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Multiple Deletions in Chromosome 3p are Associated with the Development of Head and Neck Squamous Cell Carcinoma

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KEY WORDS Head and neck squamous cell carcinoma; chromosome 3; tumor suppressor gene(s); loss of heterozygosity; microsatellite size alteration.

ABSTRACT Detailed deletion mapping was done in the chromosomal 3p21.2-22 and 3p12-13 regions, showing high deletions in our previous study, using 13 highly polymorphic microsatellite markers in 25 primary head and neck squamous cell carcinoma (HNSCC) to narrow down the candidate tumor suppressor genes' (TSGs) loci. Deletions in the different regions of chr.3p were seen to increase significantly with the progression of the clinical stages as detected by the fractional regional loss (FRL) index analysis. Five discrete areas with the following order of deletion frequency i.e. D3: 3p21.31 > D2: 3p21.32 > D1: 3p21.33 > D4: 3p21.2-21.1 > D5: 3p12.1, had been identified. Among these regions, the onset of deletions during progression of the tumor i.e. from stage I to stage IV, was suggested to occur in the following order i.e. D3 → D1& D2 → D4 & D5. The deletion in the D5 region was significantly associated with the progression of the clinical stages. Microsatellite size alterations (MAs) were seen to be high in and around the highly deleted regions. Also loss of normal copy / interstitial alterations of chr.3 in the late stages of the tumor as well as rare biallelic alterations around the highly deleted regions were seen in our samples. The Human Papilloma Virus (HPV) infection was found to be associated with the MAs in D3 region, whereas nodal involvement of the tumor was correlated with the deletions in D1, D2, D4 and D5. Thus, this study indicates that multiple tumor suppressor genes (TSGs) may be present in chr.3p whose differential deletions are associated with the development of HNSCC.

INTRODUCTION

The incidence of HNSCC tumors is fourth for men and sixth for women in the world (Parkin et al. 1993). In India, about 100,000 individuals suffer from the disease at any given time (Jayanth et al. 1991). Several etiological factors particularly tobacco / alcohol consumption and HPV infection are shown to be linked with the disease (Wu et al. 1994; Gillison et al. 1999). Among the HPVs, the high risk HPV-16 and 18 are most prevalent in the HNSCC tumors (Gillison et al. 2000). But the molecular genetic events associated with the initiation and progression of the disease, are poorly understood. The karyotypes of HNSCC tumors are complex and the chromosomal aberrations have been shown to increase during progression of the tumor (Dasgupta et al. 2002). Among the chromosomal alterations the nonrandom deletions in several chromosomal regions have been suggested to play major role in the development of this tumor (Dasgupta et al. 2002). In a microcell hybrid system, it has been shown that the normal chr.3 could suppress the tumorigenecity of oral cancer cell lines indicating the presence of at least one tumor suppressor gene in this chromosome (Dasgupta et al. 2002). In a preliminary HNSCC progression model the deletion in the chr.3p has been suggested to be necessary for the development of dysplastic head and neck lesions (Califano et al. 1996), while others have reported that deletions in chr.3p might be the late event in this tumorigenesis (El-Naggar et al. 1995). Also there is ambiguity in identification of common deleted regions in chr.3p among the different investigators (Wu et al. 1994; Maestro et al. 1993; Field et al. 1994; Roz et al. 1996; Partridge et al. 1996).

In our previous study of HNSCC tumors from Indian patients two broad chromosomal regions 3p12-13 (9.7cM) and 3p21.2-22 (16.1cM) with

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high deletions were identified and these regions overlap with the regions of deletion described in other tumors including HNSCC (Dasgupta et al. 2002). However, no one yet precisely identified the candidate TSGs' loci in chr.3p in HNSCC. Thus in order to find out how the chr.3p deletions occur during progression of the tumor and to narrow down the candidate TSGs' loci in the highly deleted regions (3p12-13 and 3p21.2-22) detailed deletion mapping was done in this study using 13 highly polymorphic microsatellite markers from these regions in 25 primary HNSCC tumors at different stages.

lesions from previously untreated and unrelated individuals with corresponding normal tissue were taken for this analysis (Table 1). The tissues were obtained from the patients treated at the hospital section of "Chittaranjan National Cancer Institute" and "Cancer Center and Welfare Home, Calcutta", after appropriate informed consent by the patients and approval of the protocol by the hospital authorities. The samples were frozen immediately after collection and stored at -80^{O}C until use.

The detail clinical history of the patients has been presented in Table 1. Clinically, the tumors were graded and staged according to the UICC TNM classification. The patients were considered as tobacco habituated if they consumed at least 10-15 cigarettes/bidis or equivalent amount of chewable tobacco per day for a minimum of 10 years.

Table 1: Clinicopathological features and FRI. index of the head and neck lesions.

Tumors	*Age/ Sex/ Religion*	*Site*	*Histo-pathology*	*Node*	*Tobacoo*	*HPV status*	*FRL index*	*Mean FRL Index*	*P-Value of mean FRL index*
Stage I									
4976	45/F/m	Alveolus	WDSCC	-	-	A	0		
7524	34/F/m	Tonsil	WDSCC	-	-	16	0		
1895	65/F/h	Buccal mucosa	WDSCC	-	+	16	0	0.12	
3970	46/M/h	Mandible	WDSCC	-	-	A	0		
2937	60/F/h	Buccal mucosa	WDSCC	+	+	16	0		
Stage II									
1649	45/F/m	Tongue	MDSCC	-	+	16	0.16		
4762	70/F/h	Tongue	WDSCC	-	-	A	0.25		
663	70/M/h	Alveolus	MDSCC	+	+	A	0.5	0.414	
2073	40/M/h	Maxilla	MDSCC	+	-	16	0.5		
1128	56/M/h	Bucall mucosa	WDSCC	-	+	16	0.66		
Stage III									
5266	40/M/h	Alveolus	WDSCC	-	+	16	0		
6946	59/M/h	Tongue	MDSCC	+	+	16	0.16		<0.05
1068	58/M/h	Buccal mucosa	WDSCC	-	+	16	0.2	0.432	
4786	55/F/h	Lip	WDSCC	-	-	16	0.4		
1332	85/F/h	Alveolus	WDSCC	+	+	A	0.83		
1665	55/M/h	Larynx	WDSCC	+	+	A	1		
Stage IV									
2878	69/M/h	Larynx	WDSCC	-	+	A	0		
5151	57/M/m	Alveolus	WDSCC	-	-	16	0.16		
1640	47/M/h	Buccal mucosa	WDSCC	+	+	A	0.5		
2785	60/M/h	Buccal mucosa	MDSCC	+	+	A	0.5		
5398	50/M/h	Buccal mucosa	WDSCC	-	+	16	0.66	0.628	
1542	48/M/h	Larynx	PDSCC	+	+	16	0.83		
5966	48/M/m	Larynx	WDSCC	+	-	16	1		
5219	48/M/h	Larynx	MDSCC	+	+	16	1		
7072	43/M/m	Maxilla	PDSCC	+	+	16	1		

Age: Years; *F:* Female, *M:* Male *h:* Hindu, *m:* Muslim, *WDSCC:* Well differentiated squamous cell carcinoma, *MDSCC:* Moderately differentiated squamous cell carcinoma;
–: Absence of nodal involvement of the tumor/No tobacco habit of the patient;
+: Presence of nodal involvement of the tumor/Patient habituated with tobacco.
A: HPV absent, *16:* HPV-16 present.

MATERIALS AND METHODS

Sample Collection and Clinical Data

Twenty-five freshly operated head and neck

Microdissection and DNA Extraction

The normal cells present as contaminants in the primary head and neck lesions were removed by microdissection procedure (Dasgupta et al. 2002). Samples containing < 60% tumor cells were not taken for analysis. The high molecular weight genomic DNA was extracted from the tissues according to the standard procedure (Dasgupta et al. 2002).

Selection of Microsatellite Markers

In order to perform high-resolution deletion mapping of the 3p21.2-22 and 3p12-13 regions, we have taken total 13 highly polymorphic microsatellite markers in and around these regions (Table 2). All the markers have shown ≥ 69% informativeness in our samples (Data not shown). There is a discrepancy in the localization of some markers between the Genome Database and the http://www.ensembl.org. In this study we have followed mainly http://www.ensembl.org in the localization of the microsatellite markers.

Microsatellite Analysis and Interpretation of LOH and MA

A standard polymerase chain reaction (PCR) analysis containing [γ-^{32}P] ATP end labeled forward primer was done in a 20 µl reaction volume as described by Dasgupta et al (Dasgupta et al. 2002). The DNA samples were amplified through 30 cycles in a thermal cycler (Techne, UK) comprising 1 min at 95^0 C, 1 min at appropriate annealing temperature ($50\text{-}60^0$ C) and 2 min at 72^0 C followed by final extension at 72^0 C for 7 min. Analysis of PCR products on denaturing polyacrylamide sequencing gels and scoring of LOHs and MAs on autoradiograms were performed as described by Dasgupta et al (Dasgupta et al. 2002). This LOHs/MAs analysis procedure could detect LOHs in presence of 50% tumor DNA whereas MAs in presence of 10-30% tumor DNA (Dasgupta et al. 2002).

Detection of HPV-16 and 18

The presence of HPV in the head and neck lesions was detected by performing PCR using primers from the consensus L1 region (D'Costa et al. 1998). The typing of HPV-16/18 in the L1 positive samples was done by PCR using specific primers from the E6 region of HPV-16 (Park et al. 1999) and E7 region of HPV-18 (Balaram et al. 1995). The PCR products were electrophoresed in 2% 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 membrane for southern hybridization with 32p-labelled HPV type specific probes (Gillison et al. 2000). As a positive control for HPVs, the DNAs from SiHa (for HPV-16) and HeLa (for HPV-18) cell lines and the HPV type specific plasmids were used.

Statistical Analysis of the Clinical Data

We utilized multiple polymorphic markers (n=13) to examine the LOH at six chromosome 3p regions viz. 3p21.33, 3p21.32, 3p21.31, 3p21.2, 3p21.1 and 3p12.1. For data analysis, we used two approaches: (1) For individual samples we determined the frequencies of LOH at individual chromosomal regions; (2) To determine whether the deletions in chr.3p were associated with the progression of the tumor, we determined frequencies of loss of individual markers using a Fractional Regional Loss (FRL) Index as defined below by Wistuba et al. (Wistuba et al. 1999):

$$\text{3p Fractional Regional Loss (FRL) index} = \frac{\text{Total number of chr.3p regions with LOH}}{\text{Total number of informative 3p regions}}$$

To correlate regional loss with the progression of the tumor, we calculated the mean FRL index for each histological category (Table 1). The 't- statistic' has been used for comparison of the mean FAL index of these histological groups.

Chi-square analysis was performed to determine the association between the tumors' genetic profile (LOHs/MAs) and different clinicopathological features like tobacco habits, nodes at pathology, tumor stages and HPV infection (Table 3 and Table 4). P-value < 0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

To understand the role of chr.3p deletions in the development of HNSCC we have studied the FRL index in six different regions of chr.3p i.e. 3p21.33, 3p21.32, 3p21.31, 3p21.2, 3p21.1 and 3p12.1, using 13 highly polymorphic microsatellite markers in 25 primary HNSCC tumors from Indian patients (Table 1 and Table 2). All the markers studied showed LOHs and/or MAs in the HNSCC samples in more than one tumor (Fig. 1 and Table 2). It was evident that the

allelic losses in these six chr.3p regions gradually increased with progression of the clinical stages, and there was a significant correlation of the mean FRL index (P value<0.05) with progression of the tumor (Table 1, Fig. 2). Thus it indicates that the accumulated deletions in the six regions are necessary for progression of the tumor. Similar phenomenon of chr.3p deletions has also been seen in the development of lung carcinoma (Wistuba et al. 1999).

Among the six chromosomal regions the high LOHs have been seen in five discrete areas with following frequency of LOHs i.e. 41-50% in D1 (D3S1561-D3S1611), 60% in D2 (D3S3685), 77% in D3 (D3S3560), 40-57% in D4 (D3S3616-D3S1295) and 29-42% in D5 (D3S3508-D3S1274) (Table 2). The deletions in the D1-D5 areas overlap with the reported deletions seen in HNSCC, lung carcinoma, breast carcinoma etc. (Dasgupta et al. 2002). Among these five areas (D1-D5), the deletion in D3 was seen to be highest and more or less constant (75-80%) throughout stage I/II and stage III/IV tumors (Fig. 3). Thus it indicates that deletion in D3 is prerequisite for the development of at least stage I/II tumors. This region is within the broad 3p14 - 25 where deletion has been suggested to be necessary for the initiation of dysplastic oral lesions (Jiang et al. 2001). In the nearby region of D3 some ESTs (Genome Database) and CDC25A gene were seen to be present (Demetrick and Beach 1993). The CDC25A is a positive cell cycle regulator (Sanchez et al. 1997). The ESTs in this region should be characterized to find out if there is any candidate TSG. The second highest frequency of deletion in the stage I/II tumors has been seen in the D1 and D2 regions with 44% and 50% LOHs respectively. But unlike deletions in the D3 region, the frequency of deletion in the D1 and D2 regions considerably increased in stage III/IV tumors with 57% and 64% LOHs respectively. Thus it seems that the deletions in these regions occur after the deletion in the D3 region and is necessary for the progression of the tumor. Among the two markers in the D1 region, the D3S1611 is intragenic of the mismatch repair gene MLH1 (Kok et al. 1997) and 50% LOHs observed in this locus has indicated a probable involvement of this gene in the HNSCC development. The DLC1, candidate TSG of lung and other cancer (Daigo et al. 1999) is localized at least 1 Mb away from this region. The role of DLC1 in the development of HNSCC has not been explored. Among the genes characterized in D2 region none have any tumor suppressor function. The CTNNB1 gene localized in this region is shown to positively regulate the cell proliferation (van Hengel et al. 1995). Unlike D1 and D2, the deletion frequencies of D4 and D5 regions in stage I/II tumors were as low as 29% and 20% respectively. But in stage III/IV tumors the deletion frequencies in D4 and D5 were comparable to that in D1 and D2 (Table 2 and Fig.3). Interestingly, there was a significant association (P value- 0.027) of the increase in deletion frequency in D5 from stage I/II to stage III/IV (Table 3). This indicates that the deletions in these regions might have some cumulative effect along with other deletions in chr.3p during the late stages of the tumor progression. The LOHs in the D5 region have been seen in different tumors eg. lung carcinoma, renal cell carcinoma etc (Xian et al. 2001). In the D5 region the candidate TSG, DUTT1/ROBO1 is found to be localized (Sunddaresan et al. 1998). The absence of this gene has been shown to affect the development of lung causing bronchial epithelial hyperplasia in a transgenic mice model system (Xian et al. 2001). However no point mutation has been detected in lung carcinoma. Also the tumor suppressor function of this gene is yet to be studied. Another candidate TSG NRC1, proposed to be involved in the different types of renal cell carcinoma, has been localized in the nearby region of D5 (Lott et al. 1998). But its involvement in the development of HNSCC has not yet been studied. However, except some ESTs no genes have been characterized in details in the nearby region of the D4. Between D3 and D4, a cluster of candidate TSGs eg. SEMA3B, RASSF1, FUS1 etc. have been found to be localized (Tse et al. 2002; Dreijerink et al. 2001; Lerman and Mirra 2000). However, low incidence of promoter hypermethylation in the RASSF1 gene as well as LOHs in this locus in HNSCC tumors has indicated that RASSF1 gene may not be involved in this tumor development (Hogg et al. 2002). Thus we have to take more markers from these regions to find out if there are any candidate TSGs' loci.

The high frequency of MAs has been seen either in the highly deleted regions or in the flanking regions (Table 2). The increase in the frequency of MAs in the later stages of tumor development indicates that MAs might provide some growth advantage during progression of the tumor. Interestingly, the prognostic

Table 2: Allele status of the chr.3 markers in the invasive HNSCC samples

Marker	*Locus*	*Distance (Mb)*	*4976*	*7524*	*1895*	*3970*	*2937*	*1649*	*4762*	*663*	*2073*	*1128*	*5266*	*6946*	*1068*	*4786*	*1332*	*1665*	*2878*	*5151*	*1640*	*2785*	*5398*	*1542*	*5966*	*5219*	*7072*	*% of LOH*	*% of MA*	
D3S 1561	3p21.33	39.3fromp-ter										-																41%, (7/17)	12%, (2/17)	D1
D3S 1611	3p21.33	0.5																										50%, (7/14)	11%, (2/18)	
D3S 3521	3p21.32	3.7																										36%, (5/14)	15%, (3/20)	
D3S 3685	3p21.32	1.6							-																			60%, (9/15)	12%, (2/17)	D2
D3S 3582	3p21.31	3.1								-																		29%, (6/21)	11%, (2/19)	
D3S 3560	3p21.31	2.7																										77%,(10/13)	13%,(12/16)	D3
D3S 1289	3p21.31	6.2																-										20%, (4/20)	0%, (0/20)	
D3S 3616	3p21.2	3	-	-		-							-						-									57%, (8/4)	8%, (1/11)	D4
D3S 1295	3p21.1	0.8																										40%, (4/10)	11%, (2/19)	
D3S 3553	3p21.1	1.6	-	-		-			-				-						-		-				-			14%, (2/14)	20%, (3/15)	
D3S 3508	3p12.1	23.9																										33%, (6/18)	10%, (2/20)	
D3S 3681	3p12.1	0.3							-	-											-							29%, (5/17)	21%, (4/19)	D5
D3S 1274	3p12.1	0.4																										42%, (8/9)	6%, (1/17)	
TNM stage			I	I	I	I	I	II	II	II	II	II	III	III	III	III	III	III	IV	IV	IV	IV	IV	IV	IV	IV	IV			

LOH, MA-1, MA-2, LOH/MA, RH, NI, ND

LOH: Loss of heterozygosity,
MA-1: Microsatellite size alteration of one allele,
MA-2: Microsatellite size alteration of both alleles,
LOH/MA: Loss of one allele and size alteration of the other,
RH: Retention of heterozygosity,
NI: Non-informative,
ND: Not done
D3S1561 is 39.3 Mb from chr.3p telomere. Then on distance of each marker is given from its preceding marker mentioned in the table.

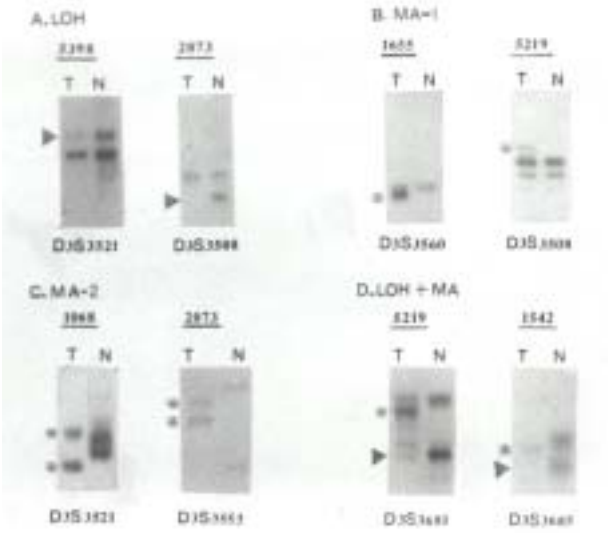

Fig. 1. Representative autoradiograph showing LOHs and MAs at different marker loci on chr.3p in different samples.

Legends to the Figures:

T: DNA of the dysplastic / tumor cells after microdissection; *N:* corresponding normal tissue or PBL. Sample numbers are same as in Table-2 indicates loss of the corresponding allele; * indicates size alteration of one or both alleles.

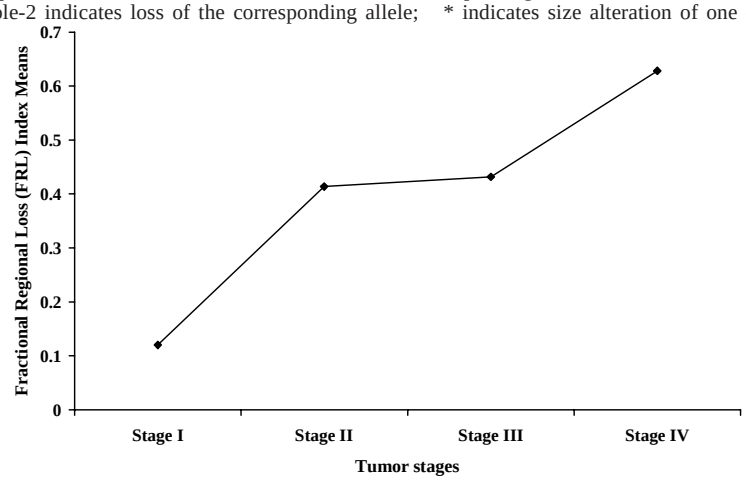

Fig. 2. LOH expressed in terms of FRL index

significance of MAs has been shown in HNSCC tumors (Arzimanoglou et al. 1998; Oh and Mao 1997). However, the actual significance of MAs in the tumor development is not clear. But the MAs have been suggested to be important parameter for molecular detection of cancer (Mao et al. 1994).

Four tumors at stage II-IV (#2073, 1542, 5966, 5219) showed either homozygosity or molecular abnormalities in all the markers indicating probable loss of normal copy of chr.3 (Table 2). Interestingly, 5 tumors at stage I-IV (#1128, 663, 1655, 1332, 7072) showed interstitial alterations (LOHs and/or MAs) particularly in some or all of the high LOHs regions (Table 2).

Like our previous study, we have seen high (28%, 7/25) bi-allelic alterations mainly in and around the highly deleted regions (Table 2). The presence of these rare biallelic alterations indicate that the LOHs/MAs in one allele might impose some selective pressure on the other allele for deletion or size alteration for growth advantage

Table 3: Clinicopathological correlation of the high LOHs regions

Characteristics	*D1 region*		*Total*	*P*	*D2 region*		*Total*	*P*	*D3 region*		*Total*	*P*	*D4 region*		*Total*	*P*	*D5 region*		*Total*	*P*
	LOH+	*LOH-*		*Value*	*LOH+*	*LOH-*		*Value*	*LOH+*	*LOH-*		*Value*	*LOH+*	*LOH-*		*Value*	*LOH+*	*LOH-*		*Value*
Tobacco+	10	8	18		8	10	18		8	11	19		8	10	18		11	7	18	
				0.443				0.465				0.923				0.348				0.309
Tobacco-	2	5	7		1	5	6		2	4	6		1	6	7		2	5	7	
HPV+	7	9	16		7	9	16		7	9	16		6	10	16		9	7	16	
				0.88				0.654				0.932				0.821				0.88
HPV-	5	4	9		2	6	8		3	6	9		3	6	9		4	5	9	
Node+	9	3	12		7	2	9		6	6	12		8	4	12		10	2	12	
				0.028				0.035				0.567				0.007				0.008
Node-	3	10	13		5	10	15		4	9	13		1	12	13		3	10	13	
StageI/II	4	6	10		2	7	9		4	6	10		2	7	9		2	9	10	
				0.806				0.446				0.676				0.349				0.027
StageIII/IV	8	7	15		7	8	15		6	9	15		8	8	16		11	4	15	

Table 4: Clinicopathological correlation of the high MAs regions

Characteristics	*D1 region*		*Total*	*P*	*D2 region*		*Total*	*P*	*D3 region*		*Total*	*P*	*D4 region*		*Total*	*P*	*D5 region*		*Total*	*P*
	MA+	*MA-*		*Value*	*MA+*	*MA-*		*Value*	*MA+*	*MA-*		*Value*	*MA+*	*MA-*		*Value*	*MA+*	*MA-*		*Value*
Tobacco+	3	9	12		2	15	17		2	16	18		2	16	18		4	14	18	
				0.526				<1				<1				<1				<0.2
Tobacco-	1	12	13		0	6	6		0	7	7		0	7	7		0	7	7	
HPV+	3	13	16		2	14	16		0	16	16		2	14	16		4	12	16	
				0.945				<1				<0.05				<1				<0.2
HPV-	1	8	9		0	7	7		2	7	9		0	9	9		0	9	9	
Node+	3	9	12		2	9	11		2	10	12		2	10	12		3	9	12	
				0.526				<0.2				<0.2				<0.2				0.526
Node-	1	12	13		0	12	12		0	13	13		0	13	13		1	12	13	
StageI/II	1	9	10		1	7	8		0	10	10		1	9	10		2	8	10	
				0.911				0.702				<1				0.651				0.911
StageIII/IV	3	12	15		1	14	15		2	13	15		1	14	15		2	13	15	

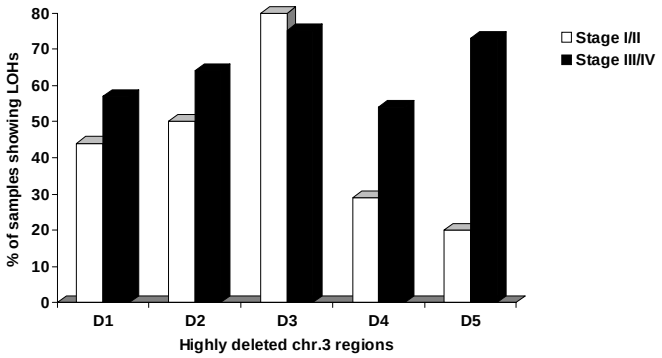

Fig. 3. Pattern of deletion in the D1-D5 regions of chr.3p during HNSCC development.

of the tumor. In the later stages of the tumor the loss of normal copy of the chr.3 and interstitial alterations suggest that these chromosomal alterations are needed for progression of the tumor. These types of chromosomal abnormalities have also been seen in the allelotyping of chr.9 in our HNSCC samples (Tripathi/ Bhar et al. in press). Similar to our analysis loss of chr.3 and chr.17 has also been reported in primary HNSCC tumors (Scholes et al. 1998; Adamson et al. 1994). In this analysis 64% of the samples (16/25) were found to be positive for HPV-16 infection by using L1 specific primers (Table 1). HPV infection was not found to be significantly associated with any of the clinicopathological parameters (Data not shown). Only LOHs in the D5 region was significantly associated with the progression of the tumor whereas allelic losses in all the regions except D3 were significantly correlated with the nodal involvement (Fig.3 and Table 3). MAs in D3 region were found to be significantly correlated with HPV infection (Table 4).

Thus, it can be concluded from our analysis that the differential deletions in the five highly deleted regions (D1-D5) seen in chr.3p in the head and neck lesions are needed for the development of specific stages of HNSCC tumor progression. The loss of function of TSGs located in these regions may have sequential cumulative effect in this tumor development. Also Huebner K suggested about the necessity of the compound mutations in different TSGs located in different chr.3p regions for the development of lung, kidney and other cancers (Huebner 2001). Along with these deletions, other molecular changes in the chromosome like MAs, interstitial alterations and loss of normal copy of chr.3 may have some role in this tumor progression by imposing some selective pressure for growth advantage of the tumor.

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