Characterization of the 11q13.3 amplicon in head and neck squamous cell carcinoma
Gibcus, Johan Harmen

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Chapter 3

High-resolution mapping identifies a commonly amplified 11q13.3 region containing multiple genes flanked by segmental duplications

Johan H. Gibcus, Klaas Kok, Lorian Menkema, Mario A. Hermsen, Mirjam Mastik, Philip M. Kluin, Jacqueline E. van der Wal and Ed Schuuring

ABSTRACT

DNA amplification of the 11q13 region is observed frequently in many carcinomas. Within the amplified region several candidate oncogenes have been mapped, including cyclin D1, TAOS1 and cortactin. Yet, it is unknown which gene(s) is/are responsible for the selective pressure enabling amplicon formation. This is probably due to the use of low resolution detection methods. Furthermore, the size and structure of the amplified 11q13 region is complex and consists of multiple amplicon cores that differ between different tumor types. We set out to test whether the borders of the 11q13 amplicon are restricted to regions that enable DNA breakage and subsequent amplification. A high resolution array of the 11q13 region was generated to study the structure of the 11q13 amplicon and analyzed 29 laryngeal and pharyngeal carcinomas and 9 cell lines with 11q13 amplification. We found that boundaries of the commonly amplified region were restricted to 4 segments. Three boundaries coincided with a syntenic breakpoint. Such regions have been suggested to be putatively fragile. Sequence comparisons revealed that the amplicon was flanked by two large low copy repeats known as segmental duplications. These segmental duplications might be responsible for the typical structure and size of the 11q13 amplicon. We hypothesize that the selection for genes through amplification of the 11q13.3 region is determined by the ability to form DNA breaks within specific regions and, consequently, results in large amplicons containing multiple genes.
INTRODUCTION

Genomic instability is an important step in generating the multiple changes required for cancer. It plays a role in tumor initiation, development and progression, and confers various biological features to cancer cells such as response to therapy. DNA amplification is one of the manifestations of genomic instability and is frequently found in human cancer. It is one of the mechanisms by which cells can accomplish overexpression of oncogenes and occurs throughout the human genome at different frequencies. The sequence characteristics of the loci where DNA amplification can occur remain largely unknown. For several decades, the mechanisms of DNA amplification have been extensively studied mainly for genes that are involved in the resistance to cytotoxic drugs. Several models explaining the mechanism of gene amplification have been proposed, including translocation–excision–deletion–amplification, deletion–plus–episome, sister–chromatid–exchange, onionskin, extrachromosomal–doubling–rolling circle and the chromatid–breakage–fusion–bridge (BFB) cycle model [17, 47, 59, 193, 254–257]. One of the main initiators of the proposed mechanisms is DNA double strand breakage.

DNA amplification of the 11q13 region is observed in several types of solid tumors, but most frequently (36%) in carcinomas of the head and neck region (HNSCC) [107]. The involvement in several types of carcinoma and the frequent occurrence in head and neck cancer implicate a selective advantage for cells containing increased mRNA levels for genes within the amplified region. Although the amplification is abundantly present, the clinical significance for increased mRNA levels of a specific target gene is not yet found. Efforts to narrow down the overlapping region of amplification resulted both in the identification of different independent amplicon cores (depending on tumor type) as well as several target genes within each core [107, 114, 173, 174, 178]. We propose that DNA breakage occurs at specific regions explaining the position and size of the amplicon.

Using information from the completed human genome sequence, high–resolution DNA copy number analysis to determine the structure and exact location of an amplicon has only recently become available. Whole genome array CGH (WGA) brought the resolution of conventional chromosomal CGH to a higher level, but the use of high–resolution CGH arrays (HR–aCGH) containing oligonucleotides or genomic (BAC) clones that completely cover and overlap a certain locus improved the analysis enormously [19].

We generated a CGH array designed specifically for the 11q13 region with a higher resolution than any previously described method [173, 174, 176, 178] which enables the accurate detection of amplicon structures. We sought to find out whether the formation of 11q13 amplicons is solely due to gene selection or is the result of variation in chromosome structure as reflected in repeat sequences, DNA flexibility and evolutionary breakpoints. For this purpose, we mapped the boundaries of the 11q13 amplicons in a series of 29 HNSCCs and 9 head and neck cell lines with previously
identified DNA amplification of the 11q13 region using this high density HR–aCGH array. By comparing the boundaries of all 38 cases, we found 4 regions flanking the 11q13 amplicons that were common in numerous cases. In silico analysis of the nucleotide sequences at these four common boundaries revealed that 3 of the boundaries coincided with syntenic breakpoints and the identification of 2 inverted large low copy repeats (LCRs), known as segmental duplications (SDs) flanking the commonly amplified 11q13.3 region. We hypothesize that the selection of genes in the 11q13.3 region that become amplified, is determined by the ability to form DNA breaks within specific regions. This will result in the formation of large amplicons containing many genes that are not all necessarily relevant for tumorigenesis and tumor progression.

MATERIAL AND METHODS

HEAD AND NECK CARCINOMAS AND CELL LINES

For this study, we used primary carcinomas of the larynx (n=22) and pharynx (n=8) from 30 patients diagnosed at the University Medical Center Groningen, Leiden University Medical Center (The Netherlands) and the Hospital Valle del Nalon (Oviedo, Spain) (Table 1). These carcinomas were included because all contained 11q13 DNA amplification as determined previously using conventional CGH, Southern blotting or whole genome array analysis (chapter 2 and Table 1). The percentage of tumor cells present in the DNA sample used for FISH and CGH was established by staining parallel sections with hematoxylin and eosin. DNA from these tumors was isolated using a standard high salt extraction method. All patient samples were primary tumors that had received no therapy prior to surgery.

We also included 9 HNSCC cell lines (UMSCC2, UMSCC11A, UMSCC14A, UMSCC14B and UMSCC22B as well as VUSCC59, VUSCC96a, VUSCC147 and VU947T (resp. C01–C09) that contained 11q13 amplification as reported previously [258, 259]. The cell lines were cultured in Dulbecco’s Modified Eagle Medium (DMEM; GIBCO BRL, Gaithersburg, MD) supplemented with 10% FCS (Cambrex Bio Science, Walkersville, Maryland), L–Glutamine (GIBCO BRL) and penicillin/streptomycin (50 units/ml and 50µg/ml) at 37°C in 5% CO2. DNA from these cell lines was isolated using a standard high salt extraction method.

GENERATION OF A HIGH–RESOLUTION CGH ARRAY OF THE 11q13 REGION

We have selected 350 overlapping BAC–clones mapped to chromosome 11q13, resulting in an average two–fold coverage of the entire 11q13 region (BOX 2). To increase the resolution within the different 11q13 amplicon cores, various cosmid and PAC clones that were previously mapped within these loci were added to the set [114,260]. In addition, some BAC clones (n=109) along the chromosome 11q–arm were included on the array. The positioning of the clones was determined using
High-resolution mapping identifies a commonly amplified 11q13.3 region

NCBI build 35.1 (June 2004). The BAC clones were purchased from either BACPAC (CHORI, Oakland, USA) or Invitrogen (Breda, Netherlands). All genomic 11q13 clones on this array are listed in Supplementary Table 1. Methods to construct this CGH array were previously described [97]. Briefly, all clones were grown and isolated in a 96–well format, adapted from Dr. M.Rocchi, University of Bari, Italy; (http://www.biologia.uniba.it/rmc/). A 3–DOP–primer based amplification [95] was applied to the DNA extracted from the genomic clones (BOX 3). These PCR products were

Table 1: Patient inclusion

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Previous, method used previously to detect amplification. CGH, conventional Comparative Genome Hybridization on metaphase spreads; WGA, Whole Genome Array analysis (unpublished; using WGA-arrays as described elsewhere [214]); SB, Southern blot performed with probes for cyclin D1 and EMS1/cortactin [143]; the amplification levels were confirmed by interphase FISH (unpublished); 11q13 amp, amplification (+) detected by HR-aCGH; case p05 contained gain but no amplification (-).
At the beginning of this PhD project, the sequencing of the genome was ongoing and resulted in the release of new builds on a monthly basis (http://may2005.archive.ensembl.org/Homo_sapiens/stats/status.html) (Figure 1). Over 350 overlapping sequenced BACs mapped to the 11q13 region were used to generate an array CGH platform referred to as the 11q13 array.

The initial 11q13 array was based on clones positioned in Build 27, which was established in October 2001. At the first remapping of the 11q13 array clones in June 2002 (Build 30), more of the genome was sequenced and the number of contigs on chromosome 11 was reduced from 128 to 65 (Figure 1), indicating a twofold reduction of sequence gaps. With the “completion” of the human genome in April 2003 (Build 33) an additional 100 BACs were added to fill large gaps in the clone library for the 11q13 array. Furthermore, the position of FISH mapped clones relative to sequenced BACs was determined using clones which contained both FISH-mapping and sequence information [307] (Figure 2). An additional number of (smaller) PAC clones and cosmids was added to the 11q13 array clone-set to further increase the density of clones to an average of 1 clone per 50 kb [207]. Since most clones were (partially) sequenced at Build 33, further remapping was...
High-resolution mapping identifies a commonly amplified 11q13.3 region performed based on sequence information. The finished sequence of Build 33 was also available at other genome browsers such as the UCSC (http://genome.ucsc.edu/cgi-bin/hgGateway) and ENSEMBL (http://www.ensembl.org/Homo_sapiens/index.html). We have used the UCSC LiftOver tool (http://genome.ucsc.edu/cgi-bin/hgLiftOver) to convert clone positions from NCBI Build 35.1 to Build 36.2.

spotted 5 times on epoxy coated slides (Schott Nexterion, Mainz, Germany) using a Biorobotics Microgrid II Arrayer (Isogen Life Science, IJsselstein, The Netherlands). The array also contains a series of control spots (each 5 times) including DOP–PCR products from human Cot–1 DNA (Roche), total human DNA DH10B–bacterial DNA, DNA derived from BACs with Drosophila DNA inserts and spot buffer. Furthermore, the array contained 18 human chromosome X–specific clones and three chromosome Y–specific BAC clones as described previously [261]. The slides were stored at 4°C until further use. Spot quality was verified by hybridization using a random panomer probe (Molecular Probes, Invitrogen, Breda).

**Hybridization**

Hybridization was performed using previously described protocols [96] with small adaptations. Using the manufacture’s protocol the slides were blocked using Nexterion Block E (Schott Nexterion) prior to the hybridization procedure. Reference

Figure 2: The remapping of original clones to a new position. Clones annotated in previous builds only (red) were mapped to new builds using overlapping clones that were mapped in both builds (black). Clones could relocate several Megabases from their original position and sometimes a whole group of clones shifted and changed orientation (blue).
BOX 3: CONSTRUCTION OF THE 11q13 ARRAY

BAC Isolation protocols

In order to determine the best protocol for BAC-isolation and amplification, an array CGH test slide was generated containing a BAC clones that were prepared for spotting using 4 different procedures. Clones were either grown in large quantities without PCR-based amplification or BAC DNA isolated from smaller cell cultures were amplified using either ligation-mediated PCR [96] or degenerate oligonucleotide primer based PCR (DOP-PCR) with one [98] or three different primers [95, 308]. Test hybridizations using cell lines with 11q13 copy number aberrations showed that DOP-PCR using 3 different primers (3xDOP PCR) detected the known copy number aberrations. Furthermore, this random amplification method showed a smaller standard deviation over duplicate clones compared to ligation mediated PCR.

Generation of the 11q13 array

The 11q13 array was designed to cover the q arm of chromosome 11 with the highest clone density at the 11q13 region (Figure 1). BAC DNA was isolated in 2ml of culture medium in a 96-well format. The isolation procedure contained an exonuclease treatment to remove remnant E.coli DNA (adapted from dr. Reinhard Ullman, Max-Planck Institute, Berlin). Isolated BAC DNA was cut using HindIII and MseI restriction enzymes to assess the quality of DNA isolation and enable visualization of DNA after etidium bromide staining. BAC DNA was amplified using the 3xDOP-PCR protocol. Hereafter the PCR products were pooled and spotted on epoxy-coated glass slides (Quantifoil, Jena) using a Microgrid II arrayer. The array consisted of 16 subgrids and each subgrid contained 5 replicates of a single BAC clone (Figure 2). A second generation 11q13 array containing the same clones was constructed in December 2003.

Figure 1: The distribution of clones used to construct the 11q13 array. The overlap of clones (top) and individual BAC clones (bottom) are shown in blue. PAC and cosmid clones are shown in brown. The position was determined after remapping the clones to NCBI build 35. The graph is generated by the UCSC genome browser depicting the remapped clones as “custom tracks”.

The 11q13 array was designed to cover the q arm of chromosome 11 with the highest clone density at the 11q13 region (Figure 1). BAC DNA was isolated in 2ml of culture medium in a 96-well format. The isolation procedure contained an exonuclease treatment to remove remnant E.coli DNA (adapted from dr. Reinhard Ullman, Max-Planck Institute, Berlin). Isolated BAC DNA was cut using HindIII and MseI restriction enzymes to assess the quality of DNA isolation and enable visualization of DNA after etidium bromide staining. BAC DNA was amplified using the 3xDOP-PCR protocol. Hereafter the PCR products were pooled and spotted on epoxy-coated glass slides (Quantifoil, Jena) using a Microgrid II arrayer. The array consisted of 16 subgrids and each subgrid contained 5 replicates of a single BAC clone (Figure 2). A second generation 11q13 array containing the same clones was constructed in December 2003.
DNA consisted of a pool of 20 individuals (male or female) with a normal karyotype. Selected test and reference DNA samples (500 ng) were labeled using the bioprime labeling kit (Invitrogen) in combination with either Cy3–dUTP (Perkin–Elmer/NEN, cat. no. NEL 578) or Cy5–dUTP (NEL 579) added to a buffered mixture of dUTP depleted dNTP’s and Klenow enzyme. An overnight incubation at 37°C was followed by removal of unincorporated nucleotides using micro–spin G50 columns (Amersham Biosciences, Uppsala, Sweden). The incorporation of labeled nucleotides was validated using a ND–1000 spectrophotometer (Nanodrop, Wilmington, USA). The test and reference DNA were pooled and further concentrated using Microcon YM–30 columns (Millipore, Billerica, MA, USA). Labeled DNA was resolved in 50 ul Nexterion Hyb (Schott Nexterion) complemented with dextran sulphate (5%), 200 µg Cot1 DNA (Roche) and 1 mg yeast t–RNA (Invitrogen). Slides were subjected to a 30–90 minute prehybridization at 65°C using 50 µl of hybridization buffer (Schott Nexterion) enriched with dextran sulphate (5%) and salmon sperm DNA (10 µg/ul, Invitrogen). Hereafter, slides were washed in dH2O at 37°C for 5 minutes. The hybridization mixture was denatured at 100°C and applied to the array using a lift‑erslip™ (Erie Scientific, Portsmouth, USA). The hybridization was performed at 65°C for 42 hours in a Genemachines Mica hybridization chamber (Isogen Life Science, Ijsselstein, The Netherlands). After hybridization, the slides were washed using the manufacturer’s protocol.

**Image analysis and quantification**

Slides were scanned on an Affymetrix 428 scanner using Jaguar software (Affymetrix, Santa Clara, CA, USA). The acquired images were quantified using Imagene software package 5.6 (Biodiscoveiy Inc., El Segundo, CA, USA). The mean ratio of replicate spots was quantified using our Analyze Array software version 1.6 as described by Kok et al. [261]. Briefly, a spot was included for normalization when their intensity was >2x background signal (Drosophila DNA). The median ratio of the
replicate spots from the same BAC clone was determined and spots differing more than 20% from this value were excluded. Finally, BACs with only two replicate spots were also excluded. For the remaining replicas, the average ratio was calculated. If the standard deviation from the median of these replicated BACs was more than 20% (CV<0.2) the clones were also excluded. All remaining BACs were included. However, high or low copy signals were only interpreted as possible gain or loss of DNA if at least two consecutive BACs on the array showed the same deviation from the normal ratio.

The normalized 2log ratio was used to determine the breakpoint regions with the “aCGH smooth” algorithm \[106\]. Cell lines with previously detected copy number changes have been used to determine the parameter settings of aCGH smooth. The following changes were applied: $\lambda=8.75$; pool size=150 and generations=80000. Using these settings, sex mismatch hybridizations using a pool of karyotyped normal males (46, XY) and females (46, XX) were performed to determine technical variation. Furthermore, copy number changes of unknown normalized patients and cell lines were determined. The array–based CGH profile including the aCGH smooth results of all 39 cases are shown in BOX 4 (example is shown in Figure 1A). The copy number changes (gain, amplification or loss) detected with HR–aCGH of some hybridizations were verified using interphase FISH (examples in Figure 1).

**Fluorescence in situ hybridization**

Fluorescence In Situ Hybridization (FISH) was performed on either metaphase spreads of the cell lines or interphase nuclei from snap frozen tumor samples following routine procedures \[260,262\]. For dual color FISH, probes were labeled with digoxigenin–11–dUTP (Roche) or biotin–16–dUTP (Invitrogen) by standard nick–translation. The hybridization solution contained 50% formamide, 10 % dextran sulphate, 50 mM sodium phosphate, pH = 7.0, 2x SSC, 3 ng/µl of each probe and a 50–fold excess of human C0t–1 DNA (Invitrogen). Immunodetection was performed as described earlier \[262\]. Images were captured using a Leica DMRA2 fluorescence microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Leica DC 350Fcharge–coupled device camera. Digital images were processed with Leica CW4000 software. Interfase nuclei were examined by eye and the red (Texas red) and green (FITC) signals were counted separately in approximately 100 nuclei (see Figure 1B). To confirm copy number changes detected with aCGH, a single BAC from a selected subregion labeled with digoxigenin was co–hybridized with a centromere 11 (plasmid pLC11A) FISH–probe labeled with biotin (or vice versa). The ratio between BAC and centromere signals provides an accurate estimation of copy number differences (see Figure 1B and 1c). A ratio of 1.0 indicates normal copy (or polyploidy of chromosome 11), a ratio <1 is loss and >2 is gain. Nuclei were considered to contain amplification of a particular BAC clone when the number of signals exceeded 5 (and ratio >2.5).
High-resolution mapping identifies a commonly amplified 11q13.3 region

**BOX 4: 11q13 HIGH DENSITY ARRAY CGH PROFILES**

CGH-profiles detected with high-density 11q13-specific HR-CGH array. Normalized 2log ratios from HR-aCGH (Y-axis) were smoothened with aCGH smooth [16](gray lines). The position on human chromosome 11 indicated on the X-axis is shown in megabases. By combining HR-aCGH with FISH data of numerous cases (see examples in Fig.1), a 2log ratio >0.5 using aCGH was defined as DNA amplification, whereas a 2log ratio between 0 and 0.5 as gain (thus no amplification). In three cases (L10, L12 and P05) the highest 2log ratio was around below 0.5. The amplification of L10 was confirmed by FISH showing >10 11q13 FISH copies per cell in 10% of the nuclei in the tumor specimen; because Since the percentage of tumor cells in this DNA sample was estimated 10% in agreement with FISH data, we considered the increase of the 2log ratio as amplification. Case L12 was included in this study because of DNA amplification was detected by SB (>2-fold increase) and because the specimen was estimated to contains 40% tumor cells. Case P05 was considered not to contain amplification (but gain) because conventional CGH analysis did not show copy number changes and additional MLPA-analysis only revealed a maximum two-fold increase in agreement with gain. As described in Figure 1 and confirmed by FISH in various cases, due to our normalization procedure, we defined a significant decrease of the smoothed 2log ratio (generally) below 0 as deletion. Therefore, 25 cases (marked with *) were considered to carry a 11q deletion starting immediately telomeric of the 11q13.3 amplicon, and 10 cases (marked with **) with deletion of 11q more to the telomeric end. Cases with no deletion were also marked (–).
### 11q13 high density array CGH profiles

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IN SILICO NUCLEOTIDE ANALYSIS

For this purpose, we used the the UCSC database [263](http://genome.ucsc.edu/; hg17, may 2004) and the NCBI human genome browser (http://www.ncbi.nlm.nih.gov/genome/guide/human/; build 35). The May 2004 freeze was used to enable optimal comparison between multiple sequence databases, some of which were not yet available in the March 2006 freeze. The two remaining sequence gaps within the 2004 freeze (chr11:68846378–68848982 and chr11:69437112–69454899) were also still present within the 2006. Thus, within the 11q13 region no significant changes in sequence order have been found between the two assemblies.
**Figure 1:** The detection of 11q13 amplification using array-CGH analysis. Example of two laryngeal carcinomas with both 11q13.3 amplification and 11q deletion (L05 and L01). (A) A high resolution CGH array of the chromosome 11q13 region was used to determine copy number changes. Normalized 2log ratios from HR-aCGH analysis (Y-axis) were smoothed with the aCGH smooth algorithm [106] indicated with gray bars in the CGH-profiles. Each spot represents the median ratio of various replicates from the same genomic clone (see Material and Methods). The position of the genomic clones on human chromosome 11 is indicated on the X-axis in megabases (all clones are listed in Supplementary Table 1). (B) To confirm the copy number changes observed by aCGH, dual colored fluorescence in situ hybridization (FISH) on interphase nuclei of the same two carcinomas was performed using a FITC labeled probe from a specific region (either I, II or III) combined with a Texas red labeled probe for the chromosome 11.
High-resolution mapping identifies a commonly amplified 11q13.3 region

RESULTS

DETECTION OF SPECIFIC BOUNDARIES FLANKING THE COMMONLY AMPLIFIED 11q13.3 REGION IN HEAD AND NECK CANCER

In order to determine the boundaries of the 11q13 amplicon structure we analyzed 22 laryngeal and 8 pharyngeal cancers as well as 9 HNSCC cell lines with previously identified 11q13 amplification on a high density 11q13 specific CGH array. The array was designed to contain the highest clone density surrounding the 11q13 region (64.1–74.4 Mbp; NCBI build 35), with an average coverage of one clone per 52kb resulting in average twofold coverage. In addition, various BAC clones along the q–arm outside the 11q13 region were included (Supplementary Table 1).

Accuracy of copy number and size detection was tested by hybridizing head and neck squamous cell carcinoma cell lines and patients containing copy number alterations that were previously identified by interphase FISH. Detecting copy number differences is often performed by comparing patients to a fixed ratio that has been determined by multiple self–self hybridizations [98]. Using such a method with fixed ratios hampers the accurate detection of copy number transitions, especially within an admixture of normal cells. Therefore, we preferred the detection of copy number transitions using a smoothing algorithm which enables detection of copy number changes without using a fixed ratio [106•]. Cell lines with previously detected chromosome 11q13 copy number changes were used to determine the parameter settings of aCGH smooth [106•]. Using these settings, copy number changes of unknown normalized patients were determined and verified applying FISH to interphase nuclei (see examples in Figure 1). Copy numbers exceeding a 2log ratio of 0.5 represented DNA amplification. A 2log ratio between 0 and 0.5 was not considered as amplification but defined as gain because an increase of only 1 or 2 extra DNA copies was detected. The loss of DNA copies within a profile resulted in a significant decrease of the smoothed 2log ratio, generally below zero. For example, FISH on the carcinoma cells of patient L05 (Figure 1) showed that ~20% of all nuclei had 2 signals for the centromere 11 probe and 2 signals for each of the probes from the 11q13 subregion, whereas the majority of nuclei (~70%) showed 3 signals of centromere 11 and a varying signal number for the other probes. This indicates that in this particular
Detection of specific boundaries flanking the amplified 11q13.3 region case ~70% of the nuclei represent tumor. In these tumor cells the presence of amplification is confirmed by >10 signals for the amplicon probe (probe II). Furthermore, 3 signals were observed for the proximal probe and 2 for the distal probe. Because three signals for the centromere 11 probe were detected, the 2log ratio around zero represents loss of the whole 11q–arm starting immediately telomeric of the amplicon (Figure 1, probe III). Patient L01 showed a similar pattern. Most important for this study, the smoothing algorithm enabled the accurate identification of copy number transitions as well as their location, even in the presence of an admixture of normal cells within the tumor sample. As summarized in Figure 2, in 29 patients and 9 cell lines with previously identified 11q13 amplifications, DNA amplification was also detected with HR–aCGH. One case (P05) with previously detected (low copy) 11q13 amplification, did not show amplification using 11q13 aCGH analysis (in BOX4 the

Figure 2: The location and size of the 11q13 amplicon in 30 head/neck cancers and 9 cell lines detected with 11q13 high-density array CGH. The position and size of the 11q13 amplicon were determined by 11q13 high-density array CGH for each case (in Supplementary Figure 1 all HR-aCGH-profiles are illustrated). DNA amplification (2log ratio > 0.5) is depicted as thick bars and gain (2log ratio between 0 and 0.5 and not considered as amplification) as thin bars for each of the 39 patients (see Table 1 and Material and Methods) listed on the Y-axis. The position on chromosome 11 is indicated in megabases on the X-axis relative to a representation of the chromosome 11 q-arm (according to NCBI Build 35).
Figure 3: Four common boundaries flanking the 11q13 amplicons coincide with specific repeat sequences and chromosomal transitions in other species. The amplicons within the 67-72 Mbp 11q13.3 region of the 39 cases (from Figure 2) are represented as thick black bars (thin bars represent gains). The four regions with frequent copy number transitions are indicated in vertical gray blocks and numbered boundary 1 to 4. The position and size of the segmental duplications is indicated by blue boxes. Superimposed, a synteny sequence comparison of the human chromosome 11q13 (position 67 to 72 Mbp) is shown with sequences from different organisms shown in the phylogenic tree (downloaded from the NET tab at the UCSC genome browser, http://hgwdev.cse.ucsc.edu) [263]. The synteny comparison shows a sequence comparison (by blastz) between a specific species and the human 11q13 region. Chains of sequence are shown as colored boxes referring to the homologous chromosome from the compared species. Gaps in chains of sequence are displayed as threads and angle brackets (< or >) mark the orientation. All of the tracks and color codes can be found at http://hgwdev-baertsch.cse.ucsc.edu/cgi-bin/hgTracks. The arrows mark the position of the syntenic transitions between human 11q13.3 sequences and mouse (also see Figure 4) and cow. The gaps and/or discontinuity at positions 67.2-67.5 and 71.0-71.2 mark the regions that are not present in most other organism; those regions indicate the presence of segmental duplications (see text). The position and size of the segmental duplications is indicated by blue boxes.
Amplicon boundaries are flanked by synteny transitions

In order to study whether the four common boundaries were associated with specific sequences related to DNA fragility, we performed an in silico analysis of the region containing most 11q13.3 amplifications (from position 67–72 Mb). Fusion of chromosomes during evolution might preferably occur at fragile regions and can be considered as possible breakpoint regions in the process of DNA amplification [264]. Therefore, we compared human chromosome 11 (hChr11) to homologous sequences of a number of sequenced mammals that are phylogenically close to human (Figure 3).

**Figure 4:** Schematic representation of the syntenic homology between the human chromosome 11q13.3 region and the mouse genome. Nucleotide sequence comparison revealed homology between human chromosome 11 (hChr11) and mouse chromosome 7 (mChr7) and 19 (mChr19) using the ENSEMBL synteny viewer (http://www.ensembl.org/Homo_sapiens/syntenyview). The homology between the large arm of hChr11 and mChr 7 and 19 can be subdivided into 3 regions (I, II and III). The human chromosome 11q13.3 region that is commonly amplified (between boundary 2 and 4; marked as II) shows homology with a telomeric part region of the mChr7 telomere. The sequence on the centromeric side of the human 11q13.3 amplicon (marked I) is homologous to sequence from mChr19. The sequence on the telomeric side of the human 11q13.3 amplicon (marked III) is homologous to a region on mChr19 and does not flank the region homologous to region II.

array–based CGH profiles including the aCGH smoothing analysis of all 39 cases are shown). Most amplicons were found within the same region (67–72 Mbp from the p–arm telomere). HR–aCGH and smoothing analysis allowed us to determine the boundaries of an amplicon at a resolution of approximately 52 kb. This analysis revealed that the boundaries are not randomly distributed but clustered within four specific positions (Figure 3, lower panel). On the centromeric side two boundaries are detected: one in 8/38 cases (boundary 1; -400 kb in size) and one more common in 21/38 cases (boundary 2; -200 kb in size). On the telomeric side 10/38 cases were clustered in boundary 3 (-100 kb) and another 12/38 in boundary 4 (-300 kb in size) (Figure 3).

In addition, HR–aCGH analysis revealed that 30/38 cases had 11q13 amplification with accompanying deletion of 11q–ter. This 11q–deletion started immediately adjacent to the amplicon at 11q13.3 in 25/38 cases and more distal from the amplicon in 10/38 cases (BOX 4 and examples in Figure 1).
High-resolution mapping identifies a commonly amplified 11q13.3 region

No synteny breakpoints between human and chimpanzee (Pan troglodytes) were observed because hChr11 is highly conserved in higher apes. However, when the q13 region of hChr11 was compared to the mouse genome (Mus musculus), regions of high similarity were found for mouse chromosome 7 (mChr7) and 19 (mChr19) (Figure 3 and Figure 4). Two separate regions on mChr7 were found to be homologous to a continuous region on hChr11 (region II and III in Figure 4). The transition between these mouse chromosomes is located at approximately 68.6 Mb on hChr11 (NCBI B35, UCSC hg17) that coincides with boundary 2 detected in 21/38 cases (Figure 3). The transition between mChr7 region II and mChr19 region III is located at approximately 70.9 Mb and coincides with boundary 4 observed in 12/38 cases (Figure 3). This comparison reveals that the telomeric locus in the mouse genome conserved during evolution coincides with the commonly amplified locus at human 11q13.3 between boundary 2 and 4 (region II in Figure 4). Boundary 3, which is determined by 10/38 cases coincides with a transition in the cow genome (Figure 3). Immediately flanking boundary 4 on the telomeric side and boundary 1 on the centromeric side, the region on hChr11 does not contain any homology to other species (Figure 3). After this “synteny gap”, there is a chromosomal transition between hChr11 and its homologous counterparts in macaque, mouse, rat, dog and cow. In summary, within the region between 67 Mb and 72 Mb on hChr11 we identified 4 regions containing syntenic transitions. These transitions coincided with 3 of the 4 common amplicon boundaries, implying that these regions are prone to break.

![Figure 5: The structure of the segmental duplications flanking the 11q13 amplicon.](image)

Alignment of 500kb sequence containing the centromeric segmental duplication (top; 67.1M – 67.6M) compared to the telomeric segmental duplication (bottom; 70.9M-71.4M) was performed using the MAUVE multiple genome alignment tool (http://gel.abers.wisc.edu/mauve/). The interconnecting lines between both sequences (boxes) indicate blocks with high homology located in an inversed orientation.
Segmental duplications flank the 11q13.3 amplicon

We have found two large chromosomal structures flanking the 11q13.3 amplicon that can be identified as a "synteny gap" i.e. there is no high level homology between this part of hChr11 and other species (Figure 3). Further analysis revealed that these regions (67.20–67.55 Mbp and 70.95–71.30) consisted of large repeats with an invert-
High-resolution mapping identifies a commonly amplified 11q13.3 region. Such large repeats are known as low copy repeats (LCRs) or segmental duplications (SDs) [265–267]. Both segmental duplications at 11q13.3 are part of a family of segmental duplications that is known to contain clusters of olfactory receptors [268, 269]. These clusters often reside near syntenic breakpoint regions [270, 271]. We tested for homologous regions of these SDs in silico by nucleotide sequence comparisons using the BLAT search program (http://genome.ucsc.edu) and two different fragments of 25kb within the SDs. This comparison revealed a homology >95% between both regions (Table 2a and 2b). Furthermore, the use of the segmental duplications tab at http://hgwddev.cse.ucsc.edu containing experimental tracks of the UCSC Genome Bioinformatics Group, revealed high homology between the telomeric segmental duplication at 11q13 and segmental duplications on chromosome 4p16, 8p23 and 3q21. These regions were also found in the in silico sequence comparison (Table 2b). We performed dual color FISH on metaphase preparations from lymphocytes of a healthy individual using a BAC clone from the telomeric SD (RP11–167J8) in combination with either the chromosome 11 centromere probe (Figure 6A) or the chromosome centromere 8 probe (Figure 6B). Multiple copies of the telomeric SD were detected on different chromosomes in agreement with the sequence comparison analysis (Table 2b).

Segmental duplications have been shown to be involved in the formation of genetic disorders [272], but their involvement in cancer is yet to be proven. The repeat structure of these SDs surrounding the 11q13.3 amplicon might underlie formation of secondary DNA structures creating loops and fragility. Because fragility has been...
Chromosomal amplification is initiated by double strand breaks

DISCUSSION

Chromosomal amplification is initiated by double strand breaks

Based on their finding that the 11q13 amplicon is flanked by inverted duplicated segments, Shuster and coworkers [119] suggested that 11q13 amplification is most likely to arise through the mechanism of Breakage–Fusion–Bridge (BFB) cycles. This model is used to explain the occurrence of DNA amplification of many other chromosomal loci [38**,64]. Even though BFB cycles seem to underlie formation of amplicons [64,273], the exact mechanism is yet to be proven. Many clues for the involvement of the BFB mechanism have been elucidated in in vitro systems, because it is difficult to identify and modify parts of the mechanism in vivo [22,64,273–277]. One of the most important prerequisites for the induction of amplification is the involvement of two double strand breaks surrounding the key gene(s) within an amplicon [35,38**,274,275,277,278]. Since amplification plays a potent role in carcinogenesis, we have tried to gain insight in the mechanism of amplification of the 11q13 region by investigating the genomic structure at the position of copy number transitions in head and neck cancer that frequently shows amplification of the 11q13 region [107**]. We used CGH array for the detection of 11q13 amplifications, which enabled us to map the boundaries of all amplicons at high resolution. For this purpose, we generated a CGH array for the 11q13 region (position 64.1–77.4 Mbp) using a set of overlapping genomic clones that cover this region at least twice with an average coverage of one clone per 52 kb. The resolution of our array is higher than with any mapping strategy previously reported for this region [173*,174**,176,178]. Boundaries in 29 human carcinomas and 9 cell lines clustered in four specific regions (Figure 2), implicating a local susceptibility for DNA breakage. In silico analysis revealed that three of these regions co-localized with syntenic chromosomal transitions in the mouse and cow genome. Remarkably, the region containing amplicons was flanked by two segmental duplications (SDs).

The mechanism of BFB requires the occurrence of at least two double strand breaks. The first break would lead to the loss of the telomere. The second break occurs at the bridge phase, when cell division forces the dicentric chromosome to break apart [64]. It is the position of the first break and the subsequent attempt to repair it, which determines whether BFB occurs. Therefore, telomeric loss should be detected frequently if BFB is the mechanism of amplification. Our aCGH data revealed that in 25/38 cases with 11q13 amplification telomeric 11q–sequences are
indeed deleted with a breakpoint adjacent to the region of amplification (BOX 4 and Figure 1). Using FISH and conventional CGH analysis in cell lines with amplification at 11q13.3, loss of telomeric DNA sequences were reported previously [201, 279]. This implies that the break causing telomeric loss is probably also responsible for initiating multiple rounds of amplification. Recovering from this cycle of BFB requires either the re-activation of telomerase or DNA recombination to capture telomeres from intact chromosomes [280]. Both mechanisms serve to alleviate the activation of checkpoints similar as DNA repair allows cells to re-enter growth after DNA damage [281]. Interestingly, several DNA repair genes are located on the chromosome 11q-arm including MRE11A, ATM, H2AFX and CHK1. Therefore, deletion of the 11q telomere might further enhance genomic instability by the functional loss of these DNA repair genes [282, 283].

Studies on clinical specimens have shown that overexpression of the cyclin D1 gene is associated with chromosomal abnormalities [210, 221]. Increased expression of cyclin D1 in normal hepatocytes in vitro triggered marked chromosomal abnormalities [284]. In a considerable number of head and neck carcinomas with 11q13 amplification and concomitant cyclin D1 overexpression, increased expression was also detected in the premalignant lesions adjacent to the carcinoma, however, without 11q13 amplification [285••]. This indicated that increased cyclin D1 expression is also involved in the initiation of genetic instability including gene amplification in early head and neck cancer. Because the cyclin D1 gene itself is located within the 11q13 amplicon, its overexpression due to 11q13 amplification might maintain and/or enhance further genomic instability in head and neck cancer. [210, 285••]. Thus, genetic instability could be an explanation for the frequent 11q13.3 amplification in multiple tumor types [19••].

Previously, syntenic transitions surrounding the 11q13.3 amplicon were suggested to be transitions in replication timing [286••, 287, 288•]. The change in replication timing is indicative of differences in DNA structure (e.g. GC and repeat content), but has not been assigned to specific sequences. Yet, the change in replication timing as described by Watanabe and coworkers [287] overlaps with boundary 2 in our analysis. A replication timing difference between fragile sites and surrounding regions has also been suggested [289–291]. Fragile sites are potential sites for DNA double strand breakage and possible initiators for BFB-mediated amplification [277]. Although it is known that clastogenic drugs cause DNA breakage, it is unknown why these breaks are not sequence specific and occur within regions of up to several megabases [292]. Within the chromosome 11 cytogenetic band 13 three fragile sites have been mapped previously. Even though the exact location is unknown, FRA11A and FRA11H are mapped further centromeric [293, 294] and FRA11F further telomeric to 11q13.3 [295]. We propose that these 11q13 fragile sites are not involved in the fragility for chromosomal breakage necessary for 11q13 amplification, whereas the specific chromosomal structure of the 11q13.3 region marked by the presence of both syntenic transitions and segmental duplications is.
The role of segmental duplications in amplification

The specific homology between human chromosome 11q13.3 and the telomeric part of mouse chromosome 7 provides a possible evolutionary explanation for the structural difference between 11q13.3 and flanking regions. With palindromic segmental duplications flanking the 11q13 region, large secondary DNA structures might be formed due to sequence homology. Segmental duplications could cause fragility similar to palindromic sequences and alu repeats, which have previously been implicated in double strand break mediated amplification [49,296]. Segmental duplications have been reported to be involved in many genetic aberrations [297] and in some hematological malignancies [298]. Amplification at 17p11.2–p12 has been linked to the presence of SDs [299], but a general role for SD mediated amplification is yet to be proven. However, the involvement of SDs in chromosomal deletions involved in genomic disorders [300*] as well as chromosomal duplications mediated by segmental duplications have been established [301]. For instance, congenital heart defects are associated with deletions on distal 8p (del8p) [302] and recurrent translocations have been identified between 4p16 and 8p23 [303]. Strikingly, these aberrations are found within regions that are flanked by two SDs sharing high homology with the 11q13 SDs (Table 2). Furthermore, Saunders and coworkers [63] have described dicentric chromosomes in oral squamous cell carcinoma containing 11q13 amplifications as well as translocations between chromosome 11, 4 and 8 and 3. These translocations could be mediated by homology between the segmental duplications. The mechanism underlying amplicon formation involving EGFR [257], MYCN [304] and ERBB2 [59*•] seems structurally different from the complex amplicon that is formed at 11q13 [19*•]. The involvement of the segmental duplications might thus be a unique feature of amplification at 11q13. This genomic structure at the chromosomal 11q13 region will have important implications for the identification of a key gene in the 11q13 amplicon. Since the amplicon size of the 11q13 region in head and neck cancer is determined by unique genomic structures, not a single gene but a set of genes will be selected in most carcinomas. This is reflected by the fact that our current analysis does not lead to a region containing a specific gene of interest (Gibcus et al., unpublished data) in agreement with previous findings by others [173*,174*•,178] including a recently published paper by Huang and coworkers [305]. Therefore, several genes remain candidate for driving the amplification, including cyclin D1, TAOS1, FGF19, FADD, PPFIA1 and cortactin. Functional analysis of the candidate genes is needed to identify the gene(s) relevant for tumor progression.

In this manuscript, we have compared the breakpoint patterns of 38 head and neck carcinomas and cell lines with 11q13 amplification in detail. Within the 11q13.3 region amplifications occur at specific regions that coincide with regions of synteny transition in the mouse and cow genome. Furthermore, the transitions are flanked by segmental duplications. Other studies have reported enrichment of segmental duplications near synteny breaks [306], indicating that the same DNA structure that enabled chromosomal breakage during evolution and the insertion of a SD might
also be involved in the initiation of amplification.

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http://www.springerlink.com/content/w187387865q31030/439_2006_Article_299_ESM.html