA of another (LOH+MA) or MA of both alleles (MA-2) particularly in the highly deleted regions (Fig. 2).

**Analysis of allelic alterations (LOH/MA) in the dysplastic head and neck lesions:** Ten samples from dysplastic lesions from the head and neck region were analysed using the 4 markers (D8S277, D8S265, D8S261 and LPL). But no cases of LOH or MA were detected by these markers (data not shown), indicating that the deletion in the chr 8p21.3-23 region was not a prerequisite for the development of dysplastic lesions of the head and neck.
HPV type 16/18 infection in the head and neck lesions: HPV positivity was seen in 53 per cent (28/52) samples (Table). Among the HPV positive samples, 96 per cent (27/28) of these were found to be positive for HPV type 16 and the other sample was HPV type 18 positive (Fig. 2). HPV infection was absent in the dysplastic lesions but prevalent in different HNSCC tumours irrespective of primary sites, gender, lymph node involvement and tobacco habit (Table). HPV infection had no significant association with the clinicopathological parameters except differentiation grades of the tumour (P <0.05) HPV infection was higher in males (P < 0.05).

No significant association was found between HPV positivity and the highly deleted chr. 8p21.3-23 region in the 3 groups of tumours (orofacial, larynx and oral cavity). Interestingly, a significant association was also observed...
between HPV infection and the number of alleles in chr.8 showing LOH and/or MA ($P < 0.05$).

**Clinicopathological association with the highly deleted region:** No significant clinicopathological association was found between the highly deleted region in chr.8p21.3-23 with different parameters like tobacco habits, HPV status, nodes at pathology and tumour stages (stage I + II vs. stage III + IV) and site of the lesion.

**Discussion**

In the present study, we have mapped the allelic alterations (LOH and MA) on chr.8 to locate the candidate TSGs loci associated with the development of HNSCC in Indian patients. The chromosomal 8p21.3-23 region was found to be highly deleted only in the HNSCC samples with no allelic alterations in this region in the dysplastic head and neck lesions, indicating the importance of this region in the development of HNSCC tumours. Similar results have been observed in carcinomas of the head and neck, prostate, lung, breast etc., in Western patients. However, we did not find high LOH in the chromosomal 8p21 (D8S137) and 8p12 (D8S87 and D8S283) regions though others have detected high LOHs in these regions in HNSCC tumours. This might be due to the differences in etiology, ethnicity, sample preparation and the methodologies used.

The microcell hybrid experiments by Tanaka et al. have shown that chromosomal 8p12-p.ter could suppress the tumourigenecity and invasiveness of colon carcinoma cells, whereas Gustafson et al. showed that 8p22-23 could suppress only the tumourigenecity of different tumours. It can be concluded that the chromosomal 8p21.3-23 region could harbour at least one TSG responsible for the development of these tumours. The deletion mapping of chr.8 by Ishwad et al. and Sunwoo et al. in HNSCC tumours detected an overlapping high deleted region in chr.8p23, 2.88 Mb telomeric to the D8S277 marker used in our study. Similarly, Wistuba et al. reported high LOHs in D8S277 locus in lung carcinoma. Comparable to our results using the marker D8S261, Califano et al. detected highest LOHs (40%) in invasive HNSCC samples, but unlike us they also detected 13 per cent LOHs in the hyperplasia/dysplasia lesions. E1-Naggar et al. reported a high frequency of deletion at the locus LPL (48%) though we only found 29 per cent LOHs in this locus. Several candidate TSGs associated with the development of different cancers i.e., liver, ovary, prostate, colon, bladder, breast etc. have been localized in the chr.8p21.3-23 region. Among them Pin X and D8S267, GATA4 (0.26 Mb centromeric to D8S265), DLC1 (1.26 Mb telomeric to D8S265), N33 (2.5 Mb telomeric to D8S261), PRLTS (0.5 Mb telomeric to D8S261), PCM1 (0.28 Mb telomeric to LPL) are quite important. However the associations of these TSGs have not yet been tested with the development of HNSCC. The minimal deleted region seen in our analysis is about 13.12 Mb region in chr.8p21.3-23, thus more markers from this region should be studied to narrow down the candidate TSG loci.

The significant association of HPV infection with TNM stages and histopathological grades, and the absence of HPV infection in the dysplastic lesions studied indicated that HPV might be associated with the progression of some HNSCC tumours. In the Indian context, similar to our results, D’Costa et al. detected 15 per cent HPV type 16 positivity but not HPV type 18 in their samples, though Balaram et al. found a more or less equal frequency of HPV types 16 and 18 in their samples (74%). The significantly high allelic alterations (LOH/MA) in the HPV positive samples compared to the HPV negative samples indicated that HPV infection may interfere in the differentiation pathway of the basal epithelial cells leading to longer survivability of these cells mainly due to interaction of viral E6 and E7 proteins. As a result there is abrogation of cell cycle control, chromosomal alterations, telomerase in activation and cell immortalization.

The presence of a comparatively high frequency of rare biallelic alterations in the highly deleted region indicated that the LOH or MA in one allele of this region may impose selective pressure on the other allele of the same locus for deletion or size alteration. The losses of wild type chr.8 as well as its interstitial alterations seen in two tumours may be due to non-disjunction with or without reduplication. The differences in allelic alterations in some of the markers in the tumours 5090A and 5090B might be due to the subclonal evolution of a particular clone in the secondary site (5090B) compared to the primary site (5090A) though they had originated
from the same clone as evidenced by the change of same allele of the marker D8S277. Similar type of phenomenon has been seen in the analysis of CDKN2A gene in oral epithelial dysplasias and carcinomas.

Thus, it can be concluded that the deletion in chr.8p21.3-23 region may be associated with the development of invasive HNSCC. The occurrence of MAs and bi-allelic alterations, loss of normal copy of chr.8 and HPV infection might have some role in the development of HNSCC tumours. The highly deleted region could harbour candidate TSG(s) associated with the progression of the invasive HNSCC tumour.

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