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Molecular Cytogenetic Characterization of Esophageal Cancer Detected by Comparative Genomic Hybridization

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Aim: Detection of cytogenetic alterations in esophageal cancer (EC). A total of 40 cases of primary EC and their paired nearby nontumor tissues were collected. The comparative genomic hybridization (CGH) is the technique that brings out the gains and losses of chromosome fragments and was applied to determine the aberrations from the tissue DNA. In noncancer tissues, the gains were at 19p (5/40, 13%), 20q (5/40, 13%), and losses at 9p (13/40, 33%), 2q (10/40, 25%), 12q (10/40, 25%), 13q (10/40, 25%), 5q (9/40, 23%), 6q (9/40, 23%), 7q (9/40, 23%), and 8p (9/40, 23%). Two cases in nontumor tissues showed no CGH change. In the 40 cases of primary EC, the gains were at 8q (10/40, 25%), 3q (9/40, 23%), 2q (7/40, 18%), and 13q (7/40, 18%), and the losses were at 1q (8/40, 20%), 4q (8/40, 20%), 3p (7/40, 18%), 5q (7/40, 18%), and 18q (7/40, 18%) in comparison with paired nearby noncancerous tissues. We found that the loss aberrations were on 1q, 2p, 3p, 5q, 6q, 9p, 11p, 15q, 16q, 18q, 21q and gains on 20p in both tumor and nontumor tissues; nevertheless, −4p, −7q, −8p, −10q, −12q, −13q, −14q and +17p, +19q, +22q were only found in nontumor tissues and +1q, +2pq, +3q, −4q, +4q, +5q, 7p, +8q, +10q, +12q, +13q, +14q −17p, −19pq, −22q in EC. From these results, we suggest that most of the tissues near the cancer parts of EC may be considered as a precancerous region. The alteration between cancer and noncancer tissues may play a role in the development of EC. J. Clin. Lab. Anal. 24:167–174, 2010.

Key words: comparative genomic hybridization (CGH); esophageal cancer (EC); nearby nontumor tissues

INTRODUCTION

The incidence of esophageal carcinoma (EC) ranks the ninth in the world (1). In western countries, the mortality of EC ranges between 10,000 and 13,000 people annually (2–11). EC is one of the cancers associated with a poor 5-year survival rate (1,3,5). There have been several epidemically changes of EC in the past few decades. Between 1997 and 2005, several studies had demonstrated that there was a correlation between the incidence of EC and reflux, and the incidence of EC and the development of Barrett's esophagus, and they also confirmed that the number of the incidence of EC was on the rise (5). In Asian populations, however, the incidence of EC, which is not as prevalent as that in the United States and Europe, is between 80 and300 cases per 100,000 people (7). Nevertheless, there has been a tendency of increase in the number of cases outside these regions; among them the highest incidence rates are in Northern China, in a particular area of northern Iran, and in southern Russia (8,9). In Taiwan, according to the annual statistical reports of cancer by the National Health Bureau in 1998, the incidence of EC was 7 per 100,000 males and ranked the ninth. The mortality rate was 6 per 100,000 men and ranked the sixth, and the

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ratio between males and females was 9.4:1.0. There was a clear correlation among people chewing betel quid, smokers, and alcohol drinkers (11). The major pathological type of EC in Taiwan is squamous cell carcinoma (SCC), which accounts for 90% of the EC cases. The minor is adenocarcinoma, which accounts for 6%; it is very different from that in the United States and Europe. The causes of the difference are still unknown. At present, only a few studies of EC have been conducted in Taiwan (10). In particular, no study has been done on nearby noncancerous tissues.

Conventional chromosome study by banding analysis may provide an overall view of the structural and chromosome changes, but these changes may not provide molecular significance. The comparative genomic hybridization (CGH) is the technique that brings out the gains and losses of chromosome fragments, which can provide possible molecular defects. The CGH has been used to study the changes of chromosome fragments in EC, and the results have shown that the common losses were −1p, −1q, −3q, −4q, −5q, −6p, −7q, −8p, −9p, −10q, −11p, −13q, −14q, −15q, and −18q, and the common gains were +6q, +8q, +13q, and +19q. In order to understand the molecular cytogenetic changes in EC and its nearby noncancer tissues in Taiwan, we studied 40 primary EC and their nearby noncancer tissues by CGH (12). The identification of chromosome changes may eventually improve in understanding the carcinogenesis of EC, and provide a basis for further molecular study of the mechanism.

MATERIALS AND METHODS

Samples and Controls

A total of 40 ECs and their paired nearby nontumor tissues were collected from 40 patients at the department of surgery of the Changhua Christian Hospital from February, 1994 to December, 2000, and all the ECs are SCC. The patients had not received any preoperative chemotherapy or radiotherapy when their tissues were collected. The samples were freshly delivered to the Department of Pathology in the hospital after surgery, and the samples were dissected by a pathologist to obtain the cancer and noncancer tissues. The noncancer tissues were collected as fast as possible to avoid the contamination of cancer cells. The tissues were frozen quickly by using liquid nitrogen, and then stored in −80°C refrigeration. The genomic DNA was extracted from cancerous and nearby noncancerous frozen tissues by using a commercial DNA isolation kit (Promega, Madison, WI). The reference DNA was pooled from the normal amniocentesis samples which have been proved with normal chromosomal karyotyping. This study was approved by the Institute Review Board of Kaohsiung Medical University Hospital.

Cell Culture and Slide Preparation for CGH

Cell cultures followed by the methotrexate working solution synchronization procedure were treated with thymidine working solution as well as Colcemid (Gibco, New York, NY) before reaching confluence and incubated at 37°C. Cells were given 0.1 ug/ml Colcemid for 10 min. They were then harvested and treated with hypotonic solution (0.54% KCl) for 20 min, fixed in three changes of fixative solution (3:1, methanol:acetic acid) and stored at −20°C overnight.

Comparative Genomic Hybridization

CGH was performed as described (2,12–14). Labeling of tumor DNA with Fluorescein-12-dUTP NEL-413 (PerkinElmer, Boston, MA) and normal reference DNA with Texas Red-5-dUTP NEL-417 (PerkinElmer) was performed by standard Nick translation. The denatured DNA probes containing each 500 ng of tumor DNA and reference DNA, and 46 ul of COT-1 DNA were hybridized for 48 hr to normal metaphase spreads (Invitrogen, Carlsbad, CA). Then, slides were washed completely and also DAPI (4,6-diamidino-2-phenylindole) (1.25 μg/ml) was used for counter stain. Image was performed on a Nikon E600 microscope equipped with three combined filters for DAPI, FITC, and a Texas red spectrum with a coupled device (CCD) camera. For each analysis, the averaged chromosome-specific green-to-red fluorescence ratios from at least 10–20 metaphases were plotted by using the Quips CGH software (Imaging, Newcastle, UK). The corresponding ratio for amplifications was 1.5, and the corresponding ratio for losses was less than 0.85. The cases were classified as a trend gain or loss when the 95% confidence interval varied beyond the ratio of 1.0.

RESULTS

CGH Analysis of the Nearby Noncancerous Cells

CGH data for 40 pairs of noncancer tissues of esophageal tissues were collected after CGH analysis. The results showed that the individual chromosomal aberration pattern has clustered on some specific regions in comparison with the amniotic cells of the normal cases.

In the nearby nontumor group, DNA gains were mainly found in +1p (5%), +1q (5%), +17p (10%), +19q (13%), +20p (13%), and +22q (7.5%) (Table 1) and DNA losses were mainly found in −1q (15%), −2p (25%), −3p (15%), −4p (7.5%), −5q (23%), −6q (23%), −7q (23%), −8p (23%), −9p (33%), −10q

TABLE 1. The Results of the Regions With Chromosome Losses of the CGH Analysis in Noncancerous Parts of 40 Esophageal Tissues in Comparison With Normal Cells[a]

Gain and loss	Cases/40	%
−1q24–q31	6	15
−2q22	10	25
−3p24/p21	6	15
−4p14–p16	3	7.5
−5q21–q32	9	23
−6q24	9	23
−7q32–q36	9	23
−8p15–p24	9	23
−9p12–p22	13	33
−10q21.3–q26.2	8	20
−11p12.3–p13	7	18
−12q14–q25	10	25
−13q21.1/q33	10	25
−14q22–q31	8	20
−15q21.1–q22.3/q26.3	9	23
−16q23	4	10
−18q11.1–q12.3	5	13
−21q12.2–q13.2	2	5
Normal	2	5

[a]Normal cells are amniotic cells collected from the normal cases.

TABLE 2. The Results of the Regions with Chromosome Gains of the CGH Analysis in Noncancerous Parts of 40 Esophageal Tissues in Comparison With Normal Cells[a]

Gain and loss	Cases/40	%
+1p13–21	2	5
+1q21	2	5
+17p11.1	4	10
+19q13.1–2	5	13
+20q11.2–p12	5	13
+22q11.1–q22.1	3	7.5

[a]Normal cells are amniotic cells collected from the normal cases.

(20%), −11p (18%), −12q (25%), −13q (25%), −14q (20%), −15q (23%), −16p (10%), −18q (13%), and −21q (5%) (Table 2). In this group, the most frequent gains detected were +19p13 (13%) and +20q11–12 (13%) and the most frequent loss detected was −9p12–22 (33%). There are two cases without any gain or loss in this group. The X and Y chromosomes were excluded from the CGH analysis as samples and reference DNA were not sex matched.

We showed two representative cases in Figures 1 and 2. Case N1 with fewer abnormalities showed chromosome changes at −1q24, −9p12, +11p11, +11q12–13.2, −13q13, −17p11–q11, and +19q13.1 (Fig. 1). Case N2 showed chromosome changes at −2p12, −2q14.3–22, −4q13.3, −4q22, −6q24, −7p13; −7q31.3, −8p21.2, −9p13, −9p12, −10p12.3, +11p12.3, −15q12–q22.2, and −18q11.2 (Fig. 2).

CGH Analysis of EC Cells

From case T1 to case T40, there were different changes on each case. In the cancerous tissues, DNA sample gains were mainly found in +8q21.1–24 (10/40, 25%), +17p, +19q, +20p, and +22q (Table 3) and losses were mainly found in −1q, −2q, −3p, −4p, −4q, −5q, −6q, −7q, −8p, −9p, −10q, −11p, −12q, −13q, −14q, −15q, −16p, −18q, and −21q (Table 4). The most frequent gain detected was at +8q21.1–q24 (10/40, 25%) and the most frequent losses detected were at −1q21 (25%) and −4q32. The X and Y chromosomes were excluded from the CGH analysis as samples and reference DNA were not sex matched, which was the same as those of the first group. The representative case T1 showed chromosome changes at +1q41–q44, −3p14.3–22, −4p16, −5q12–21, +6p22.3, −6q23, −7p14, −8p23.1–p21.2, +8q21.3–q24.3, −10q21.1–21.3, +11p15.5–p13, +12p13.1, −13q13–q32, +14q12–q21, −15q22.3–q23, −16q21–q24, +17p12, +17q12, −18q12.3∼q22, −22q11.2, and +22q12.1 (Fig. 3). The representative case T2 showed chromosome changes at +2q12, +2q14.3, +3q22, +4q32, −6q16–q25.1, +10q23.2 −10q25.3, −11p12, +14q21, +14q31∼q32.3, and+17q22 (Fig. 4).

Comparison of the EC Group and Nearby Nontumor Group

After comparing both groups, we found the same gain on chromosome +20p11.2–12, and the same losses on chromosome −3p21, −5q21–32, −6q24, −9q12–22, −11p12.3–p13, −15q22.3, −18q11.1–q12.3, and −21q12.2–q13.2. If we combined both groups, the chromosome numbers of the ranking with the highest frequency were: −1q, −2q, −3q, −5q, −6q, −7q, −8p, +8q, −9p, −10q, −12q, −13q, −14q, and −15q.

DISCUSSION

We compared our results with other studies in Taiwan; all data have shown that frequent losses of chromosomes in EC are −2q, −3p, −4q, −5q, −9p, −13q, and −18q and frequent gains are chromosomes +3q, +8q, and +20q (12,15,16). We also compared the results between Taiwanese population and those of other populations in the world including China; the gain of chromosome +8q and losses of chromosomes −3pq, −4q, −5q, −9p, −13q, and −18q are the common findings (13,14,17–26). The most significant finding is the gain of chromosome +8q with a minimum high-level amplification region at +8q21–24 and +20q with a minimum high-level amplification region at +20q11.2–12 (13,18,27). In other populations, the US, for example, chromosome changes were frequently seen at −1q, −2q, −3p, +3q, −4q, −5q, −6q, 8q, −12pq,

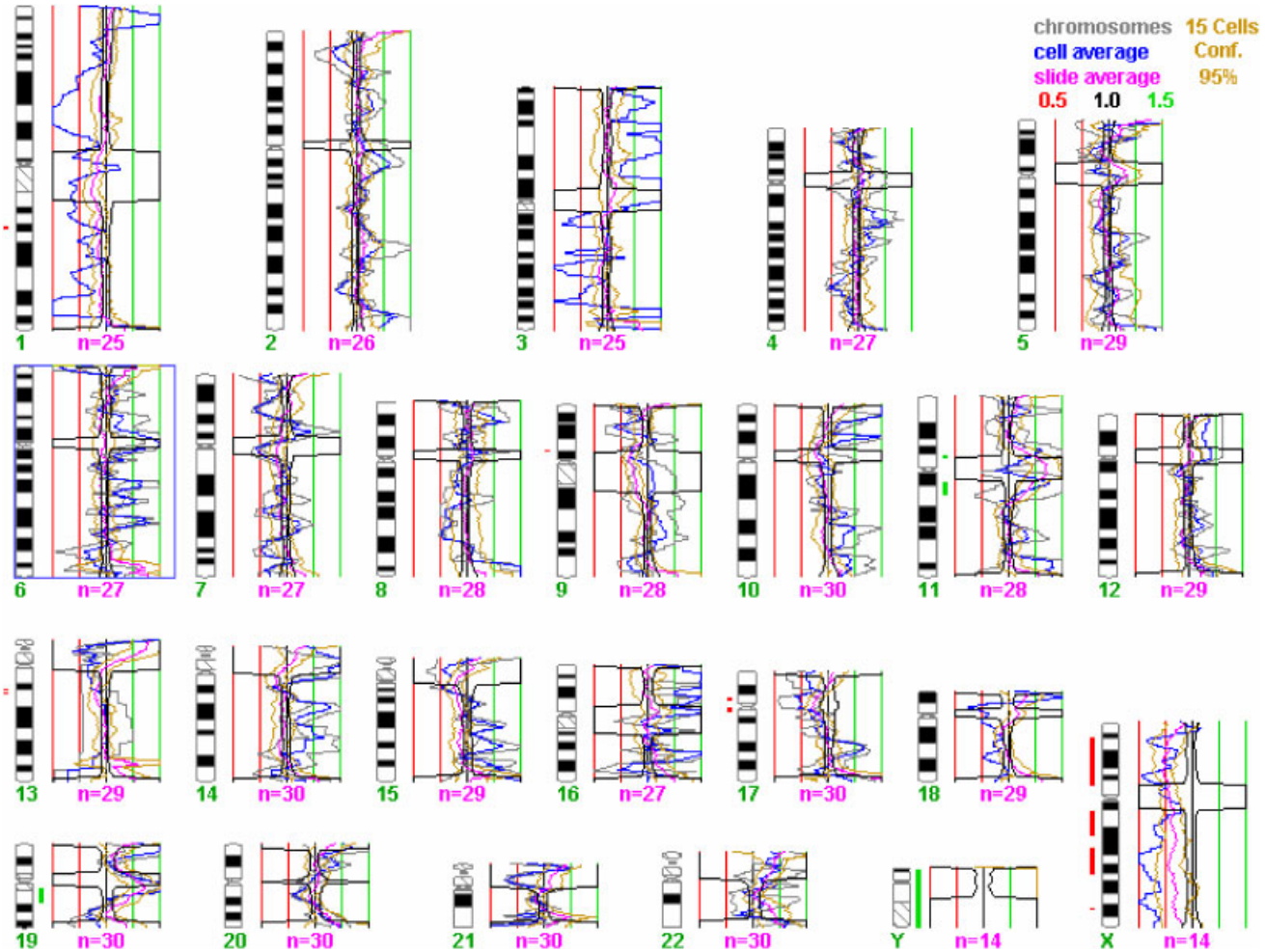

Fig. 1. Summary of comparative genomic hybridization abnormalities identified in one of the paired nontumor tissues (case N1) is shown. Each vertical line represents a single genetic aberration observed in a single specimen. Losses are shown on the left and gains on the right.

−13q, −14q, +16q, +17pq, −18q, +19pq, +20q, and +22q. The clinical pathological studies showed that gains of +5p and +7q and deletions of −4p, −9p, and −11q were significant prognostic factors (20,27–29). All the populations have the same losses and gains on chromosomes −3p, +3q, −4q, −5q, +8q, −9p, −13q, +17p, −18q, +19p, and +20q but different losses and gains on chromosomes 10q and 15q.

From the results of our study and others, the most common losses are found in −3p14, −4q32, −5q21, −9p12–22, and −18q21 and the common gains in +8q21–24 and +20q13 in EC. Chromosome 3p14 has a tumor suppressor gene called the Fragile Histidine Triad (*FHIT*) gene which spans the common fragile site FRA3B. This gene is frequently inactivated by carcinogen-induced intragenic deletions in many types of cancers. Rare silent point mutations were found in esophageal adenocarcinomas (30). The other two frequent losses are described at chromosome 3p25–26 encompassing two candidate tumor suppressor genes: the Von Hippel-Lindau (*VHL*) gene and the peroxisome proliferators activated receptor-gamma (*PPARg*) gene. Both genes have been reported to be mutated in VHL disease and colon carcinomas (30). LOH analysis revealed the 3p25–26 loss in 67% of 36 distal esophageal and gastric cardiac carcinomas without detected mutations (30). On chromosome 5q21, there are two tumor suppressor genes—Mutated in Colorectal Cancer (*MCC*) and Adenomatosis Polyposis Coli (*APC*) located at this region, and dysfunction of these genes have been found in several cancers (30). Chromosome 9p is known to harbor a tumor-suppressor gene, which is *p16*, and it is one of the most common genetic abnormalities in cancers (31–34). *P16* encodes a cell cycle regulatory protein that inhibits cyclin-dependent kinases 4 and 6 preventing phosphorylation to result in chromosome changes. In esophageal adenocarcinoma (EAC), frequent *p16* deletion has been described but point mutation in

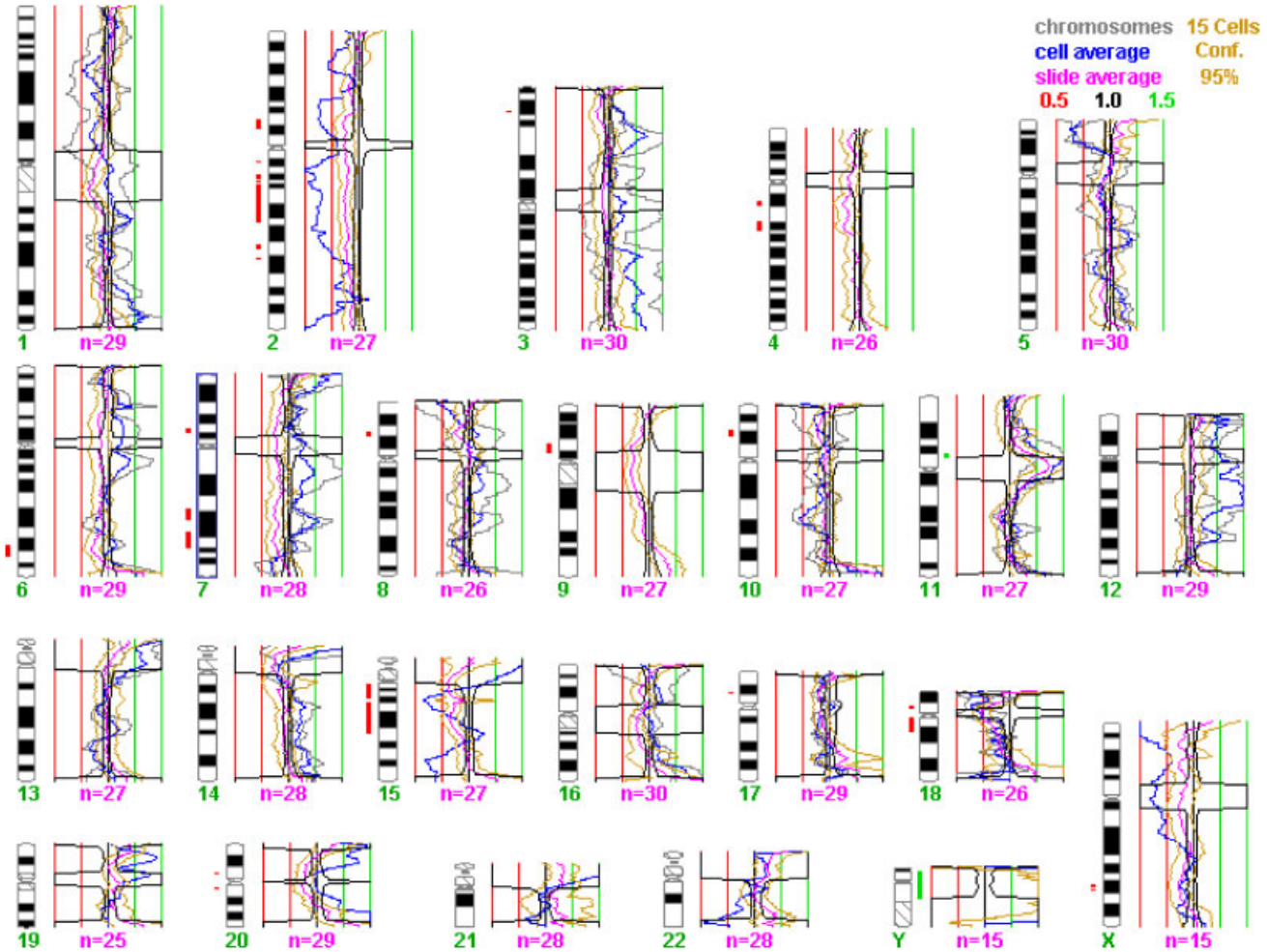

Fig. 2. Summary of comparative genomic hybridization abnormalities identified in EC tissues (case N2) is shown. Each vertical line represents a single genetic aberration observed in a single specimen. Losses are shown on the left and gains on the right.

TABLE 3. The Results of the Regions With Chromosome Gains of the CGH Analysis in 40 Esophageal Tissues in Comparison With Noncancerous Tissues

Gain and loss	Cases/40	%
+1q31–44	5	13
+2q12–31	7	18
+2p22	2	5
+3q13–29	9	23
+4q31–32	2	5
+5q23–34	2	5
+7p14–p22	4	10
+8q21.1–q24	10	25
+10 q21.1–q23.2	3	7.5
+12p13.1	3	7.5
+13q13–q34	7	18
+14q21–q22/q23	4	10
+20q11.2–p12	3	7.5

TABLE 4. The Results of the Regions With Chromosome Losses of the CGH Analysis in 40 Esophageal Tissues in Comparison With Noncancerous Tissues

Gain and loss	Cases/40	%
−1q21.2	8	20
−2p24/p22	4	10
−3p14 - p22	7	18
−4q32	8	20
−5q23.1	7	18
−6q16–q25	6	15
−9p13/p13–p21	5	13
−11p12–P15.5	4	10
−16p12–12.2	2	5
−18q11.2–q22/q23	7	18
−19q13.2–q13.4	3	7.5
−21q21./q22.1/22.3	4	10
−22q11.2~q12.2	3	7.5

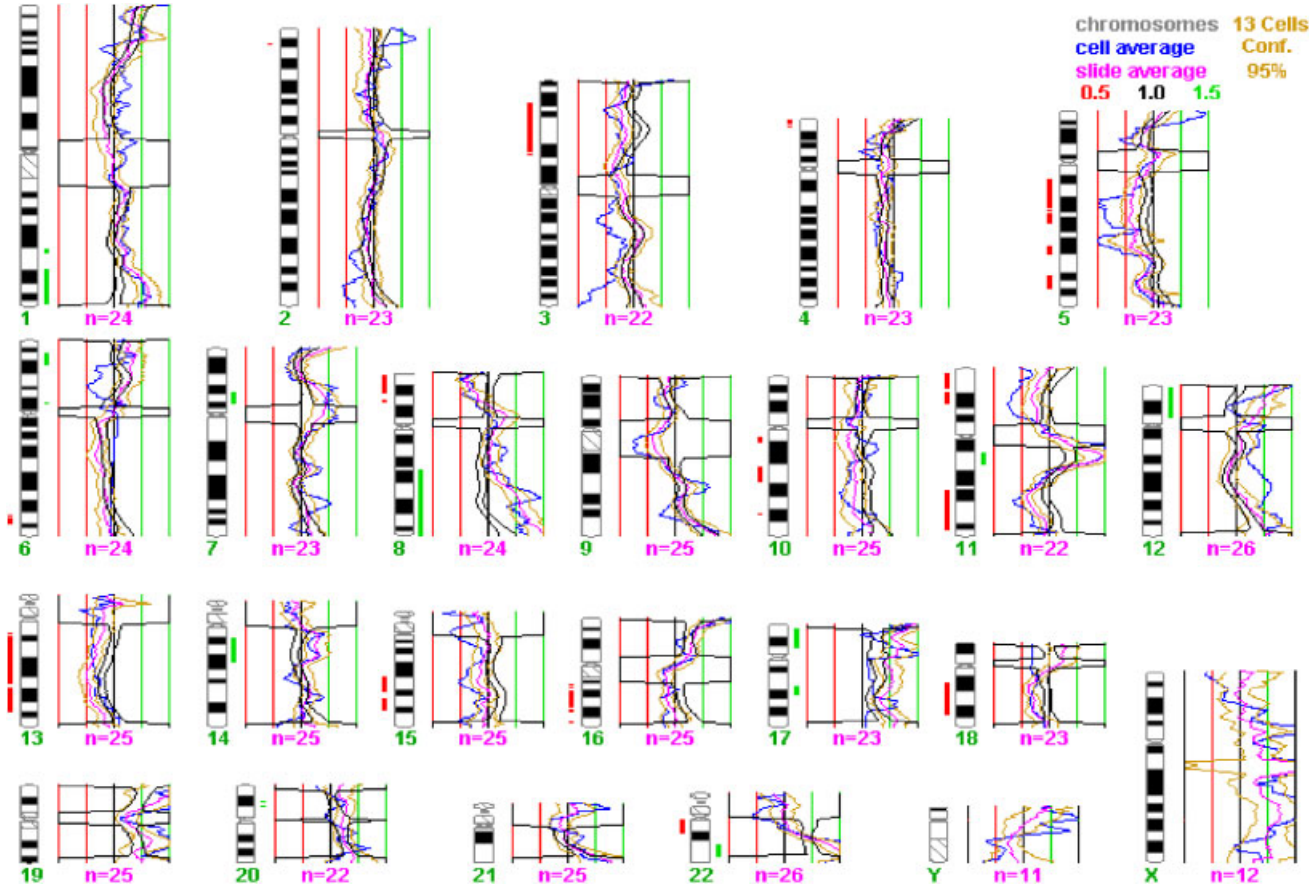

Fig. 3. Summary of comparative genomic hybridization abnormalities identified in paired nontumor tissues (case T1) is shown. Each vertical line represents a single genetic aberration observed in a single specimen. Losses are shown on the left and gains on the right.

exons 1 and 2 were rare in EAC. These results are well correlated with the results of CGH (35). The loss on chromosome 18q has been reported in 63–70% of EACs (30,35). There are two tumor suppressor genes, which are *DCC* and *SMAD4*, located at this region; 46% of Barrett's esophagus patients have abnormalities in this area (30).

C-myc is located on chromosome 8q24 and the activation may contribute to tumor progression by preventing cells from entering Go phase. The progression of EAC is often characterized by the accumulation of genetic abnormalities. Amplifications of the *C-myc* loci or gains of this loci might be of value as prognostic markers because they are already present in nondysplasia cases and may precede the following event of the amplification as observed in high-grade dysplasia and EAC (35). The oncogene *SRC* gene is located on chromosome 20q11–13; over expression of the *SRC* gene in NIH 3T3 cells caused reduction of cell-to-cell transmission of molecules in the 400- to 700 Da range. Down regulation was enhanced by point mutation of tyrosine-527, whereas mutation of tyrosine-416 suppressed both the down regulation of communication by the tyr-527 mutation and that by gene over expression. The regulation of communication by *SRC* may be important in the control of embryonic development and cellular growth. But in EC, the real function results from this gene effect may not be clear yet (35). The other high frequencies are on chromosome 2q, which may involve *TGF*-α and *VEGF* genes. These genes have been strongly related to the development of abnormal tissues in EC, and the aberration of our CGH data may be involved in the loci of these critical genes which form EC (2,36,37).

We performed CGH on these cases with at least 13–20 cells on each case except a few cases which were under this limitation but still above ten cells. The same specific band regions must be present at least two times to be regarded as being counted. According to these limitations, the strict rule may erase arbitrariness and bias. Therefore, the specific region is statistically recorded in our results. So the other regions which were not specific will be discarded. This is the reason why there were no abnormalities in a few regions in our

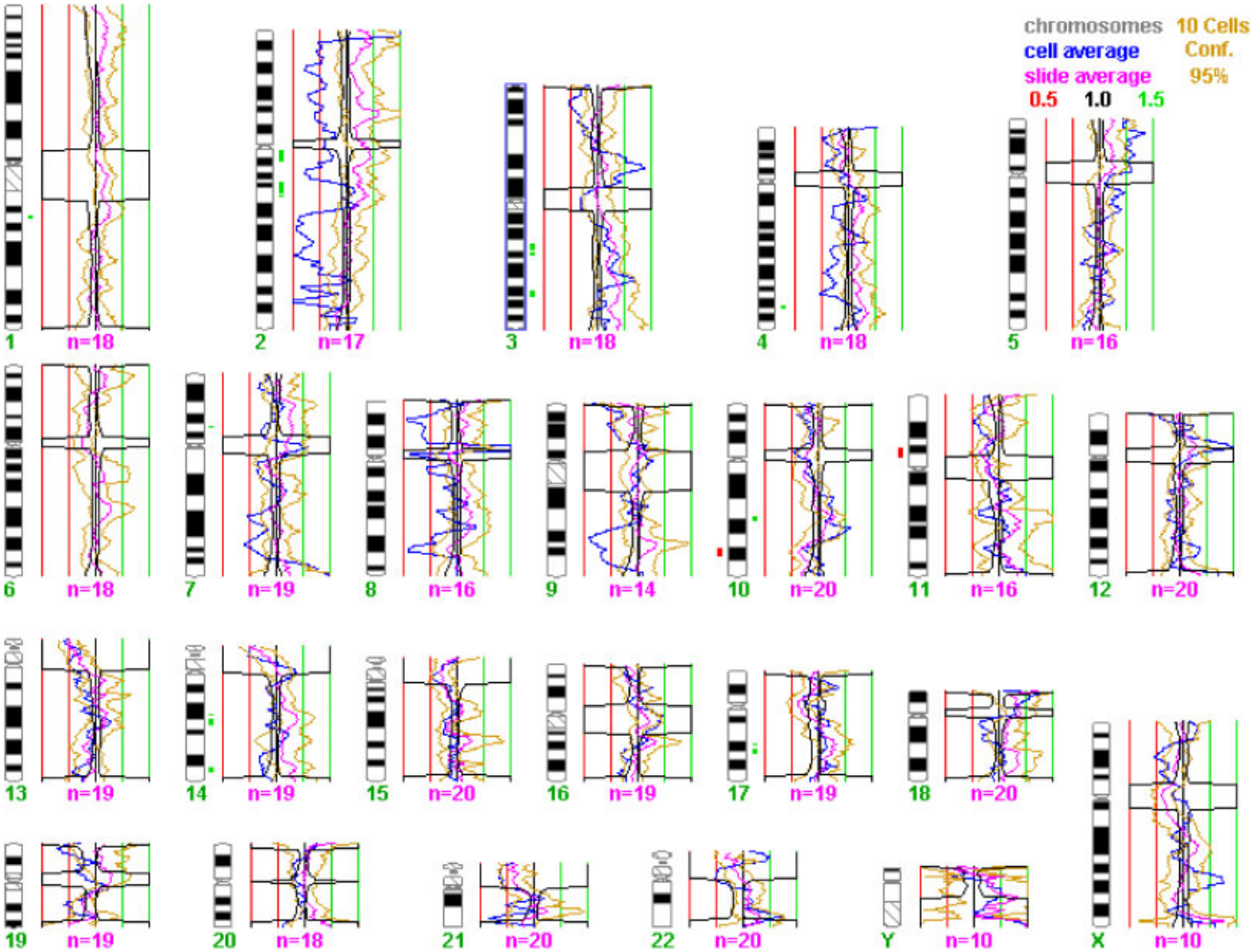

Fig. 4. Summary of comparative genomic hybridization abnormalities identified in paired nontumor tissues (case T2) is shown. Each vertical line represents a single genetic aberration observed in a single specimen. Losses are shown on the left and gains on the right.

research but there were in the research conducted in China, Japan, and the USA. However, there were abnormalities in 10q, 15q, and 21q in our research, but they were not present in theirs, which might result from the difference of the area of a specific gene and the dietary uniqueness.

After using CGH to analyze the two established EC groups, we found that the weakness of CGH is that we cannot locate the specific sequence, but chromosome regions can be approximately located if the genes are oncogene or suppressor genes, and it is more cost-efficient when compared with the SNP array, which is more scalable and costly.

The changes on the noncancerous area and the frequent area of EC may be related to the formation of cancer; hence, those cases must be regarded as precancerous changes. The unique changes of the noncancerous tissues, which were not manifested on the EC tissues, may not be directly related to the formation of the cancer, but they may be instrumental in cancer formation at the pre-cancerous period or the development of inhibitive and harmful cancer cells at the post cancerous period.

In conclusion, the carcinogenesis of EC may begin in the nearby noncancer tissues, and there are similar molecular cytogenetic changes in EC and its nearby noncancerous tissues. We suggest that nearby noncancer tissues of the tumor should not be regarded as normal tissues.

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