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Identification and functional characterization of isocitrate dehydrogenase 1 (IDH1) mutations in thyroid cancer

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ABSTRACT

Mutations in the genes for isocitrate dehydrogenase 1 (IDH1) and isocitrate dehydrogenase 2 (IDH2) have been recently identified in glioblastoma. In the present study, we investigated *IDH1* and *IDH2* mutations in follicular thyroid cancer (FTC) and anaplastic thyroid cancer (ATC), with the latter, like glioblastoma, having a rapidly aggressive and lethal clinical course. By direct genomic DNA sequencing, we analyzed exon 4 of the *IDH1* and *IDH2* genes that harbored the mutation hot spots codon 132 and 172 of the two genes in glioblastoma, respectively, in 12 thyroid cancer cell lines, 20 FTC, and 18 ATC tumor samples. A novel homozygous G367A *IDH1* mutation, resulting in a G123R amino acid change in codon 123, was identified in a case of ATC. A previously described *IDH1* V71I mutation was found in a case of FTC and a case of ATC and no mutations were found in the cell lines. The overall prevalence of mutations was thus 1/20 (5%) in FTC and 2/18 (11%) in ATC. We did not find mutation in the *IDH2* gene in these thyroid cancer cell lines and tumor samples. Sequence alignment analysis of 16 species revealed that the novel *IDH1* G123R mutation was located in a highly conserved region, raising the possibility of a serious functional consequence as could also be predicted by the occurrence of a positively charged amino acid from this mutation. To test this, we created a G123R mutant by site-directed mutagenesis and demonstrated a decreased enzymatic activity of IDH1, similar to the expected reduction in the enzymatic activity of the previously described R132H IDH1 mutant measured as a control. Thus, functionally relevant *IDH1* mutations can also occur in thyroid cancer, particularly ATC, suggesting a potential tumorigenic role of the IDH1 system that could represent a new therapeutic target for thyroid cancer.

Introduction

Mutations in the genes for the enzymes isocitrate dehydrogenase 1 (IDH1) and isocitrate dehydrogenase 2 (IDH2) have been recently identified in brain glioblastomas, particularly secondary glioblastomas, with a high frequency (>70%) particularly in the *IDH1* gene [1–4]. IDH is a key player in the tricarboxylic acid cycle, or Krebs cycle, and catalyzes the oxidative decarboxylation of isocitrate to produce alpha-ketoglutarate (α-KG). The activity of IDH is dependent on nicotinamide adenine dinucleotide phosphate ($NADP^+$) and the biochemical reaction catalyzed by IDH leads to the production of NADPH, which plays an important role in the cellular control of oxidative damage [5]. *IDH1* mutations were found to be exclusively in codon 132 [1]. Several different *IDH1* mutations have been identified, all affecting the amino acid arginine at codon 132 and resulting in various amino acid substitutions (R132H, R132S, R132C, and R132G). *IDH1* mutations were relatively uncommon in other cancers, with 8% in acute myeloid leukemia [6], 2.7% in prostate cancer, and 1.7% in B-acute lymphoblastic leukemia [7]. All the *IDH2* mutations were found in codon R172 and tumors without mutations in *IDH1* often had mutations affecting the analogous amino acid (R172) of the *IDH2* gene [3]. The R132 residue is highly conserved over evolution and is localized in the substrate binding site of IDH1 where hydrophilic interactions between R132 and the α- and β-carboxylates of isocitrates occur [8]. Codon 132 IDH1 mutants have been shown to be associated with reduced catalytic activity of the IDH1 enzyme [3,9,10].

Recently, the IDH1 gene was examined for mutations in medullary thyroid cancer (MTC) and papillary thyroid cancer (PTC), but no mutations were found in these types of thyroid cancer [4]. Thyroid cancer is the most common endocrine malignancy, which, in addition to MTC and PTC, also includes follicular thyroid cancer (FTC) and anaplastic thyroid cancer (ATC). These two latter thyroid cancers, particularly ATC, are more aggressive. In fact, ATC, like glioblastoma, is an extremely aggressive and rapidly lethal human cancer [11]. Given the common *IDH1* and *IDH2* mutations in glioblastoma and its somehow similar aggressiveness with ATC, it remains an interesting question whether *IDH1* and *IDH2* mutations also occur in ATC. In the present study, we analyzed the *IDH1* and

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IDH2 mutation in both ATC and FTC since they have not been examined for mutations in the two genes.

Materials and methods

Cell lines and tumor samples. A total of 50 samples, including 12 thyroid cancer cell lines (K1, K5, OCUT-1, OCUT-2, FB-1, SW1736, BCPAP, HTh7, HTh74, KAT 18, FTC133, and C643), 20 FTC tumors, and 18 ATC tumors were used for mutational analysis of the *IDH1* and *IDH2* genes as shown in Table 1. Cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, streptomycin (100 μg/mL), and penicillin (100 U/mL). Tumor samples were from an IRB-approved study [12]. Genomic DNA from cell lines and tumors was isolated by standard phenol–chloroform extraction and ethanol precipitation procedures, using MaXtract high density gel tubes (Qiagen, Valencia, CA).

PCR amplification and sequencing. PCR amplification of exon 4 of *IDH1*, which was the hot spot of *IDH1* mutation in other cancers, was performed using the primers and PCR conditions as described previously [4]. In addition, we used a reverse primer (5′-CATGCAAAATCACATTATTGCC-3′) to sequence the amplicon for reverse orientation. The amplification of exon 4 of *IDH2* was performed using the forward primer IDH2-4F 5′-TGCACTCTAGACTCTACTGCC-3′ and reverse primer IDH2-4R 5′-ACAAAGTCTGTGGCCTTGTAC-3′ with the annealing temperature at 60 °C. The PCR amplified products were directly sequenced using a Big Dye terminator v3.1 cycle sequencing ready reaction kit (Applied Biosystems) and ABI PRISM 3730 automated next generation genetic analyzer (Applied Biosystems). The GenBank accession numbers of *IDH1* and *IDH2* are NM_005896.2 and NM_002168.2, respectively.

Multiple amino-acid sequence alignment. Amino-acid sequences of *IDH1* of various species were obtained from NCBI database (http://www.ncbi.nlm.nih.gov/protein/) as follows: H_sapiens (NP_005887.2), P_troglodytes (XP_001142197), C_lupus (XP_536047.2), B_taurus (NP_851355.2), M_musculus (NP_034627.2), R_norvegicus (NP_113698.1), G_gallus (XP_421965.2), D_rerio (NP_958907.1), D_melanogaster (NP_652044.1), A_gambiae (XP_001688948), O_sativa (NP_001043749), A_thaliana (NP_176768.1), K_lactis (XP_451683.1), E_gossypii (NP_984921.1), N_crassa (XP_323176.1), and S_pombe (NP_594105.1). These sequences were compared using a computer based multiple sequence alignment program (http://pir.georgetown.edu/cgi-bin/multialn.pln).

Expression vector construction. The wild-type clone of IDH1 (Catalog No. SC322129) was obtained from OriGene Technologies, Inc. (Rockville, MD). The DNA fragment containing the entire open reading frame of *IDH1* was PCR amplified using a forward primer containing BamH1 and Kozak consensus sequences (IDH1 WT-F1 5′-CGCGGATCCGCCACCATGTCCAAAAAAATCAGT-3′) and a reverse primer containing Not1 sequence (IDH1 WT-R1 5′-CGTGCGGCCG CCAAGTTTGGCCTGAGCTAG-3′). The amplified fragment was digested with BamH1 and Not1 and cloned into the pcDNA4TOmyc-His(B)™ (Invitrogen, CA). The IDH1 ORF was re-amplified from the pcDNA4TOmyc-His(B)™ using the forward primer (IDH1 WT-F1) and reverse primer containing EcoR1 (IDH1 R2-5′-GCTGA ATTCTCAATGGTGATGGTGATG-3′). The resultant fragment was digested with BamH1 and EcoR1 and subsequently cloned into a mammalian expression vector pcDNA3.1 (+)™ (Invitrogen, CA) between the BamH1 and EcoR1 sites, which produced a myc tag fused to the COOH terminus of the IDH1gene. The integrity of the plasmid was verified by restriction enzyme digestion and sequencing.

Table 1
Mutational analysis of *IDH1* and *IDH2* genes in thyroid cancers.

Samples	*IDH1*		*IDH2*	
	Analyzed samples	Mutations	Analyzed samples	Mutations
Cell lines				
FTC	2	0	2	0
ATC	8	0	8	0
PTC	2	0	2	0
Tumors				
FTC	20	1 (5%)	20	0
ATC	18	2 (11%)	18	0

Site-directed mutagenesis. The expression vector pcDNA3.1 (+)™ carrying IDH1_myc was used to generate the IDH1 mutants, G123R and R132H, with a Quick Change XL Site-Directed mutagenesis kit according to the instruction of the manual. The primers were designed using template specific mutagenic primer design program. The primer sequences are as follows: G123R: sense, IDH1-G367A_F 5′-CCCCCGGCTTGTGAGTAGATGGGTAAAACCTAT-3′; antisense, IDH1-G367A_R 5′-ATAGGTTTTACCCATCTACTCACAAGCCGGGGG-3′; R132H: sense, IDH1-G395A-F 5′-GTAAAACCTATCATCATAGGTCATCATGCT TATGGGGATCAATAC-3′; antisense, IDH1-G395A-R 5′-GTATTGAT CCCCATAAGCATGATGACCTATGATGATAGGTTTTAC-3′. All the mutations were confirmed by sequencing with primer IDH1-VEC_F 5′-ATGCAAGGAGATGAAATGACACG-3′. Plasmid DNAs for the transfection experiments were purified using an Invitrogen mini prep kit (K2100-11).

Cell transfection. HEK293T cells were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Gibco). Cells were transiently transfected with an empty vector, wild-type or each of mutant IDH1 expression vectors using the Lipofectamine 2000 transfection reagent per manufacturer's instructions (Invitrogen Life Technologies, CA). Cells were harvested 48 h after transfection and subjected to lysis for Western blotting and IDH1 enzymatic assay.

Western blotting. Western blotting was performed as described [13]. Briefly, about 20 μg of cell lysate proteins prepared for IDH1 enzymatic assay was separated on 10% SDS/PAGE and transferred to PVDF membrane (Millipore Co., Bedford, MA). After transfer, the membrane was blocked with 5% skim-milk/PBS containing 0.1% Tween 20 (PBST) for 1 h at room temperature, and incubated overnight at 4 °C with anti-Myc antibody (SC-40). After washing four times with PBST the blots were incubated with anti-mouse HRP conjugated antibody (SC-2005) for 1 h at room temperature. After washing with PBST, protein bands on the membrane were detected with enhancement chemiluminescence (ECL) reaction and exposure to X-ray film. To ensure the equality of protein loading, the same membrane blot was stripped off and re-probed with an anti-β-actin antibody (SC-1616-R).

IDH1 enzymatic assay. The IDH1 enzymatic assay was performed as described previously [3,14,15]. Briefly, cells were collected 48 h after transfection and centrifuged at 1000*g* for 10 min at 4 °C. After washing with cold phosphate-buffered saline, cells were lysed in mammalian cell lysis buffer containing 0.1% Triton X-100. Cells were then disrupted by ultra sonication and incubated on ice for 30 min. Cell lysates were then centrifuged at 12,000*g* for 10 min at 4 °C. The supernatants were collected and protein concentration was measured using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). For each enzymatic reaction, an equal volume of cell lysate containing the same amount of protein was added to the assay solution containing 33 mM Tris buffer (pH 8.0), 0.33 mM EDTA, 0.1 mM $NADP^+$ (Sigma Catalog No. N-0505), 1.33 mM manganese chloride and 1.3 mM isocitrate (Sigma Catalog No. I-1252). Isocitrate dehydrogenase activity was measured spectrophotometrically at 27 °C by monitoring the formation of $NADP^+$ to NADPH at 340 nm.

Results

Relatively common IDH1 mutations in thyroid cancer

We analyzed exon 4 of the *IDH1* and *IDH2* genes for mutations in FTC and ATC. This exon was chosen to be analyzed because it