

Deletion of chromosome 4q predicts outcome in stage II colon cancer patients

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Abstract. *Background:* Around 30% of all stage II colon cancer patients will relapse and die of their disease. At present no objective parameters to identify high-risk stage II colon cancer patients, who will benefit from adjuvant chemotherapy, have been established. With traditional histopathological features definition of high-risk stage II colon cancer patients is inaccurate. Therefore more objective and robust markers for prediction of relapse are needed. DNA copy number aberrations have proven to be robust prognostic markers, but have not yet been investigated for this specific group of patients. The aim of the present study was to identify chromosomal aberrations that can predict relapse of tumor in patients with stage II colon cancer.

Materials and methods: DNA was isolated from 40 formaldehyde fixed paraffin embedded stage II colon cancer samples with extensive clinicopathological data. Samples were hybridized using Comparative Genomic Hybridization (CGH) arrays to determine DNA copy number changes and microsatellite stability was determined by PCR. To analyze differences between stage II colon cancer patients with and without relapse of tumor a Wilcoxon rank-sum test was implemented with multiple testing correction.

Results: Stage II colon cancers of patients who had relapse of disease showed significantly more losses on chromosomes 4, 5, 15q, 17q and 18q. In the microsatellite stable (MSS) subgroup ($n = 28$), only loss of chromosome 4q22.1–4q35.2 was significantly associated with disease relapse ($p < 0.05$, FDR < 0.15). No differences in clinicopathological characteristics between patients with and without relapse were observed.

Conclusion: In the present series of MSS stage II colon cancer patients losses on 4q22.1–4q35.2 were associated with worse outcome and these genomic alterations may aid in selecting patients for adjuvant therapy.

Keywords: DNA copy number changes, stage II, colon cancer, prognosis

Abbreviations

CGH	Comparative Genomic Hybridization;
FFPE	Formalin fixed paraffin embedded;
MSS	Micro satellite stable;
MSI	Inmicro satellite instable;
CRC	Colorectal cancer.

1. Introduction

Worldwide, colorectal cancer (CRC) is the third most common cancer affecting more than 940,000 pa-

tients annually. Nearly 500,000 patients die from the consequences of CRC each year (www.who.int) [43]. In The Netherlands in 2006, 11,231 patients were diagnosed with CRC and 4709 CRC related deaths occurred [40]. The incidence of CRC is relatively high in the western world and steadily increases each year [40]. Two thirds of all CRCs occur in the colon and one third is located in the rectum. Patients with colon tumors are treated differently and have a different prognosis compared to rectal cancer patients.

Staging is traditionally based on pathologist's evaluation of tumor extent, lymph nodes and distant metastasis, all included in the TNM classification [34] and therapeutic decisions are based on this system. Upon resection of the primary tumor, some 40% of all colon cancers appear to be stage II (TNM/UICC) [34]. Of

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patients with stage II colon cancer 20–30% will relapse, i.e. develop distant metastasis, and these patients will die of their disease. Five-year survival rates for stage II colon vary between 50% and 90%. Patients with stage III colon cancer will face relapse of tumor during follow-up in 60% of cases and chemotherapy can reduce recurrence rates with 31–41% [45]. Although postoperative chemotherapy is standard for stage III colon cancer it is not for stage II colon cancer [5,14]. Nevertheless, it has been shown that subgroups of patients with stage II colon cancer can benefit from adjuvant chemotherapy [24]. In fact, high-risk stage II colorectal cancer patients have a worse prognosis than single node positive stage III colorectal cancer patients [28]. Better prediction of relapse as an indication for the need of adjuvant chemotherapy is thus required for stage II colorectal cancer [5]. Histopathological and clinical variables are most frequently used for identifying high-risk stage II colon cancers. Peritoneal involvement, extramural vascular invasion, tumor perforation, male gender, bowel obstruction, number of nodes harvested and presence of lymph node micro metastasis have all been identified as negative prognostic factors in stage II colon cancer [16,22,30]. Notwithstanding this extensive body of work, these histopathological factors have not yet provided an established basis for accurately identifying patients at high-risk for relapse in a clinical setting. Molecular markers can help to select stage II colon cancer patients with worse prognosis. Gene expression profiles have been able to identify stage II colon cancer patients at high-risk for relapse [4,15]. However, RNA is an instable molecule and its expression subject to environmental and circadian rhythms. In contrast, DNA is more stable and can be obtained for copy number analysis from formalin fixed paraffin embedded (FFPE) material which is frequently the only material available in routine clinical practice. DNA copy number aberrations are a hallmark of cancer. These chromosomal aberrations are less frequent in micro satellite instable (MSI) tumors compared to micro satellite stable (MSS) tumors. The latter group is also referred to Chromosomal Instable (CIN) and accounts for 85% of all colorectal tumors. MSS CRCs have a worse outcome than MSI CRCs [41].

In CRC, chromosomal aberrations, important for tumor progression and prognosis, have been studied [12, 18–20,23,29,32], but to which extent specific chromosomal changes have prognostic value specifically in stage II colon cancer patients has only been analyzed in limited detail. Therefore, the present study analyzed genome wide DNA copy number changes using high

resolution oligonucleotide based array CGH for identifying chromosomal aberrations that may be used as prognostic markers for patients with stage II colon cancer.

2. Materials and methods

2.1. Patient and sample selection

Forty patients operated between 1990 and 2000 for stage II colon cancer (pT3 or pT4, pN0, pM0, R0 TNM classification, fifth edition [34]) were selected for this study, 16 with and 24 without relapse. Seventeen patients underwent resection of their primary tumor at the John Goligher Colorectal Unit, Leeds General Infirmary (UK) and 23 patients at the Zaans Medical Centre (The Netherlands). Medical records of all patients were reviewed retrospectively to obtain clinical data, patient characteristics and follow-up data. None of the patients received postoperative chemotherapy or radiotherapy. Tumor relapse was defined as the occurrence of distant metastasis, confirmed by ultrasound, CT scan and/or histology within 36 months. Haematoxylin and eosin (H&E) stained sections were reviewed by a pathologist for TNM tumor stage [34], differentiation grade, number of nodes, assessed tumor perforation, extramural vascular invasion (EMVI) and peritoneal involvement. All samples were used in compliance with the respective institutional ethical regulations for surplus material and use of material from Leeds General Infirmary (UK) was approved by the Leeds (West) research ethics committee, unique identifier CA02/014.

2.2. DNA isolation from formalin-fixed and paraffin-embedded (FFPE) tissue blocks

DNA was isolated from FFPE colon cancer tissue of 40 samples. Corresponding normal mucosa DNA was isolated from 37 of the samples as a reference and was obtained from the resection margins or at least 1 cm distance from the tumor. For 3 cases no normal mucosa was available and a pool of reference DNA isolated from blood obtained from eighteen healthy males was used. For each tumor an area containing at least 70% of tumor cells was marked on the slides. Of the FFPE blocks 4–6 10 µm sections were cut, deparaffinized and macro dissected. Isolation of DNA was performed as previously described [42], including incubation with sodium thiocyanate (1 M, CNNaS), proteinase K treatment and purification using a column based method (QIAamp microkit; Qiagen, Hilden, Germany).

2.3. Microsatellite stability

All samples were analyzed for Microsatellite instability (MSI) using MSI Analysis System, version 1.1 according to manufacturer's instructions (Promega, Madison, WI, USA). This PCR based assay is using 5 mononucleotide markers to determine MSI status. PCR products were separated by capillary electrophoresis using ABI 3130 DNA sequencer and output data were analyzed using the accompanying package GeneScan 3100 (Applied Biosystems, Foster City, CA, USA). Tumors were classified as micro satellite instable (MSI) when instability of two or more markers was seen. When a single or no instable markers were seen, tumors were considered as micro satellite stable (MSS).

2.4. Chromosomal copy number analysis by array comparative genomic hybridization (array CGH)

Labeling and hybridization was done as previously described [6]. Briefly, 500 ng of genomic DNA of tumor and reference was labeled with either Cyanine 3-UTP (Cy3) or Cyanine 5-UTP (Cy5) nucleotide mixture, respectively, according to manufacturer's instructions (CGH labeling Kit for Oligo Arrays, Enzo Life Sciences, Farmingdale, NY, USA) and purified using the QIAquick PCR Purification Kit (Qiagen, Westburg, Leusden, The Netherlands). Labeled tumor and (matched) reference DNAs were mixed prior to hybridization onto Agilent 4 × 44 K oligonucleotide arrays (Agilent Technologies, Palo Alto, CA, USA). Each slide consists of 4 arrays, each containing 45,220 *in situ* synthesized 60-mer oligonucleotides representing 42,494 unique biological features distributed over the genome. Immediately after hybridization the slides were scanned using microarray scanner G2505B (Agilent technologies, Palo Alto, CA, USA) and image analysis was performed using feature extraction software (version 9.1, Agilent Technologies, Palo Alto, CA, USA). The Agilent CGH-v4_91 protocol was applied using default settings. Oligonucleotides were mapped according to the human genome build NCBI 36 (May 2006). Of both Cy3 and Cy5 channels, local background was subtracted from the median intensities. The log₂ tumor to normal intensity ratio was calculated for each spot and normalized against the median of the ratios of all autosomes.

2.5. Statistical analysis of genomic profiles

Analysis of array CGH data was done in the statistical computing language R, version 2.6.1 (<http://www.r-project.org>). Chromosomal copy number losses and gains were identified using the package CGHcall [37] with cellularity set to 0.7 and median normalization. To reduce the dimension of the array CGH data set without loss of information, regions were defined as previously described [39].

To calculate significance of DNA copy number differences between patients who did and did not relapse, a Wilcoxon–Mann–Whitney two sample test was used, implemented in the software package CGHmultiarray, that provides multiple comparison correction [38]. Differences were analyzed for all patients and for MSS colon cancer patients separately. *p*-values of <0.05 and false discovery rates, i.e. the expected proportion of false positives among the DNA copy numbers claimed to be correlated with outcome, of <0.15 were considered to be statistically significant [13].

Statistical analysis of associations with histopathological and clinical variables was performed using SPSS software (SPSS for Windows, version 15.0, SPSS Inc., Chicago, IL, USA). Mann–Whitney *U*-test and chi-square test were used for analyzing differences in continuous or categorical variables, respectively. For correlations with overall survival (OS) and disease free survival (DFS) univariate survival analysis with log rank statistics was used and Kaplan–Meier curves were constructed. To determine independent effects of clinical variables and DNA copy number changes, multivariate Cox proportional hazard analysis was performed. OS and DFS were defined as time from surgery to date of death due to all causes or to date of first evidence of distant metastasis. *p*-values <0.05 were considered as statistically significant.

3. Results

3.1. Patient and tumor characteristics

Patient and tumor characteristics of 40 stage II colon cancer patients are listed in Table 1. Twenty-eight tumors were MSS and 12 MSI. Relapse of tumor occurred in 14 MSS patients and 2 MSI patients (*p* = 0.05). No significant differences in age, gender, number of nodes assessed, differentiation grade, T-stage and other histopathological variables between patients who relapsed and who did not were seen (Table 1).

Table 1
Patients and tumor characteristics of stage II colon cancers ($N = 40$)

	No relapse ($n = 24$)	relapse ($n = 16$)	
Mean age (years) (range)	72 (49–91)	73 (54–90)	ns [§]
Mean number of nodes assessed (range)	8.5 (3–17)	7.7 (1–22)	ns [§]
Gender			
Female	12	10	ns [§]
Male	12	6	
Location			
Right-sided*	18	10	ns [§]
Left-sided [#]	6	6	
T-stage			
T3	23	15	ns [§]
T4	1	1	
Differentiation			
Poor	2	3	ns [§]
Moderate	22	13	
Tumor perforation			
Yes	0	0	ns [§]
No	24	16	
Peritoneal involvement			
Yes	1	1	ns [§]
No	23	15	
EMVI			
Yes	2	2	ns [§]
No	9	4	
Unknown	13	10	
MSI			
Yes	10	2	0.05 [§]
No	14	14	

Notes: *Right (caecum, ascending colon, transverse colon, hepatic flexure); #left (splenic flexure, descending colon, sigmoid colon); EMVI – Extramural vascular invasion; Ns – not significant; [§]Mann–Whitney U -test; [§]chi-square.

Mean follow-up time for all patients was 73 months (range 2–155). A trend was seen for DFS of MSI patients being better compared to MSS patients, although this was not significant (5-year DFS 80% vs. 50%, $p = 0.06$) (Fig. 1). Survival time for MSS patients with ($n = 14$) or without ($n = 14$) relapse was 23 months (range 2–59) versus 104 months (range 5–155), respectively.

3.2. DNA copy numbers aberrations in MSS and MSI stage II colon cancer patients

CGH profiles of 40 stage II colon cancers patients showed copy number aberrations consistent with those reported in literature [7,8,12,35,36]. The mean frac-

tion of the genome altered was 15.3% (range 0–44%), and was significantly higher for MSS tumors (19.4%, range 0–44%) compared to MSI tumors (5.9%, range 0–18%) ($p < 0.001$). The amount of losses versus gains was balanced in the MSS tumors, but MSI tumors showed more gains than losses; gains 4.8% (0–16.2%) versus losses 1.1% (0–3.1%). Gains in MSI tumors predominately occurred at 8q24.3 (4 out of 12) and 9p21.3 (4 out of 12) and losses at 6p22.1 (4 out of 12), 16p13.2 (5 out of 12) and 17p13.1 (3 out of 12). For MSS tumors the highest frequency of aberrations (in more than 12 out of 28 patients) were gains of chromosome 7p22–p11, 7q11, 7q22, whole chromosome 13 and 20 and losses of chromosome 8p23.1–p12, 15q13.3–q26.1, 17p13.2–p11.2 and 18p11.3–q22.3. DNA copy number changes of MSI and MSS tumors are summarized in Fig. 2A and B.

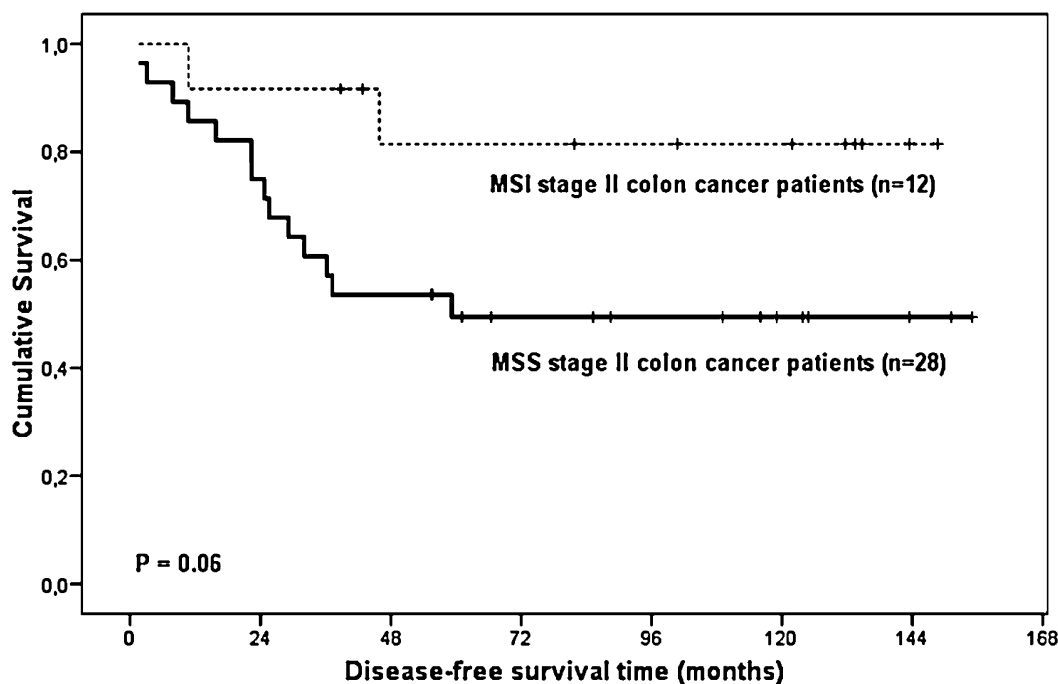


Fig. 1. Kaplan–Meier analysis of disease-free survival for 40 stage II colon cancer patients stratified by MSI/MSS status. MSI colon tumors showed a trend towards better disease-free survival compared to MSS patients ($p = 0.06$).

3.3. Differences in DNA copy numbers aberrations between stage II colon cancer patients with and without relapse

Overall, significant differences were observed between patients without or with relapse, with 11.2% vs. 21.4% of the genome altered ($p = 0.005$). This was primarily reflected in the amount of losses which was 10.9% of the genome for the patients which relapsed versus 3.9% for the patients which remained disease free ($p = 0.002$). The chromosomal region(s) which significantly differed between patients with or without relapse were losses on chromosome 4p16.1–p12 (30 Mb), 4q12–q35.2 (127 Mb), 5p14 (9 Mb), 5q11.2–q14.2 (21 Mb), 5q32–q34 (0.86 Mb), 15q11.2–q26.1 (21 Mb), 17q21.1 (0.48 Mb), 18q12.1–q12.3 (15 Mb) and 18q21.1–q22.13 (6 Mb). No significant difference was observed in the amount of gains, 7.3% of the genome altered for patients who did not relapse versus 10.5% for patients that did ($p = 0.11$).

Also among the 28 MSS patients, more aberrations were observed in patients who relapsed ($p = 0.03$), being mainly losses. Loss of chromosome 4q22.1–4q35.2 (90.5 Mb) was the only significant aberration, being more frequently deleted in patients with relapse of tumor. Loss of 4q22.1–4q35.2 was seen in 43–50%

($p = 0.01$ to $p = 0.004$) of patients who relapsed, depending on which boundaries for the lost region were used. An overview of DNA copy number changes in MSS patient who either relapsed or not, is presented in Fig. 3. The number of MSI patients was too small to further stratify and analyze differences between either or not relapse occurred.

Both OS and DFS were significantly worse for patients with loss of chromosome 4q22.1–4q35.2 or part of that region. Considering any loss on chromosome 4q22.1–4q35.2 predicted worse OS and DFS survival (5-year OS 70% vs. 5%, $p < 0.001$; 5-year DFS 75% vs. 5%, $p = 0.004$) (Fig. 4). Cox proportional hazard model demonstrated that any loss on chromosome 4q22.1–4q35.2 was an independent prognostic factor for worse DFS (HR = 15.4, $p < 0.001$). Age >72.5 year (HR = 6.7, $p = 0.04$) and any loss on chromosome 4q22.1–4q35.2 (HR = 16.9, $p < 0.001$) were independent prognostic factors for OS.

4. Discussion

A substantial part of stage II colon cancer patients currently develop distant metastasis after resection of their primary tumor and subsequently die of their dis-

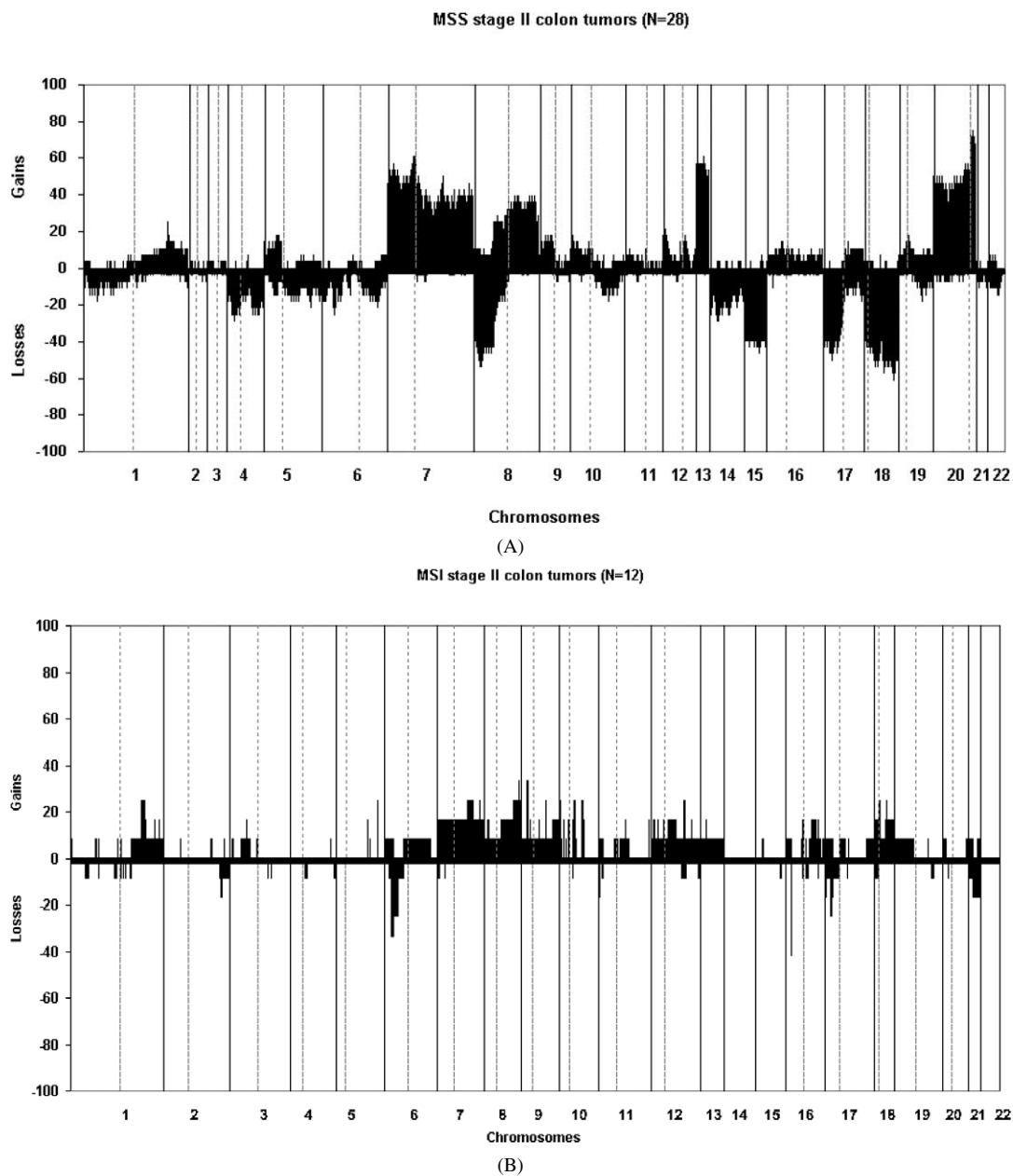


Fig. 2. Percentage of chromosomal gains and losses measured by array CGH in (A) 28 MSS and (B) 12 MSI stage II colon cancers patients. X-axis displays 44,000 oligo-nucleotides in genomic order (chromosomes 1–22). Y-axis displays percentage of tumor with gains (>0) or losses (<0). Boundaries of chromosomes are indicated by black vertical lines and locations of centromeres are indicated by dotted lines.

ease. Nevertheless, the American Society of Clinical Oncology (ASCO) [5] and the National Comprehensive Cancer Network (NCCN) [44] independently recommended against routine administration of adjuvant therapy in stage II disease, due to a lack of evidence for the benefit of survival [5,14,17,24,45]. Notwithstanding, it has been suggested that subgroups of stage II

patients will benefit from adjuvant therapy. At present no objective parameters for selecting these patients are established [10,28]. Hence, objective and robust prognostic markers are desirable to select and stratify stage II colon cancer patients at high-risk for relapse. The present study found in stage II colon cancer patients deletion of chromosome 4q to be associated with

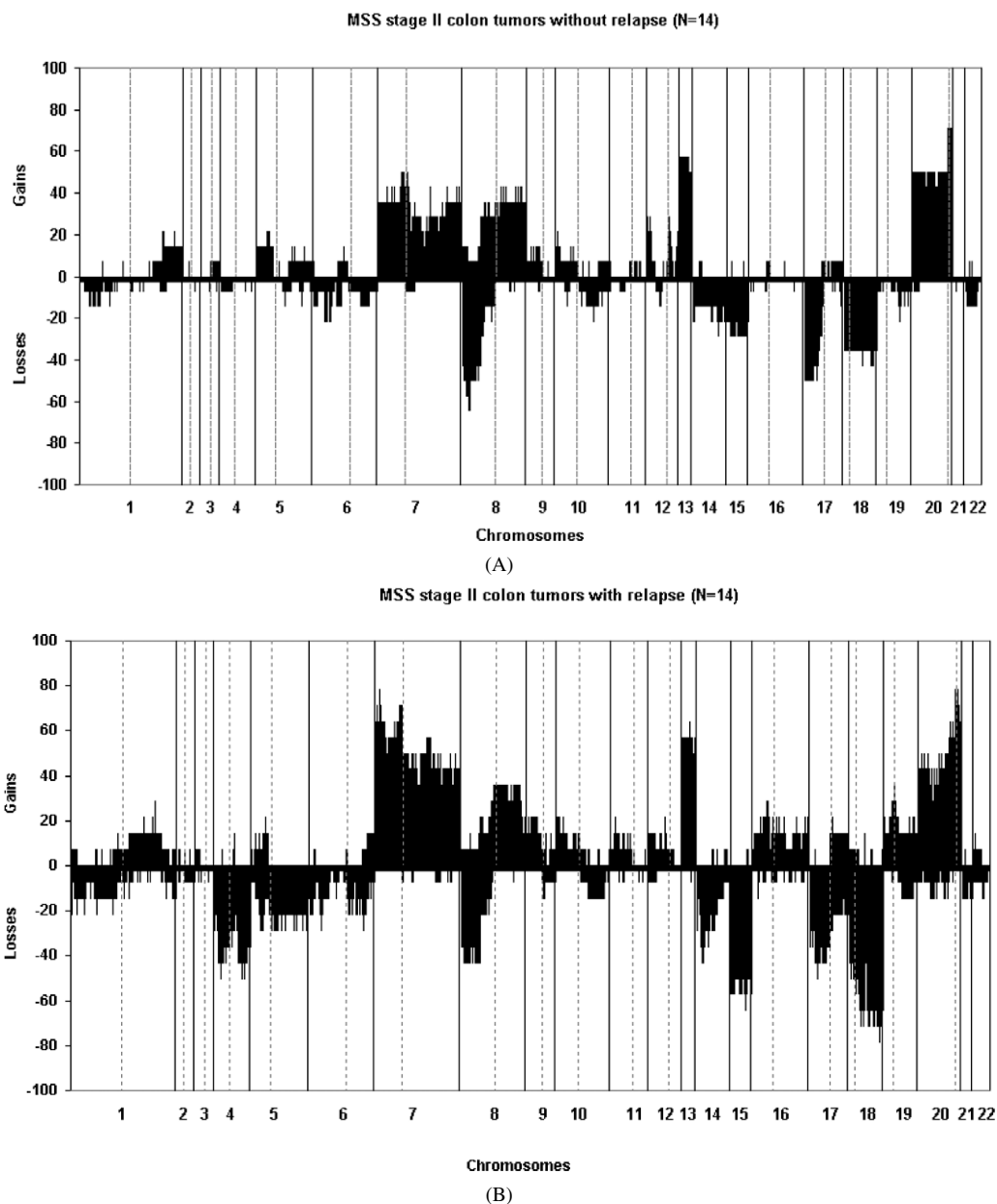


Fig. 3. Percentage of chromosomal gains and losses measured by array CGH in 28 MSS stage II colon cancer patients without (A) and with (B) relapse of disease. X-axis displays 44,000 oligo-nucleotides in genomic order (chromosomes 1–22). Y-axis displays percentage of tumor with gains (>0) or losses (<0). Boundaries of chromosomes are indicated by black vertical lines and the location of centromeres are indicated by dotted lines.

relapse of disease. This conclusion is consistent with literature [1–3,11,12,19] and suggests that it could be worthwhile further exploring whether patients with loss of chromosome 4q should be considered for adjuvant therapy.

Moreover, in the present study significantly more losses on chromosome 4, 5, 15q, 17q and 18q were

seen in tumors of patients who had cancer relapse. This observation is validated by the results published by Sheffer et al. who linked worse prognosis to a simultaneous deletion of 4p,15q and 18q in colorectal cancer [32]. Forty percent (12 of 40) of tumors were MSI and had 5.9% of their genome altered, which were primarily gains (4.8%). This is in accordance with re-

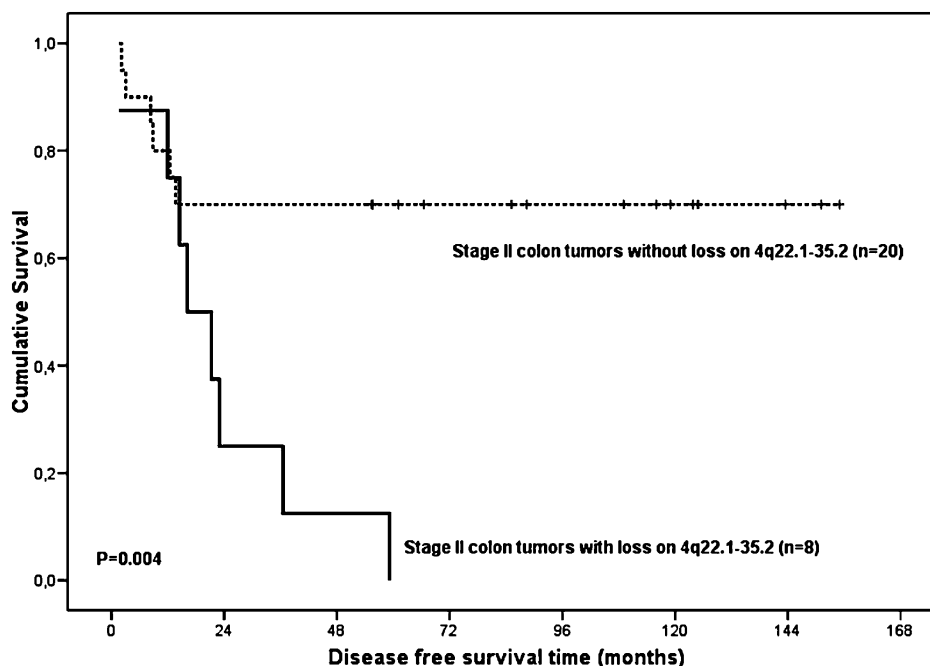


Fig. 4. Kaplan-Meier disease-free survival plot of 28 MSS stage II colon cancer patients stratified for any loss of chromosome 4q22.1–q35.2. Stage II colon tumors with any loss on chromosome 4q22.1–q35.2 showed worse disease-free survival than without losses on 4q22.1–q35.2 ($p = 0.004$).

sults reported by Trautman et al. who reported 2.3% gain versus 0% loss in 23 MSI and 17.2% gain versus 19.9% loss in 23 MSS sporadic colon cancers analyzed by BAC array CGH [35].

When MSS patients were analyzed, only losses in the region of 4q22.1–35.2 occurred significantly more frequent in patients who relapsed compared to those who did not. Losses of 4p, 5p, 5q, 15q, 17q and 18q were associated but not significantly related to relapse when only MSS patients were analyzed. A likely explanation for this could be a limited sample size and hence lack of power to detect associations with, e.g., 18q loss at a statistically significant level. Loss of 18q as prognostic marker in stage II CRC have been reported previously based on loss of heterozygosity analysis [21,25]. As no aberrations on chromosome 4 were seen in MSI tumors, loss of 4q appears particularly relevant for predicting relapse in patients with stage II MSS colon tumors.

Numerous studies have analyzed the relation of DNA copy number changes and clinical outcome in colorectal cancer patients. In only a small number of studies the prognostic value of chromosomal aberrations limited to patients with early stage colon cancer have been analyzed. In 70 stage I and II MSI and MSS colorectal cancer patients, loss of chromosome

4p was an independent prognostic factor, and loss of chromosomes 4 and 14q were associated with a worse prognosis [2]. This study used BAC array CGH and reported minimally deleted regions at 4q24–28 and 4q32–35, which is consistent with the findings in the present study [2]. Additional detailed analysis of this group of patients using low resolution loss of heterozygosity (LOH) analysis showed that in particular loss of 4p16, 4q31.1 and 4q33.1 was related to prognosis, further validating our findings [1]. When chromosomal aberrations were studied with CGH in stages I–IV colorectal tumors, loss of chromosome 4q resulted in shorter survival times [11], loss of 4q32–34 was associated with metastasis [3] and loss of 4q35 and 4q31.3 correlated with shorter disease-specific survival [19]. A meta-analysis of 31 CGH studies encompassing copy number profiles of 373 colorectal tumors and 102 liver metastasis, showed loss of chromosome 4 to be related to progression from primary tumor to distant metastasis [12]. Loss of chromosome 4q in relation to survival does not seem to be CRC specific, but has also been reported for other epithelial tumors; i.e., bladder [31], head and neck [9], prostate [26] and breast cancer [33]. Deletion of 4q has also been associated with micro metastasis in lung cancer [46].

The critical region 4q22.1–35.1 encompasses 90.5 Mb of chromosomal region with 265 coding genes. The size of the region thus makes it difficult to pin-point the candidate tumor suppressor genes or non-coding RNA. Integration of array CGH and gene expression array or extensive mutation analysis by next generation sequencing could possibly further narrow down the number of candidate driver genes.

In summary, loss of chromosome 4q22.1–35.1 may predict relapse of disease in stage II colon cancer patients. Chromosome 4q may thus have important prognostic value for patient tailored therapy.

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