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Cisplatin as monotherapy or in combination with 5-Fluorouracil (5FU) is currently the first-line treatment of stage III or IV colorectal cancers. Predicting the efficacy of these drugs in individual patients before the establishment of a treatment regimen is essential. The aim of the present study was to develop a method to predict the response to cisplatin/5FU therapy in colorectal carcinomas using the minimum required number of cancer cells from each patient. Single cell gel electrophoresis (COMET assay) was used to assess the effect of cisplatin treatment, and quantitative real-time PCR (qRT-PCR) was used to detect the expression of thymidylate synthase (TYMS), dihydrofolate reductase (DHFR), serine hydrate methyltransferase (SHMT1) and dihydropyrimidine dehydrogenase (DPYD) in human colon carcinoma cell lines. The application of these methods for predicting the response to combination drug therapy in a simple and rapid manner would help physicians tailor treatment strategies to individual patients, even those with the same type of cancer.

Key Words: Colorectal carcinomas, Cisplatin, 5-Fluorouracil, qRT-PCR, COMET assay.

Introduction
Cisplatin (CDDP) is a platinum-based drug used for the treatment of various types of cancer. 5-Fluorouracil (5FU) is a pyrimidine analog that is also commonly used as an anticancer drug. Using these two drugs as monotherapy is inefficient due to both acquired and intrinsic resistance mechanisms. Cisplatin and 5FU combination therapy or each drug as monotherapy is the first-line treatment for colorectal cancers, although combination treatment shows better efficacy.

The ability to predict the response to treatment in individual patients is essential, and evaluating the response to chemotherapy using methods with few requirements is important to assist clinical or medical oncologists in the determination of adequate treatment strategies.

Single cell gel electrophoresis is a rapid and sensitive technique commonly used to measure the efficacy of antitumour treatments by evaluating DNA damage in individual cells. The principle of the COMET assay is that unfragmented DNA maintains a well-organised structure in the nucleus, but when the cell is damaged, this organisation is disrupted. When an electric field is applied to the DNA, the unfragmented parts are too large and move slowly, while the fragmented parts move faster due to their molecular weight and dense conformation. The amount of genetic material in the nucleus (called “head”) and the amount included in the fragmented pieces (called “tail”) reflect the effect of a drug on the DNA chains. During measurement, the percentage from the total DNA of a single cell distributed to the tail part reflects the fragmented DNA, and that distributed to the head part reflects the unfragmented DNA. Thus the final outcome is a percentage with no units. 5FU is a thymidylate synthase (TYMS) inhibitor. Dihydropyrimidine dehydrogenase (DPYD) is the main enzyme involved in the degradation of 5FU. TYMS, dihydrofolate reductase (DHFR) and serine hydrate methyltransferase (SHMT1) are the three enzymes that constitute the de novo thymidylate synthesis pathway in mammals. In the present study, quantitative real-time PCR was used to analyse the gene expression pattern of these enzymes in human cancer cell lines derived from colorectal carcinomas in response to treatment with 5FU according to a previously published method.

Materials and Methods
The human colon carcinoma cell lines LoVo, HCT-116, HT55 and HCT-15 used in the present study were obtained from the European Collection of Cell Cultures (ECACC, UK). The COMET assay and qRT-PCR were used to predict the response to 5FU/cisplatin chemotherapy in these cell lines.

Cell Culture: Cells were cultured in 75cm2 flasks (Orange Scientific, 5520200, Belgium) in the medium indicated for each line with the appropriate amount for each cell line of heat inactivated fetal bovine serum (FBS, Invitrogen, 10106-169, California) and 2mM L-Glutamine (Sigma, G5792, Germany), and incubated at 37oC, in a 5% CO2 atmosphere. Cells were divided into three 75cm2 flasks that contained no added drug, cisplatin (1μg/ml) (P4394, Sigma-Aldrich, Germany) or 5-FU (750μg/ml) (Teva Pharma B.V., Netherlands). After 24 h of incubation, cells were detached by trypsinisation (Trypsin-0.25% EDTA, Invitrogen, 25200-072, California).

COMET Assay:
Single cell gel electrophoresis was performed using the IKZUS COMET assay kit (Cat. No. 0905-050-K, Italy) with alkaline lysis. CDDP-treated and untreated cells were seeded onto slides specifically designed for the COMET assay using low-melting-point agarose, and incubated with lysis solution for 1 h, followed by alkaline solution incubation for 30 min. Slides were then subjected to electrophoresis for 20 min at 25V in 0.5X TBE and cells were fixed with 70% ethanol in phosphate buffered saline (PBS) (P3813, Sigma-Aldrich, Germany). Slides were then subjected to electrophoresis for 20 min at 25V in 0.5X TBE and cells were fixed with 70% ethanol in phosphate buffered saline (PBS) (P3813, Sigma-Aldrich, Germany). Slides were stained with staining solution and then observed in a UV light microscope. Data were analysed using the Comet Score software (TriTek Corp., USA).

qRT-PCR
RNA from 5FU-treated and untreated cells was extracted using the RNase mini kit (74104, Qiagen, Germany) in the QIAcube system (9001293, Qiagen, Germany) and 2mM L-Glutamine (Sigma, G5792, Germany), and incubated at 37oC, in a 5% CO2 atmosphere. Cells were divided into three 75cm2 flasks that contained no added drug, cisplatin (1μg/ml) (P4394, Sigma-Aldrich, Germany) or 5-FU (750μg/ml) (Teva Pharma B.V., Netherlands). After 24 h of incubation, cells were detached by trypsinisation (Trypsin-0.25% EDTA, Invitrogen, 25200-072, California).
Results

Table 2 shows the results of single cell electrophoresis in the different cell lines analysed. The results varied according to the cell line and the differences were statistically significant with the exception of the LoVo cell line. The statistical evaluation was performed by measuring the difference of the mean. The percentages of DNA in the head and the tail are shown in Table 2. Cisplatin treatment had a significant effect in all cases, suggesting that these cancer cell lines respond to this chemotherapeutic agent. Figures 1 and 2 illustrate the efficacy of CDDP in the HT55 cell line.

The results of the qRT-PCR analysis in cells treated with 5FU are shown in Table 3 and show variable effects of 5FU on the different genes in the different cell lines. The data are expressed as the threshold cycle (CT) parameter, which is an indicator of the expression of a gene. Cycle threshold is called the cycle at which the fluorescence from a sample crosses the threshold, and is proportional to the expression of the gene studied. The key gene affected by 5FU is TYMS, while the other genes played secondary roles. In the cancer cell lines LoVo, HCT-15 and HCT-116, 5FU treatment had an effect on the TYMS gene, by decreasing the gene expression, that was not observed in the HT55 cancer cell line, which indicates resistance to this chemotherapeutic agent. Expression of the SHMT1 gene was not significantly affected by 5FU treatment, while the expression of the DPYD gene was altered by 5FU in all the cell lines studied. Statistical analysis was performed by measuring the difference of the mean.

Discussion

One of the most commonly used strategies for the treatment of colorectal carcinomas is a combination of cisplatin and 5FU. Because of the toxicity associated with this line of treatment, assays capable of predicting the response of individual patients to these drugs are essential for oncologists before drug administration.

Cisplatin reacts with DNA in vivo, causing cross-linking of DNA, which ultimately leads to programmed cell death (apoptosis). The single cell electrophoresis assay (COMET assay) can measure the effect of cisplatin treatment using only a few flowing cancer cells from each patient. Single cell gel electrophoresis can detect the damage to DNA as single strand and double strand breaks. This technique allows the evaluation of the effect of platinum on the integrity of the DNA. Because the effect of different drugs vary even in the same type of tumour cell lines, COMET assay can determine the effect of each drug on the DNA.

On the other hand, 5-fluorouracil causes cell cycle arrest in the post-G1/pre-S phase and induces apoptosis by inhibiting DNA synthesis and by interacting directly or indirectly with several enzymes. qRT-PCR is used to determine the effect of 5FU in different cell lines. This technique enables the comparison of the gene expression patterns of reference and endogenous genes. In the present study, the endogenous gene analysed was glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which catalyses the conversion of glyceraldehyde 3-phosphate during glycolysis, and it is used as an endogenous-housekeeping gene in qRT-PCR reactions. This method is very sensitive, requires only small amounts of RNA, and is based on the detection and quantification of a fluorescence reporter (SYBR Green). The parameter that was studied was the CT. The enzymes analysed in the present study were TYMS, SHMT1, DPYD and DHFR. TYMS catalyses the conversion of glyceraldehyde 3-phosphate during glycolysis, and it is used as an endogenous-housekeeping gene in qRT-PCR reactions. This method is very sensitive, requires only small amounts of RNA, and is based on the detection and quantification of a fluorescence reporter (SYBR Green). The parameter that was studied was the CT. The enzymes analysed in the present study were TYMS, SHMT1, DPYD and DHFR. TYMS catalyses the conversion of glyceraldehyde 3-phosphate during glycolysis, and it is used as an endogenous-housekeeping gene in qRT-PCR reactions. This method is very sensitive, requires only small amounts of RNA, and is based on the detection and quantification of a fluorescence reporter (SYBR Green). The parameter that was studied was the CT.
nucleic acid biosynthesis, and DHFR reduces dihydrofolic acid to tetrahydrofolic acid. Tetrahydrofolate and its derivatives are essential for purine and thymidylate synthesis.

The two techniques described in the present study are simple and have few requirements, and their use will allow physicians to evaluate patient response to drug treatment in advance, thus facilitating the selection of appropriate therapeutic strategies.

Conclusion
The heterogeneity and genetic instability of cancer make it necessary to personalise treatment. The results of the present study suggested that the response of cell lines to cisplatin and 5-FU treatment vary, even in cell lines derived from the same type of cancer. In this pilot study, a new robust and effective method to predict the response of individual patients to a drug is described. Further investigations using additional drugs and a wider population of tumour cell lines would be of great value.

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