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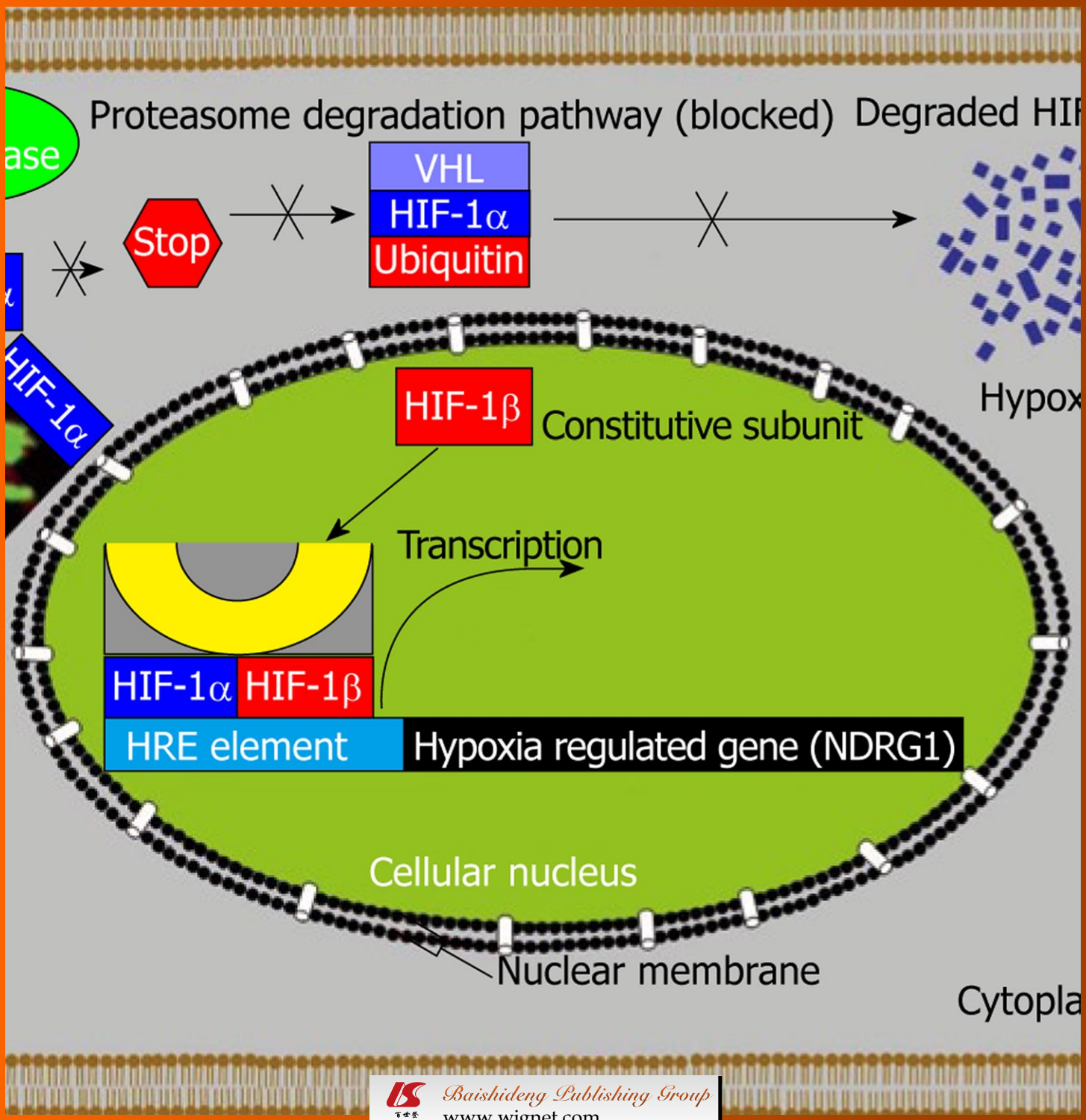

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TOPIC HIGHLIGHT

Harun M Said, PhD, *Series Editor*

Inhibition of carbonic anhydrase IX as a novel anticancer mechanism

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Abstract

Carbonic anhydrases (CA, EC 4.2.1.1) catalyze the interconversion bewteen carbon dioxide and bicarbonate with generation of protons. The carbonic anhydrase isozyme IX (CA IX) is highly overexpresed in hypoxic tumors and shows very restricted expression in normal tissues. CA IX is a dimeric protein possessing very high catalytic activity for the hydration of carbon dioxide to protons and bicarbonate. Its quaternary structure is unique among members of this family of enzymes, allowing for structure-based drug design campaigns of selective inhibitors. Inhibition of CA IX with sulfonamide and/or coumarin inhibitors was recently shown to lead to a potent retardation for the growth of both primary tumors and metastases. Some fluorescent sulfonamides were shown to accumulate only in hypoxic tumor cells overexpressing CA IX, and might be used as diagnostic tools for imaging of hypoxic cancers. Sulfonamide inhibitors were also more effective in inhibiting the growth of the primary tumors when associated with irrdiation. CA IX is thus both a diagnostic and therapeutic validated target for the management of hypoxic tumors normally non-responsive to classical chemio- and radiotherapy.

Key words: Carbonic anhydrase; Hypoxia; Sulfonamides; Coumarins; Tumorigenesis; Tumor imaging; Tumor acidification

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INTRODUCTION

CO_2 is a fundamental chemical species in many biological systems, injorgqanisms all over the phylogenetic tree. Its reaction with watyer is a slow process at the physiological pH, and it needs the presence of a catalysts to become effective. These catalysts are the carbonic anhydrases (CAs, EC 4.2.1.1), a superfamily of metalloenzymes which evolved at least 5 times independently in diufferent organisms[1,2]. In mammals, including humns, only α-CAs are present[1,2], but 16 different isozymes have been characterized to date, which differ in their subcellular localization, catalytic activity, and susceptibility to different classes of inhibitors. There are cytosolic isozymes (CA Ⅰ, CA Ⅱ, CA Ⅲ, CA Ⅶ and CA XⅢ), membrane bound ones (CA Ⅳ, CA Ⅸ, CA XⅡ and CA XⅣ), mitochondrial (CA VA and CA VB) and secreted (CA Ⅵ) isoforms. Three acatalytic forms, called CA-related proteins (CARPs), CARP Ⅷ, CARP X and CARP XI, are also known[1]. In humans, CAs are present

in a large variety of tissues such as the gastrointestinal tract, the reproductive tract, the nervous system, kidneys, lungs, skin and eyes[1,2]. Most CAs are very efficient catalysts for the reversible hydration of carbon dioxide to bicarbonate and protons ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$), which is the only physiological reaction in which they are involved[1].

Many CA isoforms are involved in critical physiologic processes such as respiration and acid-base regulation, electrolyte secretion, bone resorption, calcification and biosynthetic reactions which require bicarbonate as a substrate (lipogenesis, gluconeogenesis, and ureagenesis)[1]. Two CA isozymes (CA IX and CA XII) are predominantly associated with and overexpressed in many tumors, being involved in critical processes connected with cancer progression and response to therapy[1-3]. CA IX is confined to few normal tissues (stomach and body cavity lining), but it is ectopically induced and highly overexpressed in many solid tumor types, through the strong transcriptional activation by hypoxia, accomplished *via* the hypoxia inducible factor-1 (HIF-1) transcription factor[1,3,4]. The detailed mechanism by which HIF-1α leads to the potent overexpression of CA IX in hypoxia was discussed in an earlier review[1]. In contrast to other α-CAs, CA IX is a multidomain protein formed of a short intracytosolic tail, one transmembrane segment, an extracellular CA domain and a proteoglycan (PG)-like domain composed of 68 amino acid residues[4-12]. Expression of CA IX is strongly increased in many types of tumors, such as gliomas/ependymomas, mesotheliomas, papillary/follicular carcinomas, as well as carcinomas of the bladder, uterine cervix, kidneys, esophagus, lungs, head and neck, breast, brain, vulva, and squamous/basal cell carcinomas, among others. In some cancer cells, the VHL gene is mutated leading to the strong upregulation of CA IX (up to 150-fold) as a consequence of constitutive HIF activation[13-15]. On the other hand, as this protein is present in extremely low amounts only in few normal tissues such as the gastric mucosa (whereit seems to be in a catalytically inactive state) inhibitors of CA IX may show less side effects compared to other anticancer druigs which interact with their target both in the normal and cancerous tissues[6]. As the role of CA XII in cancer is less understood at this moment, in this review only CA IX will be discussed.

CATALYTIC ACTIVITY OF CA IX PLAYS A ROLE IN TUMOR ACIDIFICATION

The expression of CA IX is strongly up-regulated by hypoxia and is down-regulated by the wild-type von Hippel-Lindau tumor suppressor protein (pVHL)[2-4,11-13]. The transcription factor HIF-1 is a heterodimer consisting of an inducible subunit (HIF-1α) and a constitutively expressed subunit (HIF-1β)[1,2,12,13]. HIF-1 activation under hypoxia is achieved by stabilization and/or expression of the α-subunit. Oxygen-dependent prolyl-4-hydroxylase domains (PHD) covalently modify a HIF-1α domain known as the oxygen-dependent degradation domain, by hydroxylating proline residues. Hypoxia attenuates proline hydroxylation due to inactivity of PHD in the absence of oxygen, resulting in HIF-1α stabilization and non-recognition by pVHL. The association of HIF-1α with the β-subunit leads to the formation of HIF-1 and expression of target genes that contain HRE (hypoxia responsive element) sites, including glucose transporters (GLUT-1 and GLUT-3), vascular endothelial growth factor, which triggers neoangiogenesis, and, last but not least, CA IX, which is involved in pH regulation and cell adhesion[13-15].

The overall consequence of the strong CA IX overexpression is the pH imbalance of the tumor tissue, with most hypoxic tumors having acidic extracellular pH (pHe) values around 6.5, in contrast to normal tissue which has characteristic pHe values around 7.4. The role played by CA IX in such acidification processes of hypoxic tumors was recently demonstrated by our and Pastorekova's groups[13]. Using Madin-Darby canine kidney epithelial cells, Svastova and colleagues proved that CA IX is able to decrease the pHe of these cultivated cells. CA IX selective sulfonamide inhibitors (of type 1 and 2) reduced the medium acidity by inhibiting the catalytic activity of the enzyme, and thus the generation of H^+ ions, binding specifically only to hypoxic cells expressing CA IX. Deletion of the CA active site was also shown to reduce the medium acidity, but a sulfonamide inhibitor did not bind to the active site of such mutant proteins[13]. Therefore, tumor cells decrease their pHe both by production of lactic acid (due to the high glycolysis rates), and by CO_2 hydration catalyzed by the tumor-associated CA IX, possessing an extracellular catalytic domain. Low pHe has been associated with tumorigenic transformation, chromosomal rearrangements, extracellular matrix breakdown, migration and invasion, induction of the expression of cell growth factors and protease activation[12,13]. CA IX probably also plays a role in providing bicarbonate to be used as a substrate for cell growth, whilst it is established that bicarbonate is required in the synthesis of pyrimidine nucleotides[14-16].

The crystal structure of the catalytic domain of human CA IX, was recently reported by this group[17]. As for other α-CAs, the CA IX catalytic domain appeared as a compact globular domain, with an ovoid shape of 47 × 35 × 42 $Å^3$ in size. CA IX has a 3D fold characteristic of other α-CAs, for which the structure has been solved earlier[17], in which a ten-stranded antiparallel-sheet forms the core of the molecule. An intramolecular disulfide bond, which is common to the other membrane-associated α-CAs (CA IV, CA XII and CA XIV), was observed between Cys23 and Cys203[1,17]. The active site details, including the Zn(II) coordination (by His 94, 96, 119 and a water molecule), proton shuttle residue (His64) as well as amino acid residues involved in binding of inhibitors are rather similar with those of other α-CAs[1,17]. Thus, Leu91, Val121, Val131, Leu135, Leu141, Val143, Leu198 and Pro202 define the hydrophobic region of ther active site, whereas Arg58, Arg60, Asn62, His64,

Ser65, Gln67, Thr69, and Gln92 identify the hydrophilic one. The crystallographic data showed the dimeric nature of the enzyme, which has been inferred from previous experiments reported by Hilvo *et al*[8]. Indeed, two identical dimers, resulting from a Cys41-mediated intermolecular disulfide bond between two adjacent monomers, were observed in the asymmetric unit of the crystals[17]. The dimer assembly, by means of an intermolecular disulfide bond, is consistent with the proposed function of the enzyme in tissues where its expression has been reported, as both active sites of the dimer are clearly exposed to the extracellular medium, being thus able to efficiently hydrate CO_2. In addition, the N-terminal regions of both monomers are located on the same face of the dimer, while both the C-termini are situated on the opposite face. This structural organization allows for concomitant positioning of both PG domains, at the entrance to the active site clefts, oriented toward the extracellular milieu to mediate cell interaction, and of both C-terminal transmembrane portions for proper CA IX anchoring to the cell membrane. Furthermore, the position of the PG portion, at the border of the active site, suggests a further role of this domain in assisting CA domain-mediated catalysis. Indeed, as shown recently by our group[18] the CO_2 hydrase activity of the CA IX full length has an optimum at a pH of 6.5 (typical of hypoxic solid tumors) whereas that of CA IX catalytic domain (similarly to that of CA I or CA II) has an optimum at pH around 7[17,18]. Thus, the PG domain, which is rich in acidic amino acid residues (26 dicarboxylic amino acids, Asp and Glu, on a total of 58 amino acid residues forming the PG domain) was postulated to act as an intrinsic buffer of this enzyme, which facilitates the CO_2 hydration reaction at acidic pH values which are one of the main features of hypoxic tumors[17,18].

CA IX INHIBITORS ACCUMULATE IN HYPOXIC TUMORS AND IMPAIR THEİR GROWTH AND METASTASİS GROWTH

Many sulfonamide/sulfamate/sulfamide and coumarin CA inhibitors (CAIs) were reported to efficiently target CA IX in recent years[19-39]. The compounds specifically designed for targeting CA IX, which were important to understand its role in tumorigenesis were, among others: (1) fluorescent sulfonamides, used for imaging purposes and for determining the role of CA IX in tumor acidification[13,16,22,24,30]; (2) positively or negatively-charged compounds, which cannot cross plasma membranes due to their charged character and thus inhibit selectively only extracellular CAs, among which CA IX[1,13,23,25]; (3) ureido-substituted benzenesulfonamides with potent antitumor effects both for the primary tumor and metastases (in animal models)[31, 36]; and (4) diverse chemotypes than the sulfonamides and their bioisosteres, such as the coumarins[32,33], which showed notable inhibition for the growth of the primary tumors and impair metastases formation in animal models of hypoxic tumors[36].

Some of the most interesting CA IX inhibitors available initially, were the compounds investigated by Svastova *et al*[13] (possessing structures 1 and 2) for their *in vivo* role in tumor acidification. These compounds present a special interest because derivative 1 is a fluorescent sulfonamide with high affinity for CA IX (K_I of 24 nmol/L)[1,13], which was shown to be useful as a fluorescent probe for hypoxic tumors[1,13,16,22,24]. This inhibitor binds to CA IX only under hypoxia *in vivo*, in cell cultures or animals with transplanted tumors[13,16,24,30,36]. Although the biochemical rationale for this phenomenon is not understood in details, these properties may be exploited for designing diagnostic tools for the imaging of hypoxic tumors[16,24]. Indeed, Dubois *et al*[16,24] showed the accumulation of 1 only in the hypoxic regions of animals with transplanted hypoxic colorectal tumors (see discussion later in the text).

Compound 2 belongs to type II mentioned above, of permanently charged, membrane-impermeant derivatives, and is also a very strong CA IX inhibitor (K_I of 14 nmol/L)[1,29]. It belongs to the class of positively charged, membrane-impermeant compounds previously reported by our group[1,29], which are highly attractive for targeting CA IX with its extracellular active site, since such compounds do not inhibit intracellular CAs, and may thus lead to drugs with less side effects as compared to the presently available compounds which indiscriminately inhibit all CAs[1].

The *in vivo* proof of concept that sulfonamide CA IX inhibitors may indeed show antitumor effects, has been first published by Neri's group[34]. By using membrane-impermeant derivatives of types 3 and 4, based on the acetazolamide scaffold to which either fluorescein-carboxylic acid or albumin-binding moieties were attached, this group demonstrated the strong tumor retardation (in mice with xenografts of a renal clear cell carcinoma line, SK-RC-52) in animals treated for one month with these CA inhibitors[34].

The same group[35] also reported the proof-of-concept study showing that human monoclonal antibodies targeting CA IX can also be used for imaging of hypoxic tumors. The generation of high-affinity human monoclonal antibodies (A3 and CC7) specific to hCA IX, using phage technology has been reported[35]. These antibodies were able to stain CA IX *ex vivo* and to target the cognate antigen *in vivo*. In one animal model of colorectal cancer studied (LS174T), CA IX imaging closely matched pimonidazole staining, with a preferential staining of tumour areas characterised by little vascularity and low perfusion. These new human anti-CA IX antibodies are expected thus to be non-immunogenic in patients with cancer and might serve as broadly applicable reagents for the non-invasive imaging of hypoxia and for pharmacodelivery applications[35] (Figure 1).

The same conclusion has been reached by our and Lambin's groups by using small molecule CA IX-selective inhibitors of the type 1[16]. Fluorescent sulfonamides 1 with a high affinity for CA IX have been developed and shown to bind to cells only when CA IX protein

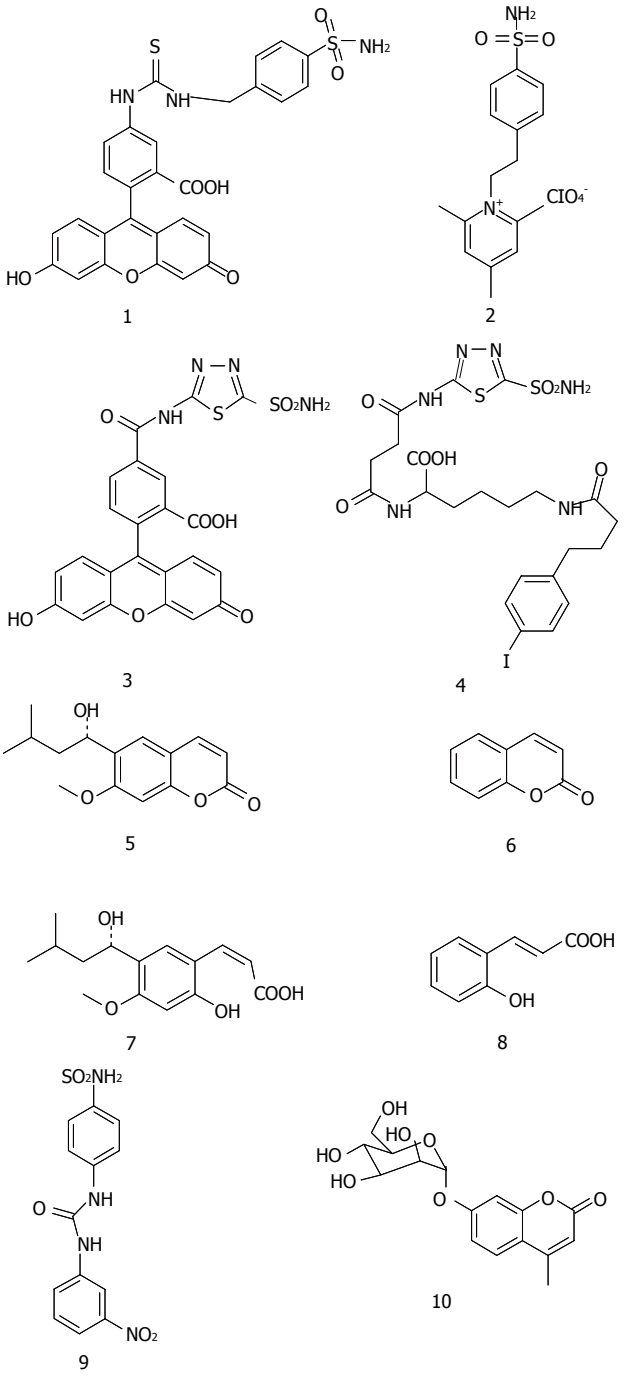

Figure 1 The strctures of 1-10. 1: KI (CA Ⅸ) = 24 nmol/L; 2: KI (CA Ⅸ) = 14 nmol/L.

was expressed and while cells were hypoxic[16]. NMRI-nu mice subcutaneously transplanted with HT-29 colorectal tumours were treated with 7% oxygen or with nicotinamide and carbogen and were compared with control animals. Accumulation of sulfonamide 1 was monitored by non-invasive fluorescent imaging. Specific accumulation of 1 could be observed in delineated tumour areas as compared with a structurally similar non-sulfonamide analogue incorporating the same scaffold (i.e., a derivative with the same structure as compound 1 but without the SO_2NH_2 moiety). Administration of nicotinamide and carbogen, decreasing acute and chronic hypoxia, respectively, and prevented accumulation of 1 in the tumor. When treated with 7% oxygen breathing, a 3-fold higher accumulation of 1 was observed. Furthermore, the bound inhibitor fraction was rapidly reduced upon tumour reoxygenation. Such *in vivo* imaging results confirm previous *in vitro* data demonstrating that CAI binding and retention require exposure to hypoxia. Fluorescent labelled sulfonamides may thus provide a powerful tool to visualize hypoxia response in solid tumors. An important step was thus made towards clinical applicability, indicating the potential of patient selection for CA IX-directed therapies[16].

Dubois *et al*[39] also recently showed that combining sulfonamide CA IX inhibitors with tumor irradiations hase an enhanced antitumor effect in mice bearing HT29 colorectal transplanted tumors.

More recently, sulfonamide 1 has also been shown to significantly decrease the growth of primary tumors in the 4T1 mouse metastatic breast cancer animal model by Lou *et al*[36]. However, an even stronger effect has been observed with an ureido sulfonamide (compound 9) which at pharmacological doses of 15-30 mg/kg strongly inhiibted both the growth of the primary 4T1 tumor, as well as the formation of lung metastases[31,36]. It is interesting to note that the 4T1 model tumors overexpress a very high amount of CA IX. The same study also used another mouse breast tumor cell line, the 67R1 line, which does not express at all CA IX. Indeed, the animals harboring these tumors were treated with sulfonamide or coumarin CA IX inhibitors but no influence of the tumor growth has been observed, which represents a clear-cut proof of concept that inhibition of CA IX is indeed responsible for the tumor/metastases growth inhibition with these compounds[36].

Coumarin and thiocoumarins were only recently discovered to act as CAIs, and their inhibition mechanism deciphered in detail by one of our groups[32,33]. We demonstrated recently that the natural product 6-(1*S*-hydroxy-3-methylbutyl)-7-methoxy-2H-chromen-2-one 5 as well as the simple, unsubstituted coumarin 6 are hydrolyzed within the CA active site with formation of the 2-hydroxy-cinnamic acids 7 and 8, respectively, which represent the de facto enzyme inhibitors[32,33]. Some other interesting facts emerged during such studies: (1) this new class of CAIs, the coumarins/thiocoumarins, binds in hydrolyzed form at the entrance of the CA active site and does not interact with the metal ion, constituting thus an entirely new category of mechanism-based inhibitors; and (2) it is possible to obtain highly isoforms-selective CAIs belonging to the coumarin/thiocoumarin class. Indeed, we reported coumarins which selectively inhibit CA IX and XII, without inhibition of CA I and II (the main offtarget isoforms)[32,36-38].

One of these derivatives, a glycosyl coumarin (compound 10) strongly inhibited the growth of the primary tumor and the formation of metastases in the same 4T1 animal model of hypoxic tumor overexpressing high

amounts of CA IX, whereas in a breast cancer cell line with no CA IX expression (67R1) no such effects have been observed[39].

There are ongoing clinical trials with a monoclonal antibody targeting specifically CA IX-girentuximab (which is in Phase III cliniacl trials for the tretment of renal carcinomas) and several sulfonamide/coumarin CA IX ihiibtors are in advanced preclinical evaluation[40].

CONCLUSION

With its overexpression in many cancer tissues and not in their normal counterparts, CA IX constitutes an interesting target for novel approaches in the design of anticancer therapies. CA IX is crucial for tumor pH regulation contributing both to the acquisition of metastasic phenotypes and to chemoresistance. Consequently, further research needs to be done in the field of the tumor-associated CA IX in order to better understand its exact role in cancer. CA IX selective inhibitors are now available and they constitute interesting tools for studying the physiological and/or pathological effects of this enzyme. The design of CA IX selective inhibitors containing a variety of scaffolds and with interesting physico-chemical properties has been achieved. New sulfonamides and coumarins have been synthesized with some of these strongly and selectively inhibiting CA IX (over the offtarget isoforms CA I and Ii), with inhibition constants in the low nanomolar. Thus, many biochemical, physiological and pharmacological novel data point to the use of CA IX inhibition in the management of hypoxic tumors, which do not respond to the classical chemo- and radiotherapy. There are possibilities of developing both diagnostic tools for the non-invasive imaging of these tumors and therapeutic agents, that probably perturb the extratumoral acidification in which CA IX is involved. Much pharmacologic work is however warranted in order to understand whether a successful new class of antitumor drugs may be developed starting from these preliminary but highly encouraging observations, but girentuximab, a CA IX monoclonal antibody is alreday in Phase III clinical trials and several small molecule inhibitors are in advanced preclinical evaluation

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ORIGINAL ARTICLE

Inhibition of N-Myc down regulated gene 1 in *in vitro* cultured human glioblastoma cells

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Abstract

AIM: To study short dsRNA oligonucleotides (siRNA) as a potent tool for artificially modulating gene expression of N-Myc down regulated gene 1 (*NDRG1*) gene induced under different physiological conditions (Normoxia and hypoxia) modulating NDRG1 transcription, mRNA stability and translation.

METHODS: A cell line established from a patient with glioblastoma multiforme. Plasmid DNA for transfections was prepared with the Endofree Plasmid Maxi kit. From plates containing 5×10^7 cells, nuclear extracts were prepared according to previous protocols. The pSUPER-NDRG1 vectors were designed, two sequences were selected from the human NDRG1 cDNA (5′-GCATTATT-GGCATGGGAAC-3′ and 5′-ATGCAGAGTAACGTGGAAG-3′. reverse transcription polymerase chain reaction was performed using primers designed using published information on β-actin and hypoxia-inducible factor (HIF)-1α mRNA sequences in GenBank. NDRG1 mRNA and protein level expression results under different conditions of hypoxia or reoxygenation were compared to aerobic control conditions using the Mann-Whitney *U* test. Reoxygenation values were also compared to the NDRG1 levels after 24 h of hypoxia ($P < 0.05$ was considered significant).

RESULTS: siRNA- and iodoacetate (IAA)-mediated downregulation of NDRG1 mRNA and protein expression *in vitro* in human glioblastoma cell lines showed a nearly complete inhibition of NDRG1 expression when compared to the results obtained due to the inhibitory role of glycolysis inhibitor IAA. Hypoxia responsive elements bound by nuclear HIF-1 in human glioblastoma cells *in vitro* under different oxygenation conditions and the clearly enhanced binding of nuclear extracts from glioblastoma cell samples exposed to extreme hypoxic conditions confirmed the HIF-1 Western blotting results.

CONCLUSION: NDRG1 represents an additional diagnostic marker for brain tumor detection, due to the role of hypoxia in regulating this gene, and it can

represent a potential target for tumor treatment in human glioblastoma. The siRNA method can represent an elegant alternative to modulate the expression of the hypoxia induced *NDRG1* gene and can help to monitor the development of the cancer disease treatment outcome through monitoring the expression of this gene in the patients undergoing the different therapeutic treatment alternatives available nowadays.

Key words: N-Myc down regulated gene 1; Short dsRNA oligonucleotides; Human cancer diseases; Brain cancer; Radiotherapy

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Said HM, Polat B, Stein S, Guckenberger M, Hagemann C, Staab A, Katzer A, Anacker J, Flentje M, Vordermark D. Inhibition of N-Myc down regulated gene 1 in *in vitro* cultured human glioblastoma cells. *World J Clin Oncol* 2012; 3(7): 104-110 Available from: URL: http://www.wjgnet.com/2218-4333/full/v3/i7/104.htm DOI: http://dx.doi.org/10.5306/wjco.v3.i7.104

INTRODUCTION

RNA interference (RNAi) or short dsRNA oligonucleotides (siRNA) approach represents a powerful tool for drug target discovery and validation in eukaryotic cell culture approaches, *in vitro*, as well as within *in vivo* systems, such as animal disease models and human therapeutics. siRNA is a potent tool for artificially modulating gene expression through the introduction of short interfering RNAs. These molecular mechanisms that the siRNA approach is consisted of are occurring naturally as a gene regulatory mechanism having a number of advantages over other gene/antisense therapies including specificity of inhibition, potency, the small size of the molecules and the diminished risk. The systems for stable and regulated expression of these molecules emerged as well. Selective gene inhibition *via* siRNA occurs *via* two methods: (1) siRNA cytoplasmic delivery mimicking an active endogenous RNAi mechanism intermediate; and (2) nuclear delivery of gene expression cassettes that express a short hairpin RNA, which mimics the micro interfering RNA active intermediate of a different endogenous RNAi mechanism. In contrast, screens of many siRNA sequences can be accomplished rapidly using synthetic oligos. The activity of siRNA in the cytoplasm may lower the barrier and thereby accelerate the successful development of therapeutics based on targeted non-viral delivery systems. Under hypoxia, hypoxia-inducible factor (HIF)-1α is involved in the transcriptional regulation of the N-Myc down-regulated gene 1 (*NDRG1*) gene[1,2] (Figure 1) together with other transcription factors. In this relation it is of interest to investigate the expression of NDRG1 protein in human cancer[3]. This gene is necessary for *P53*-mediated apoptosis and regulated by phosphatase and tensin homologue. In several cancers, it was suggested to be a tumour suppressor gene[4].

MATERIALS AND METHODS

Cell culture, hypoxia treatment and transfection of glioblastoma cell lines

Early-passage U373, U251 and U87-MG human malignant glioblastoma from the American Type Culture Collection (ATCC, Rockville, MD, United States) and GaMG, a cell line established from a patient with glioblastoma multiforme (Gade Institut of the University Bergen, Norway)[5], were grown on glass Petri dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, non-essential amino acids, penicillin (100 IU/mL)/streptomycin (100 μg/mL) and 2 mmol/L L-glutamine. Cells were treated with *in vitro* hypoxia for 1, 6 or 24 h at 5%, 1% or 0.1% O_2 as indicated in a Ruskinn Invivo2 hypoxic workstation (Cincinnatti, OH, United States) as previously described[6,7]. For reoxygenation experiments, dishes were returned to the incubator following 24 h of hypoxia. Plasmid DNA for transfections was prepared with the Endofree Plasmid Maxi kit (Qiagen, Hilden, Germany). Stable as well as transient transfections were performed using Fugene6 transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The pSUPER constructs transfected into U373, U251, U87-MG and GaMG glioblastoma cells lines were incubated for 8 h under standard normoxic conditions (21% O_2, 5% CO_2) post transfection with further incubation under hypoxic conditions (0.1%) for 24 h.

Preparation of nuclear extracts, whole-cell lysates and immunoblotting

From plates containing 5 × 10^7 cells, nuclear extracts were prepared according to previous protocols[8] with minor modifications. Aliquots containing nuclear extracts were stored in aliquots at -80 ℃. Whole-cell lysates were prepared with 0.1 mL RIPA buffer (1 × TBS, 1% Nonidet P-40) (Amresco, Vienna, Austria), 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitors pepstatin A (1.4 μmol/L), aprotinin (0.15 μmol/L) and leupeptin (2.3 μmol/L), and 100 μmol/L phenylmethylsulfonyl fluoride (all were obtained from Sigma, St. Louis, MO, United States). To inhibit protein dephosphorylation, phosphatase inhibitor mix (Sigma) was added. Using a syringe fitted with a 21-gauge needle to shear DNA, lysates were transferred to a pre-chilled microcentrifuge tube, followed by 30 min incubation on ice. Cell lysates were cleared by centrifugation at 15 000 × *g* for 12 min at 4 ℃. Twenty microgram of whole-cell lysates were separated onto SDS 8% polyacrylamide gel electrophoresis and transferred to a 0.45 μmol/L nitrocellulose membrane (Protran BA 85; Schleicher and Schuell, Das-

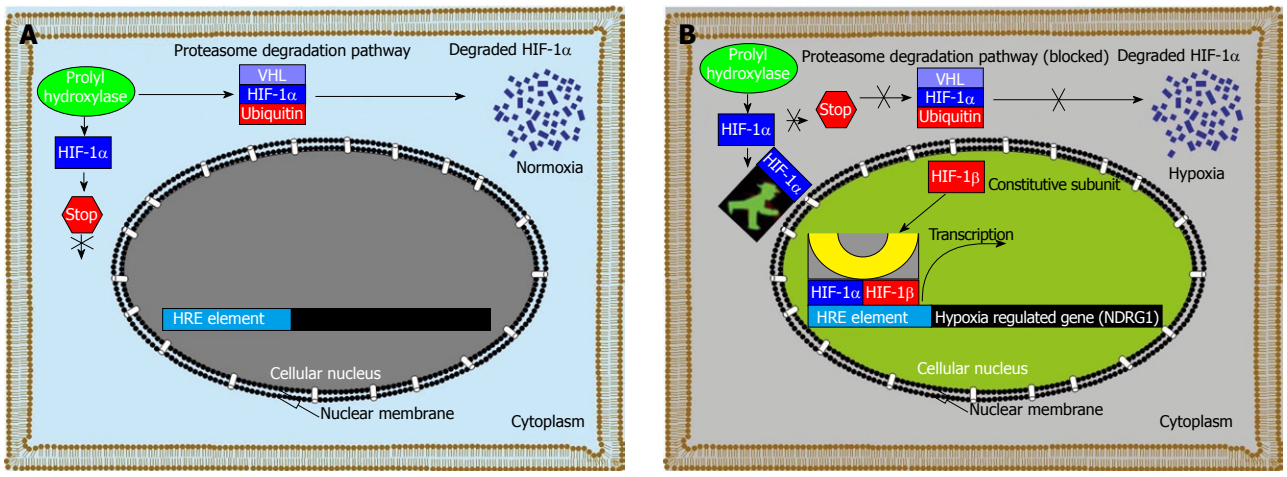

Figure 1 Hypoxia induced regulation of N-Myc down-regulated gene 1 in human brain cancer hypoxia-inducible factor-1α induced regulation of hypoxia induced N-Myc down-regulated gene 1 expression in human tumour cells. A: Under normoxic oxygenation conditions in the tumor cell microenvironment, hypoxia-inducible factor (HIF)-1α is rapidly degraded *via* the von Hippel-Lindau tumour suppressor gene product (pVHL)-mediated ubiquitin proteasome pathway; B: When the tumor environment aeration conditions shifts from normoxic to hypoxic aeration conditions, HIF-1α subunit becomes stable, translocates into the cellular nucleus and interacts with co-activators of which its transcription machinery is consisted such as p300/CBP to modulate the transcriptional activity of numerous hypoxia inducible genes, like N-Myc down-regulated gene 1 (NDRG1) in the case and about 61 other hypoxia induced genes[28,40]. HRE: Hypoxia response element.

sel, Germany). Nonspecific binding was blocked by 5% nonfat milk powder in TBS overnight at 4 ℃ followed by incubation with the NDRG1 primary antibody (ab8448, Abcam, Cambridge, United Kingdom), diluted 1:1000 in 2.5% nonfat milk powder in TBS for 1h at room temperature or with followed by incubation with the M75 mouse monoclonal antibody against CA Ⅸ (Bayer Healthcare Co., diluted 1:7200) or with HIF-1α monoclonal antibody (610959, BD Biosciences, dilution 1:500). Blots were washed twice in TBS/0.05% Tween-20 (Bio-Rad, Munich, Germany) and subsequently, three times in TBS for (5-10) min each. The secondary antibody goat anti rabbit-HRP (stock solution: 400 μg/mL); DakoCytomation, Denmark) was incubated at a dilution of 1:2000 for one additional hour at room temperature followed by five wash steps as described above. Antibody detection and development was as described in[9].

Knock-down of endogenous NDRG1 by siRNA and iodoacetate

Human glioblastoma cell lines U373, U251, U87-MG and GaMG were grown up to 50% confluence on 10 cm plates in complete medium (RPMI 1640 medium or DMEM depending on the cell line) supplemented with 10% fetal calf serum, 100 μg/mL streptomycin, and 100 units/mL penicillin). The pSUPER-NDRG1 vectors were designed as mentioned before[10-17]. To establish pSUPER-NDRG1, two sequences were selected from the human NDRG1 cDNA (5'-GCATTATTGGCATGGGAAC-3' (positions 398-416) and 5'-ATGCAGAGTAACGTGGAAG-3' (positions 601 to 619), relative to the start codon). All constructs were confirmed by sequencing. Transient transfection of siRNA constructs into the glioblastoma brain tumor cell lines exposed to extreme hypoxic aeration condition was *via* Fugene6 solution (Roche, Germany) according to the manufacturers suggested instructions. Also, cells were transfected with the empty vector pSUPER (Oligo-Engine, Seattle) and pSUPER-NDRG1. Detection of reduced NDRG1 mRNA and protein levels was performed by Northern-blotting and as well as immunoblotting, applying the Goat polyclonal anti-NDRG1 antibody (Abcam ab 21727). Iodoacetate (IAA; 50 μmol/L) was used as a glycolysis inhibitor and was added to the growth medium shortly before the respective hypoxia treatment.

Tissue biopsies were obtained surgically from two groups of patients: 15 patients with glioblastoma multiforme (GBM) and 15 patients with low-grade astrocytoma (LGA; WHO grade 2). Samples were immediately frozen at -80 ℃ and stored in liquid nitrogen before further analysis. To compare the expression of the individual genes examined, reverse transcription polymerase chain reaction was performed using primers designed using published information on β-actin and HIF-1α mRNA sequences in GenBank (accession numbers NM_001101 for β-actin, NM_001530.2 for HIF-1α, and NM_006096 for NDRG1, respectively). An aliquot of (1-5) μg of total mRNA from human gliblastoma and astrocytoma tissue or glioblastoma cell lines was transcribed at 42 ℃ for 1 h in a 20 μL reaction mixture using 200 U RevertAid™ M-MuLV RT, oligo(dT)18 primer and 40 U Ribonuclease inhibitor (all from Fermentas, Ontario, Canada). For polymerase chain reaction (PCR)-reactions primers were designed in flanking exons with Primer3 software (available online http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi): to produce a 593 bp amplification product of NDRG1, the forward primer (F1) was 5'-CTCTGTTCACGTCACGCTGT-3' and the reverse primer (R1)5'-CTCCACCATCTCAGGGTTGT-3'. To produce an 668 bp amplification product of β-actin, the forward primer (F1) was 5'-CGTGCGTGACATTAAGGAGA-3' (nucleotides 697-716)

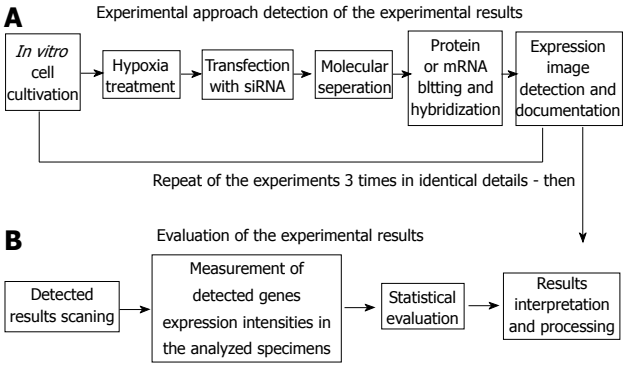

Figure 2 Detection and experimental monitoring of hypoxia induced N-Myc down-regulated gene 1 in specimens of human brain cancer. The experimental monitoring approach includes (A) experimental approach detection of the experimental results. Here, the tumor cell lines are first cultivated *in vitro* and subsequently transfected with the N-Myc down-regulated gene 1 (NDRG1) short dsRNA oligonucleotides (siRNA) construct and treated with fixed O_2 concentration in the hypoxia chamber followed by the specimens extraction, quantification, quality control and molecular seperation of tumor cells specimens. Further protein or mRNA blotting and Hybridization with subsequent NDRG1 expression image detection and documentation take place. The experimental approach with these different stages are repeated at least three times to have statistical significant results that are necessary for evaluation of the experimental results (B), where first the films with the detected results are scanned followed by the detection of the genes expression (which is in the case *NDRG1* gene and the house keeping genes or loading controls (β-actin and 18s RNA, respectively) measurement of intensities in the analyzed specimens, statistical analysis and evaluation of the obtained results.

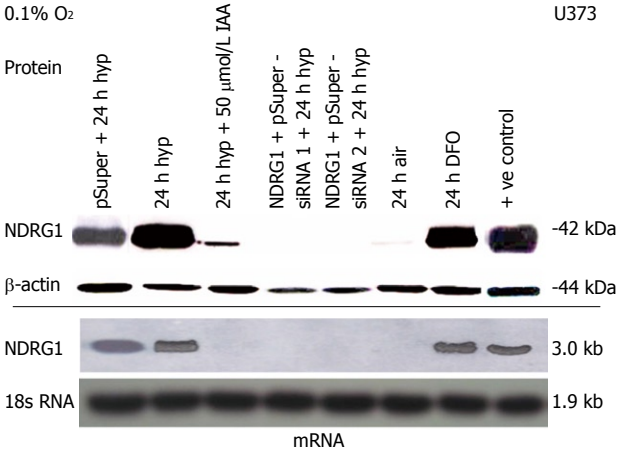

Figure 3 Inhibition of N-Myc down-regulated gene 1 protein and mRNA expression in U373 human glioblastoma cell line *in vitro via* short dsRNA oligonucleotides- and iodoacetate-mediated interference into human tumor cellular glycolysis process. Upper panel: Western blotting analysis result diagram related to the specific inhibition of hypoxia induced expression in human brain tumor cells (U373 Glioblastoma cell line as an example) on protein level *via* Western blotting analysis. Clear complete inhibition of N-Myc down-regulated gene 1 (NDRG1) induced by extreme hypoxic conditions in the tumor microenviroment (0.1% O_2/for 24 h) was present upon transfection with the one of the two variants of NDRG1 short dsRNA oligonucleotides (siRNA) construct applied in these experiments when compared to the nearly complete inhibition *via* inhibitive interaction with the tumor cell glycolysis pathway. β-actin served as a loading control. Figure shows one representative experiment out of three experiments; Lower pannel: Northern blotting analysis displaying the specific inhibition of hypoxia induced NDRG1 mRNA expression in human brain tumor cells (U373 Glioblastoma cell line as an example) on mRNA level. Complete inhibition of NDRG1 induced by extreme hypoxic conditions in the tumor microenviroment (0.1% O_2/for 24 h) was could be achieved upon transfection with the one of the two variants of NDRG1 siRNA construct applied in these experiments. Inhibitive interaction into the glycolysis pathway due to the parallel treatment with 50 μmol/L with iodoacetate for 24 h showed a similar functional effect. The 18s RNA fragment with a molecular weight of 1.9 kb served as a loading control. This is one representative experiment out of three experiments.

and the reverse primer (R1) 5'-CACCTTCACCGTTC-CAGTTT-3' (nucleotides 1345-1364) and to produce an 233 bp amplification product of HIF-1α, the forward primer (F1) was 5'-TTACAGCAGCCAGACGATCA-3' (nucleotides 2516–2535) and the reverse primer (R1) 5'-CCCTGCAGTAGGTTTCTGCT-3' (nucleotides 2729-2748). The PCR was performed and PCR products were separated on agarose gels as mentioned previously.

Visualisation, expression level evaluation and analysis

The data presented here are representative for 3 similar experiments. Densitometric evaluation of Northern blots was performed with 1D Kodak Image Analysis Software. Signals were measured in Kodak light units and divided by the corresponding signals of the house keeping gene β-actin or 18s RNA for the northern blot results. NDRG1 mRNA and Protein level expression results under different conditions of hypoxia or reoxygenation were compared to aerobic control conditions using the Mann-Whitney *U* test. Reoxygenation values were also compared to the NDRG1 levels after 24 h of hypoxia ($P < 0.05$ was considered significant). Further details are outlined in (Figure 2).

RESULTS

Hypoxia induced NDRG1 mRNA in human glioblastoma detection via Western blotting

In all four glioblastoma cell lines examined including the U373 cell line (Figure 3, upper pannel), expression of NDRG1 was either reduced or inhibited upon application of one of the two siRNA constructs, each separately showing that the inhibition of NDRG1 was 100% of it basal expression level under normoxic conditions and 97% from its expression level after hypoxic treatment (0.1% O_2) for 24 h. Also, when 50 μmol/L of the glycolysis inhibitor IAA was applied, *in vitro*, for 24 h with 0.1% hypoxia, on protein level there was an inhibition of 85% of the expression level.

Hypoxia induced NDRG1 mRNA in human glioblastoma detection via northern blotting

On mRNA level (Figure 3, lower panel), there was a complete inhibition of NDRG1 mRNA expression also when 50 μmol/L glycolysis inhibitor IAA was applied, *in vitro*, for 24 h with 0.1% hypoxia treatment that was applied to all glioblastoma cell lines exposed to 0.1% O_2 examined *in vitro*, showing that the inhibition level on the mRNA level is more effective and not depending on the nature of the option applied (Chemical treatment with 50 μmol/L IAA or the transfection with either NDRG1 siRNA construct) for NDRG1 down regulation with final inhibition of expressed NDRG1 in glioblastoma

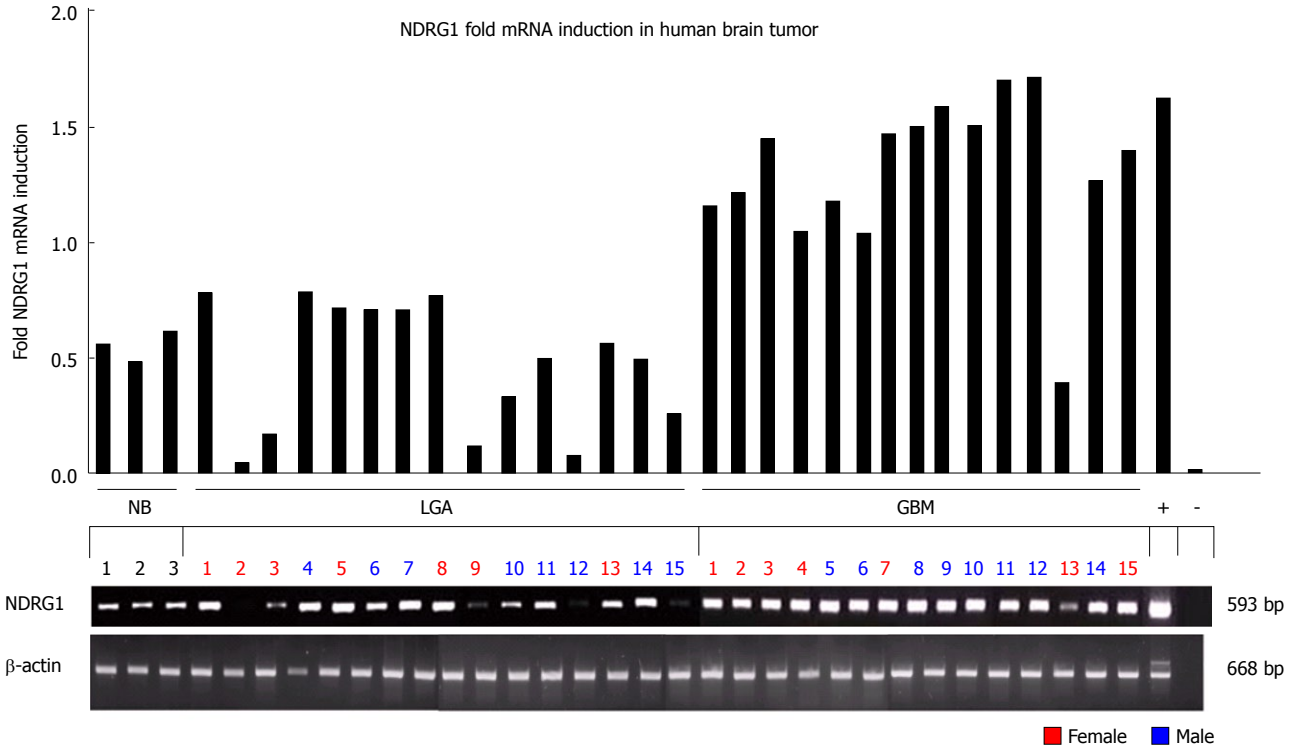

Figure 4 Expression of N-Myc down-regulated gene 1 mRNA in human brain cancer tissue *in vivo*. NDRG1: N-Myc down-regulated gene 1; NB: Nonneoplastic brain; LGA: Low-grade astrocytoma; GBM: Glioblastoma.

cells (Figure 3, lower panel).

Hypoxia induced NDRG1 expression in 2 groups of human brain tumour specimens

In vivo, mRNA expression of HIF-1α was similar in tumor specimens from patients with low-grade astrocytoma or glio-blastoma (results not shown). A tumor-grade association with NDRG1 mRNA expression was exhibited *in vivo*. No increase in NDRG1 expression was shown in low-grade astrocytoma while an increase of at least 2-fold in NDRG1 expression was shown in 10/15 patients in GBM in no patient with LGA as seen in (Figure 4).

DISCUSSION

NDRG1 protein expression has been described to be present in normal brain or brain tumor tissue[11] as well as being an important gene that is playing an active role in the regulation of a broad spectrum of human cancer diseases like human gastric cancer, squamous cell carcinomas, breast cancer, human hepatocellular carcinoma, brain tumors and leukemia[12-22]. NDRG1 was suggested to be a prognostic marker for hypoxic regions within a tumor mass because of its stability as a protein[23-25] and because it is highly expressed in malignant tumor tissues compared to normal tissue of the same origin[26].

During the different sets of experiments within our study, we observed, a brain tumor-type-dependent increase in NDRG1 mRNA expression level. NDRG1 protein and NDRG1 mRNA were generally up-regulated in response to prolonged to severe (0.1% O_2) *in vitro* hypoxia, although the effect was undetectable at the protein level in one cell line with a strong constitutive, normoxic NDRG1 expression.

In a previous approach were human tumor specimens from patients suffering from LGA or GBM where analyzed showed that tumor specimens with GBM displayed a higher level of NDRG1 than low-grade astrocytoma both at the protein and mRNA level[27-29].

As known by previous contributions that induction of NDRG1 sequence-specific posttranscriptional gene silencing in different glioblastoma celll lines, *in vitro*, by RNA interference[30,31] resulted in a strong inhibitory activity of NDRG1 expression, both on mRNA and protein level. This approach when compared to glycolysis inhibition *via* IAA application, which has previously been shown to posses HIF-1-inhibitory functions[32-36], or HIF-1α and hypoxia induced genes like NDRG1 inhibitory functions can represent one innovative option with a high potential in the monitoring of human cancer disease like brain cancer as shown by the results of this series of research experiments, since hypoxia-tolerant human glioma cells reduce their oxygen consumption rate in response to oxygen deficit, a defense mechanism that contributes to survival under moderate hypoxic conditions[37]. Overcoming the metabolic restrictions of hypoxia may allow for the progression of lower-grade tumors to GBM.

An alternative of this level when used within a framework of an integrated detection of monitoring system as shown or as it can be seen by the results of other experimental approaches used to detect different sets of cancer

disease related genes that are hypoxia induced[11,27-29,35-42] can present a therapeutic strategy targeting hypoxia-induced NDRG1. However, the success of such approaches still awaits the development of an efficient delivery system that can affect a large number of tumor cells.

Experimental inhibition of NDRG1 expression in four glioblastoma cell lines *in vitro* by either siRNA technology or interference into tumor cell glycolisis might be a potential therapeutic tool in regulating the expression of this gene in glioblastoma. Furthermore, successful inhibition of tumor cell growth by RNAi aimed at oncogenes *in vitro* and *in vivo* may represent alternative therapeutic applications for these diseases. RNAi is a molecular biology tool with a big potential as therapeutic agent of cancer in human.

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COMMENTS

Background

N-Myc down-regulated gene 1 (*NDRG1*) is a member of the NDRG family. Its induction occurs *via* diverse physiological and pathological conditions (hypoxia, cellular differentiation, heavy metal, N-myc, neoplasia) which modulate NDRG1 transcription, mRNA stability and translation.

Research frontiers

Up to this date, the complete detailed function of this protein in humans remains unknown. Hypoxia represents a common feature of solid tumors. Hypoxia-inducible factor-1 (HIF-1) is a key regulator of tumor cell hypoxia. It regulates the expression of several genes related to oxygen homeostasis in response to hypoxic stress. NDRG1 has been shown to possess more specific characteristics for clinical analysis and identification purposes and has been found to be a stable marker of acute tumor hypoxia.

Innovations and breakthroughs

Detailed understanding of how hypoxia regulates transcription of the *NDRG1* gene increase knowledge of the cellular responses of normal and cancer cells towards low oxygen tension.

Applications

Direct inhibition of NDRG1 *via* siRNA or indirect inhibition through interfering with the cancer cell glycoltic activities *via* application of iodoacetate might be a potential therapeutic tool for regulating the expression of this gene in glioblastoma.

Terminology

Hypoxia is a pathological condition where organ tissues are deprived of adequate oxygen supply due to the failure to deliver oxygen to target tissues. The difference between normal oxygen supply and the demand at the cellular level may result in an oxygenation hypoxic condition. Normoxia normal oxygen concentration as a result of the normal or adequate oxygen supply on the cellular level, which is typically 20%-21% O_2; Glioblastoma multiforme: Highly invasive brain tumors. It is the last stage of human brain tumor where patients suffering from this type of brain tumor have a maximum life expectancy of maximum 2-6 mo. Human glioblastoma multiforme (GBM) cells vary in their ability to survive under hypoxic conditions. HIF-1α subunits are highly inducible by different oxygenation conditions in human GBM cells, HIF-1 acts as a master regulator of numerous hypoxia inducible genes related to angiogenesis, cell proliferation/survival, and glucose/iron metabolism. Tumor therapy is referring to the approaches applied against various cancer diseases in human. They include application radiation therapy, surgical removal of cancer tissue, drugs or other substances that block the cancer growth and spread by interfering with specific molecules involved in tumor growth and progression including medical agents that interfere with cell growth signalling or tumor blood vessel development, cancer cells specific death promotion, stimulating the immune system to destroy specific cancer cells, and cancer cells toxic chemical agents delivery into cancer cells as well as gene therapeutic modalities; Tumor microenvironment: Extracellular environment present in a very small region of a solid tumor. It must be mentioned that cells in different areas of solid tumors will have markedly different microenvironments; Angiogenesis: The formation of new blood vessels in human body tissues.

Peer review

Due to its clear regulatory behaviour under hypoxic condition in human tumor cells, NDRG1 represents an additional diagnostic marker for brain tumor detection. Due to the role of hypoxia in regulating this gene, it can represent a potential target for tumor treatment in human glioblastoma.

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CASE REPORT

Everolimus plus long-acting somatostatin analogs in thymic epithelial malignancies

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Abstract

Although thymic epithelial tumors (TETs) are rare in the general population, they represent the most frequently diagnosed primary malignant tumor of the anterior mediastinum. Unlike localized disease, metastatic disease is invariably fatal. While several chemotherapy agents have proven to be effective in TETs, somatostatin analogs are the only targeted agents with an established role in this disease. Everolimus is an mTOR inhibitor with multiple application in oncology. In this report, we show for the first time that everolimus was effective in two heavily pretreated patients with advanced TETs, with a progression-free survival longer than 1 year and minimal toxicity.

Key words: Thymoma; Everolimus; mTOR; Mediastinum; Target

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INTRODUCTION

Thymic epithelial tumors (TETs) are rare in the general population, with an estimated incidence in Netherlands of 3.2 cases per 1 000 000 inhabitants[1]. In spite of their rarity, TETs are the most frequently diagnosed primary malignant tumor of the anterior mediastinum, accounting for about 40% of mediastinal masses in adult patients[2]. Stage and histology are predictive of survival[3]. At histological examination, TETs present both epithelial and lymphocytic cells. Epithelial cells are responsible for neoplastic growth and can regulate maturation of the accompanying lymphocytic population, which is involved in the typical associated autoimmune syndromes[4]. Surgery, radiotherapy, and chemotherapy offer a possibility of cure for patients with localized or locally advanced TETs. Metastatic disease is virtually incurable but highly responsive to chemotherapy, which can significantly prolong survival and palliate symptoms. TETs are responsive to several anti-neoplastic agents including platinum

compounds[5], anthracyclines[5], and pyrimidine analogs[6]. Among targeted drugs, somatostatin analogs, employed in combination with prednisone, provided radiological disease stabilization in TETs patients and improvement of the associated auto-immune syndromes[7].

The biology of TETs is presently unknown. Immunohistochemistry data showing expression of c-KIT and epidermal growth factor receptor (EGFR) in TETs prompted experimentation of imatinib and cetuximab in TETs[8,9]. Multi tyrosine kinases inhibitors(such as sorafenib and sunitinib) and histone deacetylase inhibitors (such as bolinostat) were also tested in TETs[10]. Unfortunately, results obtained with targeted therapy, with the exception of somatostatin analogues, were overall modest and can presently be considered inconclusive and not applicable to clinical practice.

Everolimus (Afinitor®, Novartis Pharma, Basel, Switzerland) is an oral inhibitor of the mTOR pathway with multiple applications in transplantation medicine, cardiology and oncology[11]. In two phase Ⅲ randomized, placebo-controlled trials, everolimus, administered at 10 mg daily, successfully prolonged progression free survival in patients with advanced kidney cancer and neuroendrocrine pancreatic cancer[11]. Of all retrospectively reviewed patients treated for advanced TETs at our Institution in the last five years, records of two cases who received everolimus were identified. Everolimus was obtained for compassionate use with approval of the local ethics committee on the grounds of its broad range of antitumor activity and in view of lack of medical options of established efficacy. Both patients gave their informed consent to treatment. Although they had been heavily pretreated, they obtained a remarkable, long-lasting clinical improvement with everolimus, with improved quality of life, a durable radiographic response and manageable side effects, as detailed below.

CASE REPORT

Case 1

In October 2005, the patient, a 51-year old woman from Genua, with no relevant medical history, was incidentally diagnosed with a mediastinal mass on chest X-ray. A chest computed tomography scan with contrast showed the presence of a 6 cm mass in the upper-anterior mediastinum, with multiple bilateral pleural metastases. An ultrasound-guided trans-thoracic core biopsy was performed at the "Gallino" Hospital of Genua. Histologic analysis was diagnostic of B2 thymic epithelial tumor (World Health Organization 2004). Additional histologic analyses, performed at the Department of Pathology of Regina Elena (Rome), revealed strong EGFR expression on immunohistochemistry. An ^{111}In-DTPA-pentetreotide scintigraphy (Octreoscan) showed intense radioactivity uptake in the mediastinal and pleural lesions.

Patient was completely asymptomatic at the time of the diagnosis, with an Eastern Cooperative Oncology Group performance status of 0. In view of the advanced stage of the disease (stage Ⅳa according to the Masaoka staging system), patient was judged to be inoperable and suitable for medical therapy. Since November 2005 to August 2010, patient underwent several lines of treatment, which included cisplatin-doxorubicin-cyclophosphamide, carboplatin-etoposide, capecitabine-gemcitabine and cisplatin-doxorubicin-cyclophosphamide-prednisone. Patient also received single agent cetuximab, single agent imatinib, high dose prednisone and single agent octreotide. Patient received all of these anti-neoplastic regimens at the medical Oncology Department of the "Gallino" Hospital (Genua), except for imatinib, which was delivered at the Department of Molecular and Clinical Oncology and Endocrinology of University Federico Ⅱ of Naples. Since January 2005 to the present time, patient has uninterruptedly received long-acting somatostatin analogs (octreotide LAR 30 mg or lanreotide LAR 60 mg delivered intramuscularly every month). No unexpected or life-threatening side effects were reported. Heart, liver, kidney and bone marrow functions were periodically monitored during treatment administration. Due to intolerance to iodine contrast, treatment response was periodically evaluated with PET-TAC with FDG, without contrast, approximately every three-four months while receiving treatment and every 6 mo during follow-up. In August 2010, patient had complained about mild dyspnea at rest for the past month which worsened on exertion. Her Eastern Cooperative Oncology Group performance status was 1. With respect to her last scan performed in April 2010, positron emission tomographic/computed tomographic (PET-CT) scan performed in July 2010 showed increased size and SUV of the mediastinal mass (SUVmax = 6.3 *vs* 4.5) and of the bilateral pleural lesions (SUVmax = 4.5 *vs* 3.5). Given the amount of chemotherapy received, patient was no longer considered suitable for cytotoxic therapy. She was started on everolimus on August 4, 2010 at the dose of 5 mg/d, which was increased to 10 mg/d after a week. Follow-up during treatment was performed with a complete blood count every two weeks and a complete blood chemistry every month, history and physical examination every two months, with phone contact at need, PET-CT scan approximately every three months, as well as ecocardiographic assessment of heart function every 6 mo.

After two weeks' treatment, patient developed grade 2 oral mucositis with thrush, which resolved with appropriate antifungal and coating agents. Patient also developed grade 1 hyperglycemia, which was easily managed with diet recommendations. After two months' treatment, patient referred a marked improvement of dyspnea. Her performance status had improved to 0. On October 25th, 2010 patient underwent a PET-CT scan, which showed disappearance of pleural effusion and decreased SUV of all pathologic lesions (Figure 1). Patient underwent subsequent scans in January 2011, May 2011 and August 2011 which indicated lack of progression and progressive decrease in the metabolic activity of the lesions. During treatment with everolimus, patient has

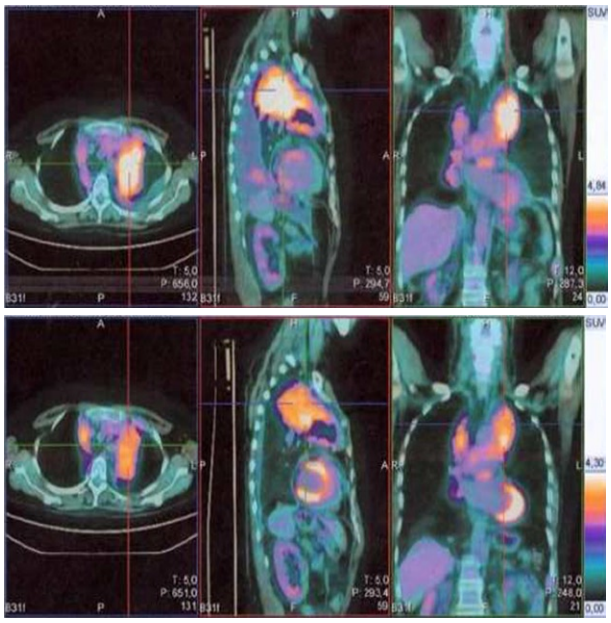

Figure 1 The marked decrease in fluoro-2-deoxy-D-glucose uptake in positron emission tomography/computed tomography performed in October, 2010 (below) in comparison to positron emission tomography/computed tomography performed in August, 2010 (above) in patient 1. Color scales are approximately the same.

reported several episodes grade 1/2 diarrhea, stomatitis, anemia, rash, hypertension. All of these side effects were manageable and were responsible for a total treatment interruption of 7 wk. Dose was never reduced throughout treatment. As of November 30th, 2011 patient has been continuing to take 10 mg everolimus daily, with complete resolution of dyspnea and a performance status of 0.

Case 2

In January 2007, the patient, a 42-year old woman from Naples, complaining of severe tiredness and showing palpebral ptosis, was referred a neurologist for suspected myasthenia gravis. Diagnosis was confirmed by electromiography and serum anti-acetilcholinesterase antibodies levels and patient was started on pyridostigmine. A whole body CT scan with and without contrast showed a mediastinal mass (4.2 cm × 6.5 cm), which extended from the right paratracheal space to the aorto-pulmonary window and was attached to the esophagus and the left atrium. A pericardial effusion was detected along with several pleural lesions in the right costovertebral space and on diaphragmatic pleura. Multiple biopsies of the mediastinal lesion were obtained via thoracotomy, performed at the "Antonio Cardarelli" Hospital in February 2007. Histology analysis performed at the same Institution was diagnostic of B3 thymoma. Immunohistochemistry was positive for p53, ki67, CD1a and CD5 and negative for TTF1 and CK7. Additional immunohistochemistry analysis performed at the Department of Pathology of Regina Elena (Rome) was negative for c-kit and positive for EGFR. Given the presence of multiple pleural metastases, patient was not considered suitable for surgery or radiotherapy. Since February 2007 to July 2007, patient underwent eight cycles of cisplatin-doxorubicin-cyclophosphamide, achieving disappearance of all pleural lesions and shrinkage of the mediastinal mass on CT scan. Given the partial response obtained, surgery was deemed to be feasible, but it was refused by the patient, so she received single agent octreotide LAR as maintenance treatment (Octreoscan was positive). In September 2008, a CT scan with contrast showed progressive disease, with enlargement of the mediastinal mass (55 mm × 12 mm), and recurrence of several pleural lesions. Since October 2008, she underwent 17 cycles of capecitabine-gemcitabine, which was interrupted in November 2009 for unacceptable toxicity. Since January 2010 to July 2010, patient was enrolled in the TETIMAX study and was treated with imatinib. Imatinib was interrupted for progressive disease on CT scan, which showed a mediastinal mass measuring 18 mm × 59 mm, with multiple pleural metastases (largest diameter, 27 mm × 38 mm). Patient subsequently received everolimus, according to the same dosing and schedule adopted for case 1. After one month's treatment pyridostigmine dose was slowly tapered and was suspended after three months. A CT scan was scheduled for October 2011, but a subsequent CT scan was performed in February 2011, due to patient's poor compliance. Stable disease was achieved, with the mediastinal mass measuring 18 mm × 51 mm, shrinkage of the largest pleural metastasis, which measured 16 mm × 9 mm, and shrinkage or stability of the others. Subsequent CT scans performed in June and November 2011 confirmed stable disease. As of November 30th, 2011 patient is free of progression and continuing treatment with everolimus.

DISCUSSION

Platinum-based chemotherapy is the standard approach for TETs not amenable to loco-regional treatments. Cisplatin-doxorubicin-cyclophosphamide[12], cisplatin-etoposide[13] and carboplatin-paclitaxel[14] are among the combination regimens tested as first-line treatment for thymomas, with a response rate of 42.9%-56% and a PFS of 16.7-26.4 mo. Poor evidence about second-line treatment is available in literature. We showed that combination of capecitabine and gemcitabine was able to provide a PFS of 11 mo (95% confidence interval 4-17), with an overall response rate of 40% and excellent tolerance in cisplatin-pretreated patients[6]. Single-agent pemetrexed also provided a promising PFS of 45.4 wk in patients with previously treated thymoma[15].

Somatostatin analogues, employed either alone or in combination with prednisone, were the first targeted agents to prove to be active in TETs[16], with a PFS of 9.2 mo (95% CI, 8.1 mo to 13.9 mo), obtained with the combination regimen in a phase Ⅱ trial[7]. Although several other targeted agents, including cetuximab, cixutumumab, sorafenib and sunitinib, yielded promising results in TETs

in either single cases or small series, no conclusive evidence is presently available for any of these drugs[10]. Of note, one of the largest phase Ⅱ trial on targeted agents was conducted on hystone deacetilase inhibitor belinostat in 25 thymoma patients, with a response rate of 8% only, but a promising TTP of 11.4 mo[10].

The mTOR pathway plays a critical role for neoplastic growth *via* phosphorylation of the p70 S6 kinase, a key enzyme in protein synthesis, and it is also implicated in regulation of actin cytoskeleton, angiogenesis and cellular response to hypoxia and energy depletion[11]. The mTOR complex mediates downstream signaling of many soluble factors, including cytokines and growth factors, such as the epidermal growth factor and the insulin-like growth factor-1 (IGF-1)[11]. Both of these soluble factors may have a role in TETs biology. In fact, a retrospective analysis of 111 histology samples showed that 22 cases (20%) were positive for IGF-1R on immunohistochemistry[17]. Similarly, EGFR was detected in 23 of 31 of TETs on immunohistochemistry[18]. Conversely, anti-IGF-1R antibody cixutumumab and anti-EGFR antibody cetuximab demonstrated activity in TETs[9,19]. Although unsupported by *in vitro* experiences, these findings suggest that stimuli transduced by TK receptors, and consequently by mTOR, are important for thymoma growth, thus providing the biological rationale to support the use of everolimus in TETs. Of note, investigational mTOR inhibitor ridaforolimus recently provided a prolonged disease stabilization (> 16 wk) in a patient with thymic carcinoma in a phase Ⅰ trial[20].

The two cases reported here present several interesting points worthy of discussion. First, the PFS longer than one year and the incontrovertible and marked improvement of clinical conditions and quality of life obtained in both of these two patients strongly suggest that everolimus merits additional clinical investigations in TETs patients. Furthermore, as cixutumumab may increase signaling *via* the mTOR pathway, experimentation of the combination of everolimus plus cixutumumab appears to be of interest[19]. Second, patient 2 also showed remission of myasthenia gravis, and pyridostigmine could be suspended. Such effect may be due to the immunosuppressant properties of everolimus coupled with its anti-tumor efficacy. Third, patient 1 was evaluated with PET/CT without iodine contrast during treatment with everolimus and decrease in FDG uptake as shown in Figure 1 was concordant with clinical benefit. We previously reported that PET results were concordant results of CT scans with iodine contrast in 6 of 9 patients for whom both examinations were performed in the TETIMAX trial[8]. PET/CT could be useful for evaluation of response in TETs, but additional data are required.

In conclusion, these two are the first reported cases of everolimus in patients with TETs. We believe that the prolonged clinical benefit shown in these patients should encourage experimentation of everolimus in this disease, either alone or in combination with other promising agents, such as cixutumumab.

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January 25-26, 2012
Multi-Disciplinary Appoaches to Cancer Therapy
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3rd National Conference: Renal and Bladder Cancer 2012
London, United Kingdom

January 30-31, 2012
2nd Annual Clinical Trials in Oncology
Rome, Italy

February 2-3, 2012
Stem Cells 2012 Conference and Exhibition
San Diego, CA, United States

February 6-8, 2012
Mahidol International Conference on Infections and Cancers 2012
Bangkok, Thailand

February 12-17, 2012
Keystone Symposia: Cancer and Metabolism
Alberta, Canada

February 22-25, 2012
Excellence in Oncology
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March 8-10, 2012
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March 9-10, 2012
13th European Congress: Perspectives in Lung Cancer
Amsterdam, Netherlands

March 14-16, 2012
BTOC-11 Biological Therapy of Cancer
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April 13-15, 2012
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April 20-22, 2012
The 9th Meeting of Asian Society for Neuro-Oncology
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April 26-28, 2012
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April 28, 2012
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Eurasian forum on the management of patients with tumors of the gastrointestinal tract
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July 25-27, 2012
5th Latin American Conference on Lung Cancer
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September 6-8, 2012
The 8th International Jordanian Oncology Society Conference
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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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