

High Resolution Allelotype of Microdissected Primary Nasopharyngeal Carcinoma¹

Kwok-Wai Lo,² Peter M. L. Teo, Angela Bik-Yu Hui, Ka-Fai To, Yuen-Shan Tsang, Sylvia Yat-Yee Chan, Ko-Fung Mak, Joseph C. K. Lee, and Dolly P. Huang

Departments of Anatomical and Cellular Pathology [K-W. L., A. B-Y. H., K-F. T., Y-S. T., S. Y-Y. C., K-F. M., J. C. K. L., D. P. H.], and Clinical Oncology [P. M. L. T.], Prince of Wales Hospital, The Chinese University of Hong Kong, Hong Kong SAR, China

Abstract

Nasopharyngeal carcinoma (NPC) is a common cancer in South China but is rare in other parts of the world. To better understand the molecular basis of this cancer, we performed high-resolution allelotyping on 27 microdissected primary tumors using 382 microsatellite markers. We have detected high frequencies of allelic imbalance on 3p (96.3%), 9p (85.2%), 9q (88.9%), 11q (74.1%), 12q (70.4%), 13q (55.6%), 14q (85.2%), and 16q (55.6%). Nonrandom allelic changes of 12q and 16q were revealed for the first time. In addition, loss of heterozygosity on chromosomal arms 1p (37.0%), 5q (44.4%), and 12p (44.4%) were also common in NPC. Multiple minimally deleted regions, 7–40 cM, were identified at 3p14–24.2, 11q21–23, 13q12–14, 13q31–32, 14q24–32, and 16q22–23. Frequent deletions of these minimally deleted regions implied the presence of tumor suppressor genes that may be involved in the development of NPC. Consistent loss of heterozygosity on 3p, 9p, and 14q in almost all tumors suggested that such changes are critical events in NPC tumorigenesis.

Introduction

NPC³ poses one of the serious health problems in Hong Kong. It is the third common cause of cancer deaths in our male population. This cancer has a peculiar geographic and ethnic distribution. NPC is prevalent among southern Chinese, including those in Hong Kong, with the incidence rate 25-fold higher than most other countries. Radiotherapy is an effective treatment for NPC, and >80% of patients with early disease are curable. Unfortunately, most of the NPC patients are diagnosed at later stages, where treatment is much less effective and difficult. If these patients can be diagnosed earlier or if relapses can be predicted sooner, clinical management would be improved greatly.

Previous etiological studies demonstrated that the development of NPC might be attributable to a complex interaction of genetic factors, dietary exposure to chemical carcinogens, and EBV infection (1). Relatively little information of the NPC-associated genetic alterations is known. Hence, identification of genetic changes in this cancer is crucial in revealing the molecular basis of NPC tumorigenesis. This may lead to the development of molecular markers for early diagnosis, prognosis, and monitoring of disease progression. Previous studies have shown that mutations of the common tumor suppressor, *Rb* and *p53* genes, are rare in NPC (1). LOH studies unveiled frequent allelic losses on chromosomes 3p, 9p, 11q, 13q, and 14q (2–9). The highest

frequencies of allelic deletion were found on 3p and 9p. By detailed mapping and investigation of candidate tumor suppressor genes in 9p, we illustrated the inactivation of the *p16* gene to be important in the tumorigenesis of NPC (4, 10, 11). A functional tumor suppressor locus has also been identified on 3p21 by monochromosome transfer (12). Recently, CGH study has illustrated high incidences of loss and gain of multiple chromosome arms (3p, 9q, 11q, 12q, and 14q) in this cancer (13). To further investigate the critical genetic events leading to the tumor evolution, we performed a comprehensive allelotype analysis of microdissected primary NPC using 382 microsatellite markers. We aim to determine the frequency of chromosomal deletion and the extent of deleted regions on all autosomal arms. The subsequent high-resolution allelotypes will identify MDRs of the genome that may harbor candidate tumor suppressor genes important for the development and progression of NPC.

Materials and Methods

Patients. Primary NPC biopsies were obtained from 27 patients with consent prior to treatment at the Department of Clinical Oncology in the Prince of Wales Hospital. The corresponding peripheral blood sample of each patient was also collected as constitutional DNA control. The tissue samples were embedded in OCT compound and stored at -80°C until used. All of the NPC tumors were histologically confirmed by a pathologist. Among these 27 NPCs, 24 cases were classified as undifferentiated carcinoma (WHO III), 2 were poorly differentiated (WHO II), and one was well differentiated (WHO I), according to the WHO (1978) classification (14). The mean age of the patients was 48.5 ± 11.5 . The sex ratio (male:female) was 2.9:1. The clinical disease stage of patients at presentation ranged from stage I to IV (Union International Centre Cancer staging classification; Ref. 15).

Microdissection and DNA Extraction. For each primary tumor, 40–60 serial frozen sections (5 μm thick) were subjected for microdissection manually or by laser capture microdissection, under the guidance of a pathologist. All sections were lightly stained with hematoxylin. Neoplastic cells of the tumor samples were isolated and collected for DNA extraction. Isolated tumor cells and blood samples were digested with proteinase K and extracted with phenol/chloroform as described (2).

High-Resolution Allelotyping. Three hundred and eighty-two microsatellite loci derived from the 22 autosomes were examined in this high-resolution allelotyping study. Fluorescent dye-labeled primer pairs flanking polymorphic microsatellite loci were obtained from Applied Biosystems (Foster City, CA). All primer pairs used were from the ABI PRISM Linkage Mapping Set MD-2.⁴ The average interval of the loci is about 10 cM. Multiplex PCR was set up to examine two loci in each reaction. Each PCR reaction mixture contained 50 ng of DNA, 0.33 μM each primer, 10 mM Tris (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 250 μM each deoxynucleotide triphosphate, and 0.6 unit AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA) in a total volume of 7.5 μl . The PCR reactions were performed in an ABI PRISM 877 integrated thermal cycler (Applied Biosystems). Following the directions of the manufacturer, amplification was started with 15 min at 95°C , followed by 10 cycles composed of 15 s at 94°C , 15 s at 55°C , and 30 s at 72°C , and then 23 cycles composed of 15 s at 89°C , 15 s at 55°C , and 30 s at 72°C . Amplified PCR products for multiple loci were pooled, electrophoresed on an ABI PRISM 377

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² To whom requests for reprints should be addressed, at Department of Anatomical and Cellular Pathology, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong SAR, China. Phone: 852-26321136; Fax: 852-26497286; E-mail: kwlo@cuhk.edu.hk.

³ The abbreviations used are: NPC, nasopharyngeal carcinoma; LOH, loss of heterozygosity; MSI, microsatellite instability; FAL, fractional allelic loss; MDR, minimally deleted region; CGH, comparative genomic hybridization.

⁴ Internet address: <http://www.pebio.com/ab/apply/dr/lmsv2/>.

automated DNA sequencer (Applied Biosystems), and analyzed with Genescan 2.1 software (Applied Biosystems).

Assessment of LOH. For each informative locus of the NPC samples and its corresponding normal control, the allelic ratio (*AR*) was calculated. The *AR* was determined by measuring the peak height of the smaller allele (allele 1) relative to that of the large allele (allele 2). The LOH value was defined as follows: LOH value = *AR* of normal/*AR* of tumor. Depending on which allele is lost, allele 1 or allele 2, the LOH values of <0.5 or >1.5 were considered to be indicative of LOH. Both allelic loss and gain can be presented as LOH in microsatellite analysis. We have used CGH to confirm whether our allelic imbalance refers to gain or loss. CGH analyses have been performed in 15 of 27 cases of these NPCs and reported previously (13). Gains of chromosomes 1q, 8q, 12p, and 12q were detected frequently in these cases. As a result, LOH on these chromosome arms were considered as allelic imbalance. MSI was defined as shifts of bands of tumor sample when compared with the corresponding normal control.

Results

We have comprehensively investigated the allelic status for each tumor using a panel of 382 microsatellites mapping to 22 autosomes. The average interval of the markers is about 10 cM. All 39 nonacrocentric chromosomal arms were assessed at five or more loci for LOH. An average of 264 (69.2%) informative loci/case was detected in the NPC tumors. All nonacrocentric chromosomal arms were informative for all tumors in this high-resolution allelotype analysis. Sensitivity of LOH study of NPC tumor tissues is limited by the purity of tumor specimens, which are inevitably contami-

nated to various degrees by nonneoplastic elements. The contamination may mask the true genetic alterations and their frequencies. To avoid such problems, microdissection of the tumor specimens has been used to enrich the tumor cells. In the current study, the content of the neoplastic cells was >90% in each sample after microdissection. LOH was clearly observed in these microdissected tumor samples. Representative examples of LOH are shown in Fig. 1. LOH of each locus was scored according to the criteria mentioned in "Materials and Methods." Figs. 2 and 3 summarize the percentage of LOH at each chromosomal arm. LOH affecting loci on multiple chromosomes were observed in all of the NPC samples. Frequencies of LOH for individual chromosomal arms varied between 7.4% (21q) and 96.3% (3p). The mean percentage of LOH was $32.8\% \pm 19.4\%$. In the present study, 52.2% (mean percentage + SD: $32.8 + 19.4\%$) was chosen to be a significant percentage of LOH in NPC. This represents the 99% confidence upper limit for the overall rate of random chromosome loss or imbalance in tumors. Frequencies of LOH on 3p (96.3%), 9p (85.2%), 9q (88.9%), 11q (74.1%), 12q (70.4), 13q (55.6%), 14q (85.2%), and 16q (55.6%) were higher than this baseline level (52.2%). Among these eight chromosome arms, the remarkable high frequency of LOH on 3p, 9p, 9q, and 14q indicated that such alterations play critical roles in NPC. The high incidences of LOH on 3p, 9p, 9q, 11q, 13q, and 14q had also been implicated in previous studies (2-9). However, the frequent microsatellite ab-

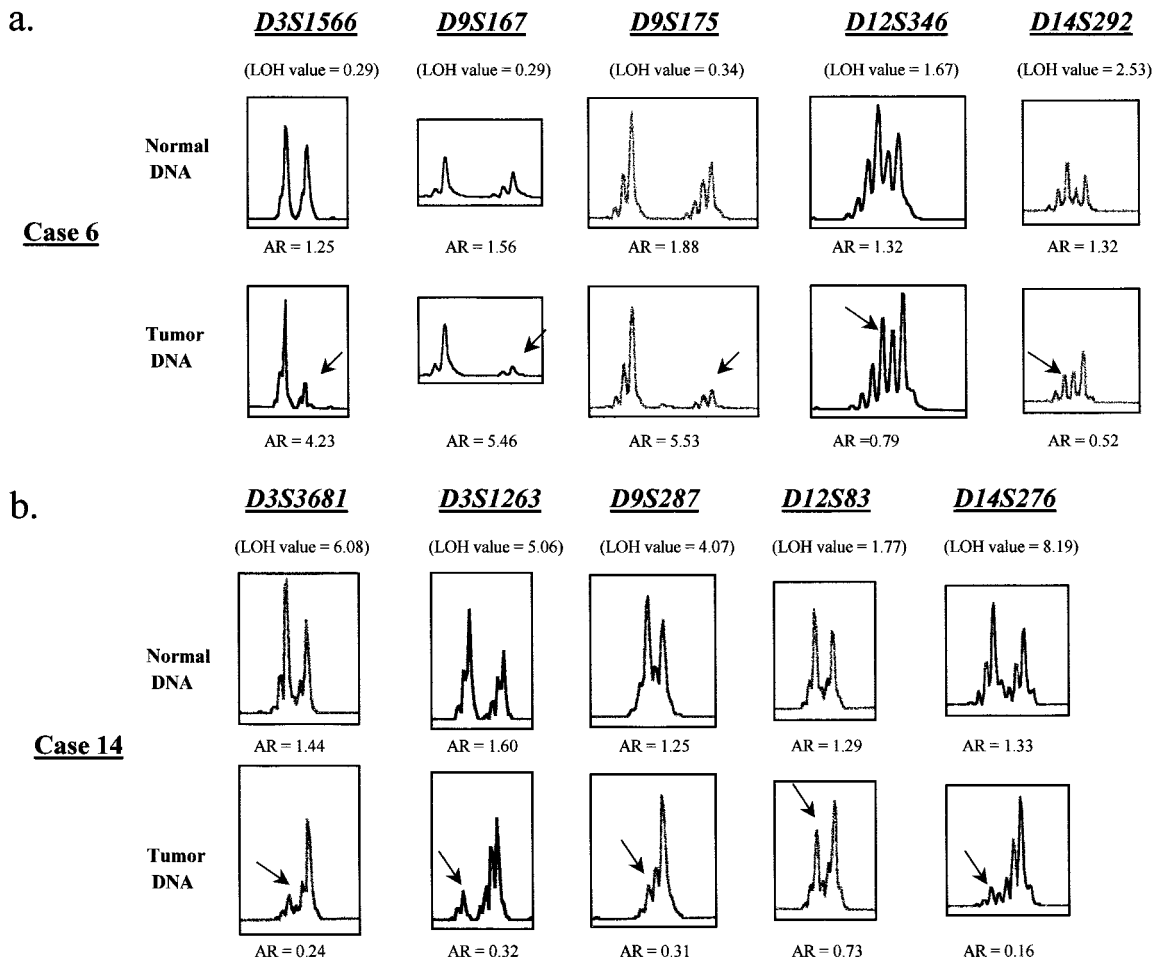


Fig. 1. Allelotype analyses of microdissected NPCs. Representative examples of LOH at different loci showing allelic imbalance are: a, NPC case no. 6; and b, NPC case no. 14. Left, case numbers of the tumors. Top, microsatellite markers. Arrows, alleles that showed loss or imbalance in the tumor samples. The LOH value is indicated for the specific locus of each case. *AR*, allelic ratio.

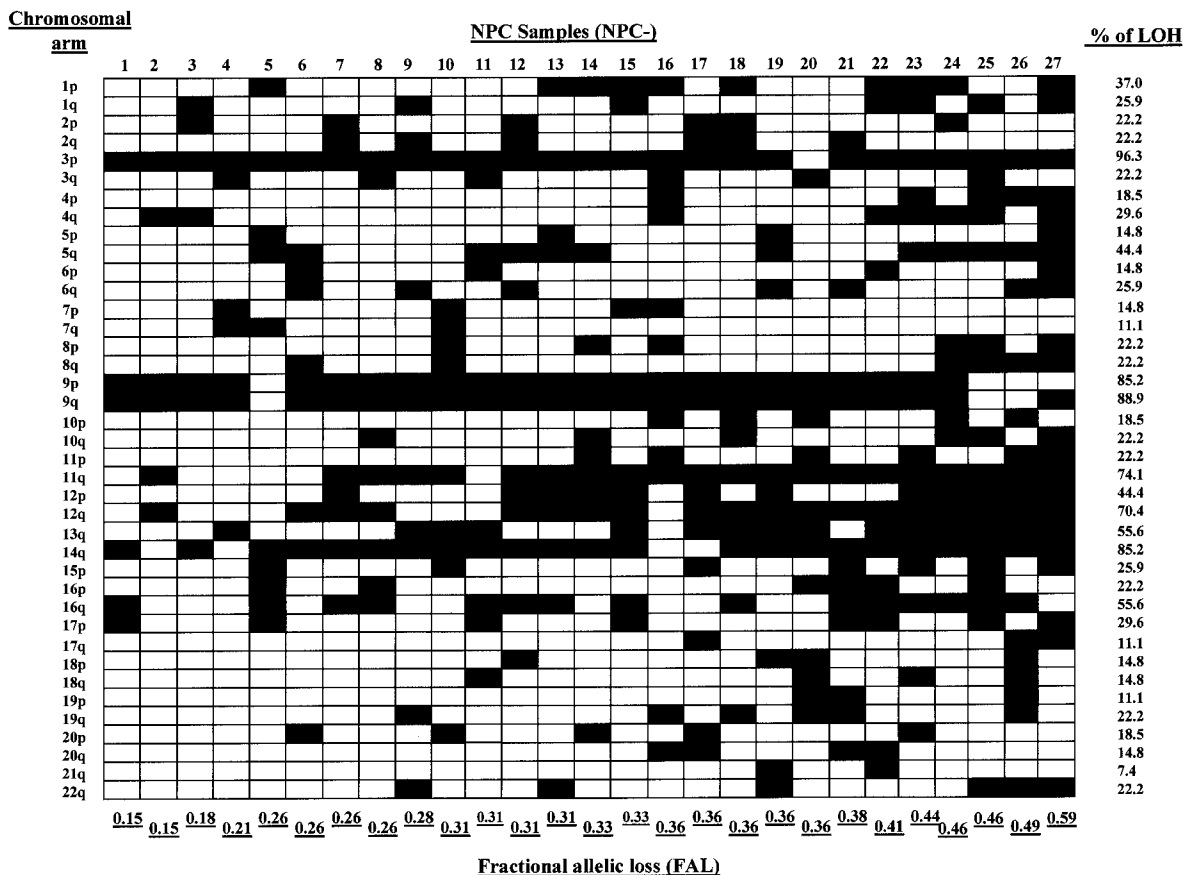


Fig. 2. Allelotype for each of the 27 microdissected nasopharyngeal carcinomas. Top, case number of each tumor. ■, LOH; □, retention of heterozygosity. All chromosomal arms of these 27 tumors are informative. The FAL value of each tumor is shown in the bottom of each column. Tumors are arranged from low to high FAL values. Right, frequency of LOH for each chromosomal arm.

normalities of chromosomes 12q and 16q are novel findings in NPC and appear as new candidates in the search for tumor suppressor genes or amplicons. In addition, LOH on chromosomes 1p (37.0%), 5q (44.4%), and 12p (44.4%) were also higher than the

mean percentage of LOH (32.8%) in NPC. Because only a limited number of tumor samples were examined in the current study, no significant association of LOH on specific chromosomal arms with the staging of NPC was identified.

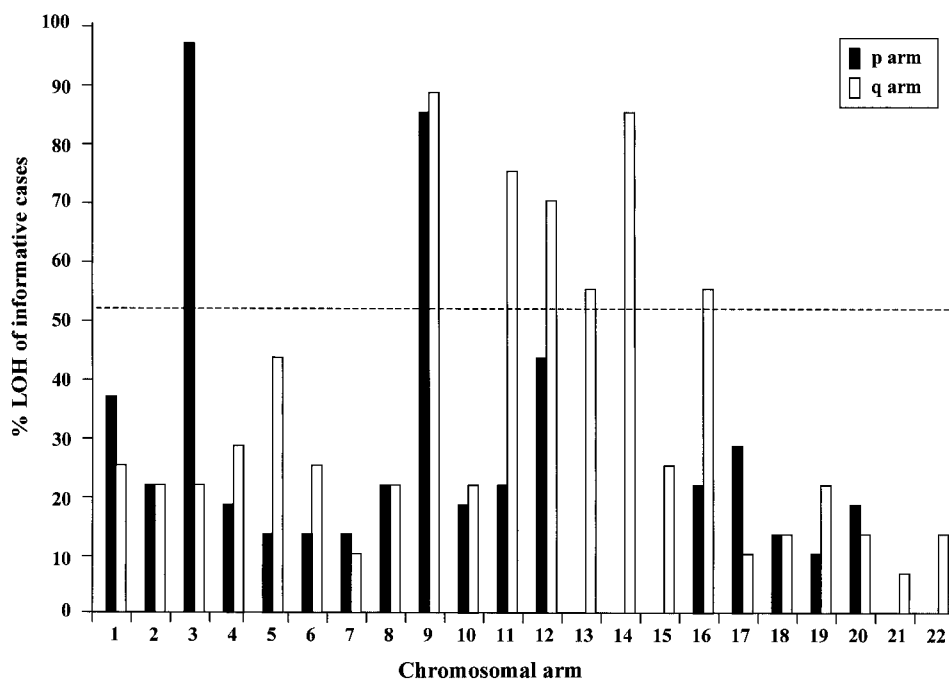


Fig. 3. Frequencies of LOH for 39 nonacrocentric autosomal arms in the microdissected NPCs. Allelic imbalances were evaluated with microsatellite analyses on 382 loci. ■, p arm; □, q arm.

In this high-resolution allelotype, we have identified several MDRs on the chromosomal arms that were frequently deleted in NPC, such as 3p, 11q, 13q, 14q, and 16q. Fig. 4 shows the five MDRs that were defined by the tumors containing deletion on these chromosomal arms. On chromosome 3p, a 43.2-cM MDR between *D3S1266* (3p22–24.2) and *D3S1285* (3p14–21) was delineated from the 27 microdissected NPCs. Deletion of this region was found in 88.9% of tumors. A MDR on chromosome 11q was confined to a 20.6-cM region that lay between *D11S898* (11q21–22) and *D11S925* (11q23). Twenty of 27 (74.1%) primary NPCs showed loss of such region. Two MDRs

were identified on chromosome 13q. A 32.8-cM region at 13q12–14.3 was flanked by the loci *D13S217* (13q12) and *D13S153* (13q14.1–14.3). Another deletion region is about 7.2 cM and located at 13q31–32, between *D13S170* (13q31) and *D13S265* (13q31–32). The frequencies of deletion at the regions 13q12–14.3 and 13q 31–32 were 51.9 and 48.1%, respectively. A MDR on chromosome 14q with frequency of 23 of 27 (85.2%) in NPC was delineated. The 28.3-cM region was mapped between *D14S280* (14q24.3–31) and *D14S292* (14q32.1–32.3). In chromosome 16q, the MDRs identified were flanked by the loci *D16S515* (16q22.3–23.1) and *D16S3091*

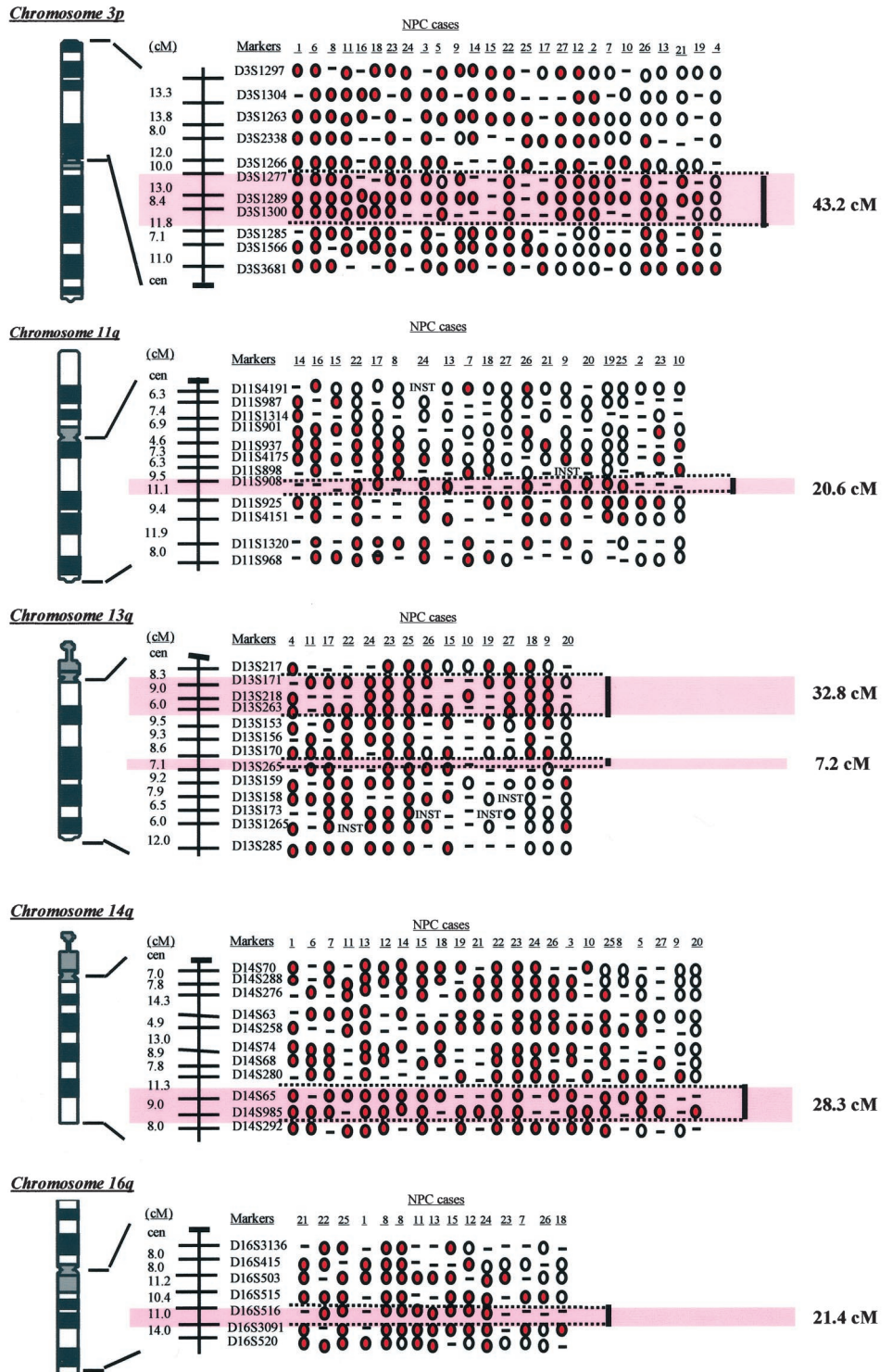


Fig. 4. The MDRs on chromosomes 3p (a), 11q (b), 13q (c), 14q (d), and 16q (e) in primary NPC tumors. Top, case number of each tumor. Left, microsatellite markers and their intervals (cM). Black bar, MDRs; red circle, LOH; white circle, retention of heterozygosity; -, noninformative; INST, microsatellite instability.

(16q22.3–23.1). The size of this MDR is 21.4 cM, and the frequency is 55.6% (15 of 27).

The FAL for each tumor was calculated. FAL is defined as the fraction of the total number of informative chromosome arms that shows LOH. FAL of the 27 NPCs ranged from 0.154 to 0.590 with a mean of 0.331 ± 0.103 . No association between FAL value and the age, sex, and staging of the patients was found. Correlations between several LOH at specific chromosome arms and the FAL value were observed. On the basis of the Mann-Whitney *U* test, LOH was significantly associated with high FAL value for chromosome 11q ($P = 0.002$), 12q ($P = 0.01$), and 13q ($P = 0.005$).

MSI was observed in 17 of 27 (63.0%) cases of NPC. The number of loci with MSI in these tumors ranged from 1 to 4. No specific loci susceptible to these genetic alterations were observed in this cancer. On the basis of the guidelines in a recent report by Boland *et al.* (16), all NPC cases were considered either as low-frequency MSI or microsatellite stable in this study. No tumors with high-frequency MSI were found.

Discussion

In the present study, we have examined a total of 382 loci at 22 autosomes of 27 NPC tumors. The high-resolution allelotyping on the microdissected tumor samples has generated a more accurate and clear-cut profile of the chromosomal abnormalities of NPC than previous LOH studies (2–9, 17). This allelotyping has also mapped several putative tumor suppressor loci to multiple chromosomal regions with a size from about 7 to 40 cM.

Comprehensive genomic screening has demonstrated the involvement of nonrandom chromosome abnormalities on 3p, 9p, 9q, 11q, 12q, 13q, 14q, and 16q in NPC tumorigenesis. Aberrations of tumor suppressor genes or cancer-related genes on these chromosomal arms play important roles in the development and progression of NPC. The identification of multiple genetic changes in each of the NPC tumor is consistent with the multiple-step model of tumorigenesis as in most other solid tumors. One of the unique features of NPC is the consistent presence of the EBV in all malignant cells. The expression of several viral genes, such as the *EBNA-1* and *LMP-1* genes, may contribute to the transformation of the nasopharyngeal epithelial cells (1). The close association of the EBV with NPC may imply different tumorigenesis processes in NPC when compared with the other human cancers. Compared with the allelotypes of other head and neck cancers, a lower incidence of LOH on 17p and higher frequency of LOH on 16q were found in NPC (18). It is proposed that a distinct pathway for the genesis of NPC may occur in this viral-related cancer at the head and neck region.

In concordance with our present findings, frequent LOH on 3p, 9p, 11q, 13q, and 14q have also been reported in a previous allelotyping study of NPC in Thailand, where a median incidence of this cancer is observed (17). They have examined for genome-wide chromosome deletions in 27 primary tumors by using 56 microsatellite markers. The frequent allelic alterations on these regions have also been reported in multiple LOH studies (2–9). However, the frequencies of LOH detected on most of these reports were lower than those of the current study. Our results demonstrated that the sensitivity of LOH study was highly increased with the use of microdissection and a large panel of microsatellite markers. In addition to confirming most of the formerly reported genetic alterations, we have found frequent LOH on chromosome 16q and significant allelic imbalance on 12q. Moreover, the common chromosomal abnormalities on 1p, 5q, and 12p were first illustrated in this cancer.

Our NPC allelotyping illustrated similar genetic changes to the previously reported CGH study. In CGH study, frequent losses of 3p, 9p,

9q, 11q, 13q, 14q, and 16q and gain of chromosome 12 were reported. Among the 27 microdissected tumors, 15 cases have also been subjected to CGH analysis (13). The findings of these two analyses on the 15 samples were consistent. However, we noted that deletion of several chromosomal regions, such as 9p and 3p, were detected by allelotyping analysis but not by CGH in some of the cases. Failure in the detection by CGH might be attributable to the small size of deletion or resulting from duplication of the remaining chromosome after loss of the functional alleles. Comparison of the allelotype and CGH results indicated that the allelic imbalance in chromosome 12q and 1q were attributable to an increased copy number of these chromosome regions. It is suggested that the combination of allelotyping and CGH analysis will be the most effective approach for the identification of the genetic changes in human cancers.

The most striking finding in this study is the LOH of chromosome 3p in almost all NPC samples (96.3%). This is in concordance with previous reports of genetic alteration in NPC (2, 3, 17). The frequency of 3p LOH varied from 67 to 100% in different studies. Our recent LOH study of 3p on 40 microdissected NPCs from different geographic regions have also shown a similar frequency (95%). Moreover, a high incidence of LOH of 3p was also detected in the normal epithelial cells and precancerous lesions of the nasopharynx (data not shown). These results implied that the inactivation of tumor suppressor genes on 3p is an important and early event during the development of NPC. According to our high-resolution allelotype, we delineated a 43.2-cM (or ~29.2 Mb) MDR region on 3p14 to 3p24.2, flanked by *D3S1285* and *D3S1266*. The region overlapped with the small deletion regions identified in previous studies (2, 3). One of the candidate tumor suppressors located at 3p14.2 is the *FHIT* gene. The gene overlapped with the locus *D3S1300* and closed to *D3S1285*. Alterations of this gene have been demonstrated in several NPC cell lines, suggesting that the aberrant *FHIT* gene may be associated with the development of NPC (19). Cheng *et al.* (12) have demonstrated a region with ~11 Mb at 3p21 (between *D3S1298* and *D3S1578*) that showed tumor suppressor function in a NPC cell line HONE-1 by the monochromosome transfer. These loci were located within our MDR region and overlapped with the tumor suppressor loci identified in a variety of human cancers, such as breast cancer, head and neck cancers, lung cancers, and renal cancers (20). To identify the major target gene(s) involved in NPC tumorigenesis, we will perform a detailed deletion mapping on a larger panel of microdissected tumors, cell lines, and xenografts. A high incidence of LOH was detected on chromosome 9p (85.2%). This is similar to our former studies on the frequent deletion of 9p21 and inactivation of the *p16* gene in NPC (4, 10). The tumor suppressor function of the *p16* gene in NPC was further confirmed by inhibiting tumorigenic potential with restoration of p16 in a NPC cell line (11). Because the homozygous deletion of 9p21 was common in NPC, the inactivation of other candidate genes such as *p15* and *p14^{ARF}* may also involve in the development of this cancer. Our preliminary result has also shown aberrant methylation of the *p15* gene in some NPC primary tumors. Although LOH of 9q was found in 88.9% of NPCs in this study, almost all of these cases showed the deletion of 9p. The loss of both arms of chromosome 9 or the entire chromosome appeared to be a common event in NPC.

In this study, we targeted an important tumor suppressor locus on 14q to a 28.3-cM (or 10-Mb) MDR that was between *D14S280* (14q24.2–31) and *D14S292* (14q32.1–32.2). The high frequency of LOH of 14q (85.2%) implicated that inactivation of this tumor suppressor may play a critical role in NPC as well as those on 3p and 9p. LOH on chromosome 3p, 9p, and 14q in almost all NPC tumors suggested that inactivation of the tumor suppressor genes on these three chromosomal arms are essential events for the development of this cancer.

In this genome-wide study of NPC, we have detected high incidence of loss on 11q (74.1%) and 13q (55.6%). The association of these deletions with high FAL value also suggested that such changes might be correlated with the progression of NPC. The MDR region identified at 11q21–23 (20.6 cM or 18 Mb) overlapped with those we reported previously. Hence, the candidate tumor suppressor genes, *ATM* and *SDHD*, located within this region may be associated with the development of NPC. *SDHD* encodes a mitochondrial respiratory chain protein, the small subunit of cytochrome *b* in succinate-ubiquinone oxidoreductase (cybS) and mutated in hereditary paraganglioma (21). This gene is considered as a candidate tumor suppressor involved in NPC tumorigenesis. We also identified two distinct MDRs on chromosome 13q. The first region on 13q12–14.1 included the tumor suppressor genes *Rb* and *BRCA2*. The other deletion region on 13q31–32 was refined to 7.2 cM (or 8.5 Mb) in this study. No candidate tumor suppressor was mapped to this region.

Frequent LOH of 16q on NPC is a novel finding in this allelotyping study. We have mapped a 21.4-cM (5.7-Mb) MDR to 16q22.3–23.1. The region is adjacent to the *E-cadherin* gene, which encoded a cell adhesion molecule and is associated with tumor invasiveness and metastasis. Zheng *et al.* (22) showed that loss of E-cadherin expression was common in this cancer and significantly associated with advanced stages of this disease. The other candidate tumor suppressor at 16q, *pRb-2/p130*, is located at 16q12 and mapped between *D16S415* and *D16S503*. This region was also deleted in 48.1% NPC tumors. Claudio *et al.* (23) has reported the mutation of the *pRb-2/p130* gene in 30% of NPC from North Africa. To confirm the involvement of this gene in NPC, we will investigate the genetic and epigenetic changes of this gene in our primary tumor samples in the near future.

Gain of chromosome 12 is a major finding in our previous CGH analysis. In the current study, we have found a high incidence (70.4%) of allelic imbalance on 12q in NPC. These findings suggest that activation of proto-oncogenes in this region may be involved in the development of this cancer. Oncogenes mapped to chromosome 12q include *CDK4*, *INT1*, and *MDM2*. However, our microsatellite analysis has shown multiple distinct regions of allelic imbalance. The target oncogene(s) involved in NPC tumorigenesis require further investigation.

In summary, our study revealed the existence of a multiplicity of genetic changes in NPC tumorigenesis. The identification of novel MDRs on multiple chromosome arms allows us to locate the target genes involved in the development of this cancer. Moreover, consistent allelic deletion of chromosomes 3p, 9p, and 14q were detected in almost all tumors in the current study. These findings suggest that inactivation of tumor suppressor genes on these regions are critical events for the development of NPC. It is believed that early diagnosis of NPC can be achieved with examination of 3p, 9p, and 14q deletions in high-risk groups. The microsatellite polymorphic markers on these chromosomal arms may be useful for the detection of NPC-specific genetic changes in the plasma or brushed nasopharyngeal biopsies of the patients. The high-resolution allelotype allows us to better understand the molecular basis of NPC and also enables the development of new tools for the early detection of this cancer.

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