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Chromosomal imbalances associated with acquired resistance to fluoropyrimidines in human colorectal cancer cells

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Abstract

The chromosomal aberrations underlying the development of resistance to fluoropyrimidines have not yet been identified. To characterise the genomic changes that induce the development of resistance to fluoropyrimidines, we used comparative genomic hybridisation (CGH) to analyse and compare the parent DLD-1 human colorectal cancer cell line and two cell lines, DLD-1/5-FU and DLD-1/FdUrd, which were resistant to 5-fluorouracil (5-FU) and 5-fluoro-2′-deoxyuridine (FdUrd), respectively. Both resistant cell lines showed a genetic aberration derived from the parental cell line DLD-1. Losses of 3p and 3q were also detected as additional genetic changes in the two resistant cell lines. Both resistant cell lines showed decreased orotate phosphoribosyltransferase (OPRT) activity, which is associated with the activity of the uridine monophosphate (*UMP*) synthase gene (3q13). These results suggested that the loss of 3q might be a genetic change responsible for the decreased OPRT activity and fluoropyrimidine cytotoxic response in cancer cells. Amplification of 18p11.2-p11.3 containing the thymidine synthase (*TS*) gene (18p11.32) was observed only in the DLD-1/FdUrd-resistant cell line, which overexpresses TS. These findings suggested that 18p amplification represents a genetic change associated with the overexpression of the TS protein. Our results indicate that chromosomal aberrations identified by CGH could explain, at least in part, acquired fluoropyrimidine resistance.

Keywords: Comparative genomic hybridisation; Drug resistance; 5-Fluorouracil; 5-Fluoro-2′-deoxyuridine; Colorectal cancer; Thymidine synthase; Orotate phosphoribosyltransferase

1. Introduction

Fluoropyrimidines, such as 5-fluorouracil (5-FU) and 5-fluoro-2′-deoxyuridine (FdUrd), are used widely for the treatment of gastric and colorectal cancers. However, the development of acquired resistance is a serious obstacle to the successful treatment of cancers. The traditional molecular methods used for detection of drug resistance have focused on the gene expression of various enzymes. However, the genetic changes that occur during the development of drug resistance are poorly understood. Comparative genomic hybridisation (CGH) is a powerful molecular cytogenetic DNA-based technique that enables global screening analysis of chromosomal gains and losses, and identification of oncogene and tumour suppressor genes in solid tumours. Moreover, CGH is useful for characterising genomic changes associated with chemoresistance. For example, several studies have used CGH for analysis of acquired chemoresistance to cisplatin, topoisomerase II inhibitor, vindesine and other anticancer drugs [1–3]. With regard to fluoropyrimidines, only one study used CGH analysis to demonstrate that the gain of the 18p-containing thymidylate synthase (*TS*) gene was a common chromosomal aberration in cell lines resistant to TS inhibitors (raltitrexed and 5-FU) [4]. Previous studies also showed that the gene expression and protein level of TS were associated with the response to 5-FU [5,6]. However, other studies have also reported a significant decrease in 5-FU metabolising enzymes including orotate

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phosphoribosyltransferase (OPRT) and uridine kinase (UK) in human cancer cell lines treated with high concentrations of 5-FU [7, 8]. Therefore, it is possible that other chromosomal aberrations could be associated with fluoropyrimidine resistance. In the present study, we used CGH to analyse human colorectal carcinoma DLD-1 cells and two resistant sublines to identify the genomic changes that induce the development of resistance to fluoropyrimidines (5-FU and FdUrd).

2. Materials and methods

2.1. Cell lines and cultures

The human colorectal carcinoma DLD-1 cell line was provided by Yuko Murakami (The Second Cancer Laboratory, Taiho Pharmaceutical Co., Tokyo, Japan). The 5-FU-resistant subline of human colorectal carcinoma DLD-1 cell line (DLD-1/5-FU) and FdUrd-resistant DLD-1 cell line (DLD-1/FdUrd) were established by stepwise escalation of each drug at the Second Cancer Laboratory, Taiho Pharmaceutical Co., as previously described in Ref. [9]. DLD-1/5-FU cells were repeatedly exposed for 120 h to step-wise increased concentrations (from 0.2 to 100 μM) of 5-FU. DLD-1/FdUrd cells were established in a similar manner, using concentrations of 0.01–0.1 μM of FdUrd. It was confirmed that all drug-resistant cell lines retained resistance after at least 3–4 months under drug-free culture conditions. All cell lines were cultured with Roswell Park Memorial Institute (RPMI)-1640 supplemented with 10% fetal calf serum (FCS). The cytotoxic effects (IC_{50}) of 5-FU and FdUrd against drug-resistant DLD-1/5-FU and DLD-1/FdUrd cells were 65.2- and 9.7-fold, respectively, lower than those against the respective parental cells, as previously described in Ref. [9]. High molecular weight DNA of the cell lines was isolated using a DNA isolation kit (QIAamp DNA Mini Kit, Qiagen Inc., Santa Clarita, CA, USA). Normal DNA was extracted from peripheral blood lymphocytes of healthy men following their informed consent.

2.2. Comparative genomic hybridisation and digital image analysis

CGH was performed according to a procedure previously described in Ref. [10]. For conventional CGH, normal DNA was examined as the reference DNA, and cell line DNA was examined as the target DNA. In addition, to identify only new chromosomal aberrations in the resistant cell lines, direct comparison using 'modified CGH' of the resistant cell line versus the parental cell line was performed as previously described in Ref. [11]. The parental cell line DNA was examined as the reference DNA, and the resistant cell line DNA was examined as the target DNA for the modified CGH. Target DNA was labelled with Spectrum Green-dUTP (Vysis Inc., Downers Grove, IL, USA), and reference DNA was labelled with Spectrum Red-dUTP (Vysis Inc.) by nick translation. Each 200 ng of ethanol-precipitated tumour- and reference-labelled DNA, and 20 ng unlabelled Human *Cot-1* DNA (Gibco BRL, Gaithersburg, MD, USA) were mixed. The DNA was denatured at 73 °C for 3 min. Slides with normal metaphase spreads were denatured in 70% (v/v) formamide/2× saline sodium citrate (SSC) at 73 °C for 3 min. Labelled DNA was then hybridised in a humidified chamber at 37 °C for 72 h. The slides were washed three times for 12 min each in 50% (v/v) formamide/2×SSC at 45 °C, twice in 2×SSC, once in 2×SSC at room temperature, and once in distilled water at room temperature for 10 min. The slides were counterstained with 0.2 μg/μl of 4′,6-diamino-2-phenylindole (DAPI) in antifade solution (Oncor Inc., Heidelberg, Germany). Images were collected using a charge-coupled device (CCD) camera mounted on a fluorescence microscope (Olympus Inc., Tokyo, Japan) equipped with a single band pass filter. The 8–10 images of metaphases with good quality hybridisation were analysed using a digital image analysis system (FineStar^{TM} FISH-CGH analysis system; TOYOBO Inc., Tokyo, Japan). A target to reference ratio of >1.2 was considered as a gain, a ratio <0.8 was considered as a loss, while a ratio >1.4 was considered amplification. These cut-off values were determined by positive and negative control CGH experiments. Normal DNA from a healthy man was used as a negative control, and DNA from the MPE-600 breast carcinoma cell line was used as a positive control. The heterochromatic regions, the p arms of the acrocentric chromosome and Y chromosome were excluded from the analysis because of suppression hybridisation with *Cot-1* DNA in these regions. In order to control for the difference in hybridisation between Spectrum Green-dUTP and Spectrum Red-dUTP labelled probes, we performed inverse hybridisation by a target DNA labelled with Spectrum Red-dUTP, and a reference DNA labelled with Spectrum Green-dUTP as previously described in Ref. [11]. We confirmed the presence of the DNA copy number aberration by comparing the results of standard and inverse CGH.

3. Results

Conventional CGH analysis, in which DNA of normal cells was used as the reference DNA, was performed in each resistant and parental cell lines. Fig. 1 and Table 1 summarise the chromosomal aberrations in all three cell lines. The gain of 2p16-p22 was observed in the parental and two resistant cell lines. Furthermore, the two resistant cell lines showed a few additional chromosomal changes. Losses of 3p and 3q were identified in

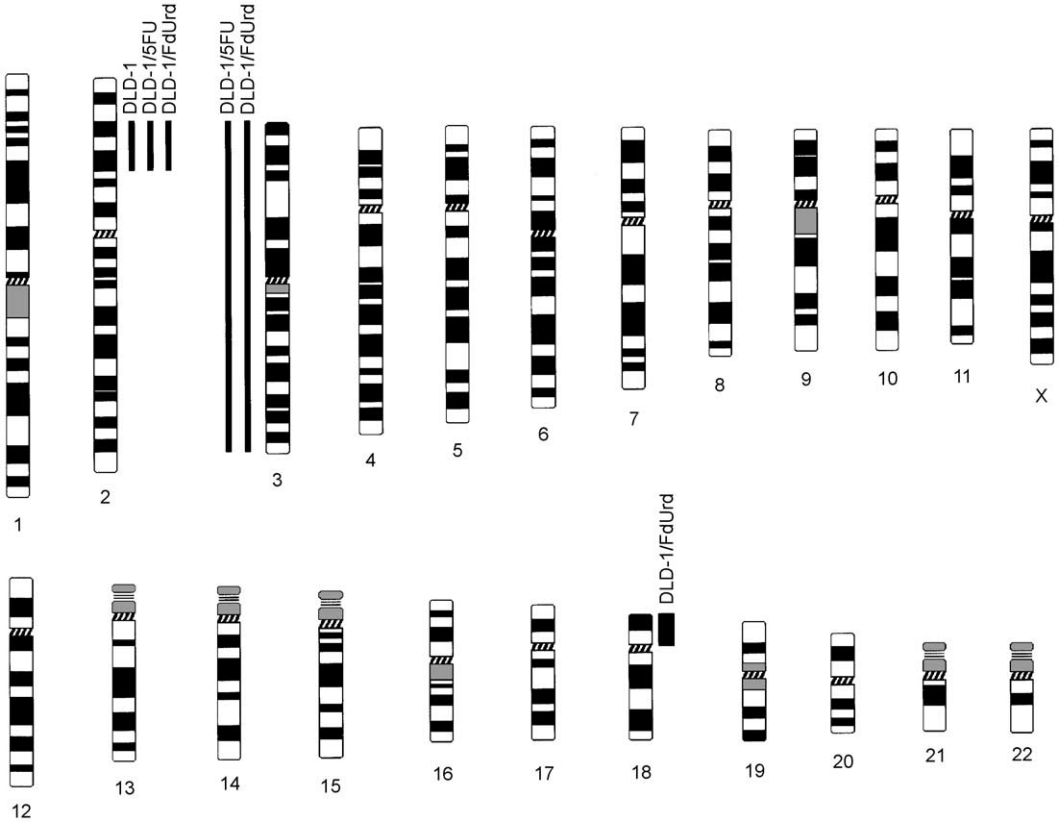

Fig. 1. Summary of the gains and losses of DNA copy number detected by conventional comparative genomic hybridisation (CGH) in a parental cell line and the two resistant cell lines. The chromosomal localisation of gains is represented on the right side of the chromosome ideograms and that of losses on the left side. Thick lines indicate the amplified regions.

Table 1
Chromosomal imbalances in colorectal cancer cell lines

	Gains (amplifications)[a]	Losses
Conventional CGH		
DLD-1	2p16-p22	–
DLD-1/FdUrd	2p16-p22, 18p11.2-p11.3	3p, 3q
DLD-1/5-FU	2p16-p22	3p, 3q
Modified CGH		
DLD-1/FdUrd versus DLD-1	18p11.2-p11.3	3p, 3q
DLD-1/5-FU versus DLD-1	–	3p, 3q

CGH, comparative genomic hybridisation; 5-FU, 5-fluorouracil; FdUrd, 5-fluoro-2′-deoxyuridine.
[a] Amplification is underlined.

both resistant cell lines, DLD-1/5-FU and DLD-1/FdUrd. However, amplification of 18p11.2-p11.3 was observed only in the DLD-1/FdUrd resistant cell line. In addition, direct comparison was performed using modified CGH to identify differences in DNA copy number between the parental and resistant cell lines. Certain chromosomal aberrations found in the modified CGH were similar to the changes noted in resistant cell lines (Table 1, Fig. 2). The ratio of target to reference CGH profiles was markedly higher in 18p11.3 on the telomeric side of 18p (Fig. 2).

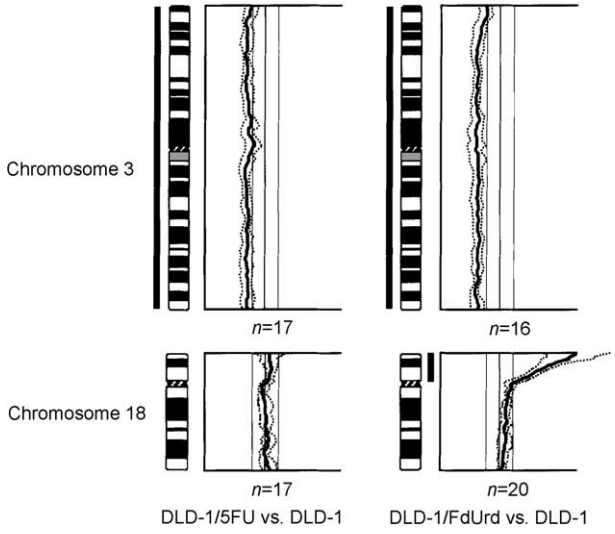

Fig. 2. Profiles of direct comparison modified comparative genomic hybridisation (CGH) between the parental cell line and resistant cell lines. The vertical middle line represents the baseline as a ratio of 1.0. A left-sided shift indicates a ratio of 0.8 and a right-side shift indicates a ratio of 1.2. Average target/reference fluorescence intensities (thick lines) ±1 standard deviation (thin lines) are shown. The *n* number underneath each profile indicates the number of chromosomes counted to provide this profile. The solid bars to the left and right of chromosome ideograms indicate loss and gain of DNA sequences, respectively.

4. Discussion

In the present study, we used CGH to compare parental and resistant cell lines and identified the genetic changes induced during the development of resistance to fluoropyrimidine. The chromosomal change identified in the parental cell line, DLD-1, was a gain of 2p16-p22. Both resistant cell lines also showed the gain of 2p16-p22, and thus this change was considered as a genomic change derived from parental cell line, DLD-1. Loss of 3q was observed in both resistant cell lines. These findings were not reported in previous studies using CGH analysis in other resistant cell lines [4].

OPRT is an important enzyme involved in the conversion of pyrimidines into the active nucleotide form, and the sequential activities of the bifunctional enzyme, uridine monophosphate (UMP) synthase, whose gene is located on 3q13 [12]. Genetic deficiency of UMP synthase activity causes the inherited human disease orotic aciduria. Previous studies using Epstein–Barr virus (EBV)-transformed lymphocytes derived from patients with hereditary orotic aciduria demonstrated that the cytotoxic effects of 5-FU on homozygotes were milder than those on the normal controls which were EBV-transformed lymphocytes derived from healthy volunteers [13]. However, the cytotoxic effects of 5-FU on the heterozygotes were intermediate between the homozygote and controls. The level of OPRT activity in the two resistant cell lines used in the present study was lower than that of the parental cell line reported in a previous study [9]; and OPRT activity was significantly lower in DLD-1/5-FU cells than in the parental cells. These results suggest that the loss of 3q may be genetic in nature to reduce OPRT activity and decrease the fluoropyrimidine cytotoxic response in cancer cells.

Losses of 3p were also observed in the two resistant cell lines used in the present study. No such findings were observed in previous CGH studies of resistant cell lines [4]. Interestingly, it was reported that the introduction of chromosome 3, which contains *hMLH1* mapped on 3p21.3 [14], to DNA mismatch repair deficient cells with homozygous *hMLH1* mutations, reduced the resistance to DNA damaging agents such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, 6-thioguanine, and cisplatin [15–17]. Previous studies also revealed that DNA mismatch repair-deficient colon cancer cell lines were more resistant to 5-FU in vitro than DNA mismatch repair-proficient cell lines [18,19], and that mismatch repair deficiency mediated by hMLH1 played a role in G2 cell cycle arrest response and cytotoxicity after exposure to fluoropyrimidines [20]. Although parental DLD-1 cells exhibit microsatellite instability with *hMSH6* mutations [21,22], the loss of the 3p-containing *hMLH1* gene could potentially induce the acquisition of a more rigid resistance to fluoropyrimidines. However, several recent clinical studies have reported that the microsatellite instability phenotype is associated with excellent survival in patients with advanced colorectal cancers receiving 5-FU-based chemotherapy [23,24]. It has been reported that microsatellite instability phenotype was associated with a Crohn's-like lymphoid reaction and intraepithelial lymphocytosis [25,26]. Therefore, the inconsistency between *in vitro* and *in vivo* may be due to host immune responses. Further studies and assessments are necessary to unravel the inconsistency.

Amplification of 18p11.2-p11.3 was observed in the DLD-1/FdUrd-resistant cell line. This finding was consistent with previous CGH studies in a TS inhibitor-resistant cell line [4]. A previous study demonstrated overexpression of the *TS* gene and *YES1* gene, which has been mapped on 18p11.32 [27], in TS inhibitor-resistant cell lines, which was detected by a cDNA microarray [28]. The same study also demonstrated overexpression of TS mRNA and protein in all TS inhibitor-resistant cell lines [28]. In this regard, TS overexpression is strongly associated with fluoropyrimidine resistance [29]. Although the gains of 18p were consistently detected in all TS inhibitor-resistant cell lines in the above study, we found 18p amplification only in the DLD-1/FdUrd-resistant cell line. While the exact reason for the difference between the results of the two studies is not clear at present, it could be due to different mechanisms being involved in the acquired resistance to fluoropyrimidines in the resistant cell lines used in the two studies. In this regard, further characterisation of these resistant cell lines demonstrated *TS* gene amplification in DLD-1/FdUrd-resistant cell line and overexpression of TS protein compared with the parental cell line [9]. In contrast, these features were not observed in another DLD-1/5FU-resistant cell line [9]. These findings suggest that 18p amplification could be the genetic change that induced the overexpression of TS protein in the DLD-1/FdUrd-resistant cell line. Several studies have reported that the significant decrease of fluoropyrimidine incorporation into RNA could be another mechanism for 5-FU resistance in human tumour cells treated with high concentrations of 5-FU [7,8]. Therefore, the cytotoxic effect of DLD-1/5-FU may be predominantly due to the incorporation of fluorouridine triphosphate (FUTP) into RNA and subsequent interference in RNA processing, because the DLD-1/5-FU cell line was established using high concentrations of 5-FU. The results indicate that 18p amplification could be one of the underlying mechanisms responsible for acquisition of resistance to fluoropyrimidines, although it appears to be a genetic change that is not common to all resistant cells.

Several studies reported that not only *TS* gene expression, but also the gene expression of dihydropyrimidine dehydrogenase (*DPD*), a pyrimidine catabolism enzyme, was associated with sensitivity to 5-FU [30,31]. cDNA microarray analysis of 60 human cancer

cell lines also demonstrated that reduced *DPD* gene expression was associated with the sensitivity to 5-FU [32]. Previous studies reported that the *DPD* gene is mapped on the chromosomal region 1p22 [33]. No additional chromosomal aberrations on 1p22 were observed in all of the resistant cell lines used in our CGH study, as well as in the study of Rooney and colleagues [4]. Moreover, the resistant cell lines used in our study did not show overexpression of DPD mRNA or increased DPD activity (data not shown). Interestingly, Kirihara and colleagues [34] reported that treatment with 5-FU was associated with a significant increase in the *TS* gene expression level compared with that of DPD in 5-FU-sensitive gastrointestinal cancer cell lines. They also demonstrated that the DPD expression level could predict primary 5-FU sensitivity, and that TS expression might be a potent predictor of 5-FU resistance after 5-FU treatment. These findings, and the results of the present study, suggest that amplification of 18p11 including the *TS* gene may have a more detrimental effect on resistance after fluoropyrimidine treatment.

In summary, the loss of 3p and 3q and amplification of 18p11.2-11.3 observed in our study could be considered as potentially relevant genomic aberrations with regard to the acquired resistance to fluoropyrimidines. It seems that chromosomal aberrations in tumour cells could potentially include more than one resistance mechanism in order to acquire a more rigid resistance during the growth of tumour cells under fluoropyrimidine exposure. However, because the resistant cell lines used in our study were established *in vitro* by exposure to the test drug, their acquired resistance may differ from those of resistant cancer cells growing *in vivo* and also may differ to innate resistance. Therefore, further studies involving not only resistant cell lines, but also a large number of clinical specimens, treated under clinical schedule settings, are necessary for a fuller understanding of the mechanisms involved in the resistance to fluoropyrimidines.

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