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Folate receptor expression in carcinomas and normal tissues determined by a quantitative radioligand binding assay

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Abstract

The folate receptor (FR) is a valuable therapeutic target that is highly expressed on a variety of cancers. The current development of folate-targeted cancer therapies has created the need for quantitating functional FRs in clinical specimens. In this article, we report on the creation of a highly sensitive radioactive binding method for quantitatively measuring FR expression in frozen tissue homogenates. Expression was positive in approximately 89% of human ovarian carcinomas but was negligible in both mucinous ovarian carcinomas and normal ovary. Expression was also significant in carcinomas of the kidney, endometrium, lung, breast, bladder, and pancreas. Normal tissues from humans and six different laboratory species were also analyzed; surprisingly, some interspecies variability in FR expression (especially in kidney, spleen, and lung tissue) was found. Interestingly, normal human lung tissue displayed high expression levels, whereas expression in normal lung of the other species was negligible. However, considering that folate–drug conjugates fail to accumulate in the lungs of patients, the consequence of this finding was not considered to be of clinical concern. Overall, this new methodology is reliable for determining functional FR expression levels in tissues, and it could possibly be a useful clinical test to determine patient candidacy for FR-targeted therapeutics.

Keywords: Folate receptor; Radioligand binding assay; Cancer; Tumor marker

The α- and β-isoforms of the folate receptor (FR)[1] are glycosylphosphatidylinositol-anchored membrane proteins that bind folic acid with high affinity ($K_d \sim 10^{-10}$ M) and mediate the cellular uptake of this vitamin and drug conjugates thereof via receptor-mediated endocytosis [1]. The significance of this receptor as a tumor marker was discovered in 1991 when amino acid sequence analysis of a protein enriched on the surface of a human ovarian carcinoma cell line was shown to be the FR [2]. Monoclonal antibodies (MOv18 and MOv19) that cross-react with the FR revealed that it was expressed on a majority of nonmucinous ovarian carcinomas; however, little to no reactivity was detected on nonepithelial tumors and normal tissues [3]. Subsequent analyses have shown elevated FR expression in approximately 90% of ovarian carcinomas as well as numerous other cancers, including endometrial, kidney, lung, mesothelioma, breast, brain, and myeloid leukemia [4–14]. A few normal tissues have also been found to express the FR, although most express the protein at much lower levels than that detected in FR-positive carcinomas. Significant FR expression is, however, measurable in the proximal tubules of the kidney, the choroid

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[1] *Abbreviations used:* FR, folate receptor; RT-PCR, reverse transcriptase polymerase chain reaction; NIH, National Institutes of Health; BCA, bicinchoninic acid; PBS, phosphate-buffered saline; cpm, counts per minute; IHC, immunohistochemistry; DTPA, diethylenetriamine pentaacetic acid.

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plexus, intestinal brush-border membranes, type 1 and type 2 pneumocytes of the lung, and placental tissue [15–20].

Exploiting the fact that many cancers overexpress FRs, whereas most normal tissues express low to negligible levels, could prove to be of clinical significance, particularly in regard to the current development of folate-targeted imaging and therapeutic agents [21]. To date, various folate–drug conjugates have demonstrated possible therapeutic value in animals [22]. Likewise, folate-targeted imaging agents are currently being evaluated in phase 2 clinical trials, and phase 1 trials have also been initiated for an FR-targeted immunotherapy [23].

The need for determining which types of cancers are FR positive (i.e., those that could be potential targets for FR-directed therapies) has necessitated the development of methods to measure FR expression in cancer tissue specimens. Various quantitative and semi-quantitative methods have been employed for measuring FR expression in tissue biopsies, including immunohistochemistry, radioimmunoassays, quantitative autoradiography, and cytofluorometric analysis (all of which use anti-FR antibodies) as well as quantitative reverse transcriptase polymerase chain reaction (RT-PCR), fluorescent in situ hybridization, and radioligand binding assays [4,5,9–14]. Unfortunately, results from antibody-based methods can be hampered by factors such as (i) nonspecific binding to tissues, (ii) inaccessibility to the antigen, (iii) heterogeneous antigen expression and/or poor tissue sampling, and (iv) the possibility that the antibody might not distinguish between functional FR and nonfunctional FR, both of which have been demonstrated on human cells [24]. Likewise, assays that measure mRNA levels are not always accurate indicators of functional protein levels, and they are often tedious and expensive to perform. In contrast, radioligand binding assays have the distinct advantage of sensitively measuring functional receptors within a sample; this is important for most FR-targeted therapies because they typically require receptor binding via a folate-based ligand.

We have developed a simple and rapid radioligand binding assay, modeled after a previously described procedure [4], to quantitate functional FR from frozen tissue specimens. This new method involves the immobilization of solubilized membrane protein from homogenized tissues onto a 30,000-MW cutoff microfiltration device, which is then incubated with [^{3}H]folic acid after an acid pretreatment step (to release endogenously bound folates). Our studies include the optimization of critical steps of this radioligand binding assay for the purpose of increasing the assay's sensitivity and reliability. Once it had been optimized, we applied this method to determine the FR distribution pattern among (i) a selection of human cancer tissues and (ii) normal tissues from a variety of animal species to understand the interspecies FR expression variability. Our data show that FR expression (frequency of occurrence and net tissue levels) is significant among many human cancers and that substantial differences exist for FR tissue expression among many commonly used laboratory animals.

Materials and methods

Human tissues

Frozen human tissue specimens were obtained from the NIH Cooperative Human Tissue Network (funded by the National Cancer Institute) and the National Disease Research Interchange (funded by the National Institutes of Health, NIH).

Normal animal tissues

Sprague–Dawley rats, Balb/c mice, and New Zealand white rabbits were obtained from Harlan. Animals were housed and euthanized at Purdue University in accordance with a protocol approved by the institutional animal care and use committee; these animals were 6–7 weeks old at the time their organs were harvested. Each animal was rapidly euthanized by CO_2 asphyxiation, and major organs were immediately collected and frozen on dry ice. Organs were stored at −80 °C until the FR assay was performed. Frozen Dunkin–Hartley guinea pig organs were obtained from Harlan, frozen dog tissues (beagle) were obtained from Marshall Farms, and frozen primate tissues (*Macaca fascicularis*) were obtained from the NIH-funded National Primate Research Center at the University of Washington.

FR assay

All sample preparation procedures used for the FR assay were performed at 4 °C. Tissue samples were homogenized in homogenization buffer (10 mM Tris, pH 8.0, 0.02 mg/ml each of leupeptin and aprotinin; 1 ml buffer/50 mg tissue) using a PowerGen 125 homogenizer (Fisher Scientific). Large debris was removed by mild centrifugation (3000*g* for 15 min). Membrane pellets were then collected by centrifugation at 40,000*g* for 60 min and resuspended in solubilization buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 25 mM *n*-octyl-β-D-glucopyranoside, 5 mM EDTA, and 0.02% sodium azide). Insoluble material was removed by a second 40,000*g* 60-min centrifugation, and the total protein concentration of the supernatants was determined by the bicinchoninic acid (BCA) method (Pierce Chemical). Each sample was then diluted to 0.25 mg/ml in solubilization buffer, and 100 μl was placed inside each of two Microcon-30 microconcentrators (30,000-MW cutoff, Millipore). Samples were then centrifuged at 14,000*g* for 10 min at room temperature to pass all of the liquid through the membrane