Characterization of the 11q13.3 amplicon in head and neck squamous cell carcinoma
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Chapter 2

Amplification of the 11q13.3 region coincides with 17p21.1 and 20q13.31 amplification in HNSCC

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Submitted
ABSTRACT

Head neck squamous cell carcinoma (HNSCC) includes laryngeal (LSCC), pharyngeal (PSCC) and oral squamous cell carcinoma (OSCC) and is characterized by recurrent genomic abnormalities. We applied array based comparative genomic hybridization (aCGH) to 34 PSCC and LSCC and detected a high frequency of 11q13.3 amplification.

To study 11q13 amplification in LSCC, PSCC and OSCC specifically, we complemented our data with aCGH data on 89 OSCC and CGH data on 82 LSCC and 124 PSCC and we determined the the most frequent aberrations per tumor subsite and co-aberrant regions. The most frequent aberrations (in >25% of the cases) in this series of 329 HNSCC in total, were loss of 3p13–p25, 8p23.1–p23.3, 9p21.1–9p24.3 and 18q21.1–18q23 and gain/amplification of 3q12.1–3q29, 8q21.3–8q24.3 and 11q13.1–11q13.5. Comparison of the recurrent aberrations in 132 PSCC, 89 OSCC and 108 LSCC revealed that OSCC are characterized by more frequent loss of 8p23 and 18p11.32, and less frequent gain of 3q and 17q. PSCC contained more frequently increased copies of chromosome 3q, 7q11–q31, 19p11–q13 and 20q11–q13, whereas in LSCC loss of 13q14–q31 is more frequent. Furthermore, the median number of aberrant chromosomal bands was significantly higher in PSCC (342/811) compared to LSCC (210/811) and OSCC (156/211). Not only 11q13.3 gain but also high-level amplification was found most frequent in PSCC. Using CGHMultiarray statistical analysis to chromosomal bands, copy number increases at 17q12–q21.2 and 20q13.3 were related to increased copies of band 11q13.3.

The frequent co-amplification of 11q3.3 and 17q12–22 suggests that amplified genes within these regions, including CCND1 and ErbB2, might underlie genomic instability.
INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC), including oral (OSCC), pharyngeal carcinoma (PSCC) and laryngeal squamous cell carcinoma (LSCC), is a significant cause of morbidity and mortality throughout the world [126,195]. Overall 5–year survival rates for HNSCC has remained unchanged at approximately 50% over the past 30 years [195–197]. Currently, clinical markers such as T–stage and lymph node metastasis are used to determine treatment strategies. However, the predictive value of these clinical markers is limited and therefore new molecular prognostic markers are needed. Although the different HNSCC sub–sites (i.e. OSCC, PSCC and LSCC) are often combined to ensure statistical power in different studies, there are considerable differences in disease progression and patient survival in the separate sub–sites. These biological differences have previously been explained by anatomical variations, e.g. the variability of the lymphatic drainage [198,199] or exposure to environmental factors [200]. The pharynx has an extensive lymphatic drainage system and an increased incidence of lymphatic metastasis at the time of diagnosis whereas specific areas of the larynx e.g. glottis have significantly poorer drainage and are less frequently associated with nodal metastasis [199]. For a better understanding of the genetic alterations in HNSCC and to identify new molecular biomarkers that predict clinical outcome, various studies reported such alterations using multi–probe FISH [201‘], restriction landmark genomic scanning (RLGS)[176], classical cytogenetics [202] and classical/conventional CGH [134‘,203,204], and more recently high–resolution and whole–genome array CGH [173‘,175,205–208‘]. In most of these studies, a mixture of HNSCC subsites was investigated assuming that HNSCC at the various subsites have a common profile of somatic genetic abnormalities. However, few studies, although with relative low numbers of cases from each of these subsites, suggested that the genetic aberrations differ between the subsites [198,209,210]. In agreement with the genetic differences, also immunohistochemical studies showed a clear difference in expression of certain markers such as cyclin D1 and EGFR [211].

Gain/amplification of the chromosome 11q13.3 region is the most frequent aberration in HNSCC [212] and high–level amplification has been reported to be associated with shorter time to recurrence [140] and the presence of lymph node metastasis [138,143,144‘]. In Amplification of 11q13.3 and overexpression of amplified 11q13 genes has also been related to increased genomic instability [131‘,210]. Presently, it is not known whether 11q13 amplification coincides with specific recurrent aberrations or genomic instability in general.

We performed aCGH on a set of 26 laryngeal and 8 pharyngeal carcinomas. To compare genomic alterations in HNSCC of different subsites, we combined our aCGH data with previously published CGH derived from OSCC (n=89)[175] and from PSCC (n=124), and LSCC (n=82)[212]. Furthermore, we investigated the genetic profile of recurrent aberration between carcinomas with/without 11q13 amplification.
MATERIAL AND METHODS

PATIENT MATERIAL
We have used snap frozen material from 26 laryngeal and 8 pharyngeal patients to perform aCGH to either BAC arrays (3 pharyngeal, 15 laryngeal carcinomas from University Medical Center Groningen) or oligo arrays (5 pharyngeal, 11 laryngeal carcinomas from the Hospital Universitario Central de Asturias, Oviedo, Spain). These patients had previously been studied for 11q13 amplification using a dedicated 11q13 array [207].

aCGH PLATFORMS
For the detection of genomic aberrations two whole genome aCGH platforms (WGA) were used. The whole genome oligonucleotide array was generated at the microarray core facility from the VU Medical Center in Amsterdam and contained 28,355 carefully selected probes dispersed throughout the genome [213].

The Whole genome BAC–array was constructed at the microarray core facility of the University Medical Center Groningen (UMCG) in Groningen. The WGA BAC array contained 3500 BAC clones obtained from Dr Nigel Carter (Wellcome Trust Sanger Institute, UK), supplemented with clones from the Human BAC Resource Consortium–1 Set (Dr Pieter de Jong, Children's Hospital Oakland Research Institute, Oakland, California), dispersed throughout the genome with an average resolution of 1 clone per megabase (Mb) [95]. The BAC DNA isolation procedure was adapted from the protocol published at the website of Dr. M Rocchi (http://www.biologia.uniba.it/rmc/) and carried out in 96–well plates (adapted from Dr. Reinhard Ullman, Max Planck institute, Berlin) as described previously [97]. Array hybridization, signal processing and normalization have been described elsewhere [213, 214].

DATA SMOOTHING AND SEGMENTATION
Clones from both array platforms were remapped to the latest build of the human genome (NCBI build 36.2/UCSC build 18) by batch conversion using UCSC Liftover (http://genome.ucsc.edu/cgi-bin/hgLiftover). Log2 ratios of individual clones were smoothed into levels of similar ratios using circular binary segmentation, as implemented in the CGHcall package for “R” [215] (Supplementary Figure 1). The smoothed levels were applied to the mergeLevel procedure to validate whether segments exist with “similar mean” levels [216, 217]. CGHcall further provided the regions that were significantly aberrant in the tumor DNA compared to normal DNA (losses, gains and amplicons). Chromosome X and Y were used as an internal (gender mismatch) control and were excluded from further aberration evaluation.
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Gains from amplifications.

**WGA aberration confirmation**

For the analysis of whole genome chromosomal aberrations in patients with HNSCC, array data from 16 patients were combined with data obtained from 18 patients analyzed with an oligo array or a BAC array respectively. Although the sensitivity of the oligo array differed from the BAC array, aberrations detected with both platforms could be verified by comparing them with a BAC array specific for the long arm of chromosome 11q (Supplementary Figure 2)[207]. Copy number ratios obtained from WGA CGH were compared to ratios obtained with the High-Resolution 11q-specific CGH array as reported previously [207] to discern gains from amplifications. To enable the combination of array data from multiple platforms we translated the position of clones into chromosomal bands using the Progenetix database (http://www.progenetix.net)[218] (Supplementary Figure 3).

**Supplementary Figure 1: Genomic aberrations determined by CGHcall for 34 HNSCC and 89 OSCC analyzed with aCGH.** Illustrated are 3 carcinomas as an example (cases L20, L04 en X4118). The chromosomal location of array clones (black dots) is shown from chromosome 1 to 22 along the x-axis. Log2 ratios of are shown on the right Y-axis. The probability score determined by CGHcall, for the detection of gains (red) and losses (blue), are shown on the left Y-axis. Smoothed log2 ratios are illustrated by gray lines.
Progenetix analyses

Aberration frequencies from raw data were obtained by applying the tools available at the Progenetix data repository (http://www.progenetix.net) to both arrays separately [218]. Progenetix enabled platform comparison by relocating array probes, for both platforms separately, to the nearest of 862 cytogenic bands available. The CBS smoothed and called data of all aCGH platforms were uploaded to Progenetix.

Previously published aCGH data of 89 OSCC [175] were uploaded to Progenetix (http://www.progenetix.net) after smoothing and segmenting the data (see below). From the Progenetix database we selected all 124 pharyngeal (C10, C13 and C140) and 82 laryngeal tumors (C32) except carcinoma in situ and nasopharyngeal carcinoma (C11), which were considered as different entities.

After uploading data of all 329 carcinomas to Progenetix, the genomic aberrations were analyzed per band (for 826 bands available). Progenetix calculated the number of aberrant chromosomes and the number of aberrant bands per case.

Supplementary Figure 2: Whole genome array CGH analysis compared to 11q13-specific array CGH analysis. Gray areas display the amplicon boundaries. Dark gray lines indicate merge level values of circular binary segmentation [216]. The analysis of the these carcinomas using the 11q13-CGH array was reported previously [207].

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CLUSTER ANALYSIS

Cluster analysis for the number of aberrant chromosomes and the genome position was performed by applying Pearson clustering to the Progenetix output data in Genesis 1.7.2 (Graz University of Technology) [219].

STATISTICS

We determined the genomic regions (chromosomal bands) enriched in two dichotomized subsets of patients with CGHMultiarray [220]. The web–implemented CGHMultiarray applies a Wilcoxon two–sample statistic with ties. We applied a Bonferroni correction for the number of chromosomal bands tested (n=811 for 826 bands without chromosomes X and Y). Chromosomal instability between different HNSCC subsites was tested by one–way ANOVA and a Bonferroni correction for the columns tested using Prism 5 (GraphPad Software, Inc). Further statistical analyses were performed using Statistical Package for the Social Sciences version 14.0.0 (SPSS, Chicago, USA). All tests were two–sided and P values of < 0.05 were considered significant.

RESULTS

RECURRENT GENETIC ALTERATION IN HSCC USING CGH.

We performed aCGH on 27 LSCC and 7 PSCC of which gains, losses and amplicons are shown in Figure 1A. We compared our aCGH data with those from a previously

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published aCGH study on 89 oral squamous cell carcinomas (OSCCs)[175](Figure 1B) and 206 HNSCCs analyzed with conventional CGH, available at the Progenetix database (Figure 1C)[218]. The most frequently lost region in all 329 HNSCC cases from the combined data of these 3 studies (Figure 1D) was 3p14.1–p14.2 (40%). Other regions lost in more than 25% of cases were found at 3p13–p25 (>35%), 8p23.–p23.3 (>26%), 9p21.1–9p24.3 (>26%) and 18q21.1–18q23 (>26%). The most frequently detected gain/amplification was located at 3q26.31–3q27.1 (61%) and regions with gain in more than 25% were limited to 3q25.1–3q29 (-51%), 8q24.11–8q24.3 (>40%) and 11q13.1–11q13.5 (>30%) (Figure 1D). High-level amplification was most frequently observed at 3q27 (6%) 11q13.3–q13.4 (9%).

Figure 1: Whole genome data derived from a combination of 329 HNSCC cases. Cases were combined from (A) our aCGH (n=34) and (B) aCGH on OSCC published by Snijders et al [175](n=89) and (C) CGH data from the Progenetix database (n=206) [218]. (D) results of CGH analysis of all 329 HNSCC. Orange horizontal indicate aberrations present in more than 25% of cases. Chromosomal losses are shown in blue and gains are shown in red. Large copy number increases (amplifications) are colored dark red.
**Recurrent genetic alterations differ between HNSCC from various subsites.**

To investigate the presence of subsite–specific recurrent genetic alterations, we compared the genomic profiles of 108 LSCC, 132 PSCC and 89 OSCC (Figure 2). Comparison of carcinomas originating from these subsites showed that loss of 8p23 (p = 1.3x10⁻⁸) and 18p11.32 (p = 0.0003) was found more frequently in OSCC, whereas gain of 3q and 17q were significantly less frequent. In PSCC gain/amplification of the entire 3q arm and chromosome 7q11–q31, 19p11–q13 and 20q11–q13 were observed more frequently, whereas LSCC were characterized by loss of 13q14–31 (Figure 2). Gain/amplification of the 11q13.3 region was present in LSCC (25%), PSCC (51%) and OSCC (36%).

In order to estimate the degree of genetic instability in HNSCC at these three subsites, we determined the mean number of aberrant chromosomal bands. Of the defined 811 chromosomal bands and 21 chromosomes tested, the total number of aberrant bands and chromosomes was significantly higher in PSCC (342/811 and 9/21) compared to OSCC (210/811 and 6/21; p = 0.001) and LSCC (156/811 and 6/21; 23/21).

**Figure 2:** Whole genome data derived from 329 HNSCC cases, depicted by cancer sub-site. Larynx: n = 108; Pharynx: n = 132; Oral: n = 89. Significantly different regions, detected by CGH Multiarray, are marked by circles and text on top of each graph. Chromosomal losses are shown in blue and gains are shown in red. Large copy number increases (amplifications) are colored dark red.

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Supplementary Figure 4: Euclidean clustering of different platforms using Genesis. Cases are clustered to indicate similar patterns of aberrant chromosomes. There are no obvious differences between cases analyzed with aCGH cases (blue: UMCG; red UCSF) and cases analyzed with conventional CGH (obtained from Progenetix, green).
Amplification of 11q13.3 coincides with amplification of 17p21.1 and 20q13.31 (p=0.01). To ensure that these differences were unrelated to the use of either aCGH or classical CGH we performed hierarchical clustering (Supplementary Figure 4). Cases analyzed with CGH, derived from the Progenetix database, did not cluster separately from cases analyzed with aCGH.

Figure 3A: Clustering of 329 HNSCC cases by the presence of 11q13.3 amplification. Chromosomal aberrations are shown along the genome (vertically) per case (horizontally). For every case, chromosomal gains are shown in red and chromosomal losses are shown in blue. Chromosomes are numbered on the right (top to bottom) and depicted as p-arm (gray) and q-arm (black). Cases are color coded according to tumor origin: LSCC (blue), PSCC (red) and OSCC (green). The horizontal gray bars depict regions that are associated with 11q13.3 amplification.
Amplification of the 11q13.3 region coincides with amplification of 17q21.1 and 20q13.31.

The 11q13 region is one of the most frequently amplified regions in HNSCC [212] [this study], and has been suggested that a non-random co-amplification with other chromosomal regions frequently occurs [221]. To investigate what alterations coincide with 11q13.3 gain/amplification, alignment of 11q13.3 positive cases (n=126) versus 11q13.3 negative cases (n=203) revealed that significant co-aberrant regions included gains of chromosome 17 and the long arms of chromosome 1 and 20 (Figure 3A). Statistical analysis, using CGHMultiarray, revealed that the most significant relations were found for 17q21.1 (p=6.3 x10^-7) and 20q13.31 (p=8.7x10^-5) (Figure 3B). These same regions were also overrepresented in PSCC (Figure 2).

Figure 3B: Statistical analysis by CGHMultiarray showing aberrant chromosomal regions related to the presence of 11q13.3 amplification. The level of significance (y-axis) between gain of 11q13.3 and gain or loss of other regions (x-axis) is shown. The significance for 11q13.1-q13.5 is a consequence of comparing 11q13.3 negative tumors to 11q13.3 positive tumors.

Discussion

Squamous cell carcinomas, derived from the different sub-sites of the head and neck region, are often treated as a single entity. Most studies on the molecular genetic analysis of HNSCC were performed on a mixture of SCC originating from different subsites. By combining our data with those of two large (a)CGH series, our analysis showed that although many similarities between OSCC, LSCC and PSCC exist, the frequency of various specific recurrent genomic alterations differed significantly between these sites (Figure 2). These differences might provide new tools to explain the biological differences between HNSCC originating from these sites. In addition, we confirm the high frequency of many previously reported aberrations that were determined in a sub-band resolution. This analysis limits the amount of putative target genes within the most frequently aberrant regions.
Recurrent aberrations in HNSCC

The analysis of 329 HNSCC carcinomas revealed that the most frequent chromosomal losses are found at 3p14.1–14.2, 8p23.1–p23.3, 9p21.1–9p24.3 and 18q21.1–18q23 confirming previously reported whole genome screens [222] (reviewed by [223]). The loss of these regions have been suggested to attribute to the loss of the residing tumor suppressor genes FHIT (at 3p14.3), CSMD1 (at 8p23)[224], and CDKN2A (at 9p21.1) and DCC (at 18q21.1)[225], respectively. The most frequent chromosomal gains included 3q12.1–3q29 (Cyclin L and PIK3CA)[226, 227], 8q21.3–8q24.3 (MYC and LRP12)[206] and 11q13.1–11q13.5 (TPCN2, CCND1, ORAOV1, PPFIA1, FADD and CTTN)[174**,208**,228]. In addition, high-level DNA amplification of 11q13.3, 3q26–q27 and 8q24 are within the top 25% of aberrations in HNSCC (Figure 1D). Amplification of 11q13.3 has been described for many carcinomas and is one of the most abundant aberrations in HNSCC [107**,212].

Different recurrent aberrations at specific HNSCC subsites

Comparison of carcinomas originating from LSCC (108), PSCC (132) and OSCC (89) (Figure 2) revealed various recurrent aberrations that might be characteristic for a particular subsite. In these chromosomal regions numerous genes are located some of which might be involved in the disease. In PSCC gain of the chromosome 3q arm, 7q11–q31 (MDR1/ABCB1)[229], the 19q arm (CCNE1, TGFBI) and 20q11–q13 (E2F1, MMP9, CYP24, ZNF217) were observed more frequently, whereas LSCC were characterized by loss of 13q14–31 (RB1). In OSCC loss of 8p23 (CSMD1) and 18p telomere was found more frequently, whereas gain of 3q (CCNL, BCL6, PIK3CA) and 17q (TOP2A, ERBB2) were significantly less frequent. Increased copies of 19q and 20q have previously been associated with lymph node metastases [230] and PSCC have an increased incidence of lymph node metastasis [199] and a worse 5–year relative survival compared to LSCC and OSCC [197]. There are many genes located within these differentially aberrant regions. Thus, although some genes might be functionally related to these aberrations, it is difficult to pinpoint them based on these genomic associations.

In this manuscript we showed that gain/amp of 11q13.3 was most prominent in PSCC (51% of the cases) compared to LSCC/OSCC (Figure 3) in agreement with this study and others [209,231]. Since CGH analysis also revealed that PSCC are the most genomically unstable HNSCC as reported by others [209,210], 11q13.3 amplification might be related to the genomic instability. Overexpression of the 11q13.3 gene cyclin D1, has directly been related to genomic instability [131*,210], but within the 11q13.3 region other candidate oncogenes have been identified [148,173*,174**,208**,232*]. High expression of FADD [232*], cortactin [233**] and PPFIA1 [234] have independently been shown to correlate with lymph node metastasis, overall survival and/or in cell migration and invasion. This strongly suggests that amplification of 11q13.3 and concomitant overexpression of these genes mediates the invasive potential of PSCC.

Amplification of 11q13.3 coincides with amplification of 17p21.1 and 20q13.31
Gain/amplification of 11q13.3 coincides with gain of 17q13–q25 and 20q13.3

We showed that gains of 17q13–q25 and 20q13.3 are more common in tumors with 11q13.3 amplification. Recurrent homologous staining regions within these bands have been detected in breast carcinomas (BC) [235] and overrepresentation of these regions in HNSCC has been found using conventional CGH [134*, 204, 236] and restriction landmark genome scanning [176]. Amplification of 20q13 has been related to adverse patient outcome in BC [237] and is a frequent event in many carcinomas including BC and HNSCC [176, 238]. Amplification of 20q13.3 has previously been fine–mapped to two different amplicons containing CYP24 and ZNF217 respectively [171**].

We found the most significant relation between gain of 11q13.3 and 17q at 17q–2–q21.2. Centered in this region are TOP2A and ERBB2 (HER–2/neu) [239, 240]. TOP2A and ERBB2 might both affect chemotherapy in BC [241]. However, specific deletion of the TOP2A gene in cases that harbor amplification of the nearby ERBB2 locus, favors a role ErbB2, rather than TOP2A in 17q12–q21.2 amplification in BC [242]. In HNSCC using immunohistochemistry ErbB2/Her2/neu overexpression rates varied between 0 and 47 % [243] but only low percentages of amplification of ERBB2 have been reported yet [244, 245]. Hyper activation of the ErbB pathway, resulting in a potent growth signal, is often found in human cancer [246]. Amplification of ERBB2 results in overexpression of ErbB2 and is directly related to hyper activation of the ErBb pathway [247, 248]. Yet, neoplastic transformation involving ErbB2 is enhanced by cyclin D1 expression [249], indicating that co–amplification of both genes confers to a worse patient prognosis.

Amplification of the epidermal growth factor receptor (EGFR/ErbB1, 7p11.2) also leads to activation of the ErbB–pathway [246] and has been related to adverse patient prognosis in HNSCC [250]. Previously, Timpson et al. have shown that overexpression of cortactin (EMS1/CTTN; 11q13.3) led to sustained signaling of EGFR via attenuated ligand–induced down–regulation [251] and we speculated that 11q13 amp/ cortactin overexpression would be mutually exclusive with EGFR overexpression in HNSCC [252]. Our CGH analysis of 329 HNSCC revealed gain of 7p11.2 (containing EGFR/ErbB1) in 17% of cases and there was no relation to increased copies of 11q13.3 (data not shown). These results imply that amplification of 11q13.3 and 7p11.2 could independently lead to sustained signaling of EGFR, and once more underline that EGFR activation in HNSCC can be triggered via different mechanisms. EGFR amplification predicts sensitivity to the EGFR inhibitor gifitinib, whereas overexpression of ErbB2 and ErbB3 associated with gefitinib resistance [253]. These results indicate that amplification of specific ErbB–family members might affect patient prognosis.

In conclusion we found that 11q13.3 is the most frequently amplified region in HNSCC and PSCC specifically. Significant co–amplification of 20q13 and 17q–2–q21.2 might indicate a specific genomic makeup that requires specific treatment. Profiling of genomic alterations might be a valuable tool in HNSCC to determine treatment strategies.
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