

Original Paper

Determination of amplicon boundaries at 20q13.2 in tissue samples of human gastric adenocarcinomas by high-resolution microarray comparative genomic hybridization

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Abstract

Comparative genomic hybridization (CGH) of gastric adenocarcinomas frequently shows gains and amplifications of chromosome 20. However, the underlying genetic lesion is unknown and conventional CGH results do not allow specification of the target region. In order to investigate this chromosomal aberration with a higher resolution and sensitivity, microarray-based CGH was performed with both scanning and high-resolution arrays of chromosome 20 in a series of 27 gastric adenocarcinomas. Locus-specific fragments of genomic DNA from bacterial artificial chromosome (BAC) clones were spotted as microarrays. A scanning array contained a set of 27 BAC clones covering chromosome 20q. A high-resolution array contained 27 overlapping BAC clones at 20q13.2. This high-resolution array was used to narrow down the amplicon at 20q13.2 in tumours showing amplification of this chromosomal region with the scanning array. Positive copy number changes on chromosome 20q were detected in 12 of 27 cases (44%). These changes included gain of the whole arm of chromosome 20q in 8 of 27 (30%) cases, amplification restricted to 20q12.1 in one case, and amplifications restricted to 20q13 in three cases (11%). The three tumours showing amplification restricted to 20q13 were analysed further using the high-resolution array. In one tumour, the whole contig was amplified at a constant level. One of the other two tumours had a clear proximal breakpoint, while the other tumour had a clear distal breakpoint within the 20q13.2 region. The proximal and the distal breakpoint were approximately 800 kb apart. In the present study, an amplicon at 20q13.2 has been narrowed down to 800 kb which is likely to harbour one or more putative oncogenes relevant to gastric carcinogenesis, for which ZNF217 and CYP24 are good candidates. Copyright © 2003 John Wiley & Sons, Ltd.

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Introduction

Gastric adenocarcinoma is a very common disease, ranking fifth as a cause of cancer death in The Netherlands, with approximately 2100 new cases annually. It is the second most common cancer worldwide [1]. *Helicobacter pylori* is a major aetiological factor that probably interacts with dietary factors and the Epstein–Barr virus may also play a role in a subset of cases [2–4]. The pathogenesis of the intestinal type of gastric adenocarcinoma morphologically follows a sequence of *H. pylori* infection causing active and subsequent gastritis, leading to mucosal atrophy and intestinal metaplasia, and in a small number of cases followed by dysplasia and

ultimately adenocarcinoma [5]. Despite the high incidence, knowledge of the genetic events leading to gastric adenocarcinoma is still limited. An essential early step in the pathogenesis of most cancers is loss of one of the defence mechanisms that control the integrity of the genome. This makes it possible for a cell to acquire genomic changes more rapidly, a situation called genomic instability [6]. In a subset of gastric adenocarcinomas, genetic instability occurs at the DNA level, caused by a failing DNA mismatch repair system [7], but in the majority of gastric adenocarcinomas, genomic instability occurs at the chromosomal level, affecting numerous genes and thereby causing tumour progression. Only recently have techniques

become available to study the patterns of these chromosomal changes in more detail. Chromosome-based comparative genomic hybridization (CGH) allows a genome-wide analysis of DNA sequence copy number at the chromosomal level in a single experiment [8] and has provided important information about the chromosomal changes involved in gastric adenocarcinoma. Apart from loss of a number of chromosomal regions harbouring known or putative oncogenes (including 17p and 18q), a number of areas of chromosomal gain have been documented [4,9–16]. These include gains of 1q, 8q, 13q and 20q. In particular, the 20q region has been found to show gains (range 23–100%), and sometimes amplification, in a number of CGH studies on gastric adenocarcinoma [9,10,12–14,16–21]. Based on the chromosome CGH findings, the q12–13 region was considered to be the relevant area on chromosome 20 [12]. Gain of 20q was also frequently found in other types of adenocarcinoma [22–27]. This indicates the presence of one or more important oncogenes on the long arm of chromosome 20, which could be involved in gastric adenocarcinoma. In order to identify the candidate oncogenes in this still rather large region harbouring many genes [28], it is important to focus further on these 20q amplifications.

Chromosome-based CGH is not the method of choice for this purpose, since it has a limited sensitivity for small chromosomal aberrations (10–20 Mb). Furthermore, the use of metaphase chromosomes as a template for hybridization limits the resolution of closely spaced aberrations and makes direct linkage of ratio changes to specific genomic/genetic markers impossible. Hybridization to an array of mapped DNA sequences overcomes the limitations of conventional CGH. With this microarray-based approach, copy numbers are related to the test/reference fluorescence ratio on the arrayed DNA segments. The genomic resolution is determined by the map distance between the arrayed clones and by the length of the cloned DNA segments. This can be minimized using a contig consisting of short (10 kb) overlapping clones. This technique therefore permits the detection of gains and losses at a much higher resolution [22,29,30]. Such microarray CGH across amplified regions in breast cancer has facilitated oncogene identification by providing precise information on the locations of amplicon boundaries [22].

The purpose of the present study was to narrow down the region of amplification of chromosome 20 using high-resolution microarray CGH in tissue samples of gastric adenocarcinomas.

Materials and methods

Material

Twenty-seven patients with sporadic primary gastric adenocarcinoma of the intestinal or diffuse type who

had undergone (partial) gastrectomy were included in the study. Of the 27 patients, 21 were male and six were female, with a mean age of 63 years (range 27–85 years). Twenty tumours were of the intestinal type and seven were of the diffuse type [31]. DNA was isolated from snap-frozen tissue samples from these gastric resection specimens. All samples were obtained from the archives of the Department of Pathology of the VU University Medical Centre and the study followed the local ethical guidelines of the Institutional Review Board of the VU University Medical Centre. In each case, a tumour content of more than 75% was certified on two sandwich sections. Normal human male genome DNA was isolated from lymphocytes obtained from a blood bank. DNA isolation was performed following the manufacturer's instructions (Qiamp Tissue Kit, QIAGEN Inc, Valencia, CA, USA), with some modifications as described before [32]. Chromosome-based CGH has been performed previously on a subset ($n = 18$) of these tumours [14].

Microarrays

Microarrays were produced as described previously [33]. In short, DNA isolated from bacterial artificial chromosome (BAC) clones was amplified using ligation-mediated PCR to generate representations of these human BAC DNAs. The DNAs were spotted on chromium-coated microscope slides using a custom-built arrayer. Scanning and high-resolution arrays were used as described previously [22,29,33]. The scanning array comprised 27 clones distributed over chromosome 20 and 52 clones throughout the rest of the genome. The high-resolution array for 20q13.2 contained a contig of 27 overlapping clones, five of which were also present in the scanning array.

Comparative hybridization

Test and reference genomic DNA (1 μ g of each) were labelled by nick translation with Cy3 dCTP (Amersham Pharmacia Biotech) and fluorescein dCTP (DuPont NEN NEL424), respectively. Non-incorporated nucleotides were removed using a Sephadex G-50 spin column. Labelled DNA (500–1000 ng) was mixed with Cot-1 DNA (35–70 μ g; Gibco BRL) and ethanol-precipitated. The precipitated DNA was dissolved in hybridization mix (10 μ l) to achieve a final composition of 50% formamide, 10% dextran sulphate, $2\times$ SSC, 2% SDS, and 100 μ g of tRNA. The hybridization solution was heated to 70 °C for 10 min to denature the DNA and then incubated at 37 °C for approximately 60 min to allow blocking of the repetitive sequences. A wall enclosing the array was made with rubber cement and the resulting well was filled with hybridization mix. Slides were placed in a small hybridization chamber containing 50% formamide (200 μ l) and $2\times$ SSC to prevent evaporation. Hybridization proceeded at 37 °C for 16–24 h.

After hybridization, slides were washed once in 50% formamide, $2\times$ SSC, pH 7, at 45 °C for 15–30 min and once in PBS/0.05% Tween at room temperature for 5–10 min. Excess liquid was drained from the slides and the array was mounted in an antifade solution containing DAPI (1 µg/ml) to counterstain the DNA targets.

Image acquisition and data analysis

Image acquisition and analysis, and data extraction were performed as described previously [29]. In short, DAPI, Cy3, and fluorescein images were captured using a CCD-based imaging system. After segmentation and background correction, mean signal ratios of Cy3 to fluorescein were calculated for every clone. Clones with a high standard deviation (>0.3) of the average integrated fluorescence for either Cy3 or fluorescein over the triplicate/quadruplicate spots and/or a low correlation (<0.8) between pixels within spots were discarded. Normalization was based on the mode of the integrated fluorescence for Cy3 and for FITC, respectively, for all clones except those on chromosomes 20 and X. Chromosomal copy number changes were classified as follows. The term 'positive copy number changes' is used for both gains and amplifications. An amplification is a relatively narrow and sharply defined positive copy number change, usually with a multiple-fold amplitude. A gain is a larger chromosomal region with increased copy number, usually corresponding to approximately one or two copies (also depending on the ploidy of the tumour). Fluorescence ratios of 0.8 and 1.2 were used as thresholds for losses and gains, respectively, based on normal versus normal experiments. Analysis of duplicate experiments yielded consistent results.

Fluorescence *in situ* hybridisation

To verify the presence of gains of specific loci at the tissue level, fluorescence *in situ* hybridization (FISH) analysis with a locus-specific probe (LSI 20q13.2 amplicon SpectrumOrange Vysis, Inc, Downers Grove, IL, USA) was performed on 4 µm sections derived from paraffin-embedded tissue, as described by Bastian *et al* [34]. This probe hybridizes to a segment within the 20q13.2 region containing the *ZNF217* gene, which is covered by the contig used for microarray CGH. Image acquisition was done with a confocal laser-scanning microscope (Leica TCS-SP, $\times 100$ objective [PLAN APO]), using the 'Z-stack' option.

Results

Twenty-seven patients treated with partial or complete gastrectomy for primary gastric adenocarcinoma were included in the study. Twenty-one of them were male and six female, with a mean age of 63 years (range 27–85 years). Twenty tumours were of the intestinal type and seven were of the diffuse type [31].

DNA obtained from each tumour was first screened with a medium-resolution scanning microarray. Using this scanning array, positive copy number changes of chromosome 20q were detected in 12 cases (44%). These changes included gains of the whole arm of chromosome 20q in eight (30%) of the cases, amplification restricted to 20q12 in one case (4%), and amplifications restricted to 20q13 in three cases (11%) (Figure 1). The one tumour with amplification at 20q12 was a 9.5 cm, TNM stage 3, poorly differentiated intestinal-type adenocarcinoma in a 32-year-old male patient. The three tumours with 20q13 amplification measured 3.5, 6, and 14 cm, respectively, two being TNM stage 3 and one TNM stage 4. They were all poorly differentiated intestinal adenocarcinomas. The patients were 65, 69, and 81 years old, respectively, and all were male.

To obtain information on the presence of copy number changes at 20q13 at the tissue level, we performed FISH on formalin-fixed, paraffin-embedded tissue sections of the three tumours that showed amplifications of this region with microarray CGH. In all three tumours that showed 20q13 amplification, areas were present in which the tumour cells showed a clear copy number increase for the locus at 20q13 (Figure 2). However, since large clumps rather than individual spots were seen, no formal counts could be performed.

Subsequently, the three tumours that showed amplification of chromosomal band 20q13 were investigated with a high-resolution microarray with a contig of 27 clones spanning this region. In contrast to the scanning array, which has an average genomic distance of 1.4 Mb between the spotted clones, the high-resolution microarray contains a series of overlapping clones that specifically cover 20q13.2. With this microarray it is possible to define the exact boundaries of the amplified region, thus potentially giving more precise information on the candidate genes located in this specific chromosomal region. The results are shown in Figure 1. In one tumour (tumour 27), the whole length of the contig was amplified at a constant level, indicating that in this case the amplicon boundaries are outside the contig region. In tumour 14, a clear breakpoint proximal in the contig (at clone D20S854) was seen. Tumour 16 showed a breakpoint distal (at clone D20S120) in the region under study, with an additional, yet less prominent, proximal change. This proximal amplicon boundary was located at the same position as the clear breakpoint in tumour 14. These proximal and distal breakpoints flank a region of approximately 800 kb within the 20q13.2 region between 52.800 and 53.600 kb (ie between D20S854 and D20S120; see website UCSC Human Genome Project Working Draft at <http://genome.ucsc.edu/goldenPath/aug2001Tracks.html>), which contains among others the genes *ZNF217* and *CYP24*.

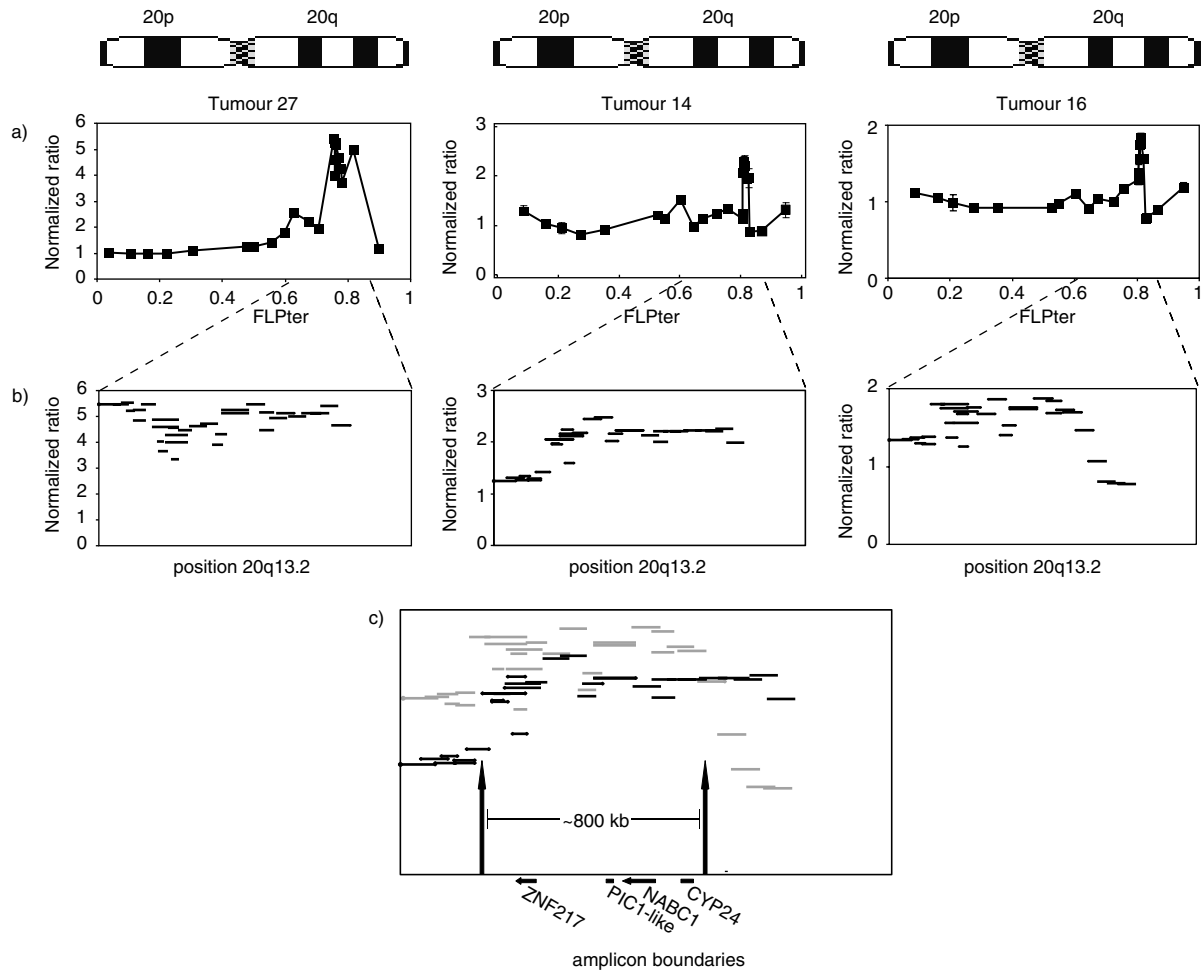


Figure 1. Three gastric adenocarcinomas (tumours 14, 16, and 27) with amplification at 20q13.2 analysed by microarray comparative genomic hybridization. (a) Data from the scanning array (whole chromosome 20) for every tumour are presented as line graphs. The relative position of each clone (represented by a dot in the graph) is given along the x-axis, ranging from 0 (20pter) to 1 (20qter). An ideogram of chromosome 20 is displayed for orientation at the top of each graph. Cy3 to Cy5 fluorescence ratios, representing tumour to normal copy number ratios for each clone, are plotted along the y-axis. (b) The corresponding results of the high-resolution arrays with overlapping clones at 20q13.2. Each array target clone is represented by a horizontal bar indicating the location and length of the clone as determined by STS content mapping. The vertical position indicates the measured Cy3 to Cy5 fluorescence ratio. Two gastric adenocarcinomas (tumours 14 and 16) showed variation in copy number across the region, indicating chromosomal breakpoints as the boundaries of the amplicons. (c) Projecting the contig array graphs from tumours 14 and 16 on top of each other clearly shows the marked variation in Cy3 to Cy5 fluorescence ratios indicating the amplicon boundaries. The positions of the *ZNF217*, *PIC1-like*, *NABC1*, and *CYP24* genes within the amplicon are indicated

Discussion

Chromosomal instability is a major mechanism of genetic damage in gastric adenocarcinoma, as has been demonstrated by classical CGH studies showing chromosome abnormalities in almost all gastric tumours analysed. A number of chromosomal losses, causing loss of tumour suppressor gene function, have been identified in sporadic gastric adenocarcinoma, for example *p53* on 17p13 and *E-cadherin* on 16q22. Also, amplifications of *cyclin D1* on 11q13, *C-Met* on 7q31, and *HER2-neu/C-erbB2* on 17q21–q22 have been described. However, the picture is still far from complete. Studies using chromosome-based CGH have indicated the involvement of multiple chromosomal copy number changes in gastric adenocarcinoma, including gain of the long arm of chromosome 20 [4,9–16]. These recent CGH studies, including

those in gastric adenocarcinomas, have detected high frequencies of chromosomal gains, causing renewed interest in the role of oncogenes. Although chromosome CGH increased our possibilities to analyse the complete genome of primary tumour samples in one experiment, it has certain limitations in resolution for chromosomal aberrations. Array-based CGH has overcome this problem to a large extent and is, in this respect, probably the most sophisticated technique [29]. Recently, this strategy has proven to be successful in analysing the 20q13 amplicon in breast cancer [22]. In the present study, we therefore set out to study the role of chromosome 20q copy number changes in gastric adenocarcinoma in more detail, using high-resolution microarray CGH.

In the present study, positive copy number changes on chromosome 20q were detected in 12 of 27 cases (44%), including whole chromosome arm gains in

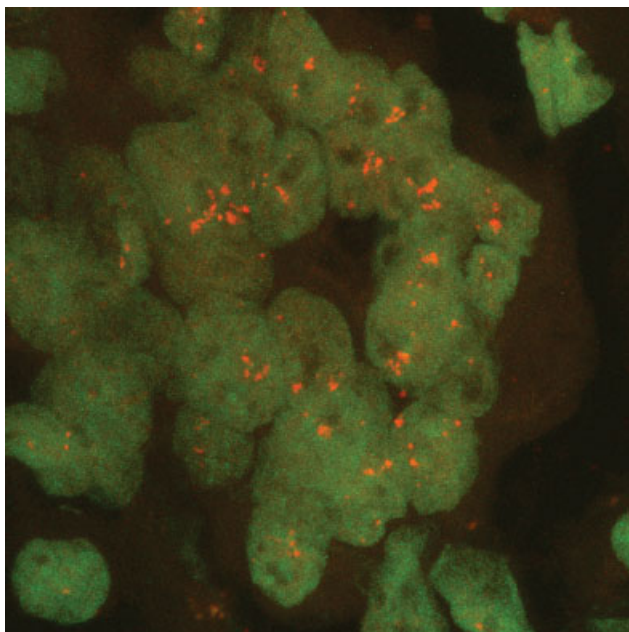


Figure 2. Example of a FISH experiment with a locus-specific probe for 20q13.2 in a gastric adenocarcinoma that showed amplification of this region with microarray CGH (tumour 16). A high copy number for this locus is clearly visible in the tumour cells

eight cases (30%), an amplification restricted to 20q12 in one case (4%), and amplifications restricted to 20q13 in three cases (11%). Since these positive chromosomal copy number changes are highly prevalent and clearly show a non-random distribution, it is likely that they harbour oncogenes that play an important role in the pathogenesis of gastric adenocarcinoma. Identification of the actual oncogenes in these regions of chromosomal gain, however, is complicated. This can be facilitated by studying cancers that within the same region of positive copy number changes, show amplifications pointing at the loci of putative oncogenes that drive the selection of tumour cells during carcinogenesis. These amplifications are therefore of major importance for our understanding of the biology of gastric adenocarcinoma. Given the fact that amplifications in adenocarcinomas are rare, the prevalence of 11% for amplification of 20q13.2 in gastric adenocarcinoma certainly is to be considered a frequent event. The three gastric primary adenocarcinomas that showed amplification of 20q13 were investigated in more detail with an array of overlapping BAC and P1 clones. This array was previously designed to study amplicon boundaries and copy-number profiles across a 2 Mb region of recurrent amplification at 20q13.2 in breast cancer [22]. Using this contig array, two discrete amplicon boundaries could be determined in two different gastric adenocarcinomas (tumours 14 and 16) flanking a region of approximately 800 kb, while the third adenocarcinoma (tumour 27) showed amplification of the whole contig (Figure 1c). The observation that, in two tumours, the breakpoints are clearly within the contig region is a very strong indication that the relevant oncogenes lie inside this ~800 kb region

at 20q13.2. Previously, chromosome-based CGH of these tumours [14] (and own unpublished results) had revealed low-level gains of larger parts of 20q, but the narrow amplification detected by microarray CGH had remained undetected. Amplifications of 20q13 have been reported in adenocarcinomas from different sites including hepatocellular carcinoma, gastric adenocarcinoma [13,18,19] and cancers of the gastro-oesophageal junction, colorectal cancer [35], ovarian cancer [36], and especially breast cancer (~18%) [37]. In addition, a region slightly proximal to 20q13 (20q11.2–q12) has been reported to be amplified in gastric adenocarcinoma [9,12]. Most breast cancers and cell lines with amplifications at 20q13.2 show elevated and slightly varying copy numbers across the entire region [22,29,38]. However, some breast cancers showed narrow peaks that may point to the locus of genes that drive selection during carcinogenesis. Interestingly, the amplicon boundaries found in gastric primary adenocarcinoma in the present study coincide with those found previously in breast cancers (S21, S59) [22] and a breast cancer cell line, MCF7 (unpublished data). This is a strong indication that the same chromosomal breakpoints are involved in both gastric and breast carcinogenesis, and possibly in other adenocarcinomas as well. This region has also been found to be involved in genomic instability and immortalization in model systems [39,40].

The complete sequence of human chromosome 20 has recently been unravelled [28], but still little is known about the expression of genes at 20q in gastric adenocarcinoma. *BTAK* (*STK6*) at 20q13.31 was found to be amplified (5%) and overexpressed (50%) in primary gastric adenocarcinomas and amplification of *BTAK* correlated with aneuploidy and poor prognosis [41]. Another study reported amplification (7%) and overexpression (40%) of *AIB1* (at 20q13.12) in gastric adenocarcinoma [42]. However, both *BTAK* and *AIB1* are located outside the amplicon described in the present study, indicating that still other genes at chromosome 20q may be involved in the development of gastric adenocarcinoma.

A number of putative oncogenes are located within the amplicon boundaries found, including *ZNF217*, *ZNF218*, *NABC1*, *PIC1L*, *prefoldin*, and *CYP24*, as well as a pseudogene, *CRP* [38,43]. Of these, *ZNF217* and *CYP24* are particularly interesting candidates. Knowledge about the function of these genes is still limited. *ZNF217* is a transcription factor, which is associated with immortalization, and is overexpressed in breast cancer [38,43,44]. With the AmpliOnc I genomic array (Vysis, Downers Grove, IL, USA), amplification of the *ZNF217* locus was found in three of five cell lines derived from adenocarcinomas of the gastro-oesophageal junction [45]. *CYP24* is involved in vitamin D metabolism and down-regulates the levels of the active form of vitamin D that, among others, inhibits cell growth, eg of colon cancer cells *in vitro* [46,47]. Dietary intake of vitamin D has been found to be associated with a reduced risk

of colon cancer [48] and overexpression of *CYP24* due to amplification may abrogate vitamin-D-mediated growth control. However, data on vitamin D growth control in gastric adenocarcinoma are scarce. In breast cancer, both *ZNF217* and *CYP24* expression (relative to the expression of the vitamin D receptor) was correlated with copy number [22,38]. So far, no such data are available for gastric adenocarcinoma.

In conclusion, in the present study of gastric adenocarcinomas, the amplicon on 20q13.2 has been narrowed down to an 800 kb candidate locus potentially harbouring one or more oncogenes important in gastric carcinogenesis. We hypothesize that these include *ZNF217* and *CYP24*.

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