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Genetically Altered Fields as Origin of Locally Recurrent Head and Neck Cancer: A Retrospective Study

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ABSTRACT

Purpose: Surgeons treating patients with head and neck squamous cell carcinoma (HNSCC) rely heavily on histology to decide whether the resection margins are tumor free and subsequent adjuvant treatments can be omitted. However, despite the presence of tumor-free margins, 10–30% of HNSCC patients still develop a locally recurrent tumor. Evidence is available that recurrent cancer develops from either (*a*) outgrowth of a relatively small number of tumor cells that have not been detected by the pathologist or (*b*) a precursor lesion in which additional genetic alterations have led again to invasive cancer.

Experimental Design: In a retrospective study on 13 HNSCC cases, we analyzed the primary tumor, its surrounding histologically tumor-free resection margins, and local recurrences for loss of heterozygosity (22 microsatellite markers on 6 chromosomes) and *TP53* mutations to determine the origin of the recurrent cancer.

Results: A precursor lesion was absent in 5 of 13 (39%) cases, and the genetic similarity of the primary and recurrent cancer was high, providing evidence that residual cancer cells were the origin of recurrence. For the remaining eight cases (61%) a genetically related precursor lesion (field) was detected, and for five of these cases, evidence was found that both the primary and recurrent carcinoma originated from this field. The remaining three cases were less conclusive.

Conclusions: This study explains the pathobiology of locally recurrent HNSCC in patients with histologically tumor-free resection margins and indicates that the development of novel therapies to decrease the local recurrence rates in HNSCC should not only be focused on eradicating residual cancer cells but also on the precursor lesions that are left behind.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) comprises approximately 5–10% of all newly diagnosed cancer cases in Europe and the United States (1). Despite significant advances in surgery and radiotherapy over the last decades, the 5-year survival rates of HNSCC patients improved only moderately, partly due to the relatively high rate of locally recurrent cancer. Even when the surgical margins are diagnosed as tumor free by histopathology, the local recurrence rate is still 10–30% (2–4). A local recurrence is defined according to clinical criteria as the occurrence of another carcinoma within 3 years after and <2 cm away from the primary carcinoma (5). There are two theoretical explanations for these unexpected local recurrences. First, cancer cells have remained in the patient, which were not detected in the resection margins by the pathologist. This is likely to concern a relatively small number of cancer cells, and this source of local recurrence can therefore be designated local minimal residual cancer [MRC (6)]. Second, it has recently been suggested that tumor-related mucosal precursor lesions, "fields" of genetically altered cells surrounding the tumor, may be left behind and might give rise to new invasive carcinomas (7–10). These new carcinomas are clonally related to the primary tumor because they develop from a common genetically altered field and have therefore been designated "second field tumors" [SFTs (5)].

These fields can be large: a diameter of >7 cm has been reported for the oral cavity and oropharynx (11). These dimensions indicated that second primary tumors, which are defined according to clinical criteria as tumors developing >2 cm away from the primary tumor or at least 3 years after occurrence of the primary tumor (5), might develop in these fields left behind. Indeed, it was shown that the majority of second primary tumors in the same or adjacent anatomical region develop from such a precursor field left behind (11).

Most patients present with invasive carcinomas, but there is ample evidence to suggest that HNSCC develops through a number of histologically defined precursor lesions designated as mild, moderate, and severe dysplasia. Califano *et al.* (12) demonstrated that the progressing stages characterized by these histological changes run more or less parallel with an increase in the number of genetic alterations. Within this progression model, loss of heterozygosity (LOH) of chromosomes 3p, 9p, and 17p could be defined as early alterations, and LOH of 8p, 18q, and 13q could be defined as late alterations (7, 12–14). Responsible tumor suppressor genes or oncogenes have been identified on some of these chromosomal regions, such as the *TP53* gene on 17p and the *CDKN2A* locus on 9p21. Mutations in the *TP53* tumor suppressor gene are present in the majority of HNSCCs (15), and evidence has been provided that *TP53* mu-

tations are early genetic alterations in HNSCC (9, 14, 16). In a recent publication (14), the fields of genetically altered cancer cells were placed in the perspective of multistep HNSCC genesis. The process of clonal selection is continuing in a field, and eventually a subclone develops into carcinoma, characterized by invasive growth and metastatic potential. Hence, a clonal relationship exists between a field and the carcinomas that develop within the field.

Knowledge of the type and sequence of genetic alterations involved in HNSCC genesis and the involvement of field as a precursor lesion allows us to reconstruct the origin of recurrent cancer after resection of HNSCC with curative intent. The aim of the present study was to reveal the origin of locally recurrent cancer. With the help of genetic markers, we set out to determine whether local MRC or a precursor field was the cause of recurrent cancer.

MATERIALS AND METHODS

Patients and Tissue Samples. Clinicopathological information was obtained from patient records and pathology reports. Patient records were screened between 1993 and 2000. Inclusion criteria were as follows: (*a*) the primary tumor was located in the oral cavity or oropharynx and treated by surgery (with or without postoperative radiotherapy), and resection margins were histologically tumor free; (*b*) the local recurrence developed within 2 cm of the treated area and within 3 years, according to previously published criteria (5, 17); (*c*) the local recurrence was biopsied or treated by surgery; and (*d*) sufficient paraffin-embedded material was available to allow molecular studies. In total, 13 cases fulfilling all criteria were collected (Table 1).

Genetic Analyses. Microsatellite analysis at 3p, 9p, 17p, 8p, 13q, and 18q and mutations in the *TP53* gene were used as genetic markers of clonality. DNA was sequenced for *TP53* mutations in exons 4–10 as described previously (18). LOH analysis was performed as described previously (11, 19). In short, 22 microsatellite markers on chromosomes 3p, 9p, 17p, 8p, 13q, and 18q were selected for LOH analysis. Paraffin-embedded tissue blocks were used that had been prepared for routine histopathology. Neoplastic areas were manually micro-dissected from both primary tumors and local recurrences. Moreover, all resection margins (a minimum of four and a maximum of seven) surrounding the primary tumor were screened for dysplasia by an experienced pathologist (J. A. K.). All areas with signs of dysplasia were manually microdissected. When no morphological changes were found for a particular patient, the epithelium of each margin was divided randomly and dissected. DNA of the stroma was used as control to assess LOH and was taken from the same paraffin block that the tumor or margins were dissected from.

Establishment of Common Genetically Altered Field. We used genetic markers to discriminate SFT from the MRC-derived local recurrence. We started the analysis by defining whether the primary tumor was surrounded by a field and whether this had a clonal origin common with both the primary tumor and the local recurrence. To establish a clonal relationship, previously published criteria were used (20): the primary tumor, local recurrence, and the epithelial layer of one of the margins of the primary tumor should contain a similar *TP53* mutation and/or a similar microsatellite shift, an alteration in the length of a microsatellite also referred to as microsatellite instability (MSI), and/or a similar pattern of LOH (based on all markers and at least 2 informative markers/arm) in a single chromosomal arm. The probability that any of these alterations occur by chance in all these three lesions can considered to be low. With respect to the analysis of the margins, when more than one margin showed concordant losses, the one with the highest level of concordance was selected for further comparison and calculation.

Assessment of Clonal Relationship in the Absence of Field. The absence of a genetically altered field was considered an indication that the recurrence developed from MRC. It could not, however, be excluded that the field remained unnoticed, and therefore additional confirmation based on comparison of the genetic profiles of primary tumor and recurrence was

Table 1 Patient characteristics and origin of local recurrence

Patient no.	Gender	Age (yrs)	Site	TNM[a]	RT	Dysplasia[b]	Interval (mo)
1	F	50	Mobile tongue, L	T_1N_0	N	Mild	10
2	M	75	Mobile tongue, R	T_1N_0	N	Mild	9
3	M	46	Mobile tongue, L	T_2N_{2B}	Y	No	6
4	F	52	Floor of mouth, Ant	T_2N_0	N	No	6
5	M	65	Floor of mouth, L	T_1N_0	N	Mild[c]	25
6	F	67	Base of tongue, L	T_2N_1	Y	No	9
7	M	74	Proc alv sup, R	T_2N_0	N	Mild	10
8	M	81	Mobile tongue, L	T_2N_0	N	Severe	30
9	M	72	Base of tongue, R	T_3N_{2B}	N[d]	No	5
10	F	83	Mobile tongue, L	T_1N_0	N	No	7
11	F	86	Mobile tongue, R	T_1N_0	N	Mild	29
12	F	64	Floor of mouth, Ant	T_4N_{2C}	Y	No	11
13	M	44	Floor of mouth, R	T_3N_1	N	No	15

[a] TNM, tumor-node-metastasis; RT, treated with postoperative radiotherapy; Interval, time to local recurrence; L, left; R, right; Ant, anterior; Proc alv sup, processus alveolaris superior.

[b] The grade of dysplasia indicated corresponds to the margin that was considered to contain the tumor-related field. When other margins showed a higher grade, it was indicated separately.

[c] Margin A (see Fig. 1) was graded as moderate dysplasia. However, the genetic profile of the mild dysplastic lesion of margin E showing more concordance is shown.

[d] Patient refused postoperative radiotherapy.

Table 2 Genetic patterns to decide whether a local recurrence has developed from MRC[a] or precursor lesions (based on data from Fig. 1)

| Patient no. | Presence of tumor-related field | | Comparison of genetic patterns of primary tumor and local recurrence | | | Decision origin of local recurrence |
| | | | Other than LOH; similar markers | LOH | | |
	Yes/no	If yes, based on		Chance of similarity by coincidence (%)	If similar, based on	
1	Yes	MSI at 13q, LOH at 3p, 17p, and 8p	NA	No similarity	NA	Precursor lesion
2	Yes	*TP53* mutation, LOH at 9p and 17p	NA	9	LOH at 3p and 13q	Precursor lesion
3	No		MSI at 8p, *TP53* mutation	<1	LOH at 3p, 9p, and 17p	MRC
4	Yes	MSI at 8p, LOH at 9p and 17p	*TP53* mutation	11	LOH at 3p, no LOH at 13q	MRC or precursor lesion
5	Yes	MSI at 3p, LOH at 9p	NA	52	No LOH at 13q	Precursor lesion
6	Yes	*TP53* mutation, LOH at 3p and 9p	NA	1	LOH at 8p, no LOH at 17p, 18q, and 8p	MRC or precursor lesion
7	Yes	*TP53* mutation, LOH at 17p	MSI at 8p	8	LOH at 3p and 18q	MRC or precursor lesion
8	Yes	LOH at 9p, 17p, and 8p	NA	18	LOH at 18q	Precursor lesion
9	No		*TP53* mutation	<1	LOH at 3p, 9p, 17p, and 8p	MRC
10	No		*TP53* mutation, MSI at 3p and 18q	1	LOH at 17p, 8p, and 18q, no LOH at 13q	MRC
11	Yes	LOH at 9p		14	LOH at 3p and 17p	Precursor lesion
12	No		*TP53* mutation	<1	LOH at 3p, and 9p, no LOH at 17p, 13q, 18q, and 8p	MRC
13	No		*TP53* mutation	<1	LOH at 3p, 9p, 17p, 13q, and 8p, no LOH at 18q	MRC

MRC, mean residual cancer; LOH, loss of heterozygosity; MSI, microsatellite instability; NA, not applicable.

performed. An identical *TP53* mutation or an identical microsatellite shift in the primary and recurrent tumor was considered proof that MRC was the origin of the recurrence (19, 20). In addition, LOH profiles of the primary tumor and local recurrence were compared for this purpose, and we calculated the chance that the genetic pattern of the local recurrence was identical to that of the tumor by coincidence. Starting point for the calculation was the reported LOH frequency for the different chromosome arms (3p, 76%; 9p, 82%; 17p, 74%; 13q, 46%; 18q, 35%; 8p, 70%). These frequencies have been determined in primary HNSCCs (median number of carcinomas, 50; range, 40–81) from previous studies (9, 11, 19). Microsatellites on a given chromosome arm do not detect LOH in an independent manner; therefore, all markers on a chromosome arm, not the individual microsatellite markers, were considered to represent one locus. Information was also obtained from the arms that showed no LOH at all. The chance (in percentage) that a similar pattern (yes or no LOH in multiple arms) occurred in both lesions by coincidence was calculated. If this was <5%, the genetic concordance between primary tumor and local recurrences was considered high enough to support the option that MRC was the origin of the recurrence.

Assessment of Clonal Relationship in the Presence of Field. In cases in which a precursor lesion (a genetically altered field surrounding the tumor) was observed, the genetic patterns of the primary tumor and the local recurrence were compared. Even when such a field is present, a MRC origin of the recurrent cancer is an option. A genetic similarity between a primary tumor and recurrent tumor can be considered evidence for a MRC origin of the recurrent cancer, whereas a genetic dissimilarity indicates that the recurrent cancer has progressed independently in the preneoplastic field. *TP53* mutations, microsatellite shifts, and LOH profiles of the primary tumor and local recurrence were compared for this purpose. To allow for this comparison, the genetic markers that defined the presence of the common precursor lesion had to be excluded because these lack discriminative power on theoretical grounds. A concordant MSI or *TP53* mutation (not shared with the field) was judged to be in favor of the vision that the local recurrence originated from local MRC. Without concordant *TP53* mutations or MSI, the probability was calculated that the LOH pattern of the local recurrence was identical to that of the tumor by coincidence. For this calculation, we used the reported LOH frequency for the different chromosome arms, as discussed above. All microsatellites at one arm were considered to represent one locus, and information was also obtained from the arms that showed no LOH at all. A value of <5% was considered statistical evidence to assume that the LOH pattern of the recurrence was concordant with that of the primary tumor and that MRC was the source of the local recurrence.

RESULTS

Local Recurrence in the Absence of Tumor-Related Field. The different profiles are indicated in Fig. 1 and further details on the motivation for decisions can be found in Table 2. In 5 of the 13 cases (38%; patients 3, 9, 10, 12, and 13), we could not detect a genetically altered field that was clonally related to the primary tumor. No concordant genetic aberrations encompassing a whole chromosome arm could be found in the
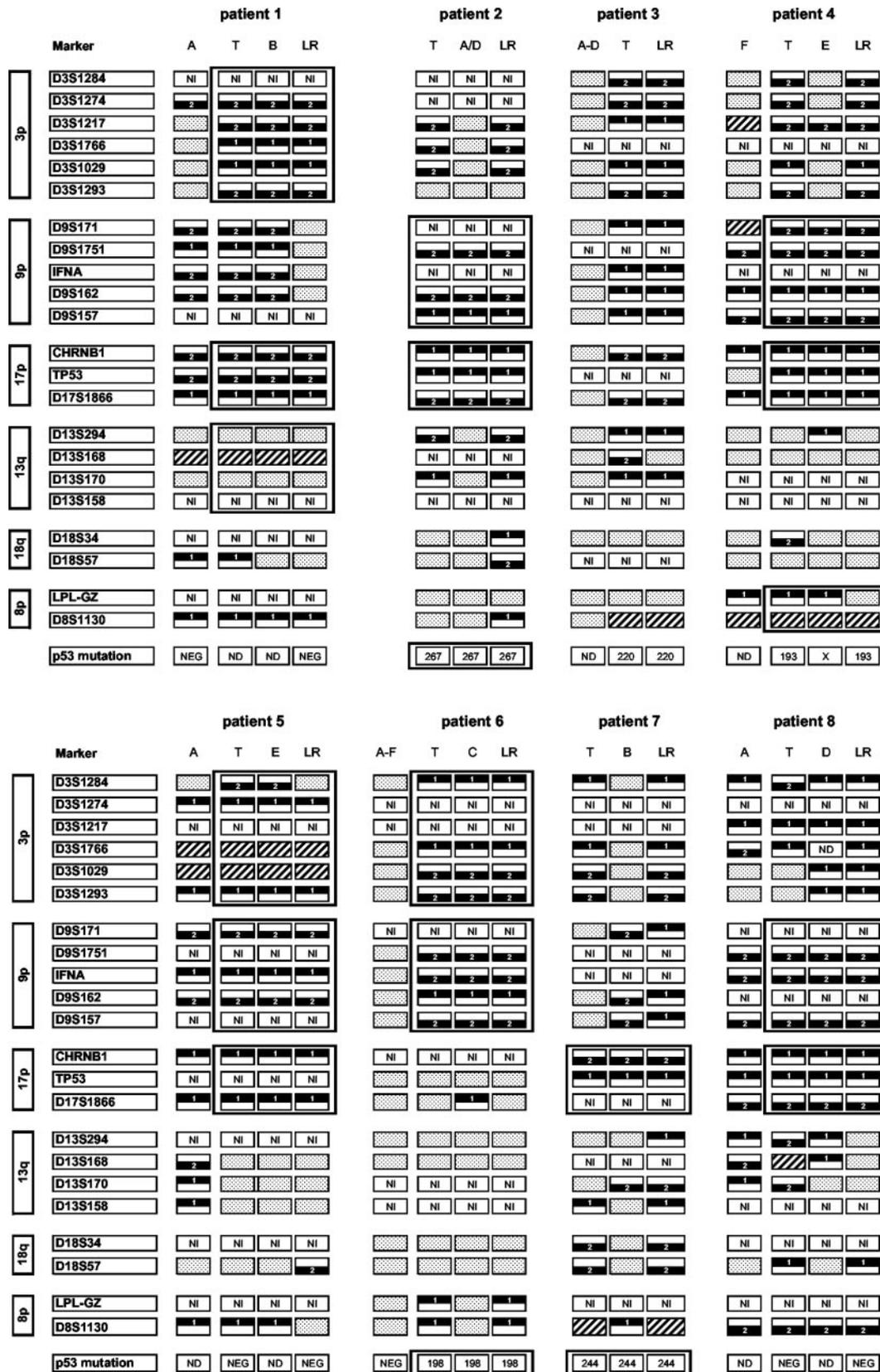
Fig. 1 Genetic profiles of the primary tumors, corresponding resection margins, and local recurrences. In the *first column* the chromosome arms are shown, and in the *second column*, the microsatellites located at those arms are shown. For each patient, the pattern of the primary tumor (*T*) and of the local recurrence (*LR*) is shown. *A–F* indicate the margins of the primary tumor (*A–E* stands for *A* through *E*, meaning that five margins have been
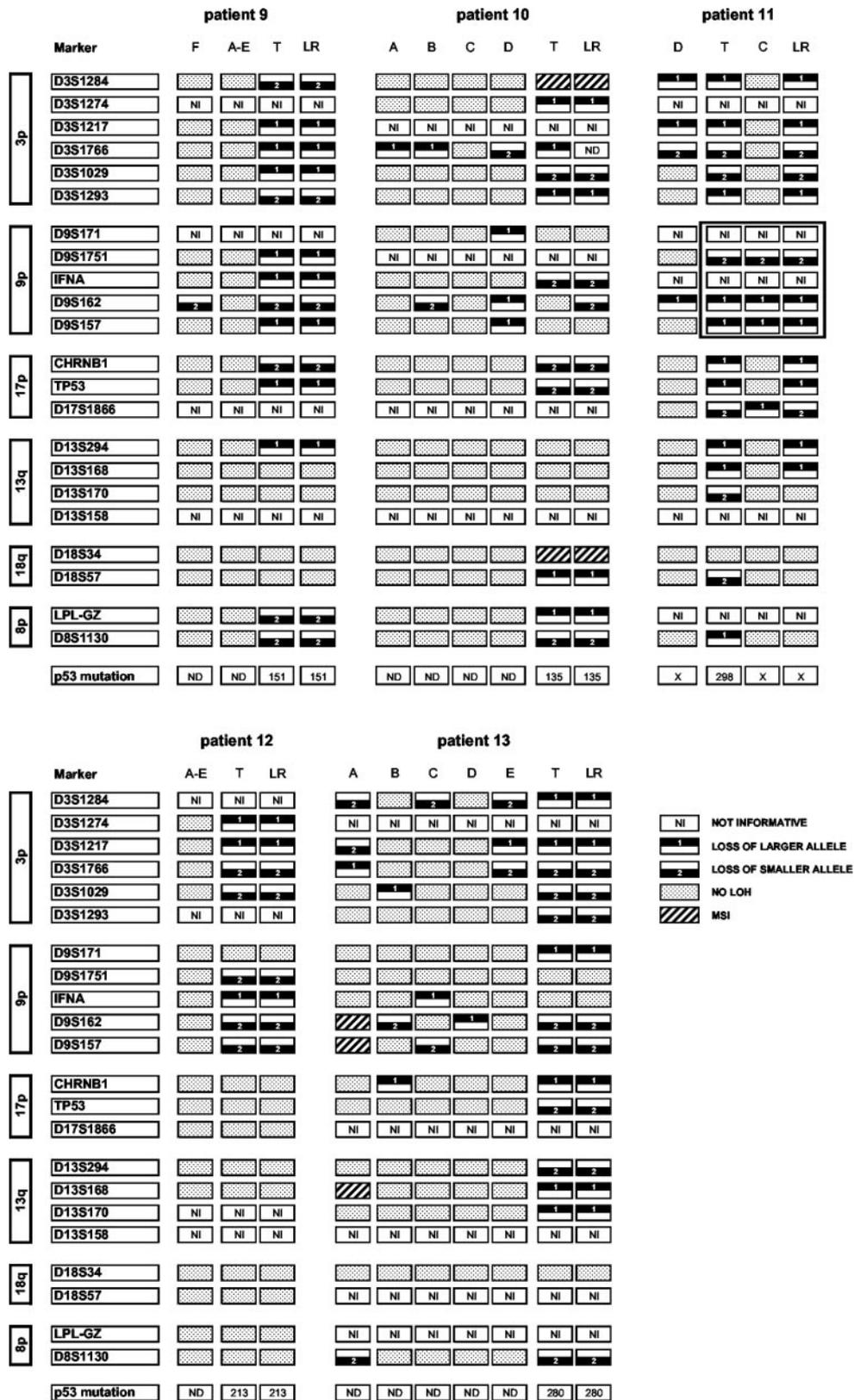
analyzed, and *A/D* means that margins A and D are shown). When a tumor-related field is found (according to the criteria in "Materials and Methods"), the genetic profile of the margin corresponding to that field was depicted between those of the primary tumor and the local recurrence. A *box* emphasizes the genetic alterations that have led to decide in favor of a tumor-related field. *NI,* not informative; *ND,* not determined; *NEG,* no mutation in the *TP53* gene; *X,* no confirmation of the *TP53* mutation as found in the corresponding tumor.

margins. The absence of field was a strong (but not conclusive) indication that these recurrences were derived from local MRC. When comparing the genetic profiles of the recurrent and the primary tumors, however, no or only minor differences were observed. All five pairs share an identical *TP53* mutation, two share a common MSI pattern, and in all combinations, the probability that the LOH pattern was identical by coincidence was ≤1%. These results were considered to be evidence that in all five cases, the local recurrences originated from MRC.

Local Recurrence in the Presence of a Tumor-Related Field. In 8 of 13 cases (62%), a tumor-related genetically altered field could be detected. In three of these cases (patients 2, 6, and 7), the decision was based on the common *TP53* mutation in primary tumor, one of its margins, and the local recurrence. In three cases (patients 1, 4, and 5), a common MSI was observed, and in the remaining two patients (patients 8 and 11), a common LOH pattern of at least one chromosomal arm indicated the presence of a field clonally related to the tumor.

Precursor Field as Origin of Local Recurrence. It should be noted that in the presence of a tumor-related field, the local recurrence could still could have developed from MRC. To distinguish local recurrences originating as result of histologically undetected MRC cells from new tumors arising in a tumor-related genetically altered field, we used the concordance of genetic markers between tumor and recurrence that were not shared with the field. In five cases, we could not find evidence for a concordant pattern of the remaining set of genetic markers (patients 1, 2, 5, 8, and 11). There were neither common *TP53* mutations nor microsatellite shifts. In addition, we could not exclude that a common LOH pattern in a given chromosomal arm had originated by coincidence. Based on the observed differences, we therefore concluded that these five cases are likely to be local recurrences that have developed from a precursor field. As an example, we discuss the characteristics of patient 8. Margin D is similar to the primary tumor and the local recurrence based on the pattern of LOH at 9p, 17p, and 8p, confirming the presence of a clonally related field. When comparing primary tumor and local recurrence, a similar LOH pattern was only present at 18q. The probability that this has occurred by chance is 50% (LOH may occur at one of the two alleles) multiplied by 35% (the chance of observing LOH in this arm) is 18%. The primary tumor and recurrent tumor might therefore have this similar pattern by chance. The local recurrent tumor was therefore not considered to originate from MRC and, consequently, most likely arose as new tumor from the unresected genetically altered field.

For three additional patients (patients 4, 6, and 7) it was difficult to decide the origin of the recurrence. The recurrent tumor was genetically very similar to the original tumor [a common *TP53* mutation (patient 4), a common MSI (patient 7), and a convincingly similar LOH pattern (patient 6)]. These alterations were not shared with the detected field. Despite the presence of a precursor lesion surrounding the primary tumor, the genetic profiles indicate that MRC is the likely origin of recurrent cancer. The possibility that the recurrences of these three cases were derived from a precursor lesion, however, could not be excluded conclusively.

DISCUSSION

The aim of this study was to reveal the pathobiology of locally recurrent HNSCC. Patients were selected who had locally recurrent cancers defined by present clinical criteria: the development of another cancer within a period of 3 years after the first tumor and at a distance of <2 cm from the first tumor. Only patients who had tumor-free margins when the primary tumor was resected were included.

The present study suggests that in approximately 40% of patients, a limited number of cancer cells (MRC) are left behind after surgery, and this leads to the outgrowth of a new, genetically identical carcinoma at the same site. An important finding is that in the other 60% of cases, fields of genetically altered cells were detected that had the same clonal origin as the primary and recurrent tumor. It is known that new carcinomas develop in such a field (7, 9, 14). Indeed, for the majority of cases with a tumor-related field surrounding the primary tumor, the genetic dissimilarity between the primary and recurrent tumor provided evidence that the local recurrence has originated from the field with genetically altered cells. Thus, approximately half of the local recurrences (as defined according to clinical criteria) originate from genetically altered fields and may be considered SFTs (5). In a previous study, we could show that a similar proportion (60%) of second primary tumors had developed from a precursor field (11). In that study, the clinical criteria for a second primary tumor were used: it should develop >2 cm away from the primary tumor or >3 years after the primary tumor. The other 40% of second primary tumors in that study were genetically completely unrelated to the primary tumor.

The data of the present study suggested a relation between the origin of locally recurrent tumor and the time interval between the development of the primary and recurrent cancer. The five cases with a likely MRC origin showed an average interval of 8.8 months, whereas this interval was 20.6 months for the five cases for which a precursor lesion was the most likely cause. Apparently, a few cancer cells take less time to grow into a clinically detectable cancer than a field of preneoplastic cells needs for further progression to invasive cancer.

For some cases we studied here, available data were too limited for reliable classification, and misclassification may have occurred. Despite the presence of an unresected tumor-related precursor lesion, for three cases we could not find definitive evidence that the preneoplastic cells were indeed the source of recurrent cancer. The primary and recurrent tumors were genetically very similar, suggesting that the locally recurrent tumor might well have developed from a few cancer cells left behind after resection that were not detected by the pathologist. Nonetheless, for these three cases, it also remains possible that the tumor-related precursor lesion was the origin for the recurrent tumor because the presently used technique may not be sensitive enough to detect differences between preneoplastic and neoplastic cells. In addition, when chromosomal breakpoints would have been taken into account, the local recurrence of patient 2 could also be classified to originate from local MRC. The details of the 3p alterations have not been included in our evaluation, but the absence of LOH at D3S1293 suggests a breakpoint in the 3p arm that is present in the primary tumor

and the local recurrence, but not in the field. Because we do not know the likelihood of a break between that particular microsatellite and D3S1029, we have not been able to use this observation in the evaluation.

The studied precursor lesions, the fields with genetically altered cells, are macroscopically not visible to the surgeon, which explains in part why they are often left behind. The pathologist, however, can recognize most of these lesions as dysplasia. It is important to note that in most cases (four of five cases, 80%) the SFTs arose in tumor-related precursor lesions that were graded as mild dysplasia, confirming previous reports that histology seems suitable for identification of genetically aberrant mucosal epithelium but of very limited use for risk assessment (21, 22). Because it is known that fields can be large (11), some of the second primary tumors defined according to the clinical criteria should also be classified as SFTs. These molecular studies have led to new insights, and a new classification of local recurrences, SFTs, and second primary tumors based on genetic criteria has been proposed (5).

Our observations indicate that reliable methods should be developed for molecular grading of the margins of surgically treated HNSCC patients. In previous studies addressing the risk for progression of leukoplakia lesions in the oral cavity, it was shown that a specific pattern of allelic imbalance and ploidy are important markers to predict progression (22–24). These approaches may also be very well suited for accurate risk assessment of fields surrounding the tumor that are not clinically visible. Once a more reliable risk assessment has been developed, it can be exploited to identify the high-risk fields. The presence of such a high-risk field in a surgical margin should be followed by life-long surveillance at regular intervals. Moreover, it could indicate a more conservative approach with regard to adjuvant radiotherapy as far as the primary site indications are concerned. Patients with a preneoplastic field are at risk for SFTs, and this therapeutic modality should thus be kept in reserve, if at all possible. In addition, current treatment modalities (surgery and/or chemotherapy/radiotherapy) are very effective to eradicate tumor cells, but these may not be indicated for the treatment of relatively large fields of preneoplastic cells. There is a need for the development of targeted treatment strategies for patients at risk for SFTs (25).

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