A highly malignant case of neuroblastoma with substantial increase of single-nucleotide variants and normal mismatch repair system

A case report

Lin-Qing Yuan, MDa, Jin-Hu Wang, MDa, Kun Zhu, MDa, Min Yang, MDa, Wei-Zhong Gu, MDa,c, Can Lai, MDa, Hao-Min Li, PhDb,c, Qiang Shu, MD, PhDc, Xi Chen, MD, PhDb,c,*

Abstract

Rationale: Neuroblastoma is a common abdominal malignancy in children. The chemoresistant and relapsed cases have poor prognosis. The genetic background and the mechanism of resistance remain uncleaved. Next-generation sequence (NGS) is becoming a popular tool to unravel the genetic background and to guide precision medicine in oncology studies as well as in clinical practice.

Patient concerns: Here we report a neuroblastoma case of a boy aged 2 years and 8 months when first diagnosed, with multiple metastatic sites found in both lungs. The metastatic tumors were resistant to chemotherapy and the patient suffered from severe bone marrow suppression. NGS of the whole exon revealed somatic mutations including 9666 single-nucleotide variants (SNVs) from 5148 genes, 55 copy number variations (CNVs), and 140 insertion–deletion variations. The high frequency of SNVs makes it distinguished case. However, no mutation of key tumor driver genes with functional significance was identified. No abnormality was found in nucleic acid synthesis enzymes. No amplification of c-Myc and n-Myc was found by fluorescence in situ hybridization (FISH). Both NGS and immunohistochemistry (IHC) analysis indicated that DNA mismatch repair (MMR) system was intact.

Interventions: After initial diagnosis, the patient received combinational chemotherapy, which includes vindesine, an analogue of angiotension-converting enzyme inhibitors suggested by NGS data, for 4 months. Radical surgery of the tumor together with the left kidney and the left adrenal gland was performed 5 months after diagnosis. Postsurgical chemotherapy protocols was similar with the previous.

Outcomes: The patient died 2 years after initial diagnosis after 8 relapses following combinational chemotherapy.

Lessons: This case of neuroblastoma is with pronounced somatic mutations but unidentified driver gene and therapeutic target. Although NGS is a potentially powerful tool to guide precision medicine, at current stage, its application in the clinic certainly has its limits. The underlying mechanism of the substantially increased SNV number, as well as the malignant behaviors of the tumor, is yet to be revealed.

Abbreviations: ACEI = angiotension-converting enzyme inhibitors, ALK = anaplastic lymphoma kinase, CDS = coding sequence, CMMRD = constitutional DNA mismatch repair deficiency, CNV = copy number variation, CT = computed tomography, EGFR = epidermal growth factor receptor, EMA = epithelial membrane antigen, ENQUA = European neuroblastoma quality assessment, FISH = fluorescence in situ hybridization, HE = hematoxylin and eosin, hMLH = human MutL homologs, hMSH = human MutS homologs, IHC = immunohistochemistry, INDEL = insertion–deletion variation, INSS = neuroblastoma staging system, MDR = multiple drug resistance, MMR = DNA mismatch repair, ncRNA = noncoding RNA, NGS = next generation sequencing, SNP = single-nucleotide polymorphism, SNV = single-nucleotide variant, SYN = synapsin, TSS = transcription start site, TTS = transcription termination site, UTR = untranslated region, WT1 = Wilms’ tumor 1.

Keywords: DNA mismatch repair, Myc, multiple drug resistance, neuroblastoma, next-generation sequencing, precision medicine, somatic mutation

Editor: N/A.

L-QY and J-HW contributed equally to this study.

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers’ bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or nonfinancial interest (such as personal or professional relationships, affiliations, knowledge, or beliefs) in the subject matter or materials discussed in this manuscript.

This research is supported by National Science Foundation of Zhejiang Province, China (LY15H160024) and National Science Foundation of China (No. 81202021). The authors have no conflicts of interest to disclose.

* Departments of Central Laboratory, Pathology, Oncology and Radiology, The Children’s Hospital of Zhejiang University School of Medicine, Institute of Translational Medicine, Zhejiang University; † The Key Laboratory of Diagnosis and Treatment of Neonatal Diseases of Zhejiang Province, Hangzhou, Zhejiang, China.

Correspondence: Xi Chen, The Children’s Hospital of Zhejiang University School of Medicine, No. 3333 Binsheng Road, Hangzhou 310052, Zhejiang Province, China (e-mail: chchen@zju.edu.cn).

Copyright © 2017 the Author(s). Published by Wolters Kluwer Health, Inc.

This is an open access article distributed under the terms of the Creative Commons Attribution-Non Commercial License 4.0 (CCBY-NC), where it is permissible to download, share, remix, transform, and buildup the work provided it is properly cited. The work cannot be used commercially without permission from the journal.

Medicine (2017) 96:50(e8845)

Received: 26 August 2017 / Received in final form: 29 October 2017 / Accepted: 2 November 2017

http://dx.doi.org/10.1097/MD.0000000000008845
1. Introduction

Neuroblastoma is a common abdominal malignancy in children with relatively poor prognosis compared with other childhood tumors. Choice of treatment procedure is made according to the risk stratification based on clinical manifestations and the pathological type. In the international neuroblastoma staging system (INSS) commonly used in the clinic, any primary tumor with dissemination to distant organs is defined as stage IV that requires chemotherapy before and after radical surgery. Common migration sites include the lymph nodes, bone marrow, bone, liver, and skin. Metastasis of neuroblastoma to the lung is rare, with a reported occurrence rate of 3% to 4%, and diffused dissemination to the lung is extremely rare.

The histological manifestation of neuroblastoma is highly heterogeneous. Its mutational dynamics is to be investigated for the discovery of applicable therapeutic targets.

2. Materials and methods

2.1. Sample collection

This research was approved by the institutional ethical committee in 2014 and was done in accordance with the Helsinki Declaration of 1975. Signed informed consent forms were obtained from the patient’s guardian. Tissue samples were taken after surgery. Neuroblastoma with poor differentiation was diagnosed by postsurgical pathological examinations. Whole blood with anticoagulant was also collected.

2.2. Whole exome sequencing

NGS was performed by Novogene, Beijing, China. Total DNA was extracted from the tissue and the blood. After building the DNA library, target exome sequences were captured by liquid-phase chips (Agilent) and were sequenced by NGS (HiSeq 2000; Illumina). Somatic SNV was analyzed by muTect (muTect-1.1.4.jar) and by COSMIC plus DBSNP online databases.

2.3. Pathological analysis

Tissues were fixed in 4% paraformaldehyde, embedded in paraffin and cut into 4μm sections, then subjected to hematoxylin and eosin (HE) staining. Immunohistochemistry (IHC) analysis was performed by primary antibodies against hMSH2 and hMLH1 (Protein Tech) and with a biotinylated peroxidase-conjugated streptavidin system (Bio-Genex Laboratories). IHC of SYN, CD56, vimentin, CD99, Wilms’ tumor 1 (WT1), epidermal growth factor receptor (EMA), and desmin (primary antibodies from Dako, Denmark) were also performed. All specimens were scanned by 3DHistech (Pannoramic, Hungary) with companion software.

2.4. Fluorescence in situ hybridization (FISH)

Amplification of Myc was detected by commercial kit (Vysis, Abbott) using fluorescent probe against the c-Myc and n-Myc genes in sectioned paraffin-embedded tissue. Based on the guidelines of European neuroblastoma quality assessment (ENQUA), over 100 cells were counted. As the diagnostic criteria, samples with <5% cells with >4 probes bound to the chromosomes were considered negative.

3. Case report

3.1. Clinical data and treatment procedure

The patient was a boy aged 2 years and 8 months when first admitted to the hospital due to palpable left-side abdominal mass. Needle biopsy and pathological examinations diagnosed the patient as neuroblastoma with poor differentiation. No migration to the bone marrow was detected. Computed tomography (CT) revealed multiple migration loci in the lung and pleural fluid (Fig. 1A), but no migration to the liver or to the bone. The patient received chemotherapy for 4 months by

---

Figure 1. CT scans of the metastatic tumors in the lung. (A) Image of a representative window at primary diagnosis. (B) Image of the same window after chemotherapy and surgery. (C) Image of the same window in the follow-up examinations after tumor relapses. Arrows indicate the loci of the metastatic tumors.
combination of vindesine+ifosfamide+etoposide and vindesine +sprubicine+carboplatin, topotecan, and gemcitabine were also used. Then radical section of the tumor together with the left kidney and the left adrenal gland was performed 5 months after diagnosis. Pathological examinations confirmed neuroblastoma with poor differentiation and massive necrosis. A later CT showed that after first session of chemotherapy, both the number and the size of the high-density loci in the lung were reduced (Fig. 1B). The patient received several postsurgical chemotherapy protocols similar with the previous. He was always developing severe bone marrow suppression upon chemotherapy with low white blood cell and platelet counts, and severe respiratory inflammation. Follow-up CT scans found no relapse of the original tumor in the adrenal gland, but multiple metastatic loci in both lungs, with new loci developed after surgery (Fig. 1C). No metastasis in other organs was detected. The patient died 22 months after diagnosis, 17 months after surgery. The timeline of the reported case from initial diagnosis to death is summarized in Table 1.

### 3.2. Pathology and FISH

In the tissue from needle biopsy of the mass from left kidney by HE staining (Fig. 2A), necrosis was commonly seen. Clusters of tumor cells with poor differentiation and with round nuclei could be observed; no anaplastic cells were observed; spindle-like tumor cells with poor differentiation and with round nuclei could be observed. The postsurgery specimen was the sectioned left kidney, left adrenal gland together with the tumor. The tumor could be observed. The postsurgery specimen was the sectioned left kidney, left adrenal gland together with the tumor. The tumor was located above the kidney, with a size of 7 cm and massive necrotic area of 5.5 cm. The tumor had invaded the envelope of the kidney, but not into the renal pelvis and the ureter. Blood congestion, edema, and partial hyaloid degeneration were observed in the fat and fibrous tissues surrounding the tumor mass. Postsurgical pathological examinations revealed similar cellular morphology with significant necrosis and poor differentiation (Fig. 2A).

IHC showed positive staining for SYN, CD56, vimentin, with Ki-67 >30%, and negative staining for CD99, WT1, EMA, and desmin. FISH showed negative for both c-Myc and n-Myc amplification (Fig. 2B).

IHC revealed positive staining of MMR markers, hMSH2 and hMLH1, in the nucleus region in normal kidney in this case. This was the same result as obtained from tissues of another neuroblastoma case without identified mutation (Fig. 2C), as well as other control tissues (data not shown). No significant decrease in hMSH2 and hMLH1 levels was found in this case.

### 3.3. Somatic mutations revealed by NGS

A total of 9666 somatic SNVs from 5148 genes were detected. The number of somatic SNSV detected in different regions of the genes is shown in Table 2. Among the 3111 mutations in the exonic coding CDS region, 1469 mutations of missense, stoploss, and stopgain mutations that would cause change of amino acid sequences or the length of proteins. A total of 1171 genes were affected, and 1008 (85.9%) were mapped to 1386 distinct proteins in ConsensusPathDB. In pathway analysis, a total of 608 genes (53.4%) were present in at least one of 19 pathways identified. The 19 pathways enriched in mutated genes/proteins are listed in Table 3. NGS also revealed 55 copy number variations (CNVs) and 140 insertion–deletion variations (INDELS) in this case.

Table 4 contains the druggable gene targets predicted by detected mutations. Potentially effective drugs in targeted therapy were anti-EGFR antibodies, and anthracyclines/related substances such as adriamycin. It was also suggested that the tumor cells could be sensitive to HMG-CoA and ACEI, which are nonrelevant to tumor chemotherapy.

Mutation of *MSH2* is shown in Table 5. A heterozygous mutation C23T from exon 1 of this gene was identified, leading to a mutation of T8M in the protein level. The mutation rate was 6.3% (2/34) in this sample.

### 4. Discussion

Here we report a case of highly malignant neuroblastoma with diffused metastatic loci in the lung. Pathological examination

| Table 1 | The timeline of the reported case from initial diagnosis to death. |
|---------|---------------------------------------------------------------|
| **Age** | **Summaries from initial and follow-up visits** | **Diagnostic testing** | **Interventions** |
| 2 years and 8 months | The patient was a boy aged 2 years and 8 months when first admitted to the hospital for palpable left-side abdominal mass. | Needle biopsy and pathological examinations diagnosed the patient as neuroblastoma with poor differentiation. | Five times of chemotherapy in 4 months by combination of vindesine+ifosfamide+etoposide and vindesine+sprubicine+carboplatin, topotecan, and gemcitabine were also used. |
| 3 years and 1 month (before surgery) | The patient gained relapse following combined chemotherapy. | CT revealed multiple migration loci in the lung and pleural fluid, but no migration to the liver and the bone. | Radial section of the tumor together with the left kidney and the left adrenal gland was performed. |
| 3 years and 1 month (after surgery) | The patient developed severe bone marrow suppression following postsurgical chemotherapy for 4 times, with low white blood cell and platelet counts, and severe respiratory inflammation. | Pathological examinations confirmed neuroblastoma with poor differentiation and massive necrosis. | Eleven times of postsurgical chemotherapy protocols similar with the previous. |
| 4 years and 6 months | The patient died. | Follow-up CT scans found no relapse of the original tumor in the adrenal gland, but multiple metastatic loci in both lungs, with new loci developed after the operation. | |


revealed typical undifferentiated neuroblastoma cells. MDR of the metastatic tumors, as well as bone marrow suppression after chemotherapy, has made it an obstinate case in pediatric oncology. To study the genetic background of the tumor and to search for drug targets, NGS was performed to analyze the somatic mutations in the exon region. A substantially increased number of somatic mutations, especially SNVs, were identified. However, the underlying mechanism of the malignancy remained undefined. It is known that severe neuroblastoma cases are frequently associated with amplification of the Myc gene. Other

Figure 2. Expression of Myc gene and MMR proteins in the tumor tissues. (A) HE staining of tumor tissues obtained by needle biopsy and by surgery (200×). (B) IHC of CD56 and Syn, and FISH analysis of c-Myc and n-Myc of tumor tissue sectioned after chemotherapy (400×). (C) IHC of hMLH1 and hMSH2 in this case and a control neuroblastoma case without identified MMR mutation (200×).
Table 2

The number of somatic SNV detected in different regions of the genome.

| Type of mutation         | Number |
|--------------------------|--------|
| CDS                      | 3111   |
| synonymous_SNP           | 1612   |
| missense_SNP             | 1451   |
| stopgain                 | 14     |
| stoploss                 | 3      |
| unknown                  | 31     |
| Intron                   | 4780   |
| UTR3                     | 261    |
| UTR5                     | 171    |
| Splicing                 | 60     |
| ncRNA_exonic             | 211    |
| ncRNA_intronic           | 253    |
| ncRNA_UTR3               | 10     |
| ncRNA_UTR5               | 4      |
| ncRNA_splicing           | 1      |
| Upstream TSS             | 100    |
| Downstream TSS           | 45     |
| Intergenic               | 659    |
| Total                    | 9666   |

CDS = coding sequence, ncRNA = noncoding RNA, SNP = single-nucleotide polymorphism, TSS = transcription start site, TTS = transcription termination site, UTR = untranslated region.

Table 3

The pathways enriched in mutated genes/proteins analyzed by ConsensusPathDB.

| Pathway name                                      | Set size | Candidates contained | P       | q       | Pathway source |
|---------------------------------------------------|----------|----------------------|---------|---------|----------------|
| Extracellular matrix organization                  | 264      | 33 (12.5%)           | 5.71e-06| 0.0063  | Reactome       |
| Thyroid hormone synthesis                          | 9        | 5 (55.6%)            | 4.88e-05| 0.0216  | SMPDB          |
| Antigen processing and presentation—Homo sapiens   | 77       | 14 (18.2%)           | 5.85e-05| 0.0216  | KEGG           |
| ECM-receptor interaction—Homo sapiens             | 87       | 14 (16.1%)           | 0.0023  | 0.0631  | KEGG           |
| Immuno regulatory interactions between a Lymphoid and a non-Lymphoid cell | 146 | 18 (12.6%) | 0.0072 | 0.134 | Reactome |
| Olfactory signaling pathway                       | 427      | 36 (8.5%)            | 0.0528  | 0.411   | Reactome       |
| Pregnane X receptor pathway                       | 33       | 7 (21.2%)            | 0.0167  | 0.231   | KEGG           |
| ABC transporters—Homo sapiens                     | 44       | 8 (18.2%)            | 0.0225  | 0.277   | KEGG           |
| Interaction between L1 and Ankyrins                | 29       | 6 (20.7%)            | 0.0407  | 0.386   | Reactome       |
| Cell adhesion molecules (CAMs)—Homo sapiens       | 142      | 16 (11.3%)           | 0.0437  | 0.386   | Reactome       |
| Irinotecan pathway                                | 14       | 4 (28.6%)            | 0.0557  | 0.411   | PharmGKB       |
| Graft vs host disease—Homo sapiens                | 41       | 7 (17.1%)            | 0.0603  | 0.417   | KEGG           |
| Constitutive androstane receptor pathway           | 32       | 6 (18.8%)            | 0.0676  | 0.423   | WikiPath       |
| Collagen formation                                | 87       | 11 (12.6%)           | 0.0724  | 0.423   | Reactome       |
| CDG association with the ORC origin complex       | 8        | 3 (37.5%)            | 0.0727  | 0.423   | Reactome       |
| Anchoring of the basal body to the plasma membrane| 90       | 11 (12.4%)           | 0.0857  | 0.446   | Reactome       |
| Assembly of collagen fibrils and other multimeric structures | 44 | 7 (15.9%) | 0.0893 | 0.446 | Reactome |
| Cobalamin (B12) transport and metabolism         | 16       | 4 (25.0%)            | 0.0929  | 0.446   | Reactome       |
| Saturated fatty acids beta-oxidation               | 25       | 5 (20.0%)            | 0.0998  | 0.446   | EHMN           |

Table 4

Therapeutic targets predicted by analysis of mutated genes.

| Gene(s)                                      | Drug target(s)                   |
|----------------------------------------------|----------------------------------|
| ROS1                                         | Anti-EGFR antibodies             |
| FGGR3A                                       | Cetuximab (anti-EGFR antibody)   |
| UGT1A10, SLC2B8A3                            | Anthracyclines and related substances (Adriamycin) |
| SLC01B1                                      | HMG-CoA reductase inhibitors     |
| NR3C2                                        | Angiotension converting enzyme inhibitors (ACE) |

to the Lynch syndrome, previously called HNPCC. However, in this case, the genes of all MMR components do not carry mutations except for MSH2 that carried a heterozygous missense SNV mutation. Based on a recent study of functional screening to identify pathogenic MSH2 mutation, the T8M mutation was proved to be nonpathogenic. Further IHC analysis did not produce evidence of MMR deficiency in the protein level.

Although NGS can be a powerful tool to guide personalized precision treatment, at current stage, even without consideration of the relatively high cost, its application in the clinic still has its limits. First, the procedure to obtain representative tumor tissue has intrinsic difficulty because tumors are highly heterogeneous. Sites like lung metastatic loci are not readily accessible for biopsy. In this case, some lung migration sites were responsive to treatment, whereas others manifested different response. Theoretically, it would be informative to compare the genetics of different loci, but is difficult to practice. Second, the number of druggable target molecules is limited. In this case, drug target analysis based on NGS data suggested that the tumor might be sensitive to anti-EGFR antibodies and adriamycin. Although anti-EGFR antibodies are potentially effective, they are not readily available in the clinic of pediatric oncology in China and in many developing countries. Furthermore, there are many potential targets without effective compounds. It is somewhat perplexing that in our clinical protocol, vindesine, an analogue of adriamycin predicted as an effective treatment option, did not result in improved clinical outcome, suggesting the current data
interpretation tools could be inadequate. The last but not the least, our knowledge toward the genetics background of neuroblastoma is still very limited. The case reported is an example of pronounced somatic mutations but without identified driver gene and therapeutic target. Deeper understanding of the oncogenetics, as well as optimization of analytical tools, must be achieved before NGS data can provide more diagnostic and therapeutic insight in the clinic.

Acknowledgments

The authors thank professor Jian Chen from Institute of Translational Medicine, Zhejiang University, for his help with the analysis of DNA MMR.

References

[1] Brodeur GM, Pritchard J, Berthold F, et al. Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. J Clin Oncol 1993;11:1466–77.

[2] Dubois SG, London WB, Zhang Y, et al. Lung metastases in neuroblastoma at initial diagnosis: a report from the International Neuroblastoma Risk Group (INRG) project. Pediatr Blood Cancer 2008;51:589–92.

[3] Theruvath J, Russo A, Kron B, et al. Next-generation sequencing reveals germline mutations in an infant with synchronous occurrence of nephro- and neuroblastoma. Pediatr Hematol Oncol 2016;33:264–75.

[4] Sammartin E, Munoz L, Piqueiras M, et al. Deletion of 11q in Neuroblastomas Drives Sensitivity to PARP Inhibition. Clin Cancer Res 2017;23:1–3.

[5] Lasorsa VA, Formicola D, Pignataro P, et al. Exome and deep sequencing of clinically aggressive neuroblastoma reveal somatic mutations that affect key pathways involved in cancer progression. Oncotarget 2016;7:21840–52.

[6] Pugh TJ, Morozova O, Attiyeh EF, et al. The genetic landscape of high-risk neuroblastoma. Nat Genet 2013;45:279–84.

[7] Eleveld TF, Oldridge DA, Bernard V, et al. The genetic landscape of high-risk neuroblastoma. Nat Genet 2013;45:279–84.

Table 5
Characteristics of MSH2 mutations identified.

| GeneName | MSH2 |
|----------|------|
| CHROM    | 2    |
| POS      | 47630353 |
| REF      | C    |
| ALT      | T    |
| Func     | exonic |
| ExonicFunc | missense SNP |
| AACHange | MSH2:NM_000251:exon1:c.C23T:p.T8M |
| cytoBand | 2p21 |
| FORMAT   | GT:AD:DP:BQ:FA:SS |
| CX05N    | 0:48:0:48:0:00:0 |
| CX05T    | 0/1:45:3:42:48:0:063:2 |

AD = allelic depths for the ref and alt alleles in the order listed, BQ = average base quality for reads supporting alleles, DP = approximate read depth (reads with MQ = 255 or with bad mates are filtered), FA = allele fraction of the alternate allele with regard to reference, GT = genotype, homozygous =0/0 or 1/1, heterozygous = 0/1, SS = variant status relative to nonadjacent normal, 0 = wild type, 1 = germline, 2 = somatic, 3 = LOH, 4 = posttranscriptional modification, 5 = unknown.