ORIGINAL ARTICLE

Isocitrate dehydrogenase mutation hot spots in acute lymphoblastic leukemia and oral cancer

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Abstract   Isocitrate dehydrogenase (IDH) encodes a nicotinamide adenine dinucleotide phosphate+-dependent enzyme for oxidative decarboxylation of isocitrate and has an essential role in the tricarboxylic acid cycle. Mutations of IDH1 and IDH2 have been identified in patients with glioma, leukemia, and other cancers. However, the incidence of IDH mutations in acute myeloid leukemia in Taiwan is much lower than that reported in Western countries. The reason for the difference is unknown and its clinical implications remain unclear. Acute lymphoblastic leukemia (ALL) is a heterogenous hematopoietic malignancy. Oral squamous cell carcinoma (OSCC) results from chronic carcinogen exposures and is highly prevalent in trucking workers, especially in southern Taiwan. Subtypes of both diseases require specific treatments, and molecular markers for developing tailored treatments are limited. High-resolution melting (HRM) analysis is now a widely used methodology for rapid, accurate, and low-cost mutation scanning. In this study, 90 adults with OSC and 31 children with ALL were scanned by HRM analysis for IDH1 and IDH2 mutation hot spots. In ALL, the allele frequency was 3.23% in both IDH1 and IDH2. In OSCC, the allele frequency was 2.22% in IDH2. A synonymous mutation over pG313 (c.939A > G) of IDH2 was found in both pediatric ALL and adult OSCC. Therefore, we concluded that mutations of IDH are uncommon in ALL and OSCC and are apparently not a major consideration when selecting treatment modalities.
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Introduction

Isocitrate dehydrogenase (IDH) encodes a nicotinamide adenine dinucleotide phosphate (NADP)+-dependent enzyme for oxidative decarboxylation of isocitrate and produces 2-oxoglutarate and carbon dioxide in the tricarboxylic acid cycle. The recent discovery of somatic heterozygous mutations of IDH in glioblastoma is considered a major advance in oncology [1,2]. Mutations of IDH impair the ability to catalyze the conversion of isocitrate to α-ketoglutarate (α-KG), which decreases conversion of α-KG to the metabolite D2-hydroxyglutarate. Of the three IDH isoforms (IDH1, IDH2, and IDH3), two isoforms (IDH1 and IDH2) are associated with brain tumors [3−10], acute myeloid leukemia (AML) [11−15], myelodysplastic syndromes [16,17], prostate cancer [9], acute lymphoblastic leukemia (ALL) B [9], paraganglioma [18], thyroid cancer [19], and colorectal cancer [3,20]. Like tumor suppressors, heterozygous missense mutations of IDH1 residues act in a dominant negative fashion [18].

Most studies agree that mutational status in IDH1 and IDH2 is associated with poorer prognosis [11,21,22], while some studies report no correlations [12,23]. In low-grade glioma, however, IDH has a higher incidence of mutations [5,24], i.e., 68% in diffuse astrocytoma, 69% in oligodendroglioma, and 78% in oligoastrocytoma [24]. Grade IV secondary glioblastoma (GBM) also has a high incidence of IDH mutations (88%). Some evidence indicates that the IDH mutation is an early indicator of oncogenesis in secondary GBM [2].

Invasive oral squamous cell carcinoma (OSCC) accounts for most malignant disorders of the oral cavity. Histologic grading has limited value for predicting treatment outcomes. Despite recent advances in surgical, radiotherapy, and chemotherapy treatment protocols, the long-term survival of patients with OSCC has not substantially improved, and responses to different treatment modalities are difficult to predict [25]. Since OSCC apparently develops during several steps in the process of carcinogenesis after chronic carcinogen exposure, tailored treatments based on specific markers are needed [25,26]. Since many molecular markers have already been proposed e.g., p53, epidermal growth factor receptor, c-erbB2, c-Jun, Ki67, telomerase, cyclooxygenase-1 and -2, vascular endothelial growth factor, [26,27], this study investigated whether IDH has a role in the development of OSCC.

AML is a hematopoietic malignancy characterized by failure of the myeloid precursors to mature. Because AML is a heterogeneous disease, special therapeutic considerations are needed for its varied phenotypes. In addition to morphology and karyotype, World Health Organization classifications of molecular markers, such as genetic mutations of nucleophosmin, FMS-related tyrosine kinase, and CCAAT/enhancer-binding protein alpha, must be considered when selecting treatment strategies. ALL is also a heterogenous disease comprising multiple subtypes with different genetic alterations and varied treatment responses [28].

Whole genome sequencing performed in searches for new molecular markers of AML have revealed arginine (R132) mutations in IDH1 [29,30]. A study of patients who have AML with a normal cytogenic status found that 16% had mutated IDH1 [30]. Studies by different research groups have also confirmed significant recurrence in IDH1 [11−15] and IDH2 mutations [13,16,21].

However, a recent National Taiwan University Hospital (NTUH) study of 493 patients with AML revealed a much lower (5.5%) incidence of IDH mutation [31]. Although a possible explanation for this low occurrence rate may be the differences in species between Western and Eastern countries, the sensitivity of the current detection method is insufficient to confirm this theory. This study compared the clinical and biological features of IDH mutations in AML and in other cancers (i.e., oral cancer) that are highly prevalent in betel nut users in southern Taiwan. The IDH1 and IDH2 mutations of ALL and oral cancer were analyzed in patients treated in Kaohsiung Medical University Hospital (KMUH). Additionally, samples from four patients with AML were analyzed to compare the difference between the KMUH and NTUH groups.

ALL is another form of leukemia associated with high rates of immature lymphocytes. In southern Taiwan, oral cancer is common in construction workers and truck drivers who habitually chew betel nut. Instead of the direct sequencing method used to detect mutations in the NTUH study [31], this study applied high resolution melting (HRM) analysis, which has proven highly effective for detecting mutations [32,33], polymorphisms, and epigenetic differences [34], and it is rapidly becoming the standard mutation scanning methodology. The polymerase chain reaction (PCR) amplification and analysis were performed in a well by closed-tube method [32,33]. The values obtained by real-time PCR (LightCycler® 480, Roche Applied Science, USA) in the HRM analysis were evaluated for use in identifying hot spots of IDH1 and IDH2 gene mutations.

Methods

Patients

Ninety patients who were recently diagnosed with oral cancer at Kaohsiung Medical University Hospital and Changhua Christian Hospital were recruited. Mononuclear cells were obtained from four pediatric patients with AML and 35 with ALL in accordance with protocols approved by the Institutional Review Boards of the Kaohsiung Medical University and Changhua Christian Hospital. Of the 35 pediatric patients with ALL, four were excluded from the final statistical analysis due to insufficient DNA to complete both the IDH1 and IDH2 surveys.

Mononuclear cell samples from AML and ALL patients were centrifuged and stored at −80°C before use. Biopsy samples were obtained from the oral cancer patients during surgery and before systemic treatment or radiotherapy using a protocol approved by the Institutional Review Board at Kaohsiung Medical University. All tissue specimens were classified histopathologically as squamous cell carcinoma and stored immediately after resection in liquid nitrogen before DNA extraction.

Design and synthesis of PCR primers

The genomic region spanning the wild-type V71, G97, G123, and R132 of IDH1 at Exon 4 was amplified by PCR with sense

and antisense primers 5′-AACGACCAAGTCACCAAGGA, and 5′-GCCAACATGACTTACTTGATCC, respectively. Exon 4 and Exon 7 of IDH2, where a single nucleotide polymorphism (SNP) is normally found at R140, R172, and V294, were amplified by upstream primers of 5′-GGGGTTCAAATTCTGGTTGA and 5′-GTCAGCCTTCTGCCCTCTC and downstream primers of 5′-CAGTGGATCCCCTCTCCAC and 5′-TCCAGCCAGAGAAGAC CAAC, respectively.

DNA extraction

Blood DNA was extracted by first drawing 8 mL of blood into a Vacutainer with ethylene diamine tetra-acetic acid anti-coagulant. After mixing with red cell lysis buffer (1 mM NH_4HCO_3, 115 mM NH_4Cl), cell pellets were collected by centrifuge at 4°C. The cell pellets were resuspended in white cell lysis buffer (100 mM Tris-Cl pH 7.6, 40 mM EDTA pH 8, 50 mM NaCl, 0.2% SDS (sodium dodecyl sulfate), 0.05% sodium azide). Protein precipitate solution (∼6 M NaCl) was added and spun to collect supernatant. The supernatant was then mixed with 100% isopropanol and centrifuged. The DNA was obtained by resuspending the pellet in TE buffer.

Fresh tumor tissues were frozen and stored in liquid nitrogen before analysis. The DNA was collected by first washing 60−80 mg of tissue with PBS (phosphate buffered saline). The dissected tissue was resuspended in DNA buffer (1 M Tris pH 8.0, 0.5 M EDTA) and incubated overnight with proteinase K (10 mg/ml) and 10% SDS at 45°C overnight. The DNA was purified using standard procedures for phenol extraction and ethanol precipitation as described previously [35].

HRM analysis

The IDH1 and IDH2 mutations were identified by HRM analysis as described previously [32,33,36]. The LightCycler 480 High Resolution Melting Master kit (Reference 04909631001, Roche Diagnostics, USA) was used according to manufacturer instructions, and LightCycler 480 Gene-Scanning Software version 1.0 (Roche Diagnostics) was used for all data analysis. The PCR reactions were performed in duplicate in 20 μl final volume using the Light-Cycler 480 High-Resolution Melting Master 1X buffer, containing Taq polymerase, nucleotides, ResoLight dye, and 30 ng DNA. For detecting IDH mutations, primers and $MgCl_2$ were used at a concentration of 0.25 μmol/l and 2.5 mmol/l.

A SYBR green I filter (533 nm) was used in the PCR program, which included an initial denaturation−activation step at 95°C for 10 minutes and then 45 cycles of the following program: denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds and extension at 72°C for 15 seconds, fluorescence detection in single acquisition mode. The melting program included three steps: (1) denaturization at 95°C for 1 minute, (2) renaturization at 40°C for 1 minute, and (3) subsequent melting by continuous fluorescent reading from 60 to 90°C at a rate of 25 acquisitions/°C. The curve shape and peak height in each curve were checked to confirm good reproducibility.

Direct sequencing

After identifying SNPs based on HRM analysis and normalized and temperature-shifted melting curves, the samples were subjected to direct sequencing using the sense and antisense primers as described previously [32,33,35]. Sequencing was performed in the ABI Prism 310 Genetic Analyzer (Applied Biosystems, USA) according to the manufacturer instructions. After HRM analysis, the samples were purified with PCR-M™ for system clean up (VIOGEN, Sunnyvale, CA USA) and then directly sequenced. The sequence reaction was performed in a final volume of 10 μl, including 1 μl of the purified PCR product, 2.5 μmol/l of one of the PCR primers, 2 μl of ABI PRISM terminator cycle sequencing kit version 3.1 (Applied Biosystems, USA) and 2 μl 5X sequence buffer. The sequencing programme started from 96°C for 1 minute and was followed by 25 cycles of the following PCR program: denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds, and extension at 60°C for 4 minutes.

Results

This study recruited 90 adults with OSCC and 31 pediatric patients with ALL. Four pediatric with AML were also analyzed. The primers were designed to provide sufficient specificity within a range of sequences in which four IDH1 mutation hot spots (V71, G97, G123, andR132) and three IDH2 (R140, R172, and V294) have been reported (Fig. 3). The mutational screening included one IDH1 exon and two IDH2 exons, which included 866 codons. Normalized temperature-shifted data were clearly differentiated between the mutation and normal samples.

Four heterozygous changes were found in ALL samples. One (s.22) resided in bp 315 at Exon 4 of IDH1. Fig. 1A (upper panel) shows the melting profile of s.22. A single nucleotide polymorphism of C > T was confirmed by direct sequencing (Fig. 1A, lower panel). No mutations were found in Exon 4 of IDH2 after direct sequencing of the suspected s.35 (Fig. 1B, left panel). Two ALL (s.18, s.21) and two AML specimens (s.34, s.37) specimens showed heterozygous changes in Exon 7 of IDH2 (Fig. 1B, right panel). Direct sequencing confirmed the occurrence of the same SNP at bp (base pair) 939 (Fig. 1C). Although one base substitution was noted, the same protein was encoded, which indicated a synonymous mutation. The estimated allele frequency of ALL was 3.23% in both IDH1 and IDH2 (two of 62).

Finally, 90 oral cancer squamous cell carcinomas were screened. Fig. 2 shows the normalized and temperature shifted difference plots. No mutations were found within Exon 4 in either IDH1 (Fig. 2A) or IDH2 (Fig. 2B). No nucleotide polymorphism was found in o.12 after direct sequencing (sequence chromatogram not shown). In Exon 7 of IDH2, the melting curve showed differences in normalized temperature-shifted data between mutation and normal samples. The c.21, a previously recognized single nucleotide polymorphism in ALL, was used as a positive control and mixed with OSCC samples for analysis. The o.11 and o.128 showed similarly shifted curves (Fig. 2C). They harbored the same SNP (A > G) at codon 313, which indicated a synonymous change. After sequencing, comparison
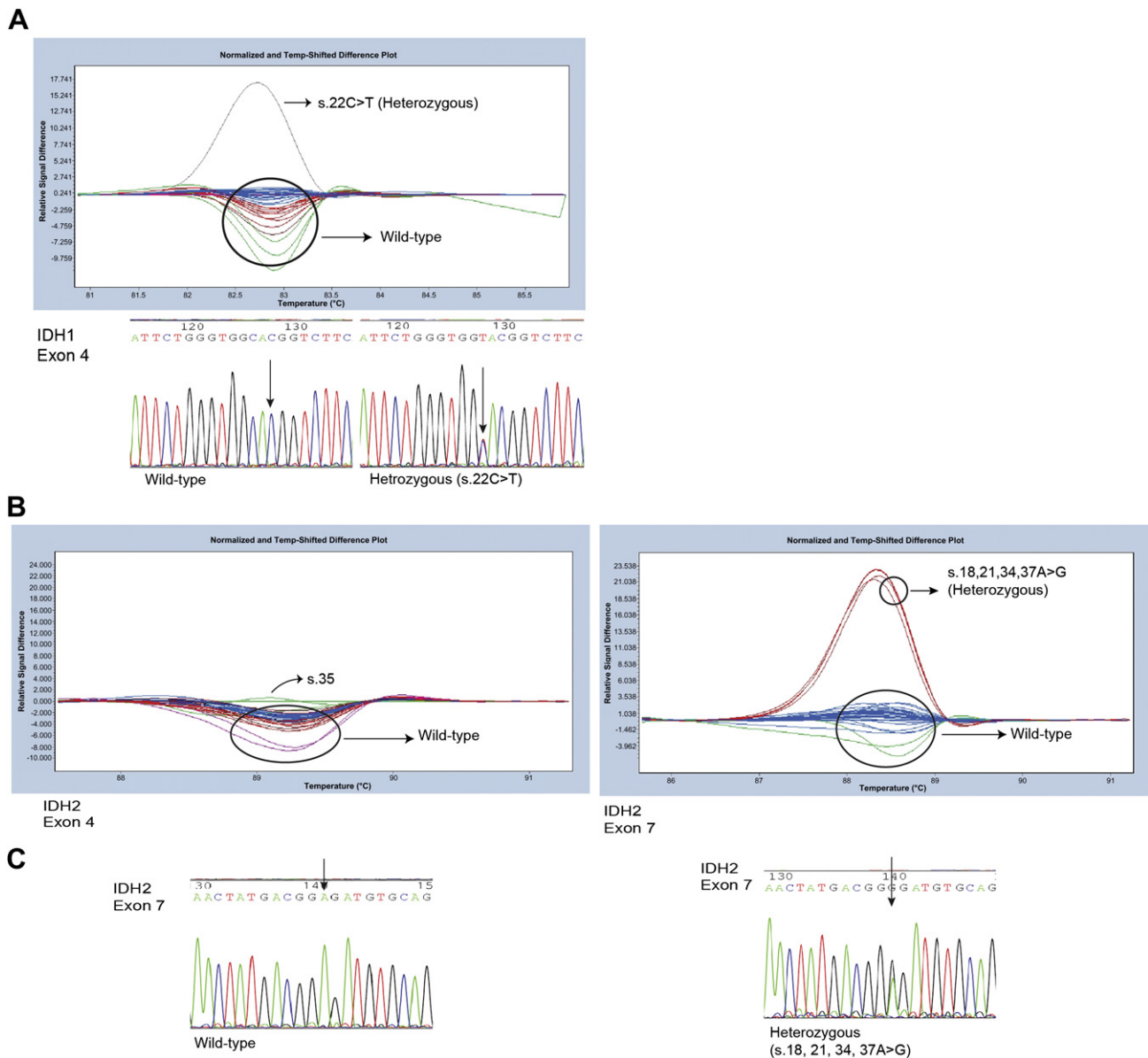
Figure 1. Detection of SNP in ALL and AML by HRM analysis and confirmation by direct sequencing. Normalized and temperature shifted difference plots for IDH1 and IDH2 genes. A. Mutation screening over exon4 of IDH1. The DNA samples of leukemia carrying heterozygous variation were distinguished after grouping of different melting curves by shapes (arrowhead: wild-type and heterozygous). Direct sequencing over the range of codons of interests revealed a rapid base change in bp 315 from Cytosine to Thymine. B. Left panel: Mutation screening over exon4 of IDH2. After obtaining the melting profile of s.35, direct sequencing revealed no mutation. Right panel: Mutation screening over exon7 of IDH2. The melting profiles for s.18, s.21, s.34 and s.37 clearly distinguished A > G. C. Representative sequencing chromatograms of wild-type (left panel) and IDH2 mutations (right panel) spanning the codon 939. Nucleotide variation from Adenine to Guanine is visible (arrowhead).

of melting profiles of o.123 and o.84 revealed no mutations. The estimated allele frequency was 2.22% (two of 180).

Compared with data reported in the literature, the number of allele changes observed in this survey was lower than those reported in earlier studies. However, allele change in pG313 might be seen as a novel finding (Fig. 3). Possible explanations are differences in tumour genetics and the different age range of the population. Another possibility is that the IDH mutations of interest were beyond the range of hotspots investigated in this study.

Discussion

The importance of mutation screening increases as treatment options become available for cancers that have different phenotypes but similar pathology. The main concerns when selecting mutation detection techniques are cost, mutation type, sample source, detection sensitivity, detection specificity, and template quality. Some sequencing methods detect mutations by identifying individual nucleotides with stands that are complementary
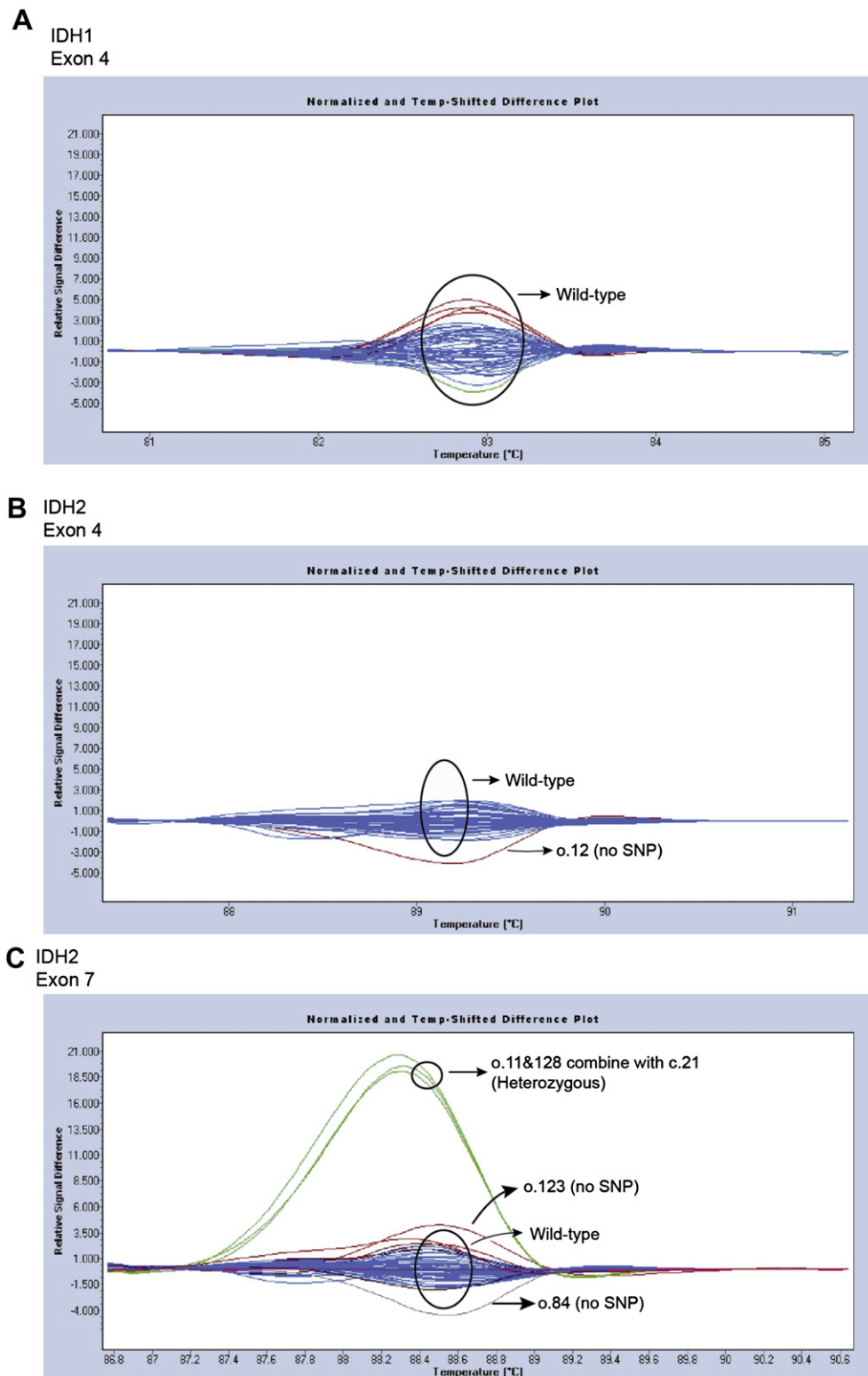
Figure 2. Detection of SNP in oral cancer by HRM analysis and confirmation by direct sequencing. Normalized and temperature shifted difference plots for IDH1 and IDH2 genes. Panel A, direct sequencing of exon 4 of IDH1 showing no mutations. Panel B, further direct sequencing of suspected heterozygous melting profile of o.12 showing no mutations. However, no mutation was found. Panel C, HRM screening of o.11 and o.128 and c.21 from a previous ALL specimen showing heterozygous curve pattern apparently harboring the same nucleotide polymorphism. Direct sequencing revealed no nucleotides mutations in o.123 and o.84.
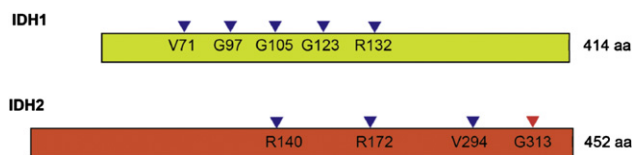
Figure 3. IDH mutation hot spots. Blue triangles indicate reported mutations at specific residues. Red triangles indicate SNPs discovered in this study.

strand to the DNA sample. Such methods include direct sequencing [37], pyrosequencing [38], nested sequencing [39], mutant-enriched sequencing [40], cold sequencing [41], and HRM analysis. Although direct sequencing is currently considered the gold standard for detecting DNA mutations, its drawbacks are its high cost and the need for high-quality templates. Prescreening can minimize the need for downstream sequencing. HRM analysis, however, is relatively fast and cost-effective for both gene scanning and SNP detection. This robust and highly sensitive method is currently the preferred method of mutation detection in most diagnostic laboratories [42]. However, inconsistent quantities of DNA inputs can cause curve deviation and false positive results [32]. Poor sample amplification can also produce aberrant melting curves [32]. Therefore, direct sequencing is recommended for additional confirmation of melting curves.

This study revealed similar single nucleotide polymorphisms in pG313 (c.939A > G) and pG105 (c.315C > T). The latter is also found in adult patients with AML [43] and in pediatric patients with Wilms tumor [44]. This observation confirms that HRM is a useful technique in clinical settings. However, no amino acid mutation occurs in this context since both the GGA and GGG codon polymorphism are indicators of glycine translation, which is considered a synonymous change. Therefore, the clinical relevance of this codon variation was not investigated further. Allele frequency in IDH2 is 2.22% in oral cancer and 3.23% in ALL. Allele frequency of IDH1 is 3.23% in ALL and no mutation hotspots are detectable in OSCC. Since the observed nucleotide polymorphism is the transition of GGC to GGT, both of which encode glycine, this base change is assumedly neutral.

The only silent mutation observed in the four AML specimens was over pG313 (c.939A > G) of IDH2. The allele frequency is 25% (two of eight). No nucleotide polymorphisms were identified in IDH1. However, this may have been due to the limited numbers of specimens analyzed. This synonymous change occurred in the same codon as that observed in the ALL patients in this study. The biologic implications are unclear. However, since the patients in both groups were pediatric, the base change could be an early indication of the oncogenesis of pediatric malignancies.

Oral cancer is believed to result from multiple processes involving many genetic alterations. The most common cause is believed to be exposure to carcinogens associated with chronic use of betel nuts, tobacco, and alcohol. Although demographic and phenotypic presentations are generally similar, responses to standard treatment vary widely. Current therapies are not tailored to specific

genotypes. The discovery of novel gain-of-function IDH mutations in GBM and AML in the current study has promising therapeutic considerations. However, further studies are needed to identify associations among IDH, oral cancer, and even ALL. To date, no IDH mutations of OSCC have been reported. Possible explanations for the lack of R132C mutations observed in this study are the very low incidence of this mutation (reportedly 1.7%) and the small number of samples screened in this study. In short, this study found that HRM is a cost-effective method of mutation screening and that IDH is not a reliable molecular marker of oral cancer or ALL.

Acknowledgment

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