Journal of Pathology and Translational Medicine 2015; 49: 257-261
http://dx.doi.org/10.4132/jptm.2015.04.14

■ ORIGINAL ARTICLE ■

IDH Mutation Analysis in Ewing Sarcoma Family Tumors

Ki Yong Na · Byeong-Joo Noh[1]
Ji-Youn Sung[2] · Youn Wha Kim[2]
Eduardo Santini Araujo[3]
Yong-Koo Park[2]

Department of Pathology, Central Physical Examination Agency, Daegu; [1]Department of Pathology, Graduate School of Medicine, Kyung Hee University, Seoul; [2]Department of Pathology, Kyung Hee University College of Medicine, Seoul, Korea; [3]Orthopedic Pathology Laboratory, Buenos Aires, Argentina

Received: April 2, 2015
Accepted: April 14, 2015

Corresponding Author
Yong-Koo Park, M.D.
Department of Pathology, Kyung Hee University Hospital, 23 Kyungheedae-ro, Dongdaemun-gu, Seoul 130-872, Korea
Tel: +82-2-958-8742
Fax: +82-2-958-8740
E-mail: ykpark@khmc.or.kr

Background: Isocitrate dehydrogenase (IDH) catalyzes the oxidative decarboxylation of isocitrate to yield α-ketoglutarate (α-KG) with production of reduced nicotinamide adenine dinucleotide (NADH). Dysfunctional IDH leads to reduced production of α-KG and NADH and increased production of 2-hydroxyglutarate, an oncometabolite. This results in increased oxidative damage and stabilization of hypoxia-inducible factor α, causing cells to be prone to tumorigenesis. **Methods:** This study investigated IDH mutations in 61 Ewing sarcoma family tumors (ESFTs), using a pentose nucleic acid clamping method and direct sequencing. **Results:** We identified four cases of ESFTs harboring IDH mutations. The number of *IDH1* and *IDH2* mutations was equal and the subtype of IDH mutations was variable. Clinicopathologic analysis according to IDH mutation status did not reveal significant results. **Conclusions:** This study is the first to report IDH mutations in ESFTs. The results indicate that ESFTs can harbor IDH mutations in previously known hot-spot regions, although their incidence is rare. Further validation with a larger case-based study would establish more reliable and significant data on prevalence rate and the biological significance of IDH mutations in ESFTs.

Key Words: Isocitrate dehydrogenase; Sarcoma, Ewing; PNA clamping

Ewing sarcoma (ES) is the second most common primary bone sarcoma of those that typically develop in children and young adults. It is also called ES family tumor (ESFT) and includes extraskeletal ES and primitive neuroectodermal tumor.[1] ESFT is an aggressive tumor with metastases present at diagnosis in 20%–25% of cases. With current therapeutic options, the 5-year survival rate for non-metastatic disease is as high as 70%. However, survival for patients who have metastasis is approximately 20%, and for those who develop relapsed or refractory disease, the survival rate is less than 10%. There is a need to identify alternative therapeutic agents that appropriately target the biomolecular mechanisms of this disease.[1]

There is a group of tumors and tumor syndromes that carry mutations in metabolic enzymes involved in the tricarboxylic acid cycle, especially enzymes in the isocitrate dehydrogenase (IDH) family. IDH catalyzes the oxidative decarboxylation of isocitrate to yield α-ketoglutarate (α-KG) with production of reduced nicotinamide adenine dinucleotide (NADH). Dysfunctional IDH leads to reduced production of α-KG and NADH and increased production of 2-hydroxyglutarate, an oncometabolite. Together, this results in increased oxidative damage and stabilization of hypoxia-inducible factor α, causing cells to be prone to tumorigenesis.[2] A functional study proved that *IDH2* mutations in mesenchymal cells can induce malignant transformation.[3] Mutations in *IDH1* are reported to cluster at a single hotspot locus (R132), whereas *IDH2* mutations occur primarily at two loci (R140 and R172).[2] Recurrent somatic *IDH1/2* mutations have been described in gliomas and secondary glioblastomas.[4] Similar *IDH1/2* mutations have been detected in acute myeloid leukemia[5] and myelodysplastic disorders.[6] Recently, IDH mutations have been reported in a large proportion of cartilaginous tumors,[7] a small number of osteosarcomas,[8] and in giant cell tumors.[9] The fact that these mutations appear to be present in these relatively common bone tumors led us to investigate *IDH1/2* mutations in ESFTs.

pISSN 2383-7837
eISSN 2383-7845

MATERIALS AND METHODS

Patient and tissue samples

Formalin-fixed, paraffin-embedded tissue samples from 61 patients with primary localized ESFTs were obtained in Korea, Brazil, and Argentina. Fifty-five of the 61 tissue samples were obtained by surgical biopsy and the other six were obtained by surgical excision. At the time of tissue sampling, none of the patients had a history of chemotherapy or radiation therapy and there was no evidence of metastatic disease. The disease was diagnosed according to World Health Organization (WHO) criteria.[10] Briefly, they are small round cell sarcomas showing diffuse membranous CD99 immunostaining, cytoplasmic periodic acid–Schiff staining, and *EWSR1* gene translocation as demonstrated with fluorescence *in situ* hybridization (ZytoLight SPEC EWSR1 Dual Color Break Apart Probes, ZytoVision, Bremerhaven, Germany). However, lack of an *EWSR1* gene translocation was not considered as grounds for exclusion if a tumor showed the typical immunophenotypes, which are inconsistent with other small round cell tumors in the differential diagnosis of diseases such as lymphoma and rhabdomyosarcoma.

After histological diagnosis, the patients received standard multidrug chemotherapy in combination with surgery. Data, including the follow-up period and overall survival, were available for 48 patients. During follow-up, assessment of distant metastasis was available in 38 patients. The patients were grouped into dead of disease, alive with disease, and no evidence of disease (NED). The classification of patients as NED was established when the follow-up period had passed more than 24 months. Our study protocol was reviewed and approved by the Kyung Hee University Institutional Review Board.

Pentose nucleic acid–mediated clamping polymerase chain reaction for detection of *IDH1/2* mutations

IDH1/2 mutations were tested using the pentose nucleic acid (PNA) Clamp *IDH1/2* Mutation Detection Kit (Panagene, Daejeon, Korea). All reactions had a total reaction volume of 20 μL and contained template DNA, primer and PNA probe sets, and fluorescent polymerase chain reaction (PCR) master mix. All required reagents were included with the kit. Real-time PCR reactions of PNA-mediated clamping PCR were performed using a CFX 96 (Bio-Rad, Hercules, CA, USA). PCR cycling conditions were as follows: 5 minutes at 94ºC followed by 40 cycles of 94ºC for 30 seconds, 70ºC for 20 seconds, 63ºC for 30 seconds, and 72ºC for 30 seconds. In this assay, PNA probes and DNA primers were used together in the clamping reaction. Positive signals were detected by intercalation of fluorescent dye. The PNA probe, which is complementary to the wild-type sequence, suppresses amplification of the wild-type target. This suppression results in preferential amplification of mutant sequences by competitively inhibiting the binding of DNA primers to wild-type DNA. PCR efficiency was determined by measuring the threshold cycle (Ct) value. Ct values for control and mutant assays were obtained from fluorescent amplification plots. Calculations of the delta Ct (ΔCt) value were done as follows: ΔCt1=(Standard Ct)−(Sample Ct), ΔCt2=(Sample Ct)−(Non-PNA mix Ct). The gene was considered to be mutated when ΔCt1 values were more than 2.0. When ΔCt1 values were between 0 and 2, a ΔCt2 value was then calculated. The gene was considered to be mutated if the calculated ΔCt2 value was ≤4.

Direct sequencing

Genomic PCR for sequencing was performed in 20-μL volumes using 30 ng of template DNA and 2× Taq PCR Smart Mix (Solgent, Daejeon, Korea). The PCR primers used for *IDH1/2* amplification were as follows: *IDH1* forward primer (5'-CGGTCTTCAGAGAAGCCATT-3') and *IDH1* reverse primer (5'-GCAAAATCACATTATTGCCAAC-3'). *IDH2* forward primer (5'-CCAATGGAACTATCCG-3') and *IDH2* reverse primer (5'-CTCCACCCTGGCCTACCTG-3'). PCR cycling commenced with a 10 minutes hold at 95ºC, followed by 40 cycles of 95ºC for 30 seconds, 58ºC for 40 seconds, and 72ºC for 60 seconds, terminating with 72ºC for 5 minutes. Each amplified product was purified using a PCR clean-up kit (Macherey-Nagel, Duren, Germany) and sequenced in duplicate, in both the forward and reverse directions, using a BigDye Terminator Kit (Applied Biosystems, Carlsbad, CA, USA) on an ABI Prism 3100 station (Applied Biosystems), according to the manufacturer's instructions. Sequences were compared with the GenBank-archived sequence of human *IDH1/2*.

Immunohistochemistry

The primary antibody that is specific for the *IDH1* R132H point mutation (1:200, Histonova DIA-H09, Dianova, Ham-

Table 1. Results of *IDH1/2* mutation analysis using PNA clamping and direct sequencing

	PNA clamping	Direct sequencing
Wild type	57	59
Mutant		
IDH1	2	1
IDH2	1	1
Equivocal	1	0

PNA, pentose nucleic acid.

http://dx.doi.org/10.4132/jptm.2015.04.14

Table 2. Summary of four cases bearing *IDH1/2* mutations, including clinicopathologic characteristics

Case No.	PNA clamping	Direct sequencing	DIA-H09	Race	Age (yr)	Sex	Site	F/U (mo)	Met
1	R132	R132H	(+)	Korea	47	M	Face	NED 32	No
2	R132	Wild	(–)	Brazil	12	F	Foot	DOD 48	No
3	R172	Wild	(–)	Korea	14	F	Ilium	NED 72	No
4	Equivocal	R172K	(–)	Korea	19	M	Femur	NED 48	No

R132H, CGT>CAT; R172K, AGG>AAG.
PNA, pentose nucleic acid; F/U, follow-up; Met, metastasis; M, male; NED, no evidence of disease; F, female; DOD, dead of disease.

Table 3. Clinicopathologic analysis according to *IDH1/2* mutation status

Characteristic	Gene profile		
	Wild	Mutant	p-value
Race			
Argentina	12	0	.009
Brazil	37	1	
Korea	8	3	
Age (yr)			
<20	29	3	.614
>20	28	1	
Sex			
Female	24	2	>.999
Male	33	2	
Tumor site			
Central	21	1	>.999
Peripheral	36	3	
Distant metastasis			
Yes	9	0	.554
No	25	4	

burg, Germany) was used for the samples bearing *IDH1* mutations, revealed by either direct PCR or PNA clamping. The Bond Polymer Intense Detection System (Vision Biosystems, Melbourne, Australia) was used according to the manufacturer's instructions with minor modifications. Nuclei were counterstained with hematoxylin. Paraffin-embedded tissues of brain astrocytomas were used as a positive control.

Statistics

Statistical analyses were performed using SPSS Software (SPSS Inc., Chicago, IL, USA). Pearson's chi-square test or Fisher exact test were performed to determine correlations between IDH mutation status and clinicopathological parameters. Statistical significance was defined as a p-value less than .05.

RESULTS

Using the PNA clamping method, *IDH1/2* mutations were detected in three of the 61 patients (5%). Of these three samples, two were *IDH1* mutants and one sample was an *IDH2* mutant. By direct sequencing, *IDH1/2* mutations were detected in two of the 61 patients (3%), of which, one sample was an *IDH1* mutant and one sample was an *IDH2* mutant. In total, four cases out of 61 (6%) harbored *IDH1/2* mutations by at least one of the two methods employed, and the numbers of *IDH1* and *IDH2* mutants were equal (Table 1).

Table 2 summarizes the clinicopathologic characteristics of the four mutant cases. In one of four cases, the *IDH1* mutation was found by both the PNA clamping method and direct sequencing (case No. 1) (Fig. 1A). In two of four cases, the *IDH1/2* mutation was found only by the PNA clamping method (cases Nos. 2 and 3). In one of four cases, examination by the PNA clamping method showed equivocal results, but direct sequencing showed an *IDH2* mutation (case No. 4) (Fig. 1C). The overall concordance rate of both methods was over 95% (58 of 61) and the discordance rate was less than 5% (3 of 61). In mutant cases, the concordance rate was 25% (1 of 4) and the discordance rate was 75% (3 of 4), although case No. 4 showed equivocal results by the PNA clamping method. Immunohistochemistry with antibody to DIA-H09 in the four cases bearing *IDH1/2* mutations showed positive reactions only in case No. 1 (Fig. 1B).

In the four cases bearing *IDH1/2* mutations, three patients were Korean and one patient was Brazilian, and the male/female ratio was 1:1. Three of the four patients were in their second decade and one patient was in the fifth decade. There was no evidence of distant metastasis in all patients and only one patient died during follow-up.

Statistical analyses showed that *IDH1/2* mutant cases had stronger associations with Korean patients than with South American patients (p=.009). There was no significant association between *IDH1/2* mutations and any of the other characteristics of tumors or patients (Table 3).

DISCUSSION

Research on *IDH1/2* mutations in human tumors has been active in recent years and has revealed that various tumors of different origins bear *IDH1/2* mutations.[2] Following increased in-

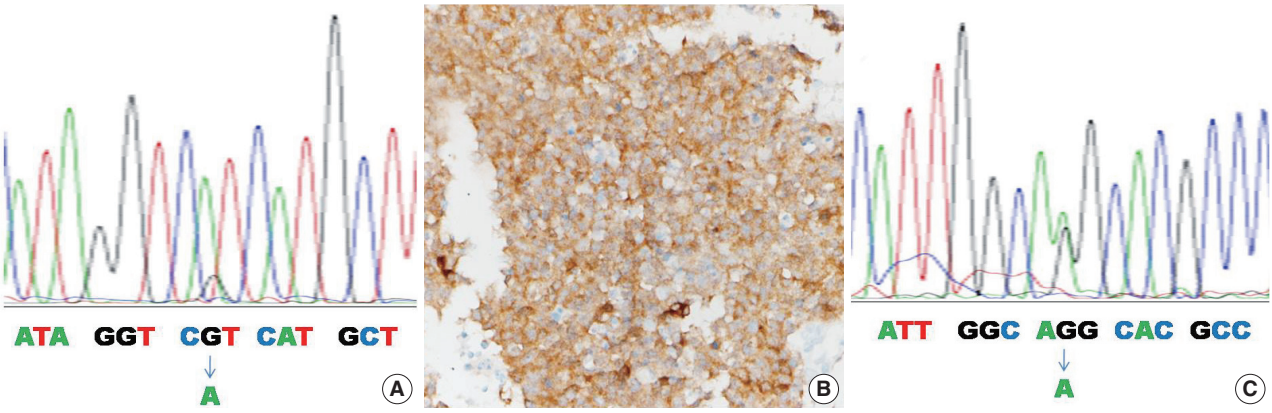

Fig. 1. The case No. 1 sample shows *IDH1* R132H mutation by direct sequencing (A) and positive immunoreactivity with antibody clone H09 (B). The case No. 4 sample shows *IDH2* R172K mutation (C).

terest in *IDH1/2* mutations in soft tissue tumors, a rudimentary study on sarcoma cell lines demonstrated IDH mutations in fibrosarcoma.[11] Subsequently, a study on chondrogenic tumors demonstrated *IDH1/2* mutations in 81 of 145 (56%) cases with an *IDH1*:*IDH2* mutation ratio of 10.6:1. This study also included the evaluation of *IDH1/2* mutations in 222 osteosarcomas, 79 chordomas, and 25 ESFTs, and no mutations were found.[7] Therefore, IDH mutations are considered to be found exclusively in chondrogenic tumors. Furthermore, previous studies support the value of examining IDH mutations for the purpose of differentiating chondrosarcoma from chondroblastic osteosarcoma[12] and chordoma.[13] However, a recent study in Japan showed that three of 12 osteosarcomas (25%)[8] and 16 of 20 giant cell tumors (80%) harbor *IDH2* mutations,[9] suggesting the possibility of *IDH1/2* mutations in various soft tissue tumors in addition to chondrogenic tumors.

We demonstrated that four of 61 ESFTs (6%) possessed IDH mutations. The PNA clamping method is known to be sensitive, rapid, and simple to perform and can detect mutant alleles when present at levels 100-fold lower than those of wild-type alleles. In contrast, the minimum percentage of mutant DNA required for analysis by direct sequencing is more than 25%.[14] The two cases harboring IDH mutations, found only by PNA clamping, might have had less than 25% mutant DNA. It is worth noting that three osteosarcomas bearing IDH mutations were found in Japanese,[8] and three ESFT bearing IDH mutations were found in Korean patients. However, evaluation of the same tumors from American patients revealed no mutations.[7] Although it is still early to remark on the background responsible for these findings, it is possible that Asian populations may be predisposed to IDH mutations in these tumors and therefore should be further evaluated.

Variation in the most prevalent mutation type according to tumor has been observed. *IDH1* R132H represents the most common type in gliomas,[4] and *IDH2* R140Q is exclusively found in acute myeloid leukemia.[5] Whereas *IDH1* R132C represents the most common type in cartilaginous tumors,[13] *IDH2* R172S is the dominant type in osteosarcomas[8] and giant cell tumors.[9] In our study, ESFTs demonstrated equal numbers of *IDH1* and *IDH2* mutations in which one case of R132H and one case of R172K were found. A previous study on cartilaginous tumors demonstrated that IDH mutations are frequent in acral-based tumors without any other association with other factors.[7] IDH mutations in osteosarcomas and giant cell tumors did not show any association with other clinical parameters.[8,9] Our study also did not find a significant association between IDH mutations and clinical parameters of ESFTs.

In conclusion, our study is the first report to demonstrate IDH mutations in ESFTs. It provides evidence that ESFTs can harbor IDH mutations in previously known hot-spot regions, although its incidence is rare. To provide generalized knowledge, our study is still lacking enough data about these mutations in ESFTs. Further validation with a larger case-based study would establish more reliable and significant data on prevalence rate and the biological significance of *IDH1/2* mutation status in ESFTs.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

REFERENCES

1. van Maldegem AM, Hogendoorn PC, Hassan AB. The clinical use of biomarkers as prognostic factors in Ewing sarcoma. Clin Sarco-

http://dx.doi.org/10.4132/jptm.2015.04.14

ma Res 2012; 2: 7.
2. Schaap FG, French PJ, Bovée JV. Mutations in the isocitrate dehydrogenase genes *IDH1* and *IDH2* in tumors. Adv Anat Pathol 2013; 20: 32-8.
3. Lu C, Venneti S, Akalin A, *et al*. Induction of sarcomas by mutant *IDH2*. Genes Dev 2013; 27: 1986-98.
4. Yan H, Parsons DW, Jin G, *et al*. *IDH1* and *IDH2* mutations in gliomas. N Engl J Med 2009; 360: 765-73.
5. Abbas S, Lugthart S, Kavelaars FG, *et al*. Acquired mutations in the genes encoding *IDH1* and *IDH2* both are recurrent aberrations in acute myeloid leukemia: prevalence and prognostic value. Blood 2010; 116: 2122-6.
6. Abdel-Wahab O, Manshouri T, Patel J, *et al*. Genetic analysis of transforming events that convert chronic myeloproliferative neoplasms to leukemias. Cancer Res 2010; 70: 447-52.
7. Amary MF, Bacsi K, Maggiani F, *et al*. *IDH1* and *IDH2* mutations are frequent events in central chondrosarcoma and central and periosteal chondromas but not in other mesenchymal tumours. J Pathol 2011; 224: 334-43.
8. Liu X, Kato Y, Kaneko MK, *et al*. Isocitrate dehydrogenase 2 mutation is a frequent event in osteosarcoma detected by a multi-specific monoclonal antibody MsMab-1. Cancer Med 2013; 2: 803-14.
9. Kato Kaneko M, Liu X, Oki H, *et al*. Isocitrate dehydrogenase mutation is frequently observed in giant cell tumor of bone. Cancer Sci 2014; 105: 744-8.
10. Fletcher CD, Bridge JA, Hogendoorn PC, Mertens F. WHO classification of tumours of soft tissue and bone. 4th ed. Lyon: IARC Press, 2013.
11. Rasheed S, Nelson-Rees WA, Toth EM, Arnstein P, Gardner MB. Characterization of a newly derived human sarcoma cell line (HT-1080). Cancer 1974; 33: 1027-33.
12. Kerr DA, Lopez HU, Deshpande V, *et al*. Molecular distinction of chondrosarcoma from chondroblastic osteosarcoma through *IDH1/2* mutations. Am J Surg Pathol 2013; 37: 787-95.
13. Arai M, Nobusawa S, Ikota H, Takemura S, Nakazato Y. Frequent *IDH1/2* mutations in intracranial chondrosarcoma: a possible diagnostic clue for its differentiation from chordoma. Brain Tumor Pathol 2012; 29: 201-6.
14. Lee HJ, Xu X, Kim H, *et al*. Comparison of direct sequencing, PNA clamping-real time polymerase chain reaction, and pyrosequencing methods for the detection of *EGFR* mutations in non-small cell lung carcinoma and the correlation with clinical responses to EGFR tyrosine kinase inhibitor treatment. Korean J Pathol 2013; 47: 52-60.