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SHORT REPORT

DNA amplification at 11q13.5–q14 in human breast cancer

Pierre Szepetowski[1], Vincent Ollendorff[2], Josiane Grosgeorge[1], Anouk Courseaux[1], Daniel Birnbaum[2], Charles Theillet[3] & Patrick Gaudray[1]

[1]*LGMCH, CNRS URA 1462, Faculté de Médecine 06107 Nice, France;* [2]*INSERM U 119, 27 Boulevard Leï Roure, 13009 Marseille, France* [3]*CNRS URA 1191, Université de Montpellier II, 34095 Montpellier, France*

Band q13 of chromosome 11 is frequently amplified in human breast cancers, but the gene(s) responsible for the emergence of this amplicon remain(s) elusive as yet. As a tribute to the complexity of the amplification events involving 11q13 sequences in human breast cancer, we have now studied a more telomeric region at 11q13.5–q14 defined by a new transcription unit, D11S833E. We have observed that amplicons present in cell lines and primary tumors amplified for both BCL1 and D11S833E could be interrupted between these two loci. Such discontinuities were demonstrated by using a probe for the *KRN1* gene, which we have localized between the BCL1/*FGF4* region and D11S833E. In fact, *KRN1* was not present in 4 out of 10 amplicons bearing both BCL1 and D11S833E. Furthermore, we have observed tumors in which D11S833E could be amplified in the absence of amplification of other known markers of 11q13. Therefore, D11S833E defines a new and independent amplification unit in this region.

While initial reports about amplification of 11q13 in human breast carcinomas emphasized a potential role for the *FGF3* (*INT2*) and *FGF4* (*HST*) proto-oncogenes (Lidereau *et al*., 1988; Theillet *et al*., 1989), a first degree of complexity arose from the observation that sequences surrounding the BCL1 major t(11;14)(q13;q32) translocation breakpoint cluster were more frequently amplified than *FGF3*/*FGF4* (Theillet *et al*., 1990). Accordingly, a candidate gene, *CYCD1 (PRAD1)*, situated *ca* 150 kb telomeric to BCL1, has been shown to be amplified and overexpressed in breast cancers (Lammie *et al*., 1991). However, D11S97, an anonymous probe located about 500 kb centromeric to BCL1, can be amplified in tumors amplified for neither BCL1 nor *FGF3*/*FGF4* (Szepetowski *et al*., 1992a). Therefore, amplification of 11q13 DNA sequences may be more complex than first suspected, and the number and identity of target gene(s) remain open questions. Understanding the 11q13 amplification events relies on a general description of the amplification units in various tumors. As a first step toward this goal, we have recently localized several 11q13 markers with respect to regional breakpoints (Hagemeijer *et al*., 1991; Szepetowski *et al*., 1992b), and described a new transcription unit (D11S833E) distal to *FGF3*/*FGF4* on band 11q13.5–q14, which was amplified in a breast carcinoma-derived cell line, MDA-MB134, together with other loci of 11q13 (Ollendorff *et al*., 1992; Lafage *et al*., 1990; 1992). This result suggested that amplification units could extend up to 11q13.5–q14, and led us to investigate the genetic organization of this region in more detail.

In a previous study, we assigned several 11q13 markers, including D11S833E, to the telomeric side of *FGF3* and *FGF4*, but we could not map them more precisely (Szepetowski *et al*., 1992b). Since then, a new gene, *KRN1*, has been mapped to 11q, centromeric to the t(11;16) translocation breakpoint present in the CF52 somatic hybrid containing 11q sequences distal to *FGF4* (MacKinnon *et al*., 1991). This hybrid is known to contain D11S833E (Ollendorff *et al*., 1992), demonstrating that this probe is telomeric to *KRN1*. Another somatic hybrid called CE4 contains 11q13–qter sequences, distal to the 11q13 break of the t(11;14)(q13;q32) major translocation breakpoint, in a mouse background (Figure 1a). Hybridization to the DNA of CE4 and fluorescent *in situ* hybridization performed on metaphase chromosomes of the Rec-1 cells containing the t(11;14)(q13;q32) translocation (Figure 1b) showed that *KRN1* is telomeric to BCL1. Moreover, a 1500-kb NruI restriction fragment containing sequences extending from D11S97 to *FGF3* (Szepetowski *et al*., 1992b) does not include *KRN1*. This result proves that *KRN1* is not situated between BCL1 and *FGF3*, and thus is telomeric to *FGF3*. In addition, the largest restriction fragment of the *KRN1* gene which is found in neither *FGF4* nor D11S833E indicates that the distance between these two is more than 500 kb (data not shown). The map derived from these results is presented in Figure 1c.

This location is contradictory to that found by Richard *et al*. (1991). Using radiation hybrids, these authors have mapped *KRN1* to the centromeric side of BCL1/*FGF3*/*FGF4*. We have no definitive explanation for this discrepancy. However, since our results are based on two different physical mapping strategies, i.e. hybridization on DNA from a non-reduced somatic hybrid and from metaphase chromosomes of a human cell line, we believe that radiation reduction might not be totally random throughout the genome and thus bias mapping in regions highly susceptible to DNA damage.

Several cell lines, amplified or not for 11q13, were screened for the presence of amplified loci on the telomeric side of BCL1. D11S97 was also included in this study since it has been observed to be amplified

Correspondence: P. Gaudray, LGMCH, CNRS URA 1462, Faculté de Médecine, Avenue de Valombrose 06107 Nice cedex 2, France
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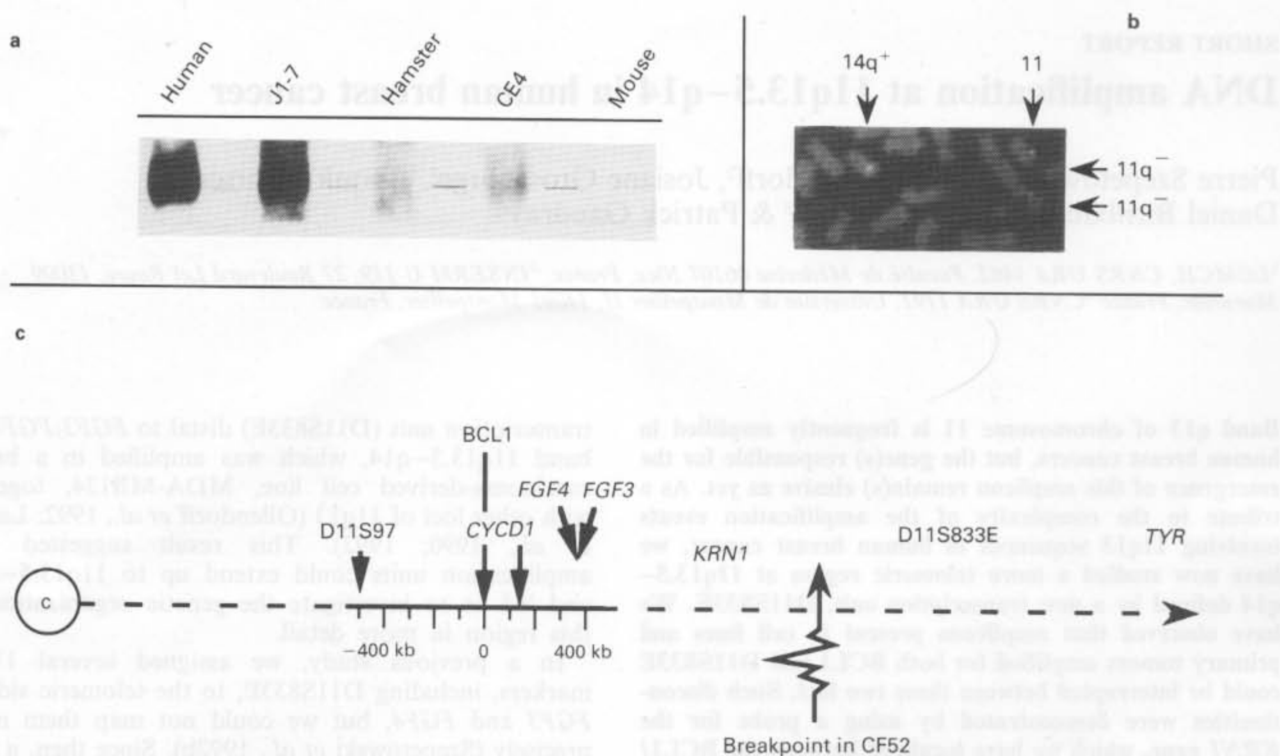

Figure 1 *KRN1* is located between *FGF3* and D11S833E. (**a**) DNA isolated from normal human leukocytes, hybrid cell lines and their normal counterparts, hamster corresponding to J1-7 and mouse to CE4, was digested by HindIII restriction enzyme and analysed according to Szepetowski *et al.* (1992b). The *KRN1* probe used was the polymerase chain reaction (PCR) fragment highly specific for the bona fide *KRN1* gene located on 11q13 (Richard *et al.*, 1991). (**b**) Fluorescent *in situ* hybridization on Rec-1 cell line chromosomes was performed according to Szepetowski *et al.* (1992b). Normal 11, der14q$^+$ and two der11q$^-$ chromosomes are indicated by arrows. The probe was *KRN1* cDNA-containing plasmid. Therefore, the *KRN1*-like sequence known to be situated on 11p15 (MacKinnon *et al.*, 1991) is detected on the normal chromosome 11 as well as on the der11q$^-$. (**c**) Physical map of 11q13 derived in part from **a** and **b** (BCL1 represents the breakpoint in the CE4 somatic hybrid). The mapping of the breakpoint in the CF52 hybrid relies on both MacKinnon *et al.* (1991) for the absence of *KRN1* and Szepetowski *et al.* (1992b) for the presence of D11S833E in this cell line. Distances betwen BCL1, *CYCD1* and *FGF3*/*FGF4* were reported in Lammie & Peters (1991)

separately from BCL1 (Proctor *et al.*, 1991; Szepetowski *et al.*, 1992a). Normal peripheral blood leukocytes were used as control cell DNA, and *TYR*, which is located on 11q14–q21 and has never been found amplified in human tumors, was a control for diploidy of 11q DNA sequences. The three cell lines A431, CAL39 and MDA-MB134 bore 11q13 amplicons which contained at least *FGF3*/*FGF4* (data not shown), BCL1, *CYCD1* and D11S97, although the last-mentioned was amplified to a lower level in A431 (Figure 2). The CAL39 amplicon extended at least to *KRN1* on the telomeric side of BCL1. The MDA-MC134 mammary cancer cell line had a D11S833E locus amplified at a level similar to the other amplified loci (10- to 15-fold), whereas *KRN1* was indistinguishable from the conrol (Figure 2). In this cell line, unamplified *KRN1* was framed by two amplified regions, thus defining a discontinuity.

We have shown previously that BCL1 is the 11q13 locus most frequently amplified in mammary carcinomas, perhaps because it is situated between *CYCD1* and D11S97, which define two amplicons close enough to overlap in many instances (Theillet *et al.*, 1990; Szepetowski *et al.*, 1992a). We then studied the amplification status of 11q13 probes telomeric to BCL1 in a subset of DNA samples bearing an amplification of BCL1, consisting of 22 primary breast carcinomas and three cell lines. Ten BCL1-amplified samples (40%) also showed amplification of D11S833E (Figure 3). Since it is known that amplification levels may vary along an amplified region, we distinguished three classes of gene copy numbers in tumors where such a gradient was noticeable: no amplification detectable, highest level of amplification and intermediate level (white, black and hatched rectangles in Figure 3 respectively). In most instances (9 out of 10), the level of amplification of D11S833E was similar to the highest levels observed in a given sample, as in the case of MDA-MB134 (Figures 2 and 3).

Of the 10 samples amplified for D11S833E, four (N83, N101, M281, MDA-MB134) had a normal copy number of *KRN1*. In tumor N104, BCL1 and D11S833E were amplified in the absence of *CYCD1*, *FGF3* and *FGF4* amplification (Figure 3). Therefore, in half of the tumors or cell lines amplified for both BCL1 and D11S833E, non-amplified sequences, including *KRN1* in most cases, separated these two loci.

The occurrence of discontinuities could be the result of several processes. First, reamplification of non-contiguous sequences could develop from a primordial amplification unit covering the whole area. Such a mechanism has been proposed in *in vitro* model

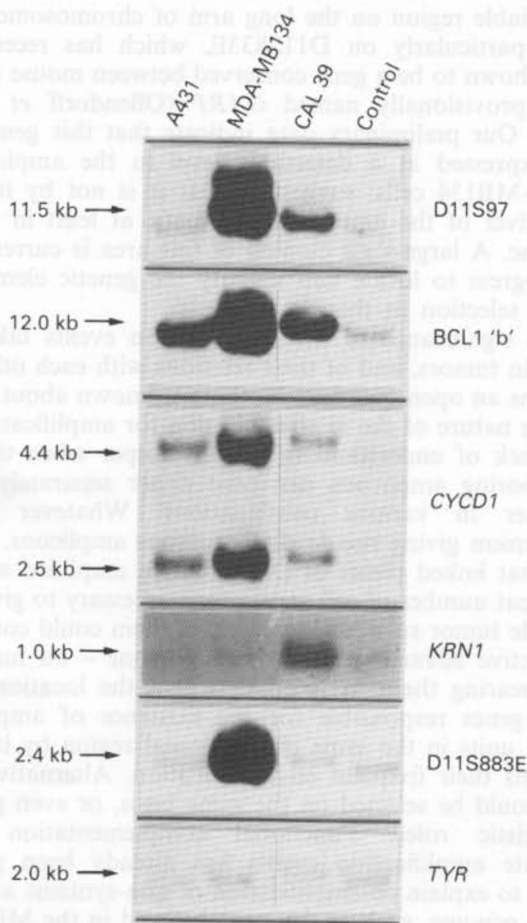

Figure 2 Amplification at 11q13–q14 in human cancer cell lines. The amplification status of various 11q13–q14 markers was compared in three cancer cell lines and control leukocytes from a healthy donor. A431 and MDA-MB134 11q13-amplified cell lines have been described previously (Wada *et al.*, 1988; Lafage *et al.*, 1990; Szepetowski *et al.*, 1992b). CAL39 is a squamous cell carcinomas cell line of the vulva, which was established and generously given to us by J. Gioanni. DNAs were digested by EcoRI and analysed by Southern blotting. Amplification levels determined by densitometric scanning were 15–20 for every amplified marker in MDA-MB134 and 4–6 CAL39 and in A431, except for D11S97, which was amplified only ~twofold in the latter. The CYCD1 probe used in this study was the 1.8-kb BamHI fragment corresponding to the 'C' probe in Arnold *et al.* (1989)

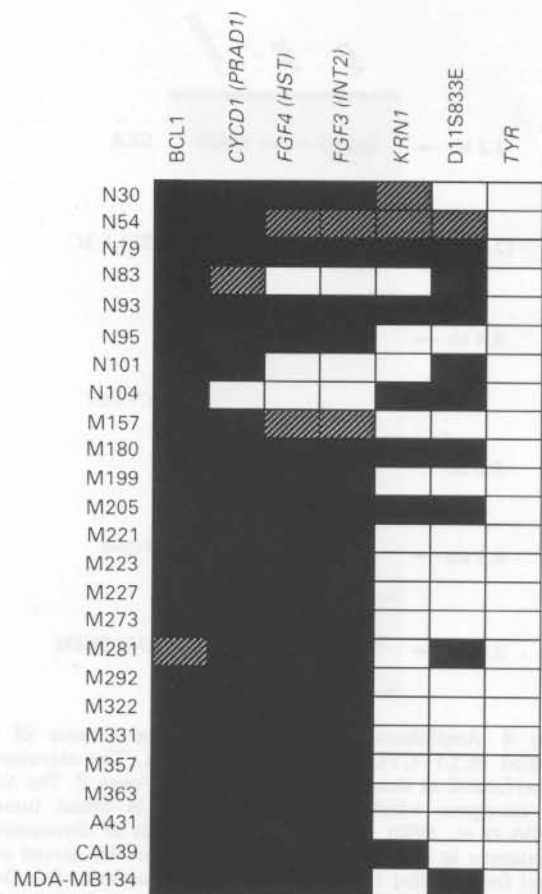

Figure 3 Discontinuities in a population of DNA samples bearing an amplification of BCL1. EcoRI-digested DNAs of 22 primary breast cancers and three cell lines, all amplified for BCL1, were analysed according to Figure 2. In order to avoid blot-to-blot variability, the same Southern blots were successively hybridized with every probe indicated. A black rectangle indicates that the marker was amplified at the highest level observed for amplification at 11q13 in the given sample, a white one that it was not amplified at all and a hatched one that it was definitively amplified but that other 11q13 markers have been found amplified at higher level (see, for example, D11S97 *vs.* BCL1 amplification of A431, in Figure 2)

Table 1 Amplification of D11S833E in breast carcinomas

Amplification of		*D11S833E*	
BCL1	*FGF3 + FGF4*	*n*	*%*
Total population		27/155	17.4
+	+	14/34	41.2
+	−	4*/6	66.7
−	−	9†/115	7.8

*Three out of four were amplified for *CYCD1*
†One out of nine was amplified for D11S97, and the same sample was also the only one amplified for *CYCD1* (out of six tested for this marker)

systems (Saito *et al.*, 1989; Stark *et al.*, 1989). This would lead to a gradient between high (BCL1 and D11S833E) and low (*KRN1*) levels of amplification. Such a gradient could be hidden by a dilution of the amplification signals as a result of stromal contamination in primary tumors. However, *KRN1* was totally absent from the amplicon present in every cell of the MDA-MB134 cell line, and not only present in lesser amount than BCL1. Second, 'pieced amplicons' could be the result of a strong counterselection of the amplification of certain sequences, which would lead to their deletion from the area to be (re)amplified. Third, it cannot be ruled out that different cells bear different amplicons in a single tumor, although this explanation is not suitable for MDA-MB134 cells, which are clonal. Finally, discontinuities could result not only from secondary rearrangements of existing amplicons, but also from simultaneous amplification of non-contiguous parts of the same genomic region.

We thus investigated the amplification status of D11S833E in 155 breast cancer DNAs, this time chosen at random. The results are summarized in Table 1 and illustrated in Figure 4. Of the 155 tumors, 27 (17.4%) were amplified for D11S833E, as compared with 40 (25.8%) for BCL1. By itself, this high frequency of D11S833E amplification is striking since it is

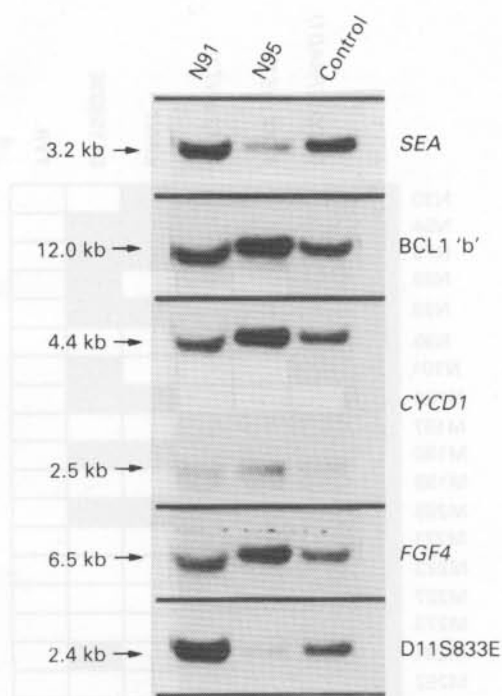

Figure 4 Amplification of D11S833E in the absence of an amplified BCL1–CYCD1–FGF3–FGF4 area. The experiment was performed as described in the legend to Figure 2. The *SEA* proto-oncogene, which is seldom amplified in breast tumors (Theillet *et al.*, 1990), indicates the basal ploidy of chromosome 11 sequences in each sample. DNA from tumor N95 served as a control for amplified 11q13 loci and was thus underloaded. Densitometric scanning of this autoradiogram indicated that D11S833E was amplified threefold in tumor N91

located far from what is considered to be the core of the 11q13 amplification units (i.e. the area comprising BCL1, *CYCD1*, *FGF3* and *FGF4*). In this panel, 6 out of 37 BCL1-amplified tumors did not bear any detectable amplification of *FGF3* and *FGF4*. Four of those were amplified for D11S833E, confirming the existence of the frequent interruption of the amplicons between BCL1 and D11S833E, as reported above. Moreover, nine tumors amplified at the D11S833E locus were not amplified for either BCL1 or *FGF3* and *FGF4*. Out of these nine tumors, eight were not amplified for D11S97 and five out of six which could be analysed for *CYCD1* were not amplified for this marker either (Table 1, Figure 4). It is worth noting that in both cases the only exception was tumor M351. The existence of at least five breast tumors amplified solely for D11S833E strongly suggested the existence of a new amplicon, distinct from the two identified previously, which were centered around BCL1 and D11S97.

We now have evidence for three subregions of 11q13 which can be amplified separately from each other in human breast cancers. The definition of a third amplifiable region on the long arm of chromosome 11 relies particularly on D11S833E, which has recently been shown to be a gene conserved between mouse and man, provisionally named *GARP* (Ollendorff *et al.*, 1992). Our preliminary data indicate that this gene is not expressed at a detectable level in the amplified MDA-MB134 cells, suggesting that it is not by itself the driver of the amplification events, at least in this cell line. A large-scale cloning of this area is currently in progress to locate and identify the genetic element under selection in this process.

The significance of the amplification events taking place in tumors, and of their relations with each other, remains an open question. Nothing is known about the precise nature of the *in vivo* selection for amplification. Our lack of understanding is even deeper when three neighboring amplicons can exist either separately or together in various combinations. Whatever the mechanism giving rise to discontinuous amplicons, the fact that linked pieces of DNA remain amplified after the great number of cell generations necessary to give a sizeable tumor suggests that each of them could confer a selective advantage – even a slight one – on tumor cells bearing them. It is possible that the location of three genes responsible for the existence of amplification units in the same chromosomal region by itself explains their frequent co-amplification. Alternatively, they could be selected on the same basis, or even play synergistic roles. Functional complementation of separate amplification events has already been proposed to explain co-amplification of non-syntenic areas of the genome, such as the one observed in the MDA-MB134 cell line, in which 11q13 and 8p12 markers have been found to be amplified within the same structure, tentatively named 'transplicon' (Gaudray *et al.*, 1992; Lafage *et al.*, 1992). The complexity of the amplification events involving sequences at 11q13 is so striking that one may wonder whether it is the attribute of this genomic area or could apply to other amplifiable regions of the genome, such as the ones containing *ERBB2*, *MYC* or *MYCN*, where variability of the amplification events has long been recognized (Shiloh *et al.*, 1985). Answers to these questions will come from the unambiguous identification of the driver genes of these amplicons and from the study of their biological function.

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