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Validation of a hypoxia-inducible factor-1 alpha specimen collection procedure and quantitative enzyme-linked immunosorbent assay in solid tumor tissues

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ABSTRACT

Hypoxia-inducible factor-1 alpha (HIF-1α) is an important marker of hypoxia in human tumors and has been implicated in tumor progression. Drugs targeting HIF-1α are being developed, but the ability to measure drug-induced changes in HIF-1α is limited by the lability of the protein in normoxia. Our goal was to devise methods for specimen collection and processing that preserve HIF-1α in solid tumor tissues and to develop and validate a two-site chemiluminescent quantitative enzyme-linked immunosorbent assay (ELISA) for HIF-1α. We tested various strategies for HIF-1α stabilization in solid tumors, including nitrogen gas-purged lysis buffer, the addition of proteasome inhibitors or the prolyl hydroxylase inhibitor 2-hydroxyglutarate, and bead homogenization. Degassing and the addition of 2-hydroxyglutarate to the collection buffer significantly increased HIF-1α recovery, whereas bead homogenization in sealed tubes improved HIF-1α recovery and reduced sample variability. Validation of the ELISA demonstrated intra- and inter-assay variability of less than 15% and accuracy of 99.8 ± 8.3% as assessed by spike recovery. Inter-laboratory reproducibility was also demonstrated (R^2 = 0.999). Careful sample handling techniques allow us to quantitatively detect HIF-1α in samples as small as 2.5 μg of total protein extract, and this method is currently being applied to analyze tumor biopsy specimens in early-phase clinical trials.

Tumor hypoxia is a common feature of a wide spectrum of solid tumors and has long been recognized to drive tumor progression and treatment resistance [1]. One of the most important regulators of the hypoxia response is hypoxia-inducible factor-1 (HIF-1),[1] which mediates transcription of numerous genes involved in biological processes such as angiogenesis, invasion, metastasis, and tumor metabolism [2,3]. HIF-1 alpha (HIF-1α) is overexpressed in many human cancers, including malignancies of the brain, breast, colon, lung, ovary, pancreas, prostate, and kidney, and is associated with resistance to treatment and poor prognosis [4–7]. As a result, HIF-1α is an attractive therapeutic target for cancer therapy, and several drugs that inhibit HIF-1α are undergoing clinical evaluation. Validation of HIF-1 inhibitors in relevant in vivo models is essential to move these potential therapeutic agents to the clinic, but this effort has been hindered by the absence of established methods to reliably and reproducibly quantify changes in HIF-1α protein in tumor tissues. Along with the intratumoral heterogeneity observed during hypoxia [8], HIF-1α protein instability in the presence of oxygen limits reliable measurement in samples that are acquired and processed under normoxia [9,10]. To date, HIF-1α protein expression has been most commonly assessed by immunohistochemistry (IHC) or Western blot, but the semiquantitative nature of these techniques or the requirement for relatively large amounts of protein limits

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[1] *Abbreviations used:* HIF-1, hypoxia-inducible factor-1; HIF-1α, HIF-1 alpha; IHC, immunohistochemistry; mRNA, messenger RNA; VEGF, vascular endothelial growth factor; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; NCI, National Cancer Institute; H-CEB, HIF-1α cell extraction buffer; BCA, bicinchoninic acid; SD, standard deviation; CV, coefficient of variation; NCTVL, National Clinical Target Validation Laboratory; PADIS, Pharmacodynamic Assay Development and Implementation Section; LLQ, lower limit of quantitation; 2-HG, 2-hydroxyglutarate; 3BSC, 3B Scientific Corporation; PHD, prolyl hydroxylase; QD×*n*, treated once daily for *n* days; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase.

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their usefulness as sensitive and qualified biomarker assay methods, especially in human biopsy specimens. Alternatively, HIF-1α activity has been assessed indirectly through protein or messenger RNA (mRNA) expression levels of HIF-1 target genes, such as vascular endothelial growth factor (VEGF) and carbonic anhydrase IX, or surrogate markers, such as angiogenesis and microvessel density.

Here we report a rigorous process for optimizing specimen collection and processing and the development and analytical validation of an enzyme-linked immunosorbent assay (ELISA) for HIF-1α that addressed sample extraction methods, assay reproducibility across different laboratories, fitness-for-purpose testing in relevant preclinical models, and clinical validation in human specimens [11,12]. Our method preserves and stabilizes HIF-1α in solid tumor tissues, allowing quantitation of HIF-1α. The assay has been used to measure drug effect on HIF-1α protein levels in human xenograft models. Finally, data from human biopsy specimens are presented.

Materials and methods

Cell lines

Human cell lines PC-3 (prostate adenocarcinoma), DU145 (prostate adenocarcinoma), SiHa (cervical squamous cell carcinoma), A375 (melanoma), and HCT-116 (colon adenocarcinoma) were purchased from and authenticated by American Type Culture Collection (ATCC) and cultured in appropriate media supplemented with 10% fetal bovine serum (Lonza) and 50 mg/L gentamicin sulfate (Lonza). The identity of each cell line was confirmed using Identifiler STR genotyping (Applied Biosystems). Cells were cultured for less than 6 months before renewal from early passage frozen stocks. Cells were maintained at 37 °C in a humidified incubator containing 21% O_2 and 5% CO_2 in air (referred to as normoxic conditions). For hypoxic culture, PC-3 cells were placed in a CO_2 incubator flushed with a mixture of gas containing 1% O_2, 5% CO_2, and 94% N_2 for 24 h (see details in Online supplementary material). Cells were grown to superconfluence in T75 (75 cm^2) flasks, washed in cold phosphate-buffered saline (PBS), and immediately lysed in the flasks on ice. To increase baseline HIF-1α levels prior to lysis, SiHa cells were treated with 1 μM bortezomib (Fisher Scientific) for 4 h.

Animal models and drug administration

Female athymic nu/nu (NCr) mice (Frederick National Laboratory for Cancer Research Animal Production Program, Frederick, MD, USA) were implanted with A375, PC-3, DU145, or HCT-116 cells as reported previously [13]. Mice developing tumors served as donors; tumors were maintained by serial in vivo passage using tumor fragment transplantation when the donor tumors reached 10 to 15 mm in diameter. Tumors were staged to a preselected size (weight = 150–300 mg) calculated using the following formula: weight (mg) = (tumor length × tumor width^2)/2 [14]. Mice were housed in sterile, filter-capped polycarbonate cages (Allentown Caging) maintained in a barrier facility on a 12-h light/dark cycle and were provided with sterilized food and water ad libitum. Mice were randomized into groups before initiation of treatment using a commercial software program (Study Director, Studylog Systems).

Topotecan (NSC 609699), the indenoisoquinoline NSC 743400, and selumetinib (AZD6244) were obtained from the Developmental Therapeutics Program of the National Cancer Institute (NCI). Topotecan was administered intraperitoneally (IP) in a sterile water vehicle. NSC 743400 was administered intravenously (IV) in a vehicle composed of 10 mM citric acid/5% dextrose (1:3). Selumetinib was administered orally by gavage in a vehicle composed of 10% dimethyl sulfoxide (DMSO). Dose volume was defined as 0.1 ml/10 g body weight. All drugs were administered as a single dose in 0.1 ml vehicle/10 g body weight.

The Frederick National Laboratory for Cancer Research is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and follows the U.S. Public Health Service Policy for the Care and Use of Laboratory Animals. All of the studies were conducted according to an approved animal care and use committee protocol in accordance with the procedures outlined in the Guide for Care and Use of Laboratory Animals published by the National Research Council in 1996.

Xenograft collection

Mice were anesthetized by isoflurane gas inhalation before biopsy or tumor resection. When surgical anesthesia was reached (no toe pinch), the skin was disinfected with Nolvasan (Fort Dodge Laboratories) and a 2- to 5-mm incision was made through the skin adjacent to the subcutaneous tumor being biopsied. An approved 18-gauge human biopsy needle (Temno, Allegiance Healthcare) was passed through the skin incision into the tumor. The wound was closed with a surgical wound clip after sample collection. Collected material (~1 × 5 mm) was immediately flash-frozen either in an empty, sterile O-ring sealed, screw-capped cryovial that was precooled on ice or a tube that was prefilled with degassed lysis buffer. Frozen specimens were stored at −80 °C until use. Xenograft tumor quadrants were collected by standard dissection methods, cut into four equal pieces with fine-point scissors, and placed into either empty cryovials or tubes prefilled with degassed lysis buffer, precooled on ice as described above. Shallow needle biopsy collections refer to needle passes near the surface of the tumor, whereas deep collections refer to needle collections that passed through the core of the tumor.

Extract preparation

Lysis of all samples used HIF-1α cell extraction buffer (H-CEB: 50 mM Tris [pH 7.4], 300 mM NaCl, 10% [w/v] glycerol, 3 mM ethylenediaminetetraacetic acid [EDTA], 1 mM $MgCl_2$, 20 mM β-glycerophosphate, 25 mM NaF, and 1% Triton X-100). Just before lysis, H-CEB buffer was supplemented with complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA) and 1 mM phenylmethanesulfonyl fluoride (Sigma–Aldrich, St. Louis, MO, USA). Degassed buffer was prepared for specimen collection by bubbling nitrogen gas through the buffer at the bottom of the tube using a pipette tip for 3 min. The pipette was withdrawn slowly from the tube while filling the head of the tube with nitrogen. The tube was immediately sealed to minimize oxygen seepage back into the tube and then flash-frozen in liquid nitrogen. Validation studies of pre- and post-degassed buffer dissolved oxygen content can be found in the Supplementary material.

Specimens that were collected dry flash-frozen in empty tubes were processed by adding degassed H-CEB directly to the frozen tissue; samples that were collected in degassed H-CEB-containing tubes followed by flash-freezing were thawed on ice. Needle biopsy samples were collected in 0.25 ml of degassed H-CEB, whereas tumor quadrants and cell pellets were collected into 0.15 to 0.4 ml, depending on sample size. Three different sample homogenization techniques were tested. For sonication, tissue was minced with fine-point scissors and then disrupted by sonication at an output of 2–3 W for 15–30 s three times (Sonic Dismembrator 550, Fisher Scientific) while the specimen tube was kept on ice. For grinding, cell samples were processed with a Bio-Gen PRO200 homogenizer (PRO Scientific, Oxford, CT, USA) with a 7-mm probe for 15 to 30 s while the specimen tube was kept on ice. Samples homogenized using bead-beating (Precellys 24, Bertin