Revised version

A copy number gain of the 6p arm is linked with advanced hepatocellular carcinoma

Yasuyo Choch[1],
Shigeto Kawauchi[1], Motonao Nakao[1], Tomoko Furuya[1],
Kiichiro Hashimoto[2], Atunori Oga[1], Masaaki Oka[2], and Kohsuke Sasaki[1]*

Departments of Pathology[1] and Surgery[2],
Yamaguchi University Graduate School of Medicine, Ube 755-8505, Japan

*Correspondence: Kohsuke Sasaki, M.D., Department of Pathology, Yamaguchi University Graduate School of Medicine, Ube 755-8505, Japan
Phone: +81-836-222222, Fax: +81-836-222223
e-mail: kohsuke@yamaguchi-u.ac.jp

Competing interests

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Keywords

Hepatocellular carcinoma (HCC), array CGH, Pathological stage, genomic stage, DNA copy number aberrations (DCNAs)

Abstract

Background: With tumor progression, genomic aberrations accumulate in cancer cells in a stepwise fashion. However, it remains not been clarified whether there are genomic changes linked with tumor progression. The purpose of this study is to elucidate the relationship between genomic alterations and disease stage in hepatocellular carcinoma (HCC). **Methods:** A technology of array CGH with DNA chips spotted with1440 BAC clones was applied to 42 surgically removed HCCs to examine the DNA copy number aberrations. **Results:** A frequent copy number gain was detected on chromosomal regions 1q, 8q and Xq. In particular, gains of 1q42.12, 1q43 and 8q24.3 were detected in more than 65% of tumors. A frequent copy number loss was detected on chromosomal regions 1p, 4q, 6q, 8p and 17p. Losses of 8p21, and 17p13 were detected in more than 55% of HCCs. However, the DNA copy number gains of clones on 6p and 8q24.12 were more frequent in stage-III /IV tumors than in stage-I /II tumors ($p<0.001$). In particular, the gain of the whole 6p was virtually limited to advanced-staged HCCs. **Conclusion:** The gain of the whole 6p is suggested as a genomic marker for the late stages in HCCs. These observations therefore support the concept of genomic staging in HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world and approximately 600,000 patients die of HCC every year [1]. Now, the incidence of HCC is rising even in Western countries [2]. The strong association of HCC with chronic liver diseases such as viral hepatitis B and C is well recognized. An accurate diagnosis and staging are critical issues in not only estimating the prognosis for each HCC patients but also determining an optimal treatment.

HCCs as well as other solid tumors develop and progress as a consequence of the stepwise accumulation of genetic alterations and subsequent clonal selection. It is also though that the genetic aberrations accompanied by hepatocarcinogenesis occur in a specific order [3]. This means that we can depict a genetic pathway to HCC development and progression. Many investigators have made attempts to identify the genetic alterations underlying hepatocyte carcinogenesis and furthermore to draft the genetic pathway to HCC [4]. However, the elucidation of a genetic pathway to HCC development and progression is not a simple matter, and information on the genetic pathway to hepatocarcinogenesis has been as yet limited [5]. In order to have a clue to the demonstration of the genetic pathway to HCC progression, a comprehensive analysis of the genomic alterations for each tumor with the known clinicopathological features is necessary. Although we applied the chromosomal comparative genomic hybridization (cCGH) to HCCs in order to have a sketchy description of the genomic detailed changes during the process of hepatocarcinogenesis [6][7], more detailed information regarding the genomic changes is necessary to identify the genomic alterations linked with HCC progression. Array-based CGH (aCGH) has a potential to draft a genetic pathway to HCC development and progression. However, the application of aCGH to human HCCs

is still limited and the relationship between the genomic changes and the stage of HCC remains to be clarified [8 – 11]. In this study, we identified late genomic events in HCC by aCGH. Advanced HCCs were characterized by DNA copy number gains of the whole 6p. The present study also supported the concept of a genomic stage as well as that of a pathological stage in HCC.

Materials and methods

Tumor Tissue Specimens and DNA Extraction

We examined 42 surgically removed HCCs in this study (**Table 1**). The patients consisted of 31 men and 11 women with an average age of 64 years (range, 39- 75 years). The patients were positive for the anti-HBV and/or anti-HCV antibody (6 HBV positive, 34 HCV positive, and 2 HBV and HCV positive). The pathological staging of these tumors was made pursuant to the tumor-node-metastasis (TMN) classification of the International Union against Cancer, and this series included 14 stage-I, 18 stage-II, 8 stage-III, and 2 stage-IV HCCs. In this study, we divided these tumors into three groups for convenience, stage-I, -II, and -III/IV. Histologiical differentiation was made according to 'The General Rules for the Clinical and Pathological Study of Primary Liver Cancer' (12). The tumor tissue specimens were stored frozen at -80˚C until use. The study protocol was approved by the Institutional Review Board for Human Use Yamaguchi University Graduate School of Medicine, and informed consent for this study was obtained from all patients. High-molecular-weight DNA was extracted from each tumor specimen with a DNA extraction kit (SepaGene, Sankojyunyaku Co., Tokyo, Japan) according to the manufacturer's instructions as previously described [6 – 9]. Control DNA was obtained from Promega (Madison, WI) and was used for reference.

Array CGH

The BAC DNA array used in this study consisted of 1440 human bacterial artificial chromosome (BAC) clones, including 356 cancer-related genes, which are spaced approximately 2.3Mb across the whole genome (Macrogen, Inc., Seoul, Korea). The BAC chip information together with information of end-sequenced BAC clones is provided through the following website: http://www.macrogen.co.kr/eng/biochip/karyo_summary.jsp. The experiments were performed as previously described [13–15]. Briefly, tumor DNA and gender-matched reference DNA (Promega, Madison, WI) were labeled with Cy5 and Cy3-dCTP (PerkinElmer Life Science, Inc.), respectively, with a random primer labeling kit (BioPrine® DNA Labeling System, Invitrogen™). For hybridization, labeled DNA was mixed with Cot-1 DNA (50 mg, Gibco BRL, Gaithersburg, MD) and ethanol precipitated. The precipitated DNA was dissolved in 40μl of hybridization mix. The probe mixture was denatured at 75˚C for 5 minutes and incubated at 37˚C for 60 minutes for blocking of repetitive sequences. The arrays were prehybridized with salmon sperm DNA to reduce nonspecific background staining. The probe mixture was applied to the array. The arrays were placed in a moist chamber at 37˚C for 72 h for hybridization. The array slides were washed 2 times in 2x standard saline citrate (2XSSC), 50% formamide, pH 7.0, at 45˚C. Then, the array slides were washed well in phosphate buffer with 0.1% NP-40, pH 8.0, in 2xSSC at room temperature.

Imaging and Analysis

After hybridization, the slides were scanned on a GenePix 4000A scanner (Axon Instruments, Union City, CA) and the 16-bit TIFF images were captured using the

GenePix Pro 5.0 software. The fluorescence images were analyzed with MAC Viewer™ software (Macrogen Inc.) optimized for analysis of the array as previously reported [13 – 15]. The fluorescence spots were defined with the automatic grid feature and adjusted manually. Then the ratio of the red/green channel of each clone was calculated and all CGH ratios were converted to log base 2 ($\log_2$ ratios). The clones with $\log_2$ ratios that exceeded ± 0.25 were considered gain and loss of the copy number. We defined a $\log_2$ ratio >1.0 as amplifications. In order to define chromosomal regions with DCNAs, the adaptive weights smoothing (AWS) procedure with the GLAD (gain and loss analysis of DNA) algorithm [16] was applied to the ratio data, as previously described [17].

Statistical analysis

The differences in the prevalence of common gains and losses between the groups were determined with the chi-square test. Differences in the total number of changes and frequency were tested by Student's *t*-test, Welch's *t*-test or nonparametric Mann-Whitney's U test. A difference was considered significant when the P-value was less than 0.05.

Results

Stage-I tumors consisted of well and moderately differentiated HCCs, and well differentiated HCCs were not detected in stage-III/IV tumors as shown in **Table 1**. In contrast, stage-III/IV HCCs consisted of moderately and poorly differentiated tumors, and poorly differentiated HCCs were not detected in stage-I tumors. However, the correlation coefficient between disease stage and histological differentiation was as low as 0.39.

The gain of 19q13.41 and loss of 4q21.22 were linked to histological differentiation of HCC ($P<0.01$) (**Table 2**). These aberrations were frequently detected in poorly differentiated HCCs but were not detected in well differentiated tumors (**Table 2**). However, the number of copy number gain and loss was not statistically affected by histological differentiation of HCC; 111.3 ± 53.2 and 127.5 ± 46.8 for well differentiated tumors, 140.8 ± 63.4 and 128.2 ± 73.6 for moderately differentiated tumors, and 134.1 ± 88.0 and 135.9 ± 69.9 for poorly differentiated tumors, respectively.

DNA copy number aberrations (DCNAs) were detected at various chromosome regions in all tumors. Although the number of DCNAs varied from tumor to tumor, the average number of clones affected was 264 per tumor; 135 gains (10.3% of clones spotted) and 129 losses (9.9%). The BAC clones with recurrent gains and losses in the HCCs are summarized in **Table 3**. A frequent copy number gain was detected on chromosomal regions 1q, 6p, and 8q. In particular, gains of 1q42.12, 1q43, and 8q24.3 were detected in more than 65% of tumors (**Fig. 1-a**). A frequent copy number loss was detected on chromosomal regions 1p, 4q, 6q, 8p, and 17p. Losses of 8p21.3, 17p13, and 22q11.21 were detected in more than 55% of HCCs (**Fig. 1-a**). The number of clones with DNA copy number gains was 128.4 ± 48.6 for stage-I tumors, 128.9 ± 67.3 for stage-II tumors, and 157.1 ± 75.2 for stage-III/IV tumors. The number of clones with DNA copy number losses was 139.4 ± 67.2 for stage-I tumors, 126.7 ± 71.3 for stage-II tumors, and 120 ± 59.1 for stage-III/IV tumors (**Fig. 2-a**). The disease progression was accompanied by a slight increase in the number of clones with DCNA, but no significant difference in the number of DCNAs was found between tumors with different stages (**Fig. 2-a**). In addition, concerning about the number of recurrent DCNAs, no correlation was observed

between the pathological stage and the average number of clones with DCNAs detected in >50% of tumors (**Fig. 2-b**). DCNAs extending to the whole chromosomal arm were not infrequent in HCC, as shown in Fig. 1-b.

Gains of 1q, 6p, 8q, and Xq, and losses of 6q, and 8p were frequent in stage-III/IV HCCs. The comparisons of the aCGH profiles with the pathological stage of the HCCs revealed that gains of almost all the clones on 6p and 8q24.12 were much more frequent in stage-III/IV tumors than in stage-I/II tumors ($p<0.001$). For example, the gain of 6p23 (clone ID 814) was detected in 0/10 (0%), 1/16 (6.3%), and 7/9 (77.8%) for stage-I, -II, and -III/IV tumors, respectively (**Table 4**). The frequency of the 6p23 gain was significantly different between the stage-I and -III/IV tumors (P=0.00045), the stage-II and –III/IV tumors (P=0.00023), and the stage-I/II and –III/IV tumors (P=0.0000053) (Table 3). In particular, the gain of the whole 6p arm was virtually limited to the advanced HCCs. The AWS procedure with the GLAD algorithm highlighted this feature (Fig, 3). Although the frequency of a gain of the entire length of chromosome 2 was not high, the chromosome 2 gain was exclusive for stage-III/IV HCCs (**Fig. 3**). The results in this study were not affected by any difference in the hepatitis virus type. The gain of BAC clones on 6p was infrequently associated with histological differentiation.

DNA amplification was a rare event in HCC, however it was exceptionally detected at Xq23 (clone ID; 326) with a frequency as 4/14 (28.6%) for stage-I, 7/18 (38.9%) for stage-II, and 4/10 (40.0%) for stag-III/IV tumors.

Detailed information of aCGH for 42 HCCs was available through a supplementary data file (♦).

Discussion

Many genes including cancer-related genes are involved in HCC as well as in other solid tumors. The genetic alterations depend on the organ and type of cancer, and the same goes for the chromosomal regions with DCNA [18, 19]. In this study, the 1.4 K BAC DNA array, which may be a low-density array, allowed drawing of sketch of DCNAs on chromosomes, and the AWS procedure with the GLAD algorithm for CGH data provided interesting information on chromosomal regions with genomic aberrations. The particular genomic alterations that occur in early cancers may be responsible for tumor development, and the genomic alterations that are detected exclusively in advanced cancers are connected to the tumor progression part from the matter of a cause or a consequence [20, 21]. Nonrandom genomic aberrations that occur at the early stage of carcinogenesis usually persist even in an advanced stage of tumors [22, 23]. It follows that early genomic events are frequently found in both early and advanced tumors, and that late genomic events are detected preferentially in advanced tumors. Accordingly, it is legitimate to assume that the recurrent DCNAs independent of disease stage are implicated in HCC development, and that the recurrent events preferentially detected in advanced tumors are responsible for HCC progression. In this context, it is likely that gains of 1q, 8q, and Xq, and losses of 1p, 4q, 6q, 8p, and 17p correspond to genomic changes associated with HCC development. The gains of clones on 6p and 8q preferentially detected in advanced HCC seem to be linked with progression of HCC. In particular, the gain of the whole 6p was virtually limited to advanced-staged HCCs; in other words, advanced HCCs were characterized by DNA copy number gains of the entire 6p arm. DCNAs extending to broad chromosomal regions are not unusual for HCC [6[]7][9][24]. The AWS procedure with the GLAD algorithm clearly defined the chromosomal regions involved. There have so far only been a few reports concerning

the relationship between genomic alterations and tumor progression in HCC, and, in addition, the controversy as to the relationship exists between these studies [3][6][22][25]. The chromosomal regions involved in advanced HCCs are different from our data. The cause of the difference is clearly unexplained, though the concept that DNA copy number gains on some particular chromosomal regions contribute to the progression of HCC is common to these studies. The high-level gain of 6p21 harboring VEGFA of which activation stimulates hepatocyte proliferation was detected in 14 of 210 HCCs [24], though a BAC clone harboring VEGFA was not spotted on the array used in the present study.

Since the disease stage largely affects the prognosis of a cancer patient, the treatment strategy usually depends on the disease stage in an individual patient. Therefore, it is of practical importance to differentiate between early cancers and advanced cancers prior to the start of treatment. To this end, attempts have been made to distinguish genomic alterations for HCC development from those for HCC [7][10][22][26]. The present study revealed that the CGH profiles were generally mirrored by the pathological stages. As mentioned above, some genomic changes such as gains of 6p and 8q are directly linked with the genomic stage and may be an alternative to the pathological stage. The genomic stage can be determined in biopsy specimens. This means that the disease stage can be estimated by an analysis of the genomic alterations on the particular chromosomal regions prior to the initiation of treatment.

It is known that histological differentiation and stage are roughly related in HCC, and this relation was apparent in the present study as well. However, the relationship between DCNAs and histological differentiation of HCC had a less impact than that between DCNAs and disease stage. In addition, the gain of BAC clones on 6p was barely

associated with histological differentiation of HCC in this study. Moinzadeh and colleagues who made CGH meta-analysis also revealed no linkage between 6p gain and histological differentiation [27].

In conclusion, advanced HCCs were characterized by DNA copy number gains of the entire 6p arm. The present data support the concept of a genomic stage in HCC.

Supplementary material

The aCGH data for 42 HCCs are available through a supplementary file.

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Abbreviations

HCC: hepatocellular carcinoma; BAC: bacterial artificial chromosome; CGH: comparative genomic hybridization; AWS: adaptive weights smoothing; GLAD: gain and loss analysis of DNA

Competing interests

The authors declare that they have no competing interests.

Authors contributions

The authors read and approved the final manuscript.

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Table 1. Clinicopathological fetures on 42 hepatocellular carcinomas

	All	TNM stage		
		I	II	III-IV
Gender				
Male	31	10	13	8
Female	11	4	5	2
Age (year)				
average (range)	64 (39-78)	65.5 (55-74)	61.5 (49-75)	63.5 (39-78)
Viral infection				
HBV-positive	6 (14.3%)	0 (0%)	3 (16.7%)	3 (30%)
HCV-positive	34 (81.0%)	12 (85.7%)	15 (83.3%)	7 (70%)
HBV- and HCV-positive	2 (4.7%)	2 (14.3%)	0 (0%)	0 (0%)
Tumor size				
Average diameter (cm)	4.62	3.0	4.4	8.47
Grade of differentiation				
Well	6 (14.3%)	4 (28.6%)	2 (11.1%)	0 (0%)
Moderately	29 (69.0%)	10 (71.4%)	13 (72.2%)	6 (60%)
Poorly	7 (16.7%)	0 (0%)	3 (16.7%)	4 (40%)

Table 2. Chromosomal regions with copy number aberrations of which frequency is different between HCCs with different histological differentiation

Chromosome region	Gene	Histological differentiation			P-value
		Well	Moderate	Poor	
Copy number gain					
19q13.41	ZBRK1		3 / 28	5 / 7	0.000622595
		0 / 6		5 / 7	0.008315262
19q13.41	KLK2		5 / 29	5 / 6	0.001106195
		0 / 6		5 / 6	0.003414791
Copy number loss					
4q21.22	-	0 / 6		5 / 7	0.008315262
		0 / 6	18 / 29		0.005622921

This table contains only data of P< 0.01

Table 3. Chromosomal regions with highly recurrent DNA copy number aberrations in HCC

Chromosomal region	Frequency (%)	Candidate gene
DNA copy number gain		
1q23.3	71	-
8q24.3	71	REC Q4
1q25.2	70	ASTN
1q23.2	69	-
Xq23	69	
1q42.12	68	LBR
1q43	67	RYR2
1q43	67	D1S204
1q23.3	64	PBX1
1q42.12	64	ITPKB
1q25.3	63	-
1q44	62	(1q terminal)
1q23.1	60	PRCC
1q44	60	Akt3, DKFZp434N0250
1q32.1	57	-
1q32.2	57	M69199
1q32.3	57	SLC30A1, NEK2
1q41	57	TGFB2
1q42.13	57	M-ABC2
1q44	57	TAZ-1
17q25.3	57	MAFG
8q24.3	56	-
1q42.12	55	A009B17
Xq23	54	TED
1q31.1	52	RP11-71C11
8q23.3	52	-
8q24.11-8q24.12	52	EXT1
8q24.23-8q24.3	52	(8q terminal)
1q23.3	50	CD48
1q42.2	50	KIAA1389
5p15.33	50	(5p terminal)
8q23.1	50	WI-13991
8q24.22	50	D8S1108
DNA copy number loss		
17p13.2	60	-
17p13.3	57	ABR
17p13.1	57	SCO1
17p12	57	-
8p21.1	57	EXTL3
17p13.1	56	GAS7
4q21.22	55	-
15q25.2	55	WI-22674
8p21.3	55	-
17p12	50	-
17p13.1	50	ALOX12
8p12	50	WS-3
22q11.21	50	BCR

Table 4. Chromosomal regions where the frequency of copy number gain is statisitically different between disease stages. P<0.001)

Region	Genes*	Stage I	Stage II	Stage I/II	Stage III/IV	P-value**
6p23	RNF182	0 / 10			7 / 9	0.000449377
			1 / 16			0.000233166
				1 / 26		5.30244E-06
6p12.1	DST	0 / 14			6 / 10	0.000817973
6p11.2	-	0 / 14			6 / 10	0.000817973
				1 / 32		2.5258E-05
6p24.3	BMP6		0 / 18		6 / 10	0.000209327
				1 / 30		4.42194E-05
6p22.3	ATXN1		0 / 18		6 / 10	0.000209327
				3 / 32		0.000660325
6p21.2	-		2 / 18		8 / 10	0.000267124
				6 / 32		0.000335232
6p24.3	-		0 / 17		6 / 10	0.000293053
				2 / 30		0.000260730
6p22.2	TRIM38			4 / 32	7 / 10	0.000306363
6p21.1	PTK7			4 / 32	7 / 10	0.000306363
8q24.12	SAMD12			8 / 29	9 / 10	0.000598645
6p21.31	PPARD			7 / 32	8 / 10	0.000812847
6p21.1	-			7 / 32	8 / 10	0.000812847

*-: Candidate genes are not identified in the relevant BAC clone
**: P-values between Stage I, II,or I & II and Stage III & IV tumors

Figure legends

Fig. 1. (a) Overall frequency of DCNAs for each clone/gene in 42 hepatocellular carcinomas. A DNA copy number gain is frequent for clones on chromosomal regions 1q, 6p, and 8q, and in particular, gains of 1q42.12, 1q43, and 8q24.3 were detected in more than 65% of tumors. A DNA copy number loss is frequent for clones/genes from 1p, 4q, 6q, 8p, 13q, 16q, 17p, and Y chromosome, and losses of 8p21.3, 17p13.1, 17p13.3, and 22q11.21 were detected in more than 55% of tumors. The upper part of the figure indicates the frequency of clones with relative increase in DNA copy number and the lower part of the figure indicates the frequency of clones with relative decrease in DNA copy number. The numbers on a horizontal axis denote chromosome number (corresponding to BAC clones spotted). The numbers on a vertical line denote the frequency of DNA copy number aberration for each clone (The minus (-) indicates the frequency of DNA copy number loss).

(b) An aCGH profile of stage II HCC from a 63-year-old male. A DNA copy number gain is observed at 10p, 13, and14q, and a loss is observed at 14q, 4, 10q, 11, 12p, 13q, 17q, and 22. It is obvious that DCNAs extend to the whole chromosomal arm.

Fig. 2. (a) The average number of DNA copy number gains (gray columns) and losses (black columns) for pathological stage-I, -II and -III/IV HCCs. The number of clones with DNA copy number gains is 128.4±48.6 for stage-I tumors, 128.9±67.3 for stage-II tumors, and 157.1±75.2 for stage-III/IV tumors. The number of clones with DNA copy number losses is 139.4±67.2 for stage-I tumors, 126.7±71.3 for stage-II tumors, and 120±59.1 for stage-III/IV tumors. There is no significant difference in the average number of gains and losses between different staged tumors. Vertical bars, standard deviation. Ordinate,

the average number of clones with DCNAs. Abscissa, pathological stage of HCC. (b) The average number of DNA copy number gains (gray bar) and losses (black bar) detected in more than 50% of tumors for pathological stage-I, -II and -III/IV HCCs. The number of clones with DNA copy number gains is 21.0±7.0 for stage-I tumors, 16.8±9.6 for stage-II tumors, and 20.6±9.6 for stage-III/IV tumors. The number of clones with DNA copy number losses is 7.6±5.0 for stage I tumors, 7.9±4.5 for stage-II tumors, and 7.0±4.0 for stage-III/IV tumors. There is no significant difference in the average number of clones with copy number gain or loss between different staged tumors. Vertical bars, standard deviation. Ordinate, the average number of clones with DCNAs.

Fig. 3. The AWS procedure using the GLAD algorithm is applied to aCGH data. The procedure makes clear chromosomal regions with DCNA. (a) The frequency of DCNAs for stage-I HCCs. In 14 stage-I HCCs, gains of 1q and 8q, and losses 4q and 17p are frequent, and in particular the 1q gain and 17p loss are detected in approximately 70% and 60% of stage I HCCs, respectively. (b) The profile of aCGH in 18 stage-II HCCs is similar to that in stage-I, though the number of chromosomal regions with DCNA appears less in stage-II tumors than in stage I ones. (c) The frequency of DCNAs for 32 HCCs in stage-I or -II. The profile is similar to those from HCCs in stage I or stage II. (d) The frequency of DCNAs for 10 HCCs in stage-III or -IV. The profile is considerably different from those of HCCs in stage-I or -II. Gains of 1q, 6p, and 8q, and losses of 6q, and 8p were distinct. In particular, a DNA copy number gain covering the entire length of 6p is a characteristic features in stage-III/IV HCCs (solid arrow). Although the frequency of a gain of the whole chromosome 2 is low, the gain of the chromosome is also a characteristic chance in stage III/IV HCCs (open arrow).

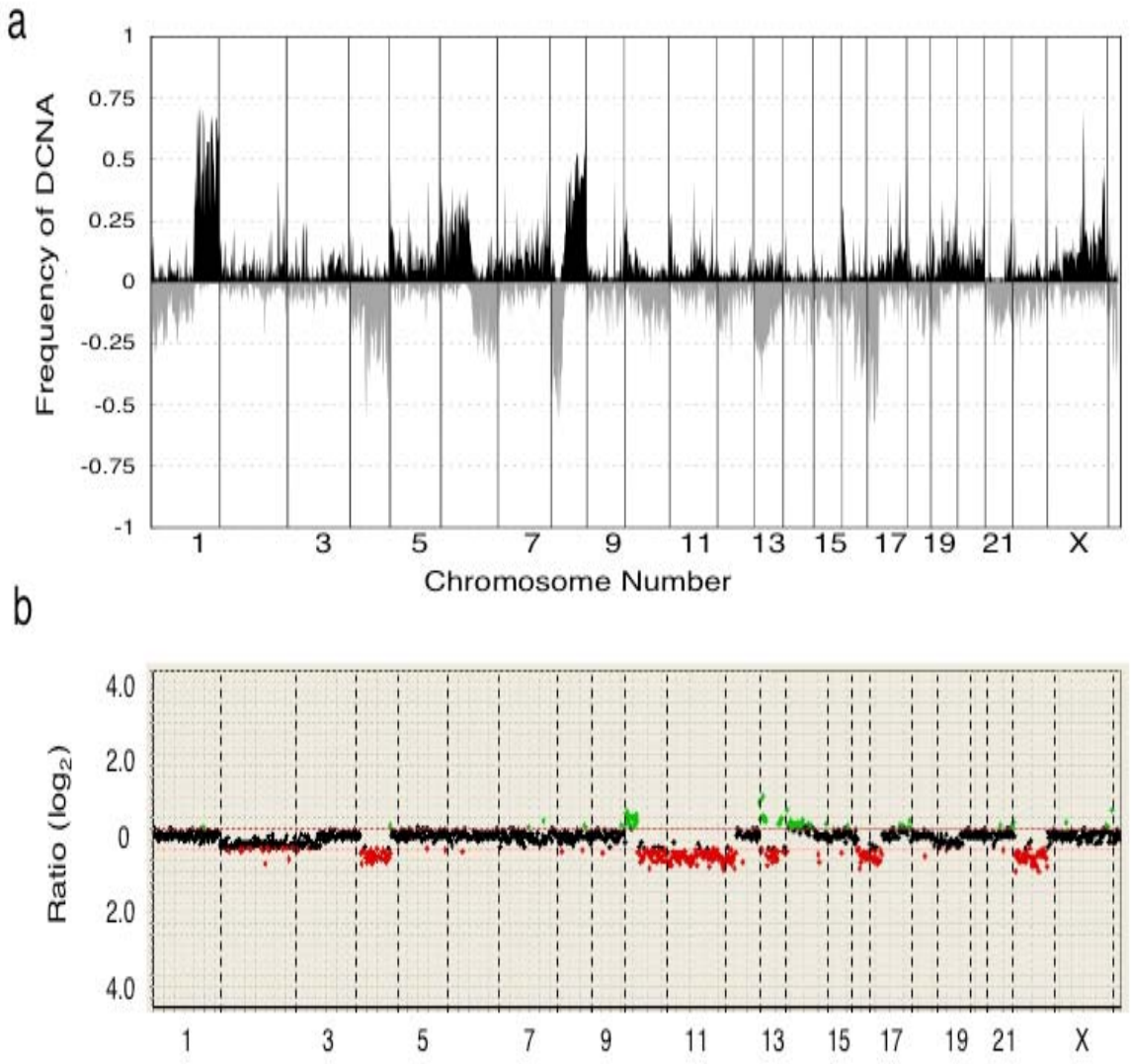
a
Frequency of DCNA
1
0.75
0.5
0.25
0
-0.25
-0.5
-0.75
-1
1 3 5 7 9 11 13 15 17 19 21 X
Chromosome Number
b
Ratio (log2)
4.0
2.0
0
2.0
4.0
1 3 5 7 9 11 13 15 17 19 21 X

Fig. 1

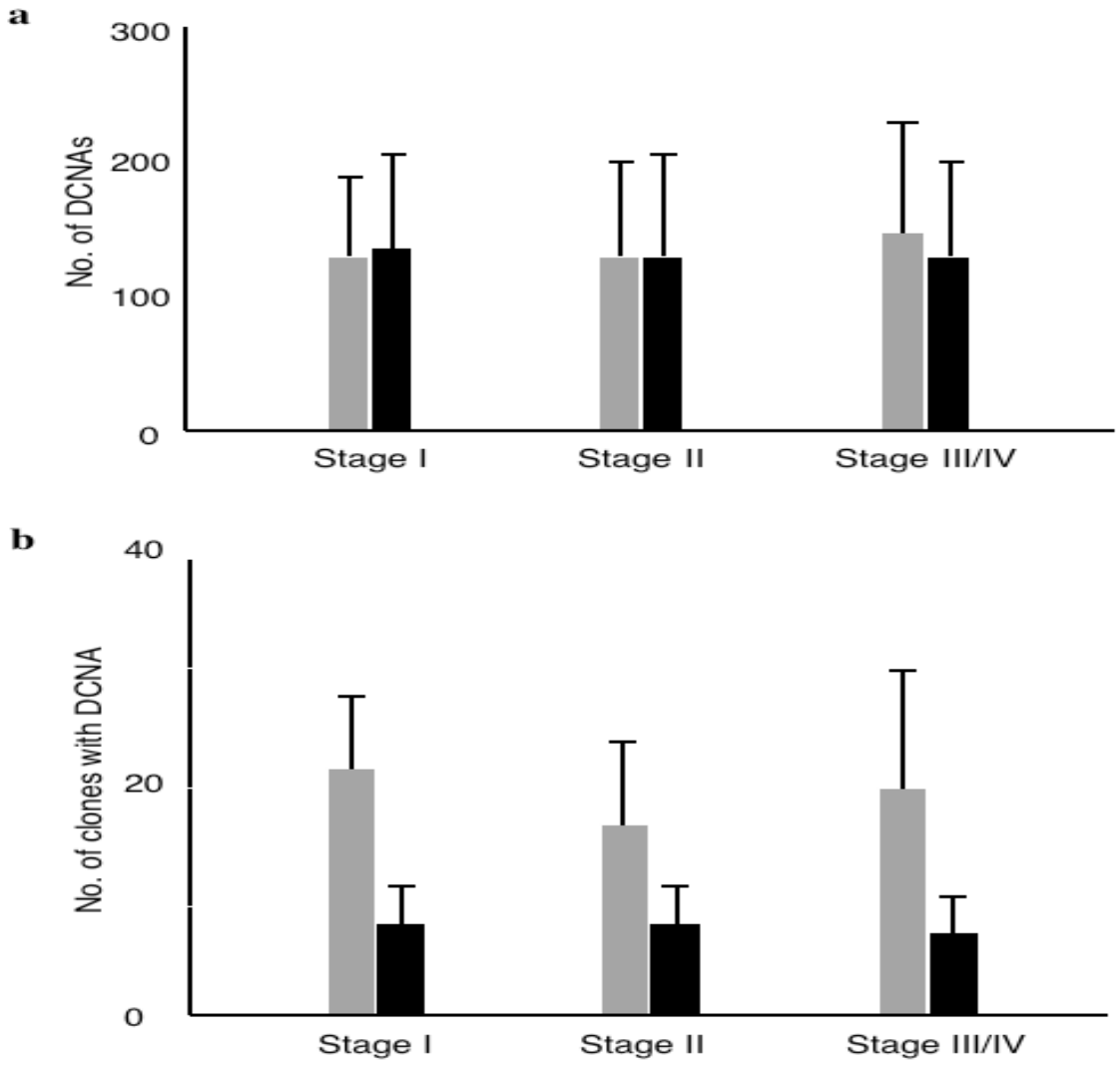
a
300
200
100
0
No. of DCNAs
Stage I
Stage II
Stage III/IV
b
40
20
0
No. of clones with DCNA
Stage I
Stage II
Stage III/IV

Fig. 2

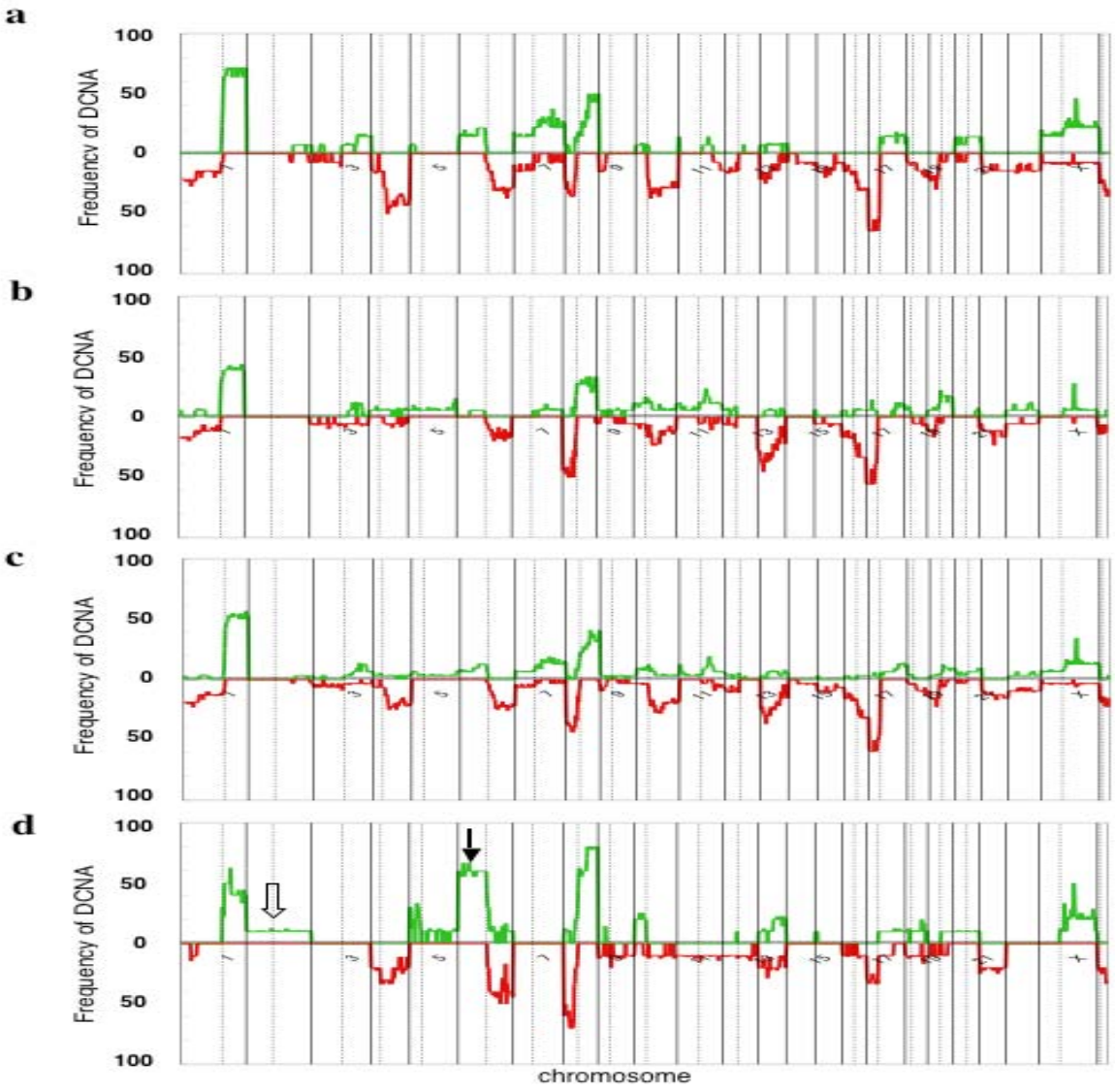

Fig. 3