Irreversible Electroporation Therapy in the Liver: Longitudinal Efficacy Studies in a Rat Model of Hepatocellular Carcinoma

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Abstract

Irreversible electroporation (IRE) is an innovative local-regional therapy that involves delivery of intense electrical pulses to tissue to induce nanoscale cell membrane defects for tissue ablation. The purpose of this study was to investigate the feasibility of using IRE as a liver-directed ablation technique for the treatment of hepatocellular carcinoma (HCC). In the N1-S1 rodent model, hepatomas were grown in 30 Sprague-Dawley rats that were divided into treatment and control groups. For treatment groups, IRE electrodes were inserted and eight 100-μs, 2,500-V pulses were applied to ablate the targeted tumor tissues. For both groups, magnetic resonance imaging scans were performed at baseline and 15-day follow-up intervals to determine tumor sizes (one-dimensional maximum diameter, D_max; estimated two-dimensional cross-sectional area, C_max) as a tactic to assess longitudinal outcomes. Additional groups of treated animals were sacrificed at 1-, 3-, and 7-day intervals posttherapy for pathology assessment of treatment response. Magnetic resonance images showed significant tumor size reductions within 15 days posttherapy (32 ± 31% D_max and 52 ± 39% C_max decreases compared with 110 ± 35% D_max and 286 ± 125% C_max increases for untreated tumors). Pathology correlation studies documented progression from poorly differentiated viable HCC tissues before treatment to extensive tumor necrosis and full regression in 9 of 10 treated rats 7 to 15 days after treatment. Our findings suggest that IRE can be an effective strategy for targeted ablation of liver tumors, prompting its further evaluation for HCC therapy. Cancer Res; 70(4); 1555–63. ©2010 AACR.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the third most common cause of cancer death (1, 2). Resection, liver transplantation, and percutaneous ablation treatments are three potentially curative therapies for early-stage HCC; effective utilization of these therapies is increasing as a result of widely implemented surveillance programs in at-risk patient populations (3). However, most patients are not candidates for resection or transplantation due to advanced disease stages and/or donor shortages (4).

According to the guidelines of the American Association for the Study of Liver Diseases, percutaneous ablation is the best treatment option for nonsurgical patients with early-stage HCC (5). These ablation techniques include percutaneous ethanol injection (6), percutaneous acetic acid injection (7), radiofrequency ablation (8, 9), microwave coagulation therapy (10), laser interstitial thermal ablation (11), and cryoablation therapy (12). Percutaneous ethanol injection is the most well-known and widely studied approach; it can offer a safe, effective, and inexpensive treatment for small HCC, achieving tumor necrosis rates of 90% to 100% for HCC smaller than 2 cm. However, for HCC between 3 and 5 cm, this rate drops to 50% (13), and the local tumor recurrence rate is up to 17% when treating HCC ≥ 5 cm (14). Radiofrequency ablation is the most widely used thermal ablation technique providing improved local disease control compared with percutaneous ethanol injection in both small and larger HCC (5, 15). However, radiofrequency ablation has significant potential limitations, including local tumor progression and a higher rate of adverse events (intrahepatic bleeding, tumor seeding, hepatic abscess, bile duct injury, and hepatic decompensation; refs. 16–18). Furthermore, depending on the location of the targeted tumor, radiofrequency ablation may be contraindicated due to the potential damage to adjacent tissues and blood vessels (19). Therefore, the development of a more effective HCC ablation technique is warranted to achieve superior tumor necrosis rates while reducing the likelihood of adverse events.

Irreversible electroporation (IRE) is an innovative locoregional therapy that was first introduced as a potential tissue...
ablation technique in 2005 (20). IRE involves targeted delivery of short (microsecond to millisecond duration) intense electrical pulses to induce cell death through permanent cell membrane defects. These pulses elevate the trans-membrane potential to an extent that causes permanent defects within the lipid bilayer of the cell membrane for those tissues contained with the targeted treatment region. These pulses are applied through electrodes positioned within the targeted tissues. Previous animal model studies have shown that IRE can ablade substantial volumes of tissue. IRE has the potential to serve as an independent new modality for targeted tissue ablation that is based on the application of strong electrical fields rather than the deposition of heat or chemical agents (21–23). Rigorous preclinical studies in human HCC cells (HepG2; ref. 24), normal liver (25), and prostate tissues (26), as well as cutaneous tumor models (22), have each shown the feasibility of using IRE as a new ablation option with negligible thermal side effects (27).

The purpose of our current study was to investigate the efficacy of IRE approaches for targeted ablation of HCC. We tested the hypothesis that IRE procedures would lead to tumor necrosis in a transplanted rodent hepatoma model. We provide serial magnetic resonance imaging (MRI) and follow-up histopathologic evidence, showing the potential longitudinal efficacy of IRE for the treatment of HCC.

**Materials and Methods**

**Tumor Cell Line and Culture**

The N1-S1 rat hepatoma cell line (American Type Culture Collection, CRL-1603) was obtained and cultured in DMEM (ATCC) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 90 μg/mL gentamicin. Cells were maintained in suspension culture flasks at 37°C in a humidified atmosphere containing 5% CO₂. This cell line was initially established from a HCC induced in a male Sprague-Dawley rat by ingestion of carcinogen 4-dimethylaminoazobenzene (28). Before each implantation procedure, the viability of the cells was tested with trypan blue staining (confirming >90% cell viability for each tumor implantation procedure).

**Animal Model**

All studies were approved by our institutional animal care and use committee and were performed in accordance with institutional guidelines. Forty-four adult male Sprague-Dawley rats (Charles River Laboratories) weighing initially 301 to 325 g were used for these experiments. After anesthesia, a mini-laporatomy was performed and the left medial lobe of the liver was exposed. N1-S1 rat hepatoma cells (1 × 10⁸) were visually injected under the hepatic capsule into this lobe. Following initial implantation, ~6 to 10 d were required for tumor induction and growth to desired pretreatment size (diameter <1.60 cm). Thirty rats from the initial 44 implanted animals produced hepatoma (1.29 ± 0.18 cm diameter) suitable for subsequent IRE treatment procedures. These rats were randomly divided into six groups: group 1, six rats for an untreated baseline control group; group 2, six rats for an untreated 15-d end-point control group; group 3, six rats for an IRE-treated 15-d end-point group and four rats each for IRE-treated 1-d (group 4), 3-d (group 5), and 7-d (group 6) end-point groups. IRE procedures were performed shortly after baseline MRI measurements; these MRI measurements were repeated at baseline and 15-d follow-up time intervals to measure tumor sizes (groups 2 and 3) with animals subsequently euthanized for histology at different study end points for each group (1-, 3-, 7-, or 15-d intervals after original baseline scan).

**IRE Procedures**

**IRE apparatus and dosing plan.** A BTX Electroporator (ECM830; Harvard apparatus) function generator and a parallel two-needle electrode array were used for all rat IRE procedures. The electrode array was constructed using two MR-compatible platinum-15% iridium needles (each 35 mm in length with a diameter of 4 mm); these were inserted through a plastic block to maintain a 1-cm spacing between the two parallel needles. We elected to use an IRE ablation protocol that included the application of 2,500-V square wave pulses, a total of eight pulses of 100-μs length with 100-ms spacing between pulses (identical to the protocol used for prior IRE studies in cutaneous tumor tissues; ref. 22). Before in vivo IRE procedures, we used a commercial finite element modeling (FEM) software package (COMSOL Multi-Physics, version 3.3) to simulate the anticipated ablation zone based on the above-described IRE protocol parameters, electrode spacing, and anticipated lethal electrical field potential for hepatic tissues of 637 V/cm (29). Our simulation closely followed those described in previous IRE studies solving the Laplace equation to calculate induced electrical field potentials based on anticipated tissue and electrode conductivities (20, 30). Based on our chosen IRE parameters, the FEM simulations suggested that we should anticipate ablation zones of roughly 1.6 cm × 1.2 cm (Fig. 1B), sufficiently large for treatment of the induced N1-S1 tumors. Also, use of a short duration for application of the electrical pulses should lead to negligible heating effects (relatively short 100-μs integration interval for Arhennius relation describing anticipated thermal damage Ω = ∫ ξ e⁻𝐸/𝑅 dξ, where ξ is the frequency factor, E is the activation energy, and R is the universal gas constant; refs. 20, 27).

**Tumor IRE procedure.** Before both imaging and IRE procedures, rats were anesthetized with a high limb injection of ketamine (75–100 mg/kg) and xylazine (2–6 mg/kg). After baseline imaging for tumor confirmation, each rat was fixed in a supine position within a restraint apparatus (rats strapped to form-fitting back board). Next, a minilaparotomy incision was performed to expose and visually locate the N1-S1 tumor within the left hepatic lobe. Before electrode placement, the tumor-bearing liver lobe was digitally palpated between thumb and forefinger to approximate the configuration of the tumor mass. For each animal, we positioned the bipolar IRE electrode array such that the two-needle insertion positions (a) essentially straddled the centroid of the tumor mass and (b) were aligned along the axis of the largest tumor dimension (for optimal treatment, tumor should be located midway between the two parallel electrodes; Fig. 1). Finally,
the electrodes were connected to the electroporation function generator and IRE pulse train was applied (requiring <1 s for application of the complete IRE pulse train). Following IRE procedure, the abdominal incisions were closed with two-layer technique followed by topical application of antibiotic ointment and Metacam injection (1–2 mg/kg s.c.). Animals were returned to storage facilities for the duration of the follow-up delay interval before end-point imaging studies. During these follow-up delay intervals, each animal was observed daily to determine the presence of any postoperative complications (incision infection or abscess formation); at necropsy, each animal was inspected for additional procedural complications, including injuries to adjacent organs, tumor seeding, and intraperitoneal bleeding.

MRI Measurements

MRI protocol. All MRI studies were performed using a 3-T Magnetom Trio clinical scanner (Siemens Medical Solutions) with custom-built rodent receiver coil (Chenguang Med. Tech. Co.). Along both coronal and transverse orientations, T2-weighted, T1-weighted, and proton density–weighted turbo spin echo (TSE) scans were performed with a multislice acquisition providing complete coverage of the entire liver volume (31). All scans were performed with a 150-mm FOV, 2.0-mm slice thickness, three signal averages, 256 matrix (0.6 × 0.6 mm² in-plane voxel size), repetition and echo time (TR/TE) = 3,500/60 ms for T2-weighted scans, TR/TE = 300/8 ms for T1-weighted scan, and TR/TE = 3,500/8 ms for proton density–weighted scan. These MRI measurements

Figure 1. A, photograph showing the IRE electrode placement within the targeted hepatic lobe. B, finite element modeling simulation of the anticipated IRE ablation zone with the selected IRE parameters. C, H&E staining showing an ablation region of coagulative necrosis and a well-delineated margin between treated and untreated liver tissues for Sprague-Dawley rat euthanized 24 h post-IRE procedure (>25).

Figure 2. H&E staining of N1-S1 rat HCC at increasing intervals posttherapy (×200). A, untreated HCC, showing viable tumor. B, 1 d posttherapy, showing that most of the tumor remains viable while adjacent liver tissue is necrotizing. C, 3 d posttherapy, showing heterogeneously necrotizing tumor and liver tissue. D, 7 d posttherapy, showing extensive necrotizing tissue debris, histocyte/lymphocyte reaction, microcalcification, and no viable tumor. E, 15 d posttherapy, showing no viable tumor but showing giant cell reaction, hemosiderin-laden histocyte reaction, and scaring fibrosis.
were performed at baseline and at the end point of the study for each respective animal.

Image analysis. Measurements were performed offline using the ImageJ software package. All coronal and axial orientation DICOM format T2-weighted TSE images collected for each animal were reviewed according to Response Evaluation Criteria in Solid Tumors (RECIST) and WHO criteria to (a) measure the maximum lesion diameter ($D_{\text{max}}$) along the orientation bearing the largest tumor diameter; ref. 32) and (b) provide an estimate of the two-dimensional cross-sectional area of the tumors at these same locations ($C_{\text{max}}$, calculated as the cross-product of the maximum lesion diameter $D_{\text{max}}$ and largest diameter measured perpendicular to $D_{\text{max}}$; ref. 33). These measurements were performed for both baseline and 15-d follow-up interval scans.

Histology

After follow-up MRI measurements, each rat was euthanized with i.v. injection of Euthasol at a dose of 150 mg/kg and bilateral thoracotomy. Two to three sections across the lesion were sampled and fixed in 10% formaldehyde solution; these tissue sections were then embedded in paraffin for H&E staining. Resulting histology slides were deidentified (w.r.t. treatment group) and reviewed by an attending surgical pathologist with specialization in gastrointestinal oncology (>10 years experience). The percentage of viable tumor tissue was separately evaluated for each animal.

Representative tissue sections from each group were selected for immunohistochemistry evaluation. CD34 staining was used as a malignant tumor neovascularization marker (34, 35) to highlight regions of sinusoidal capillarization; caspase-3 staining (previously shown during induction of hepatocyte apoptosis both in vitro and in vivo) was used as a marker of active apoptosis (36, 37).

Statistical Analysis

All statistics were performed using the SPSS statistical software package (SPSS, version 17). Lesion size increases based on one-dimensional $D_{\text{max}}$ measurements and two-dimensional $C_{\text{max}}$ measurements were compared between group 2 and group 3 animals (comparison between untreated and treated rats after 15-d follow-up interval) by non-parametric Mann-Whitney $U$ test. Test was considered statistically significant with a $P$ value of <0.05.

Results

Thirty-two of 44 rats implanted with N1-S1 cells developed hepatoma (73% tumor induction rate similar to previously reported N1-S1 induction rates; ref. 38). One rat was euthanized before IRE procedures due to suture failure and wound dehiscence, and one additional rat was excluded from the study due to excessive tumor growth before IRE procedure (detected during baseline MRI scan). No postoperative complications were observed in the IRE-treated rats (groups 3–6).

H&E staining. The ablation zones anticipated based on our FEM simulations were well correlated to in vivo IRE ablation zones observed within H&E pathology slides.

Figure 3. Representative axial (top row) and coronal (bottom row) T2-weighted (T2W), T1-weighted (T1W), and proton density–weighted (PD) TSE images for a 1.5-cm diameter N1-S1 HCC during a baseline control scan (group 1). N1-S1 HCC were hyperintense within T2-weighted images, hypointense within T1-weighted images, and typically isointense (relative to adjacent normal liver parenchyma) within proton density–weighted images. Arrows within images indicate tumor position.

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7 http://rsb.info.nih.gov/ij/
postnecropsy (Fig. 1C). Within tumor animals, H&E staining showed a clear progression from poorly differentiated viable hepatoma tissue pretherapy, to heterogeneously viable tumor tissues early post-IRE (1–3 days) and extensive tumor necrosis at delayed intervals (7–15 days) post-IRE treatment (Fig. 2). A 95% viable hepatoma is shown from a representative baseline control animal in Fig. 2A. One day post-IRE, ablation zones showed mostly viable tumor and adjacent necrotizing liver tissue (Fig. 2B). Three days post-IRE, ablation zones included both heterogeneously necrotizing tumor and liver tissues (Fig. 2C). In the 7-day post-IRE treatment animals (group 6), four of four treated lesions showed extensive necrotizing tissue debris, histocytes/lymphocytes reaction, microcalcification, and no viable tumor tissue (Fig. 2D). In the 15-day post-IRE treatment animals (group 3), five of six treated lesions showed no remnant viable tumor but showed giant cell reaction, hemosiderin-laden histocyte reaction, and scarring fibrosis (Fig. 2E). One of these six treated lesions contained a volume of <5% viable tumor tissue, compared with 68 ± 8% viable tumor tissue for the end-point control animals (group 2) with 32 ± 8% central necrosis due to tumor ischemia. Histologically determined tumor viability characteristics (percentage of viable tumor tissue estimated for each lesion at necropsy) for all animals in groups 1, 2, and 3 are shown in Fig. 4C.

**MR imaging.** T2-weighted, T1-weighted, and proton density–weighted TSE images for baseline control (group 1) 1.3-cm-diameter N1-S1 rat hepatoma are shown in Fig. 3. These tumor masses were consistently hyperintense within T2-weighted images, hypointense within T1-weighted images.
and typically isointense within proton density–weighted images. MRI images showed significant tumor size reductions post-IRE (−32 ± 31%, $D_{\text{max}}$ decrease) for treated rats (group 3), whereas all untreated tumors (group 2) increased in size (+110 ± 35%, $D_{\text{max}}$ increase; Fig. 4D, left). Similarly, corresponding two-dimensional $C_{\text{max}}$ measurements also showed significant decreases (−52 ± 39%) for treated rats (group 3), whereas all untreated tumors (group 2) showed increases in these two-dimensional size measurements (+286 ± 125%; Fig. 4D, right). There was a statistically significant difference between both one-dimensional ($D_{\text{max}}$) and two-dimensional ($C_{\text{max}}$) lesion size changes between these two groups ($P = 0.004$ for both comparisons). Representative T2-weighted baseline and follow-up MRI images from a control rat and a 15-day post-IRE treatment rat are shown in Fig. 4A and B, respectively. Corresponding H&E histology slides for these rats showed mostly viable tumor tissue for the end-point control rat but no remnant viable tumor in the treated animal.

Immunohistochemistry. For immunohistochemistry CD34 staining, diffuse sinusoidal CD34 reactivity was observed for untreated rat hepatoma (Fig. 5A); on the other hand, 1-day post-IRE CD34 staining showed mild vascular dilation and congestion (Fig. 5B). Remnant vessel skeletons with inflammatory cell infiltration and fibrotic tissue formation over a necrotic background was observed for 7- and 15-day post-IRE treatment lesions (Fig. 5C and D); limited caspase-3 staining was shown in the untreated tumors (typically within central ischemic areas), whereas the most viable tumor tissues showed no caspase-3 activation (Fig. 6A). Extensive caspase-3 activation was observed 1 day post-IRE treatment (Fig. 6B). At a delayed post-IRE follow-up interval (7–15 days post-IRE), caspase-3 was no longer visible within the treated lesion over the necrotic background (Fig. 6C).

Discussion

These animal model studies showed the potential efficacy of IRE as a targeted ablation technique for the treatment of HCC. MR images showed a significant tumor size reduction within 15 days posttherapy, and histology correlation studies showed a clear progression from poorly differentiated viable hepatoma tissue pretherapy to extensive tumor necrosis and complete tumor regression in 9 of 10 treated rats 7 to 15 days after treatment. Our study is the first to show the efficacy of IRE for targeted treatment of liver tumors in a transplanted rodent hepatoma model.

Relatively early post-IRE therapy (within 1 day posttreatment), we observed homogeneously necrotizing tissues within treated normal liver parenchyma with clear margins between the treated and untreated tissues. However, we observed somewhat different responses within N1-S1 tumor tissues; specifically, tumor tissues tended to exhibit heterogeneously necrotic characteristics at the early intervals (1–3 days) posttherapy with limited viable tumor trapped within the necrotic tissues. Eventually, all treated tumors progressed from these early-interval stages of partial necrosis to essentially complete necrosis with fibrotic scar formations 7 to 15 days later. One potential explanation for these heterogeneous delays in cell death could be that some of the treated tissues were destroyed due to the alternative me-
chanisms of ischemia and associated hypoxia (due to entrapment within surrounding necrotic tissue) as opposed to the direct effect of IRE. Additional studies will be required to rigorously investigate the mechanism of these observed temporally dependent necrosis events associated with IRE ablation procedures. For tumor tissues, although no significant changes were observed on H&E and CD34 staining 1 day posttherapy (group 4), we observed extensive caspase-3 activation that might indicate an alternative underlying cell death mechanism (i.e., tumor cell apoptosis initiation) in addition to solely cell membrane permeabilization. Delayed interval results (15 days posttherapy) consistently showed the longitudinal efficacy of this targeted IRE approach; MRI scans depicted significant lesion size reductions for each treated animal, whereas significant tumor growth occurred for untreated animals. These imaging results were well correlated to delayed interval histopathologic results that showed no viable tumor tissue within the lesion along with inflammatory cell reaction, fibrotic scar formation, remnant vascular skeleton CD34-positive staining (depicting system of damaged blood vessel walls within the treated tissue region), and an absence of caspase-3 activation.

Our study specifically showed the feasibility of using IRE as a therapeutic modality for the treatment of HCC. All treated tumors showed significant size reductions within 2 weeks posttherapy and there were no adverse events (i.e., peritoneal bleeding, tumor seeding, liver failure, or mortalities) observed for any of the 18 treated animals. For the IRE protocol selected for our study, we used far fewer pulses than prior cutaneous tumor model studies (eight square wave pulses as opposed to 80 pulses at 0.3Hz) while continuing to achieve effective treatment response. Our study showed the feasibility of using IRE to ablate HCC; however, further studies to optimize IRE parameters are certainly warranted.

The efficacy of conventional radiofrequency ablation approaches is often limited in larger tumors due to perfusion-mediated cooling, which can limit thermally induced coagulation necrosis (39). The extent of the treated tissue volume can be difficult to control due to blood circulation with heat-sink effects, leading to indistinct margins between treated and untreated tissues and/or undertreatment of the targeted tissues (40). IRE results in a distinct margin between ablated and viable tissues at the position where the magnitude of the electrical field falls below a lethal dose threshold (23). Importantly, IRE does not suffer from the heat-sink effect, which is commonly problematic for thermal ablation methods (21). Additional potential advantages for IRE methods include tumor-specific immunologic reaction (41), little impact on the collagen network within treated tissues, and the potential to abate tumor tissues near large vessels (42). Finally, application of the electroporation pulses during IRE procedures requires less than 1 second. This feature contrasts significantly with the duration required for radiofrequency ablation methods, which typically involve application of thermal energy for upward of 8 to 20 minutes per ablation to achieve sufficient temperatures for coagulative necrosis (43). Recently, a commercially developed electroporation device received 510 k approval from the Food and Drug Administration (NanoKnife; AngioDynamics, Inc.). Given the promising results of our current IRE ablation studies on the N1-S1 rat hepatoma model, future studies are warranted to further investigate the efficacy of such devices for targeted treatment of liver tumors as well as additional tumor etiologies that can be difficult to treat with conventional ablation methods.

One limitation of our study was the lack of intraprocedural imaging guidance to optimize the placement of IRE.
electrodes; suboptimal electrode placement could conceivably have led to the incomplete response observed for one rat in group 3.

During previous studies, ultrasound imaging methods were used for intraprocedural visualization of IRE ablation procedures (44). In future HCC IRE studies, ultrasound, MRI, or computed tomography techniques could be used to optimize placement of IRE electrodes to ensure that the targeted tumor mass is entirely contained within the anticipated IRE ablation zone. Functional imaging methods (dynamic contrast–enhanced computed tomography/MRI and/or diffusion-weighted MRI) may prove useful for immediate or early detection of IRE treatment response.

For these initial studies, we did not individually tailor the IRE protocol (voltage, electrode spacing) to produce an ablation zone specific to each individual tumor size. We simply used a single IRE protocol producing an ablation zone size that we anticipated would be sufficiently large to cover all tumors below one given size. For our studies, we did not experience any gross complications due to damage to adjacent liver parenchyma. However, we would anticipate that the use of much larger ablation zones would lead to decomposition and subsequent liver failure. An individualized, patient-specific approach could be important for clinical IRE applications given a desire to spare normal liver tissues to preserve function. As shown during prior studies (30), optimization of the IRE ablation volumes should be possible using preprocedural FEM simulations. Further studies are warranted to rigorously investigate the potential to individually tailor the size of IRE ablation zones to ensure complete treatment of targeted tumors while sparing as much surrounding normal liver tissue as possible.

In conclusion, this preclinical study showed the feasibility of using IRE as a novel ablation modality for targeted treatment of hepatoma in the N1-S1 rat model. Follow-up MRI images showed significant tumor size reductions and histology correlation studies showed extensive tumor necrosis within 7 to 15 days posttherapy. IRE is a promising new approach for liver-directed treatment of HCC and may offer multiple potential benefits over conventional ablation methods.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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25. Lee EW, Loh CT, Kee ST. Imaging guided percutaneous irreversible
Irreversible Electroporation of Liver Tumors

Correction: Online Publication Dates for Cancer Research April 15, 2010 Articles

The following articles in the April 15, 2010 issue of Cancer Research were published with an online publication date of April 6, 2010 listed, but were actually published online on April 13, 2010:


Dudka AA, Sweet SMM, Heath JK. Signal transducers and activators of transcription-3 binding to the fibroblast growth factor receptor is activated by receptor amplification. Cancer Res 2010;70:3391–401. Published OnlineFirst April 13, 2010. doi:10.1158/0008-5472.CAN-09-3033.


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