

DNA copy number changes at 8q11–24 in metastasized colorectal cancer

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Abstract. *Background:* C-Myc, a well-known oncogene located on 8q24.12–q24.23, is often amplified and over-expressed in both primary and metastasizing colorectal cancer. In addition, PRL-3 (also known as PTP4A3), a tyrosine phosphatase located on 8q24.3, is amplified in colorectal cancer metastasis. Beside PRL-3 and c-myc, other oncogenes located on the 8q23–24 region might be involved in this process. Therefore, the present study aims to correlate DNA copy number status of a series of genes at 8q23–24 in colorectal cancer at high resolution in correlation to metastatic disease. *Materials and methods:* Thirty-two cases of colorectal cancer, 10 stage B1, 10 B2 and 12 D (Astler–Coller) with their corresponding liver metastasis and one colorectal cell line (colo205, previously analyzed by array-CGH), were included in this study. A chromosome 8 specific MLPA probe mixture was used to analyze the presence of DNA copy number changes. The probe mixture contained 29 probes covering 25 genes on chromosome 8, as well as 6 control probes on other chromosomes. *Results and discussion:* MLPA results obtained of the colo205 colorectal cell line were comparable with previous array-CGH results, thus validating the MLPA probe mixture. Astler–Coller B1 and B2 colorectal cancers differed significantly in DNA copy number of the genes, MOS ($p = 0.04$), MYC ($p = 0.007$), DDEF1 ($p = 0.004$), PTK2 ($p = 0.02$) and PTP4A3 ($p = 0.04$). When comparing these with Astler–Coller D primary tumors, significant differences were seen for several genes as well (MYC ($p < 0.000$), DDEF1 ($p < 0.000$), SLA ($p < 0.000$), PTK2 ($p < 0.000$), PTP4A3 ($p = 0.002$), and RECQL4 ($p = 0.01$)). When comparing primary Astler–Coller D tumors and their corresponding liver metastases, a similar pattern of gains and losses was observed. Most of the liver metastases showed higher DNA copy number ratios than the corresponding primary tumors, but this difference was only significant for TPD52 ($p = 0.02$) and EIF3S6 ($p = 0.007$). *Conclusion:* In addition to c-myc, multiple genes on chromosome 8 differed significantly between primary colorectal cancers with and without liver metastases. This observation is consistent with the concept that clinical behaviour, like risk of liver metastasis, is determined by the genomic profile that is already present in the primary tumor.

Keywords: Colorectal cancer, chromosome 8, gene amplification, metastatic disease, MLPA

1. Introduction

Colorectal cancer is the second leading cause of death in the western world, accounting for more than 10% of cancer mortality in the US [12]. In the majority of patients the tumor has already metastasized to lymph nodes and or liver at the time of diagnosis [6,24], which has a severe impact on the prognosis of these patients [6,18]. A number of genetic changes

have been described in association with metastatic colorectal cancer, yet the exact biological processes responsible for metastasis still remain unclear [24]. One of the main chromosomal changes in colorectal cancer is gain of distal chromosome 8q. Gains of 8q, together with gain of 13q and 20q as well as loss of 8p, 17p and 18q are involved in adenoma to carcinoma progression [8,15]. Moreover, 8q gain, often together with 8p loss, appeared to be more frequent in metastatic lesions than in primary colorectal tumors [18]. The 8q23–24 region is of special interest because it harbors c-Myc and PRL3 [6]. The well-known oncogene c-Myc, located on 8q24.12–24.13, is often amplified and over-expressed in both primary and metastasizing

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colorectal cancer [23]. In addition, the tyrosine phosphatase PRL-3 on 8q24.3, also known as PTP4A3, has been shown to be over-expressed in colorectal cancer metastases [1,14,24]. These findings suggest that genes located at 8q23–24 might influence the metastatic process of colorectal cancer. However, beside PRL-3 and c-Myc, more oncogenes located on the 8q23–24 region might be involved in metastasis. Therefore the present study aimed to correlate DNA copy number status of a series of genes at 8q23–24 in colorectal cancer to the metastatic status.

To this end, we applied a new high resolution technique for studying DNA copy number changes, called Multiplex Ligation-dependent Probe Amplification (MLPA). MLPA can detect DNA copy number changes up to 40 different gene specific DNA sequences in a single experiment, with the use of just one pair of PCR primers [7,25].

2. Materials and methods

2.1. Materials

Thirty-two cases of colorectal cancer were included in this study, consisting of 12 Astler–Coller D primary colorectal cancer samples with their corresponding liver metastases and 20 colorectal cancers that did not have clinically apparent metastases at the time of operation (10 Astler–Coller B1 tumors, 10 Astler–Coller B2 tumors). All tumor samples were obtained from the archives of the department of Pathology of the VU University Medical Center. Normal tissue samples of liver, kidney and spleen of three different individuals were used as a reference. The colorectal cancer cell line colo205, previously analyzed by microarray CGH, was used to validate the MLPA probe set.

2.2. DNA isolation

DNA was isolated from 32 paraffin-embedded colorectal tumors and their corresponding liver metastases, as described previously [28]. Briefly, an area containing at least 70% of tumor cells was demarcated with a felt tip marker on a 4 μm hematoxylin and eosin stained tissue section. Fifteen to twenty adjacent serial sections of 10 μm were sectioned. At the end, a 4 μm “sandwich” section was made and stained with hematoxylin and eosin, to compare with the first slide as a control. After deparaffinating with xylene and washing with methanol to remove all traces of the xylene,

the 10 μm sections were hematoxylin stained and areas corresponding those demarcated on the 4 μm slide, were scraped off with a surgical blade. This material was collected in an Eppendorf tube for DNA extraction. One ml 1 M sodium-thio-cyanate (CNNaS) was added and incubated overnight at 38–40°C to reduce DNA crosslinking. After drying the tissue-pellet, the sample was incubated for three days at 50–60°C with lysis buffer and proteinase K. Forty microliters proteinase K (10 mg/ml) was added twice a day, leading to a final concentration of 2 $\mu\text{g}/\mu\text{l}$. Next, 40 μl RNase A (20 mg/ml) and 400 μl lysis buffer (QIAamp kit) were added. After incubation for 10 minutes at 65–75°C, 420 μl 100% ethanol was added. DNA was extracted by a column-based method (QIAamp-kit; Qiagen, Westburg, Leusden, The Netherlands) and collected in an Eppendorf tube.

2.3. Array-CGH

Array-CGH was performed as described in detail by Snijders et al. [26]. Briefly, tumor and reference DNAs were differently labeled by random priming. Six hundred μg test and reference DNA were hybridized to an array containing approximately 2500 DNA clones evenly spread across the whole genome, with an average resolution of 1.4 Mb.

Image acquisition, analysis and data extraction were performed as described previously [20].

2.4. MLPA

The principles of MLPA are explained in Fig. 1. For analysis of DNA copy number changes, MLPA was performed using a chromosome 8 specific MLPA probe mixture with 29 probes, covering 25 genes on chromosome 8 including 2 probes for c-Myc and 3 probes for PRL-3. The probe mixture also included 6 control probes, one probe on chromosomes 4, one on 5, and two probes on chromosomes 11 and 12 each.

Approximately 50 ng of DNA in a volume of 5 μl was denatured at 98°C for 5 minutes. Next, 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 EDTA)), and 1.5 μl MLPA buffer (1.5 M KCl, 300 mM Tris-HCl pH 8.5, 1 mM EDTA) was added. This mixture was heated at 95°C for 1 minute followed by 16 hours incubation at 60°C to let the MLPA hemiprobe hybridize. Then, 32 μl ligase-65 mixture (dilution buffer containing 2.6 mM MgCl_2 ,

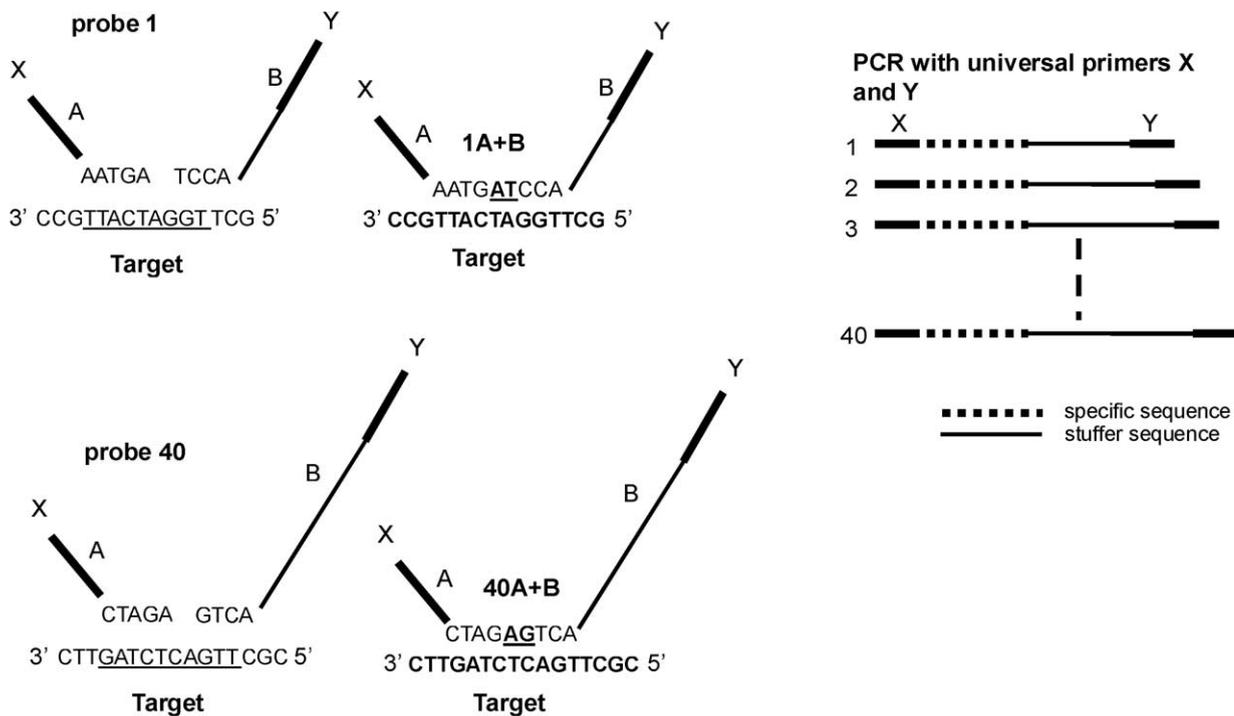


Fig. 1. Each MLPA probe consists of a ~60-mer synthetic oligonucleotide with a target-specific sequence, a stuffer sequence, and two end sequences that are recognized by PCR primers. The probe is divided in two parts, when hybridized to the target sequence, the two parts can be ligated and amplified by PCR. All probes have the same end sequence for recognition by the universal primer pair, but different total lengths due to variable length of the stuffer sequences. Therefore, PCR products of all individual clones differ in length and can be sorted by capillary electrophoresis.

5 mM Tris-HCl pH 8.5, 0.013% non-ionic detergents, 0.2 mM NAD, and 1 U Ligase-65 enzyme) was added to each sample for ligation of hybridized hemiprobe, followed by a 10–15 minutes incubation at 54°C and 5 minutes at 98°C to inactivate the ligase enzyme.

PCR was performed with 10 μ l polymerase mixture, containing the PCR primers (10 pmol), dNTPs (2.5 nmol) and 2.5 U Taq polymerase (promega), 4 μ l PCR buffer (2.6 mM MgCl₂, 5 mM Tris-HCl pH 8.5, 0.013% non-ionic detergents, 0.2 mM NAD), 26 μ l water and 10 μ l MLPA ligation reaction. As MLPA is more sensitive to contaminants in DNA preparations than ordinary PCR reactions, normal DNA was treated in exactly the same way as the DNA from the tumor samples. Experiments were carried out in triplicate.

Analysis of the amounts of the MLPA PCR products per gene was done on an ABI 3100 capillary sequencer (Applied Biosystems) according to the manufactures instructions with a mixture of 8.5 μ l deionised formamide (Applied Biosystems; product number 4311320; Quantity per Package: 25 ml), 1 μ l PCR product and 0.5 μ l marker and a ROX-labeled

internal size standard (ROX-500 Genescan; Applied Biosystems, Warrington, United Kingdom).

2.5. Data analysis

For each tumor, peak area values for every probe were derived from the ABI output and used for further analysis. For each probe, the median peak area obtained from at least three different PCR reactions was calculated. As a reference, normal tissue samples from three different individuals were used, each of which were also analyzed in triplicate.

In every sample, for every probe, a tumor to normal DNA copy number ratio was obtained by dividing the median area under the peak for each gene in the tumor tissue by the median value of the same peak for 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.

2.6. Statistical analysis

Heatmap Builder Version 1.0 software (<http://quertermous.stanford.edu/HeatmapBuilder.pdf>) was used for present descriptive data.

Between the different tumor categories, DNA copy number changes of chromosome 8 genes were evaluated with the Kruskal–Wallis and Mann–Whitney *U* non-parametric test for independent samples (SPSS 10.0 for Windows, SPSS Inc. Chicago, IL, USA). Differences between stage D primary tumors and their corresponding liver metastases were evaluated with a *t*-test for paired samples (WINKS 4.651, Texasoft, TX, USA). *P*-values less than 0.05 were considered to be significant.

3. Results

To validate the chromosome 8 MLPA probe set, we compared previous results of array CGH of the colo205 cell line with MLPA results of the same cell line. The two techniques produced very similar results (Fig. 2), which confirms the reliability of this MLPA probe set for measuring DNA copy number changes.

An overview of the DNA copy number ratios in all tumor samples is presented as a heatmap in Fig. 3. An overview of the mean values per tumor category and a statistical analysis of the differences found are given in Table 1.

Astler–Coller B2 colorectal tumors showed significant higher DNA copy number ratios compared to B1 tumors for MOS (8q11), MYC (8q24.12–q24.13), DDEF1 (8q24.12), PTK2 (8q24qter) and PTP4A3 (8q24.3). DNA copy number ratios for MYC (8q24.12–q24.13), SLA (8q24), PTP4A3 (8q24.3), PTK2 (8q24qter), and RECQL4 (8q24.3), were significantly higher in stage D tumors compared to stage B (i.e. B1 and B2 taken together) adenocarcinomas, while DDEF1 (8q24.12) was significantly lower. Boxplots of genes that differed significantly are shown in Fig. 4. When comparing primary Astler–Coller D tumors and their corresponding liver metastases, approximately the same patterns of gains and losses were observed. However, some metastases showed higher levels of amplifications than primary tumors, although for most genes these differences were not statistically significant. Only TPD52 and EIF3S6 were significantly different between the primary tumor and the corresponding liver metastases.

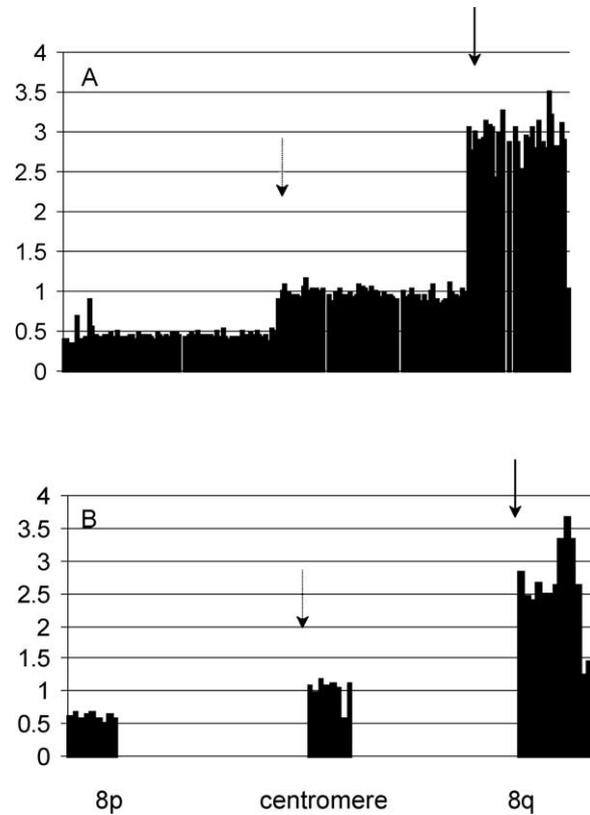


Fig. 2. (A) Array-CGH based DNA copy number ratios (Y-axis) ordered by megabase position at chromosome 8 (X-axis) for the colo205 cell line. The dashed line arrow indicates the position of the centromere. The solid arrow indicates the 8q23.1 position. (B) DNA copy number ratios of the colo205 cell line, obtained with the 8q MLPA probe set, plotted along the chromosome axis, in alignment with the array CGH copy number ratios for corresponding positions.

4. Discussion

Metastatic tumor growth is a frequent cause of cancer death. Most colorectal cancers have already metastasized by the time of diagnosis, which substantially reduces the chances of cure. Better insight in the biological processes, including DNA alterations, involved in the dissemination of colorectal cancer is a first step towards the development of new diagnostic and therapeutic strategies. Here we report a detailed analysis of DNA copy number on chromosome 8 in a series of locally confined as well as metastatic colorectal cancers.

Chromosome 8 DNA copy number changes, losses of chromosome 8p and gains of chromosome 8q have a high prevalence in colorectal tumors and were found to be more pronounced in advanced tumor stages [15].

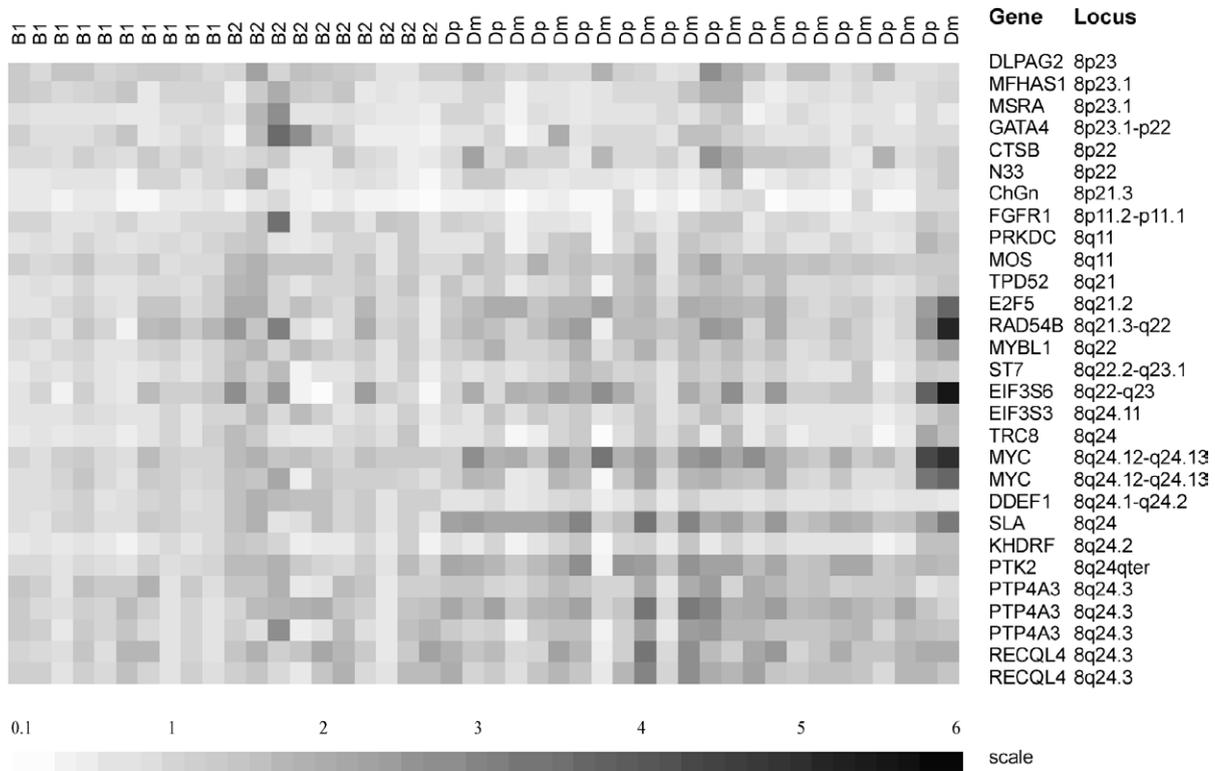


Fig. 3. Heatmap of DNA copy number ratios for 25 genes on chromosome 8p and 8q obtained by MLPA for 32 primary colorectal cancers and 12 liver metastases. Darker squares indicate higher DNA copy number ratios. Position of the genes on chromosome 8 was determined according to Mapview Build 34 version 2.

Table 1

Genes at chromosome 8 with significantly different DNA copy number ratios between the different stages of colorectal cancer. Mean ratio values and range are given in the first four columns; Astler–Coller B1, Astler–Coller B2, Astler–Coller D primary tumor (D prim), and Astler–Coller D liver metastasis (D meta). In the last three columns of the table, *p* values are given for comparison of Astler–Coller B1 and B2 tumors (B1 vs B2), Astler–Coller B and D primary tumors (Bp vs Dp), and Astler–Coller D primary tumor and corresponding liver metastasis (Dp vs Dm)

Genes		Astler–Coller B1	Astler–Coller B2	Astler–Coller D prim	Astler–Coller D meta	B1 vs B2	Bp vs Dp	Dp vs Dm
		<i>n</i> = 10	<i>n</i> = 10	<i>n</i> = 12	<i>n</i> = 12			
MOS	8q11	1.1 (0.9–1.4)	1.4 (0.8–1.9)	1.4 (0.7–2.2)	1.5 (1.1–1.9)	<i>p</i> = 0.04	N.S.	N.S.
TPD52	8q21	1.1 (0.7–1.5)	1.4 (0.6–2.1)	1.6 (0.9–2.4)	1.9 (1.0–3.7)	N.S.	N.S.	<i>p</i> = 0.02
EIF3S6	8q22–q23	1.0 (0.4–1.7)	1.3 (1.0–2.8)	1.6 (0.8–3.8)	2.2 (1.1–5.6)	N.S.	N.S.	<i>p</i> = 0.007
MYC	8q24.12–q24.13	1.1 (0.9–1.7)	1.5 (1.1–1.9)	2.1 (1.2–4.4)	2.4 (1.1–5.0)	<i>p</i> = 0.007	<i>p</i> < 0.000	N.S.
DDEF1	8q24.1–q24.2	1.0 (0.8–1.2)	1.4 (0.8–1.9)	0.8 (0.6–1.1)	0.8 (0.5–1.2)	<i>p</i> = 0.004	<i>p</i> < 0.000	N.S.
SLA	8q24	1.0 (0.8–1.3)	1.2 (0.7–1.9)	2.0 (1.4–3.0)	2.3 (1.2–3.4)	N.S.	<i>p</i> < 0.000	N.S.
PTK2	8q24qter	1.0 (0.8–1.2)	1.4 (0.8–1.9)	1.9 (1.3–2.7)	1.9 (0.4–2.6)	<i>p</i> = 0.02	<i>p</i> < 0.000	N.S.
PTP4A3	8q24.3	1.1 (0.7–1.7)	1.5 (0.6–2.1)	1.9 (1.0–2.9)	2.0 (1.1–3.4)	<i>p</i> = 0.04	<i>p</i> = 0.002	N.S.
RECQL4	8q24.3	1.1 (0.7–1.6)	1.3 (0.6–1.9)	1.6 (1.0–2.3)	1.6 (0.6–3.0)	N.S.	<i>p</i> = 0.01	N.S.

N.S. = non-significant.

Gain of chromosome band 8q23–24 was more often seen in metastasized tumors than in the non-metastasized tumors, indicating that amplification of genes in this region appears to affect the metastatic potential of tumor cells [6].

For one of these genes, PTP4A3 (8q24.3), expression levels have previously been shown to be higher in metastatic than in non-metastatic tumors [14]. PTP4A3 is a tyrosine phosphatase associated with several membrane structures involved in cell movement and PTP4A3

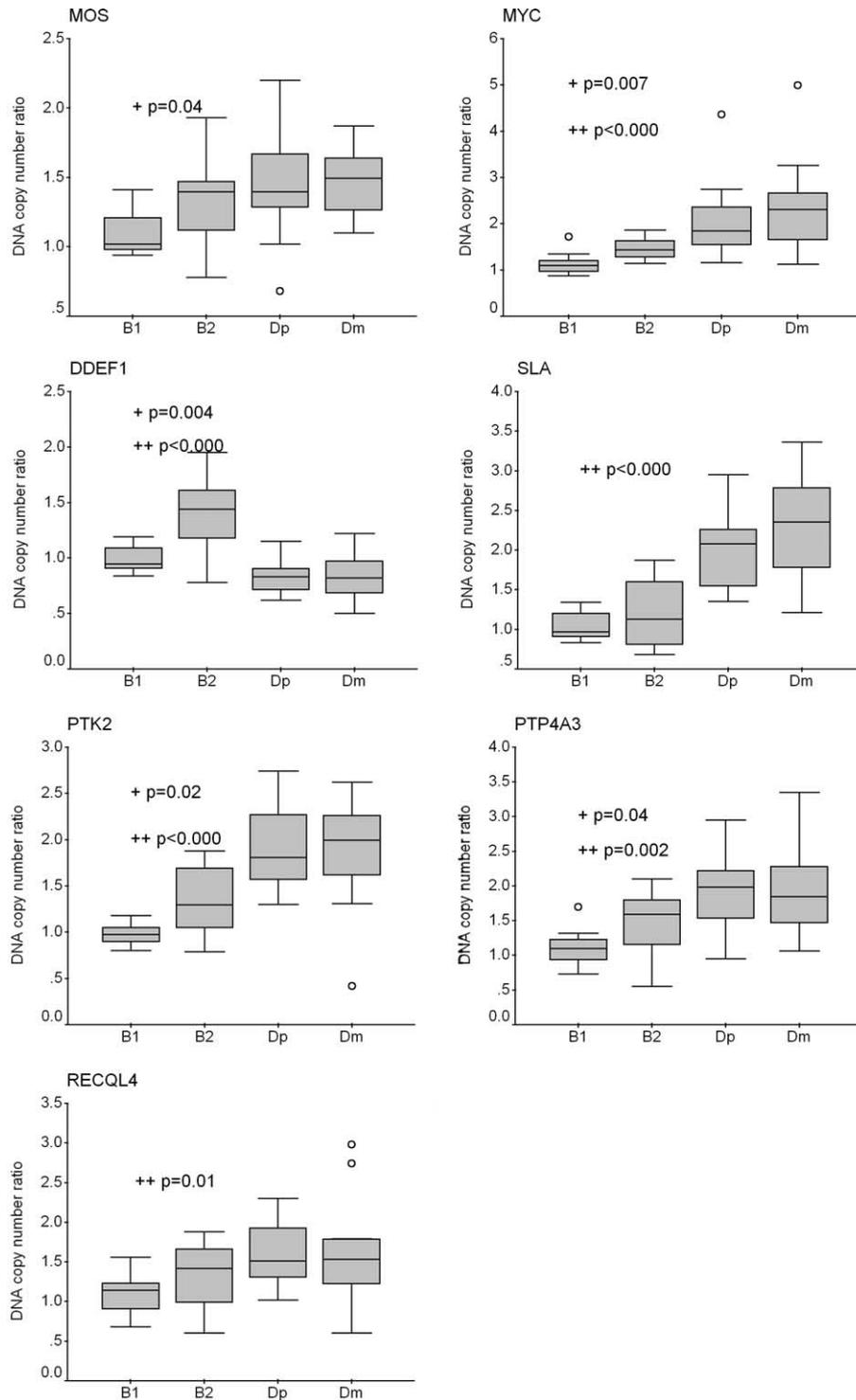


Fig. 4. Box plots of DNA copy number ratios of 8q genes that differ significantly between different stages of colorectal cancer. The central box covers the middle 50% of the data values, between the upper and lower quartiles. The line across the box indicates the median. The whiskers extend from the box to the minimum and maximum values with the exception of outliers, that are marked by circles. + Significant differences between Astler–Coller B1 and B2 tumors; ++ significant differences between primary Astler–Coller B and D tumors.

expressing cells have been shown by Zeng et al. [30] to migrate much faster than control cells. This PTP4A3 motility-enhancing activity has been coupled to its phosphatase action site. In the same study [30], of 18 colorectal cancer metastases, PTP4A3 was the only gene out of 144 genes that was over-expressed in all metastases, undetectable in normal colorectal epithelial and intermediately expressed in advanced primary colorectal cancers. This suggests the possibility that an excess of PTP4A3 phosphatase is a key alteration contributing to tumor metastases [30]. The present study demonstrates that PTP4A3 is frequently changed at the DNA level, suggesting that gene amplification is the mechanism for overexpression.

Amplification and overexpression of c-myc, located on chromosome band 8q24.12–q24.13, is frequently observed in many malignancies. C-Myc is a transcription factor which promotes cell proliferation and transformation by activating growth promoting genes and repressing growth arrest genes [13]. Previous studies have shown amplification and overexpression of this gene in the majority of primary colorectal carcinomas, especially in the more advanced tumor stages [23], and the present study confirms this.

Since gain of a broader chromosome region than 8q24.3 alone has been demonstrated previously [6, 8], we hypothesized that in addition to PTP4A3 and c-myc, other genes in this area are involved in the metastasizing process. Indeed we found that a number of other genes located at 8q23–24, i.e. PTK2, SLA, and RECQL4, showed significantly higher DNA copy numbers in Astler–Coller D than in stage B colorectal cancers.

PTK2, a cytoplasmic tyrosine kinase located on 8q24qter, was amplified in 11 out of 12 metastasized colorectal cancers examined. PTK2 is stimulated in response to cell interactions with extracellular matrix components and by exposure to a variety of agonists, including neuropeptides [5]. Interaction between PTK2 and p130-Cas, a crk-associated tyrosine kinase substrate, is suggested to be a key element in integrin-mediated signal transduction and to represent a direct molecular link between the Src and Crk oncoproteins [21]. Since this tyrosine kinase is associated with these oncogenes [10], it seems likely that activation of Src and Crk also elevates levels of PTK2.

Src-like adaptor (SLA), located on 8q24, showed significantly higher DNA copy number ratios in stage D than in stage B carcinomas. It is a negative regulator of T cell receptor signaling, and has been shown to be involved in down regulating T and B cell mediated im-

mune response [27]. Escape from the immune system is a prerequisite for metastatic tumor cells to survive and SLA might therefore be involved in the metastasizing process of colorectal cancer.

RECQL4 (RECQ protein-like 4), located on 8q24.3, encodes a DNA helicase. RecQ helicase genes are known as caretakers of the genome and are assumed to maintain genomic stability by functioning at the interface between DNA replication and DNA repair. A germline mutation of this gene gives rise to a rare autosomal-recessive disorder, the Rothmund–Thomson syndrome, which is associated with an elevated incidence of cancer, especially mesenchymal tumors [9, 11]. Cells derived from individuals with this disorder, show inherent chromosomal instability [11,17]. In the present study, two different probes were used for detecting RECQL4. In some Astler–Coller B tumors and most Astler–Coller D tumors, a gain of this gene was observed with both probes, which seems contradictory to the function of this gene as caretaker of the genome. Although an amplification of this region is seen in the present study, as seen in previous studies [6,18], these findings might indicate that RECQL4 has an additional function, gain of which could be associated with tumor metastasis. Another possible explanation might be that although this gene region is amplified, expression of this gene is not affected.

Statistically significant differences were not only seen between stage B and D primary tumors, but also between B1 and B2 tumors. Most of the chromosome 8 genes analyzed showed comparable DNA copy number levels in the two groups, but copy number ratios of the human proto-oncogene MOS, located on 8q11, were significantly higher in B2 primary tumors compared to B1 primary tumors ($p = 0.04$). This gene is known as the cellular counterpart of the viral oncogene v-mos isolated from Moloney murine sarcoma virus. It is a component of the mitogen-activated protein kinase transduction pathway and known to be involved in the control of meiosis en mitosis [19]. Given the fact that an increase in MOS DNA copy number ratio is seen between stage B1 and B2, this gene may be involved in an early step in progression of colorectal cancer.

The present study also aimed to detect any genes within the chromosome 8q region that would show different copy number ratios between the primary tumors and the corresponding liver metastases. For most genes, no such differences were observed (Table 1). Only two genes on chromosome 8, TPD52 and EIF3S6, showed significantly higher levels in the liver metastases than in the corresponding primary tumors ($p = 0.02$ and $p = 0.007$, respectively).

TPD52, tumor protein D52, is located on chromosome 8q21, and is thought to be involved in a calcium-sensitive signaling pathway mediating or associated with cellular proliferation. At the protein level, it has been found to be overexpressed in approximately 40% of breast carcinomas [2]. In the present study, amplification of this gene was observed in two thirds of the metastasizing tumors examined, which could indicate that TPD52 might play a role in the metastasizing process.

EIF3S6, also known as Int 6, is located on the 8q22–q23 region. It encodes for the subunit 6 of the eukaryotic translation initiation factor 3 (eIF3) [16]. Cells with an Int 6 deletion, have been shown to grow more slowly than wild type cells, which implicates that Int 6 is required for maintenance of a normal growth rate [3]. In the present study, amplification of this gene was more often seen in the metastasizing tumors compared to non-metastasizing tumors, although this difference did not reach a significant level ($p = 0.2$). However, in stage D tumors, a significantly higher amplification is seen in the liver metastases compared to the primary tumor (1.6 versus 2.2, $p = 0.007$), implicating a possible role in the metastasizing process of colorectal cancer.

The finding that only two genes, located on 8q21–23 and not on 8q24, were significantly different between the primary metastasizing tumor and the metastatic lesion suggests that the metastatic potential is determined in early stages of tumor development, and is already determined in the genomic profile of the primary tumor [22]. According to the classic paradigm, progression from premalignant to invasive tumor and subsequent metastasis is caused by, or at least associated with, an ongoing accumulation of genomic changes and subsequent selection of proficient subclones of tumor cells [4]. On the other hand, recent microarray based multi-variable studies suggest that metastasizing and non-metastasizing tumors have different genomic profiles throughout [29]. The present findings are consistent with the latter theory, although apparently copy number changes can still occur between primary tumors and their metastases.

The question remains whether all these amplified genes are important for tumor growth, or that some genes merely are co-amplified together with neighboring genes that are crucial, and drive the selection of the amplification. Knocking out the expression of the individual genes in a model system and evaluating the effect of this intervention on the tumorigenic phenotype could provide some answers in this respect.

In summary, we present a detailed analysis of DNA copy number changes of 25 genes on chromosome 8 in a series of locally confined as well as metastatic colorectal cancers using multiplex ligation-dependent probe amplification (MLPA). In addition to PRL3 and c-Myc, three other genes (PTK2, SLA and RECQL4) showed significantly higher DNA copy number levels in the stage D tumors compared to stage B tumors. Moreover, for TPD52 and EIF3S6 higher DNA copy number levels were found in the liver metastases than in the corresponding primary tumors.

Since metastatic colorectal cancer frequently leads to death of the patient, it is of clinical interest to predict the metastasizing potential of the primary tumor. Genomic profiling of colorectal cancer could be helpful in this respect and distinguish a subgroup of patients with high risk of developing liver metastasis. This may prove to be clinically highly relevant for determining the indication for adjuvant chemotherapy in colorectal cancer patients. In this respect, the fact that MLPA is a cost effective method suitable for high throughput molecular analysis of tumors samples in a routine diagnostic setting, is a particular advantage that could facilitate clinical implementation of this test.

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