Exome sequence analysis of Kaposiform hemangioendothelioma: identification of putative driver mutations*

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Abstract: Background: Kaposiform hemangioendothelioma is a rare, intermediate, malignant tumor. The tumor’s etiology remains unknown and there are no specific treatments.

Objective: In this study, we performed exome sequencing using DNA from a Kaposiform hemangioendothelioma patient, and found putative candidates for the responsible mutations.

Method: The genomic DNA for exome sequencing was obtained from the tumor tissue and matched normal tissue from the same individual. Exome sequencing was performed on HiSeq2000 sequencer platform.

Results: Among oncogenes, germline missense single nucleotide variants were observed in the TP53 and APC genes in both the tumor and normal tissue. As tumor-specific somatic mutations, we identified 81 candidate genes, including 4 nonsense changes, 68 missense changes and 9 insertions/deletions. The mutations in ITGB2, IL-32 and DIDO1 were included in them.

Conclusion: This is a pilot study, and future analysis with more patients is needed to clarify: the detailed pathogenesis of this tumor, the novel diagnostic methods by detecting specific mutations, and the new therapeutic strategies targeting the mutation.

Keywords: Exome; Hemangioendothelioma; Mutation

INTRODUCTION

According to the International Society for the Study of Vascular Anomalies (ISSVA) classification, vascular anomalies are classified into vascular tumors with proliferative changes in endothelial cells and vascular malformation characterized by abnormal dilation of blood vessels without proliferative change. The former includes infantile hemangioma, tufted angioma and Kaposiform hemangioendothelioma (KHE).

The disease concept of KHE was first proposed by Zukerberg in 1993.¹ The intermediate malignant tumor is a rare, locally aggressive, vascular tumor that usually occurs during childhood. KHE may be associated with Kasabach-Merritt syndrome (KMS), a severe condition characterized by profound thrombocytopenia and hemorrhage. Because its mortality has been reported as 24%, it is necessary to clarify this tumor’s detailed pathogenesis to develop early diagnostic methods and new therapeutic strategies.²

No treatment guidelines exist for KHE. Previous reports have indicated that complete surgical excision is the most reliable treatment.³ When surgical excision is impossible, combined therapy, including high-dose steroids, cytotoxic agent (vincristine or interferon-α) and cyclophosphamide, is required. Furthermore, a small number of studies have reported the effects of sirolimus, β-blocker, radiotherapy and embolization.⁴ However, the tumor is basically resistant to such treatments and complete remission is usually difficult to achieve.

Recently, advances in sequencing methods have enabled the identification of driver genes in many cancers. For example, mutations in BRAF are found in malignant melanoma, and BRAF inhibitors have already been utilized as novel treatments. Nonetheless, no studies exist on the causative genes in KHE. In this study, we performed exome sequencing of KHE and found putative candidates for the responsible mutations.
METHOD

Case

A 2-year-old Japanese boy visited our hospital to treat eruptions. His parents had noticed erythemas on his lower abdominal area at birth. His eruption was monitored at another hospital but the lesion gradually became larger, violaceous and painful.

On physical examination at the first visit, multiple, indurated nodules and erythemas merged to become geographical (Figure 1). His general condition was good and there was no record of any similar condition in his family history. Laboratory data including platelet count, prothrombin time, activated partial thromboplastin time and international normalized ratio were within the normal range. T2-weighted magnetic resonance imaging (MRI) revealed multiple subcutaneous nodules, enhanced by intravenous contrast agent (Figure 2). No apparent invasion into the muscle or fascia was noted. Hematoxylin and Eosin (H&E)-stained sections of skin biopsy specimen from the lesion showed multiple nests of tumor cells in the dermis and fat tissue without epidermal change (Figure 3). The tumor cells were spindle-shaped or round, containing interspersed capillaries with slit-like lumens and red blood cells. Slight nuclear variation was observed, but no significant nuclear mitosis, atypia or necrosis. Histopathologically, these tumor cells were positive for CD31 and CD34, but negative for GLUT-1. In addition, D2-40 was positive in the peripheral area of tumor nests and negative in the surrounding dilated vessels (Figure 3).

Based on the above findings, a diagnosis of KHE was made. Since the tumor was limited to the lower abdomen without muscle infiltration or distant metastasis, and because the patient was not accompanied with KMS, the decision was taken to treat the tumor by serial excision. Three times resection removes almost all the lesions and the pain disappears.

DNA purification

The genomic DNA for exome sequencing was obtained from the tumor tissue and matched normal tissue from the same individual, using the DNA mini kit (Qiagen, Valencia, CA). Institutional review board approval and informed consent were obtained, conforming to the Declaration of Helsinki.

Library preparation and sequencing

Exome sequencing was performed in accordance with the protocol provided by InfoBio (Tokyo, Japan). DNA was treated with the TruSeq DNA Sample Prep kit and TruSeq Exome Enrichment kit (Illumina, San Diego, CA) to provide libraries, which were sequenced on the HiSeq2000 sequencer platform (Illumina) in a paired-end 100bp configuration. Image analysis and base calling were performed using the Illumina pipeline.

The clean and trimmed reads were aligned to the reference human genome (UCSC hg19) using Burrows-Wheeler Aligner (BWA) on default settings. The bioinformatic analysis for detecting single nucleotide variants (SNVs) and inserts/deletions was performed using the Samtools (v1.0) software program and annotated according to dbSNP.
RESULT

High-quality DNAs were isolated from excised tumor tissue and matched normal tissue was derived from the same individual; these were then analyzed using paired-end exome sequences on the Illumina HiSeq2000 platform.

When compared with the reference human genome (UCSC hg19), there were 80,062 nucleotide changes in the normal tissue, and 73,878 changes in the tumor tissue. Among them, 68,342 changes were common to both tissues. We tried to extract SNVs and inserts/deletions likely to be associated with the pathogenesis.

First, we focused on oncogenes. Among APC, BCL2, TP16, FOS, MYC, TP53, RAS and VHL, as shown in table 1, the missense SNVs with a T-to-A transition or a G-to-C transition were observed in the TP53 or APC genes of tumor tissue, respectively. However, these SNVs were not tumor-specific changes because they were also noted in the matched normal tissue. The APC sequence change rs459552 is present in 86% of the population, according to allele frequency from the 1,000 genomes project of October 2011, indicating that SNV represents a common variant. However, although TP53 T-to-A transition rs104252 is reportedly found in 60%, it was reported in several malignant tumors, and germline mutations in rs104252 are thought to be associated with Li–Fraumeni syndrome, which is characterized by a hereditary predisposition to several cancers.6,7 Accordingly, this germline SNV may be the pathologic change.

Subsequently, the team tried to uncover tumor-specific somatic mutations. Among the nonsynonymous SNVs, stop/gain SNVs or insertions/deletions observed only in tumor tissue and not in normal tissue, 81 candidate genes were selected by the following criteria: 1) frequency of under 1%; and 2) alternative depth/reference depth ratio of above 0.5 in heterozygous changes (Tables 2-4). Missense changes were seen in 68 genes (Table 2). In addition, PTRF, OLFML2A, WDR81, or DIDO1 were detected as stop/gain SNVs (Table 3). Eight out of the 9 insertions/deletions did not cause frameshift and only G insertion in the IL-32 gene resulted in frameshift (Table 4).

Table 1: Changes observed in oncogenes

| Gene | Chr | Chr_start | Region | Homo/Hetero | Ref | Alt | Amino acid change | Genetic status |
|------|-----|-----------|--------|-------------|-----|-----|------------------|----------------|
| APC  | 5   | 112176756 | exonic | Homo        | T   | A   | V→D             | germline       |
| TP53 | 17  | 25358943  | exonic | Homo        | G   | C   | R→P             | germline       |

Table 2: Missense somatic changes

| Gene   | Chr  | Chr_start | Ref | Alt | Homo/Hetero | Ref_depth | Alt_depth |
|--------|------|-----------|-----|-----|--------------|-----------|-----------|
| AQP11  | chr11| 77301121  | G   | A   | Homo         | 0         | 2         |
| CHST6  | chr16| 75513276  | G   | T   | Hetero       | 3         | 2         |
| DHP5   | chr19| 12792439  | C   | A   | Hetero       | 3         | 2         |
| GAL3ST2| chr2  | 242742895 | C   | A   | Hetero       | 3         | 2         |
| PTPN21 | chr14| 88945674  | G   | T   | Hetero       | 3         | 2         |
| P2DC1  | chr16| 31228226  | C   | A   | Hetero       | 3         | 2         |
| ZBTB4  | chr17| 7366209   | A   | G   | Hetero       | 3         | 2         |
| SELRC1 | chr01| 53158524  | A   | C   | Hetero       | 10        | 8         |
| FAM135A| chr06| 71187020  | A   | C   | Hetero       | 9         | 11        |
| CEMP1  | chr16| 2580996   | T   | G   | Hetero       | 4         | 4         |
| GLB1L  | chr02| 220107628 | C   | A   | Hetero       | 2         | 2         |
| NFIC   | chr19| 3435089   | G   | T   | Hetero       | 2         | 2         |
| R3HDM4 | chr19| 899473    | C   | G   | Hetero       | 2         | 2         |
| RASSF1 | chr03| 50375431  | T   | G   | Hetero       | 5         | 7         |
| PLEKHH3| chr17| 40824327  | G   | T   | Hetero       | 1         | 2         |
| ZNF512B| chr20| 62594752  | C   | A   | Hetero       | 1         | 2         |
| GPRCS5 | chr16| 19883282  | G   | A   | Hetero       | 1         | 3         |
| ITPRIPL2| chr16| 19126384  | C   | A   | Homo         | 0         | 2         |
| ITPRIPL2| chr16| 19126388  | C   | A   | Homo         | 0         | 2         |
| LRR2C4 | chr08| 145749537 | C   | A   | Homo         | 0         | 2         |
| MB2D3L5| chr19| 7032880   | A   | G   | Homo         | 0         | 2         |
| PTPMT1 | chr11| 47587479  | C   | A   | Homo         | 0         | 2         |
| TFR2   | chr07| 100228635 | T   | C   | Homo         | 0         | 2         |
| TTL4   | chr02| 219603798 | A   | C   | Homa         | 0         | 3         |
| ZAR1L  | chr13| 32885737  | G   | T   | Homo         | 0         | 2         |
| ABCB11 | chr02| 169828367 | T   | G   | Hetero       | 15        | 8         |
**Table 2: Missense somatic changes**

| Gene    | Chr  | Chr_start | Ref | Alt | Homo/Hetero | Ref_depth | Alt_depth |
|---------|------|-----------|-----|-----|--------------|-----------|-----------|
| RETSAT  | chr02| 85571228  | G   | C   | Hetero       | 13        | 7         |
| CLIP1   | chr12| 122812693 | G   | T   | Hetero       | 7         | 4         |
| FAM75A6 | chr09| 43627675  | C   | A   | Hetero       | 44        | 26        |
| DNAH12  | chr03| 57438710  | C   | A   | Hetero       | 13        | 8         |
| CRLF1   | chr19| 18705064  | C   | T   | Hetero       | 3         | 2         |
| GPIHP1B | chr08| 144297240 | C   | A   | Hetero       | 3         | 2         |
| GRID2IP | chr07| 6542766   | C   | A   | Hetero       | 3         | 2         |
| ITGB2   | chr21| 46309368  | C   | A   | Hetero       | 3         | 2         |
| PABPC1  | chr08| 101719004 | G   | A   | Hetero       | 35        | 24        |
| OR1I1H1 | chr22| 16449784  | C   | A   | Hetero       | 31        | 23        |
| CATSPERG| chr19| 38851455  | A   | C   | Hetero       | 4         | 3         |
| CLEC18B | chr16| 74451970  | G   | C   | Hetero       | 4         | 3         |
| PABPC1  | chr08| 101719201 | A   | G   | Hetero       | 25        | 20        |
| RASAL1  | chr12| 11354922  | A   | C   | Hetero       | 5         | 4         |
| CDC27   | chr17| 45234417  | A   | G   | Hetero       | 41        | 34        |
| SPATA20 | chr17| 48626182  | A   | C   | Hetero       | 6         | 5         |
| MUC7    | chr04| 71347171  | C   | T   | Hetero       | 20        | 17        |
| BCOR    | chrX | 39931672  | C   | A   | Hetero       | 2         | 2         |
| C19orf57| chr19| 14001212  | C   | A   | Hetero       | 2         | 2         |
| CARD9   | chr09| 139264769 | G   | T   | Hetero       | 2         | 2         |
| CRB1    | chr01| 197313422 | G   | A   | Hetero       | 3         | 3         |
| DDX18   | chr02| 118522361 | A   | C   | Hetero       | 3         | 3         |
| IL22RA1 | chr01| 24469556  | G   | T   | Hetero       | 2         | 2         |
| MAD1L1  | chr07| 2108930   | G   | T   | Hetero       | 2         | 2         |
| MRGPRE  | chr11| 3249491   | A   | C   | Hetero       | 2         | 2         |
| OBSCN   | chr01| 228400288 | G   | T   | Hetero       | 2         | 2         |
| SNED1   | chr02| 241974126 | G   | T   | Hetero       | 2         | 2         |
| ARSH    | chrX | 2936675   | T   | G   | Hetero       | 5         | 8         |
| CACNA11 | chr22| 40045803  | G   | T   | Hetero       | 1         | 2         |
| LRFN4   | chr11| 66627620  | G   | T   | Hetero       | 1         | 2         |
| MRC2    | chr17| 60767030  | C   | A   | Hetero       | 1         | 2         |
| SHROOM2 | chrX | 9862832   | G   | T   | Hetero       | 1         | 2         |
| LG3     | chr12| 6884651   | A   | C   | Hetero       | 3         | 7         |
| OBSL1   | chr02| 220422281 | C   | T   | Hetero       | 1         | 3         |
| TTN     | chr02| 179419226 | A   | C   | Hetero       | 1         | 3         |
| USP49   | chr06| 41774685  | C   | G   | Hetero       | 1         | 3         |
| GPRIN2  | chr10| 46999604  | A   | G   | Hetero       | 2         | 12        |
| ADAMTS7 | chr15| 79058378  | A   | G   | Homo         | 1         | 8         |
| FAM83E  | chr19| 49116421  | G   | T   | Homo         | 0         | 2         |
| LILR83  | chr19| 54725745  | A   | G   | Homo         | 0         | 4         |
| NKX6-2  | chr10| 134598908 | C   | A   | Homo         | 0         | 2         |
| PFKL    | chr21| 45744745  | G   | T   | Homo         | 0         | 2         |

**Chr:** chromosome, **Chr_start:** chromosome start site, **Ref:** reference allele, **Alt:** alternative allele, **Homo/Hetero:** heterozygosity status, **Ref_depth:** read depth of reference allele, **Alt_depth:** read depth of alternative allele.

**Table 3: Nonsense somatic changes**

| Gene | Chr  | Chr_start | Ref | Alt | Homo/Hetero | Ref_depth | Alt_depth |
|------|------|-----------|-----|-----|--------------|-----------|-----------|
| PTRF | chr17| 40557025  | C   | A   | Hetero       | 3         | 2         |
| OLFML2A | chr09| 127549304 | C   | A   | Hetero       | 2         | 2         |
| DIDO1 | chr20| 61542820  | C   | A   | Homo         | 0         | 2         |
| WDR81 | chr17| 1636925   | G   | T   | Homo         | 0         | 2         |

**Chr:** chromosome, **Chr_start:** chromosome start site, **Ref:** reference allele, **Alt:** alternative allele, **Homo/Hetero:** heterozygosity status, **Ref_depth:** read depth of reference allele, **Alt_depth:** read depth of alternative allele.
DISCUSSION

KHE is a rare, vascular tumor experienced in childhood that invades skin and cutaneous tissues locally. The prognosis of KHE accompanied with KMS is rather poor. Specific driver mutations of the tumor remain unknown. This is the first study to investigate mutations using the exome sequence, and we described several putative causing mutations of KHE.

For instance, the putative germline mutation seen in both tumor tissue and matched normal tissue was TP53 rs1042522. Given that TP53-deficient mice developed several spontaneous tumors including angiosarcoma, another malignant vascular tumor, TP53 mutation may play a role in the tumorigenesis of vascular tumors. However, since this SNV is seen in about 60% of the population, there is a possibility that "second-hit" somatic mutation is also necessary for tumorigenesis. Another vascular anomaly, mucocutaneous venous malformation is caused by the combination of germline substitutions in the endothelial cell tyrosine kinase receptor TIE2 and the somatic ‘second hit’ lesion-restricted mutation of TIE2. Similarly, we previously indicated germline heterozygous SNV in KDR and TEM8 as the risk mutations for infantile hemangioma. As infantile hemangioma typically appears on the head or face around the second week of life, the hypothesis proposed is that the clonal expansion of endothelial cells within the lesions may be a consequence of somatic events such as microvessel trauma during delivery.

We also identified 81 genes as candidates for somatic mutation. For example, ITGB2 has been strongly implicated in angiogenesis, suggesting the possible association with the pathogenesis of KHE. IL-32, which possesses heterozygous frameshift in KHE tissue, has also been known as an angiogenesis-related cytokine. Furthermore, we found 4 nonsense mutations only in tumor tissue, including the DIDO1 gene, which is up-regulated by apoptotic signals and encodes a cytoplasmic protein that translocates to the nucleus upon apoptotic signal activation. DIDO1 is considered a tumor suppressor gene in myeloid cells, and thought to be significantly involved in the pathogenesis of myelodysplastic syndrome, a malignant blood disorder. Accordingly, the DIDO1 gene may also be involved in the tumorigenesis of KHE.

CONCLUSION

These mutations have not been reported so far, which is consistent as driver mutations of the rare tumor. Potentially, multiple genetic abnormalities - rather than a single abnormality - may be involved in KHE. For instance, germline TP53 SNV and the somatic DIDO1 gene change may cooperate to induce tumorigenesis. As limitations to this study, ‘germline’ mutation of TP53 may be due to the contamination of normal tissue and tumor tissue, because the parent DNA is not determined. In addition, the result of exome analysis was not confirmed by the sanger method. Since KHE is a rare tumor, the team was unable to collect other samples. This is a pilot study, and future analysis with more patients is needed to clarify: this tumor’s detailed pathogenesis, the novel diagnostic methods by detecting specific mutations, and the new therapeutic strategies targeting the mutation.

Table 4: Insertion/deletion somatic changes

| Gene     | Chr | Chr_start | Ref | Alt | Homo/Hetero | Ref_depth | Alt_depth |
|----------|-----|-----------|-----|-----|-------------|-----------|-----------|
| IL32     | chr16| 3119303   | -   | G   | Hetero      | 25        | 15        |
| FAM48B1  | chrX | 243382426 | GCT | -   | Homo        | 0         | 2         |
| ATXN1    | chr6 | 16327915  | ATG | -   | Hetero      | 1         | 3         |
| HAVCR1   | chr5 | 156479571 | CATTGGAACAGTCGT | - | Homo | 0 | 20 |
| PCDHB10  | chr5 | 140574177 | GGCCGA | - | Homo | 0 | 4 |
| POLI     | chr18| 51795967  | CGA | -   | Homo        | 0         | 5         |
| CCDC66   | chr19| 56650056  | -   | TCT | Homo        | 31        | 1         |
| FAM83G   | chr17| 18874687  | -   | GGG | Homo        | 0         | 2         |
| NR1H2    | chr19| 50881831  | -   | CAG | Homo        | 0         | 7         |

Chr, chromosome, Chr_start: chromosome start site, Ref: reference allele, Alt: alternative allele, Homo/Hetero: heterozygosity status, Ref_depth: read depth of reference allele, Alt_depth: read depth of alternative allele.
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