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ORIGINAL ARTICLE

High resolution analysis of DNA copy-number aberrations of chromosomes 8, 13, and 20 in gastric cancers

Tineke E. Buffart · Nicole C. T. van Grieken · Marianne Tijssen · Jordy Coffa · Bauke Ylstra · Heike I. Grabsch · Cornelis J. H. van de Velde · Beatriz Carvalho · Gerrit A. Meijer

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Abstract DNA copy-number gains of chromosomes 8q, 13q, and 20q are frequently observed in gastric cancers. Moreover gain of chromosome 20q has been associated with lymph node metastasis. The aim of this study was to correlate DNA copy-number changes of individual genes on chromosomes 8q, 13q, and 20q in gastric adenocarcinomas to clinicopathological data. DNA isolated from 63 formalin-fixed and paraffin-embedded gastric adenocarcinoma tissue samples was analyzed by whole-genome microarray comparative genomic hybridization and by multiplex ligation-dependent probe amplification (MLPA), targeting 58 individual genes on chromosomes 8, 13, and 20. Using array comparative genomic hybridization, gains on 8q, 13q, and 20q were observed in 49 (77.8%), 25 (39.7%), and 49 (77.8%) gastric adenocarcinomas, respectively. Gain of chromosome 20q was significantly correlated with lymph node metastases (p=0.05) and histological type (p=0.02). MLPA revealed several genes to be frequently gained in DNA copy number. The oncogene c-*myc* on 8q was gained in 73% of the cancers, while *FOXO1A* and *ATP7B* on 13q were both gained in 28.6% of the cases. Multiple genes on chromosome 20q showed gains in more than 60% of the cancers. DNA copy-number gains of *TNFRSF6B* (20q13.3) and *ZNF217* (20q13.2) were significantly associated with lymph node metastasis (p=0.02) and histological type (p=0.02), respectively. In summary, gains of chromosomes 8q, 13q, and 20q in gastric adenocarcinomas harbor DNA copy-number gains of known and putative oncogenes. *ZNF217* and *TNFRSF6B* are associated with important clinicopathological variables, including lymph node status.

Keywords DNA copy-number · Gastric cancer · Lymph node status · MLPA

T. E. Buffart · N. C. T. van Grieken · M. Tijssen · J. Coffa · B. Ylstra · B. Carvalho · G. A. Meijer (✉)
Department of Pathology, VU University Medical Center, PO Box 7057, 1007 Amsterdam, The Netherlands
e-mail: ga.meijer@vumc.nl

J. Coffa
MRC-Holland, Amsterdam, The Netherlands

H. I. Grabsch
Pathology and Tumour Biology, Leeds Institute of Molecular Medicine, University of Leeds, Leeds, UK

C. J. H. van de Velde
Department of Surgery, Leiden University Medical Center, Leiden, The Netherlands

Introduction

Gastric cancer is a major health problem and ranks second as a cause of cancer death worldwide [1]. The only possible curative treatment is complete surgical resection. One of the hallmarks of solid cancers, including gastric cancer, is chromosomal instability leading to gains and losses of parts of, or even whole chromosomes [2]. The mechanisms underlying this instability phenotype in gastric cancers are still poorly understood. Patterns of DNA copy-number aberrations can be analyzed at high resolution by array comparative genomic hybridization (CGH). Gains of chromosomes 3q, 7p, 7q, 8q, 13q, 17q, and 20q and losses of chromosomes 4q, 5q, 6q, 9p, 17p, and 18q have been consistently described in gastric cancer studies using CGH or array CGH analysis [3–11]. In addition, gains of chromosomes 7q, 8q, 9q, 11q, 13q, and 20q and losses of chromosomes 4p, 5q, 6, 9p, 17p, and 18q can already be

detected in gastric cancer precursor lesions with direct malignant potential, indicating these as early events in the pathogenesis of gastric cancer [12–16]. Results from array CGH studies have shown that patterns of chromosomal aberrations can be correlated with clinicopathological variables. In previous studies, we have shown different patterns of chromosomal instability to correlate with lymph node status and with age of onset of the disease [17, 18].

DNA copy-number gains of chromosomes 8, 13, and 20 have been described to play an important role in colorectal adenoma to carcinoma progression [19, 20]. Also in gastric adenomas and adenocarcinomas, gains of these chromosomes have frequently been detected, indicating the importance of genetic events on these chromosomes for gastric cancer pathogenesis. Moreover, gain of chromosome 20q has been described to correlate with lymph node metastasis in gastric cancer and poor clinical outcome in colorectal cancer [3, 21, 22]. The most widely used array CGH platforms, so far, use spotted bacterial artificial chromosomes (BACs), which means that the probe sequences on the arrays cover up to 1 kb of DNA. This approach is very suitable for detecting patterns of DNA copy-number changes but has limitations in pinpointing individual genes whose normal function is disrupted due to these chromosomal aberrations. Multiplex ligation-dependent probe amplification (MLPA) [23] allows to determine in a single experiment DNA copy-number ratios of up to 40 individual genes and can be used to zoom in on chromosomal areas that show aberrations with array CGH [24, 25]. The aim of the present study is a detailed analysis of DNA copy-number changes of individual genes within gained areas on chromosomes 8, 13, and 20 in gastric adenocarcinomas and to correlate gene specific alterations to clinicopathological data.

Materials and methods

Materials and DNA isolation

DNA of 63 formalin-fixed and paraffin-embedded (FFPE) gastric adenocarcinomas of which 53 were obtained from Leeds (Leeds University Hospital, UK) and 10 were obtained from the Dutch D1/D2 trial [26], was isolated as previously described [17] using a commercial available DNA isolation kit (QIAmp DNA microkit, Qiagen, Hilden, Germany). Briefly, areas of at least 70% tumor cells were demarcated on a 4-μm haematoxylin- and eosin-stained tissue section. Adjacent serial sections of 10 μm were cut, and after deparaffination, the tumor tissue was macrodissected using a needle. After an overnight incubation with sodium thiocyanate (1 M) at 37°C, followed by proteinase K treatment, DNA was extracted. DNA concentrations were measured on a Nanodrop ND-1000 spectrophotometer (Isogen, IJsselstein, The Netherlands), and DNA quality was assessed by isothermal amplification as previously described [27]. Only DNA of excellent, good, and intermediate quality was used for further analysis.

Table 1 An overview of DNA copy number gains of chromosomes 8q, 13q and 20q detected by array CGH in 63 gastric adenocarcinomas, and the correlation to lymph node status and histological type of the tumor

Chromosome	Status	All cases	Lymph node status		*P* value	Histological type			*P* value
			LN0	LN1		Intestinal	Diffuse	Mixed	
		n=63	*n*=22	*n*=41		*n*=38	*n*=17	*n*=8	
8q	Gain	49 (77.8%)	18 (81.8%)	31 (75.6%)	0.57	29 (76.3%)	13 (76.5%)	7 (87.5%)	0.78
	No gain	14 (22.2%)	4 (18.2%)	10 (24.4%)		9 (23.7%)	4 (23.5%)	1 (12.5%)	
13q	Gain	25 (39.7%)	10 (45.5%)	15 (36.6%)	0.49	19 (50.0%)	4 (23.5%)	2 (25.0%)	0.12
	No gain	38 (60.3%)	12 (54.5%)	26 (63.4%)		19 (50.0%)	13 (76.5%)	6 (75.0%)	
20q	Gain	49 (77.8%)	14 (63.6%)	35 (85.4%)	**0.048**	33 (86.8%)	9 (52.9%)	7 (87.5%)	**0.016**
	No gain	14 (22.2%)	8 (36.4%)	6 (14.6%)		5 (13.2%)	8 (47.1%)	1 (12.5%)	
8q+13q+20q	Gain	17 (27.0%)	6 (27.2%)	11 (26.8%)	0.97	13 (34.2%)	2 (11.8%)	2 (25.0%)	0.22
	No gain	46 (73.0%)	16 (72.7%)	30 (73.2)		25 (65.8%)	15 (88.2%)	6 (75.0%)	
8q+13q	Gain	4 (6.3%)	3 (13.6%)	1 (2.4%)	0.082	2 (5.3%)	2 (11.8%)	0 (0.0%)	0.48
	No gain	59 (93.7%)	19 (86.4%)	40 (97.6%)		36 (94.7%)	15 (88.2%)	8 (100.0%)	
8q+20q	Gain	24 (38.1%)	8 (36.4%)	16 (39.0%)	0.84	14 (36.8%)	6 (35.3%)	4 (50.0%)	0.76
	No gain	39 (61.9%)	14 (63.6%)	25 (61.0%)		24 (63.2%)	11 (64.7%)	4 (50.0%)	
13q+20q	Gain	3 (4.8%)	0 (0.0%)	3 (7.3%)	0.19	3 (7.9%)	0 (0.0%)	0 (0.0%)	0.36
	No gain	60 (95.2%)	22 (100.0%)	38 (92.7%)		35 (92.1%)	17 (100.0%)	8 (100.0%)	

Gain of 20q is significantly correlated to lymph node status and histological tumor type (in *bold*)

LN0 lymph node-negative, *LN1* lymph node-positive

DNA labeling and array CGH)

DNA labeling and array CGH were essentially performed as previously described [17, 28]. In short, tumor and normal DNA were differentially labeled using random priming (Bioprime DNA Labeling System; Invitrogen, Breda, The Netherlands) and hybridized on a BAC array containing approximately 5,000 clones printed in triplicate, consisting of the Sanger BAC 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) 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. All clones were printed on Codelink™ slides (Amersham BioSciences, Roosendaal, The Netherlands) at a concentration of 1 μg/μl in 150-mM sodium phosphate, pH 8.5, using an OmniGrid 100 microarrayer (Genomic Solutions, Ann Arbor, MI, USA) equipped with SMP3 pins (TeleChem International, Sunnyvale, CA, USA), and processed according to the manufacturer's protocol.

After hybridization, images of the arrays were acquired by scanning (Microarray scanner G2505B; Agilent technologies, Palo Alto, USA), and spot analysis and quality control were automatically performed using BlueFuse 3.4 software (BlueGnome, Cambridge, UK). When the Blue-

Table 2 Overview of patient and tumor characteristics of the 63 gastric adenocarcinomas analyzed in the study

Tumor ID	Gender	Age	Histological type	T status	N status	Tumor ID	Gender	Age	Histological type	T status	N status
1	Female	78	Intestinal	T3	N1	33	Male	75	Intestinal	T2	N3
2	Female	81	Diffuse	T3	N1	34	Male	61	Intestinal	T3	N3
3	Female	54	Intestinal	T2	N0	35	Female	82	Diffuse	T3	N1
4	Male	73	Intestinal	T3	N1	36	Male	74	Mixed	T2	N1
5	Male	81	Mixed	T2	N2	37	Female	87	Diffuse	T3	N2
6	Male	58	Intestinal	T2	N1	38	Female	78	Intestinal	T1	N0
7	Female	72	Intestinal	T2	N0	39	Male	78	Diffuse	T2	N0
8	Male	86	Intestinal	T2	N2	40	Female	84	Intestinal	T2	N0
9	male	71	Intestinal	T3	N0	41	Male	85	Diffuse	T3	N2
10	Female	75	Intestinal	T4	N1	42	Male	62	Diffuse	T3	N3
11	Female	75	Intestinal	T3	N2	43	Male	68	Intestinal	T3	N1
12	Male	64	Mixed	T3	N1	44	Male	58	Intestinal	T4	N1
13	Male	81	Intestinal	T2	N2	45	Male	61	Diffuse	T3	N1
14	Male	63	Intestinal	T2	N0	46	Female	58	Intestinal	T3	N1
15	Male	63	Intestinal	T3	N1	47	Male	81	Intestinal	T3	N1
16	Male	68	Mixed	T2	N0	48	Female	65	Intestinal	T2	N1
17	Female	72	Intestinal	T2	N1	49	Female	65	Intestinal	T2	N1
18	Male	73	Intestinal	T2	N2	50	Male	82	Intestinal	T1	N0
19	Male	71	Intestinal	T3	N2	51	Female	74	Mixed	T1	N0
20	Female	67	Intestinal	T2	N0	52	Male	72	Diffuse	T3	N2
21	Male	67	Diffuse	T3	N1	53	Female	60	Diffuse	T3	N0
22	Female	64	Intestinal	T3	N2	54	Male	47	Diffuse	T2	N0
23	Female	72	Diffuse	T3	N2	55	Female	74	Diffuse	T2	N0
24	Male	74	Intestinal	T2	N1	56	Male	65	Mixed	T2	N2
25	Male	58	Intestinal	T3	N3	57	Female	49	Intestinal	T1	N0
26	Male	71	Intestinal	T1	N0	58	Male	74	Intestinal	T1	N0
27	Female	68	Mixed	T3	N1	59	Male	58	Intestinal	T1	N0
28	Male	74	Intestinal	T2	N0	60	Male	53	Diffuse	T2	N0
29	Male	57	Mixed	T3	N1	61	Male	61	Diffuse	T2	N1
30	Male	69	Diffuse	T3	N1	62	Male	69	Diffuse	T2	N1
31	Male	67	Intestinal	T2	N0	63	Male	56	Intestinal	T2	N0
32	Female	71	Intestinal	T2	N1						

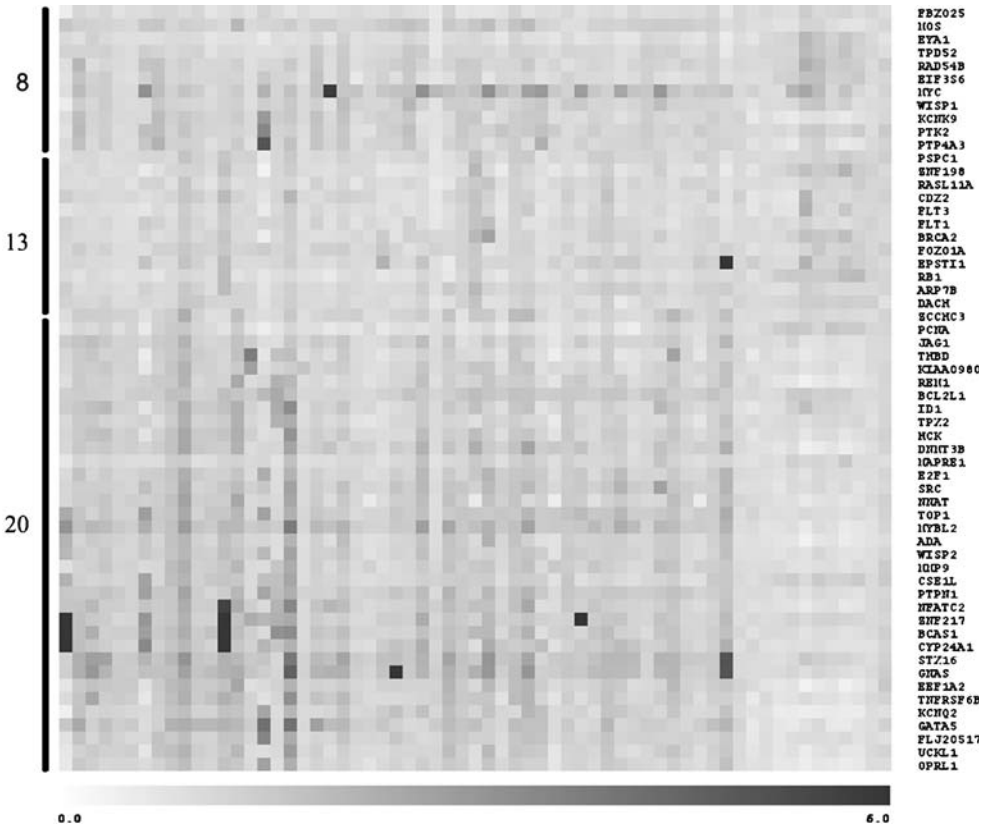

Fig. 1 Heatmap of DNA copy number ratios of 11 genes on chromosome 8, 12 genes on chromosome 13, and 35 genes on chromosome 20. The *columns* represent different gastric adenocarcinomas, and the *rows* represent the different genes. *Darker squares* indicate higher DNA copy number ratios

Fuse quality flag was below one or the confidence value was below 0.1, spots were excluded from further analysis. The $\log_2$ tumor to normal fluorescence ratio was calculated for each spot and normalized against the mode of the ratios of all autosomes. The package CGH call was used for data segmentation and defining copy-number gains and losses of each clone in the array CGH profile [29].

Multiplex ligation-dependent probe amplification

MLPA was performed, as previously described, [24] using two different probe mixes. One probemix contained 38 probes representing 31 different genes on chromosome 20 and 10 control probes located on chromosomes 2, 3, 4, 5, 12, and 16. The second probemix contained 11 probes on chromosome 8, 12 probes on chromosome 13, 16 probes on chromosome 20 representing 14 different genes, and 8 control probes located on chromosomes 2, 4, 12, and 16. Some genes on chromosome 20 are present in both probe mixes, leaving 35 different genes on chromosome 20 by combining these two probe mixes. DNA of the cell line HT29, showing a gain on chromosomes 8, 13, and 20, was used as positive control. A human pool of DNA isolated from blood of 36 healthy individuals and a pool of DNA isolated of 30 normal gastric and colon mucosa, spleen, liver, and kidney tissue samples (FFPE), were used as normal controls.

Of each sample, approximately 100 ng of DNA in a volume of 5 µl was denaturated at 98°C for 5 min. A mixture of 1.5-µl salsa probes (1–4 fmol of each short synthetic probe oligonucleotide and each phage M13-derived long probe oligonucleotide in TE (10 mM Tris-HCl, pH 8.2, 1 mM ethylenediamine tetraacetic acid (EDTA))) and 1.5 µl of MLPA buffer (1.5 M KCl, 300 mM Tris-HCl, pH 8.5, 1 mM EDTA) was added. The mixture was heated for 1 min at 95°C followed by 16 h of incubation at 60°C to allow the MLPA hemipobes to hybridize. Next, 32 µl of ligase-65 mixture (dilution buffer containing 2.6 mM $MgCl_2$, 5 mM Tris-HCl, pH 8.5, 0.013% non-ionic detergents, 0.2 mM of nicotinamide adenine dinucleotide (NAD), and 1 U of ligase-65 enzyme) was added to each sample for ligation of hybridized hemiprobes during a 10–15 min of incubation at 54°C, followed by a 5 min of incubation at 98°C to inactivate the ligase.

Polymerase chain reaction (PCR) was performed with 10 µl of polymerase mixture containing the PCR primers (10 pmol), dNTPs (2.5 nmol), and 2.5 U *Taq* polymerase (promega), 4 µl of PCR buffer (2.6 mM $MgCl_2$, 5 mM Tris-HCl, pH 8.5, 0.013% non-ionic detergents, 0.2 mM NAD), 26 µl of water and 10 µl of MLPA ligation reaction.

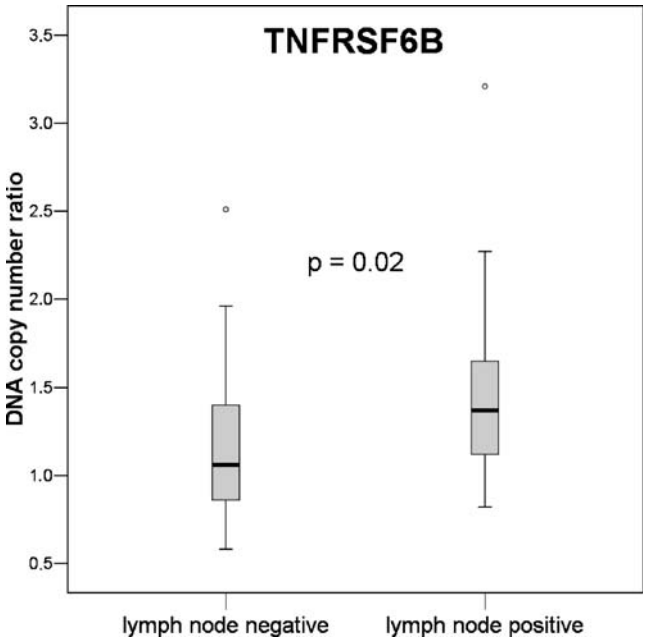

Fig. 2 Box plot of DNA copy number ratios of the gene *TNFRFS6B* between lymph node-negative and lymph node-positive gastric adenocarcinomas. Lymph node-positive gastric adenocarcinomas have significantly higher DNA copy number ratios of *TNFRFS6B* compared with lymph node-negative gastric adenocarcinomas (p=0.02). The *central box* covers the middle 50% of the data values between the upper and lower quartiles. The *line* across the box indicates the median. The *whiskers* extend from the box to the minimum and maximum values with the exception of outliers, which are marked by *circles*

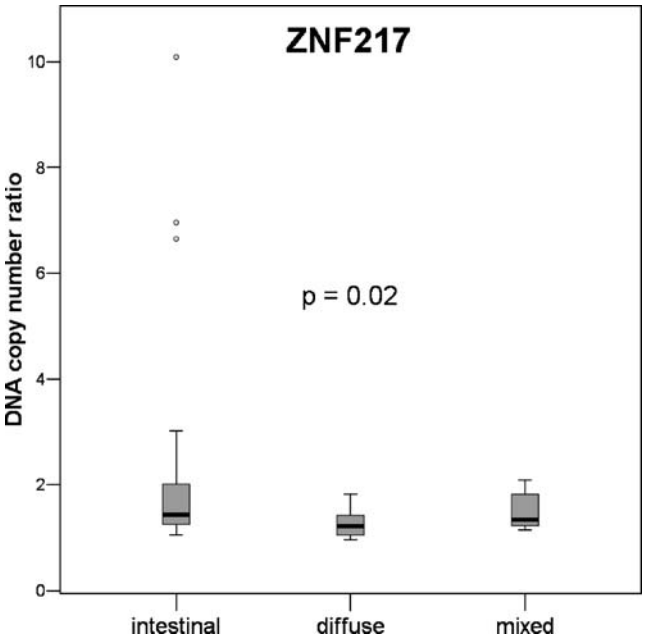

Fig. 3 Box plot of DNA copy number ratios of the gene *ZNF217* between intestinal-, diffuse-, and mixed-type gastric adenocarcinomas. DNA copy number ratios of *ZNF217* are significantly different between gastric adenocarcinomas of different histological types (p=0.02)

Multiplex ligation-dependent probe amplification data analysis

Analysis of the MLPA PCR products for each gene was performed on an ABI 3100 capillary sequencer (Applied Biosystems, Warrington, UK) in a mixture of 8.5 µl of deionized formamide (Applied Biosystems, Warrington, UK), 1 µl of PCR product and 0.5 µl marker including a ROX-labeled internal size standard (ROX-500 Genescan; Applied Biosystems, Warrington, UK). Data analysis was performed using the MLPAnalyzer version 8.0 (http://www.mlpa.com/coffalyser/) [30]. For each tumor, peak heights for every probe were derived from the ABI output and median peak heights of at least two different ligation reactions and three different PCR reactions were calculated. For each sample, tumor to normal DNA copy-number ratios was calculated per probe by dividing the median peak heights in the tumor tissue by the median peak heights in the reference DNA. All ratios were normalized by setting the median of the tumor to reference DNA copy-number ratios of the control genes in the probe mixture to 1.0. When multiple probes were present for one gene, the mean value of the probes was calculated and used for further analysis. Tumor to normal ratios below 0.7 and above 1.3 was considered as a loss or gain, respectively. TMEV software 3.1 (http://www.tigr.org/) was used to present descriptive data.

Statistical analysis

Box and scatter plots were used to present descriptive statistics. Chi-square test was used to evaluate associations of DNA copy-number gain of chromosomes 8q, 13q, and 20q with clinicopathological variables. t Test was used to evaluate differences in DNA copy-number aberrations and age of the patients. Mann-Whitney U test and Kruskal-Wallis H test were used to evaluate differences in DNA copy-number changes of each gene between lymph node status and histological tumor type according to the Laurén classification [31], respectively. Correlation coefficients between the $\log_2$ ratios for the array CGH and MLPA analysis were obtained by Spearman correlation (SPSS 12.0.1 for Windows; SPSS Inc. Chicago, IL, USA). A threshold of 0.05 for significance was used.

Results

Array CGH analysis and correlation with lymph node status and histological type

Of the 63 gastric adenocarcinomas analyzed by array CGH, 49 (77.8%) showed gains on chromosome 8, 25 (39.7%)

Table 3 A detailed overview of the frequencies of DNA copy number gains per gene, determined by MLPA analysis and mean DNA copy number ratio and range, per tumor group, i.e., lymph node-positive versus lymph node-negative gastric adenocarcinomas and intestinal- versus diffuse- versus mixed-type gastric carcinomas

Gene	Location	Gains	LN-negative	LN-positive	*p* value	Intestinal	Diffuse	Mixed	*p* value
FBXO25	8p23.3	1.6%	1.04 (0.68–1.46)	0.97 (0.48–1.26)	NS	0.98 (0.48–1.46)	1.08 (0.81–1.26)	0.87 (0.63–1.29)	0.01
MOS	8q11	39.7%	1.20 (0.78–2.02)	1.31 (0.73–1.96)	NS	1.34 (0.92–2.02)	1.15 (0.77–1.71)	1.23 (0.73–1.96)	NS
EYA1	8q13.3	9.5%	1.08 (0.55–2.07)	0.96 (0.65–1.35)	NS	1.01 (0.66–2.07)	1.05 (0.75–1.64)	0.86 (0.55–1.25)	NS
TPD52	8q21	25.4%	1.26 (0.67–1.98)	1.15 (0.68–1.59)	NS	1.20 (0.90–1.98)	1.22 (0.96–1.74)	1.04 (0.67–1.59)	NS
RAD54B	8q21.3	34.9%	1.33 (0.88–2.29)	1.22 (0.90–2.06)	NS	1.30 (0.90–2.29)	1.20 (0.88–2.06)	1.19 (0.90–1.59)	NS
EIF3S6	8q22	28.6%	1.27 (0.85–1.82)	1.12 (0.64–1.74)	0.04	1.19 (0.74–2.82)	1.13 (0.64–1.70)	1.18 (0.76–1.59)	NS
MYC	8q24.12	73.0%	1.75 (0.95–3.22)	1.76 (0.90–5.81)	NS	1.74 (0.94–3.22)	1.74 (0.90–5.81)	1.84 (1.03–2.74)	NS
WISP1	8q24	36.5%	1.26 (0.79–1.68)	1.26 (0.51–2.08)	NS	1.28 (0.51–1.88)	1.20 (0.79–1.64)	1.27 (0.79–2.08)	NS
KCNK9	8q24.3	34.9%	1.16 (0.59–2.92)	1.24 (0.58–1.90)	NS	1.22 (0.77–1.90	1.11 (0.59–1.87)	1.37 (0.58–2.92)	NS
PTK2	8q24	47.6%	1.40 (0.96–3.47)	1.29 (0.83–1.97)	NS	1.33 (0.83–1.86)	1.25 (0.91–1.97)	1.51 (0.63–5.02)	NS
PTP4A3	8q24.3	33.3%	1.37 (0.62–5.02)	1.23 (0.63–2.24)	NS	1.27 (0.86–2.20)	1.18 (0.62–2.24)	1.55 (0.63–5.02)	NS
PSPC1	13q11	22.2%	1.12 (0.65–1.48)	1.18 (0.71–1.75)	NS	1.20 (0.89–1.75)	1.15 (0.82–1.48)	1.01 (0.65–1.51)	NS
ZNF198	13q11	20.6%	1.05 (0.38–2.17)	1.08 (0.55–2.04)	NS	1.08 (0.55–2.04)	1.15 (0.74–2.17)	0.88 (0.38–1.41)	NS
RASL11A	13q12.2	11.1%	1.09 (0.87–1.35)	1.10 (0.61–1.66)	NS	1.15 (0.84–1.66)	1.08 (0.75–1.52)	0.93 (0.61–1.15)	0.03
CDX2	13q12	22.2%	1.09 (0.70–2.11)	1.19 (0.67–2.09)	NS	1.21 (0.76–2.11)	1.14 (0.70–1.73)	0.91 (0.67–1.30)	0.03
FLT3	13q12	12.7%	1.04 (0.56–2.23)	1.05 (0.65–1.70)	NS	1.08 (0.69–2.23)	1.05 (0.71–1.55)	0.87 (0.56–1.51)	NS
FLT1	13q12.2	20.6%	1.10 (0.85–1.42)	1.13 (0.67–1.85)	NS	1.17 (0.77–1.85)	1.09 (0.82–1.48)	0.98 (0.67–1.34)	NS
BRCA2	13q12.3	22.2%	1.15 (0.53–1.85)	1.22 (0.91–2.62)	NS	1.23 (0.83–2.62)	1.17 (0.93–1.85)	1.08 (0.53–1.31)	NS
FOXO1A	13q14.1	28.6%	1.18 (0.80–1.58)	1.19 (0.79–1.59)	NS	1.20 (0.79–1.59)	1.15 (0.80–1.45)	1.18 (0.89–1.46)	NS
EPSTI1	13q14.11	17.5%	1.51 (1.02–6.32)	1.21 (0.62–2.20)	NS	1.30 (0.94–2.20)	1.17 (0.62–1.68)	1.70 (0.90–6.32)	NS
RB1	13q14.2	14.3%	1.12 (0.72–1.87)	1.04 (0.60–1.97)	NS	1.06 (0.69–1.89)	1.13 (0.60–1.97	1.01 (0.72–1.55)	NS
ATP7B	13q14.2	28.6%	1.15 (0.77–1.45)	1.21 (0.90–1.99)	NS	1.24 (0.91–1.99)	1.14 (0.86–1.53)	1.05 (0.77–1.42)	NS
DACH	13q21.32	20.6%	1.13 (0.82–1.42)	1.13 (0.62–1.72)	NS	1.15 (0.62–1.72)	1.15 (0.88–1.42)	1.05 (0.71–1.36)	NS
ZCCHC3	20p13–p12.2	38.1%	1.19 (0.81–1.82)	1.28 (0.94–2.29)	NS	1.26 (0.81–2.29)	1.21 (0.91–1.92)	1.27 (0.94–1.96)	NS
PCNA	20pter–p12	17.5%	1.09 (0.48–1.65)	1.01 (0.45–1.61)	NS	1.02 (0.45–1.65)	1.06 (0.64–1.50)	1.06 (0.48–1.61)	NS
JAG1	20p12.1–p11.23	42.9%	1.24 (0.69–2.05)	1.29 (0.85–2.13)	NS	1.31 (0.69–2.13)	1.17 (0.85–1.68)	1.31 (0.97–2.05)	NS
THBD	20p12–cen	31.7%	1.04 (0.48–1.79)	1.26 (0.71–3.81)	NS	1.25 (0.48–3.81)	1.07 (0.60–1.66)	1.10 (0.74–1.34)	NS
KIAA0980	20p11.22–p11.1	42.9%	1.17 (0.70–1.76)	1.31 (0.84–2.73)	NS	1.32 (0.70–2.73)	1.12 (0.77–1.55)	1.29 (0.92–1.76)	NS
REM1	20q11.21	34.9%	1.19 (0.42–2.43)	1.24 (0.75–2.21)	NS	1.29 (0.42–2.43)	1.08 (0.60–1.51)	1.24 (0.75–1.79)	NS
BCL2L1	20q11.21	73.0%	1.44 (0.97–1.98)	1.45 (1.04–2.44)	NS	1.50 (1.10–2.44)	1.31 (0.97–1.76)	1.51 (1.19–1.98)	NS
ID1	20q11	29.2%	1.29 (0.66–2.25)	1.41 (0.80–3.33)	NS	1.48 (0.77–3.33)	1.15 (0.66–1.62)	1.32 (1.00–1.73)	NS
TPX2	20q11.2	27.0%	1.26 (0.58–2.22)	1.21 (0.71–2.35)	NS	1.28 (0.58–2.35)	1.13 (0.80–1.57)	1.21 (0.97–1.75)	NS
HCK	20q11–q12	46.0%	1.23 (0.71–2.27)	1.37 (0.90–3.12)	NS	1.40 (0.71–3.12)	1.16 (0.73–1.58)	1.28 (0.93–1.69)	NS
DNMT3B	20q11.2	54.0%	1.29 (0.43–2.26)	1.42 (0.72–2.60)	NS	1.42 (0.43–2.55)	1.20 (0.54–1.74)	1.51 (0.94–2.60)	NS
MAPRE1	20q11.21	20.6%	1.16 (0.84–1.78)	1.14 (0.70–1.78)	NS	1.15 (0.70–1.78)	1.12 (0.84–1.53)	1.17 (0.83–1.54)	NS

E2F1	20q11.2	44.4%	1.35 (0.80–2.31)	1.33 (0.93–2.26)	NS	1.37 (0.82–2.26)	1.21 (0.80–1.72)	1.47 (0.95–2.31)	NS
SRC	20q12–q13	57.1%	1.38 (0.71–2.12)	1.45 (0.77–2.70)	NS	1.51 (0.88–2.70)	1.22 (0.71–1.67)	1.48 (1.02–2.12)	NS
NNAT	20q11.2–q12	52.4%	1.30 (0.47–2.50)	1.30 (0.55–2.65)	NS	1.36 (0.47–2.65)	1.16 (0.69–1.78)	1.27 (0.91–1.91)	NS
TOP1	20q12–q13.1	63.5%	1.57 (0.85–2.78)	1.47 (0.88–2.40)	NS	1.57 (1.00–2.78)	1.29 (0.85–1.72)	1.67 (0.94–2.69)	NS
MYBL2	20q13.1	73.0%	1.49 (0.58–2.79)	1.73 (0.70–3.83)	NS	1.79 (0.58–3.83)	1.34 (0.58–2.51)	1.62 (0.95–2.65)	NS
ADA	20q12–q13.11	41.3%	1.17 (0.42–1.80)	1.28 (0.67–2.24)	NS	1.32 (0.42–2.24)	1.08 (0.53–1.74)	1.22 (0.74–1.80)	NS
WISP2	20q12–q13.1	52.4%	1.31 (0.78–2.03)	1.33 (0.78–2.62)	NS	1.37 (0.82–2.62)	1.18 (0.78–1.69)	1.40 (0.95–2.03)	NS
MMP9	20q11.2–q13.1	47.6%	1.26 (0.76–2.06)	1.33 (0.87–2.37)	NS	1.38 (0.81–2.37)	1.18 (0.76–1.72)	1.27 (0.87–1.81)	NS
CSE1L	20q13	49.2%	1.40 (0.85–2.65)	1.31 (0.88–2.54)	NS	1.37 (0.85–2.65)	1.25 (0.94–1.60)	1.43 (1.11–1.82)	NS
PTPN1	20q13.1–q13.2	65.1%	1.46 (0.85–2.76)	1.51 (0.87–2.62)	NS	1.55 (0.85–2.76)	1.32 (0.96–1.82)	1.57 (1.04–2.54)	NS
NFATC2	20q13.2–q13.3	55.6%	1.31 (0.63–2.34)	1.60 (0.77–5.60)	NS	1.61 (0.63–5.60)	1.27 (0.65–2.09)	1.45 (0.83–2.50)	NS
ZNF217	20q13.2	57.1%	1.74 (0.99–6.65)	1.79 (0.96–10.09)	NS	2.06 (1.05–10.09)	1.27 (0.96–1.82)	1.50 (1.15–2.09)	0.02
BCAS1	20q13.2–q13.3	58.7%	1.42 (0.80–2.77)	1.94 (0.95–12.17)	NS	2.03 (0.80–12.17)	1.29 (0.85–1.88)	1.47 (1.05–2.19)	NS
CYP24A1	20q13	60.3%	1.47 (0.85–3.43)	1.81 (0.80–9.16)	NS	1.92 (0.85–9.16)	1.26 (0.80–1.91)	1.52 (0.96–2.27)	NS
STX16	20q13.32	77.8%	1.78 (0.91–5.05)	1.81 (0.95–3.95)	NS	1.90 (1.11–3.95)	1.47 (0.91–2.62)	2.03 (1.09–5.05)	0.03
GNAS	20q13.3	76.2%	2.00 (0.94–7.97)	1.82 (0.87–4.68)	NS	2.02 (0.94–7.97)	1.48 (0.87–2.26)	2.12 (1.16–4.99)	NS
EEF1A2	20q13.3	47.6%	1.20 (0.41–2.52)	1.41 (0.63–2.20)	NS	1.39 (0.41–2.52)	1.21 (0.42–2.18)	1.40 (0.83–2.09)	NS
TNFRSF6B	20q13.3	49.2%	1.20 (0.58–2.51)	1.45 (0.82–3.21)	0.02	1.41 (0.67–3.21)	1.25 (0.58–2.01)	1.36 (0.82–2.13)	NS
KCNQ2	20q13.3	38.1%	1.18 (0.49–2.59)	1.30 (0.43–2.08)	NS	1.27 (0.43–2.08)	1.13 (0.52–1.85)	1.48 (0.55–2.59)	NS
GATA5	20q13.33	60.3%	1.44 (0.60–4.15)	1.58 (0.62–4.12)	NS	1.54 (0.63–4.12)	1.38 (0.60–2.46)	1.82 (0.62–4.15)	NS
FLJ20517	20q13.33	41.3%	1.28 (0.65–3.54)	1.34 (0.80–2.38)	NS	1.30 (0.80–2.38)	1.24 (0.65–1.74)	1.60 (0.95–3.54)	NS
UCKL1	20q13.33	39.7%	1.13 (0.56–2.03)	1.30 (0.67–2.86)	NS	1.29 (0.56–2.86)	1.13 (0.67–1.87)	1.24 (0.85–1.65)	NS
OPRL1	20q13.33	28.6%	1.15 (0.49–2.70)	1.19 (0.62–2.28)	NS	1.18 (0.49–2.28)	1.08 (0.51–1.57)	1.38 (0.67–2.70)	NS

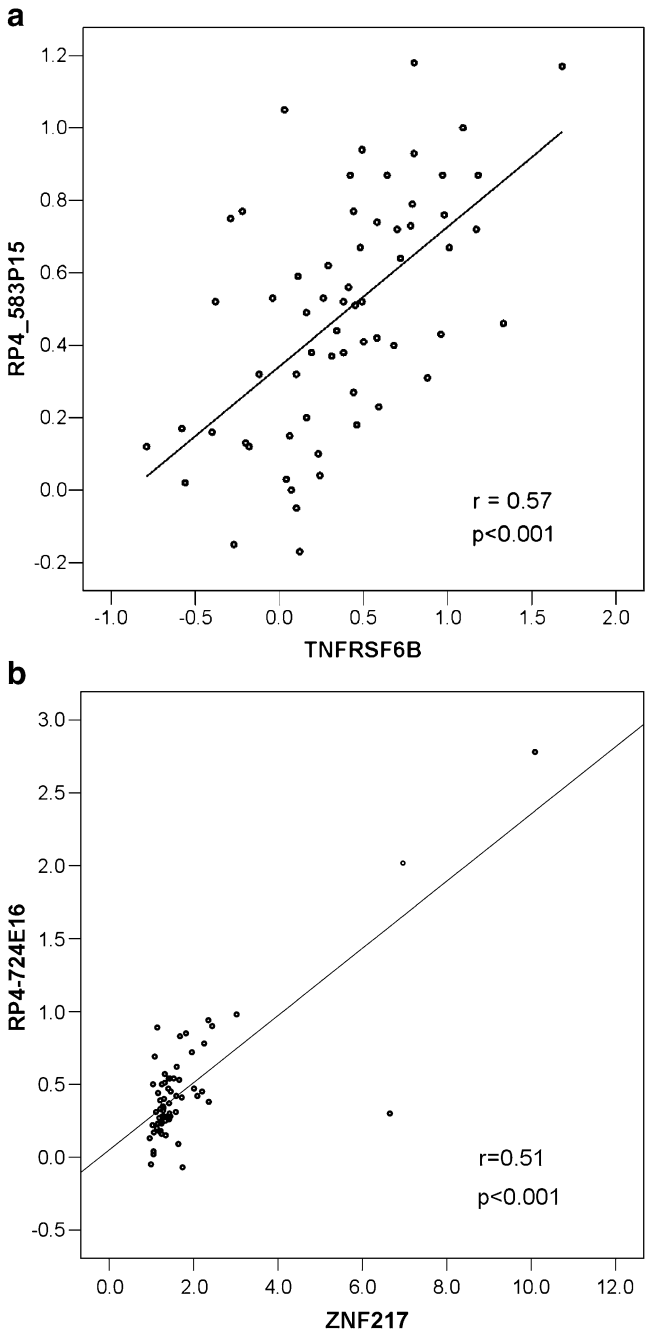

Fig. 4 Scatter plots of the $\log_2$ ratios of the BAC clone and MLPA probe of the *TNFRFS6B* (**a**) and *ZNF217* (**b**) genes. A significant correlation was detected for both genes ($p<0.001$, $r=0.57$ and $r=0.51$, respectively)

showed gains on chromosome 13, and 49 (77.8%) showed gains on chromosome 20. Concurrent gains of chromosomes 8 and 13, 8 and 20, and 13 and 20 were observed in four (6.3%), 24 (38.1%), and three (4.8%) of the gastric adenocarinomas, respectively. Concurrent gains of chromosomes 8, 13, and 20 were observed in 17 (27%) gastric adenocarcinomas. Gain of chromosome 20q was significantly correlated with lymph node status ($p=0.05$) and histological type of the tumor ($p=0.02$), being more common in lymph node-positive gastric cancers (85%) than in lymph node-negative gastric cancers (64%) and more common in intestinal-type (87%) and mixed-type (88%) gastric cancers than in diffuse-type (53%) gastric cancers. No significant (NS) correlation was found between 20q gain and age and gender of the patients and tumor size. No correlation of 8q gain or 13q gain with clinicopathological variables was found. An overview of the frequencies of copy-number gains on chromosomes 8q, 13q, and 20q detected by array CGH analysis is given in Table 1. An overview of patient and tumor characteristics is given in Table 2.

Multiplex ligation-dependent probe amplification analysis of DNA copy-number aberrations and correlation with lymph node status and histological type

All 63 gastric adenocarcinomas analyzed by array CGH were analyzed by MLPA. An overview of the DNA copy-number ratios of all individual genes on chromosomes 8, 13, and 20 in all tumor samples is presented as a heatmap in Fig. 1.

Gains of genes on chromosome 8q were observed in 9.5%–73.0% of the gastric adenocarcinomas, with the highest frequencies of gains observed in c-*myc* (73.0%). Gains of genes on chromosome 13q were detected in 11.1%–28.6%, with the highest frequencies of gains observed in *FOXO1A* and *ATP7B* (both 28.6%). Frequent DNA copy-number gains of multiple genes on chromosome 20q were observed. Gain of *TOP1*, *PTPN1*, *CYP24A1*, and *GATA5* was observed in more than 60% of the gastric adenocarcinomas, and gain of *BCL2L1*, *MYBL2*, *STX16*, and *GNAS* was observed in more than 70% of the gastric adenocarcinomas. When correlating gene-specific copy-number status to lymph node status, *EIF3S6* ($p=0.04$), located on chromosome 8q22 and *TNFRSF6B* ($p=0.02$), located on chromosome 20q13.3, were significantly different between lymph node-positive and lymph node-negative gastric adenocarcinomas. Although Mann-Whitney *U* test yielded a significant difference in copy-number of the gene *EIF3S6* between lymph node-positive and lymph node-negative gastric adenocarcinomas, mean copy-number ratio of both carcinoma groups were within the normal copy-number range (DNA copy-number ratio between 0.7 and 1.3). Lymph node-positive gastric adenocarcinomas showed gain (DNA copy-number ratio >1.3) of *TNFRSF6B* while lymph node negative gastric adenocarcinomas showed normal copy-number ratios of this gene (Fig. 2). When correlating gene-specific copy-number ratio to histological tumor type, Kruskall-Wallis *H* test yielded five significant genes. Mean copy-number ratios of *FBXO25* ($p=0.01$), located on 8p23.3, *RASL11A* ($p=0.03$), located on 13q12.2 and *CDX2* ($p=0.03$), located on 13q12, were within the normal copy-number range for all three tumor types. Mean

copy-number ratio of *ZNF217* (p=0.02), located on 20q13.2, showed gain in intestinal and mixed histological gastric cancer types versus normal in the diffuse histological types (Fig. 3). *STX16* (p=0.03), located on 20q13.32, showed mean copy-number gain in all three tumor types, but higher mean copy-number ratios in the intestinal and mixed types of gastric carcinomas compared with diffuse-type gastric carcinomas (1.90, 2.03, and 1.47, respectively). A detailed overview of the frequencies of gains per gene and mean copy-number ratio per tumor group, i.e., lymph node-positive versus lymph node-negative gastric adenocarcinomas and intestinal- versus diffuse- versus mixed-type gastric carcinomas are given in Table 3.

Correlation of copy-number status of MLPA and array CGH

The BAC clones RP4-583P15 and RP4-724E16 comprised the location of the *TNFRSF6B* and *ZNF217* genes, respectively. To evaluate the correlation between the array CGH and MLPA data, the MLPA DNA copy-number ratios were transformed to a $\log_2$ scale. Spearman correlation yielded a significant correlation between array CGH and MLPA data for both *TNFRSF6B* (r=0.57, p<0.001; Fig. 4a) and *ZNF217* (r=0.51, p<0.001; Fig. 4b).

Discussion

Gastric cancer is a common disease with a poor prognosis [1]. Chromosomal instability is a major mechanism of genetic instability in gastric cancers which has been widely studied by array CGH analysis [2, 32]. Frequent copy-number gains of chromosomes 8q, 13q, and 20q have been detected in gastric adenocarcinomas by this technique [3–10]. In this study, we report a detailed analysis of DNA copy-number changes of genes located on chromosomes 8, 13, and 20 in gastric adenocarcinomas aiming to correlate gene-specific alterations to clinicopathological data.

In the present study of 63 gastric adenocarcinomas studied by array CGH, nearly 80% of the cancers showed gain of chromosomes 8q and 20q. These frequencies of gains are consistent with previous studies where gains of chromosomes 8q and 20q were reported in 40–80% and 50–80% of gastric cancers, respectively [4, 10, 13, 14, 17, 33]. Both gains and losses of chromosome 13q have been detected in gastric cancers. Losses of chromosome 13q have been described in up to 30% of gastric cancers and gains of 13q have been described in up to 40% of gastric cancers [4, 10, 13–15, 17, 33], which is consistent with the findings of the present study.

When analyzing DNA copy-number gain at the gene level, mean copy-number ratio of two genes, *EIF3S6*E (8q22) and *TNFRSF6B* (20q12.3), correlated significantly with lymph node status. Mean copy-number ratios of *EIF3S6E* were within the normal range for both lymph node-positive and lymph node-negative gastric cancers, corresponding to the array CGH results in this study. Mean copy-number ratio of *TNFRSF6B* was significantly higher in lymph node-positive compared with lymph node-negative gastric cancers and was within the range of DNA copy-number gain and normal, respectively. Although 20q gain CGH data correlated with lymph node metastasis, of the 20q genes tested, only DNA copy-number gain of *TNFRSF6B* was significantly correlated with lymph node metastasis. *TNFRSF6B* is a member of the tumor necrosis factor receptor superfamily and is also known as decoy receptor 3 (DrR3). *TNFRSF6B* binds to FasL and inhibits FasL-induced apoptosis [34]. Overexpression of this gene has been observed in multiple cancer types, including cancers of the gastrointestinal tract. Interestingly, gastric cancers with pN2 and pN3 diseases have been shown to have significantly higher *TNFRSF6B* expression compared with gastric cancers with pN0 and pN1 diseases. Serum levels of *TNFRSF6B* were correlated with TNM stage in gastric cancers [35, 36]. We found *TNFRSF6B* DNA copy-number ratios on average to be gained in gastric cancers metastasized to the lymph nodes (pN1–3) compared with on average normal DNA copy-number ratios of *TNFRSF6B* in non-metastasized gastric cancers (pN0). Although previous studies have reported overexpression of *TNFRSF6B* without gene amplification [37], results of the present study indicate that DNA copy-number status of *TNFRSF6B* could be a valuable marker for identifying lymph node-positive gastric cancers.

DNA copy-number status of five genes, *FBXO25* (8p23.3), *RASL11A* (13q12.2), *CDX2* (13q12), *ZNF217* (20q13.2), and *STX16* (20q13.32), correlated significantly with histological tumor type, i.e., intestinal-, diffuse-, or mixed-type gastric cancer. Mean DNA copy-number ratios of *FBXO25*, *RASL11A*, and *CDX2*, however, were within the normal copy-number range for all three histological types, in consistence with the array CGH data of this study. The biological meaning of this difference, therefore, is not immediately clear, but could, e.g., relate to only a subpopulation of tumor cells being affected.

STX16 showed significantly higher mean copy-number ratios in intestinal- and mixed-type compared with diffuse-type gastric cancers; however, DNA copy-number gain was observed in all three tumor groups. *STX16* encodes a syntaxin protein which plays a role in intracellular trafficking [38], but its role in cancer still has to be elucidated.

ZNF217 encodes a transcription factor which was shown to be involved in immortalization of breast cancer cells when overexpressed [39]. High-level amplifications of *ZNF217* have been previously described in approximately 10% of gastric cancers [9, 10, 40]. In the study of Weiss et

al. on three tumors, all of the intestinal-type, out of 27 gastric adenocarcinomas, showed an amplification on 20q13. Fluorescence in situ hybridization (FISH) analysis was performed on these three tumors, confirming the copy-number increase at this locus [9]. Results from the present study suggest that DNA copy-number gain of *ZNF217* plays a more important role in intestinal- and mixed-type compared with diffuse-type gastric cancers, since gains of mean copy-number ratios are observed in intestinal- and mixed-type gastric cancers and normal mean copy-number ratio in diffuse-type gastric cancers.

In summary, we present a detailed DNA copy-number analysis of a panel of genes located on chromosomes 8, 13, and 20 in gastric adenocarcinomas. We found that DNA copy-number gain of *ZNF217* plays a role mainly in intestinal- and mixed-type but not in diffuse-type gastric adenocarcinomas. In addition, we showed that DNA copy-number gain of *TNFRSF6B* is significantly correlated to gastric cancers with lymph node metastasis, indicating a potential role for this gene as a biomarker for identifying patients at high risk of lymph node metastasis, who might, therefore, benefit from extended lymph node resection or neoadjuvant chemotherapy to improve gastric cancer outcome.

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Conflict of interest statement We declare that we have no conflict of interest.

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