DIHYDROPYRIMIDINE DEHYDROGENASE ACTIVITY CORRELATES WITH FLUOROURACIL SENSITIVITY IN BREAST CANCER

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The fluoropyrimidine drug fluorouracil (FU) is one of the most frequently prescribed chemotherapeutic drugs for the curative and palliative treatment of various cancer patients. The identification of biological factors associated with tumors either responsiveness or resistance to FU chemotherapy, including FU, is increasingly being recognized as an important field of clinical cancer research. Aim: to analyze the relationship between intra-tumoral dihydropyrimidine dehydrogenase (DPD) level and FU chemosensitivity, as DPD is the initial and rate-limiting enzyme in the catabolism of FU. Materials and Methods: The histoculture drug response assay (HDRA) was performed for 54 patients. DPD expression was examined in 81 tumor samples from breast cancer patients received two cycles of FU-based primary chemotherapy before operation. Results: We found that intra-tumoral DPD enzyme activity was inversely correlated with FU cytotoxicity. We also revealed that low DPD expression was correlated with clinical response to FU-based primary chemotherapy. Conclusions: Our study indicated that DPD is a promising molecular maker for identifying tumor cells sensitivity in breast cancer patients receiving FU-based chemotherapy.

Key Words: fluorouracil, dihydropyrimidine dehydrogenase, breast cancer, chemosensitivity.

The fluoropyrimidine drug fluorouracil (FU) is one of the most frequently prescribed chemotherapeutic drugs for the curative and palliative treatment of patients with breast, gastrointestinal, head and neck cancer [1]. The combination of FU with other anticancer agents such as cyclophosphamide and methotrexate (CMF) has been extensively used for cancer chemotherapy. Randomized clinical trial revealed that adjuvant CMF therapy significantly improved overall survival and relapse-free survival for breast cancer patients who received CMF therapy compared with those who did not [2]. Recently, FU based therapy has been employed in primary neoadjuvant chemotherapy for patients before breast-conserving treatment [3, 4]. Successful tumor downstaging by neoadjuvant chemotherapy has been associated with increased rates of breast-conserving therapy and shows better long-term outcomes [5]. Significant variability in the efficacy of chemotherapy is observed in breast cancer patients. The sensitivity of cancer drug treatment is essential for determining the most suitable strategy for individual cancer patients. However, there are no standard tools for prediction of a patient’s tumor response to chemotherapy.

The identification of molecular factors associated with either responsiveness or resistance to FU chemotherapy is increasingly being recognized as an important field of study. Dihydropyrimidine dehydrogenase (DPD) is the initial and rate-limiting enzyme in the catabolism of FU [6], DPD degrades FU to fluoro-dihydrouracil. Pharmacokinetic evaluation has further shown that DPD catalyzes > 80% of and administered dose of FU, thereby determining the amount of FU available for anabolism [7]. Early analyses of human tumor cell xenografts showed a wide range of DPD enzymatic activity among various malignant lesions [8–10]. Various human cancer cell line studies demonstrated that DPD expression is inversely correlated with FU response [11, 12]. Animal study revealed that human tumor xenografts expressing low levels of DPD mRNA and DPD activity showed a significantly better response to FU than tumors with high DPD mRNA level of DPD activity [13]. These basic studies suggested that the intra-tumoral levels of DPD may be an important factor for predicting the response of clinical tumors to FU.

Very few studies have investigated the relationship between DPD expression/activity and the effect of FU based chemotherapy for primary gastric cancer, and the conclusions are still controversial [14–16]. We assume that the controversial results may be partially due to bias from treatment strategy including surgery and postoperative adjuvant radio- or hormonal treatment. To exclude the surgical and postoperative interventions, we performed two prospective studies: (A) The histoculture drug response assay (HDRA) [17] was performed for surgically resected fresh breast tumor, intra-tumoral DPD activity was assessed and
compared with FU HDRA chemosensitivity; (B) Clinical response was evaluated for a cohort of breast cancer patients received two cycles of FU based primary chemotherapy before operation, DPD expression was compared with FU-based chemosensitivity.

MATERIALS AND METHODS

Patients and samples. Two groups of operable primary invasive breast carcinomas were included in our current in vitro and in vivo FU chemosensitivity studies. The in vitro group consisted of 54 invasive ductal carcinomas. None of the patients received chemotherapy or irradiation therapy prior to the surgery. Surgically resected breast cancer tissues were quickly divided into three samples: one was fixed in 10% buffered formaldehyde for pathological diagnosis, one was submitted for DPD enzyme activity measurement, and one for HDRA [17]. The in vivo group consisted of 81 invasive ductal carcinomas enrolled in a clinical trial. Before operation, core needle biopsy was performed to obtain tissue for pathological diagnosis and immunohistochemistry analysis, and all patients were scheduled to two cycles of CMF treatment. All tumor samples were collected with consent for tissue donation and local research ethics committee approval for use of the tissue surplus to diagnostic requirements for cellular and molecular assays.

Measurement of DPD enzyme activity. DPD activity was determined using a catalytic assay according to the method described by Ikenaka et al. [7] with modification. Breast cancer tissues were homogenized in four volumes of 50 mM Tris–HCl (pH 8.0) containing 5 mM of 2-mercaptoethanol, 25 mM of KCl, and 5 mM of MgCl₂. After homogenization, the sample was centrifuged at 105,000 g for 60 min at 4°C. The supernatant cytosol layer was collected as the enzyme source for the measurement of DPD activity. Microspin G-25 column (Amersham Biosciences, Piscataway, NJ) was used to remove internal substrate including racil and thymine. The remaining cytosolic fraction was frozen and stored at −80°C until analysis. The assay mixture (0.25 ml) consisted of 50 mM Tris–HCl (pH 8.0), 10 mM MgCl₂, 25 mM NaF, 50 mM nicotinamide, 5 mM ATP, 1 mM NADPH, [6-3H]5-FU (0.2 mCi, 20 mM), and 0.1 ml of the enzyme extract. The mixture was incubated for 30 min at 37°C, and the reaction was stopped by heating at 100°C in a water bath. After centrifugation at 3000 rpm, the supernatant (0.1 ml) was treated with 0.01 ml of 2M KOH for 30 min at room temperature. Then, the mixture was treated with 0.005 ml of 2M PCA and centrifuged. A 20ml aliquot of the supernatant was taken and spotted onto a thin-layer chromatography plate (Merck, Whitehouse Station, NJ) and developed with a mixture of chloroform, methanol, and acetic acid (17:3:1, v/v/v). The spots of 2-fluoro-alanine and 2-fluoro-ureidopropionic acid, FU degradation products, were scraped into vials and mixed with 10 mL of ACS-II scintillation fluid (Amersham, Buckinghamshire, UK). The radioactivity was measured in a Wallac 1410 liquid scintillation counter (Pharmacia, Uppsala, Sweden). Internal controls were used to compare assays.

Immunohistochemistry. For the immunohistochemical study, 4 µm thick sections on silane-coated slides were dewaxed with xylene and rehydrated through a graded alcohol series. Then, endogenous peroxidase activity was blocked in absolute methanol solution containing 0.3% hydrogen peroxide for 35 min and the slides were washed in 10 mM phosphate-buffred saline (PBS), pH 7.4. For antigen retrieval, they were immersed in 1mM citrate-phosphate buffer, and microwaved at 100°C for 15 min. After the buffer had cooled, 10% fetal serum was reacted with the slides for 15 min to eliminate non-specific immunostaining. The sections were then incubated with anti–DPD polyclonal antibodies [18] overnight at 4°C in a humidified chamber. Biotinylated goat anti-rabbit IgG was applied as a secondary antibody for 20 min at room temperature, followed by streptavidin-biotinylated peroxidase complex for 20 min at room temperature. Peroxidase activity was visualized with a diaminobenzidine as the chromagen. Replacement of the primary antibody with PBS was used as a negative control. Immunoreactivity in the cytoplasm of cancer cells was observed to evaluate DPD. When more than 25% of the cancer cells were stained, the specimen was defined as positive.

HDRA. HDRA was conducted according to previous report [19]. Briefly, FU was dissolved in RPMI-1640 medium (Sigma, St. Louis, MO) containing 2% fetal bovine serum (FCS, lansa, Mexico), penicillin-streptomycin-amphotericin B (Gibco; 100 IU/ml, 100 µg/ml and 0.25% µg/ml, respectively), and 1 ml solution per well was poured onto a 24-well plate. The cutoff concentration used to distinguish in vitro sensitivity and resistance was 300 µg/ml, which was appropriate to the plasma level achieved in vivo [19]. After the incubation for 7 days, 100 µl of 0.2% MTT (Sigma)/phosphate buffered saline (PBS) solutions, containing 50 mM sodium succinate, was added to each well. After the plates were incubated for a further 16 h, the medium was removed from each well, and 0.5 ml dimethyl sulfoxide (DMSO) per well was added to extract MTT-formazan. After 2 h, 100 µl solution was extracted from each well, transferred to 96-well multiplate, and their absorbance was read by microplate reader at 540 nm with a reference of 630 nm. The inhibition index (I.I.) was calculated using the formula: I.I. = (1-T/C) x 100, where T is mean absorbency of the treated wells per 1 g tumor, and C is mean absorbency of the control wells per 1 g tumor. As reported previously, the cut-off I.I. of 60% was employed for our study [20].

FU-based chemotherapy and evaluation. Two cycles of CMF (cyclophosphamide, methotrexate and fluorouracil) were used for patients in this group. Tumor response was determined by palpation after two cycles of treatment. If the tumor size decreased less than 50%, patients was evaluated as resistant to the chemotherapy, if the tumor size decreased more than 50%, the response were defined as sensitive to the treatment.

Statistical analysis. A computer program package (StatView 5.0, Abacus Concepts, Berkeley, CA, USA) was used for all statistical testing and management of...
RESULTS

Association between in vitro FU chemosensitivity and DPD Enzyme Activity. With the cut-off I.I. of 60%, 18 out of the 54 (33.33%) patients were resistant to FU. The DPD enzyme activity was 56.11 ± 8.45 in the chemosensitive group, and 77.44 ± 5.97 pmol/mg per min in the chemoresistant group. As shown in Fig. 1, DPD enzyme activity was significantly lower in the sensitive group than resistant group ($p < 0.0442$).

![Fig. 1.](image-url) Correlation between intratumoral DPD activity and FU in vitro chemosensitivity

Association between in vivo FU-based chemosensitivity and DPD expression. With two cycles of CMF chemotherapy, 39 out of 81 (48.15%) tumors were evaluated to be sensitive to the treatment and 42 (51.85%) were resistant. According to immunostaining, 44 out of 81 (54.32%) tumors were evaluated as immunopositive for DPD, and 37 (45.68%) were immunonegative (Fig. 2). As shown in Table, the tumors with positive DPD expression were resistant to FU, and the tumors with low DPD expression were sensitive to FU ($p < 0.0001$).

![Fig. 2.](image-url) Immunohistochemical staining of breast cancers using anti-D PD polyclonal antibody. The tumor cells showed diffuse cytoplasm staining for anti-D PD (× 400)

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DISCUSSION

Chemotherapy is one of the extremely effective modalities of breast cancer therapy before and/or after operation. No single regimen has been demonstrated to be effective in 100% of patients even with the same tumor type. Accurate prediction of an individual patient’s drug response is an important prerequisite of personalized medicine. Therefore, there is a great need to identify biological marker(s) that can predict response or resistance to chemotherapy [21]. FU is one of the most frequently prescribed chemotherapeutic drugs.

The HDRA may be an appropriate method for the culture of breast cancer since it allows the interstitial cells, which occupy the major portion of the tumor in breast cancer, to be cultured in their natural three-dimensional architecture with the cancer cells [22]. The HDRA chemosensitivity results predict clinical outcomes with high sensitivity in cancers of head and neck, stomach, colon, urinary tract, ovary, and breast [23–28]. In the present study, HDRA was performed on 54 samples. DPD activity was measured by using a catalytic assay [7]. We correlated the result of FU sensitivity in HDRA and DPD activity in tumor tissue and found that decreased DPD activity was associated with FU sensitivity. Our result is consistent with the finding from a previous study [20]. We further analyzed if intra-tumoral DPD expression was correlated with in vivo chemosensitivity in 81 patients who received two cycles of FU-based chemotherapy. As the results, decreased DPD expression was closely related with the early response to preoperative chemotherapy. Prediction of treatment effect might help to exclude patients with a low probability of a treatment benefit and improve the benefit/risk ratio in breast cancer patients receiving neoadjuvant chemotherapy. Based on our current study, DPD may be a promising molecular maker to identify patients with lower DPD expression for FU-based chemotherapy.

Interestingly, there were shown different modes of action of FU-based drugs (tegafur + uracil [UFT], tegafur + gimeracil + oteracil [S-1], 5'-deoxy-5-fluorouridine [5'-DFUR], and N4-pentyloxycarbonyl-5'-deoxy-5-fluorocytidine [capecitabine]), as well as three other drugs (cisplatin [CDDP], irinotecan hydrochloride [CPT-11], and paclitaxel) on genes expression profiles which correlated with the sensitivity of 30 human tumor xenografts in the recent study [29]. DPD mRNA expression profiles of the tumors showed a significant negative correlation with chemosensitivity to all of the FU based drugs except for S-1. S-1 has recently been developed, which consists of tegafur (FT), 5-chloro-2, 4-dihydroxy pyridine (CDHP), and potassium oxonate (Oxo) in a molar ratio of 1 : 0.4 : 1. FT is a prodrug of FU, and CDHP competitively inhibits DPD about 180 times more effectively than uracil, and leads to the retention
of a prolonged concentration of FU [30]. Therefore, the administration of S-1 might be an effective strategy for the treatment of high DPD-expressing tumors [31].

In conclusion, we analyzed the relationship between the DPD activity and the sensitivity to in vitro and in vivo FU chemosensitivity. We found that the basal level of DPD expression/activity was significantly correlated with FU sensitivity in primary breast cancer. DPD is a promising molecular marker for identifying patients for FU-based and/or s-1-based chemosensitivity.

REFERENCES


АКТИВНОСТЬ ДИГИДРОПИРИМИДИН ДЕГИДРОГЕНАЗЫ КОРРЕЛИРУЕТ С ЧУВСТВИТЕЛЬНОСТЬЮ К ФЛУОРОУРАЦИЛУ ПРИ РАКЕ МОЛОЧНОЙ ЖЕЛЕЗЫ

Препарат ряда флуоропиримидина, флуороурацил (FU), является одним из наиболее часто используемых химиотерапевтических препаратов паллиативной терапии больных онкологического профиля. Определение биологических факторов, связанных с чувствительностью либо с устойчивостью опухолей к химиотерапевтическим препаратам, в том числе и к FU, является одним из наиболее важных направлений клинических исследований в онкологии.

Цель: проанализировать взаимосвязь между внутриопухолевым уровнем дигидропиримидин дегидрогеназы (DPD) и чувствительностью клеток к FU, поскольку DPD является начальным и лимитирующим энзимом в катаболизме FU. Материалы и методы: определяли чувствительность к препаратам в гистокультуре (histoculture drug response assay, HDRA) у 54 пациентов. Экспрессия гена DPD изучена в 81 образце опухолевой ткани больных раком молочной железы, который прошел два цикла неoadъювантной химиотерапии с применением FU. Результаты: показано, что внутриопухолевая активность DPD обратно коррелирует с цитотоксичностью FU. Также выявлена, что сниженная экспрессия гена DPD коррелирует с высоким клиническим ответом на первичную химиотерапию, основанную на FU. Выводы: результаты исследования дают основание считать DPD потенциальным молекулярным маркером чувствительности клеток злокачественных опухолей молочной железы к FU.

Ключевые слова: флуороурацил, дигидропиримидин дегидрогеназа, рак молочной железы, чувствительность к химиотерапии.

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