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Mixed gastric carcinomas show similar chromosomal aberrations in both their diffuse and glandular components

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Abstract. Gastric cancer is one of the most frequent malignancies in the world. Nonetheless, the knowledge of the molecular events involved in the development of gastric carcinoma is far from complete. One of the hallmarks of gastric cancer is chromosomal instability resulting in abnormal DNA copy number changes throughout the genome. Mixed gastric carcinomas constitute a rare histological entity, containing the two main histological phenotypes (diffuse and intestinal). Very little is known about the underlying mechanisms of phenotypic divergence in these mixed tumours. To the best of our knowledge only E-Cadherin mutations were implicated so far in the divergence of these tumours and nothing is known about the involvement of chromosome copy number changes in the two divergent histological components. In this study, we compared the DNA copy number changes, in the two different components (diffuse and intestinal) of mixed gastric carcinomas by microarray – comparative genomic hybridisation (array CGH).

The analysis of 12 mixed gastric carcinomas showed no significant differences in array CGH profiles between the diffuse and intestinal components of mixed carcinomas. This supports the idea that the phenotypic divergence within mixed gastric carcinomas is not caused by DNA chromosomal aberrations.

Keywords: Mixed gastric carcinoma, chromosomal aberrations, array CGH

1. Introduction

Despite the overall decrease in incidence and mortality rates, gastric cancer remains the second most frequent malignancy worldwide [25,26]. Within the European Union, incidence and mortality rates differ between countries, Portugal having the highest incidence (31.9/100.000) and mortality (17.5%) rates attributable to gastric cancer [2]. In contrast, in The Netherlands gastric cancer ranks fifth as cause of cancer death [43], with an incidence of 15.5/100.000 [2].

Two main histological types of gastric cancer are recognized, the intestinal [22] or glandular carcinoma [5] and the diffuse [22] or isolated-cell type carcinoma [5]. Distinct genetic pathways underlie these two types of gastric cancer. Mutations in particular genes are restricted to one of the two histological types, such as mutations in *CDH1* (the gene encoding for the adhesion molecule E-cadherin) that occur only in diffuse gastric carcinoma [1,10] or amplification, and consequently over-expression, of the *ERBB2*

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oncogene, which is only observed in intestinal gastric carcinoma [42].

In addition, there is a third histological type, the mixed gastric carcinoma, with a dual pattern of differentiation, encompassing in the same tumour distinct histological components (intestinal/glandular and diffuse/isolated-cell type) [22,5]. Mixed gastric carcinomas have a poor prognosis and it was advanced that this might be due to the cumulative effect of the adverse characteristics of each of the constituents: blood-born metastases for the intestinal/glandular component and peritoneal dissemination with lymph node metastases for the diffuse/isolated-cell component [5]. Survival of patients with mixed carcinomas was shown to be significantly worse than that of patients with pure histological type tumours [6].

Analysis of *CDH1* mutations in a series of 26 gastric carcinomas (10 "pure" intestinal, 10 "pure" diffuse and 6 mixed carcinomas) showed that mutations were found in diffuse carcinomas and, within mixed carcinomas, were only detected in the diffuse component of the tumours, thus providing a genetic basis for the phenotypic divergence within mixed carcinomas [23].

Chromosomal instability is a hallmark of solid tumours [8]. In gastric cancer, DNA copy number changes constitute a major part of the genomic alterations observed, and aberrations that are consistently described by CGH are gains of chromosome 3q, 7p, 7q, 8q, 13q, 17q, 20p and 20q and losses 4q, 9p, 17p and 18q [29,11,41,17,49,12,47,28,18,34,45,20]. When comparing the copy number changes between the two histological types, some authors found differences [19,42,49] while others observed similar patterns of chromosomal copy number changes in both intestinal and diffuse carcinomas [24,18,41,47].

Although rare, mixed gastric carcinomas constitute an excellent model of nature to analyse within one tumour the pattern of copy number changes of two diverging histological types, i.e. intestinal and diffuse.

To the best of our knowledge, this is the first study that analyses DNA copy number changes in the two distinct components (intestinal and diffuse) of mixed gastric cancers by array CGH.

2. Materials and methods

2.1. Sample collection and DNA isolation

Twelve formaldehyde-fixed, paraffin-embedded gastric carcinoma tissue samples, classified as mixed carcinomas (containing distinct areas of intestinal and diffuse components) were collected. Nine cases originated from Hospital S. João, Porto, Portugal, two cases from the Dutch D1/D2 trial [3] and one case from the Academic Unit of Pathology, University Leeds, United Kingdom. All samples analysed showed metastasis in the lymph nodes at time of diagnosis and were therefore considered advanced tumours. From these cases, areas containing at least 70% tumour cells in each component were selected on a 4 μm haematoxylin and eosin stained tissue section, by a pathologist (G.A.M.). Adjacent serial sections of 10 μm were cut for DNA isolation and a final 4 μm haematoxylin and eosin stained section was made as control, to check whether there was still tumour in the marked area. After deparaffination with xylene, the areas corresponding to each component were carefully scratched from the slide with a scalpel blade. DNA was isolated as previously described [44] using a commercially available column-based method (QIAamp DNA isolation mini kit; Qiagen, Westburg, Leusden, The Netherlands). For very small tissue samples a microkit was used (QIAamp DNA isolation microkit; Qiagen, Hilden, Germany). DNA concentrations were determined using Nanodrop ND-1000 spectrophotometer (Isogen, IJsselstein, NL). DNA quality for array CGH was assessed by performing PCR for the human housekeeping gene β-globin using two primer sets that produce 209 bp (β3 forward primer acacaactgtgttcactagc and β5 reverse primer gaaacccaagagtcttctct) and 300 bp (β3 forward primer acacaactgtgttcactagc and β6 reverse primer catcaggagtggacagatcc) products. Of each archival sample, 50 ng DNA in a final volume of 5 μl was added to a PCR mixture containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 U *Taq* DNA polymerase (Applied Biosystems, Nieuwerkerk aan den IJssel, NL), 0.5 μM forward primer (β3) and 0.5 μM reverse primer (either β5 or β6). PCR reaction was performed for 40 cycles (1 minute at 94°C, 2 minutes at 58°C, 1 minute 30 seconds at 72°C) with an initial denaturation of 4 minutes at 94°C and a final extension 4 minutes at 72°C. Human DNA from placenta and water were used as positive and negative control, respectively. PCR products were visualized on a 2% agarose ethidium bromide-stained gel. This quality control is in accordance to what is published [38].

2.2. Array CGH

2.2.1. Array platform

We used a full-genome BAC array printed in the house containing approximately 5000 DNA clones

(http://www.vumc.nl/microarrays/index.html). The array comprised the Sanger 1 Mb clone set with an average resolution along the whole genome of 1.0 Mb (http://www.ensembl.org/Homo_sapiens/cytoview), the OncoBac set (http://informa.bio.caltech.edu/Bac_onc.html), containing approximately 600 clones corresponding to 200 cancer-related genes, and selected clones of interest obtained from the Children's Hospital Oakland Research Institute (CHORI) (http://bacpac.chori.org/home.htm), to fill any gaps larger than 1 Mb on chromosome 6 and to have full-coverage contigs of regions on chromosomes 8, 11, 13 and 20. Amplification of BAC clone DNA was done by ligation-mediated polymerase chain reaction (PCR) according to Snijders et al. [31]. All clones were printed in triplicate on Codelink™ slides (Amersham BioSciences, Roosendaal, NL) at a concentration of 1 μg/μl, in 150 mM sodium phosphate, pH 8.5, using a SpotArray72 printer (Perkin Elmer Life Sciences, Zaventem, BE). After printing, slides were processed according to the manufacturers protocol (Codelink™ slides; Amersham BioSciences, Roosendaal, NL).

2.2.2. *Labelling and hybridisation*

Array CGH was performed according to Snijders et al. [31], with a few modifications. Briefly, 300 ng of tumour and reference DNAs were labelled by random priming (Bioprime DNA Labeling System, Invitrogen, Breda, NL). Removal of unincorporated nucleotides was done with sephadex columns (ProbeQuant G-50 Micro Columns – Amersham BioSciences, Roosendaal, NL). Cy3 labelled test genomic DNA and Cy5 labelled reference DNA were combined and co- precipitated with 100 μg of human Cot-1 DNA (Invitrogen, Breda, NL) by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ice-cold 100% ethanol. The precipitate was collected by centrifugation at 14,000 rpm for 30 minutes at 4°C. After air-drying the pellet was dissolved in 130 μl hybridisation mixture containing 50% formamide, 2 × SCC, 10% dextran sulfate and 4% SDS. The DNA samples were denatured for 10 minutes at 73°C followed by a 60 minutes incubation at 37°C to allow the Cot-1 DNA to block repetitive sequences. The array was incubated for 38 h at 37°C with the denatured and blocked hybridisation mixture in a hybridisation station (HybArray12™ – Perkin Elmer Life Sciences, Zaventem, BE). After hybridisation, slides were washed in a solution containing 50% formamide, 2 × SCC, pH 7 for 3 minutes at 45°C, followed by 1 minute wash steps at room temperature with PN buffer (PN: 0.1 M sodium phosphate, 0.1% nonidet P40, pH 8), 0.2 × SSC, 0.1 × SCC and 0.01 × SCC. Slides were dried by centrifugation at 1000 rpm for 3 min at room temperature.

2.2.3. *Image acquisition, feature extraction and normalization*

Images of the arrays were acquired by scanning (Agilent DNA Microarray scanner – Agilent technologies, Palo Alto, USA) and Imagene 5.6 software (Biodiscovery Ltd, Marina del Rey, California) was used for automatic feature extraction (segmentation of the spots and quantification of the signal and background intensities for each spot for the two channels Cy3 and Cy5). A microsoft Excel sheet was used to subtract local background from the signal median intensities of both test and reference DNA and to calculate the tumour to reference ratios. Test to normal fluorescence ratios were normalized against the mode of the ratios of all autosomal clones. As the clones were spotted in triplicate, the median value of the corresponding three intensities was used for each clone in the array. If the standard deviation of the intensity of the three spots was greater than 0.2, clones were excluded from further analysis. Furthermore, clones with more than 20% missing values in all carcinomas were also excluded from further analysis.

All subsequent analyses were done considering the clone position from the UCSC May 2004 freeze of the Human Golden Path.

2.3. *Data analysis*

2.3.1. *DNA copy number segmentation*

To segment DNA copy number alterations (gains and/or losses), a smoothing algorithm – "aCGH-Smooth" was applied [14,15] (http://www.few.vu.nl/~vumarray/). Because there was variation of the level of noise between experiments, different cut-offs for calling gains and losses were used. To establish the most appropriate threshold in each experiment, the standard deviation (SD) computed in every case over an area without aberrations was used as input for a variable in the Array CGH Smooth (using default settings) that determines the cut off for gains and losses. Amplification was considered when the ratio (in a logarithmic scale) was above 1.0.

2.3.2. Statistical analysis

Statistical analysis comparing the intestinal and diffuse components from the same tumour was done using CGHMultiArray [40] adapted for paired analysis by using the Wilcoxon signed-rank test corrected for ties. A false discovery rate (FDR) of <0.1 was regarded as statistically significant. All the analyses were done excluding chromosomes X and Y, as in every hybridisation a sex-mismatched reference DNA was used for quality control of the experiment.

3. Results

We studied 12 gastric cancers of mixed histological type, by array CGH, analysing separately the two components (intestinal and diffuse). The mean number of aberrations observed per tumour was 10.22 (range 3–21). Overall, gains were more frequent than losses, on average the number of gains observed per tumour was 7.22 (1–16) compared to an average number of losses of 3.00 (0–12). Figure 1 gives an overview of the frequency of gains and losses in both intestinal and diffuse components of the mixed gastric cancers analysed. The most frequent aberrations (>20%; at least in 2 cases) observed in both components were gains on chromosomes 1, 6p, 7, 8, 10, 11, 12p, 13q, 16, 17q, 19q, 20 and 22q, and losses on chromosome 9p. We detected amplifications in four cases, on chromosome 8p, 8q, 12p, 15q, 17q and 20q (Table 1). On chromosomes 8p and 15q the amplified regions were large, spanning segments of approximately 7 and 11 Mb, respectively. Nevertheless, these regions include the genes *SOX7* and *CTSB* (*Cathepsin B*) on 8p and *IQGAP1* on 15q which are known to be overexpressed/amplified in gastric cancer. The amplification observed on 8q spans a region of 3.9 Mb and contains 7 genes (*TRMT12*, *RNF139*, *TATDN1*, *MTSS1*, *TRIB1*, *FAM84B and c-MYC*). The genes *K-RAS*, and *ERBB2* map to the amplified regions detected in 12p (RP11-707G18), and 17q (RP11-94L15), respectively. On chromosome 20q, two known cancer-related genes map in the amplified region, *ZNF217* and *CYP24A1*. Examples of amplifications can be seen in Figs 2A and B. In all amplicons, the amplified clones harbouring these genes were exactly the same in both histological components. As illustrated in Fig. 2B, both intestinal and diffuse components of case #1 have the same amplified clone on 17q (RP11-94L15), which harbours the oncogene *ERBB2*.

3.1. Comparison between the two components

When comparing the intestinal and diffuse components by means of CGH MultiArray for paired samples, we did not find any statistically significant difference between the intestinal and the diffuse component for every clone throughout the whole genome (Fig. 3). In addition, in order to identify and highlight any DNA copy number changes that differed between the two components, we calculated the ratio of the DNA copy number log 2 ratios from both components, diffuse *versus* intestinal [Log2 (ratio D1/I1) $=$ log 2 (ratio D1) $-$ log 2 (ratio I1)]. As expected these ratios were close to zero (Fig. 4), with the exception of a few cases with higher noise levels (Fig. 5).

4. Discussion

DNA copy number changes have been suggested to underlie some biological processes within tumours, such as metastisation [7,4,21] and acquisition of drug resistance [39]. We hypothesised that DNA copy changes might also underlie the phenotypic divergence observed in mixed gastric carcinomas. To address this hypothesis we studied 12 mixed gastric carcinomas by array CGH and analysed separately the distinct components (intestinal and diffuse) of each tumour.

In the present study, copy number changes were found on chromosomes 1, 6p, 7, 8, 10, 11, 12p, 13q, 16, 17q, 19q, 20 and 22q (gains) and 9p (losses). In 4 cases we found also amplifications, affecting chromosomes 8pq, 12p, 15q, 17q and 20q. Mapping to these regions there are genes which were described in literature to be amplified and/or overexpressed in gastric cancer [29,20,37,30,48,46,33,16,9]. These genes include: *SOX7* (8p23.1), *CTSB* (8p23.1), *C-MYC* (8q24.12-q24.13), *K-RAS* (12p12.1), *IQGAP1* (15q26.1), *ERBB2* (17q21.1), *ZNF217* (20q13.2) and *CYP24A1* (20q13.2). Although in some amplicons only one gene is present, being the obvious candidate to drive the amplicon, like *K-RAS* on 12p12.1, in other amplicons, where the region is larger, it is more difficult to pinpoint a single candidate responsible for driving the amplicon, like *SOX7* and/or *Cathepsin B* (*CTSB*) on 8p23.1. Also, the amplification on 8q harbours several known genes besides *c-MYC*, like *TRIB1* and *FAM84B*. Indeed in haematological malignancies as well as in oesophageal cancer some data exclude *c-MYC* as driving gene in 8q amplification in favour of either *TRIB1* [32] or *FAM84B* [13].

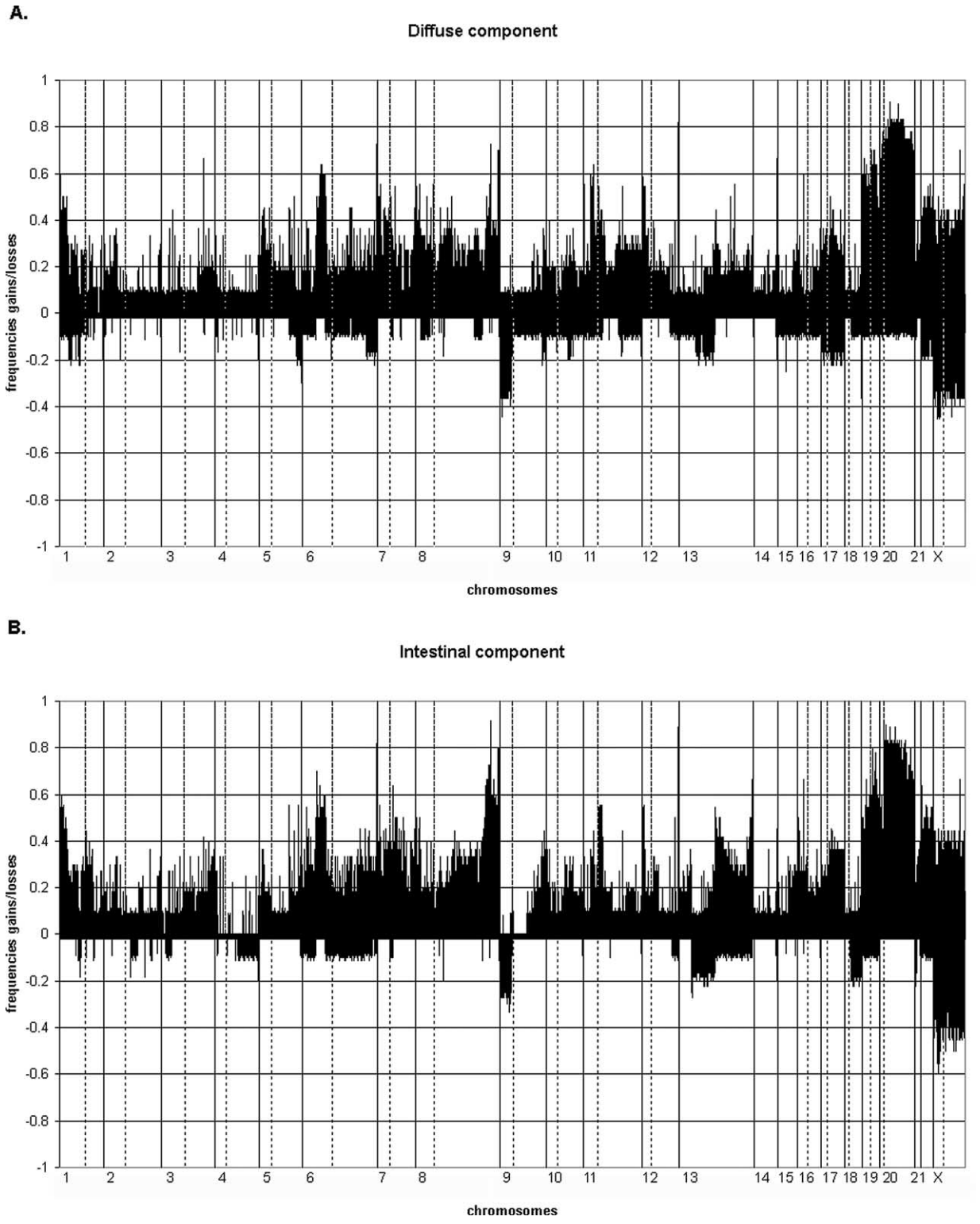

Fig. 1. Frequencies of gains and losses throughout the genome in all analysed tumours. (A) Diffuse-type component; (B) Intestinal-type component. Clones are sorted by position per chromosome (1-X). Vertical lines – transition between chromosomes; Dashed-vertical lines – centromere position.

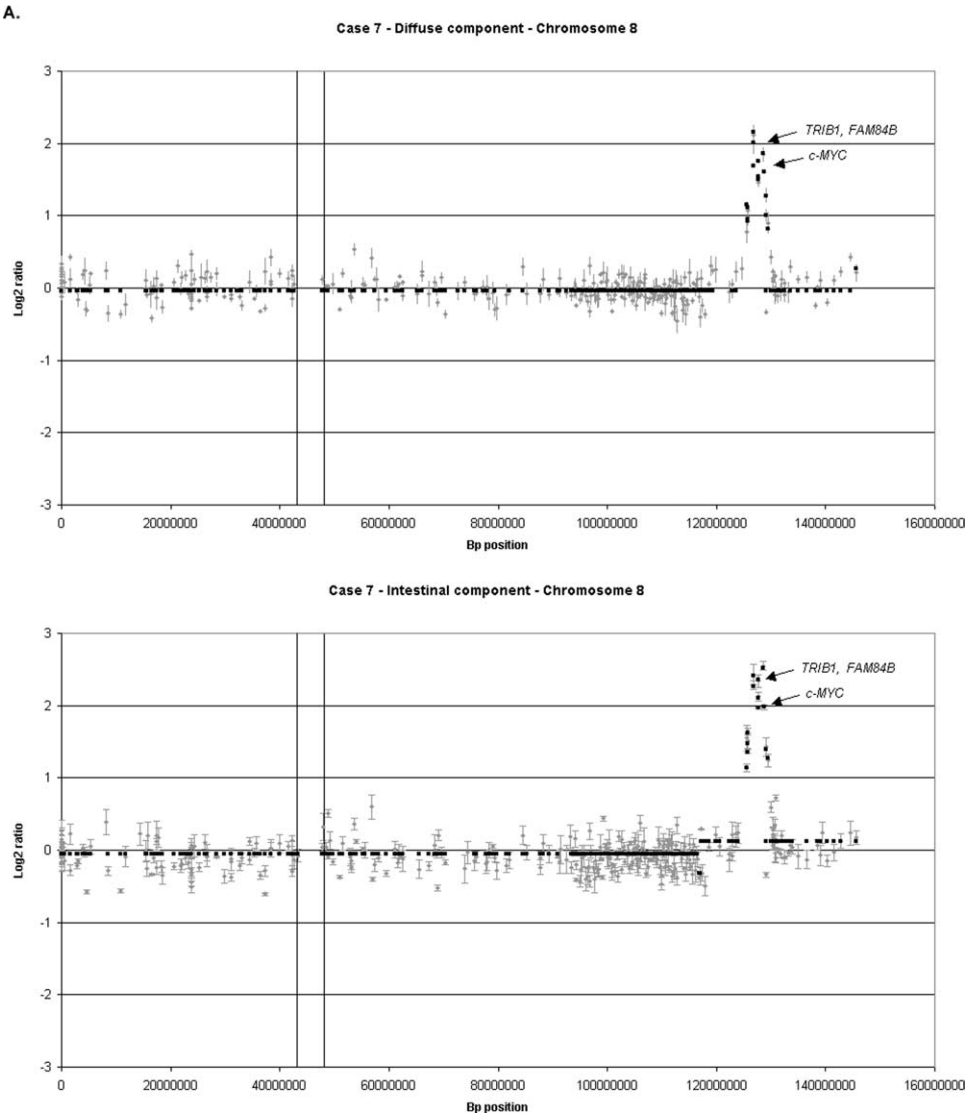

Fig. 2. Two cases with amplification of specific genomic regions. Case #7 present an amplification on chromosome 8q (A) and case #1 on chromosome 17q (B). Black squares-smoothed ratios. Two vertical lines – centromeric region.

Table 1
Mixed gastric carcinomas with one or more amplified regions

Case nr.	Chromosome	Mb location	Size amplicon (Mb)	Possible candidate genes[a]
1	17q	37.9–38.5	0.65	***ERBB2***
4	8p	4.3–11.7	7.4	*SOX7*, *CTSB*
7	8q	125.5–129.4	3.9	*TRIB1*, *FAM84B*, ***C-MYC***
	15q	88.1–99.9	11.8	***IQGAP1***
	20q	49.9–52.2	2.3	***ZNF217***, ***CYP24A1***
10	12p	25.3–25.7	0.4	***K-RAS***

[a]In bold, oncogenes known to be amplified in gastric cancer.

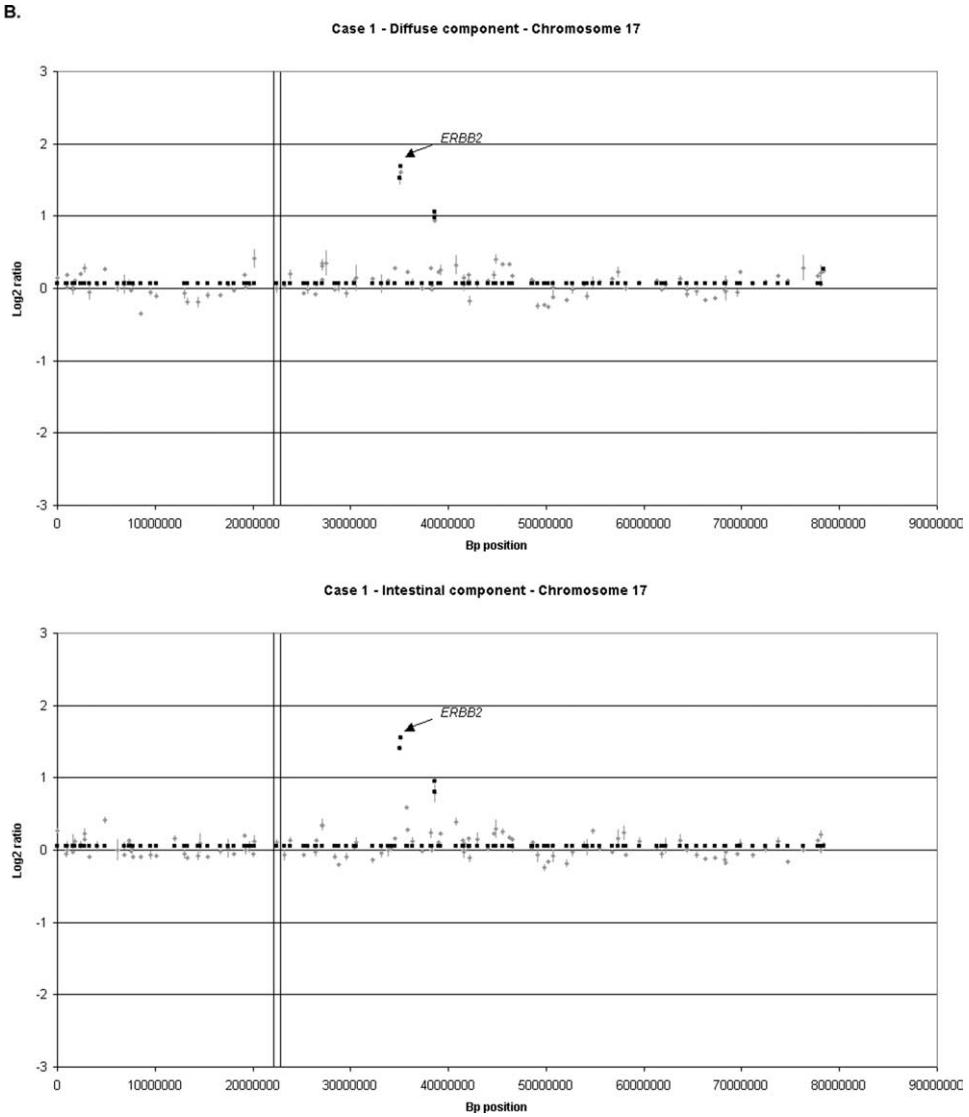

Fig. 2. (Continued).

No statistically significant differences were found between the intestinal and diffuse components within the same tumour samples. Based on the results we obtained we would expect that the ratio between the two components' ratios within the same tumour should be one, or zero in a logarithmic scale. In keeping with this hypothesis, when we subtracted the log 2 ratios we obtained a ratio close to zero. Our findings are in agreement with earlier observations in bladder tumours with mixed histology, analysed by chromosome CGH, in which a high level of concordance was found also for samples within one tumour with different histological components [36].

By CGH on 46 primary tumours, we did not find differences in copy number changes between pure intestinal and diffuse carcinomas [41]. Our results within mixed gastric carcinomas are in keeping with these findings. These observations suggest that the mechanism underlying the intestinal and diffuse histotypes of gastric cancer (within mixed or pure carcinomas) is not caused by DNA copy number changes but by other biological events, like mutation and/or promoter hypermethylation, not detectable by array CGH.

In some cases, small quantitative differences were observed in some chromosomal regions, specifically on chromosomes 8 and 20. In those cases the genomic profile of both components was similar but the aber-

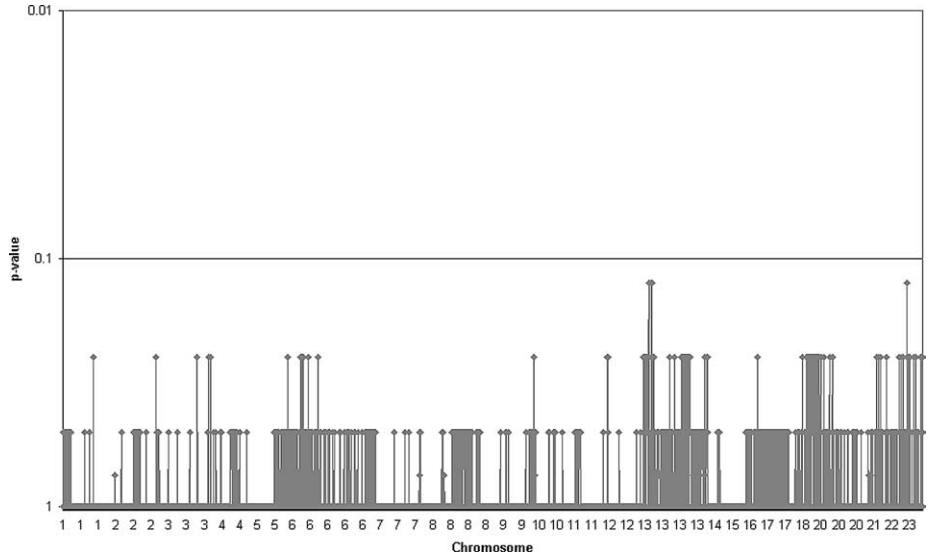

Fig. 3. Graphic view of the p-values obtained in the paired analysis where the two components (intestinal and diffuse) were compared in each tumour.

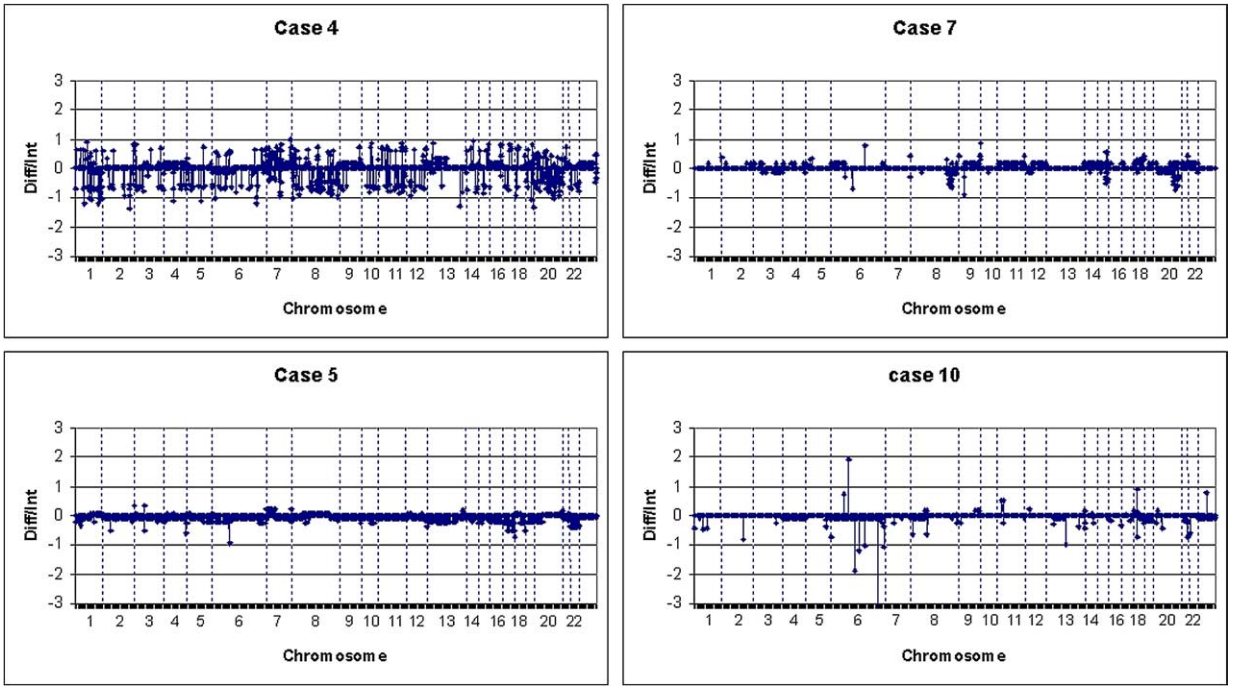

Fig. 4. Examples of ratios between diffuse and intestinal components within the same tumour (Log2 ratio Diffuse – Log2 ratio Intestinal). Dashed-vertical lines – transition between chromosomes.

rations in the intestinal component were more pronounced (higher ratios in the gained regions and lower ratios in the lost regions) when compared to the diffuse component (Figs 2A and 5B). Most probably, these differences are due to the higher amount of stromal cells in the diffuse component lowering the ratio tumour/normal reference.

Although we are assuming that the copy number changes observed are cancer specific, we can not exclude the possibility that some of these changes might

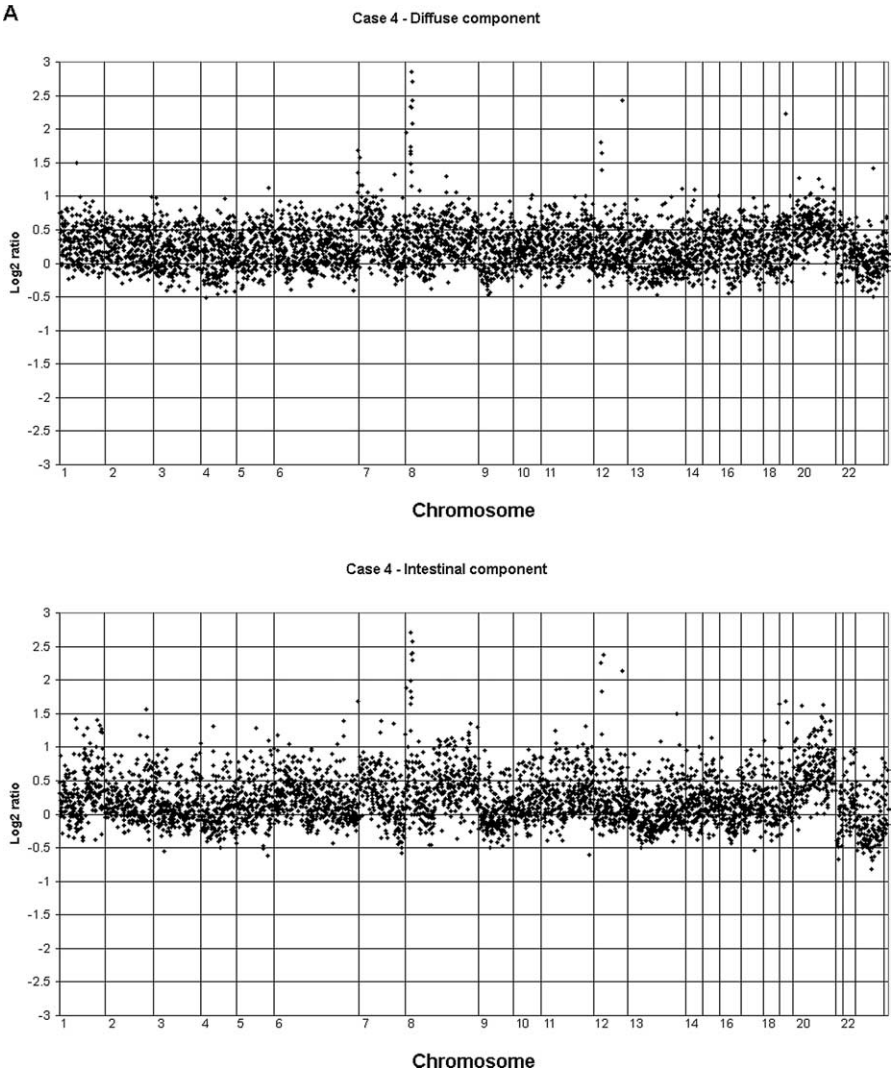

Fig. 5. DNA copy number changes profiles of two mixed gastric cancers, one with high level of noise, (A) case #4, and one with low level of noise, (B) case #7; intestinal and diffuse components analysed separately. Vertical lines – transition between chromosomes.

represent polymorphisms, which to some extent could contribute to similarities in the patterns of chromosomal aberrations seen in matched samples (i.e. the intestinal and diffuse components within a single tumour).

Some of the gains and losses observed in this study were detected in regions which are consistently altered in gastric cancer, such as losses on 9p, gains on 7, 8q, 13q, 17q and 20 [34,49,18,41,20,29,11,17,28,12, 45]. Other changes, such as gains on 1p, 6p, 11, 12p, 16, 19q and 22q, which were frequently detected in our study are also described in literature, although less frequently [49,34,24,35,27]. However, other chromosomal aberrations like 18q loss, that frequently occur in gastric carcinoma, were very rare or absent in this series of mixed gastric carcinomas [29,12]. This could be explained by the small sample size, that is a consequence of the low incidence of these lesions, although we can not rule out that mixed gastric carcinomas may show different patterns of chromosome aberrations compared to the pure histological types.

Kokkola and collaborators [19] detected amplifications of 17q only in (pure) intestinal-type gastric carcinomas. In our series of mixed gastric carcinomas we found in one case that both components harboured 17q amplification, with the same clone being involved in both components (Fig. 2B). This finding suggests a clonal origin for mixed gastric carcinomas (or at least

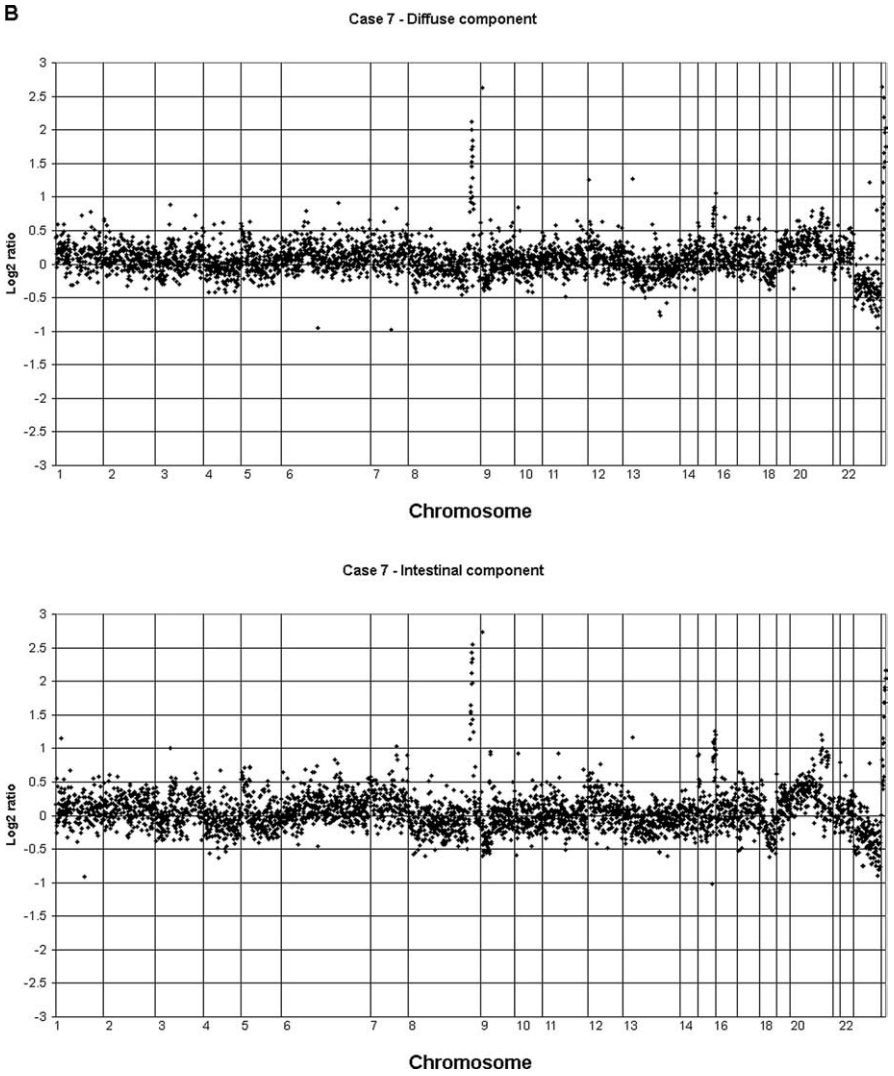

Fig. 5. (Continued).

for this tumour). According to Machado and collaborators [23], mutations of *CDH1* gene constitute the genetic basis for the phenotypic divergence of mixed gastric carcinomas. Our results suggest that after this event, no substantial DNA copy number changes take place.

In summary, in the analysis of 12 mixed gastric carcinomas by array CGH, we found the copy number changes that have been consistently reported in the literature in gastric carcinomas and, within each tumour, we found similar profiles in both components. Our results support the idea that mixed gastric carcinomas are clonal and the phenotypic divergence is not caused by chromosomal aberrations.

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