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Tumorigenesis and Neoplastic Progression

Mutant IDH1 Confers an *in Vivo* Growth in a Melanoma Cell Line with *BRAF* Mutation

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Melanoma is the most deadly tumor of the skin, and systemic therapies for the advanced stage are still limited. Recent genetic analyses have revealed the molecular diversity of melanoma and potential therapeutic targets. By screening a cohort of 142 primary nonepithelial tumors, we discovered that about 10% of melanoma cases (4/39) harbored an *IDH1* or *IDH2* mutation. These mutations were found to coexist with *BRAF* or *KIT* mutation, and all *IDH1* mutations were detected in metastatic lesions. *BRAF*-mutated melanoma cells, additionally expressing the cancer-related IDH1 mutant, acquired increased colony-forming and *in vivo* growth activities and showed enhanced activation of the MAPK and STAT3 pathways. Genome-wide gene expression profiling demonstrated that mutant IDH1 affected the expression of a set of genes. Especially, it caused the induction of growth-related transcriptional regulators (Jun, N-myc, Atf3) and the reduction of Rassf1 and two dehydrogenase genes (*Dhrs1* and *Adh5*), which may be involved in the carcinogenesis of *IDH1*-mutated tumors. Our analyses demonstrate that *IDH1* mutation works with other oncogenic mutations and could contribute to the metastasis in melanoma. ***(Am J Pathol 2011, 178:1395–1402; DOI: 10.1016/j.ajpath.2010.12.011)***

Melanoma is the most malignant tumor of the skin, and the median survival rate of patients with metastatic tumors is less than 1 year.[1] Although the incidence of melanoma has been increasing around the world, systemic therapies for the advanced stage are still limited.[2] Recent studies have provided a clearer picture of the molecular events leading to melanoma development and progression.[3,4] Since the identification of prevalent activating mutations of BRAF kinase,[5] further molecular studies have clarified the role of this pathway and others in melanomagenesis.[6] Recent genetic investigations have also demonstrated specific genotype–phenotype correlations that would be potentially informative in the context of the molecular subclassification of melanoma and therapeutic target molecules.[7] For example, the *c-kit* gene mutations have been frequently reported in acral lentiginous/mucosal melanomas and are associated with better responsiveness to the inhibitor, imatinib.[8–10]

Recently, unbiased whole-exon resequencing analysis of glioblastoma multiforme has revealed recurrent mutation of the two IDH (isocitrate dehydrogenase) isoforms, *IDH1* and *IDH2*.[11] Subsequent analysis showed that these mutations are frequent in glioma and associated with better prognosis[12,13]; furthermore, they have also been detected in a subset (about 8% to 16%) of acute myeloid leukemia (AML).[14–17] These enzymes convert isocitrate to α-ketoglutarate (α-KG) with concurrent reduction of NADPH, but IDH1 is localized in the cytosol[18] whereas IDH2 is localized in mitochondria.[19] Mutations of the two genes affect the residues responsible for hydrophilic interactions with the substrate, and have been shown to impair the enzymatic activity, and therefore they are considered to be loss-of-function alleles.[12] However, because the mutations are clustered in specific residues and only detected as heterozygous alleles, it could also be hypothesized that they are gain-of-function mutations. Recent milestone studies have revealed that mutant IDH1 or IDH2 acquires a new gain-of-function activity that results in reduction of α-ketoglutarate to 2-hydroxyglutarate (2HG) in glioma and leukemia, suggesting that *IDH1/2* mutations could be gain-of-function alterations.[20–22] Although accu-

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mulation of 2HG is associated with a risk of brain tumors including glioma,[23,24] the significance of such metabolic change in carcinogenesis remains largely unknown.

Materials and Methods

Clinical Samples and DNA Extraction

Surgical or autopsied specimens (92 cases of sarcoma, 39 cases of melanoma, and 11 cases of mesothelioma) were obtained from patients who were diagnosed and underwent surgery at the National Cancer Center Hospital, Tokyo, Japan. Tumor cells and corresponding lymphocytes or normal skin tissue were dissected out under a microscope from methanol-fixed paraffin-embedded tissues, and the DNA was extracted. High molecular weight DNA was extracted from 13 melanoma cell lines as described previously.[25] The study protocol for analysis of clinical samples was approved by the institutional review board of the National Cancer Center.

PCR and Sequence Analysis

We amplified exon 4 of the *IDH1* gene, exon 4 of the *IDH2* gene, exon 15 of the *BRAF* gene, exons 2 and 3 (covering codons 12, 13, and 61) of the *NRAS* gene, exon 3 of the *CTNNB1* gene, and exons 11, 13, and 17 of the *KIT* gene by PCR using High Fidelity Taq polymerase (Roche Diagnostic, Basel, Switzerland) as described.[26] The primers used in this study are IDH1-EX4F: 5′-AGAGAATCGTGATGCCACCAACG-3′, IDH1-EX4R: 5′-GCATAATGTTGGCGTCAAATGTGC-3′, IDH2-EX4F: 5′-ACATGCAAAATCACATTATTGCC-3′, IDH2-EX4R: 5′-CAAGTTGGAAATTTCTGGGCCATG-3′, BRAF-EX15F: 5′-AAACTCTTCATAATGCTTGCTCTG-3′, BRAF-EX15R: 5′-TAGCCTCAATTCTTACCATCCAC-3′, NRAS-EX2F: 5′-GATGTGGCTCGCCAATTAACCCTG-3′, NRAS-EX2R: 5′-GACAAGTGAGAGACAGGATCAGG-3′, NRAS-EX3F: 5′-TTACCCTCCACACCCCCAGGATTC-3′, NRAS-EX3R: 5′-AATGCTCCTAGTACCTGTAGAGG-3′, KIT-EX11F: 5′-CCAGAGTGCTCTAATGACTGAGAC-3′, KIT-EX11R: 5′-AAAGGTGACATGGAAAGCCCCTG-3′, KIT-EX13F: 5′-AGATGCTCAAGCGTAAGTTCCTG-3′, KIT-EX13R: 5′-AATAAAAGGCAGCTTGGACACGGC-3′, KIT-EX17F: 5′-GGTTTTCTTTTCTCCTCCAACCT-3′, KIT-EX17R: 5′-GTGATATCCCTAGACAGGATTTAC-3′, CTNNB1-EX3F: 5′-TATAGCTGATTTGATGGAGTTGG-3′, CTNNB1-EX3R: 5′-GCTACTTGTTCTTGAGTGAAGGAC-3′. All PCR products were purified (QIAquick PCR purification kit; QIAGEN, Hamburg, Germany) and analyzed by sequencing (Big Dye sequencing kit; Applied Biosystems, Carlsbad, CA).

In Vitro *Biological Assays*

FLAG-tagged IDH1 full-length cDNA was amplified from human normal liver cDNA using reverse transcription (RT)-PCR and subcloned into a mammalian expression plasmid (Invitrogen, Carlsbad, CA). The R132H mutant was generated by site-directed mutagenesis (Quickchange; Stratagene, Santa Clara, CA). All plasmids were validated by sequencing. G361 cells were obtained from Japanese Collection of Research Bioresources (Sennan-shi, Japan) and maintained in DMEM supplemented with 10% fetal bovine serum. Linearized plasmid was transfected by lipofectamine (Invitrogen), and stable clones were isolated after Zeocin (Invitrogen) selection. Cell proliferation was measured using the 96-well plate format by MTS assay using Cell Titer 96 AQueous One Solution Reagent (Promega, Madison, WI).[26] Colony formation assay and migration assay were performed as described.[27] To measure ROS accumulation, cells were stained with 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) (Molecular Probes, Eugene, OR), and fluorescence-activated cell sorting (FACS) analysis was performed using FACScalibur (BD Biosciences, San Jose, CA) as instructed by the manufacturer.

In Vivo *Tumorigenesis Analyses*

For assessment of *in vivo* tumorigenicity, 1×10^6 cells were subcutaneously transplanted into the trunks of nude mice. After 12 weeks, the mice were sacrificed, and the number of subcutaneous tumors as well as metastasis in other organs was examined. The mice were kept at the Animal Care and Use Facilities of the National Cancer Center under specific pathogen-free conditions, and all experiments were approved by the institutional Animal Care and Ethics Committee.

Immunoblot Analysis

For protein extraction, we used a slightly modified buffer (10 mmol/L Tris-HCl [pH 7.5], 175 mmol/L NaCl, 5 mmol/L EDTA, 0.5% Triton-X, 0.5% NP-40) with a proteinase inhibitor cocktail (Roche), and the immunoblotting procedure was preformed as described previously.[26] The antibodies used in this study are anti-FLAG peptide (clone M2; Sigma-Aldrich, St. Louis, MO), anti-MAPK, phospho-MAPK (pT202/pY204), AKT, phospho-AKT (pS473), phospho-STAT3 (pY705), p70S6K, phospho-p70S6K (pT389) antibodies (Cell Signaling Technologies, Danvers, MA), and anti-STAT3 antibody (BD Biosciences).

Gene Expression Profiling and Quantitative RT-PCR

From subconfluent G361 clones, total RNA was extracted using an RNAeasy kit (QIAGEN). Ten micrograms of total RNA was reverse-transcribed by MMLV-RT, and a Cy3-labeled cRNA probe was synthesized using T7 RNA polymerase and hybridized with a microarray covering the whole human genome (Whole Human Genome Oligo Microarray, G4112F; Agilent Technologies, Santa Clara, CA). All sample were analyzed in duplicate. After washing, the microarray was scanned by the DNA microarray scanner (Agilent Technologies). Data were normalized and statistical significance was measured by *t*-test with multiple testing correction (Benjamini and Hochberg false discovery rate) using GeneSpring software (Agilent Technologies).[27] Quantitative RT-PCR was performed in

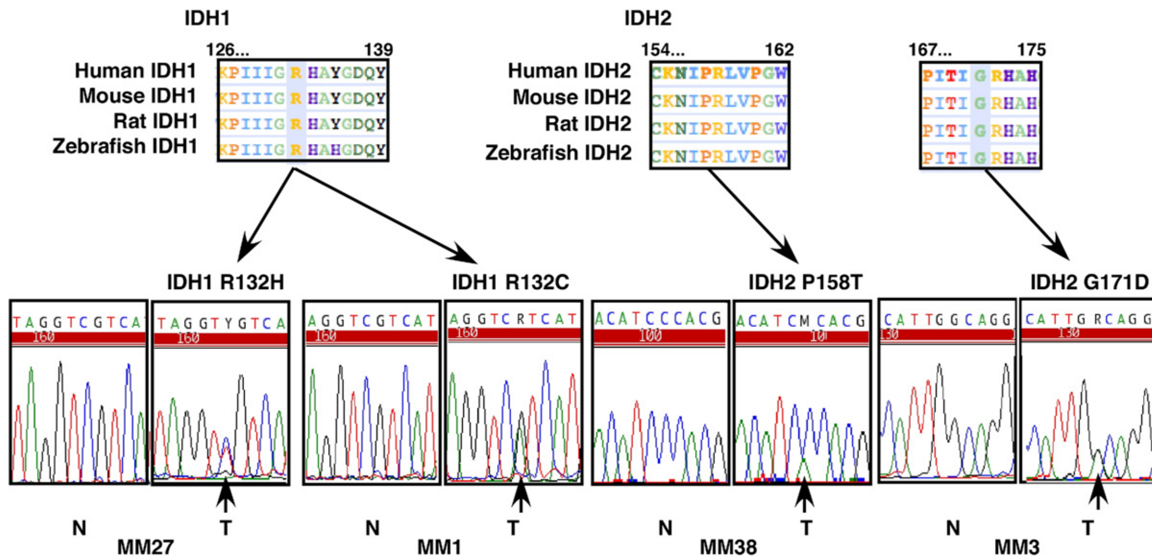

Figure 1. *IDH1* and *IDH2* mutations in melanoma. Amino acid alignments of IDH1 and IDH2 proteins among human, mouse, rat, and chicken homologues are shown in the **top panels**. Sequence chromatographies of the *IDH1* and *IDH2* genes in primary melanomas (T) and corresponding normal (N) tissues are shown in the **bottom panels**. **Arrows** indicate the heterozygous mutation in tumor samples. Note that mutated amino acids residues are well conserved among species.

triplicate and evaluated using universal probes for each amplicon and the LightCycler system (Roche). Primers designed by ProbeFinder (Version 2.45; Roche). The relative expression of each gene was determined by comparison with that of GAPDH.

Results

IDH1/2 *Mutations in Melanoma*

Since previous mutation analyses have reported that *IDH1* mutation is rare in epithelial cancers in comparison to glioma and leukemia,[28,29] we searched for the *IDH1* gene mutation in a cohort of primary tumors of nonepithelial origin (92 sarcomas, 39 melanomas, and 11 malignant mesotheliomas). Our melanoma cohort included 17 metastatic cases. After screening these 142 tumors, we found 2 melanoma cases harboring heterozygous *IDH1* mutation (R132C and R132H, 2/39) (Figure 1 and Table 1). These mutations affected exactly the same residue as that reported for glioma and AML.[11,12,16] No *IDH1* mutation was detected in sarcoma and mesothelioma cases. We then screened *IDH2* mutation in the same cohort and found two heterozygous mutations (G171D and P158T) that affected well-conserved residues among species in two MM cases (Figure 1). Especially G^{171} is located next to the most frequently altered residue (R172), but its mutation in cancer has not been reported previously.[12,14–17,28,29] These *IDH1/2* mutations were not detected in the corresponding normal tissues, and in total, we detected four somatic *IDH1/2* mutations out of 39 melanoma cases (10.3%). Three out of four *IDH1/2* mutations occurred in either mucosal or acral lentiginous subtype, and *IDH1* mutation was detected only in metastatic lesions (Table 1).

Association between IDH1/2 *Mutation and Other Mutations in Melanoma*

We next examined mutations of the melanoma-associated oncogenes (the *BRAF*, *NRAS*, *KIT*, and *CTNNB1* genes) in our study cases. In this cohort, we detected 12 *BRAF* mutations (30.8%), 8 *NRAS* mutations (20.5%), 4 *KIT* mutations (10.2%), and 3 *CTNNB1* mutations (7.7%) (Table 1). As reported previously,[4,8] the existence of *BRAF*, *NRAS*, and *KIT* mutations is mutually exclusive, and one case contains both *BRAF* and *CTNNB1* mutations. Among the *IDH1/2*-mutated cases, two had both *BRAF* and *IDH1* or *IDH2* mutations, and one had *KIT* and *IDH1* mutations. We also screened *IDH1*, *IDH2*, *BRAF*, *NRAS*, and *KIT* mutations in 13 melanoma cell lines. We observed nine *BRAF* mutations (9/13, 69.2%) and one *NRAS* mutation (1/13, 7.7%) in these cell lines, but were unable to detect any *IDH1* or *IDH2* mutation (data not shown).

Mutant IDH1-Expressing Melanoma Cells Confer a Growth Advantage in Vivo

Previous studies have shown that *BRAF* mutation occurs at the early stage of melanoma development.[30,31] Therefore, based on the above genetic analysis, we speculated that *IDH* mutation confers a growth advantage after acquiring *BRAF* or *KIT* mutation. Because the functional significance of *IDH2* mutations detected in this study remains in need of further characterization, we focused on biological roles of a well-characterized *IDH1* mutation (R132H) in melanoma. To examine the biological effect of mutant IDH1 in melanoma cells, we established clones expressing the wild or mutated (R132H) *IDH1* gene from a *BRAF*-mutated (V600E) melanoma cell line (G361) (Figure 2A). We first compared the growth of these clones, but no significant difference was observed among con-

Table 1. Clinicopathological and Mutation Profile of Melanoma Cases

Case	Primary (P) or metastatis (M) site	Primary site	Subtype	*IDH1*	*IDH2*	*BRAF*	*NRAS*	*KIT*	*CTNNB1*
MM-1	M, left thigh	Face	nd	p.R132C, hetero		p.V600E, hetero			
MM-27	M, liver	Anal	nd	p.R132H, hetero				p.K642E, homo	
MM-38	P	Abdomen	nd		p.P158T, hetero	p.V600E, hetero			
MM-3	P	Toe	NM		p.G171D, hetero				
MM-24	M, pancreas	Finger	ALM			p.D594N, hetero			
MM-4	P	Esophagus	nd			p.V600E, hetero			
MM-7	P	Skin	ALM			p.V600E, hetero			
MM-12	P	Esophagus	nd			p.V600E, hetero			
MM-14	M, brain	Forearm	nd			p.V600E, hetero			
MM-16	M, liver	Sole	ALM			p.V600E, hetero			
MM-25	M, nd	Chest wall	NM			p.V600E, hetero			
MM-6	P	Sole	ALM			p.V600E, homo			p.T41I, hetero
MM-23	M, LN	Skin	NM			p.V600E, homo			
MM-32	P	Thigh	NM			p.V600E, homo			
MM-2	P	Esophagus	nd				p.Q61H, hetero		
MM-10	M, LN	Head	NM				p.G12S, hetero		
MM-15	P	Esophagus	nd				p.G13R, hetero		
MM-26	P	Pharyngeal	nd				p.Q61H, homo		
MM-28	M, nd	Shoulder	NM				p.Q61R, homo		
MM-31	P	Heel	ALM				p.G12S, hetero		
MM-33	P	Sole	ALM				p.G12S, hetero		
MM-36	P	Sole	ALM				p.Q61R, homo		
MM-20	M, LN	Finger	ALM					p.K642E, homo	
MM-22	M, LN	Skin	NM					p.N822K, hetero	
MM-30	P	Sole	ALM					p.I817F, hetero	
MM-18	M, nd	Conjunctiva	nd						p.T40I, hetero
MM-19	M, nd	Sole	ALM						p.P44L, hetero
MM-37	P	Sole	ALM						
MM-39	P	Leg	SSM						
MM-5	P	Face	ALM						
MM-8	P	Toe	ALM						
MM-9	M, skin	ND	nd						
MM-11	P	Rectum	nd						
MM-13	M, brain	Thigh	SSM						
MM-17	M, nd	Conjunctiva	nd						
MM-21	P	Abdomen	nd						
MM-29	M, nd	Forearm	nd						
MM-34	P	Conjunctiva	nd						
MM-35	P	Sole	ALM						

Clinicopathological (primary or metastasis, primary and metastatic organ site and histological subtype) and mutation (amino acid change and zygosity) data of the analyzed cases are shown.

nd, not determined; hetero, heterozygous mutation; homo, homozygous mutation; NM, nodular melanoma; ALM, acral lentigous melanoma; LN, lymph node; SSM, superficial spreading melanoma.

trol, wild-type, and mutant IDH1-expressing clones *in vitro* (Figure 2B). We then examined colony-forming activity of these clones and found that mutant IDH1-expressing clones formed significantly more colonies (Figure 2C).

We also established wild or mutant IDH1-expressing clones from a *BRAF* wild-type melanoma cell line (GAK) (see Supplementary Figure S1A, *http://ajp.amjpathol.org*). In these clones, no significant difference in *in vitro* growth

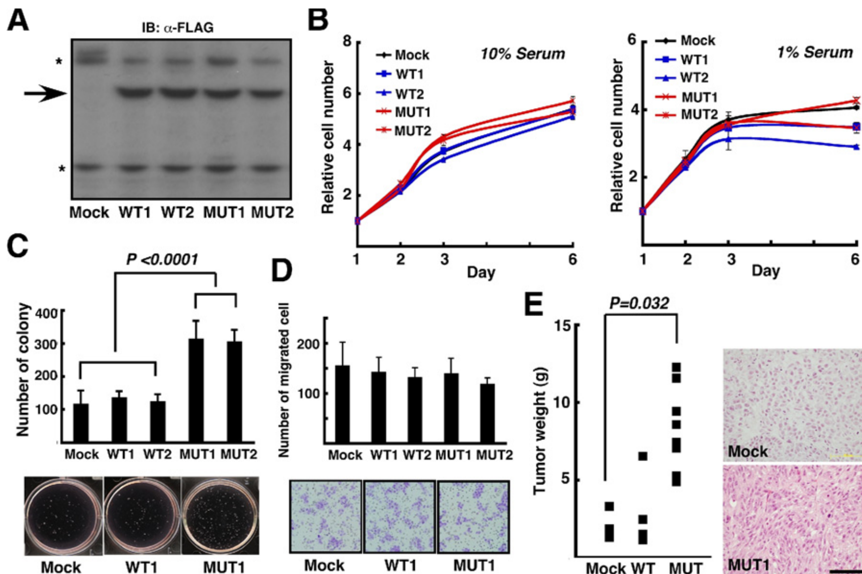

Figure 2. Growth advantage by mutant IDH1 in *BRAF*-mutated melanoma cells. **A:** Immunoblot analysis of mock, wild-type IDH1 (WT1 and 2), and mutant IDH1 (MUT1 and 2). The FLAG-tagged wild or mutant (R132H) *IDH1* genes were introduced in *BRAF*-mutated G361 cells. The cell lysates were electrophoresed and immunoblotted with anti-flag antibody. **Asterisk** indicates nonspecific signal. **B:** *In vitro* cell proliferation of mock, wild-type (WT1 and 2), and mutant (MUT1 and 2) IDH1-expressing clones under two culture conditions (DMEM supplemented with 10% or with 1% serum). **C:** Colony-forming activity of mock, wild-type IDH1 (WT1 and 2), and mutant IDH1 (MUT1 and 2). Representative plate of each clone is shown at the **bottom**. **D:** Migration activity of mock, wild-type IDH1 (WT1 and 2) and mutant IDH1 (MUT1 and 2). Representative picture of migrated cells in each clone is shown at the **bottom**. **E:** The weight of *in vivo* tumors produced by mock, wild-type (WT1), and mutant (MUT1) IDH1-expressing clones (Mock: $n = 5$, WT1: $n = 5$, MUT: $n = 8$) (**left**). Histological appearance (hematoxylin-eosin staining) of tumors formed by mock and mutant IDH1-expressing clones (**right**). Scale bar = 100 μm.

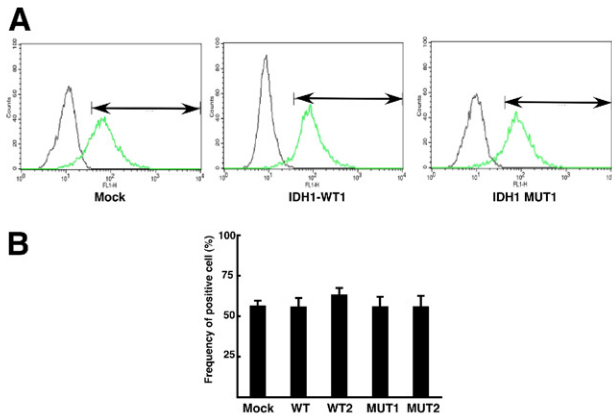

Figure 3. No significant accumulation of reactive oxygen species by mutant IDH1 in melanoma cells. **A:** Measurement of reactive oxygen species (ROS) in mock and IDH1-expressing clones by fluorescence-activated cell sorting analysis. Representative data of ROS accumulation (right peak) and control signal (left peak) are indicated. Positive cell fraction is shown by **arrows**. **B:** Frequency of fluorescence positive cells in mock, wild-type IDH1-, and mutant IDH1-expressing clones ($n = 3$).

and colony-forming activities was detected (Supplementary Figure S1, B and C, at *http://ajp.amjpathol.org*).

Because *IDH1* mutation was detected only in metastatic lesions, we next examined migration activity in these clones, but no difference was observed (Figure 2D). We finally tested the *in vivo* tumorigenicity of these clones. Mutant IDH1-expressing clones induced more frequently (8/8) than mock (5/8) and wild (5/8) IDH1-expressing clones. Moreover, mutant IDH1-expressing clones produced larger tumors than control clones (Figure 2E). Therefore, this *IDH1* mutation appears to confer a growth advantage *in vivo*. Histologically, *IDH1*-mutated clones showed spindle morphology *in vivo* compared to the tumors of mock and wild IDH1-expressing clones (Figure 2E).

No Significant ROS Accumulation in IDH1 Mutant Expressing Melanoma Cells

Recent metabolome analysis has shown that mutant IDH1 proteins, including the R132H mutant, specifically produce 2HG,[20–22] which may cause oxidative stress and induce DNA damage in affected cells. Therefore, we examined the accumulation of reactive oxygen species (ROS) in the mutant IDH1 clones in comparison to clones expressing wild-type IDH1 or the control. We did not detect any significant increase of ROS accumulation in mutant IDH1-expressing clones relative to the parental and wild-type IDH1-expressing clones (Figure 3).

Downstream Signal Pathways Affected by Mutant IDH1 in Melanoma Cells

To examine whether mutant IDH1 affects any known oncogenic pathways, we examined the activation of node proteins in the various molecular pathways (MAPK, AKT, S6K, and STAT3) in the control, wild IDH1-, or mutant IDH1-expressing clones and found that phosphorylation of MAPK and STAT3 were specifically increased in mutant IDH1-expressing clones (Figure 4A).

Finally, to further elucidate the molecular changes induced by mutant IDH1, we conducted a genome-wide gene expression profiling of control, wild-type IDH1-, and mutant IDH1-expressing clones. The only reported downstream target of mutant IDH1 is activation of the hypoxia pathway through prevention of HIF1α protein degradation by prolyl hydroxylase (PHD) activity,[32] but we did not observe any change in HIF1α targets such as Glut-1 and Pgk1 in melanoma cells (Figure 4B). We also examined the growth activity of these clones under hypoxic condition (1% O_2), but no significant difference was observed (Figure 4C). Eight genes increased significantly in mutant IDH1-expressing clones compared to the mock and wild IDH1-expressing clones. Remarkably, they include four transcriptional factors (ATF3, JUN, MYCN, and SOX8) (Table 2, Figure 4B). The expression of 47 genes decreased in mutant IDH1-expressing clones compared with the mock and wild IDH1-expressing clones. We note that mutant IDH1 reduced the expression of RASSF1, a negative regulator of RAS signaling,[33] and two dehydrogenases (DHRS1 and ADH5) in melanoma cells (Figure 4D, top 20 genes are shown in Table 2).

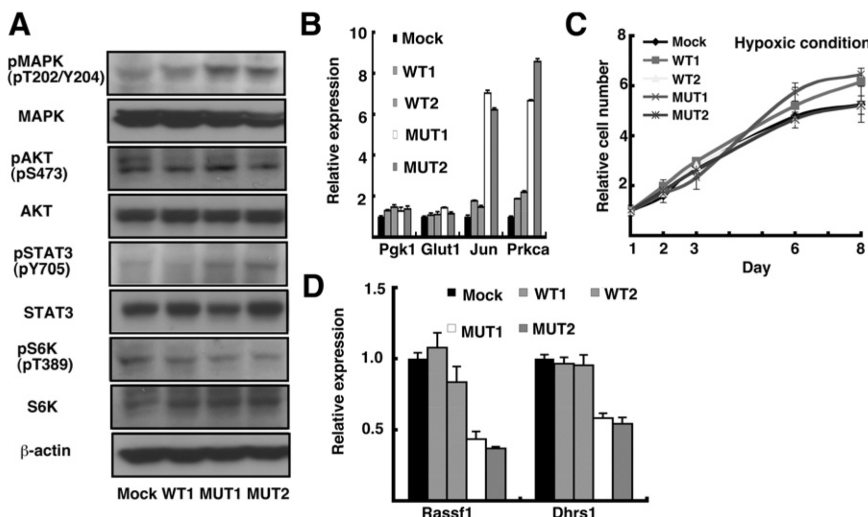

Figure 4. Downstream signal pathways of mutant IDH1 in melanoma cells. **A:** Detection of phosphorylated and non-phosphorylated forms of MAPK, AKT, STAT3, and S6K in mock, wild-type IDH1-, and mutant IDH1-expressing clones. β-actin expression was a loading control. **B** and **D:** Quantitative RT-PCR analysis of the candidate genes regulated by mutant IDH1. **C:** *In vitro* cell proliferation of mock, wild-type (WT1 and 2), and mutant (MUT1 and 2) IDH1-expressing clones under hypoxic condition (1% O_2).

Table 2. Genes Aberrantly Regulated in Mutant IDH1 Expressing Melanoma Cells

Ratio (Mut/Mock)	Gene name	Annotation
5.56	*PRKCA*	Kinase
4.47	*ATF3*	TF
4.12	*EIF5*	Translation
4.12	*JUN*	TF
4.02	*LYPD3*	Membranous
3.7	*MYCN*	TF
3.31	*ANKDD1A*	
3.26	*SOX8*	TF
0.0839	*BCL11A*	ZF
0.159	*KIF5C*	Transport
0.169	*RASSF1*	Ras signal
0.178	*HEY1*	TF
0.184	*SDC2*	Membranous
0.219	*RANBP5*	Nuclear import
0.224	*NR2F2*	TF
0.231	*TTC26*	
0.235	*PPP4R1*	Phosphatase
0.239	*ZDHHC23*	
0.25	*JAG2*	Membranous
0.261	*AHNAK2*	
0.273	*C9orf40*	
0.273	*CA13*	
0.274	*LEF1*	TF
0.276	*DHRS1*	Oxidative stress
0.279	*DOCK9*	Adaptor
0.288	*MCTP2*	Membranous
0.289	*ZNF222*	ZF
0.297	*ADH5*	Oxidative stress

Ratio of average expression (mutant IDH1 clones [Mut]/mock [Mock] clones), gene name, and annotation are shown. False discovery rate is <0.15.

TF, transcription factor; ZF, zinc finger protein.

Discussion

IDH1/2 belong to a novel family of cancer-related genes that were discovered by unbiased genome-wide exon sequence analysis.[11] Since the *IDH1/2* genes encode ubiquitous metabolic enzymes that convert isocitrate to α-KG, it remains unclear why genetic alterations of these genes occur specifically and frequently in glioma and leukemia, and are rarely detected in epithelial tumors. In the present study, we conducted focused sequence analysis of nonepithelial tumors to determine the prevalence of *IDH1/2* mutation in tumors other than glioma and leukemia. No previous studies have analyzed *IDH1/2* mutations in sarcomas, and our present analysis revealed that they are not frequent. Our analysis revealed that about 10% of melanomas (4/39) in a Japanese population harbored an *IDH1* or *IDH2* mutation. Bleeker et al analyzed 23 cases of melanoma, but did not detect any mutation.[28] Very recently, Lopez et al analyzed 78 melanoma cases and reported *IDH1* mutation in one case.[34] Interestingly, this positive case is a lung metastasis. Because our analysis also detected *IDH1* mutation exclusively in metastatic lesions, this alteration may occur in metastatic or advanced melanomas that might not have been extensively analyzed in the previous studies. It is also possible that there are ethnic or histological differences in the frequency of *IDH1/2* mutations in melanoma. The frequency of each histological subtype varies among different ethnic groups,[35] and our cohort contains a rather high percentage of the acral lentiginous subtype (15/39, 38.5%), which occurs dominantly in the Asian ethnic group. It should also be noted that three out of four *IDH1/2* mutations occurred in either the mucosal or acral lentiginous subtype. Validation analysis of an additional larger cohort should be performed to determine the exact frequency of *IDH1/2* mutations in melanoma and to see whether *IDH1*-mutated melanoma can be classified as a specific molecular or histopathological entity as recently reported in glioma and AML cases.[16,36] *IDH1* mutation has also been identified in 11% of anaplastic thyroid cancer, which is a very aggressive type of thyroid tumor and harbors *BRAF*, *NRAS*, and *CTNNB1* mutations.[37] Because melanocyte, thyroid, and glia originate from neural crest cells, it could be possible that the *IDH1* gene alteration might confer some advantageous characteristics to tumors of specific developmental origin. It is also important to characterize the biological significance of rare substitutions such as G171D and P158T of the *IDH2* gene since rare *IDH2* mutations (R140Q and R140G) are discovered as germline variations of D-2-hydroxyglutaric aciduria.[38]

Our analysis has also revealed that *IDH1* or *IDH2* mutation coexists with other prevailing mutations such as *BRAF* or *KIT* mutations in melanoma. A previously reported *IDH1*-mutated melanoma case also harbored a *BRAF* mutation (p.V600E).[34] Because *BRAF*, *NRAS*, and *KIT* mutations converge to the activation of RAS/RAF/MAPK signaling and they occur mutually exclusively in melanoma,[4,8] it can be hypothesized that mutant IDH1/2 have a biological function distinct from this signaling pathway. Chou et al have reported that *IDH1* mutation is significantly associated with *NPM1* mutation in AML.[15] It has also been reported that some AML cases harbor both *IDH1* and *NRAS* mutations.[14,15] To evaluate the biological significance of *IDH1* mutation in melanoma, we established stable clones expressing mutant IDH1 with the common *BRAF* mutation. We found that they acquired growth activity especially *in vivo* and, surprisingly, enhanced activation of the MAPK pathway relative to the parental or wild IDH1-expressing cells. To uncover the molecular signature by mutant IDH1, we then conducted genome-wide gene expression profiling of mutant IDH1-expressing cells and found that the expression of *RASSF1* mRNA was specifically down-regulated. It has been reported that RASSF1 associates with and negatively regulates the RAS signaling,[33,39] and reduced *RASSF1* expression could partly explain the MAPK activation in mutant IDH1-expressing melanoma cells. The expression of *RASSF1* gene is epigenetically silenced in a wide range of cancers including melanoma,[40] and recently, *IDH1* mutation has been shown to associate with the CpG island methylator phenotype in glioma.[41] Further study should address whether *IDH1/2* mutations have any relation to the epigenetic alterations in other tumors including melanoma. Additionally, mutant IDH1 induced the expression of other growth-related transcriptional factors, including MYCN and JUN oncoproteins and ATF3.[42] These molecular signatures could provide a key to understanding how mutant IDH1 modulates cellular signaling in cancer.

Since the discovery of *IDH1/2* mutations, their biological significance has been debated. The first comprehensive study of *IDH1/2* mutation in glioma also demonstrated that the mutant proteins lose their original enzymatic activity,[11] but the mutation profile (accumulation in specific residues and occurring only heterozygously) cast doubt on the idea that they might be tumor suppressor genes. Zhao et al have reported that mutant IDH1 heterodimerizes with wild-type IDH1 protein and diminishes the production of α-KG by a dominant-negative fashion.[32] Since α-KG is required for PHD activity, which promotes HIF1α degradation, mutant IDH1 induces HIF1α accumulation and confers resistance to hypoxia. To determine whether mutant IDH1 activates the hypoxia-responsive signaling in melanoma, we examined the expression of HIF1α target genes. However, we were unable to detect any increase in the expression of well-characterized HIF1α-regulated genes (*Glut-1* and *Pgk1*) by mutant IDH1 in melanoma cells and no growth advantage of mutant IDH1-expressing clones was observed under hypoxic condition.

Recent studies have identified that mutant IDH1/2 can convert α-ketoglutarate to 2-hydroxyglutarate, which is a completely different function from that of the wild-type enzyme.[20–22] These findings suggest that mutant IDH1/2 may have a gain-of-function effect in tumors, although the biological significance of 2HG production in human carcinogenesis remains unclear. D-2-hydroxyglutaric aciduria, caused by germline mutations of the D-2-hydroxyglutarate dehydrogenase or *IDH2* genes,[38] shows accumulation of 2HG, and its symptoms include encephalopathy and some brain tumors.[23,24] Accumulation of 2HG has been suggested to cause oxidative stress that could be associated with increased DNA damage or epigenetic alteration,[43,44] thus driving carcinogenesis. Interestingly, our gene expression profiling revealed that melanoma cells harboring mutant IDH1 significantly reduced the expression of two dehydrogenases. Although the underlining molecular mechanisms for their transcriptional regulation remain unknown, mutant IDH1 seems to affect redox status of the cell through multiple ways (accumulation of 2HG and reduction of dehydrogenases), which might increase the mutation frequency. It has been reported that STAT3 is activated by oxidative stress.[45] To evaluate the oxidative stress induced by mutant IDH1, we measured ROS accumulation in mutant IDH-expressing clones, but were unable to detect any significant increase of ROS. It is possible that 2HG may modulate the oxidative state of specific metabolites, which were not detectable under the conditions we used. Further metabolomic approach would be helpful to resolve this question and also to test whether 2HG could be used as a new diagnostic marker for melanoma as proposed in AML.[21]

In conclusion, we have identified *IDH1/2* mutations in a small subset of melanoma. *IDH1/2* mutation coexists with *BRAF* or *KIT* mutations and mutant IDH1 confers an *in vivo* growth advantage in *BRAF*-mutated melanoma cells partly through transcriptional regulation of growth-associated and dehydrogenase genes and the MAPK/STAT3 pathway activation. Further analysis to clarify the biological roles and clinical significance of mutant IDH1/2 and underlining molecular mechanisms in melanoma is warranted.

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