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Advances in Brief

An Allelotype of Squamous Carcinoma of the Head and Neck Using Microsatellite Markers[1]

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Abstract

The detection in tumors of genomic regions with a high frequency of loss of heterozygosity has led to the localization and subsequent cloning of a number of tumour suppressor genes. To identify such regions involved in the development of squamous carcinoma of the head and neck we have analyzed 28 paired normal and tumor DNA samples. Using the polymerase chain reaction to amplify 50 simple sequence repeats or microsatellite markers we have studied all 22 q limbs and 17 of the p limbs in 21 patients. In informative cases we observed a high incidence of loss of heterozygosity at five specific chromosomal regions: 3p (44%); 5q (43%); 9q (35%); 11q (45%); and 17p (31%). In addition, further analysis of tumors showing loss of heterozygosity at 5q suggests that a gene at or near the *APC* locus is involved in squamous carcinoma of the head and neck.

Introduction

SCCHN[2] accounts for up to 5% of all malignancies in the western world (1). In the Far East and India in particular the incidence is much higher with up to 40% of malignancies occurring in the head and neck region (2). Recent epidemiological evidence suggests that the incidence and mortality from this disease are increasing particularly in developed countries with young males being worst affected. The exact reasons for this trend remain obscure but changes in tobacco and alcohol habits are the most likely explanation (3, 4). A better understanding of the underlying disease process involved is essential if advances are to be made in the management of these conditions.

Cancer is a genetic disease in which several genetic events are required to induce normal cells to convert to malignancy. Among the events implicated in this process are point mutations, gene amplifications, or rearrangements leading to activation of protooncogenes, in addition to deletions, mitotic recombinations of nondisjunction events which cause functional loss of tumour suppressor genes (for reviews see Refs. 5 and 6). Studies based on somatic loss of heterozygosity as a means of identifying critical loci have already led to the discovery of several important tumor suppressor genes, including the retinoblastoma (*Rb*) gene and the genes responsible for adenomatous polyposis coli (*APC*) and neurofibromatosis type I (*NF1*) (for review see Ref. 7).

Allelotype studies demonstrating genomic regions with high frequencies of LOH have been performed on a number of tumor types (6). Up until recently these have mainly used restriction fragment length polymorphisms to detect allelic loss. The discovery of microsatellite markers has opened up a major source of highly polymorphic and abundant markers for use in allelotyping (8). To date few data are available on the possible tumor suppressor genes involved in SCCHN. We have therefore embarked on the construction of an allelotype of SCCHN using simple sequence repeats or microsatellite markers. These markers are conveniently amplified using PCR and subsequent analysis is on nondenaturing polyacrylamide gels. We have analyzed and compared normal and tumor DNA from 28 patients using a battery of 50 markers (average heterozygosity, 75%) positioned on all the autosomal chromosome limbs, excluding the p limbs of 5 acrocentric chromosomes. Five chromosomal regions, 3p, 5q, 9q, 11q, and 17p, showed a much higher frequency of LOH relative to all other limbs examined. Possible candidate genes implicated on these regions include: *APC/MCC* (5q); Ferguson-Smith/Gorlin's syndromes (9q); *PRAD-1/cyclin D* or the *MEN-1* locus (11q); *p53* (17p).

Materials and Methods

Fresh tumor biopsies were collected at the time of surgery and immediately snap-frozen in liquid nitrogen. Subsequent storage was at −70°C. At the same time 10–15 ml of lithium heparinized blood were collected from the patient as a source of normal leukocyte DNA. Tumor specimens were routinely fixed in formalin, and paraffin sections were stained with hematoxylin and eosin to confirm the histological diagnosis.

DNA was extracted from both the leukocytes and the tumor biopsies using a simplified method (9).

Microsatellite primer oligonucleotides (Table 1) were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer or were obtained commercially from Research Genetics.

PCR was performed in 12.5-μl reaction volumes with 80 ng of genomic DNA using a variety of Taq polymerase suppliers. Reaction buffers (10×) supplied with each enzyme was used. Taq (1.25 units) was used per reaction and 200 μM concentrations of each deoxynucleotide triphosphate. $MgCl_2$ (3.0 mM), 0.16 μCi [^{32}P]dCTP (Amersham), and 20 pmol of each primer were used per reaction.

Conditions were standardized for all reactions and consisted of 1 min at 95°C and 1 min at 60°C for six cycles then 25 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C. A final extension cycle of 10 min at 72°C was also included. Reaction products were loaded with 0.5 volume of bromophenol blue buffer and run on 6–8% nondenaturing polyacrylamide gels at a constant power wattage of up to 14 kW for at least 3 h.

After electrophoresis gels were vacuum dried at 80°C and exposed to Kodak or Fuji-Color X-AR film for 24–72 h at −70°C.

Results

Using a panel of 50 microsatellites all 17 chromosomal p limbs and all 22 q limbs were analyzed with at least 1 marker per limb.

Allelic imbalance or loss of heterozygosity was scored by direct visual comparison of the relative allelic ratios of the normal and tumor samples. Complete loss of an allele was frequently seen as was the reduced intensity of one allele. The latter was interpreted as contamination from normal tissue, especially infiltrating host lymphocytes as seen on paraffin sections of the tumors.

Representative examples of LOH and allelic imbalance on a number of chromosomes are shown in Fig. 1. Several tumors showed clear evidence of interstitial deletions with others showing evidence of somatic recombination. Many chromosome limbs showed allelic imbalance or LOH in a small (0–15%) number of tumors. These low

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[2] The abbreviations used are: SCCHN, squamous cell carcinomas of the head and neck; PCR, polymerase chain reaction; LOH, loss of heterozygosity.

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Table 1 *Details of the microsatellite markers used in this paper*

Symbol	Locus	Heterozygosity (size)
D1S116	*1p21–31*	65% (89–101)[a]
D1S103	*1q32–44*	88% (82–102)
FGR	*1p36.1–36.2*	71% (135–143)
CD8A	*2p12*	71% (138–170)
GCG	*2q36–37*	82% (~125)
THRB	*3p22–24.1*	50% (197–209)
D3S11	*3p14–21*	84% (135–147)
CL3-373	*3p13*	73% (~113)
D3S196	*3q27–28*	67% (86–98)
D4S174	*4p11–15*	92% (175–195)
D4S171	*4q33–35*	75% (143–161)
D5S208	*5p15.3–15.1*	69% (140–158)
D5S107	*5q11.2–13.3*	82% (133–155)
D5S82	*5q14–21*	75% (169–179)
D5S134	*5q21*	54% (167–183)
MCC	*5q21*	55% (168–176)
CFS1R	*5q33.3–34*	86% (95–127)
D6S89	*6p23–24*	92% (199–227)
IGF2R	*6q25–27*	45% (158–166)
D6S292	*6q*	83% (141–161)
EGFR	*7p11.2–12*	72% (114–128)
D7S23	*7q31–32*	80% (109–127)
D8S87	*8p12*	71% (145–157)
MYC	*8q24*	86% (87–125)
D9S54	*9p22–pter*	60% (110–126)
D9S127	*9q22.1–32*	72% (149–159)
D9S112	*9q31–34*	85% (115–135)
D10S89	*10p11.2–pter*	73% (142–156)
D10S169	*10q11.2–qter*	72% (99–117)
TH	*11p15.5*	78% (240–260)
CD3D	*11q23*	74% (85–99)
INT-2	*11q13*	85% (161–177)
F8VWF	*12p12–pter*	75% (115–129)
PLA2	*12q23–qter*	79% (122–137)
FLT-1	*13q12*	51% (164–186)
MYH6	*14q11.2–13*	81% (108–132)
FES	*15q25–qter*	75% (143–163)
D16S291	*16p13.3*	77% (~170)
D16S288	*16q11.2–12.1*	73% (154–166)
D16S289	*16q22.2–23.1*	88% (156–172)
D17S513	*17p13*	89% (183–203)
P53CA	*17p13*	90% (103–135)
D17S250	*17q11.2–12*	91% (151–169)
D18S59	*18p*	82% (148–164)
D18S34	*18q12.2–21.1*	80% (103–119)
LDLR	*19p13.3*	48% (~106)
APOC 2	*19q13.2*	80% (129–165)
D20S27	*20p12*	71% (128–138)
CSTP-1	*20q13.3*	61% (123–141)
D21S156	*21q22.3*	92% (77–107)
CYP2D	*22q11.2–qter*	80% (108–130)

[a] Numbers in parentheses, range.

frequency alterations are observed in other allelotype studies and are generally considered to be nonsignificant (10).

Several regions showed significantly higher levels of LOH (Fig. 2). The percentage of allelic loss in informative cases at these regions was: 3p, 44% (8 of 18); 5q, 43% (12 of 28); 9q, 35% (6 of 17); 11q, 45% (9 of 20); and 17p, 31% (6 of 19).

A typical example of LOH at 3p is shown in Fig. 1*A*. Using marker *D3S11* patients 2 and 6 show loss of the larger allele in both cases. The regions of LOH on chromosome 3p were further analyzed using 3 microsatellites (*THRB*, 3p22–24; *D3S11*, 3p14–21; and CT3-373, 3p13). The distribution of the losses are shown in Fig. 3*B*. Five of 8 tumors had allelic loss at CL3-373, 3 of 8 showed losses at *THRB*, and 4 of 8 had losses at *D3S11*. One tumor, from patient 20, appeared to have lost the whole p limb because all 3 markers were lost (the 3q marker retained heterozygosity).

Further analysis of 5q using a total of 5 markers revealed a common region of loss involving the *APC*/*MCC* region (5q21). Twelve of 28 tumors analyzed revealed LOH at one or more of the 5 markers used. Eight of these tumors had lost heterozygosity at the *MCC* locus. Fig. 1*B* shows examples from 4 patients. Patients 1, 3, and 4 show reduced intensity of the upper band in 1 and 3 patients and the lower band in 4 patients. Four patients showed LOH at a marker (*D5S135*) positioned 4 cM proximal to *APC*. One tumor (patient 9) appeared to have lost the whole 5q limb while the rest had evidence for deletions and mitotic recombinations (Fig. 3*A*).

Six tumors had LOH at one or other of 2 markers analyzed on 9q (Fig. 3*D*). The region of loss from 9q included 9q31 which is where the genes for the Ferguson-Smith syndrome (self-healing squamous epitheliomata of the skin) and the Gorlin's syndrome gene (basal cell nevus syndrome) have been mapped (33, 34). Fig. 1*C* reveals a reduction in intensity of one allele in patients 5, 6, and 9.

The most frequent region of allelic loss or imbalance was seen at 11q. Nine of 20 tumors (45%) had LOH at 1 of 2 markers: *Int-2* (11q13) and *CD3D* (11q23) (Fig. 3*C*). Five tumors had allelic imbalance only at the *Int-2* locus (4 of 5 retained heterozygosity distally) while 3 had LOH at the *CD3D* locus and one had lost both loci. The 11q13 region of chromosome 11 is known to harbor a number of genes of potential importance in carcinogenesis. Prominent among these is the *bcl-1/prad-1/cyclin D* locus, which has previously been shown to exhibit low-level amplification in a variety of solid tumors, including SCCHN (11). It should be noted that the PCR-based assays used in this and other reports cannot readily distinguish between duplication or low-level amplification of an allele and loss of het-

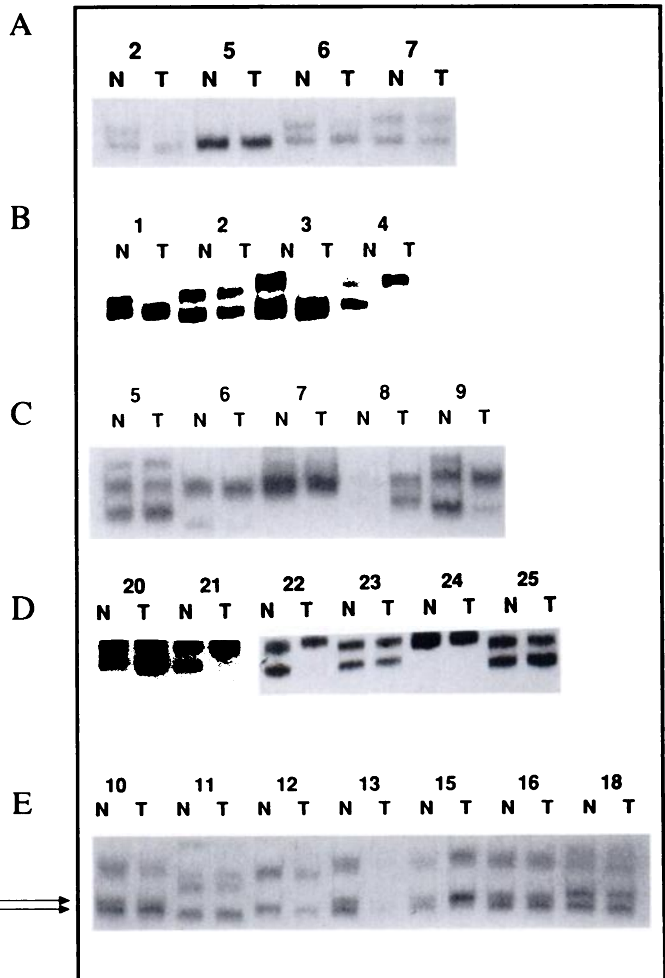

Fig. 1. Representative autoradiographs showing allelic losses/imbalances. *N*, normal DNA; *T*, tumor DNA; *A*, *D3S11*. Loss of upper band in tumors 2 and 6. *B*, *MCC*. Loss of upper band in tumors 1 and 3. Loss of lower band in tumor 4. *C*, *D9S112*. Loss of lower band in tumors 6 and 9. *D*, *Int-2*. Loss of lower band in tumors 21 and 22. Allelic imbalance in tumor 25. *E*, *p53CA*. Loss of upper band in tumor 10. Loss of lower band in tumor 15. Allelic imbalance in tumor 11. Homozygous deletion in tumor 13. *Arrows*, allelic bands. "Shadow" bands visible in upper half of the autoradiograph.

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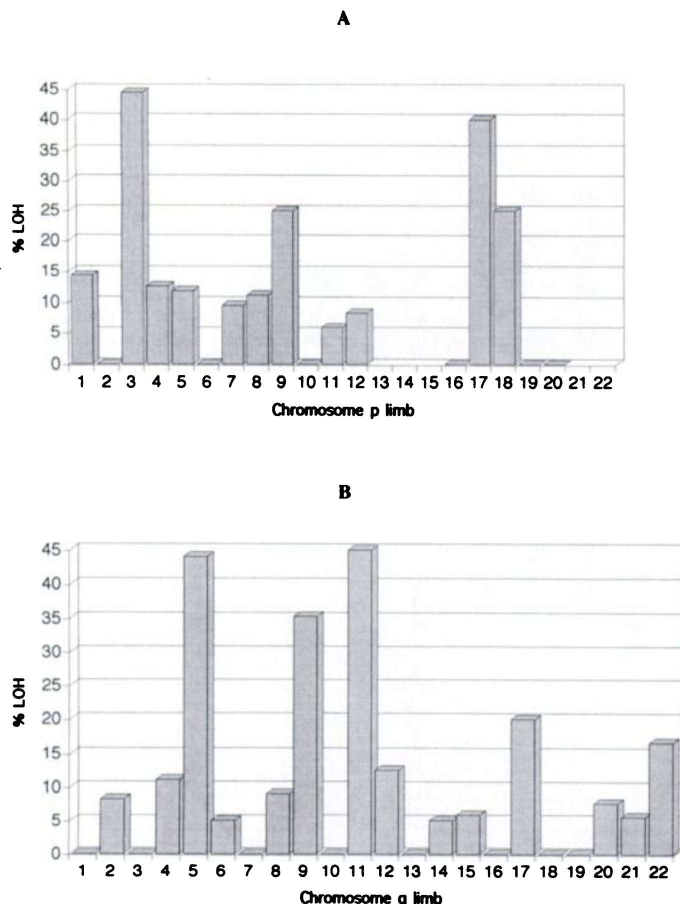

Fig. 2. Summary of the frequencies of allelic loss/imbalance. *A*, percentage of tumors demonstrating allelic loss/imbalance on the chromosomal p limbs. Acrocentric limbs 13, 14, 15, 21, and 22 not analyzed. *B*, percentage of tumors demonstrating allelic loss/imbalance on the chromosomal q limbs.

erozygosity, particularly if there are contaminating normal cells within the tumor. Both events would appear as allelic imbalance. However, we do not think that simple duplication/amplification of the cyclin D gene can account for the alterations seen on chromosome 11q. All tumors with apparent allelic imbalance on 11q were tested on Southern blots for possible amplification of the cyclin D gene (11q13) (data not shown). This analysis demonstrated clear amplification for only one tumor (patient 20). Interestingly this patient also showed clear LOH at the more distal *CD3D* locus (11q23) (Fig. 3*C*) implying that at least 2 separate genetic events have occurred at this chromosome arm. Patients 21 and 22 showed no evidence of amplification although in both cases there was clear LOH at the *Int-2* locus (Fig. 1*D*). At least for these tumors, therefore, loss of a tumor suppressor gene by deletion or mitotic recombination is a more likely scenario.

On chromosome 17 two markers positioned at 17p13, and closely linked to the *p53* locus, showed LOH in 31% (6 of 19) of informative cases. Fig. 1*E* shows allelic loss in patients 10, 11, and 15 while patient 13 reveals evidence for a homozygous deletion at the *p53* locus. The exact distance of these markers from *p53* is unavailable; however, in informative cases only 2 of 15 showed LOH at *D17S513* while 6 of 15 showed LOH at *p53CA*. Two of these 6 were the same tumors showing loss at *D17S513* while of the remaining 4, 1 tumor was uninformative but 3 retained heterozygosity at *D17S513*. This suggests a localized event at the *p53CA* region (*e.g.*, deletion). Analysis of these regions was repeated at least twice to confirm the findings. Other groups have demonstrated a high frequency of mutations in the *p53* gene in SCCHN by immunohistochemical staining and/or mutation analysis (12, 13). In the present study we have not carried out an extensive analysis of *p53* mutations in these tumors, although it has been shown that some cell lines derived from those tumors showing allelic imbalance on 17p do indeed have mutations in the *p53* gene.[3]

Correlation of the genetic findings outlined above with some clinical parameters revealed two trends. Firstly a higher number of allelic losses was found in tumors of high T (tumor) stage (tumor-nodes-metastasis classification). Three tumors in this study showed no allelic imbalance or loss at any marker studied, two were stage T_1, and the other was T2. The only other T_1 tumor showed LOH at only two loci. The mean number of allelic alterations (imbalance or LOH) for T_2 tumors was 1.8 while for T_4 tumors it was 4.9. This difference was statistically significant ($P = 0.03$, Mann-Whitney test). Secondly higher allelic losses were noted in poorly differentiated tumors (mean, 4.0) compared with well differentiated tumors (mean, 1.5). These results did not reach statistical significance ($P = 0.06$, Mann-Whitney test). All 3 T1 tumors were histologically well differentiated.

Discussion

The aim of this study was to identify the genomic regions most likely to contain important tumour suppressor genes involved in the development of SCCHN. Previous cytogenetic studies on SCCHN have demonstrated frequent chromosomal abnormalities including 3p, 9q, 11q, and 17p alterations (for review see Ref. 14). Our study has also detected genetic changes at these sites, but in addition we have shown LOH at 5q. While rearrangements on chromosome 1 are the most frequently reported cytogenetic change in SCCHN (14) we have detected allelic imbalance on chromosome 1 in only a small proportion of our tumors. Allelotype and other LOH studies have been the source of much information in the analysis of other human tumors (for review see Ref. 15). These studies have been greatly facilitated in recent years by the advent of restriction fragment length polymorphisms and more recently polymorphic microsatellite markers (8). The latter markers were used in this study for several reasons: (*a*) they are randomly distributed throughout the human genome; (*b*) they are highly polymorphic; (*c*) they are highly conserved through successive generations; and (*d*) they are simple and quick to analyze. Their main disadvantages are that they are not easy tools for quantification of allele copy numbers and that amplification by PCR is often dogged by "shadow" bands making interpretation occasionally difficult.

Our results demonstrate that several chromosomal regions show a much higher frequency of allelic loss or imbalance than the 10–15% "background" level reported by others (10). Of the candidate regions showing LOH in this study, some have previously been reported to be involved in SCCHN. For example, loss of alleles on 3p has been inferred from statistical analyses of restriction fragment length polymorphisms in head and neck carcinomas (16, 17). These results are compatible with prior indications of potential tumor suppressor genes on 3p involved in formation of tumors of the kidney (18) and lung (19). Although the existence of candidate genes on 3p has been reported (20), as yet no definitive evidence of a suppressor function for any gene in this region is available.

Similarly, chromosome 11q is implicated in SCCHN because of the putative involvement of the *cyclin D* gene in formation of squamous carcinomas (11). In addition to this potential oncogene, however, there is also evidence for a tumor suppressor gene on this chromosome arm. Data presented in this study (Fig. 1*D*) and by others (21) have shown clear loss of heterozygosity in some tumors. It should also be noted that the putative *MEN-1* tumor suppressor gene maps to 11q13 and that single chromosome transfer experiments have indicated that a suppressor function may be localized in this region (22).

[3] K. Edington and K. Parkinson, unpublished results.

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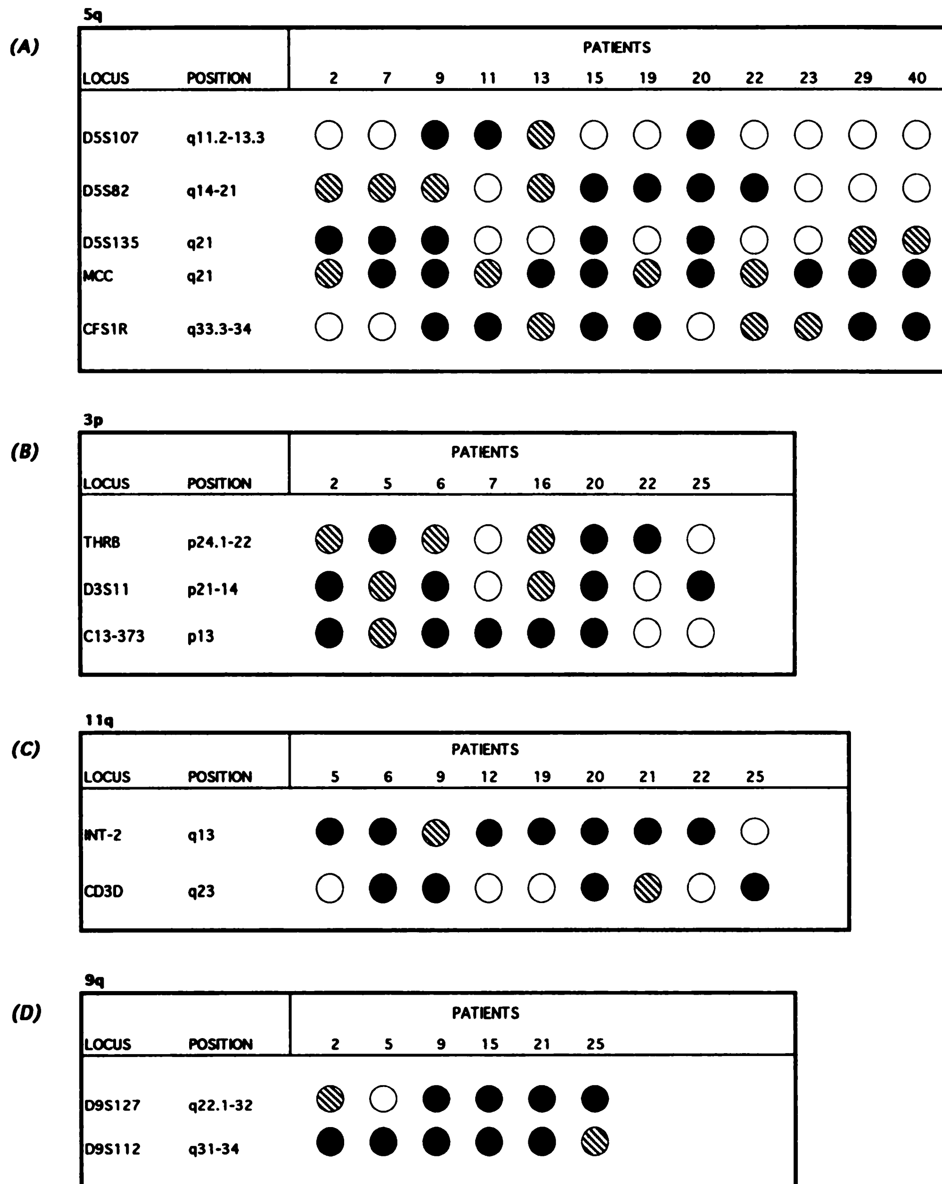

Fig. 3. Summary of allelic loss/imbalance at 5q (*A*), 3p (*B*), 11q (*C*), and 9q (*D*). ○, informative, no imbalance; ●, informative, loss/imbalance; ⊘, not informative.

p53 abnormalities remain one of the most common genetic defects found in tumors studied to date (23). Several immunohistochemical studies of the stabilized protein have been supplemented by the detection of mutations in the gene (24). Consistently high levels of *p53* overexpression in SCCHN have been found by several workers (25–27) as well as frequent mutations within the gene (13). The incidence of LOH at the *p53* locus detected in this study (31%) contrasts with a high incidence (71%) seen in oral cancers by other investigators (28), for reasons which are as yet unclear. The overall frequency of *p53* alterations detected in oral lesions to date may be a substantial underestimate of the true level, since recent investigations have shown that adjacent (uninvolved) oral tissue from patients with tumors harbors cells with clonal alterations in the *p53* gene (25). This may suggest that somatic genetic changes at the *p53* locus are in fact extremely prevalent in such patients, possibly as a consequence of repeated exposure to carcinogens in cigarette smoke and/or alcohol (12), but that such changes may not provide a strong selective growth advantage until complemented by other genetic alterations (29).

The most novel aspect of the present investigation is the detection of high frequency allelic loss/imbalance on chromosomes 5q and 9q. The initial demonstration of *APC* gene involvement in colorectal cancer (30) has been followed by work suggesting a role in esophagus (31) and breast cancers (32). The most common region of loss in SCCHN which we have detected on chromosome 5q is near the *APC/MCC* locus, implying a much broader role for this locus in squamous as well as adenocarcinoma development. However, mutation analysis of this and other candidate genes on 5q will be necessary to test this hypothesis.

Studies of familial epithelial cancers have led to the mapping of the genes for basal cell nevus syndrome (Gorlin's syndrome) (33) and multiple self-healing squamous epithelioma to chromosome 9q31 (34). It is not presently known whether these two conditions are due to distinct loci or to different mutations in the same gene. Both conditions lead to the development of multiple cutaneous tumors, indicating that the gene involved may control epithelial growth and/or differentiation. Loss of alleles at 9q31 implicates this locus also in sporadic lesions of the head and neck and suggests that genetic

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analysis of such tumors may be a useful adjunct to familial mapping studies to identify and characterize this gene.

In conclusion, the allelotype studies presented here form the basis for a more detailed dissection of the genetic alterations in SCCHN which should further our understanding of this disease and lead to novel therapeutic approaches.

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