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Partial least squares regression and fourier transform infrared (FTIR) microspectroscopy for prediction of resistance in hepatocellular carcinoma HepG2 cells

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ABSTRACT

We evaluated the feasibility of FTIR microspectroscopy combined with partial least squares regression (PLS-R) for determination of resistance in HepG2 cells. Cell viability testing was performed using neutral red assay for the concentration of cisplatin resulting in 50% antiproliferation (IC_{50}). The resistance index (RI) is the ratio of the IC_{50} in resistant HepG2 cells *vs.* parental HepG2 cells. Principal component and unsupervised hierarchical cluster analyses were applied and a differentiation of samples of cells (parental, 1.8RI, 2.3RI, 3.0RI, and 3.5RI) was demonstrated (3000–2800 cm^{-1} in the lipid and 1700–1500 cm^{-1} in the protein regions. The FTIR spectra were preprocessed with several treatments to test the algorithm. PLS-R models were built using the 1170 spectra of the HepG2 cells. Cross-validation was used to evaluate prediction of the RI value using this model. PLS-R models—preprocessed with the second derivative FTIR spectra—yielded the best model (R^2=0.99, RMSEE=0.095 and RPD=7.98). Most RI values were predicted with high accuracy (91–100%) such that the linear correlation between the actual and predicted RI values was nearly perfect (slope~1). FTIR microspectroscopy combined with chemometric analysis using PLS-R offers quick, accurate, and reliable quantitative analysis of HepG2 cell resistance.

1. Introduction

Refractoriness is an important factor resulting in failure of cancer treatment [1]. Almost 50% of all cancer cases show and/or develop resistance to chemotherapeutic drugs; this might have existed before chemotherapy (intrinsic resistance) or might have developed during treatment (acquired resistance) [2]. Hepatocellular carcinoma (HCC) is one of the most commonly diagnosed cancers in the world and it is the predominant type of HCC cancer in Asia [3]. HCC is usually identified at an advanced stage because it is difficult to detect and is resistant to conventional clinical therapies, and therefore has a poor prognosis [4]. Low drug efficacy is due to development of refractoriness to cancer treatment, leading to increasing dosages of chemotherapeutic drugs with severe side-effects [5]. Cisplatin is a platinum-based agent for hepatocellular carcinoma (HCC) treatment, however, severe side-effects (i.e., anemia, thrombocytopenia, fatigue, alopecia, infection and neutropenia, peripheral neuropathy) and developed resistance limit its usefulness [6].

The detection of early cancer drug resistance has been attempted so as to improve therapy and avoid high dosages with attendant undesirable side-effects. Such cancer biomarkers need to be verified for use as early therapeutic interventions against malignant diseases. Cancer biomarkers (i.e., carcinoembryonic anti-gen and alpha-fetoprotein) can be detected at an early stage in the blood and be used to identify colon, pancreas, breast, ovary, or lung cancer [2]. Other biomarkers have been identified that alert clinicians when cancer cells develop resistance (viz., increasing of efflux proteins, drug inactivation, alteration of drug targets, DNA damage repair, metastasis). The biomarkers for chemoresistant genes and proteins include P-glycoprotein, multidrug resistance-associated protein-1 (MRP1), its homologs MRP 2–6, and the breast cancer resistance protein (BCRP) [7].

Cancer chemoresistance and chemosensitivity assays have been evaluated for prediction of cancer response to chemotherapeutics. The assessment of drug resistance can be determined by using fresh tumor cell culture assays, cancer biomarker tests, and the nuclear medicine technique positron emission tomography (PET). The fresh tumor cell culture test is a study of the effect of drugs on tumor cell growth based on (a) isolation of tumor cells (b) treatment of isolated cells with anticancer drugs, and (c) evaluation of tumor cell viability [2]. The tests

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include (a) the lactate dehydrogenase leakage (LDH) assay, (b) the neutral red (NR) assay, (c) the methyl tetrazolium (MTT) assay, and (d) the sulphorhodamine-B (SRB) assay [8,9]. Pharmacogenetics (i.e., genomics, proteomics, and transcriptomics) were, moreover, used to detect biomarkers of chemotherapy resistance and to test the genetic factors affecting chemotherapeutic drug action [2]. The other diagnostic technique for cancer drug resistance is positron emission tomography (PET). PET is non-invasive, relatively expensive, and can be implemented in a clinical setting for cancer localization. PET is, moreover, invaluable for studying the metabolic steps in cancer cell growth [2]. By contrast, the other techniques described are invasive, expensive, and require trained laboratory personnel (for sample handling, detection, and analysis).

Fourier transform infrared (FTIR) microspectroscopy is a physicochemical analytical technique for identifying macromolecular changes in biological samples (i.e., in the relative lipid, protein, carbohydrate, and nucleic acid composition) [10]. Conventional FTIR microspectroscopy generates characteristic spectral patterns of cancer cells or varieties of cell biopsy with different modes of action [11], modes of cell death [12], and different cell states [13]. FTIR is used to (a) classify the bacterial genera [12] and (b) to discriminate resistant from sensitive cancer cells in early drug resistant human hepatocellular carcinoma (HepG2) cells [13], human melanoma cell lines [14] and human leukemia cell lines [15].

The benefits of FTIR microspectroscopy are that it is fast, uses fewer reagents, and is non-destructive and non-invasive [10]. FTIR requires minimal sample preparation, employs fast analytical techniques, and provides a fingerprint of the sample [16]. The application of FTIR combined with chemometrics is a powerful analysis approach for manipulating data in disciplinary of chemistry, biochemistry, medicine, or biology. The chemometric techniques—i.e., Principal Component Analysis (PCA) and Partial Least Squares Regression (PLS-R)—were developed from mathematical models and used for quantitative prediction based on spectroscopic data. PLS-R is based on the dimensional reduction of data and inverse calibration, in systems calibrated for the desired component while implicitly modeling other source(s) of variation [16].

PLS-R analysis is a supervised data reduction technique, which preferentially evaluates the independent variables (i.e., FTIR absorbance values) that best linearly correlate with the interest-dependent variables. Here the chemoresistance property is represented by the resistance index (RI). The cross-validation method was performed for all samples. The principle of cross-validation is sequential exclusion of factors from the calibration model: that is, each value is predicted then returned to the data pool; the process is then repeated for each sample. The best-fitting PLS-R models are evaluated according to their determination coefficient (R^2) (i.e., representing accuracy of the model fit) and the root mean square error of cross-validation (RMSECV) (i.e., an indication of the modeling error).

The current study applied FTIR microspectroscopy combined with chemometrics to predict resistance in hepatocellular carcinoma (HepG2) cells. Neutral red assay was used as the reference for chemoresistance and for calculating the resistance index (RI) value. FTIR microspectroscopy was thus tested as an alternative to pharmacogenetic techniques.

2. Materials and methods

2.1. Chemicals

The reagents in the cell culture techniques: Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and 1% penicillin-streptomycin were purchased from GIBCO, Invitrogen Corporation (Grand Island, NY, USA). Neutral red dye (NR) was purchased from Sigma Aldrich Co. (St. Louis, MO, USA). The standard chemotherapeutic drug was cisplatin bought from Boryung Pharmaceutical Co., Ltd. (Kyungki-do, Korea). Sodium chloride (NaCl) was bought form Ajax Finechem (Auckland, New Zealand).

2.2. Cell culture

Human hepatocellular carcinoma (HepG2) (ATCC#8065) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were grown in 5% CO_2 incubator at 37 °C.

2.3. Determination of half maximal inhibitory concentration of cisplatin in each cell groups

The half maximal inhibitory concentration (IC_{50} value) of cisplatin in each cell group was determined after inducing cancer cell resistance. Cell viability of either the parental or resistant HepG2 cells was evaluated using neutral red (NR) assay [13]—a cationic dye that can penetrate viable cells, accumulate, and bind with their lysosomes.

Cell viability was detected based on a colorimetric method using a spectrophotometer. Briefly, cells were seeded into a 96-well plate in the complete medium for 24 h. The cells were tested with free media containing various concentrations of cisplatin for 24 h. Untreated cells were the control containing only complete medium and cells. Neutral red was dissolved in distilled water and prepared to a final concentration of 50 μg mL^{-1} in free media. The NR solution was added to each well and incubated for 2 h at 37 °C in a 5% CO_2 incubator. After incubation, the NR solution was removed and cells were washed using 1×PBS. Cells were solubilized with 0.33%v/v hydrochloric acid in isopropanol. Cell viability was measured using a microplate reader (Tecan's Sunrise™ absorbance reader, Austria) for absorbance at dual wavelengths (*viz.*, 537 nm and 650 nm). The % cell viability was evaluated as $[A_{test}/A_{control}]\times100$; when $A_{control}$=Absorbance of untreated cells and A_{test}=Absorbance of test or treated cells. The resistance index (RI) was calculated from the ratio of the IC_{50} of cisplatin in resistant HepG2 *vs.* the IC_{50} of cisplatin in the parental HepG2.

2.4. Induction of resistance in HepG2 cells to cisplatin

The intermittent method was applied for induction resistance in HepG2 cells. The parental HepG2 cells were repeatedly induced with a stepwise increase in cisplatin concentrations [13,17]. Briefly, HepG2 cells were exposed to cisplatin at a starting low concentration of 1.0 μg mL^{-1} for 24 h. After induction, the drug in the medium was discarded. HepG2 cells were then washed by (1×) PBS and cultured in the complete medium until reaching 80% confluence. The resistance of HepG2 to cisplatin was continuously developed by increasing the concentration of cisplatin until finally generating the next resistance index. This process was repeated until 4 different groups of resistant HepG2 cells were created with different RIs (i.e., 1.8RI, 2.3RI. 3.0RI, and 3.5RI, respectively). Before all experiments, the cultures were restarted from frozen stocks (at −80 °C) and cultured to 80% cell confluence.

2.5. Preparation of sample for FTIR analysis

Preparation of cells for FTIR analysis was done as per Junhom et al., [13]. The parental and resistant HepG2 cells were trypsinized and centrifuged at 540 *g* for 5 min. These cells were washed twice using 0.9% NaCl (w/v) then re-suspended in 50 μL of 0.9% of NaCl (w/v). The re-suspended cells were transferred onto a Low-e Microscope Slide (MirrIR, Kevley Technologies, Chesterland, OH, USA) then vacuum-dried for 30 min in a desiccator. The cells on the slide were rinsed for a few seconds with distilled water then vacuum-dried. To completely remove the salt, this step was repeated. The washed and dried cell monolayer was stored in a desiccator until analysis. It should be noted