Genome-wide analysis of genetic alterations in Barrett's adenocarcinoma using single nucleotide polymorphism arrays

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We performed genome-wide analysis of copy-number changes and loss of heterozygosity (LOH) in Barrett's esophageal adenocarcinoma by single nucleotide polymorphism (SNP) microarrays to identify associated genomic alterations. DNA from 27 esophageal adenocarcinomas and 14 matching normal tissues was subjected to SNP microarrays. The data were analyzed using dChipSNP software. Copy-number changes occurring in at least 25% of the cases and LOH occurring in at least 19% were regarded as relevant changes. As a validation, fluorescence *in situ* hybridization (FISH) of 8q24.21 (*CMYC*) and 8p23.1 (*SOX7*) was performed. Previously described genomic alterations in esophageal adenocarcinomas could be confirmed by SNP microarrays, such as amplification on 8q (*CMYC*, confirmed by FISH) and 20q13 or deletion/LOH on 3p (*FHIT*) and 9p (*CDKN2A*). Moreover, frequent gains were detected on 2p23.3, 7q11.22, 13q31.1, 14q32.31, 17q23.2 and 20q13.2 harboring several novel candidate genes. The highest copy numbers were seen on 8p23.1, the location of *SOX7*, which could be demonstrated to be involved in amplification by FISH. A nuclear overexpression of the transcription factor *SOX7* could be detected by immunohistochemistry in two amplified tumors. Copy-number losses were seen on 18q21.32 and 20p11.21, harboring interesting candidate genes, such as *CDH20* and *CST4*. Finally, a novel LOH region could be identified on 6p in at least 19% of the cases. In conclusion, SNP microarrays are a valuable tool to detect DNA copy-number changes and LOH at a high resolution. Using this technique, we identified several novel genes and DNA regions associated with esophageal adenocarcinoma.

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Genomic alterations, such as amplification, deletion, translocation and loss of heterozygosity (LOH) play an important role in the pathogenesis and progression of cancer due to the activation of oncogenes or inactivation of tumor suppressor genes. Apart from numeric aberrations, such as gene amplification or deletion, which are frequently detected in carcinomas, other genomic changes in carcinogenesis, such as LOH, do not necessarily lead to DNA copy-number gain or loss. Several causes of LOH have been described, including deletion, non-disjunction and reduplication, mitotic recombination and gene conversion.¹ The two-hit model describes a tumor suppressor gene inactivation due to a loss of one allele and mutation of the other allele, resulting in an

LOH.² Besides detailed investigation of known oncogenes and tumor suppressor genes, screening for novel genomic alterations in different cancers and precursor lesions provides information of molecular mechanisms in carcinogenesis.

The incidence of esophageal (Barrett's) adenocarcinoma, mostly arising within the precancerous Barrett's esophagus, with the normal esophageal squamous epithelium changed into intestinal metaplasia, has risen in the past decades in western countries.³ Screening for chromosomal alterations has been performed to identify potential markers for development and progression of Barrett's carcinoma. Two techniques of comparative genomic hybridization (CGH), metaphase CGH (mCGH)^{4–6} and array CGH (aCGH)⁷ have

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been implemented in the study of Barrett's adenocarcinoma, revealing frequent DNA losses on 1q44, 3p14.2, 3p21.3, 4q, 5q, 7q, 9p21, 14q, 17p12–p11.2, 18q and 22q13. Repeated DNA gains have been identified at 1p13.2, 2p12–p11, 2p22.3–p22.1, 3q26, 4p15.3, 5p15.2, 7q21.1, 7q31, 8q24.12–q24.1, 9p11.2, 10q, 11q22.3, 15q12, 15q25–q26, 17q11, 17q21.3, 18q11.2 and 20q13.1–q13.3.^{5–7}

Both methods are complementary in characterizing chromosomal aberrations but they are limited to the detection within a range of 1–20 Mb.⁸ Another disadvantage is that LOH can be detected only indirectly if it is caused by deletion of one allele. The single nucleotide polymorphism (SNP) array technology, which has originally been developed for allelotyping and linkage analyses, allows a genome-wide fine mapping of copy-number changes within a range of 30–900 kb. In addition, the genotypes of the SNPs provide information about LOH throughout the genome. Regarding the copy-number changes in LOH loci it is possible to distinguish different mechanisms leading to LOH detection, such as deletion, mitotic non-disjunction or monoallelic amplification.⁹ Recently, the technique was used to investigate the progressive genomic instability in biopsies of preinvasive lesions of six patients with Barrett's esophagus, revealing interesting alterations in premalignant stages.¹⁰

We applied the SNP array technique to 27 invasive esophageal adenocarcinomas and 14 matched normal tissues for a genome-wide detailed view of copy-number changes and LOH.

MATERIALS AND METHODS

Subjects and Tissues

Frozen esophageal adenocarcinoma samples of 27 patients (24 men, 3 women) and 14 matched non-neoplastic samples of unaffected esophageal squamous epithelium were analyzed. All carcinomas have been primarily resected without preoperative radio- or chemotherapy. Histological tumor typing as proposed by the WHO classification schemes¹¹ revealed moderately or poorly differentiated adenocarcinomas of the distal esophagus in all cases. The tumors were staged according to the International Union against Cancer (UICC):¹² stages pT1, pT2 and pT3 were represented and lymph node metastases occurred in 20 of 27 patients (74%) (Table 1). The snap frozen samples were cut serially into 10-µm sections and 2 × 20 µg of each were collected in two 1.5-ml tubes. Each first and last section (5 µm) was stained with hematoxylin and eosin for histologic control to assure a relative tumor cell content of at least 70%.

DNA Extraction, Sample Processing and Array Hybridization

DNA was isolated using Qiagen DNeasy Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA quality and yield of all samples were assessed by the ratios of absorbance at 260 and 280 nm and by agarose gel electrophoresis (0.7%).

Table 1 Patient and tumor features

Case no.	Sex	Age (years)	pT	pN	pM	G
1	M	63	2	0	x	2
2	M	57	3	1	x	3
3	F	76	3	1	x	2
4	M	77	3	1	x	3
5	M	77	2	1	x	3
6	M	68	3	1	x	3
7	M	74	3	0	x	3
8	M	78	3	1	x	3
9	M	49	3	1	x	3
10	F	52	3	1	x	2
11	M	75	3	1	x	3
12	M	69	2	0	x	3
13	M	52	3	1	1	3
14	M	73	2	1	0	3
15	M	59	2	1	x	3
16	F	76	3	1	0	4
17	M	77	3	1	0	3
18	M	38	1	0	0	2
19	M	72	3	1	1	3
20	M	72	2	1	0	3
21	M	51	3	1	x	3
22	M	62	3	0	x	3
23	M	60	3	1	x	3
24	M	68	3	0	x	2
25	M	63	1	0	x	2
26	M	75	3	1	0	3
27	M	65	3	1	0	3

Clinicopathological data including sex (F, female; M, male) and age in years at the time of resection, UICC tumor stage (TNM¹²) and tumor grading (G) of the cases. 'X' in the column 'pM' means that distant metastasis could not be assessed.

Further sample processing, including digestion, adaptor ligation, amplification, fragmentation, labeling, hybridization and scanning was assayed according to the standard protocol (Affymetrix GeneChip Mapping 10K 2.0 Assay Manual). Briefly, 250 ng genomic DNA of each sample was restricted with *Xba*I before adaptor-ligated PCR amplification. After purification from free primers and nucleotides, the PCR products were checked by agarose gel (2%) electrophoresis and quantified spectrophotometrically. The purified PCR products were fragmented, labeled with biotin, added to a hybridization solution and hybridized to the 10K 2.0 Mapping Array (Affymetrix, Santa Clara, CA, USA). This array contains complementary probes of 10 204 biallelic SNPs, which are located within the amplified 250–1000 base *Xba*I fragments. For each probe, both the sense and antisense

strand in both a perfect match and a mismatch sequence are synthesized on the chip. The SNP arrays were incubated for 16 h at 48°C in the hybridization oven. The arrays were washed and stained by incubation with streptavidin, then biotinylated anti-streptavidin, followed by phycoerythrin-conjugated streptavidin using the Affymetrix Fluidics Station. Finally, the microarrays were scanned in the Affymetrix GeneChip® Scanner 3000.

Data Analysis

CEL files, containing intensity value and standard deviation for each probe on the chip were generated for each array using the GeneChip Operating Software (Affymetrix). Perfect match–mismatch average difference intensities as a signal-to-noise value and the genotypes (AA, AB and BB) were calculated for each biallelic SNP using the GeneChip DNA Analysis Software (Affymetrix). The signal detection rate, which shows the percentage that pass the discrimination filter, and the percentage of SNPs called on the array—the call rate—were determined for each array.

For further data analysis and illustration of the results, the CEL files were imported into the dChipSNP module¹³ based on the dChip software.¹⁴ All arrays were normalized to the array of median average intensity. Using a model-based method, the signal values for each SNP in each array were calculated. For copy-number estimation, signal values of each SNP locus of the tumor samples were compared to a set of 14 normal tissue samples, which were defined to have two copies per cell in all loci. A calculated copy number of more than 3 and less than 1.25 was scored as copy-number gain and loss, respectively. The total numbers of gains and losses per case were correlated with the primary tumor (pT) stage, the nodal status (pN) stage and the tumor grading (G), followed by statistical evaluation using the Student's *t*-test. A copy-number change was regarded as frequent and listed in Table 2 or 3, when occurring in at least 25% of the cases.

For LOH analysis, the software scored the LOH events with regard to the different informative marker densities in different chromosome regions. The LOH scores were then mapped to chromosome regions for each sample and a curve of the prevalence of LOH across all samples, calculated as average probability of LOH by a hidden Markov model based on the genotype calls. For each case, the LOH regions detected by this method were then reviewed in the results of copy-number analysis for differentiation between LOH, deletion and amplification, as for example, monoallelic amplification can lead to LOH detection due to a relative preponderance of the amplified allele. As non-neoplastic esophageal mucosa of 14 cases was available, manual matched pair analysis (SNP by SNP comparison of allelotypes) was performed to detect further (smaller) LOH regions. If in at least one SNP a homozygous call was seen in a tumor sample, which had a heterozygous call in the corresponding non-neoplastic sample, the locus was regarded as LOH (see exemplary illustration for chromosome 9 in Figure 1).

Retrieving annotation information about the SNP loci was carried out by using online databases NetAffx (<http://www.affymetrix.com>), Ensembl (<http://www.ensembl.org>) and Gene Ontology (<http://www.geneontology.org>).

The number of LOH loci per case was correlated with the pT, pN and the grade of differentiation (G).

Fluorescence *In Situ* Hybridization

Fluorescence *in situ* hybridization (FISH) analysis was performed using a combination of three clones (RP11-49I23, RP11-593D05 and RP11-1082H13) for visualization of the *SRY* (*sex-determining region Y*)-*box 7* (*SOX7*) gene region labeled with FITC-dUTP (green fluorescence), a locus-specific probe for *CMYC*, labeled with Cy3-dUTP (orange fluorescence) and a centromere probe 8 (CEP8), labeled with DEAC-dUTP (aqua fluorescence) (Chrombios, Raubling, Germany). Paraffin slides of 10 cases were available for FISH analysis.

Paraffin was removed from tissue sections (8 µm) by xylene (30 min) and isopropanol (3 min) and the slides were hydrated in a graded series of ethanol (100, 96, 70 and 50%) and PBS (pH 7). After pretreating the slides for 20 min in a microwave oven (180 W) in citrate buffer (pH 6) and pronase E (0.05%) digestion for 2 min at 37°C, they were washed in PBS for 2 min and dehydrated over a graded series of ethanol (70% for 2 min, 90% for 2 min and 100% for 2 min). Then the slides were air dried and denatured in 70% formamide (10 min, 73°C) before the FISH probes were added for 48 h at 37°C. After hybridization, the slides were placed in 2 × SSC/0.1% NP-40 (pH 7.4) at 73°C for 2 min, nuclear counterstaining with DAPI (10 min) was performed and the slides were embedded in antifade solution (Vectashield).

Fluorescence images were acquired using a Zeiss Axioplan2 imaging microscope (Carl Zeiss Jena, Jena, Germany) equipped with a PlanApochromat X63/NA1.4 oil objective lens and the ApoTome (Carl Zeiss Jena). The numbers of CEP8 per cell, *MYC* per cell and *SOX7* per cell were determined by evaluating at least 30 cells per case.

Immunohistochemistry

In five cases, paraffin slides were available for immunohistochemical staining. Slides (3 µm) were pretreated in a steamer for 30 min (pH 6). Staining was performed using an anti-human *SOX7* goat antibody (R&D Systems, MN, USA, diluted 1:50) and the streptavidin/AP system with biotinylated rabbit anti-goat antiserum (1:200) in an autostainer plus (Dako, Glostrup, Denmark) according to the Dako standard protocol. Nuclear and cytoplasmic staining intensity was semiquantitatively graded as negative (0), moderately positive (+) or strongly positive (++).

RESULTS

Quality Parameters and Overall Frequency of Genomic Changes

The mean genotyping call rate was 82.28% and the mean signal detection rate was 91.84%. As an estimation of the

Table 2 Results I: frequent DNA gains

Chromosome region	Position of maximum amplification (Mb)	% of cases	Maximum copy number	Candidate genes	Gene function
2p23.1	29 880	44	4.5	<i>ALK</i>	Tyrosine kinase activity
2q12.3	108 430	41	5	<i>GCC2</i>	Protein binding
2q33.3	208 501	30	3.7	<i>NP_001073944.1</i>	?
3q26.1	167 470	30	7.8	<i>BCHE</i>	Cholinesterase activity
3q27.1	185 909	30	5.6	<i>MUC13</i> ¹⁵	Cell signalling
				<i>MAGEF1</i>	?
				<i>VPS8</i>	Protein binding
5q35.2	174 064	33	5.1	<i>MSX2</i>	Transcription factor activity
6p25.3	0.779	37	8.1	?	?
6p24.1	12 997	26	5.2	<i>PHACTR1</i>	Phosphatase inhibitor activity
6p21.2	38 934	33	5.3	<i>DNAH8</i>	Nucleotide binding
6q22.33	128 074	37	4.6	<i>C6orf190, PTPRK</i>	Receptor activity
7p21.3	8462	37	5.2	<i>ICA1</i>	Neurotransmitter secretion
7p13	45 257	30	5	<i>RAMP3</i>	Receptor activity
7p12.1	53 323	30	4	?	?
7q11.22	70 078	48	7.4	<i>WBSCR17</i>	Sugar binding
				<i>CALN1</i>	Calcium ion binding
8p23.1	11 054	26	21.4	<i>SOX7</i>	Transcription factor activity
				<i>GATA4</i> ¹⁶	Transcription factor activity
8q22.3	101 477	37	5.4	<i>RNF19</i>	Transcription factor binding
				<i>ANKRD46</i>	?
8q23.2	110 449	41	5.5	<i>PKHD1L1</i>	?
8q24.13	127 867	30	19.7	<i>MYC</i> ¹⁷	Transcription factor activity
				<i>FAM84B</i>	Protein binding
9q31.2	102 851	37	5.6	<i>RP11-35N6.1</i>	Phosphatase activity
9q31.3	108 107	33	4.9	<i>Q6ZP43</i>	Nucleotide binding
				<i>ZNF462</i>	DNA binding
10p11.21	35 349	41	5.7	<i>CCNY</i>	Cell cycle
				<i>CUL2</i>	Cell cycle
10q25.3	116 126	52	6.7	<i>ABLIM1</i>	Protein binding
				<i>AFAP1L2</i>	?
12p12.1	26 206	37	14.6	<i>SSPN</i>	Dystrophin associated
12p11.23	27 478	37	9.5	<i>TM7SF3,</i>	Transmembrane protein
				<i>ARNTL2</i>	Regulation of transcription
12q22	93 707	37	5.3	<i>KRT19P2</i>	?

Table 2 Continued

Chromosome region	Position of maximum amplification (Mb)	% of cases	Maximum copy number	Candidate genes	Gene function
13q12.13	25 088	30	6	<i>ATP8A2</i> <i>CDK8</i>	Nucleotide binding Nucleotide binding
13q31.1	80 971	44	6.2	<i>SOX1</i>	Transcription factor activity
13q34	111 530	37	8.9	?	?
14q32.2	95 229	41	6.8	<i>TCL1A</i> <i>TCL1B</i>	Protein binding Protein binding
14q32.31	99 835	44	5	<i>SLC25A29</i>	Transporter activity
17q22	53 305	33	4.5	<i>CUEDC1</i>	?
17q23.2	60 350	52	5	<i>LRRC37A3</i>	Transferase activity
19p13.11	16 213	37	4.8	<i>AP1M1</i>	Protein binding
19q12	36 006	30	12.7	<i>ZNF536</i> <i>Q6NT59</i>	DNA binding ?
20p11.21	22 901	37	6.5	<i>SSTR4</i>	Somatostatin receptor activity
20q11.22	33 562	37	5	<i>CEP250</i>	Protein kinase binding
20q13.2	53 637	44	5.9	<i>CBLN4</i>	Neuromodulatory function

Recurrent DNA copy-number gains occurring in at least 26% (7/27) of the cases. Candidate genes of the amplified regions are listed with their molecular function and referenced when previously described in BCA.

genotyping accuracy, there were 0.52% heterozygous SNP calls on the X chromosome of all male patients (tumor and normal tissue). Copy-number changes occurred in all 27 carcinomas (100%) and LOH, detected by scoring, was seen in 20 of 27 cases (74%).

Copy-Number Changes

Using the high-density Affymetrix 10K 2.0. Mapping array for the detection of genomic imbalances in 27 cases of Barrett's esophageal adenocarcinoma, our data confirmed previously published data as determined by mCGH^{5,6} or aCGH.⁷ As summarized in Table 2, highest frequency of copy-number gains was seen on 2p23.3 (*ALK*; 44%), 7q11.22 (*WBSCR17*, *CALN1*; 48%), 13q31.1 (*SOX1*; 44%), 14q32.31 (*SLC25A29*; 44%), 17q23.2 (*LRRC37A3*; 52%) and 20q13.2 (*CBLN4*; 44%). The highest levels of amplification were detected on 8p23.1 (*SOX7*, *GATA4*; maximum copy number (maximum CN) 21.4), 8q24.13 (*MYC*, *FAM84B*; maximum CN 19.7), 12p12.1 (*SSPN*; maximum CN 14.6) and 19q12 (*ZNF536*; maximum CN 12.7).

As illustrated in Figure 2, the highest copy-number gain with up to 21.4 gene copies, as calculated by dChip software, was seen on 8p23.1. The least common amplified region of the seven involved cases was represented by the SNP_A-1508297 on position 11 054 000 bp and SNP_A-1513639 on position 11 422 000 bp. The amplified region harbors several candidate genes, such as *SOX7* and *GATA4*.

Correlation of the numbers per case of loci showing DNA gains revealed a tendency of pT2 tumors having more amplifications than pT1 tumors. However, as we investigated few cases and there were only two tumors classified as pT1, this tendency was not statistically significant. Correlation with the pN and grading (G) did not show significant changes.

Highest frequency of copy-number losses was seen on 3p14.2 (fragile histidine triad (*FHIT*), *LOC131691*; 48%), 9p21.3 (*KIAA1797*, *PTPLAD2*, *CDKN2A/p16*; 59%), 18q21.32 (*CDH20*, *LOC731576*; 41%) and 20p11.21 (*CST4*; 41%) as shown in Table 3.

There was no significant correlation of deletions with pT and pN categories or histological tumor grade.

Table 3 Results II: frequent DNA losses

Chromosome region	Position of minimum copy number (Mb)	% of cases	Minimum copy number	Candidate genes	Gene function
3p14.2	60 538	48	0.7	<i>FHIT</i> ¹⁸ <i>LOC131691</i>	Hydrolase activity ?
7q31.32	122 491	37	0.8	<i>TAS2R16</i> <i>SLC13A1</i>	Receptor activity Transporter activity
9p21.3	20 781	59	0.7	<i>KIAA1797</i> <i>PTPLAD2</i> <i>CDKN2A/p16</i> ¹⁹	? ? Kinase activity
9p11.2	40 744	30	0.8	<i>FAM74A3</i> <i>ZNF658</i>	? Nucleic acid binding
17p13.1	10 017	30	1.1	<i>Q8N7A4</i> <i>MYH13</i> <i>TP53</i> ²⁰	DNA binding Nucleotide binding DNA binding
17p12	12 732	37	0.5	<i>RICH2</i>	Protein binding
18q21.32	56 915	41	0.7	<i>CDH 20</i> <i>LOC731576</i>	Protein binding DNA binding
20p11.21	23 618	41	0.6	<i>CST4</i>	Cysteine protease inhibitor

Frequent DNA copy-number losses occurring in at least 26% (7/27) of the cases with candidate genes and their molecular function. References are given for genes published to show losses in BCA.

LOH

Copy-number losses were often associated with LOH and the highest frequency of LOH (Table 4) was detected on 4q31.22-qter, 5q14.2–35.1, 6p25.1-pter, 7q35-qter, 9p, 11p13–p14.3, 13q21.33-qter, 17p12-pter, 17q21.32-q24.2 and 18q22.1-qter. Many LOHs, found by LOH scoring, were large chromosomal regions, involving whole chromosome arms or even whole chromosomes, indicating that many of these LOHs were due to large deletions or monosomy. On chromosome arm 9p, a known region of high LOH frequency, only six tumors (22%) showed an LOH detected by scoring (Figure 1).

To detect smaller LOH regions, manual comparison of allelotypes of tumor tissue *vs* non-neoplastic tissue was performed in 14 cases, in which sufficient uninvolved tissue was available. This analysis revealed several additional genomic LOHs, as listed in Table 4 (*'matched pair analysis'*, *italic*). Regarding the 9p region, the LOH frequency raised up to 64% (9 of 14 matched pair cases) when manual SNP by SNP comparison was performed.

Correlating the total numbers of LOH loci with the pT stage, the pN stage and the G did not show significant differences.

FISH

For validation of the detected copy-number changes, we chose the two frequently amplified regions on chromosome 8: 8q24.21, the location of the previously identified *MYC* gene and 8p21.3, harboring the novel candidate gene *SOX7*. For visualization, we designed a combined FISH probe for *MYC*, *SOX7* and centromere 8, which was hybridized on slides of 10 carcinomas. A nearly normal copy number of *MYC* could be confirmed in case 9, a low-level gain was confirmed in six cases and a higher level amplification was confirmed in case 15 (Table 5). In contrast, in cases 14 and 17, a low-level copy-number gain, seen in FISH analysis, was not detected by SNP arrays. High-level amplification of *SOX7* could be confirmed in all three cases examined (7, 13 and 15) and nearly normal copy numbers of *SOX7* were detected in five cases by both SNP arrays and FISH analysis. In contrast, a reduced copy number of *SOX7* in case 14, as detected by SNP array, could not be confirmed by FISH. In two cases, which had normal copy numbers in SNP array analysis, FISH revealed slight copy-number gains due to polysomy (cases 1 and 17).

Table 4 Results III: loss of heterozygosity

Chromosome	Number of cases (% of 27 cases, score) (% of 14 cases, mpa)	Case no.	Alteration (score, mpa)	Least common LOH region (score)	Candidate genes
3p ^{19,21}	5 (19)	1	LOH 3p26.1–26.3	3p22.3–24.2 and 3p25.2–26.3	<i>VHL</i> , ¹⁹ <i>PPARG</i> ²²
	2 (14)	6	LOH 3p26.1–26.3, 3p14.3–24.2		
		12	Amp 3p22.3–24.2 ^a		
		15	3p-		
		23	Monosomy 3		
		25	<i>LOH 3p26.3+3p12.1</i>		
4q ²³	5 (19)	2	del 4q22.2-qter	4q31.22-qter	
	3 (21)	3	del 4q31.22-qter		
		9	Monosomy 4		
		15	Monosomy 4		
		23	Monosomy 4		
		18	<i>LOH 4q34.1–q34.3</i>		
5q ^{19,21,24,25}	5 (19)	2	del 5q12.2-qter	5q32–35.1	<i>APC</i> , ²⁶ <i>MCC</i> , ²⁶ <i>IRF-1</i> , ²⁷ <i>ECRG2</i> ²⁸
	5 (36)	9	Monosomy 5		
		11	del 5q14.2–35.1		
		15	5q-		
		23	del 5q32-qter		
		5	<i>LOH 5q11.2</i>		
		16	<i>LOH 5q12.3</i>		
		18	<i>LOH 5q12.1-q14.3</i>		
6p	5 (19)	5	LOH 6p21.31-pter	6p25.1-pter	<i>GPX5</i> , <i>GPX6</i> (6p22.1)
	5 (36)	9	Monosomy 6		
		12	LOH 6p25.1-pter		
		15	Monosomy 6		
		17	LOH 6p22.3-pter		
		16	<i>LOH 6p22.1–21.2</i> + <i>6p24.1–6p23</i>		
		18	<i>LOH 6p22.1+6p22.3</i>		
7q ²⁹	5 (19)	2	7q-	7q35-qter	
	5 (36)	5	7q-		
		11	7q-		
		13	del 7q35-qter		
		23	7q-		
		15	<i>LOH 7q21.3–q31.32</i>		
		22	<i>LOH 7q32.3-qter</i>		

Table 4 Continued

Chromosome	Number of cases (% of 27 cases, score) (% of 14 cases, mpa)	Case no.	Alteration (score, mpa)	Least common LOH region (score)	Candidate genes
9p ^{19,21,23,24}	6 (22)	2	9p-	9p	<i>IFNA</i> , ³⁰ <i>P15</i> , <i>P16</i> ¹⁹
	9 (64)	5	9p-		
		7	LOH 9		
		10	9p-		
		15	LOH 9p		
		24	LOH 9p		
		13	<i>LOH 9p21.3+9p22.3</i>		
		16	<i>LOH 9p21.3</i>		
		19	<i>LOH 9p21.3</i>		
		20	<i>LOH 9p21.3</i>		
		22	<i>LOH 9p21.3</i>		
11p ¹⁹	5 (19)	5	Del 11p13-pter	11p13–p14.3	
	5 (36)	13	Monosomy 11		
		17	del 11p13-pter		
		18	LOH 11p12-p14.3		
		23	LOH 11p13-pter		
		16	<i>LOH 11p13+11p15.3</i>		
13q ¹⁹	6 (22)	1	13q-	13q21.33-qter	<i>RB1</i> (13q14.2), ¹⁹ <i>BRCA3</i> , <i>BRCA2</i>
	5 (36)	2	del 13q21.33-qter		
		5	13q-		
		6	LOH 13q		
		9	Amp 13q ^a		
		15	13q-		
		4	<i>LOH 13q12.3+13q14.3+13q21.33</i>		
		13	<i>LOH 13q12.13</i>		
		16	<i>LOH 13q12.13-q12.3+13q14.13+13q31.3-q32.3</i>		
17p ^{19,21,23,24,25}	6 (22)	1	LOH 17p	17p12-pter	<i>TP53</i> ²⁰
	5 (36)	5	17p-		
		10	Monosomy 17		
		13	17p12-pter		
		15	Monosomy 17		
		23	Monosomy 17		
		16	<i>LOH 17p12</i>		
17q ¹⁹	5 (19)	10	Monosomy 17	17q21.32-q24.2	<i>TOC</i> (17q25) ³¹
	4 (29)	11	17q21.32–q24.2		
		15	Monosomy 17		
		23	Monosomy 17		

Table 4 Continued

Chromosome	Number of cases (% of 27 cases, score) (% of 14 cases, mpa)	Case no.	Alteration (score, mpa)	Least common LOH region (score)	Candidate genes
18q ^{19,23,24,25}	7 (26) 5 (36)	24	Amp 17q12-qter ^a	18q22.1-qter	<i>DCC</i> , ³² <i>SMAD2</i> , <i>SMAD4</i> <i>CDH7</i> , <i>CDH20</i> type2
		16	<i>LOH</i> 17q21.31		
		1	Del 18q21.2-qter		
		2	Del 18q22-qter		
		5	18q-		
		9	del 18q12.3-qter		
		15	del 1812.3-qter		
		17	del 18q22.1-qter		
		23	Monosomy 18		
		16	<i>LOH</i> 18q11.2		
		25	<i>LOH</i> 18q21.2–21.31		

mpa, matched pair analysis.

^aMonoallelic amplification leading to LOH detection.

Genomic regions with common LOH in at least 19% (5/27) of the cases were detected by LOH scoring. In each row below, frequency and type of alteration is written in italics when detected by matched pair analysis. On the right, the least common LOH regions are listed with candidate genes, which are referenced when known to be altered in BCA.

Figure 1 Schematic illustration of LOH on chromosome 9. The upper part shows the extension of widespread LOH (blue bars) detected by LOH scoring and the lower part displays single SNPs with LOH (small blue bars) and their possible extensions (white bars, spanning neighboring non-informative SNPs) in a scaled up view of 9p21.3.

Immunohistochemistry

As shown in Table 5, immunohistochemistry of five available cases revealed nuclear staining of two *SOX7*-amplified

tumors and nuclear negativity of two non-amplified tumors. However, one non-amplified tumor (case 1) also showed weak nuclear positivity. Cytoplasmic staining was seen in three cases (two amplified and one non-amplified tumor).

The results of comparative array, FISH and immuno-histochemical analyses are summarized in Table 5 and representative images of FISH and immunohistochemical staining of cases 7 and 14 are shown in Figure 3.

DISCUSSION

In this first report of combined screening for copy-number changes and LOH using SNP oligonucleotide microarrays in Barrett's adenocarcinoma, we were able to confirm many known genomic alterations. In addition, we could identify several novel altered DNA regions.

In concordance with published data, we found a DNA loss on chromosome band 3p14.3 (48% of our cases), where the *FHIT* gene is located. Frequent deletions of *FHIT* (55% of the cases) and aberrant transcripts (93% of the cases) have been described in Barrett's adenocarcinoma before.¹⁸ However, the role of *FHIT* alterations in carcinogenesis remains unclear, as alterations of *FHIT* transcripts also occur in non-neoplastic tissues.³³ In contrast to other studies reporting frequent LOH at 3p24–26 (*VHL*, *PPARγ*) in 64% of the cases,¹⁹ we found only 4 cases (15%) with LOH in this region. The frequency of 41% gains on 20q13 is similar to our own published data (33%)⁷ acquired from aCGH, and an amplification of *MYC* (8q24) in 30% of the cases is comparable to previous studies,

Figure 2 Schematic depiction of amplification on chromosome 8. On the upper part, (scaled up view of 8p23.1 (*SOX7*, *GATA4*)) green bars display the detected amplicons with their possible maximal extensions to the next non-amplified SNP (white bars). The lower part of the figure shows a zoom into 8q24.21 (*MYC*) with the same design. On the right of each bar the maximal detected copy number of the respective case is written in parentheses.

reporting a *MYC* amplification in 25% of high-grade dysplasias and 44% of adenocarcinomas.¹⁷

There was no significant correlation of genomic alterations with tumor stage and histological grading. However, this does not exclude an association between genomic instability and tumor progression as the number of investigated cases in this study was limited.

We also observed many other alterations that have not been described in Barrett's adenocarcinoma so far. The anaplastic lymphoma kinase (*ALK*), involved in the t(2;5) chromosomal rearrangement in anaplastic large cell lymphoma, has been found to be activated due to amplification in neuroblastoma.³⁴ Although gene amplification of *ALK* in esophageal carcinomas has not been reported to date, we found a low-level DNA gain at the *ALK* locus in 44% of the cases. As *ALK* expression can be detected in several tumor cell lines, Dirks *et al*³⁵ conclude that *ALK* expression may be a physiologic rather than a pathologic phenomenon. However, activation of *ALK* due to low-level copy-number gains could be relevant also in initiation and progression of carcinomas.

Another novel low-level DNA gain was seen on 3q27.1 (30% of the cases), harboring genes such as *MUC13*, coding a transmembrane protein, which is reported to be over-expressed in intestinal-type gastric cancer³⁶ and showed a trend toward higher expression in esophageal adenocarcinomas compared with normal tissue.¹⁵

The highest copy numbers were detected in two regions of chromosome 8: amplification of 8q24/*MYC* (30%) with up to 19.7 copies per cell and the amplicon on 8p23 (26%) with up to 21.5 copies per cell, involving several candidate genes, such as *GATA4*³⁷ and the gene coding the transcription factor *SOX7*, as confirmed by FISH analysis. Slight discrepancy in

Table 5 Results IV: *MYC* and *SOX7* amplification and *SOX7* expression

Case no.	Copy number of <i>MYC</i> (dChip)	Copy number of <i>MYC</i> per cell (FISH)	Copy number of <i>SOX7</i> (dChip)	Copy number of <i>SOX7</i> per cell (FISH)	Number of centromere 8 per cell (FISH)	<i>SOX7</i> nuclear IHC staining	<i>SOX7</i> cytoplasmic IHC staining
1	2.4	2.8	2.0	2.5	2.7	+	—
2	3.1	4.3	2.3	2.0	2.0		
7 ^a	3.1	3.3	15.4	12.2	1.7	++	+
9	2.1	1.8	2.0	1.9	2.0		
11	3.4	3.5	1.4	1.9	1.8		
12	4.5	3.0	2.1	1.5	1.5		
13	3.1	2.6	21.4	>20 ^b	2.6		
14 ^a	2.0	3.8	1.1	2.1	2.7	—	—
15	19.7	9.4	12.5	7.0	3.9	+	+
17	2.3	3.0	1.9	2.7	2.9	—	+

Validation of the frequent amplification on chromosome 8. The copy numbers of *MYC* and *SOX7* detected by SNP arrays (highest copy number of the amplicons) and by fluorescence *in situ* hybridization (FISH) are compared in 10 available cases. In most of the cases, there is a good accordance of both methods. The results of immunohistochemical (IHC) staining of *SOX7* protein are shown on the right.

^aDepicted in Figure 3.

^bSignal clusters.

Figure 3 Merged FISH images (**a, c**) with probes for centromere 8 (blue), *MYC* (red), *SOX7* (green) and DAPI nuclear counterstain (grey). On the right (**b, d**) immunohistochemical staining of *SOX7* protein of the same tumors is displayed. Case 7 (**a, b**) had an amplification of the *SOX7* gene, a low-level copy-number gain of *MYC* (**a**), and a strong nuclear expression of *SOX7* (**b**). In contrast, case 14 (**c, d**) had no *SOX7* amplification, a low-level gain of *MYC* copies (**c**), and no immunohistochemical *SOX7* staining (**d**). Overlying columnar (**b**) or squamous (**d**) epithelium, as well as lymphocytes and fibroblasts did not show nuclear staining.

the copy numbers of distinct genes between the array and FISH analyses could be due to the well-recognized heterogeneity of Barrett's carcinoma,³⁸ as paraffin sections from different levels of a tumor block have been used for the two techniques. However, although we used rather pure micro-dissected tumor cell populations with a tumor content of at least 70% for SNP analysis, low copy-number changes may have been missed by contaminating non-neoplastic cells. On the other hand, FISH analysis may fail to detect low copy-number changes because a portion of cell nuclei in the tissue sections are incomplete.

SOX7, which is upregulated in pancreatic cancer cell lines and in primary gastric cancer,³⁹ plays a role in parietal

endoderm differentiation⁴⁰ and is a potent activator of transcription of the proto-oncogene *Fgf-3*⁴¹ and interacts with the Wnt signaling pathway.⁴²

A frequent loss (36%)⁴ and LOH (45%)¹⁹ on 5q have been described in esophageal adenocarcinoma before. Here, 18% (score) and 36% (matched pair analysis) of our cases showed an LOH at 5q, where the candidate genes *APC*, *MCC* and *IRF-1* are located. As mutations of the retained allele of *APC* in Barrett's adenocarcinoma⁴³ and of *MCC* in gastric cancer⁴⁴ are not very frequent, there may be another target gene on 5q, such as *IRF-1*, which can induce apoptosis of esophageal adenocarcinoma cells *in vitro*.⁴⁵ However, the least common LOH area (score) in our study was 5q32–5q35.1, harboring

genes such as *ECRG2*, a candidate of tumor suppressor gene in esophageal squamous cell carcinoma.²⁸

Allelic losses of the chromosome arm 7q in Barrett's adenocarcinoma are reported in 33%.⁵ Vissers *et al*²⁹ describe frequent allelic losses in the distal part of 7q, including the marker *D7S483*, which may aid to distinguish between esophageal (Barrett's) and gastric cardia carcinomas. In our study, 19% by scoring and 36% of the carcinomas in matched pair analysis showed an LOH of 7q. Four cases had a deletion of the whole chromosome arm and one carcinoma showed a distal deletion 7q35-qter.

Mutation or loss of cadherin (*CDH*) genes have been described to occur in several carcinomas, such as E-cadherin (*CDH1*) mutations in familial gastric cancer⁴⁶ and lobular breast cancer.⁴⁷ A copy-number loss of 18q21.33, the location of *CDH20*, could be detected in 41% of the cases in our study, and LOH was observed in 26% (score) to 36% (matched pairs), which could point to a possible involvement of adhesion molecules in the progression of esophageal adenocarcinoma.

Whereas LOH on 6p has been reported to occur frequently in some other carcinomas, such as esophageal squamous cell carcinoma⁴⁸ and gastric adenocarcinoma,⁴⁹ it has not been described in esophageal adenocarcinoma. Here, 19% (score) to 36% (matched pairs) of our cases had an LOH on 6p with some variance in the extent and location of the LOH regions. However, regarding the least common LOH region on 6p22.1 (28 575–29 359 Mb), candidate genes such as *TRIM27*, *ZNF452* and *ZNF311*, as well as genes coding for glutathione peroxidases-5 and -6 (*GPX5* and *GPX6*) are to be considered. As chronic gastroesophageal reflux disease as a known risk factor for intestinal metaplasia induces oxidative stress, a loss of expression of antioxidant enzymes such as *GPX5* and *GPX6* could increase mucosal damage. Reduced expression of these enzymes has not been described in esophageal adenocarcinoma up to now, but hypermethylation and loss of expression of another glutathione peroxidase (*GPX3*) could be detected in Barrett's tumorigenesis.⁵⁰

In copy-number analysis, losses on 9p21 and 9p22 were found in 59 (*IFNA1*) and 44% (*p16*) of the cases, which is comparable to published CGH studies, showing admittedly great variance in frequency (17–53%).^{4,51} Interestingly, in our study only 21% of the cases revealed LOH on 9p as detected by LOH score in contrast to published higher frequencies of LOH studies (35–75%).^{21,24} This may be due to lower sensitivity of the LOH detection algorithm compared to copy-number analysis or other LOH detection techniques. Including manual matched pair analysis increased the LOH frequency to a proportion of 64% of the cases, which is more consistent with previous studies.

In conclusion, our results of DNA copy-number changes and LOH in Barrett's adenocarcinoma are relatively concordant with published data using other techniques such as CGH and PCR, and could partly be confirmed by FISH. Hence, SNP array technology is a valuable tool with the

opportunity of genome-wide parallel analysis of copy-number gains, losses and LOH, especially if matched pair analysis is performed to reveal smaller LOH regions.

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