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Loss of heterozygosity at 15q21.3 correlates with occurrence of metastases in head and neck cancer

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Deletions on the long arm of chromosome 15 suggesting the presence of potential tumor suppressor genes have been found in several tumors including carcinomas of the colorectum, urinary bladder, breast, lung, and head and neck. Here, we analyzed allelic imbalance on chromosome 15q in head and neck carcinomas and corresponding lymph node metastases to define common regions of aberrations with potential involvement in development and progression of these tumors. We studied a panel of 40 polymorphic microsatellite markers, spanning 15q13–15q26, in 63 head and neck carcinomas and 38 lymph node metastases. Loss of heterozygosity (LOH) could be demonstrated in 34 primary tumors (54%) and 35 metastases (92%). Aberration mapping defined three minimum regions of aberrations: a region between the markers D15S106 and D15S1029 in 15q21.3 (estimated as 3.9 Mb; region 1) was affected in the majority of tumors, whereas two other regions between D15S144 and D15S1040 in 15q13.3–14 (estimated as 2.4 Mb; region 2) and between D15S130 and D15S985 in 15q26.2–26.3 (estimated as 4.7 Mb; region 3) were less often involved. Allelic loss in region 1 correlated with T stages ($P = 0.0029$) and metastatic potential ($P = 0.0018$). LOH in regions 2 and 3 occurred predominantly in metastases ($P = 0.0129$ and $P = 0.0013$, respectively). No correlation with grading, localization, or clinical outcome could be established for any of the affected regions. Our data hint at aberrations in 15q21.3 as a possible important characteristic for head and neck squamous cell carcinomas with risk of progression.
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Head and neck cancer represents 6% of all human cancers accounting annually for approximately 500 000 new cases.[1,2] Squamous cell carcinomas are the most important histological group. The overall 5-year survival rate of patients with this cancer is comparatively low, especially because 20–30% of these patients develop other tumors in the upper aerodigestive tract[3] and due to the high incidence of recurrent disease at the primary site or in the regional lymph nodes.[4] In the last years, many efforts had been made to determine the mechanisms involved in the development of head and neck squamous cell carcinoma. In addition to external risk factors like nicotine and alcohol abuse[5] or an infection with human papillomavirus (HPV),[6] a multistep process of accumulated genetic and epigenetic alterations probably accounts for head and neck carcinoma as for most of the sporadic solid tumors.[7] Here, inactivation of tumor suppressor genes is one of the important steps. The detection of frequent deletions in a defined chromosomal region is considered to be critical evidence for the localization of tumor suppressor genes. Deletions or structural rearrangements in 15q as well as gains of genetic material from 15q have been described in a variety of studies in oral and esophageal squamous cell carcinomas.[8–15] Furthermore, a genome-wide study with microsatellite markers in head and neck squamous cell carcinomas demonstrated new hot spots for allelic losses in 15q, especially in 15q21.[4] Here, we studied 40 microsatellite markers from the long arm of chromosome 15 in 63 oral, pharyngeal, and laryngeal primary tumors and 38 lymph node metastases by multiplex PCRs.

Methods

Tumors and Patients

Tissue and patient data were obtained and used after advice of the Medical Ethics Committee of the

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University of Greifswald in accordance with the declaration of Helsinki and the International Conference of Harmonization—Good Clinical Practice. The anonymity of the patients investigated was preserved corresponding to rules of data protection of the Human Medical Faculty Greifswald and the county Mecklenburg-Vorpommern.

A total of 60 patients with head and neck squamous cell carcinomas have been included in this study. In all, 63 primary carcinomas and 38 lymph node metastases (from 21 of the 60 patients) and the corresponding normal tissues were analyzed. Dysplastic changes in the normal mucosa have been ruled out. The carcinomas comprised 50 moderately differentiated (G2) and 13 poorly differentiated (G3) tumors.[16] The carcinomas derived from the larynx (37 cases), the oropharynx (16 cases), and the hypopharynx (10 cases). All specimens underwent additional independent histopathological review (BK). Staging of all carcinomas was performed according to the criteria proposed by the International Union Against Cancer/Union Internationale Contre le Cancer (UICC)[17] and was available for all but three primary tumors (two pT1, nine pT2, 10 pT3, eight pT4, two pT1N1, one pT1N2M1, one pT2N1, three pT2N2, two pT3N1, four pT3N2, six pT4N1, and 12 pT4N2). The mean age of the patients was 59 years (range 38–85 years). Smoking history was known for 47 patients, and alcohol abuse for 24 patients.

DNA Isolation

DNA isolation from paraffin-embedded tissues was performed as follows. First, hematoxylin–eosin-stained slides were carefully inspected by light microscopy to identify areas which carry a sufficient amount (at least 3 mm^2) of tumor measured by a scaled optical adjustment. This same area was then identified on the unstained 10 μm dewaxed, rehydrated, and airdried tissue section, which was fixed in an optical installation allowing the separate isolation of predominantly neoplastic tissue without adherent nontumoros structures under microscopical control with a cannula (used for intravenous injections) as described previously.[18,19]

Molecular Genetic Analysis

A panel of 40 di-, tri-, and tetranucleotide microsatellite markers covering a greater part of the long arm of chromosome 15 was studied. They comprised D15S217 (15q13.1), D15S1013 (15q13.3), D15S1010 (15q13.3), D15S144 (15q13.3), D15S995 (15q14), D15S1040 (15q14), D15S1232 (15q14), D15S971 (15q14), D15S118 (15q14), D15S194 (15q14), D15S1044 (15q14), D15S129 (15q15.1), D15S1028 (15q21.1), D15S119 (15q21.1), D15S1017 (15q21.2), D15S982 (15q21.2), D15S1032 (15q21.2), D15S106 (15q21.3), D15S1016 (15q21.3), D15S1049 (15q21.3), D15S1029 (15q21.3), D15S962 (15q21.3), D15S117 (15q21.3), D15S1011 (15q22.2), D15S215 (15q22.3), D15S980 (15q24.1), D15S114 (15q24.3), D15S984 (15q24.3), D15S211 (15q25.1), D15S205 (15q25.2), D15S979 (15q25.3), D15S202 (15q26.1), D15S647 (15q26.1), D15S649 (15q26.1), D15S130 (15q26.2), D15S533 (15q26.2), D15S642 (15q26.3), D15S985 (15q26.3), D15S966 (15q26.3), and D15S120 (15q26.3). Primer sequences were obtained from Genome Data Base (http://www.gdb.org), cytogenetic locations and their position in megabases from 15pter (Figure 1) are according to Ensembl (http://www.ensembl.org). PCR amplification was performed in multiplex assays with fluorochrome-labeled primers (6-FAM, JOE, or TAMRA) in 12.5 ml sample volumes with 2–5ng of genomic tumor or normal DNA as template in 15 mM Tris/HCl, 50 mM KCl, with 200 μM dNTPs, 1.5 mM $MgCl_2$, 0.1 nM primers, and 1 U HotStart *Taq* Polymerase (Qiagen, Hilden, Germany). An initial denaturation and activation step of 12 min at 95°C was followed by 30–35 cycles of 1 min at 95°C, 1 min at 55–58°C and 2 min at 72°C, and a 30 min final elongation step at 72°C. PCR products were analyzed on an ABI310 genetic analyzer (Applied Biosystems, Darmstadt, Germany) with ROX-labeled internal lane standard. All PCR assays were repeated at least once. Loss of heterozygosity (LOH) was scored if one allele was $>90\%$ decreased in tumor DNA when compared with the same allele in normal control DNA in both PCR assays.

Statistical Analysis

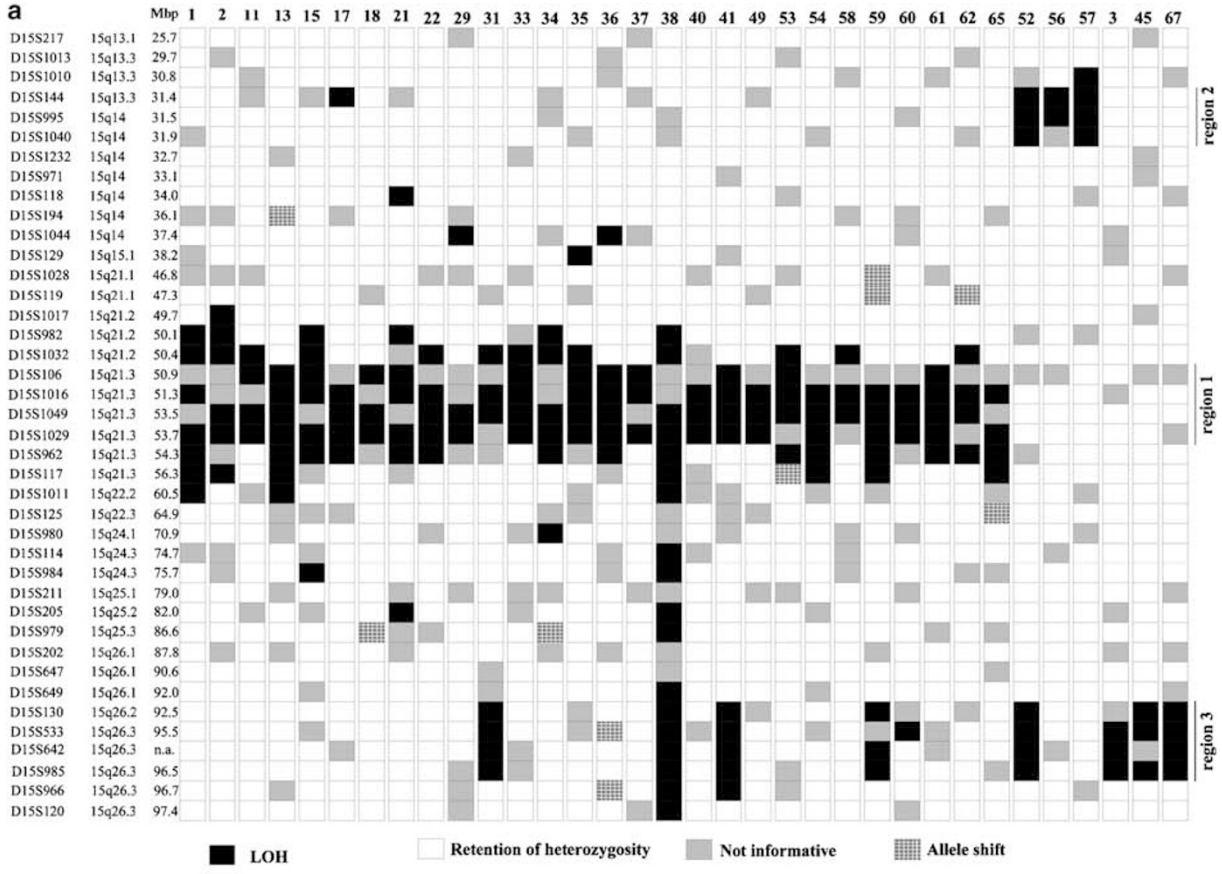
Cramer's ϕ test (χ^2) for nonparametrical data was carried out with the web (χ^2 calculator, available at http://www.georgetown.edu/faculty/ballc/webtools/web_chi.html. The *P*-value was estimated with the Graph Pad Quickcalcs, available at http://www.graphpad.com/quickcalcs/index.cfm. A *P*-value of 0.05 or less was considered as statistically significant.

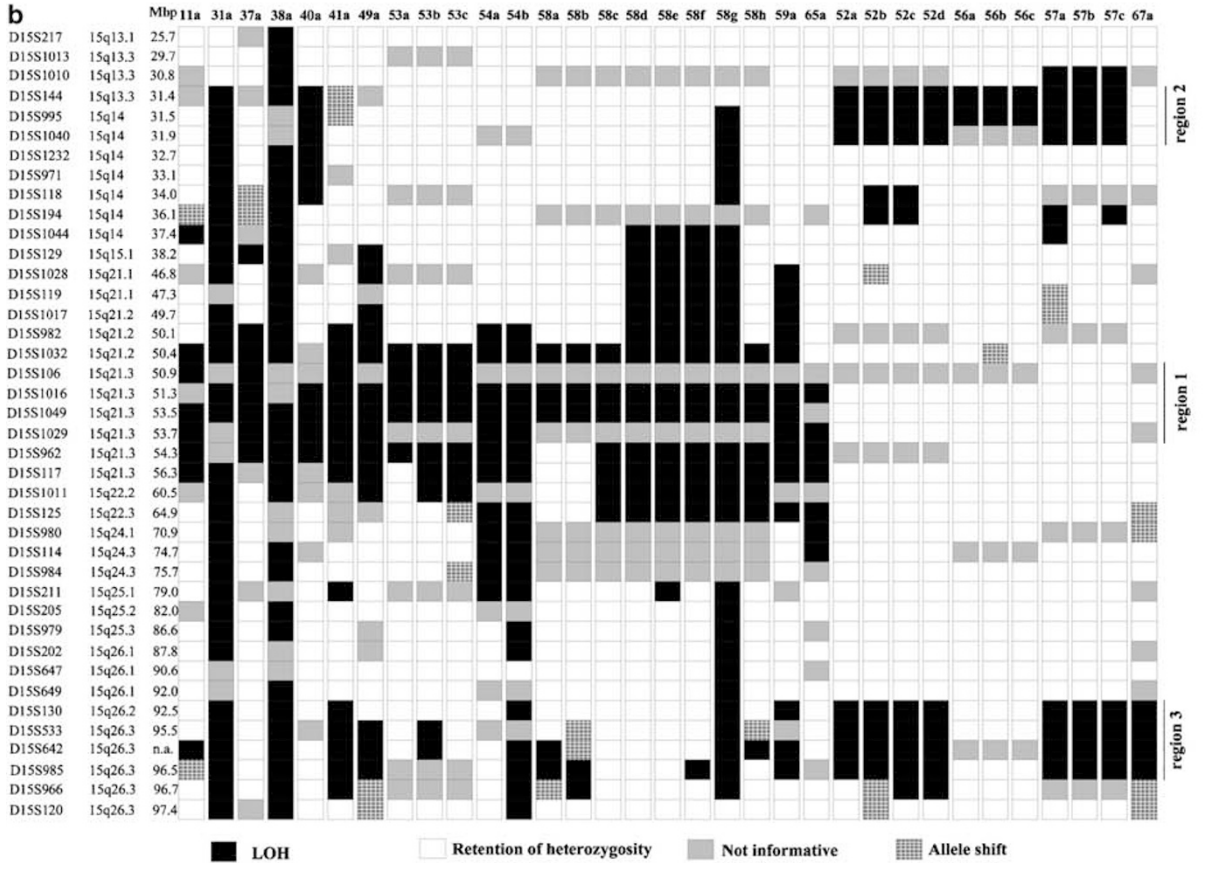
Results

Microsatellite Analysis

All head and neck carcinoma samples and lymph node metastases were informative for at least 23 microsatellite markers allowing the construction of a detailed alteration map. A total of 29 primary tumors and three lymph node metastases displayed retention of heterozygosity at all informative dinucleotide repeats (25 cases) or LOH only at one microsatellite marker outside the aberration regions discussed below (seven cases). Over all tumors, we detected 482 LOH events. In 222 events the allele with lower molecular weight was reduced, in the remaining 260 events the allele with higher molecular weight was reduced.

LOH could be demonstrated in the region 15q13–26.3 in 34 primary tumors (54%) and in 35 lymph


a
Mbp
1 2 11 13 15 17 18 21 22 29 31 33 34 35 36 37 38 40 41 49 53 54 58 59 60 61 62 65 52 56 57 3 45 67
D15S217 15q13.1 25.7
D15S1013 15q13.3 29.7
D15S1010 15q13.3 30.8
D15S144 15q13.3 31.4
D15S995 15q14 31.5
D15S1040 15q14 31.9
D15S1232 15q14 32.7
D15S971 15q14 33.1
D15S118 15q14 34.0
D15S194 15q14 36.1
D15S1044 15q14 37.4
D15S129 15q15.1 38.2
D15S1028 15q21.1 46.8
D15S119 15q21.1 47.3
D15S1017 15q21.2 49.7
D15S982 15q21.2 50.1
D15S1032 15q21.2 50.4
D15S106 15q21.3 50.9
D15S1016 15q21.3 51.3
D15S1049 15q21.3 53.5
D15S1029 15q21.3 53.7
D15S962 15q21.3 54.3
D15S117 15q21.3 56.3
D15S1011 15q22.2 60.5
D15S125 15q22.3 64.9
D15S980 15q24.1 70.9
D15S114 15q24.3 74.7
D15S984 15q24.3 75.7
D15S211 15q25.1 79.0
D15S205 15q25.2 82.0
D15S979 15q25.3 86.6
D15S202 15q26.1 87.8
D15S647 15q26.1 90.6
D15S649 15q26.1 92.0
D15S130 15q26.2 92.5
D15S533 15q26.3 95.5
D15S642 15q26.3 n.a.
D15S985 15q26.3 96.5
D15S966 15q26.3 96.7
D15S120 15q26.3 97.4
region 2
region 1
region 3
LOH
Retention of heterozygosity
Not informative
Allele shift


b
Mbp
11a 31a 37a 38a 40a 41a 49a 53a 53b 53c 54a 54b 58a 58b 58c 58d 58e 58f 58g 58h 59a 65a 52a 52b 52c 52d 56a 56b 56c 57a 57b 57c 67a
D15S217 15q13.1 25.7
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D15S118 15q14 34.0
D15S194 15q14 36.1
D15S1044 15q14 37.4
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D15S1011 15q22.2 60.5
D15S125 15q22.3 64.9
D15S980 15q24.1 70.9
D15S114 15q24.3 74.7
D15S984 15q24.3 75.7
D15S211 15q25.1 79.0
D15S205 15q25.2 82.0
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D15S202 15q26.1 87.8
D15S647 15q26.1 90.6
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D15S130 15q26.2 92.5
D15S533 15q26.3 95.5
D15S642 15q26.3 n.a.
D15S985 15q26.3 96.5
D15S966 15q26.3 96.7
D15S120 15q26.3 97.4
region 2
region 1
region 3
LOH
Retention of heterozygosity
Not informative
Allele shift

node metastases from 16 patients (76% of patients) (Figure 1). Once a microsatellite repeat was affected in the primary tumor, it was also affected in every corresponding metastasis. However, differences between primary tumor and/or metastases from the same patient could be detected. In total, 25 carcinomas showed LOH in a single overlapping region at 15q21.3 (42% of patients). This defines a region of alterations comprising the microsatellite loci D15S106 (proximal), D15S1016, D15S1049, and D15S1029 (distal), estimated as 3.9 Mb (region 1 in Figure 1). It is defined by proximal breakpoints in six tumors (no.13, no.18, no.36, no.37, no.41, no.61; Figure 1) and by distal breakpoints in six tumors (no.11, no.33, no.37, no.40, no.41, no.49; Figure 1). Examples of electropherograms demonstrating LOH in these loci are shown in Figure 2.

Three primary tumors and/or corresponding metastases (no.52, no.56, no.57) displayed LOH in 15q13.3–q14 with a single region of overlap comprising D15S144, D15S995, and D15S1040 (region 2 in Figure 1). This region is estimated as 2.4 Mb. Its proximal breakpoint could be found in three tumors with aberrations restricted to region 2 (no.52, no.56, no.57) and corresponding metastases as well as in a few metastases not related to these tumors (Figure 1).

The third region of losses found in this study encompasses D15S130, D15S533, D15S642, and D15S985 in 15q26.2–q26.3 (region 3 in Figure 1), estimated as 4.7 Mb. It is defined by proximal breakpoints in four primary tumors and the corresponding metastases (no.31, no.41, no.45, no.52) and in lymph node metastases corresponding to tumors 57 and 59 and by distal breakpoints in six carcinomas (no.3, no.31, no.45, no.52, no.59, no.67).

The relative frequency of involvement of all three regions varied widely: region 1 was affected in 25 of 60 patients with primary tumors (42%), region 2 demonstrated LOH in three of 60 patients with primary tumors (5%), and region 3 showed aberrations in eight of 60 patients with primary tumors (13%) (Figure 1a). A total of 16 patients had one or more metastases (up to eight) with LOH in 15q. Metastases from 12 patients showed losses in region 1 (57% of patients with metastases), metastases from six patients in region 2 (29% of patients with metastases), and metastases from 11 patients in region 3 (52% of patients with metastases). Three metastases displayed LOH over nearly all analyzed loci (no.31a, no.38a, no.58g; Figure 1b). Alterations with no overlap to aberration regions 1, 2, or 3 were only rare (Figure 1). Allelic instability, determined by the presence of novel alleles in the tumor tissue, could be found in eight primary tumors and 12 metastases (Figure 1).

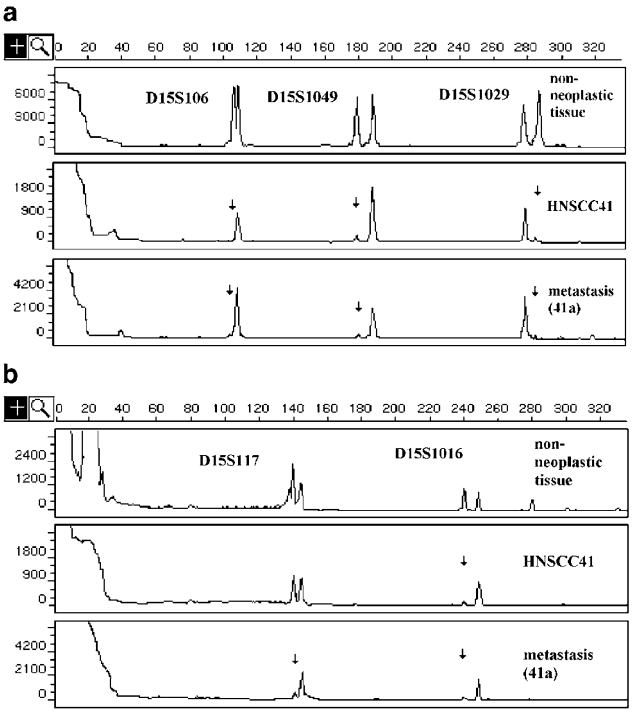

Figure 2 Electropherograms of selected microsatellite loci. Electropherogram of microsatellite loci from region 1 in a primary tumor (41) and a corresponding lymph node metastasis (41a). The *y*-axis represents the peak height in fluorescence units. The arrows mark the lost alleles. (**a**) LOH at D15S106, D15S1049, and D15S1029 in primary tumor and metastasis. (**b**) LOH at D15S1016 in both primary tumor and metastasis, LOH at D15S117 only in the metastatic tissue.

Correlations with Histological and Clinical Parameters

Comparisons between clinical parameters and alterations in region 1 are summarized in Table 1. No correlation could be established for the localization of the tumor (larynx, oropharynx, hypopharynx) ($P=0.7735$) or grading ($P=0.526$). In addition, no relation between aberrations and smoking history or alcohol abuse could be found ($P=0.0539$ and $P=0.0599$, respectively). In contrast, we detected for region 1 a significant positive correlation to T stages ($P=0.0029$) and to the occurrence of metastases ($P=0.0018$).

No correlations with localization of the tumor, grading, smoking history, alcohol abuse, or T stages were found for the other two regions (data not shown), although LOH in regions 2 and 3 shows a connection to metastases (region 2: $P=0.0129$; region 3: $P=0.0013$).

Figure 1 Pattern of LOH in head and neck primary carcinomas and lymph node metastases. Pattern of LOH in primary tumors (**a**) and lymph node metastases (**b**) with deletions in 15q. The 34 primary tumors have been taken from 34 different patients. The 33 metastases were also derived from these patients as indicated by the number (for example 53a, 53b, and 53c are three different lymph node metastases to primary tumor 53). Microsatellite markers and their position in megabases from 15pter according to Ensembl (http://www.ensembl.org) are shown on the left. Three regions with aberrations are marked by vertical bars 1, 2, and 3 (to the right).

Table 1 Correlation between clinical parameters and allelic losses in region 1

Aberrations in region 1	*T-status*					*Metastasis*	*Localization*			*Grading*		*Smoking history*	*Alcohol abuse*
	T4 (n = 26)	*T3* (n = 16)	*T2* (n = 13)	*T1* (n = 5)	*ND* (n = 3)	(n = 31)	*L* (n = 37)	*O* (n = 16)	*H* (n = 10)	*G2* (n = 50)	*G3* (n = 13)	(n = 47)	(n = 24)
Yes	17	6	1	0	1	18	20	8	5	24	9	23	6
No	9	10	12	5	2	13	17	8	5	26	4	26	19
P-value			**0.0029**			**0.0018**		0.7735		0.526		0.0539	0.0599

ND, not determined; L, laryngeal; O, oropharyngeal; H, hypopharyngeal.
Significant *P*-values are printed in bold.

Discussion

Aberrations of the long arm of chromosome 15 have been shown in a variety of studies on head and neck squamous cell carcinomas.[4,8–13] In this study, three regions of aberrations on the long arm of chromosome 15 could be identified in head and neck primary tumors and metastases. The most frequently affected region 1 stretches between D15S106 and D15S1029 in 15q21.3, region 2 lies more proximal in 15q13.3–q14 between D15S144 and D15S1040, and region 3 more distal in 15q26.2–q26.3 between D15S130 and D15S985. The aberrations found in our study could reflect two different events in the tumorigenesis. First, they could display gains of chromosomal material, which would be in line with results from Brieger *et al*[11] describing gains in 15q11.2–q15 (thus including our region 2) and in 15q25-qter (including our region 3) as an early event in oropharyngeal carcinomas. Second, the underlying alteration could be loss of chromosomal material (LOH). As only aberrations in which one allele is completely or nearly completely absent were evaluated for this study, we favor the second theory. Losses of chromosomal material from the long arm of chromosome 15 have also been reported by others. Allelic loss from 15q21 has been shown by Beder *et al*[4] in up to 62% of head and neck carcinomas, but this study—being a genome-wide LOH analysis—did not analyze a possible correlation between LOH data and clinical data. Hannen *et al*[12] demonstrated losses of the whole long arm of chromosome 15 and Squire *et al*[10] reported on losses even proximal to our region 2. Deletions in 15q21 with significance in tumor progression were shown by comparative genomic hybridization analysis (CGH).[8] Feenstra *et al*[9] detected losses in 15q21–22.3 with markers flanking the *beta-2-microglobulin* (*β2m*) gene in head and neck carcinomas and declared this as an event prior to metastasizing.

A comparison with other common regions of allelic loss on chromosome 15 in breast cancer, mesothelioma, or bladder cancer revealed little overlapping losses. Mostly, allelic losses occurred more proximal in 15q14,[20] 15q15,[21,22] 15q15–q21.1,[23] or 15q21.1.[24] However, Kee *et al*[23] showed LOH also in 15q21.3–24 and in 15q26.2–26.3 in primary small cell lung cancer and Tahara *et al*[25] demonstrated allelic losses in 37% of parathyroid adenomas in 15q26-qter. Here, at least partial overlaps to our regions 1 and 3 could be seen. Yen *et al*[15] described predominantly gains of 15q in esophageal carcinomas. A possible explanation for the differences in the specific losses between the different studies of head and neck carcinomas could be the fact that our analysis revealed in the majority of lymph node metastases and all but one primary tumor only small interstitial changes which would have been missed by cytogenetic or CGH studies. This phenomenon of interstitial deletions has also been described in a recent LOH study on transitional cell

carcinoma of the bladder[21] and in advanced breast carcinomas.[24]

Most lymph node metastases showed allelic losses covering a larger area than in the corresponding primary tumors. Different subclones in head and neck carcinomas have already been shown in cytogenetic analyses[26,27] and discordance between primary tumor and lymph node metastases could be demonstrated in recent fluorescence *in situ* hybridization (FISH) studies.[28–30] Gotte *et al*[30] reported on the highest rate of intratumoral discordance between primary tumors and corresponding metastases. Jin *et al*[31] even proposed a multicellular origin for an oral tumor with two highly complex cytogenetically unrelated clones. Intratumoral genetic heterogeneity could also be shown by LOH studies in colorectal cancers[32] and renal cell carcinomas.[33] Fukunari *et al*[34] demonstrated in colorectal carcinomas that 75% of genetically heterogeneous carcinomas but only 12.5% of genetically homogeneous carcinomas developed metastases, thus establishing a correlation between genetic heterogeneity and metastasis. Jones *et al*[35] found six bladder carcinomas (out of 24 investigated tumors) with a different LOH pattern in each of its lymph node metastases, but were able to confirm the same clonal origin. The authors conclude that the capability for metastatic spread often arises in only a single clonal population in the primary tumor, and that the variable LOH pattern reflects genetic divergence during the clonal evolution. Differences in allelic losses patterns between primary tumors and metastases could also be due to varying admixtures of normal cells. The occurrence of this admixture during the cell preparation could not be completely ruled out. Such a different pattern with interstitial deletions is referred to as a 'zebra pattern'.[36,37] The different markers studied may be affected differently by the admixture of normal cells. However, the number of deleted lower and higher molecular weight alleles is roughly the same indicating a good reliability of the data.[37]

Region 1 as presented in this study is estimated as 3.9 Mb and contains 21 known genes whose functions are mostly not yet fully determined. One interesting candidate here may be the *cell cycle progression gene* 1 (*CCPG1*[38]) which is involved in cell cycle regulation. Other examples for known genes in this region are *one cut domain family member 1* (*ONECUT1*) important for the regulation of a wide variety of genes expressed in hepatocytes,[39] *pygopus 1* (*PYGO1*) possibly involved in the activation of WNT target genes,[40] *neural precursor cell expressed developmentally downregulated 4* (*NEDD4*) probably playing a role in the regulation of epithelial sodium channel function, and *RAB27A*, a member of the RAS oncogene family. In addition, *BCL2-like 10* (*BCL2L10*) lies very close to the region of overlap and in many tumors the alterations extend to the region covered by this gene. Overexpression of *BCL2L10* is known to suppress apoptosis.[41]

Region 2 is a little smaller (estimated as 2.4 Mb) and contains 25 known genes including the caspase activation inhibitor *AVEN*,[42] *solute carrier family 12 (potassium/chloride transporters, member 6* (*SLC12A6*) with a possible role in the development of the nervous system,[43] or *ryanodine receptor 3* (*RYR3*), a calcium release channel with low Ca(2+) sensitivity. In addition, this region is in the near vicinity of the region affected in Prader–Willi and Angelman syndromes[44] and the deletion of DNA in 15q13–14 (region 2) may cause additional clinical features in these patients.[45] Region 3 is estimated as 4.7 Mb and contains 15 known genes including one (*LOC440311*) similar to glioma tumor suppressor candidate gene 2 (*GLTSCR2*[46]). *GLTSCR2* has been localized in a common deletion region for human diffuse gliomas on chromosome 19, together with other genes like *GLTSCR1*. Polymorphisms in *GLTSCR1* have been shown to correlate with the occurrence of oligodendrogliomas.[47] LOC440311 may therefore represent a candidate worthy of further analysis. Further genes located in this region are *nuclear receptor subfamily 2 group F member 2* (*NR2F2*) with possible importance in angiogenesis and *arrestin domain containing 4* (*ARRDC4*).

A comparison of the occurrence of aberrations in 15q in head and neck carcinomas with clinical parameters revealed major differences for the three affected regions. Aberrations in region 1 were found significantly more often in advanced tumor stages than in early invasive carcinomas. More importantly, we were able to demonstrate a significant correlation between alterations in region 1 and the occurrence of metastases. These results suggest that one or more gene(s) in 15q21.3 are important for the capacity to metastasize in head and neck squamous cell carcinomas. Losses of the long arm of chromosome 15 have already been described to correlate with metastasizing head and neck carcinomas[8] (including metastasizing tongue carcinomas[12]) underling the value of our results. In contrast, alterations in the regions 2 and 3 were predominantly found in metastases but only in few primary tumors with lymph node status $pN>0$. Therefore, these allelic losses could characterize a certain subset of metastases whose importance has to be determined by further investigations.

In summary, our results assign genes in 15q21.3 a high influence in the development of metastases. Alterations in this region could be judged as a possible prognostic factor to assess the metastatic capacity of a head and neck carcinoma.

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