

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

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**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

nine cell lines with 11q13 amplification. We found that boundaries of the commonly amplified region were restricted to four 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,

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deletion-plus-episome, sister-chromatid-exchange, onionskin, extrachromosomal-doubling-rolling circle and the chromatid-breakage-fusion-bridge (BFB) cycle model (Barr et al. 1996; Ciullo et al. 2002; Coquelle et al. 1997; Schwab 1999; Stark et al. 1989; Toledo et al. 2000; Van Roy et al. 2006; Vogt et al. 2004). 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) (Schuuring 1995). 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 (Freier et al. 2006; Huang et al. 2002; Hughes-Davies et al. 2003; Hui et al. 2005; Schuuring 1995). 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 (Albertson 2006).

We generated a CGH array designed specifically for the 11q13 region with a higher resolution than any previously described method (Freier et al. 2006; Huang et al. 2002; Hui et al. 2005; Lin et al. 2006), 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 nine head and neck cell lines with previously identified DNA amplification of the 11q13 region using this high density HR-CGH 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 three of the boundaries coincided with syntenic breakpoints and the identification of two 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 at all necessarily relevant for tumorigenesis and tumor progression.

## Materials 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 (Hermsen et al. 2001), Southern blotting (Takes et al. 1997) or WGA analysis (paper in preparation) (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 nine 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 (Hermsen et al. 1996; Schuuring et al. 1998). 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 50 µg/ml) at 37°C in 5% CO<sub>2</sub>. DNA from these cell lines was isolated using a standard high salt extraction method.

### Generation of a high-resolution CGH array of the chromosome 11q13 region

We have selected 350 overlapping BAC-clones mapped to chromosome 11q13, resulting in an average

**Table 1** Patient inclusion

	#	Type	Origin	Published	Previous	11q13 aCGH	11q13 amp
	P01	Pharynx	Oviedo	Hermsen et al. (2001)	CGH	Yes	+
	P02	Pharynx	Oviedo	Hermsen et al. (2001)	CGH	Yes	+
	P03	Pharynx	Oviedo	Hermsen et al. (2001)	CGH	Yes	+
	P04	Pharynx	Oviedo	Hermsen et al. (2001)	CGH	Yes	+
	P05	Pharynx	Oviedo	Hermsen et al. (2001)	CGH	Yes	–
	P06	Pharynx	Oviedo	Hermsen et al. (2001)	CGH	Yes	+
	P07	Pharynx	Oviedo	Hermsen et al. (2001)	CGH	Yes	+
	P08	Pharynx	Oviedo	Hermsen et al. (2001)	CGH	Yes	+
	L13	Larynx	Oviedo	Hermsen et al. (2001)	CGH	Yes	+
	L14	Larynx	Oviedo	Hermsen et al. (2001)	CGH	Yes	+
	L15	Larynx	Oviedo	Hermsen et al. (2001)	CGH	Yes	+
	L16	Larynx	Oviedo	Hermsen et al. (2001)	CGH	Yes	+
	L17	Larynx	Oviedo	Hermsen et al. (2001)	CGH	Yes	+
	L18	Larynx	Oviedo	Hermsen et al. (2001)	CGH	Yes	+
	L19	Larynx	Oviedo	Hermsen et al. (2001)	CGH	Yes	+
	L20	Larynx	Oviedo	Hermsen et al. (2001)	CGH	Yes	+
	L21	Larynx	Oviedo	Hermsen et al. (2001)	CGH	Yes	+
	L22	Larynx	Oviedo	Hermsen et al. (2001)	CGH	Yes	+
	L01	Larynx	UMCG	Unpublished	WGA	Yes	+
	L02	Larynx	UMCG	Unpublished	WGA	Yes	+
	L03	Larynx	UMCG	Unpublished	WGA	Yes	+
	L04	Larynx	UMCG	Unpublished	WGA	Yes	+
	L05	Larynx	UMCG	Unpublished	WGA	Yes	+
	L06	Larynx	UMCG	Unpublished	WGA	Yes	+
	L07	Larynx	UMCG	Unpublished	WGA	Yes	+
	L08	Larynx	UMCG	Unpublished	WGA	Yes	+
	L09	Larynx	LUMC	Takes et al. (1997)	SB	Yes	+
	L10	Larynx	LUMC	Takes et al. (1997)	SB	Yes	+
	L11	Larynx	LUMC	Takes et al. (1997)	SB	Yes	+
	L12	Larynx	LUMC	Takes et al. (1997)	SB	Yes	+

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 (Atayar et al. 2006)]; SB Southern blot performed with probes for cyclin D1 and EMS1/cortactin (Takes et al. 1997); the amplification levels were confirmed by interphase FISH (unpublished); 11q13 amp, amplification (+) detected by HR-aCGH; case p05 contained gain but no amplification (–)

two-fold coverage of the entire 11q13 region. 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 (Hughes-Davies et al. 2003; Janssen et al. 2000). 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 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 (Tchinda et al. 2004). 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 (Fiegler et al. 2003) was applied to the DNA extracted from the genomic clones. These PCR products were spotted five 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 five 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 (Kok et al. 2005). 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 (Snijders et al. 2001) with small adaptations. Using the manufacturer's protocol the slides were blocked using Nexterion Block E (Schott Nexterion) prior to the hybridization procedure. Reference 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 dNTPs and Klenow enzyme. An overnight incubation at 37°C was followed by removal of unincorporated nucleotides using microspin 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 µl Nexterion Hyb (Schott Nexterion) complemented with dextran sulfate (5%), 200 µg Cot1 DNA (Roche) and 1 mg yeast t-RNA (Invitrogen). Slides were subjected to a 30–90 min prehybridization at 65°C using 50 µl of hybridization buffer (Schott Nexterion) enriched with dextran sulfate (5%) and salmon sperm DNA (10 µg/µl, Invitrogen). Hereafter, slides were washed in dH<sub>2</sub>O at 37°C for 5 min. The hybridization mixture was denatured at 100°C and applied to the array using a lifter-slip™ (Erie Scientific, Portsmouth, USA). The hybridization was performed at 65°C for 42 h 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 Imagegene software package 5.6 (Biodiscovery 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. (2005). Briefly, a spot was included for normalization when their intensity was >2× 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 (Jong et al. 2004). Cell lines with previously detected copy number changes have been used to

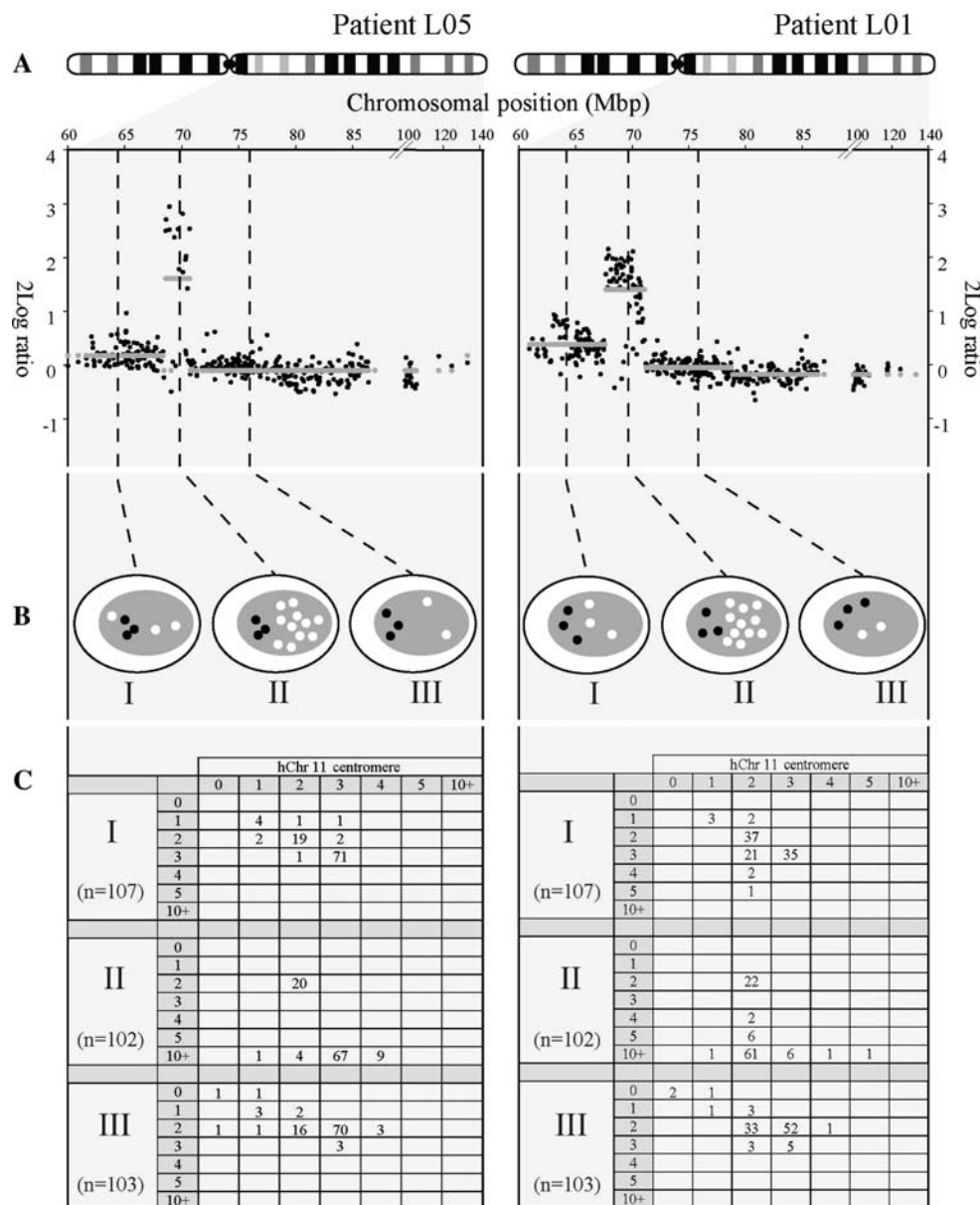
determine the parameter settings of aCGH smooth. The following changes were applied:  $\lambda = 8.75$ ; pool size = 150 and generations = 80,000. Using these settings, sex mismatch hybridizations using a pool of karyotyped normal males (46, XY) and females (46, XX) were performed to determine technical 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 Supplementary Fig. 1 (example is shown in Fig. 1a). The copy number changes (gain, amplification or loss) detected with HR-aCGH of some hybridizations were verified using interphase FISH (examples are shown in Fig. 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 (Janssen et al. 2000; Vaandrager et al. 1996). 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 sulfate, 50 mM sodium phosphate, pH = 7.0, 2 × SSC, 3 ng/µl of each probe and a 50-fold excess of human Cot-1 DNA (Invitrogen). Immunodetection was performed as described earlier (Vaandrager et al. 1996). 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. Interphase nuclei were examined by eye and the red (Texas red) and green (FITC) signals were counted separately in approximately 100 nuclei (see Fig. 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 Fig. 1b, c). 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 five (and ratio >2.5).

#### In silico nucleotide analysis

For this purpose, we used the UCSC database (Kent et al. 2002) (<http://genome.ucsc.edu/>; hg17, May 2004)



**Fig. 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 (Jong et al. 2004) indicated with gray bars in the CGH-profiles. Each spot represents the median ratio of various replicates from the same genomic clone (see Materials 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 centromere to control ploidy. The

most common FISH pattern detected with each region-specific probe (indicated with white dots) is illustrated; the signals of centromere 11-probe are represented by black dots. **c** Signals of each probe were counted and the various signals were presented as the number of nuclei containing the signal. Both carcinomas contain amplification (probe I > 10 FISH signals and aCGH 2log ratio > 0.5); in both cases a considerable number of admixed normal cells represented by nuclei with two TRITC and two FITC signals were detected (16–20% in L05 and 22–37% in L01) in agreement with the percentage of normal cells estimated in a parallel H&E section (both ~20%). The centromeric region with 2log ratio detected by aCGH between 0 and 0.5 revealed FISH signals (with probe I) with ratio of 3/3 representing gain of chromosome 11. The telomeric side with a 2log ratio close to but below 0 revealed FISH signal (with probe III) with a ratio of 2/3 representing loss of the q-arm (70% in L05 and 52% in L01) that confirmed the loss predicted by aCGH smooth

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.

## Results

### Detection of specific boundaries flanking the commonly amplified 11q13.3 region in head and neck cancer

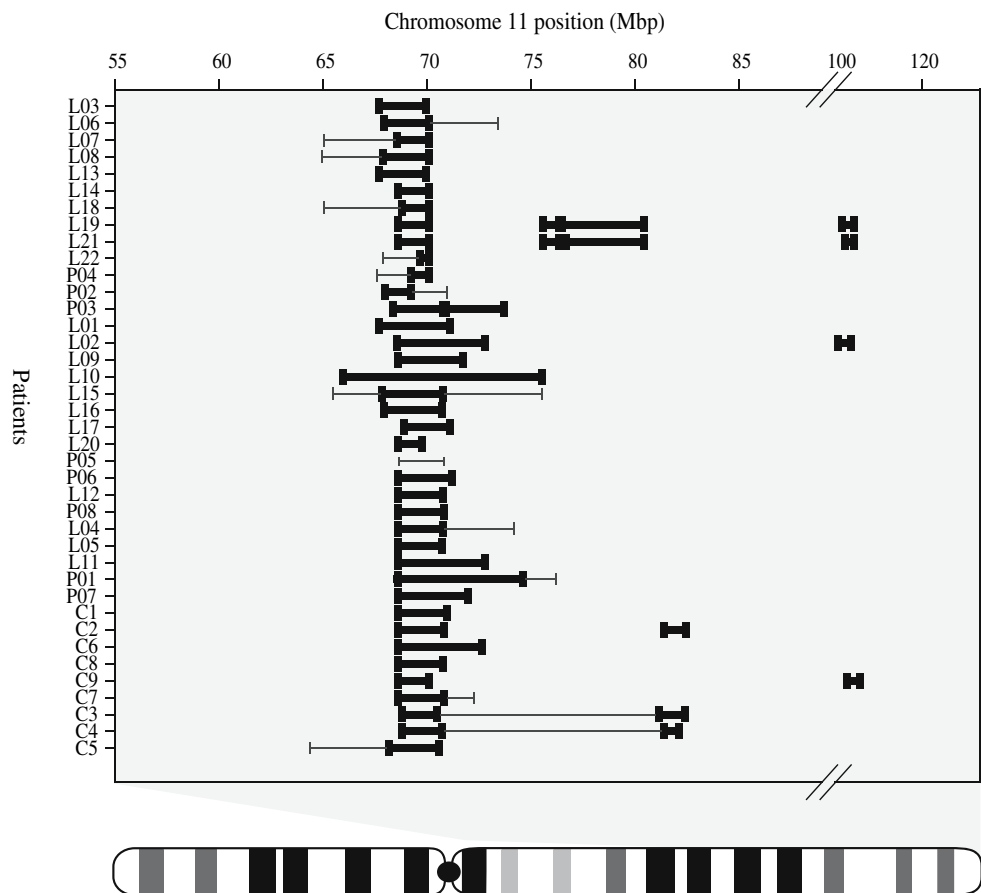
In order to determine the boundaries of the 11q13 amplicon structure we analyzed 22 laryngeal and 8 pharyngeal cancers as well as 9 HNSCC cell lines with previously identified 11q13 amplification on a high-density 11q13 specific CGH array. The array was designed to contain the highest clone density surrounding the 11q13 region (64.1–74.4 Mbp; NCBI build 35), with an average coverage of one clone per 52 kb 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 (Veltman et al. 2002). 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 (Jong et al. 2004). Cell lines with previously detected chromosome 11q13 copy number changes were used to determine the parameter settings of aCGH smooth (Jong et al. 2004). Using these settings, copy number changes of unknown normalized patients were determined and verified applying FISH to interphase nuclei (see examples in Fig. 1). Copy numbers exceeding a 2log ratio of 0.5 represented DNA amplification. A 2log ratio between 0 and 0.5 was not con-

sidered as amplification but defined as gain because an increase of only one or two 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 (Fig. 1) showed that ~20% of all nuclei had two signals for the centromere 11 probe and two signals for each of the probes from the 11q13 subregion, whereas the majority of nuclei (~70%) showed three signals of centromere 11 and a varying signal number for the other probes. This indicates that in this particular 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, three signals were observed for the proximal probe and two 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 (Fig. 1, probe III). Patient L01 showed a similar pattern. The most important finding in this study was that 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 Fig. 2, in 29 patients and nine 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 Supplementary Fig. 1, the 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 (Fig. 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) (Fig. 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 (Supplementary Fig. 1 and examples in Fig. 1).

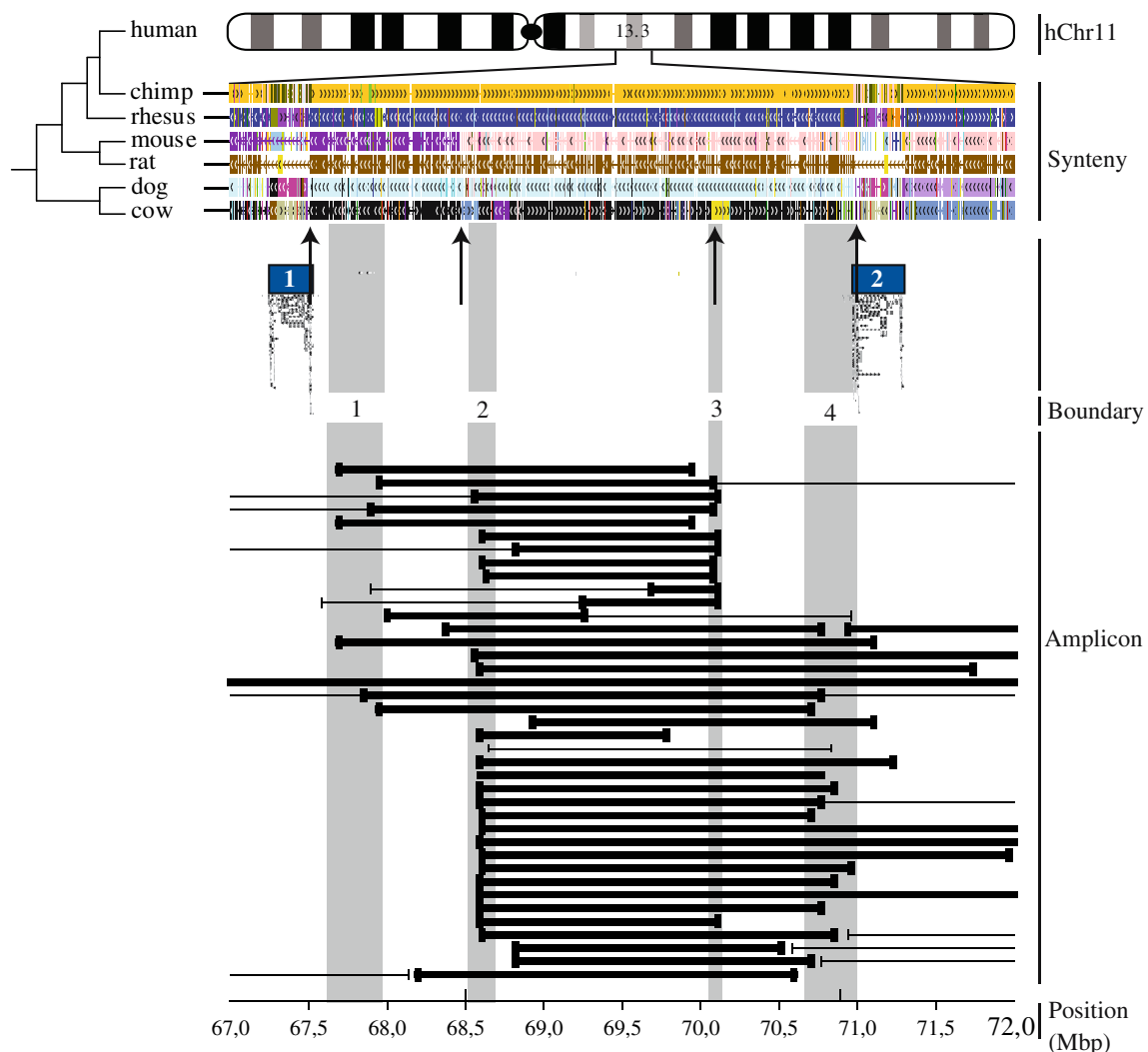
**Fig. 2** The location and size of the 11q13 amplicon in 30 head/neck cancers and nine cell lines detected with 11q13 high-density array CGH. The position and size of the 11q13 amplicon were determined by 11q13 high-density array CGH for each case (in Supplementary Fig. 1 all HR-aCGH-profiles are illustrated). DNA amplification ( $2\log$  ratio  $> 0.5$ ) is depicted as *thick bars* and gain ( $2\log$  ratio between 0 and 0.5 and not considered as amplification) as *thin bars* for each of the 39 patients (see Table 1 and [Materials 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)



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 (Yuan et al. 1997). Therefore, we compared human chromosome 11 (hChr11) to homologous sequences of a number of sequenced mammals that are phylogenetically close to human (Fig. 3). 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) (Figs. 3, 4). Two separate regions on mChr7 were found to be homologous to a continuous region on hChr11 (regions II and III in Fig. 4). The transition

between these mouse chromosomes is located at approximately 68.6 Mbp on hChr11 (NCBI B35, UCSC hg17) that coincides with boundary 2 detected in 21/38 cases (Fig. 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 (Fig. 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 boundaries 2 and 4 (region II in Fig. 4). Boundary 3, which is determined by 10/38 cases, coincides with a transition in the cow genome (Fig. 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 (Fig. 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 and 72 Mb on hChr11 we identified four regions containing syntenic transitions. These transitions coincided with three of the four common amplicon boundaries, implying that these regions are prone to break.



**Fig. 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 Fig. 2) are represented as *thick black bars* (*thin bars* represent gains). The four regions with frequent copy number transitions are indicated as *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 67–72 Mbp) is shown with sequences from different organisms shown in the phylogenetic tree (downloaded from the NET tab at the UCSC genome browser, <http://hgwddev.cse.ucsc.edu>) (Kent et al. 2002). The synteny comparison

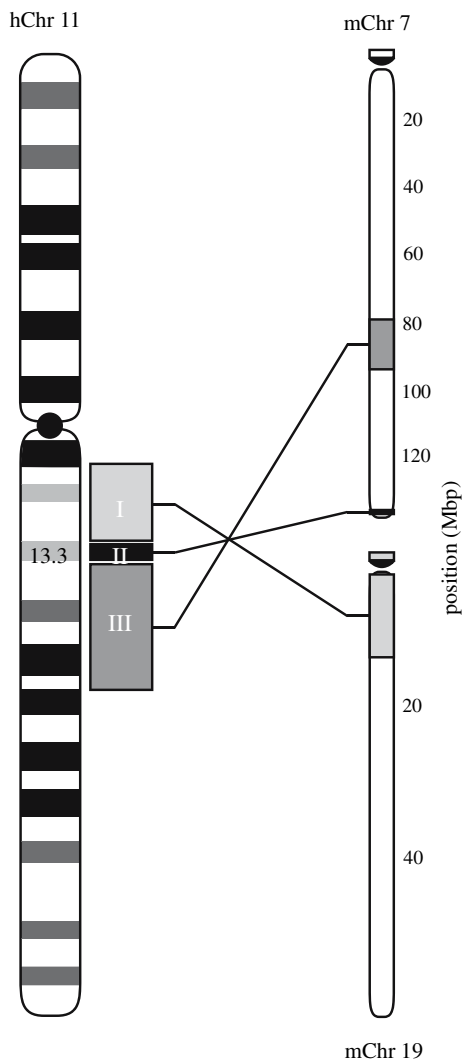
shows a sequence comparison (by blastz) between a specific species and the human 11q13 region. Chains of sequence are shown as *colored boxes* referring to the homologous chromosome from the compared species. *Gaps* in chains of sequence are displayed as *threads* and *angle brackets* (< or >) mark the orientation. All of the tracks and color codes can be found at <http://hgwddevbaertsch.cse.ucsc.edu/cgi-bin/hgTracks>. The *arrows* mark the position of the syntenic transitions between human 11q13.3 sequences and mouse (also see Fig. 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*

### 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 (Fig. 3). Further analysis revealed that these regions (67.20–67.55 Mbp and 70.95–71.30 Mbp) consisted of large

repeats with an inverted orientation (Fig. 5). Such large repeats are known as low copy repeats (LCRs) or segmental duplications (SDs) (Bailey and Eichler 2006; Locke et al. 2004; Stankiewicz et al. 2004). Both segmental duplications at 11q13.3 are part of a family of segmental duplications that is known to contain clusters of olfactory receptors (Newman and Trask 2003; Olender et al. 2004). These clusters often reside near





**Fig. 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/synteniview](http://www.ensembl.org/Homo_sapiens/synteniview)). The homology between the large arm of hChr11 and mChr 7 and 19 can be subdivided into three 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 mChr7 and does not flank the region homologous to region II

syntenic breakpoint regions (Bailey et al. 2004; Yue and Haaf 2006). 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 25 kb within the SDs. This comparison revealed a homology >95%

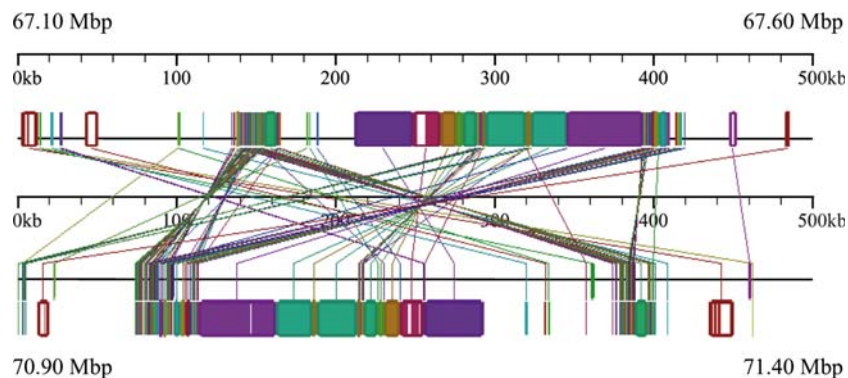
between both regions (Table 2). Furthermore, the use of the segmental duplications tab at <http://hgwdev.cse.ucsc.edu> containing experimental tracks of the UCSC Genome Bioinformatics Group revealed high homology between the telomeric segmental duplication at 11q13 and segmental duplications on chromosome 4p16, 8p23 and 3q21. These regions were also found in the *in silico* sequence comparison (Table 2, the telomeric SD). 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 (Fig. 6a) or the chromosome centromere 8 probe (Fig. 6b). Multiple copies of the telomeric SD were detected on different chromosomes in agreement with the sequence comparison analysis (Table 2, the telomeric SD).

Segmental duplications have been shown to be involved in the formation of genetic disorders (Stankiewicz and Lupski 2002), 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 associated with repeat content also, we investigated the 11q13.3 region for differences in GC-content, and the presence of LINE, SINE and SATR1/SATR2 repeats (<http://hgwdev.cse.ucsc.edu>). In this region we observed no clear differences in GC or repeat content, except that the segmental duplications at 11q13 are flanked by the clusters of SATR1 and SATR2 satellite repeats (data not shown) as has previously been reported for olfactory receptor containing segmental duplications (Newman and Trask 2003; Stankiewicz et al. 2004).

## Discussion

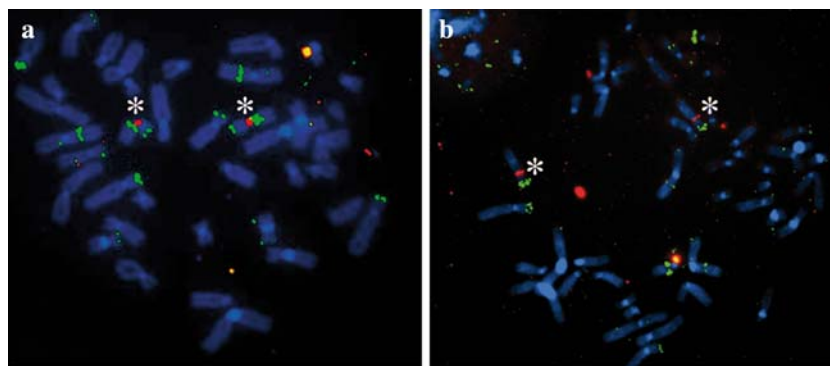
Chromosomal amplification is initiated by double strand breaks

On the basis of their finding that the 11q13 amplicon is flanked by inverted duplicated segments, Shuster et al. (2000) 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 (Toledo et al. 1992; Windle et al. 1991). Even though BFB cycles seem to underlie the formation of amplicons (Toledo et al. 1992; Trask and Hamlin 1989), the exact mechanism is yet to be proven. Many clues for the involvement of the BFB mechanism



**Fig. 5** The structure of the segmental duplications flanking the 11q13 amplicon. Alignment of 500 kb sequence containing the centromeric segmental duplication (*top* 67.1–67.6 Mbp) compared to the telomeric segmental duplication (*bottom* 70.9–

71.4 Mbp) was performed using the MAUVE multiple genome alignment tool (<http://gel.ahabs.wisc.edu/mauve/>). The interconnecting lines between both sequences (*boxes*) indicate blocks with high homology located in an inverted orientation



**Fig. 6** Segmental duplication detected by metaphase FISH. We have performed a dual color metaphase FISH on normal lymphocytes using a BAC clone located within the centromeric segmental duplication (RP11-167j8) labeled with FITC in combination with the centromere 11 probe (pLC11A) labeled with Texas red (*asterisk* in **a**) or the chromosome centromere 8 probe (D8Z2)

labeled with Texas red (*asterisk* in **b**). The segmental duplication signals are not restricted to chromosome 11 and 8. The telomeric segmental duplication BAC clone (RP11-684b2) gave similar results indicating its overlap with the centromeric segmental duplication (data not shown)

have been elucidated in *in vitro* systems, because it is difficult to identify and modify parts of the mechanism *in vivo* (Coquelle et al. 2002; Kuo et al. 1998; Okuno et al. 2004; Pipiras et al. 1998; Schimke 1988; Toledo et al. 1992; Trask and Hamlin 1989). 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 (Coquelle et al. 2002; Difilippantonio et al. 2002; Kuo et al. 1998; Mills et al. 2003; Pipiras et al. 1998; Windle et al. 1991). Since amplification plays a potent role in carcinogenesis, we have tried to gain insight into 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 (Schuurin 1995). 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 any other mapping strategy previously reported for this region (Freier et al. 2006; Huang et al. 2002; Hui et al. 2005; Lin et al. 2006). The boundaries in 29 human carcinomas and nine cell lines clustered in four specific regions (Fig. 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 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

**Table 2** Human nucleotide sequences homologous with the segmental duplications flanking the 11q13.3 amplicon

Chr	Start	End	Span	Identity (%)	Score
The centromeric SD					
11	67315001	67340000	25000	100.0	25000
11	3385217	3410946	25730	96.3	22963
3	127107548	127132342	24795	96.0	22676
11	71165608	71190755	25148	95.5	22073
16	5073512	5096800	23289	94.8	20273
4	9359821	9383022	23202	94.0	20001
4	4060936	4082754	21819	94.5	19050
3	75552575	75574387	21813	94.0	18790
12	8274264	8294371	20108	94.0	17470
8	12325476	12345633	20158	93.9	17460
3	131298506	131318592	20087	94.2	17422
8	12076199	12096495	20297	93.9	17178
8	8115994	8132795	16802	93.8	14523
7	6742303	6753608	11306	96.5	10436
3	156975109	157244854	269746	92.2	888
The telomeric SD					
11	71035001	71060000	25000	100.0	25000
11	3526539	3552211	25673	95.8	22545
4	4195660	4220589	24930	95.8	22485
12	8401893	8427626	25734	95.3	22389
8	12471239	12554434	83196	95.4	22377
8	6943825	6969603	25779	95.6	22349
8	7967284	7992669	25386	95.0	22153
8	7034696	7060125	25430	95.0	22044
4	9217041	9242864	25824	94.8	22020
11	67448689	67473035	24347	96.4	21701
7	97171923	97196391	24469	96.5	21670

Nucleotide sequence comparisons were performed with a 25,000 bp sequence from both the centromeric and the telomeric segmental duplications flanking the 11q13.3 amplicon using program BLAT (<http://genome.ucsc.edu/>). Sequences with an identity of more than 92% are listed; score indicates the fragment size of the sequence with this identity in base pairs. Indicated are the chromosomes that contain these sequences (Chr) as well as the position on each of the chromosomes from start to end (using UCSC hg17). The sequences with 100% identity are those used for comparison, respectively, the centromeric and telomeric 11q13 segmental duplication

break occurs at the bridge phase, when cell division forces the dicentric chromosome to break apart (Toledo et al. 1992). 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 (Fig. 1 and Supplementary Fig. 1). Using FISH and conventional CGH analysis in cell lines with amplification at 11q13.3, loss of telomeric DNA sequences were reported previously (Hermsen et al. 1997; Jin et al. 1998). 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 (Maser and DePinho 2002). Both mechanisms serve to alleviate the activation of checkpoints similar as DNA repair allows cells to re-enter growth after DNA damage (Maser and DePinho 2004). 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 (Lobachev et al. 2002; Reshmi and Gollin 2005).

Studies on clinical specimens have shown that overexpression of the cyclin D1 gene is associated with chromosomal abnormalities (Jin et al. 2002; Nimeus et al. 2004). Increased expression of cyclin D1 in normal hepatocytes in vitro triggered marked chromosomal abnormalities (Nelsen et al. 2005). 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 (Izzo et al. 1998). 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. (Izzo et al. 1998; Nimeus et al. 2004). Thus, genetic instability could be an explanation for the frequent 11q13.3 amplification in multiple tumor types (Albertson 2006).

Previously, syntenic transitions surrounding the 11q13.3 amplicon were suggested to be transitions in replication timing (Watanabe et al. 2002, 2004; Woodfine et al. 2004). 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 et al. (2004) overlaps with boundary 2 in our analysis. A replication timing difference between fragile sites and surrounding regions has also been suggested (Le Beau et al. 1998; Palakodeti et al. 2004; Sen et al. 1989). Fragile sites are potential sites for DNA double strand breakage and possible initiators for BFB-mediated amplification (Kuo et al. 1998). 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 (Richards 2001). 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 (Hagemeyer et al. 1991; Perucca-Lostanlen et al. 1997) and *FRA11F* further telomeric to 11q13.3 (Craig-Holmes et al. 1987). We propose that these 11q13 fragile sites are not involved in the fragility of 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 (Narayanan et al. 2006; Tanaka et al. 2002). Segmental duplications have been reported to be involved in many genetic aberrations (Lupski 1998) and in some hematological malignancies (Barbouti et al. 2004). Amplification at 17p11.2~p12 has been linked to the presence of SDs (van Dartel and Hulsebos 2004), 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 (Shaw and Lupski 2004) as well as chromosomal duplications mediated by segmental duplications has been established (De Gregori et al. 2005). For instance, congenital heart defects are associated with deletions on distal 8p (del8p) (Devriendt et al. 1999) and recurrent translocations have been identified between 4p16 and 8p23 (Giglio et al. 2002). Strikingly, these aberrations are found within regions that are flanked by two SDs sharing high homology with the 11q13 SDs (Table 2). Furthermore, Saunders et al. (2000) 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* (Vogt et al. 2004), *MYCN* (Savelyeva and Schwab 2001) and *ERBB2* (Coquelle et al. 1997)

seems structurally different from the complex amplicon that is formed at 11q13 (Albertson 2006). 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 (J.H. Gibcus et al., unpublished data) in agreement with previous findings by others (Freier et al. 2006; Huang et al. 2002; Hui et al. 2005) including a recently published paper by Huang et al. (2006). Therefore, several genes remain candidates 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 to 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 (Armengol et al. 2003), 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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