Loss of Heterozygosity on Chromosome Arm 3p in Nasopharyngeal Carcinoma

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We have examined 17 primary undifferentiated nasopharyngeal carcinoma biopsies for allelic loss on 3p, comparing the findings in tumors with those in normal lymphocyte DNA from the same patients. Ten polymorphic microsatellite markers were used between 3p13 and 3p26. Allelic loss was observed in 12 samples (70%). Two loci were most frequently affected: D3S1067 (3p21.1-14.3) in 60% and D3S1217 (3p14.2-14.1) in 58%. One tumor seemed to have a homozygous deletion at 3p26, detected by the D3S1297 marker. Analysis of the clinical data showed that an increased number of aberrations in 3p was correlated with more advanced tumor stages. *Genes Chromosom Cancer 17:118–126 (1996).*

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is the most common malignancy in males in certain populations in Southern China. The incidence per year is 32 per 10^5 in the Guangzhou region (11 × 10^6 population). NPC is also relatively frequent among the Eskimos of Greenland and Alaska, and it shows a moderate incidence in North Africa.

NPC is believed to have a multifactorial etiology. Epstein-Barr virus (EBV), environmental carcinogens, and genetic factors have been implicated. EBV DNA is detected in 100% of undifferentiated NPC (Klein et al., 1974).

Cytogenetic studies of NPC biopsy specimens and xenografts have detected structural changes in chromosome 3 (Huang et al., 1989). These researchers also found loss of heterozygosity (LOH) in the short arm of chromosome 3 in all informative cases at two sites, 3p25 (*RAF1*) in 10 of 10 cases and 3p14 (D3S3) in 9 of 9 cases, using Southern hybridization with polymorphic probes (Huang et al., 1991). Deletions in 3p are thus common in NPC.

In renal cell carcinoma of the clear cell type (RCC), 3p losses occur in nearly 90% (Kovacs et al., 1988; van der Hout et al., 1993). In the majority of the RCC, LOH has been detected at 3p25, 3p21, and 3p13-p14 (Yamakawa et al., 1991; Foster et al., 1994; van den Berg et al., 1995).

Recently, the gene responsible for von Hippel Lindau disease (*VHL;* 3p25-26) was found to be mutated in more than 50% of RCC and LOH was detected in 84% and 98% of the tumors (Gnarra et al., 1994; Shuin et al., 1994). However, mutations were not found in NPC (Sun et al., 1995b), suggesting that this gene may not be involved in the development of NPC.

In human lung cancer, allelic losses occur most frequently at regions 3p25, 3p21-p22, and 3p13-p14 (Hibi et al., 1992; Yokoyama et al., 1992; Daly et al., 1993). Homozygous deletions in human small cell lung cancer (SCLC) cell lines were also found at 3p21 (Daly et al., 1993; Yamakawa et al., 1993; Kok et al., 1994) and 3p12 (Latif et al., 1992).

In human uterine cervical cancer, a common region of LOH was identified at 3p13-p21.1 (Kohno et al., 1993; Jones et al., 1994) and in 75% of the cases allelic loss was detected at 3p13-p14.3 (Jones et al., 1992).

These findings can be taken to indicate that 3p contains several suppressor genes, with *VHL* as the most distal one and the cervical carcinoma deletion as the most proximal. The other solid tumors need more precise mapping in order to decide whether the same or closely adjacent regions are affected by deletions in 3p. As a first step toward a comparative study, we wished to map the 3p losses in NPC in more detail, using CA repeat polymorphic markers.

MATERIALS AND METHODS

Biopsies

NPC biopsies were obtained from untreated patients collected at the Ear-Nose-Throat Hospital, Guangzhou Provincial Hospital, and Shanghai

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Cancer Hospital, China. A portion of the tissue was subjected to histopathological examination, whereas the remainder was snap-frozen and kept at −70°C for subsequent DNA analysis. All tumors were undifferentiated NPC according to the World Health Organization (WHO) classification.

Clinical staging of the tumors was performed according to the tumor-nodes-metastasis (TNM) classification. The extension of the primary tumor was indicated as tumor stage T. For T0, the primary tumor was invisible, but histological examination showed a precancerous state. T1 indicated that the tumor was confined to one wall of the nasopharynx. In T2, the tumor had extended beyond two walls, but remained confined to the nasopharynx. In T3, the tumor had extended beyond the nasopharynx or involved the base of the skull. The T4 designation was used when the two alternatives of T3 occurred together. The N series of designations indicates the involvement of the regional lymph nodes. N0 indicates cases without palpable lymph nodes. N1 designates the involvement of a single, homolateral lymph node that remains smaller than 2 × 2 cm. N2 indicates contralateral, homolateral, or bilateral lymph nodes larger than 2 × 2 cm, but smaller than 8 × 8 cm, while N3 indicates lymph nodes larger than 8 × 8 cm or extending into the supraclavicular area (Hu et al., 1995).

Seventeen tumor samples from 17 patients were examined in parallel with normal lymphocyte DNA from the same donor. Twenty NPC biopsies from an equal number of patients where normal lymphocyte samples were not available were tested as well.

DNA Extraction

High molecular weight DNA was isolated from tumor tissue and blood lymphocytes from the same NPC patients by phenol extraction (Sambrook et al., 1989). To ascertain that the material contained a high proportion of tumor cells, we exploited the fact that low differentiated and anaplastic NPC carry 14–41 copies of EBV DNA per cell (Klein et al., 1974). All tumor samples were tested for presence of viral DNA by Southern blot hybridization using the 1.9 kb *Xho*1 fragment of BNLF1 (Hu et al., 1991). Raji DNA (50 copies per cell) was used as positive control. Only NPC tumor samples that contained more than 10 EBV DNA copies were included.

Microsatellite Analysis

Eighteen markers located on chromosome arm 3p were used (Table 1). Fourteen of them were polymorphic microsatellite CA repeat markers. Polymerase chain reaction (PCR) primers were obtained from Scandinavian Gene Synthesis AB, ISOGEN Bioscience, and the Nordic Primer Resource (Department of Clinical Genetics, University Hospital) in Uppsala, Sweden.

PCR was carried out in a volume of 25 μl containing 2.5–5.0 pmol of each primer, 0.2 mM of each dNTP, 50–200 ng template DNA, and 1 unit of ampli *Taq* DNA polymerase from Perkin Elmer (Norwalk, CT) or DynaZyme from Finnzymes Oy. One of the paired primers (0.25 pmol) in the reaction mixture was end-labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase (Amersham, Buckinghamshire HP7 9NA, UK). Samples were processed through 35 cycles comprising 30 sec at 94°C, 1 min at the appropriate annealing temperature, and 2 min at 72°C in a Techne PHC-3 and Perkin Elmer thermal cycler.

Aliquots of the amplified DNA were electrophoresed on denaturing 6% polyacrylamide DNA sequencing gels that were left to expose X-ray films.

RESULTS

Sensitivity of Marker

Most markers in Table 1 are included in the consensus order established at the second-generation yeast artificial chromosome (YAC)-contig map of human chromosome 3 which integrates both physical and genetic data including somatic-cell-hybrid analysis, YAC clones-end probes, ALU-PCR and fingerprint data, and the current genetic map (Gemmill et al., 1995; Naylor et al., 1996a,b). In view of the fact that each tumor sample contains an unknown quantity of normal host cells, potentially capable of giving false positive signals, the percentage LOH figures must be considered conservative, minimum estimates. In order to assess the sensitivity of our conditions for microsatellite analysis, we performed titration experiments with artificial mixtures of signal positive and negative cells. Human and mouse DNAs were mixed in different proportions and microsatellite analysis was performed at standard conditions for several of our primers. Three different primers were tested, corresponding to high, low, and intermediate frequencies of LOH in the NPC samples, as detailed below. All three primers showed approximately the same sensitivity. We found a linear relation to signal intensity up to 30% of human DNA (Fig. 1). About 5% of human DNA could be detected against a background of 95% of mouse DNA. This

TABLE 1. CA Markers in the p26-3p14 Region Used for the Allelic Loss Study

Locus	Marker used for[a]	Location	References[b]
D3S1307	A	p26.3	G1, G2, N1
D3S1297	A	p26.3-p26.2	G1, G2, N1
D3S1539	B	p26	G1, N2, Smith and Glover (1995)
D3S1620	B	p26.1-25.3	G1, G2, N1
D3S1560	B	p26.1-25.3	G1, G2, N1
D3S1304	A	p25.3	G1, G2, N1
D3S1597	B	p25.3	G1, G2, N1
VHL	B	p25.2	G2, N1
NL1Z106R	B	p25.2-25.1	Allikmets et al. (1994), N1[c]
D3S1298	A	p22.1	G1, G2, N1
D3S 966	A	p21.3	A, N1, Jones et al. (1992)
D3S1029	A	p21.31	A, G2, N1
D3F15S2	B	p21.31	G2, N1
GNAI2	B	p21.31	G2, N1
D3S1067	A	p21.1-14.3	N2, Jones et al. (1992)
D3S1228	A	p14.3-14.1	N1, N2, Jones et al. (1992)
D3S1217	A	p14.2-14.1	N1, N2, Smith and Glover (1995)
D3S1210	A	p13	G2, N1, N2

[a]A = markers used in LOH study for all samples; B = markers used only for checking the deletion region or estimating the size of deletion.
[b]The localization of markers has been placed by these authors: A: Ariyama et al. (1995); G1: according to Genethon genetic map (Gyapay et al., 1994); G2: Gemmill et al. (1995); N1: Naylor et al. (1996a); N2: Naylor et al. (1996b).
[c]The marker (also called D3S1652) is not ordered at the second-generation YAC-contig map, but mapped by hybridization using a reference panel of somatic cell hybrids containing fragments of chromosome 3 and by FISH.

means that allelic imbalance can be detected even in the presence of 30% normal DNA contamination.

Frequency of LOH in Chromosome 3

The 10 microsatellite markers used in this study are listed in Table 1. A total of 17 primary undifferentiated NPC were examined for LOH (Fig. 2). LOH was detected in 12 of the 17 tumors (70%). The remaining cases retained both alleles at all informative sites between D3S1307 and D3S1210.

LOH was particularly frequent at the D3S1067 (9/15 cases, 60%) and D3S1217 (7/12 cases, 58%) loci.

Extensive LOH was detected in four samples: T7, T24, T4, and T35. The T7 tumor showed LOH for all informative markers, indicating that the entire short arm of chromosome 3 was lost. In one sample (T24), all markers distal to D3S1217 displayed LOH, indicating the presence of a terminal deletion of 3p. T3, T8, T10, T26, and T36 showed LOH of internal markers but maintained heterozygosity for more terminal markers. This is consistent with the presence of interstitial deletions. Only 1 of 11 tested samples (9%) showed allelic loss at locus D3S1304 (3p25.3). One of 7 (14%) showed LOH for marker D3S1228 (3p14.3-p14.1).

Frequently Deleted Regions

Twelve of the 17 cases contained deletions. The pattern of allelic loss is presented in Figure 3. With the exception of the single sample mentioned above (T7) that was monosomic for the entire 3p, 11 of 12 tumors showed partial deletions. Nine tumors (T7, T4, T24, T35, T14, T13, T10, T8, and T3) showed a deletion in the 3p14.3-p21.1 region, as indicated by D3S1067 (9/15, 60%). This coincides with the results of an LOH study in RCC (Foster et al., 1994). Seven of 12 tumors (58%) that showed LOH at D3S1217 (3p14.2-p14.1) also displayed LOH at marker D3S1067 (3p21.1-p14.3).

Six tumors (T4, T35, T8, T10, T13, and T14) showed a "zebra-like" pattern of LOH in that some markers showed LOH, whereas others located between them were still heterozygous. For verification of this pattern, we performed further tests with T35. In this tumor, the markers D3S1297 (3p26.3-p26.2) and D3S1298 (3p22.1) showed LOH, whereas both alleles of the marker D3S1304 (3p25.3), located between them, were maintained without any sign of allelic imbalance. We subsequently tested two additional markers, D3S1560 on the telomeric and D3S1597 on the centromeric side of D3S1304. D3S1560 showed allelic imbalance, whereas D3S1597 had a normal

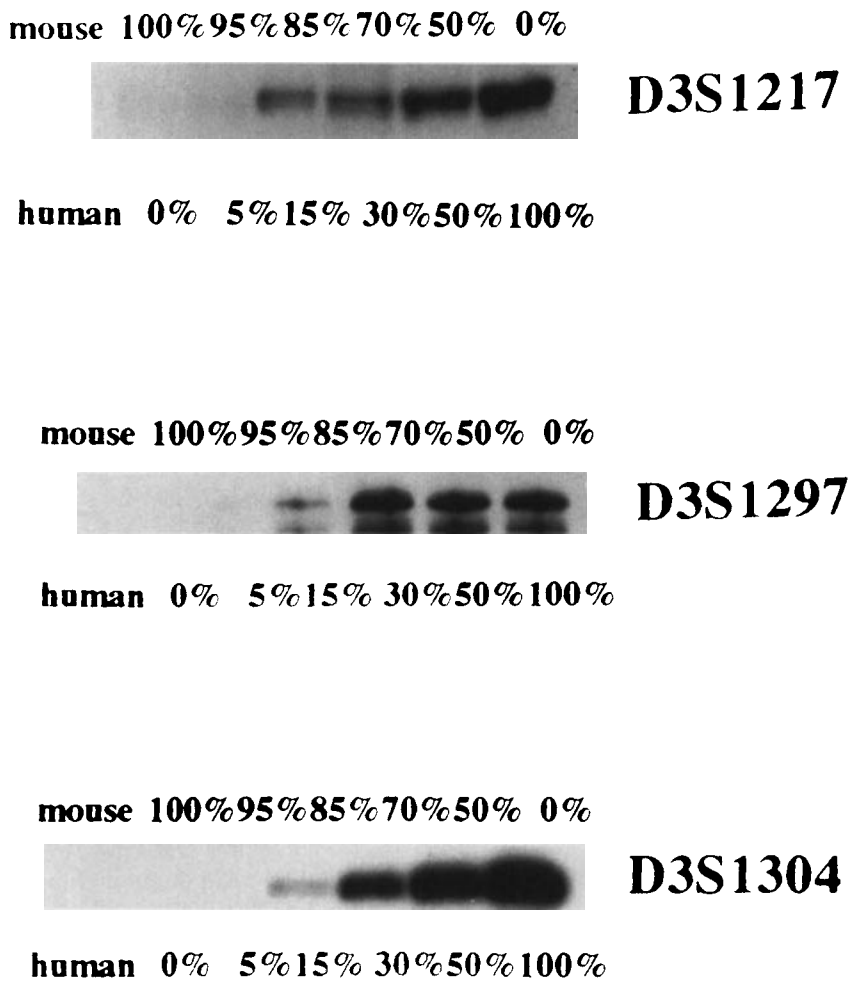

Figure 1. Model experiment to test sensitivity of different microsatellite markers demonstrating minimal (D3S1304), intermediate (D3S1297), and maximal (D3S1217) allelic imbalance in NPC. Human and mouse DNAs were mixed in different proportions, and microsatellite analysis was performed under standard conditions. The results show linear dependence of signal intensity up to 30% of human DNA. All markers show comparable sensitivity.

pattern. This result confirmed the existence of an allelic deletion at 3p26.1-25.3.

Homozygous Deletion

In addition to the samples reported above that were compared to matched lymphocytes from the same patient, we examined 26 additional NPC biopsies from patients whose lymphocyte samples were not available. In one of them, T62, the locus D3S1297, mapped at 3p26.2-p26.3, was not found (Fig. 3A). Ten other microsatellite markers were all maintained, including the marker D3S1307 that is adjacent to D3S1297. To confirm this result, the additional markers D3F15S2 (3p21.31), *GNAI2* (3p21.31), and *VHL* (3p25.2) were tested as an internal control in duplex PCR, in parallel with D3S1297 and other markers already mentioned. The primers for D3S1297 gave no PCR products, in contrast to the other three primers that produced specific bands in the same reaction mixture. D3S1297 generated a weak, specific band with T62 DNA, however, if two subsequent PCR reactions were performed, each with 30 cycles. We suggest that this PCR product was generated by contaminating normal DNA in the tumor sample. If this interpretation is correct, it excludes the possibility that the deletion of the D2S1297 marker from the tumor tissue is spurious and due to polymorphism. In order to obtain some information on the size of the deletion, the two markers closest to the deletion were subsequently tested. One was a more distal marker (D3S1539) and one a more proximal marker (D3S1620). The primers for both markers generated two bands, without any allelic imbalance. This is additional proof of homozygous

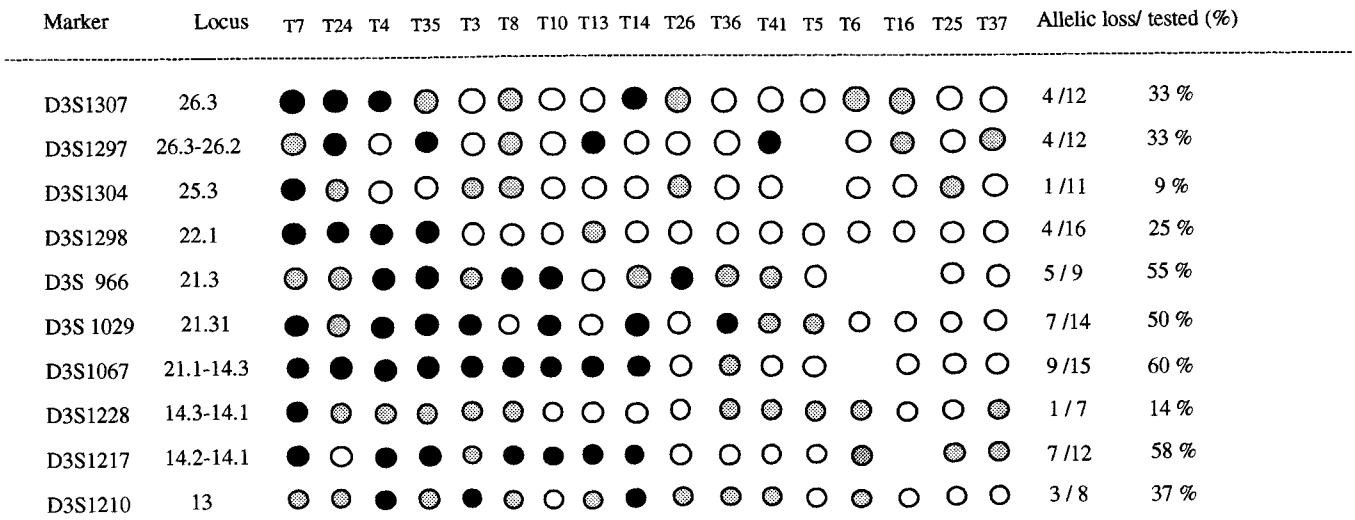

Figure 2. Schematic representation of LOH in 3p in 17 NPC. Each column corresponds to one NPC. Case numbers are shown at the top. The data were obtained by PCR over CA repeats with the markers in Table 1. In each case, the tumor DNA was tested in parallel with lymphocyte DNA from the same patient. Some alleles in some cases were non-informative due to homozygosity. Empty space indicates that this sample was not tested due to shortage of materials. (○) Both alleles retained; (●) LOH; (◎) non-informative.

deletion at this locus and indicates that the size of the deletion was less than 7 cM (Fig. 3B).

Comparison With Clinical Status

Table 2 shows the extent of LOH in relation to clinical stage. Four tumors (T7, T24, T4, and T35) that showed extensive LOH (G4, see footnote to Table 2) were stage III–IV, and none of them was classified as stage II. Eight of the nine patients that showed LOH in the 3p21.1-p14.1 region were in stage III–IV. In contrast, 7 of 8 G1 patients who showed no LOH in this region were stage II.

Four of 9 tumors with LOH at 3p21.1-p14.3 had metastases, whereas only 1 of the 8 tumors without LOH had metastases.

DISCUSSION

It has been shown that multiple loci may be deleted or mutated within a single tumor type (Van der Hout et al., 1991). This can be exemplified by Wilms' tumor, where several loci on 11p may be lost or mutated (Jeanpierre et al., 1990). Also, the same locus may be lost in more than one tumor type (Brauch et al., 1990). Deletions of the short arm of chromosome 3 are found in many tumors, particularly in carcinomas. They may affect different 3p regions in the same type of tumor. In human lung cancer, loci at 3p25, 3p21-p22, and 3p13-p14 are most frequently deleted (Hibi et al., 1992; Yokoyama et al., 1992; Daly et al., 1993). Common regions of deletion have been identified at 3p13-p21.1 in endometrial cancer and at 3p13-p14.3 in cervical cancer (Jones et al., 1992). Two separate regions, 3p14-p13 and 3p26-p24, have been found to be independently deleted in some breast cancers (Chen et al., 1994).

LOH has been found in 3p22-p21, including D3S1298 and D3S1067 and proximal to D3S1235 and D3F15S2 in both lung cancer (Kok et al., 1987; Hibi et al., 1992) and other cancers such as RCC and gastric cancer (Yamakawa et al., 1993; Schneider et al., 1995; Van den Berg et al., 1995). A second region proximal to D3S1228 has shown frequent LOH in other cancers as well, such as cancer of the larynx and breast (Chen et al., 1994; Sun et al., 1995a). These data suggest that 3p deletions may be different in different tumors.

As a step toward a comparative analysis, we attempted to map 3p losses in NPC by examining 10 polymorphic microsatellite loci for allelic imbalance or deletion. The markers spanned the region 3p13 to 3p25-26. Matched tumor-lymphocyte samples were examined from 17 patients. Twelve of the 17 analyzed samples (70%) had LOH in 3p. The most frequently changed regions were 3p21.1-14.3 (60%) and 3p14.2-14.1 (58%).

Since we relied on only clear cases of LOH, these figures may be considered minimal estimates. Admixture of normal tissue may obscure marker losses.

LOH at both the proximal and distal parts of 3p, with markers retained between them, was observed in tumors T4, T35, T8, T10, T13, and T14 and suggests the occurrence of partial deletion and sometimes multiple deletions. A similar "zebra

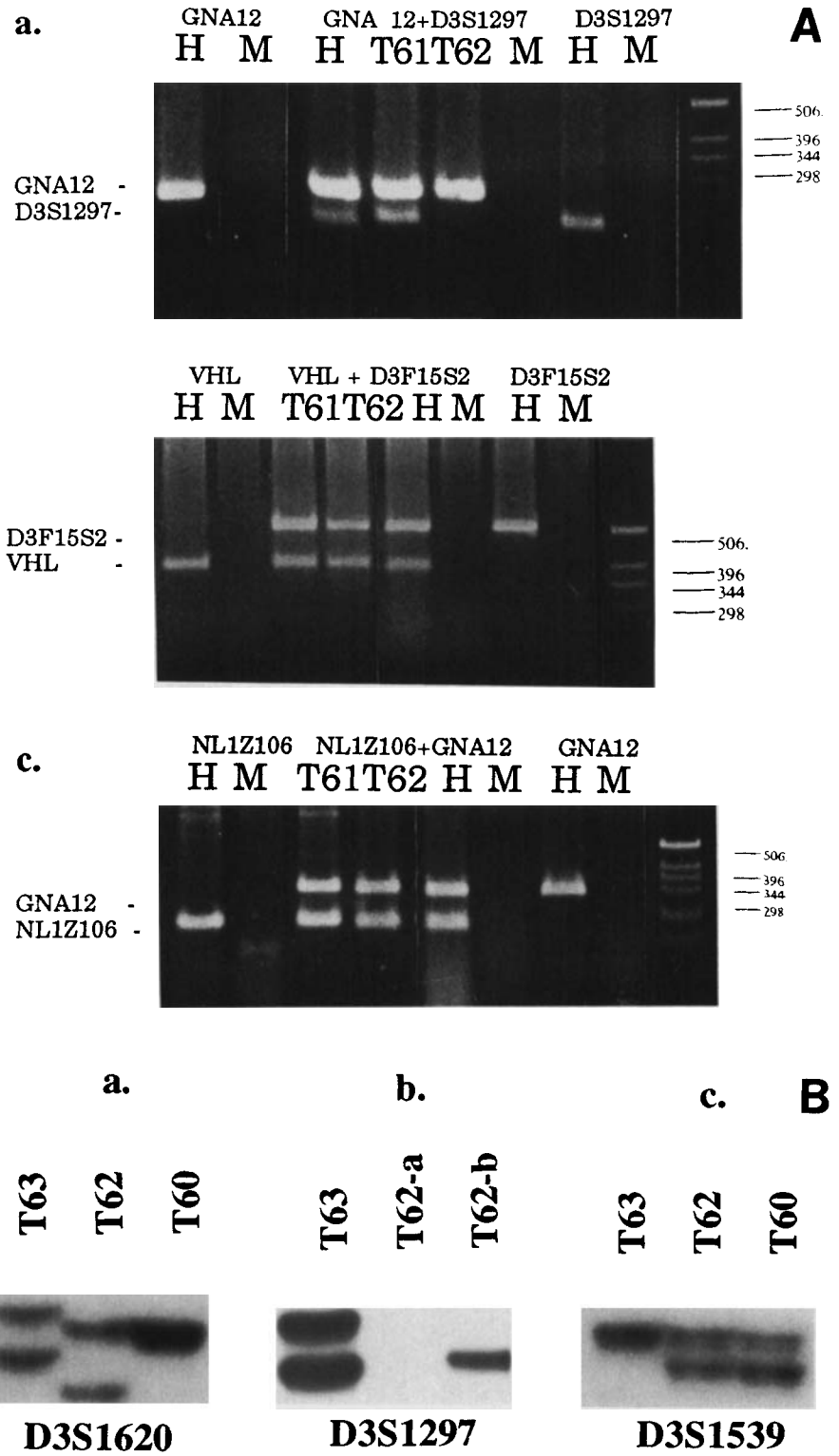

Figure 3. **A:** Detection of a homozygous deletion in tumor T62. Tumor DNA from another sample (T61), normal human lymphocyte DNA (H), and mouse DNA (M) were used as controls. Five additional markers surrounding D3S1297 were analyzed: (**a**) simultaneous test of *GNAI2* and D3S1297; (**b**) simultaneous test of *VHL* and D3F15S2; (**c**) simultaneous test of *NL1Z106R* and *GNAI2*. **B:** Homozygous deletion in tumor T62. Two markers, D3S1539 (telomeric) and D3S1620 (centromeric) adjacent to the deleted D3S1297 were used to check the deletion in three NPC samples. The D3S1297 marker was lost in the T62 tumor, whereas all markers were retained in three samples. T62-a and T62-b in the middle panel represent a single round and three rounds of 30 cycles PCR with marker D3S1297, respectively.

TABLE 2. Relationship Between Clinical Stage and Allelic Loss on 3p

Name	Gender	Age (years)	Tumor type[a]			Tumor size (T0–4)	Cervical lymph nodes	Hematogenous metastasis[b]		Clinical stages I–IV	Grade of allelic loss[c]
			C	G	M			Bone	Nerve		
T7	M	65			+	T3	N0	+	−	III	G4
T24	M	55	+			T3	N0	−	−	III	G4
T4	M	50			+	T4	N0	+	+	IV	G4
T35	M	45			+	T4	N0	+	+	IV	G4
T3	M	65			+	T3	N0	−	−	III	G2
T8	M	47	+			T2	N3	−	−	III	G2
T10	M	64			+	T2	N0	−	−	II	G3
T13	M	62	+			T3	N0	−	−	III	G3
T14	M	61			+	T4	N1	+	+	IV	G3
T26	M	56	+			T2	N2	−	−	II	G1
T36	M	58			+	T2	N0	−	−	II	G1
T41	M	52	+			T2	N0	−	−	II	G1
T5	M	65			+	T2	N0	−	−	II	G1
T6	M	19		+		T2	N0	−	−	II	G1
T16	F	46			+	T2	N0	−	+	III	G1
T25	M	53			+	T2	N0	−	−	II	G1
T37	F	39	+			T3	N0	−	−	II	G1

[a]C = cauliflower tumor; M = massive tumor; G = granular tumor.
[b]Bone = local bone infiltration; nerves = local nerve infiltration.
[c]G4: extensive loss including marker D3S1067 and marker D3S1217; G3: loss of both markers D3S1067 and D3S1217; G2: loss of one marker, either D3S1067 or D3S1217; G1: no loss or loss of neither marker D3S1067 nor D3S1217.

pattern" has been reported in primary RCC (Lubinski et al., 1994; Van den Berg et al., 1995), lung carcinoma (Hibi et al., 1992; Yokoyama et al., 1992), and NPC (Lo et al., 1994). In one previous study on RCC (Kovacs et al., 1988), only terminal deletions were found in 3p, with or without an associated non-reciprocal translocation.

That marker D3S1304 in 3p25.3 was retained in T35 was confirmed by the use of markers on both sides of the region. The more telomeric marker (D3S1560) was deleted, whereas the more centromeric marker (D3S1597) was retained.

One possible source of error might stem from a difference in the sensitivity of the different markers to normal tissue contamination. Would a marker signaling LOH in 58–60% of the tumors be less sensitive to normal tissue contamination than a marker that was lost in only 9% of the tumors? This was approached by model experiments with artificial mixtures of human and mouse DNA. These experiments indicate that LOH from tumors could be detected in the presence of 30% normal tissue contamination, with markers showing high, low, and intermediate loss frequencies from the tumor material (Fig. 1).

The homozygous deletion found in the T62 tumor is of particular interest. Genetic polymorphism is unlikely since a weak signal was detected when the number of cycles was increased, probably derived from contaminating normal tissue. In this region, allelic loss was localized between the *VHL* locus and the more distal D3S1304 marker in the T13, T14, and T41 tumors; the same region was involved in homozygous deletion at D3S1297 in T62. Frequent losses have also been found in the same region in esophageal and oral cancer (Wu et al., 1994; Ogasawara et al., 1995).

LOH in 3p in NPC tumors has been studied previously. Huang et al. (1991) reported 100% LOH at two chromosomal loci: *RAF*1 (10/10 cases at 3p25) and D3S3 locus (9/9 cases at 3p14) by Southern blot hybridization using chromosome 3 specific polymorphic probes. In another study, they showed that LOH was observed in 18 of 27 NPC biopsies (67%; Lo et al., 1994). A high frequency of LOH was found at several loci: D3S1038 (3p25) in 9/17 informative cases (53%), D3S1228 (3p14.3-p14.1) in 7/14 cases (50%), D3S659 (3p13) in 10/20 cases (50%), and D3S1076 (3p21.1) in 9/19 cases (47.4%) using CA marker analysis (Lo et al., 1994). We find the most frequent deletions at D3S1067 (3p21.1-p14.3; 60%) and D3S1217 (3p14.2-p14.1; 58%). Our results show LOH on 3p in 70% of NPC, which is in line with previous findings of 67% using

the same methodology (Lo et al., 1994). There is, however, a discordance regarding the precise localization of the most frequently deleted regions. In particular, Lo et al. (1994) found 53% allelic deletion at 3p25.

The extent of LOH appeared to be correlated with tumor progression, as tumors in the more advanced stages III and IV showed a larger frequency of LOH than did tumors in stages I and II. The difference was significant ($P < 0.005$), judged by the Fisher exact, two-tailed method. This finding is consistent with the increase of LOH associated with progression in breast cancer and other tumors (Eiriksdottir et al., 1995). In the case of NPC, this point needs confirmation in larger material.

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