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－在留中国人研究者研究助成－

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1．研究テーマ

Diffuse 型胃癌の発生と進展に重要な遺伝子を絞り込むための分子細胞遺伝学的研究

2．本年度の研究業績

(1) 学会・研究会等における発表 (有)・無 (学会名・演題)

第92回日本病理学会総会（福岡）・Comparative CGH and histological analyses in diffuse-type gastric carcinoma.

第62回日本癌学会総会（名古屋）・Comparative CGH analyses of diffuse-type gastric carcinomas with and without tubular adenocarcinoma component.

(2) 学会誌等に発表した論文 有・(無) (雑誌名・論文名)

但し、現在論文（Peng el al. Genetic lineage of poorly differentiated gastric carcinoma with tubular compnent analyzed by comparative genomic hybridization）を国際誌The Journal of Pathologyに投稿し、revise 中。

3．今後の研究計画

今回の研究では、diffuse型胃癌の発生と進展に重要な染色体レベルの異常や、染色体異常の標的候補遺伝子を明らかにした。またtubular componentのある低分化型腺癌とdiffuse型の胃癌では染色体異常のパタンがかなり異なり、両者の発生の系譜が異なることも明らかにした。今後、この研究で絞り込まれた標的候補遺伝子に注目し、cDNAマイクロアレイなどを用いて、発現異常を伴うことを証明したい。また発現異常が、ゲノムの変化を伴わずepigeneticな変化によって起こる側面についても研究したい。

4．指導責任者の意見

彭 敦發先生は、これまで研究の困難さからデータの乏しかったdiffuse型胃癌のゲノム変化をCGHとアレイCGHを用いて明らかにすることに成功されました。これはきわめてlaboriousな研究で、並みはずれた根気と努力の成果であります。今回の助成を受ける前の彭先生のデータは、既に（インパクトファクター5.06の国際誌である）The Journal of Pathology(201: 439–450, 2003)に掲載されており（Peng et al. Alterations of chromosomal copy number during progression of diffuse-type gastric carcinomas: metaphase- and array-based comparative genomic hybridization analyses of multiple samples from individual tumours.)、高い評価を得ております。この論文が学位論文となり、昨年医学博士の学位を授与されました。今回のデータも前回と同じ雑誌に投稿し（Peng el al. Genetic lineage of poorly differentiated gastric carcinoma with tubular compnent analyzed by comparative genomic hybridization)、revision後acceptableであるとの評価を得、現在revisionを行っている所です。彭先生は、研究に対する情熱もさることながら、夜遅くまで黙々と実験する集中力と体力、きちんと対照の取れたデータを出すことのできる科学的なセンスを持ち合わせておりましたが、それが今回日中医学協会の助成を得た研究によって益々磨かれ、研究者として大きく成長されたことに対し、指導させていただいた私どもは、日中医学協会に対して深い感謝の意を表するものであります。

指導責任者氏名 杉原 洋行

5．研 究 報 告 書

別紙「研究報告書の作成について」に倣い、指定の用紙で作成して下さい。

研究発表または研究状況を記録した写真を添付して下さい。

※研究成果を発表する場合は、発表原稿・抄録集等も添付して下さい。

※発表に当っては、*日中医学協会助成金による*旨を明記して下さい。

—日中医学協会助成事業—

Diffuse型胃癌の発生と進展に重要な遺伝子を絞り込むための分子細胞遺伝学的研究

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Abstract

Gastric carcinoma (GC) has been classified into the diffuse and the intestinal types. Diffuse-type GCs at advanced stages are thought to derive from intestinal-type as well as diffuse-type early GCs. In the present study, we focused on diffuse type GCs with minor tubular components (TC) to clarify their derivation. We applied comparative genomic hybridization (CGH) and array CGH to the DNAs that were taken by microdissection from multiple portions in individual tumors and amplified by degenerate oligonucleotide-primed PCR, and compared the results with those in diffuse-type GCs without TC. We found that the occurrence of the tumors with TC inclined to older, male patients. Frequent stemline changes common to the samples in each tumor were 8q+, 7p+, 3q+, 20q+ and 10p+, being basically different from those in the tumors without TC. The number of chromosomal changes was greater and 6p+, 10p+, 10q+, Xp+ and 4q- were more frequently detected in the tumors with TC than in those without TC. Within individual tumors, no significant difference was found in the frequency pattern of chromosomal changes between the samples of TC and the others, suggesting their derivation from the common precursor. We noticed that there were two subgroups in the tumors with TC: one with 5p+, 6p+, 7p+, and 10p+ and the other without them. The latter had the cytogenetic and clinicopathological aspects common to those in the tumors without TC. The analysis of clonal evolution process by constructing dendrograms in each tumor gave the results consistent with the notion that the latter subgroup may derive from diffuse-type GC without TC and the former from tubular adenocarcinoma.

Key Words　Gastric carcinoma: diffuse type, CGH, array CGH, Laser microdissection

Introduction

During the past decades, progresses have been made in understanding of the molecular events in the tumorigenesis and progression of GC [1]. Cytogenetic data have also been rapidly accumulated recently since the advent of comparative genomic hybridization (CGH), which is proved as a powerful screening method for detecting cytogenetic alterations in solid tumors [2, 3]. Despite these progresses, the reports on genetic and chromosomal aberrations in GCs were not consistent with each other.

We have recently developed the method that enables us to limit earlier events from many chromosomal changes detected by CGH [4]. Applying this method to early and advanced diffuse-type GCs, we demonstrated that advanced GC of this type may have progressed from early GCs of the same type by acquiring additional chromosomal changes [4].

In clinical practice, however, diffuse-type GC with TC is not uncommon, the cytogenetic characteristics and its relationships with intestinal type GC and diffuse-type GC without TC remain to be clarified. In the present study, we thus focused on diffuse-type GC with TC.

Materials and methods

Tumor samples

A total of 123 tumor samples were taken from 27 cases with gastric carcinomas, including 8 cases with minor tubular component (TC) (cases 20-27) and 19 cases of diffuse-type cancers without TC (cases 1-19). In the tumors with TC we examined, the tubular component accounted for less than 30 % of the tumor.

Laser capture microdissection and DNA preparation

We carried out microdissection of tumor cells from tissue section as described previously [4]. In individual tumors, we took about 500 to 5000 tumor cells from each of 2 to 8 different parts, in which tumor cells accounted for 70% or more. The films containing tumor cells were digested in 40 μl of proteinase K solution at a concentration of 200 μg/ml for about 70 hours at 42 °C.

Whole genome amplification by DOP-PCR

We amplified sample DNAs by DOP-PCR in two phases as described previously [4], which gave the PCR products of more than 2 Kb in size, being suitable for nick-translation labeling.

Probe DNA labeling, CGH and digital image analysis

DOP-PCR amplified tumor and normal DNAs were labeled with fluorescein-12-dUTP and tetramethyrhodamine-5-dUTP, respectively, by nick translation [5]. Hybridization and image analyses were carried out as described previously [4]. Gains and losses in DNA copy number were defined by green to red ratios (G/R) > 1.2 and < 0.8, respectively [4]. High-level gains (amplifications) were defined by a G/R≧1.5. Chromosomes 1p32-pter, 16p, 19, 22 and Y were excluded in the analyses.

Random priming labeling and array CGH analysis

We labeled tumor DNA and reference DNA of the same sex (100 ng each) with Cy 3-dCTP and Cy 5-dCTP respectively, by random priming reaction, and carried out array CGH following the manufacturer's protocol. GenoSensor Array 300(Vysis) which spotted with 287 target DNA clones including locus markers, proto-oncogenes and tumor suppressor genes were used. Target spots were automatically identified and analyzed by the built-in software. The loci with amplification were defined as a mean T/R ratio > 2.0, and the loci with a T/R ratio < 0.6 were considered as losses [4].

Temporal analysis of CGH results

In order to assess clonal consistency, we compared the positions of breakpoints among the samples as reported previously [4]. We defined the common alterations shared by all the samples in each tumor as stemline changes, those shared by multiple but not all the samples in each tumor were described as recurrent sideline changes, while the other changes unique to each sample as single sideline changes.

Results

1. Cytogenetic alterations detected by metaphase CGH

The chromosomal copy number aberrations detected in 54 samples of the 8 tumors with TC were summarized in Figure 1 and Table 1. The total number of chromosomal aberrations per sample was significantly higher ($P = 0.006$) in the tumors with TC (14.02±7.65 (SD)) than those without TC (10.57± 5.80).

High level gains (amplifications) were detected in totally 26 chromosomal regions in 7 of the 8 tumors with TC (Table 1, Figure 1). The mean number of amplified regions in the tumors with TC (6.00 ± 5.45) was much higher than in those without TC (1.11 ± 1.24) ($P = 0.0009$). The recurrent amplified regions in the tumors with TC were at 8q24 (5/8), 7p (4/8), 20q (4/8), 5p (3/8), and 13q (3/8). Copy number gains at 6p, 10p, 10q and Xp and copy number loss of 4q were significantly more common in the tumors with TC than those without TC ($P < 0.05$) (Figure 2A). In individual tumors with TC, there was no significant difference in the frequencies of any chromosomal changes between the samples of TC and those of SIG/POR (Figure 2B).

2. Array CGH analyses

The array CGH results of the tumors with TC are presented in Table 1 and those without TC were shown in Table 2 in Ref.4. The most frequent amplicons were at 8q24 (5/8), 7p (4/8) and 20q (4/8), followed by 5p (3/8) and 13q (3/8). In the tumors with TC, *CMYC* was involved in the amplicon at 8q24 in 4 cases, but in case 27, *PTK2* instead of *CMYC* was proved to be responsible for this amplicon. *EGFR* gene at 7p12.3-p12.1 was amplified in both groups with and without TC. In the 20q amplicons in our series, amplifications of *STK15*, *CAS*, and *ZABC1* were involved.

3. Temporal analysis of CGH results

In the tumours without TC, 8q+ (5/19), 8p+ (4/19), and 17p- (6/19) were picked up as frequent stemline changes [4]. In the individual tumours with TC examined, we also detected the stemline changes, such as gains of 8q (6/8), 7p (4/8) and 10p (2/8).

Gains at 8p and 10p were not frequent but appeared to be specific to the tumours without and with TC, respectively (Table 2).

Table 1. Frequencies of Chromosomal Aberrations of Total, Stemline and Sideline Changes in Diffuse GCs with TC

Array CGH		Metaphase CGH				Array CGH		Metaphase CGH			
Copy number gains/AMP	Candidate genes†	The number of cases with aberration/AMP	Stemline changes	Sideline changes		Copy number losses	Candidate genes¶	The number of cases with aberrations	Stemline changes	Sideline changes	
				Recurrent	Single					Recurrent	Single
8q+/q24	*MYC* #, PTK2* , E2F5* , EXT1*	8 (100%)/5	6	2	0	4q-	?	5 (62.5%)	0	4	1
3q+/q25-26	*TERC, PIK3CA* #, EIF5A2*	8 (100%)/2	2	4	2	14q-	?	5 (62.5%)	1	4	0
20q+/q12-13	*TOP1 , STK15 , CAS , ABII, ZABC1*	7 (85.7%)/4	2	4	1	3p-	*MLH1 , FHIT , VHL*	4 (50.0%)	0	2	2
Xp+/p22	?	7 (85.7%)/1	0	5	2	17p-	*TP53 , LLGL1 #*	4 (50.0%)	1	3	0
Xq+/q25	*AR 3'*	7 (85.7%)/1	0	3	4	21q-	?	4 (50.0%)	0	3	1
1q+/q31-34	*LAMC2 #, AKT3*	6 (75.0%)/1	1	4	1	5q-	*APC , (MCC), MSH3*	3 (37.5%)	0	3	0
2q+/q11.2-21.2	*CASP8, HER-4*	6 (75.0%)/1	1	2	3	9q-	*PTCH #, TSC1*	3 (37.5%)	0	2	1
6p+/p22-25	*CCND3, PIM1*	6 (75.0%)/2	0	5	1	16q-	*CDH1 #, CDH13 , CYLD*	3 (37.5%)	0	3	0
7p+/p14-22	*EGFR #*	6 (75.0%)/4	4	1	1	18p-	?	3 (37.5%)	0	1	2
10p+/p12.1-14	*?, D10S249, GATA3, D10S1260*	6 (75.0%)/2	2	3	1	7q-	?	3 (37.5%)	0	2	1
13q+/q32-34	*IDP1; ?*	6 (75.0%)/3	1	4	1	9p-	*p16 , MTAP*	3 (37.5%)	0	1	2
20p+/20p	?	6 (75.0%)/1	1	4	1	18q-	*DCC, DPC4, (MADR2)*	3 (37.5%)	1	1	1
5p+/5p	*(SKP2, CDH6, PC4), D5S23 #, D5S2064*	5 (62.5%)/3	1	4	0	2q-	*(PMS1)*	2 (25.0%)	0	1	1
8p+/p22-23	*FGFR1, CTSB**	5 (62.5%)/2	1	4	0	4p-	?	2 (25.0%)	0	2	0
2p+/p15-16	*MYCN, REL*	5 (62.5%)/1	1	1	3	10q-	*PTEN, DMBT1*	2 (25.0%)	0	2	0
10q+/q24.3-26	*FGFR2* #*	5 (62.5%)/1	0	4	1	11p-	*p57, WT1 #, KAI1*	2 (25.0%)	0	1	1
11q+/q23-24	*CCND1 , FGF3 , (BCL1)*	5 (62.5%)/2	0	3	2	11q-	*ATM, MEN1 , RDX*	2 (25.0%)	0	2	0
11p+/p12-14	*HRAS* #*	4 (50.0%)/1	0	4	0	12q-	?	2 (25.0%)	0	2	0
12p+/p11.2-12.3	*CCND2 , KRAS*	4 (50.0%)/1	1	1	2	17q-	*BRCA1, NF1*	2 (25.0%)	1	1	0
15q+/q24-26	*FES* , IGF1R , H(GAP1)*	4 (50.0%)/2	1	2	1	Xp-	?	2 (25.0%)	0	1	1
18q+/q21	*BCL2 ; ?*	4 (50.0%)/1	0	3	1	Xq-	?	2 (25.0%)	0	1	1
5q+	?	3 (37.5%)/0	0	1	2	6q-	?	2 (25.0%)	0	1	0
6q+	*MYB #*	3 (37.5%)/0	0	3	0	8p-	?	2 (25.0%)	0	0	1
7q+/q21.3-22	*MET , CDK6* , MDR1*	3 (42.9%)/1	1	1	1	2p-	*MSH2*	1 (14.3%)	0	0	1
9q+	*ABL1*	3 (37.5%)/0	1	1	1	15q-	?	1 (14.3%)	0	1	0
12q+/q15-25	*WNT1 , CDK2, MDM2, GLI , CDK4*	3 (37.5%)/1	0	2	1						
14q+	*AKT1 #*	3 (37.5%)/0	0	1	2						
18p+/18p	*YES1 , TYMS*	3 (37.5%)/1	0	2	1						
1p+	*FGR , MYCL1 , NRAS #*	2 (28.6%)/0	0	2	0						
9p+	?	2 (28.6%)/0	0	0	2						
16q+	?	2 (28.6%)/0	0	1	1						
17q+/q14-21	*HER-2* #, PBP* , TOP2A* , THRA**	2 (14.3%)/1	1	1	0						
3p+/p13-14	*RAF1*	1 (14.3%)/1	0	1	0						
4p+	?	1 (14.3%)/0	0	1	0						
4q+	*PDGFRA , EIF4E*	1 (14.3%)/0	0	1	0						
21q+	?	1 (14.3%)/0	0	1	0						

† See web site http:www.helsinki.fi/~lgl_www.html. The bold letters indicate the genes that showed amplifications in array CGH. The genes with # indicate they were detected in more than 35 % of the cases. The genes with asterisks are the putative target gene (s) corresponding to the amplifications in CGH analyses (at the loci shown in bold letters in the left end column). ¶ See web site http:www.helsinki.fi/~lgl_www/CMG.html. The bold letters indicate the genes that showed losses in array CGH. Genes in parentheses are not included in the Genosensor array 300 chip. AMP= amplification; TC= tubular adenocarcinoma component.

Table 2. Frequent Stemline Changes between the tumors with and without TC

	N	8q24+	8p22-23+	17p12-ter-	7p14-22+	3q26-ter+	20q+	10p+
Tumors without TC	19	**5 (26.3 %)**	**4 (21.1 %)**	**6 (31.6 %)**	3 (15.8 %)	2 (10.5 %)	2 (10.5 %)	0
Tumors with TC	8	**6 (75 %)**	1 (12.5 %)	1 (12.5 %)	**4 (50 %)**	**2 (25 %)**	**2 (25 %)**	**2 (25 %)**
Cases 21, 22	2	**2 (100 %)**	**1 (50 %)**	0	0	0	0	0
Cases 20, 23-27	6	**4 (66.7 %)**	0	1 (16.7 %)	**4 (66.7 %)**	**2 (33.3 %)**	**2 (33.3 %)**	**2 (33.3 %)**

We picked up the minimal overlapping regions of the chromosomal changes which were detected as stemline changes in 20 % or more of either tumor group with or without TC, as indicated in bold letters. TC = tubular component, GC = gastric cancer.

Discussion

Within individual diffuse- type GC with TC, there was almost no difference in the pattern of chromosomal copy number aberrations between the samples of SIG/POR and those of TC (Figure 2B). This finding and the presence of stemline changes common to TC and SIG/POR indicate that these components are of the same lineage and that this group of GC is also monoclonal despite its marked histological heterogeneity. Between the tumours with TC and without TC, however, the genetic lineage appeared to be different because the patients bearing the tumours with TC showed significantly higher male/female ratio and age than those without TC. This notion was supported by the findings in CGH analysis that the frequent stemline changes of the tumours with TC were 8q+, 7p+, 3q+,

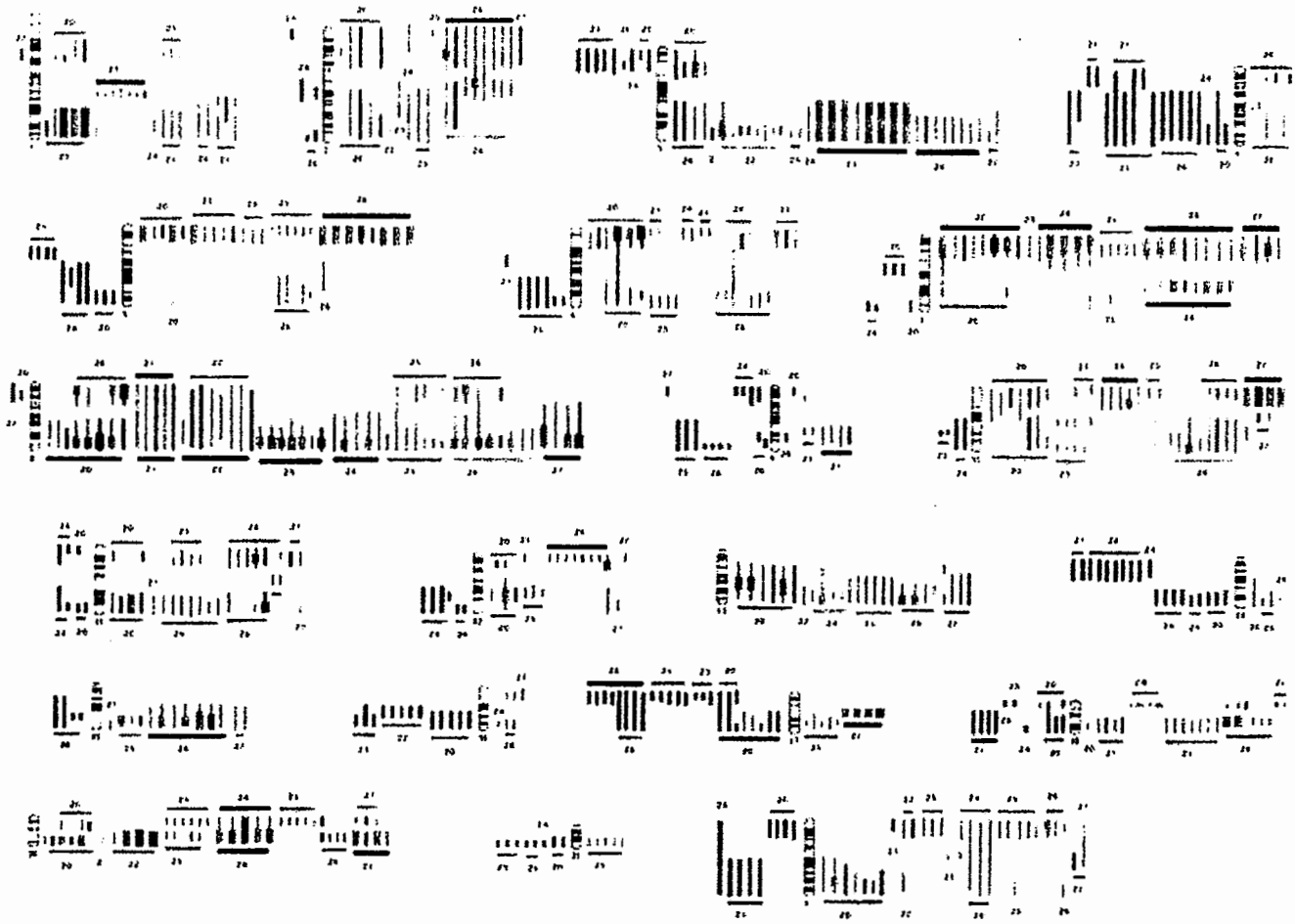

Figure 1. Metaphase CGH results in 54 samples of 8 tumours with TC. The regions of copy number gains and losses in all the samples are shown as the bars on the right and left sides of each ideogram, respectively. The thick bars mean amplifications. The results of the same case are marked with horizontal bars with case number. Black and grey bars represent stemline and sideline changes, respectively.

20q+ and 10p+, which were different from those of the tumours without TC (8q+, 8p+ and 17p-) (Table 2). Although 8q+ was included in the recurrent stemline changes in both groups, array CGH disclosed that the target genes were different; *C-MYC* was involved in the amplicon at 8q24 in 4 of the 5 tumours with TC bearing this amplicon and *PTK2* was in the other one, whereas neither *C-MYC* nor *PTK2* was involved in the amplicon of 8q in the tumours without TC [4].

Among the common sideline changes, gain of 10p was detected in 75 % of the tumours with TC as stemline changes or recurrent sideline changes (Figures 1, Table 1) but in none of the tumours without TC we examined [4]. In both intestinal and diffuse types of GC, however, 10p + was reported infrequently. At 10p, no oncogenes have been reported so far. However, *GATA3* and two loci, *D10S249* and *D10S1260*, were amplified in case 27.

Based on the chromosomal constitution, diffuse type GCs with TC examined were further classified into two subgroups: the one with 5p+, 6p+, 7p+ and 10p+ (6 cases) and the other without them (2 cases). All of the patients of the former subgroup were male. Their tumours did not show a layered structure in their mucosa lesions that is considered to be a remnant of the growth pattern in an incipient phase. Moreover, these tumours had numerous chromosomal alterations including 3 or more loci of amplification. The patients of the latter subgroup were female. Their tumours showed a layered structure and a spreading growth in the mucosa, and had fewer chromosomal alterations and amplifications than those of the former subgroup. The analysis of clonal evolution process by constructing dendrograms in each tumor gave the results consistent with the notion that the latter subgroup may derive from diffuse-type GC without TC and the former from the intestinal-type GC.

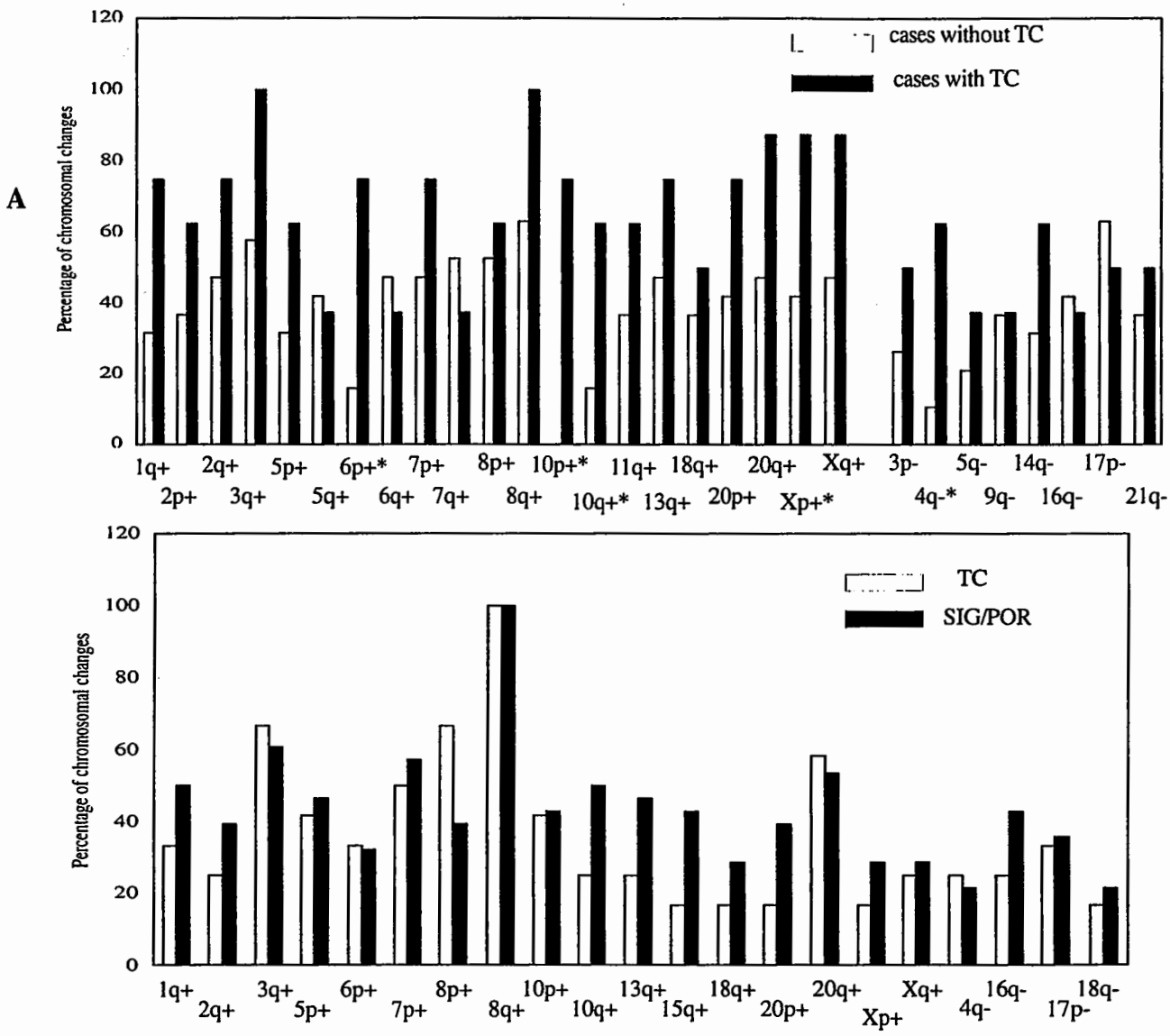

Figure 2. The pattern of frequencies of chromosomal copy number changes. The pattern was compared between the diffuse GCs with and without TC (A) and between the samples from POR/SIG components and from TC (B). The asterisks indicate statistically significant differences ($P < 0.05$).

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