Independent Amplification and Frequent Co-Amplification of Three Nonsyntenic Regions on the Long Arm of Chromosome 20 in Human Breast Cancer

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Abstract

DNA amplification at 20q13.2 is common in breast cancer, correlates with poor prognosis, and may reflect location of an important oncogene. Recently, other regions along 20q were also found to undergo amplification. Here, amplification levels and patterns of co-amplification were analyzed by interphase fluorescence in situ hybridization at 14 loci along 20q in 46 uncultured breast carcinomas and 14 cell lines. Three regions were independently amplified in uncultured tumors: RMC2OC001 region at 20q13.2 (highly amplified in 9.6% of the cases), PTPN1 region 3 Mb proximal (6.2%), and AIB3 region at 20q11 (6.2%). Co-amplifications involving two or three of these regions were seen in 11 of the 19 highly amplified tumors. The results suggest that three distinct nonsyntenic regions along 20q may be important and that complex chromosomal rearrangements underlie their frequent co-amplification in breast cancer.

Introduction

The involvement of the long arm of chromosome 20 in human breast cancer progression has been reported in numerous studies. Allelic imbalance of the 20q13 region was reported in 16% of primary breast carcinomas (1) and studies by CGH found gains and amplifications of this region in 12–18% of primary tumors and 40% of cell lines (2, 3). According to CGH, 20q13 was the most often amplified region in breast cancer that did not involve a previously known oncogene. High prevalence (44%) of 20q13 amplification was also reported by chromosomal microdissection studies of HSRs in breast cancer cell lines (4). Furthermore, 20q13 amplifications by CGH correlated with poor prognosis in node-negative breast cancer (5). Based on this evidence, the 20q13 region is likely to harbor a novel oncogene, the overexpression of which, as a result of the amplification, contributes to breast cancer progression. We and others are now studying this amplification in more detail at the molecular level with the aim to eventually positionally clone the target gene of the amplification. FISH with locus- and gene-specific as well as randomly selected cosmid and P1 probes mapped to 20q was first used to study copy number aberrations in interphase nuclei isolated from breast carcinomas. One commonly amplified region spanning ~1.5 Mb at 20q13.2 was identified, and all candidate genes in this region were excluded (6). Using a probe for the critical region, high-level 20q13 amplification was found in 9 of 132 (~7%) primary breast carcinomas by FISH (7) and in 74 of 1341 tumors (~5%) by Southern analysis (8). An association between 20q13 amplification and poor prognosis in node-negative breast cancer (7) or aggressive tumor phenotype (8) was also reported.

During the course of studies focusing at the 20q13 region, evidence on the amplification of other regions along 20q in breast cancer has emerged. Co-amplification of the PTPNI gene (located about 3 Mb proximal) was found along with the minimal 20q13.2 amplification region in primary tumors and cell lines. Two separate highly amplified regions were identified in the MCF-7 breast cancer cell line at 20q12 as well as at 20q13.2, distal to the minimal region (6). Furthermore, microdissection studies of HSRs from breast cancer cell lines indicated that besides the 20q13 region, DNA sequences originating from the 20q11 region were also often involved (4, 9). Finally, overexpressed sequences in 20q-amplified breast cancer cell lines were recently recovered from both 20q11 and 20q12 regions but not from the 20q13.2 region (9). Since the amplification pattern at 20q now appeared much more complex than previously thought and since several different regions along the long arm of the chromosome seemed to be involved, the question arose as to which region is independently selected for and, therefore, likely to harbor the target gene of the amplification? Here, interphase FISH studies of nuclei from 146 breast cancer specimens were screened for amplification at all five nonsyntenic regions along 20q that were found to be amplified in primary breast carcinomas or cell lines. Those tumors that were positive with one or more probes were subsequently screened for amplification at nine other neighboring loci and genes to assess their involvement in the amplicons.

Materials and Methods

Probes for FISH. Fourteen P1 and cosmids probes defining genes and loci along 20q were chosen for the study. The probes were physically mapped by fractional length from pter (FLpter) measurements as described (6, 10). The probes included P1 clones for the BCL-2-related BCL-X gene (FLpter 0.526), cellular transcription factor E2F (0.541), newly discovered and overexpressed genes AIB3/AIB4 at 20q11 (0.58), protein tyrosine phosphatase PTPN1/PTP1B (0.78), melanocortin-3 receptor MC3R (0.81), vitamin D-24 hydroxylase CYP24 (0.83), phosphoethanol-pyruvate carboxy kinase PCK (0.84), and guanine nucleotide-binding protein GNAS1 (0.87), as well as six cosmids clones along 20q: RMC2OC001 (0.824, defining the previously reported critical region of amplification at 20q13.2); RMC2OC002 (0.742); RMC2OC006 (0.79); RMC2OC003 (0.85); and c.k.01.109.085. Finally, a reference probe for 20p, RMC2OC039 (FLpter 0.237), was also used.

The aforementioned probes included all previously described amplification regions at 20q in breast cancer. The following nomenclature for the different amplification regions was adopted. Region A refers to our previously described critical amplification region at 20q13.2 defined by the probe RMC2OC001. Region B is the PTPNI gene region 3 Mb proximal from region A. Region C
is the 20q11 amplification site initially mapped by microdissection and subsequently shown to contain \( AiB3 \) and \( AiB4 \) genes. Region D defines a locus at 20q12 amplified in MCF-7 breast cancer cell line (probe RMC20C002), and region E, another similar MCF-7-specific amplification site at 20q13.2–13.3, is located about 3 Mb distal from region A and defined by probe cK20.10E9.

**Preparation of Cell Lines and Primary Breast Tumors for FISH.**

Fourteen established breast cancer cell lines (BT-474, BT-549, DU4475, MCF-7, MDA-134, MDA-157, MDA-361, MDA-415, MDA-436, MPE-600, SK-BR-3, ZR-75–1, UACC-812, and UACC-893), all from the American Type Culture Collection (Rockville, MD), were grown in recommended culture conditions. The cell lines were harvested at confluence to obtain interphase nuclei from cells that were predominantly in the G2 phase of the cell cycle and subsequently fixed in methanol:acetic acid (3:1) and dropped on microslide glasses. Similarly prepared foreskin fibroblasts were used as negative controls.

One hundred thirty-four primary breast carcinomas and 12 metastatic tumors diagnosed at the Tampere University or City Hospitals during 1977–1995 were used in this study. The tumors included all but five cases described in our previous study (7) as well as five additional tumors selected on the basis of the fact that they showed clear 20q13 amplification by CGH. Five cases were lobular invasive carcinomas, with the remaining ones representing ductal invasive carcinomas. Tumor specimens were first histologically examined, and only those blocks containing more than 60–70% malignant epithelial cells were considered representative for FISH analysis. Nuclei from specimens were isolated either from 100-μm thick frozen sections or from two 50-μm sections of paraffin-embedded blocks as described previously (11). After methanol-acetic acid fixation (only required for fresh specimens), the cells were placed on slides and used for FISH as described (7, 11).

**FISH Analysis.**

Two-color FISH was performed with the 20q-specific test probe labeled with biotin-dUTP or FITC-dUTP and the 20p-specific reference probe labeled with digoxigenin-dUTP as described (6, 7). After hybridization, the bound probes were detected immunochromatically with avidin-FITC (in the case of biotin-labeled probes) and anti-digoxigenin rhodamine. Slides were counterstained with 0.2 μM 4,6-diamidino-2-phenylindole in an antifade solution. Hybridization signals were evaluated using Olympus BX-50 epifluorescence microscope using criteria described previously (6, 7). The entire slide was first scanned through, and 50–100 randomly chosen individual nuclei were scored in detail to determine the mean number of signals for the test and reference probes as well as their ratio (defined as the level of amplification). Control hybridization to normal fibroblasts was done to ascertain that the probes recognized a single copy target and that the hybridization efficiencies of the test and reference probes were similar. DNA amplification was defined as described previously (7), with tumors classified into those with no amplification, low-level amplification (1.5–3.0-fold), or high-level (>3-fold) amplification.

**Results**

**Amplification Patterns at 20q in Breast Cancer Cell Lines.**

Seven of the 14 (50%) breast cancer cell lines (MCF-7, BT474, MDA-157, SKBR3, UACC-812, MDA-361, and UACC-893) showed >1.5-fold amplification with several of the 14 markers examined by FISH (Fig. 1). Altogether, five distinct regions along 20q with >3-fold amplification were found in these cell lines. These included region A at 20q13.2 (the previously defined critical region surrounding the RMC20C001 probe), region B (\( PTPN1/PTPB1 \) locus about 3 Mb proximal), region C (the \( AiB3/AiB4 \) gene locus at 20q11), region D at 20q12–20q13 (defined by cosmid RMC20C002), and region E at 20q13.2–13.3 (defined by cosmid cK20.10E9 located about 3 Mb distal from region A). The frequencies of amplification (over 3-fold) of these five regions in the 14 cell lines were: A, 21%; B, 14%; C, 21%; D, 14%; and E, 21%.

Some of the amplicons in the breast cancer cell lines were large and apparently contiguous. For example, in SKBR-3 the amplicon spanned >5 Mbs from PTPN1 to the marker cK20.10E9. On the other hand, separate amplifications of multiple regions, with no (or only low-level) amplification of the intervening regions were also found, such as the three separate regions of amplification found in BT474.

**Amplification Patterns along 20q in Uncultured Breast Carcinomas.**

In uncultured tumors, the amplification patterns were clearly different from those observed in the cell lines. Somewhat unexpectedly, the amplicons were smaller than those seen in the cell lines (Fig. 2). This resulted in better definition of the minimal regions of involvement. Altogether, 41 of the 146 tumors (28%) showed >1.5-fold amplification of one or more of the five regions tested, with region A being amplified in 26%, B in 18%, C in 15%, D in 3%, and E in 5% of the cases (Fig. 1). The mean amplification levels in the tumors ranged from 1.5- to 7.1-fold, but individual tumor cells visualized by FISH contained up to 15-fold amplification. Nineteen tumors (13%) showed over 3-fold amplification of one of the five regions with the following frequencies of involvement: A, 9.6%; B, 6.2%; C, 6.2%; D, 0%; and E, 0%.

Thus, based solely on the frequency of involvement, region A emerged as the critical region, with B and C also possibly being significant (Fig. 2). Region A was the only highly amplified region at 20q in four cases, B in two, and C in two. All three regions were simultaneously highly amplified in two cases, regions A and B in four cases, A and C in four cases, and those of B and C in 1 case (Fig. 3A). In all of the uncultured tumors with these co-amplifications, the chromosomal regions between the regions A, B, and C were not amplified. Regions D and E could be excluded as locations of important oncogenes because of the absence of high-level amplification at these sites.

Inclusion of the low-level amplifications into the statistical analysis increased the frequency of the co-amplification phenomenon considerably (Fig. 3B). Especially in the case of high-level amplification of one of the regions, the two other regions also often showed slightly elevated copy number. On the other hand, the predominance of region A became more evident in this analysis, since there were several cases where only this region showed low-level amplification.

**Discussion**

The present extensive FISH analysis indicates that three separate regions along the long arm of chromosome 20 can be independently amplified in uncultured breast carcinomas, and that their co-amplification is strikingly common. Two other regions that were also implicated by the cell line data were not found to be highly amplified in any of the uncultured tumors. The data suggest that the most important region of amplification in breast cancer is at 20q13.2, the site of our previously described critical region defined by the probe RMC20C001 (6). However, the two other nonsyntenic amplification regions, 3 Mb and 20 Mb proximal, clearly also appear important, and may contain genes whose amplification is selected for independently or by virtue of their additional contribution to the effects achieved by amplification of the critical 20q13.2 region.

The independent amplification of each region and the consistency of the three co-amplification sites suggest that random co-amplifications of irrelevant chromosomal segments along with an important target gene are unlikely to explain the findings. Thus, alternative hypotheses to account for the frequent co-amplification of two or three widely separated regions along the same chromosome arm must be considered. It is possible that the specific genes located in these three regions may provide such a strong selective advantage to breast cancer progression that they all tend to become amplified independently. All known cloned genes have now been excluded from region A, including the vitamin D hydroxylase (\( CYP24 \)) gene recently implicated as a candidate (Ref. 6 and present data). A

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Fig. 1. Amplification of the 14 different probes along 20q in breast cancer cell lines and uncultured breast carcinomas. Only those cases that showed >1.5-fold amplification with at least one of the probes were included in the plot. Chromosome ideogram and the FLpter scale are given as an approximate reference for the location of the probes. The five different regions of amplification implicated previously in breast cancer are indicated at the bottom of the figure as A, B, C, D, and E.

ampl., amplification; LA, level of amplification.

PI/BAC contig for the most frequently amplified region spanning 1.5 Mb at 20q13.2 is approaching completion, and identification of expressed sequences is under way.\(^4\) Region B encompasses the \(PTP1B/PTPN1\) gene, which is a nonreceptor tyrosine phosphatase involved in growth regulation by insulin receptor-mediated signal transduction (12). Although \(PTPN1\) has recently been reported to be overexpressed in 72% of breast carcinomas (13), our studies indicate a lack of correlation between amplification and overexpression of this gene.\(^5\) Finally, region C at 20q11, originally discovered by microdissection, has already been found to contain at least two expressed sequences, \(AIB3\) and \(AIB4\), but the data do not yet prove that these genes are the definitive target genes for the 20q11 amplicon (9). It is interesting to note that cDNA selection did not recover any overexpressed clones from the 20q13.2 region, although this was represented in the original HSRs that were dissected. The cellular apoptosis susceptibility (CAS) gene was recently also mapped to 20q13 (14). Having tested the gene for amplification in cell lines and selected primary tumors showing high level 20q13 amplification by CGH,\(^5\) we agree with the authors on the low-level (2–3-fold) amplification in some cell lines, but the absence of high level amplification in cell lines and selected primary tumors clearly excludes this functionally interesting gene as a target for 20q amplifications. Overall, the complexity of the gene amplification phenomenon as shown here for 20q illustrates the difficulty in implicating a specific gene or region as the target for amplification without thorough knowledge of the amplification status of the neighboring genes and loci.

The co-amplification of nonsyntenic sites along the same chromo-

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\(^4\) C. Collins, unpublished data.

\(^5\) M. M. Tanner, unpublished data.
The somatic arm observed in this study is not an uncommon event. The 11q13 amplification in breast cancer involves several nonsyntenic cores and at least two distinct target genes, EMS-I and Cyclin-D1 (15, 16). The 12q12–q14 amplicon in sarcomas is also discontinuous and involves the CDK4 gene at the proximal region and MDM2 about 2 Mb distal from it, with the intervening region often not showing any amplification (17). L-myc and rlf genes, both located at 1p32 but less than one-half of a megabase apart, may form a functional fusion gene when amplified in small cell lung cancer (18). CGH results also indicate that co-amplifications of different regions along the same chromosome arm are common, although many of these target genes are still unknown. These include co-amplifications of 17q12 (ERBB2) and 17q23 regions, 8q24 (MYC) and 8q22 regions in breast cancer, as well as that of NMYC at 2p24 and one or two proximal regions at chromosome 2 in neuroblastomas (2, 4, 19). Finally, co-amplifications of important oncogenes from different regions of the genome may also occur and localize to the same HSRs in cancer (4, 19). For example, NMYC (2p24) and MDM2 (12q13-q14) were found to be co-amplified first in double minutes and then integrated into chromosomes in neuroblastoma (20). Thus, there may be underlying mechanisms that predispose to co-amplifications of multiple important genes in the tumor cells and to localization of such initially nonsyntenic sequences into the same chromosomal structures. Alternatively, co-amplifications of important genes in the tumor cells and to localization of such initially nonsyntenic sequences into the same chromosomal structures. Alternatively, co-amplifications of important genes in the tumor cells and to localization of such initially nonsyntenic sequences into the same chromosomal structures. Alternatively, co-amplifications of important genes in the tumor cells and to localization of such initially nonsyntenic sequences into the same chromosomal structures.
though such chromosomal integration sites could not be studied by interphase FISH studies of clinical tumor specimens, the co-amplified sequences originating from 20q13.2 and 20q11 clearly resided in the same HSRs in some of the breast cancer cell lines studied here (4, 9).

In conclusion, the results indicate an extremely complex amplification process involving almost the entire long arm of chromosome 20 in breast cancer. The consistent involvement of three distinct loci in the co-amplifications, as well as their independent amplification, suggest that all of them could be important for breast cancer progression. Thus, several target genes at 20q are likely to be selected for amplification. Finally, extensive chromosomal re-arrangements and multiple breaks must underlie generation of this amplification. Further studies of this and other similar co-amplification events may give important clues to the mechanisms of gene amplification and ampiclon evolution in human solid tumors in vivo.

References
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