Metagenomic Next-Generation Sequencing In Febrile Neutropaenia of Children Hematological And Malignant Diseases

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Research

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Abstract

Objective: To evaluate the diagnostic value of metagenomic next-generation sequencing in febrile neutropaenia of Hematological and malignant tumor children.

Methods: We retrospectively studied the diagnostic value of metagenomic next-generation sequencing in febrile neutropaenia of Hematological and malignant tumor children in the Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University from July 2018 to December 2019. Children with neutropenia and fever for more than 72 hours were sent for mNGS analysis together with traditional pathogen detection tests. The positive rate of pathogen detection and the following change of clinical treatment strategy was analyzed. Furthermore the duration of fever was compared between patients receiving both mNGS and traditional tests and those only had pathogen detected by traditionally methods.

Results: The positive rate of mNGS was 75.6% (28/37cases), and that of the two methods combined was 86.4% (32/37cases). In these patients with long duration of fever, the detection positivity for bacteria, fungi, and viruse detection was high, and mycoplasma and rickettsias were also found. Among all the cases reported, clinical treatment strategies were changed in 9 cases due to the pathogen detection. The duration of fever of patients receiving mNGS together with traditional tests was shorter than those only had traditional ones (7.84d±0.17 vs. 8.39d±0.25, respectively, $P<0.001$) .

Conclusion: Due to high positive rate of mNGS in pathogen detection, active treatment can be taken in time, infections can be controlled earlier. It has a positive effect on the outcome of febrile neutropaenia patients.

Introduce

Febrile neutropaenia in Hematological and malignant diseases had always be a threatening disease[1,2].Despite the advance of the treatment these years, unknown pathogen infection remains a problem for clinicians. Almost any bacteria or fungus can become a potential pathogen in immunocompromised patients. Strong infection and undiscovered pathogens are often fatal to these patients. To such patients, comprehensive detection of pathogen is very important[3,4,5,6].

Metagenomic next-generation sequencing (mNGS), of plasma cell-free DNA, as to its wide pathogen coverage, non-invasive performance and fast detecting, is now more and more used in clinical. In recent years, many articles have reported that mNGS is used for pathogen detection in undiagnosed cases of infection at different sites[7,8,9]. In Matt s ziner, et al's research, hidden pulmonary infection pathogens in half of 31 children with immune deficiency from three children's hospitals were re-detected by mNGS, including bacteria, fungi and viruses[4]. Another study from Ying Li showed that owing to its re-detection mNGS prompted changes in treatment strategy for more than one-third of patients[10]. However, there are also articles showed that the value of mNGS in clinical practice is quite limited. Some just take it as a last

try. Unnecessary treatment, additional diagnostic investigations and even longer length-of-stay were caused in some cases which might lead to excessive medical and financial burden.

Since July 1, 2018, mNGS was Introduced to our department. Febrile neutropaenia of children with hematological and malignant tumor diseases were received with this detection together with traditional pathogen detection tests (we use TT for short in our articles) including blood culture, G test, galactomannan test, specific antibody detection, chest CT and other targeted imaging tests, if the fever lasts for three days long. We retrospectively examined the positive rate of pathogen detection and the change in clinical decisions of these patients, and also compared the duration of fever of these patients with those without this detection before, so as to find how much it really contributed.

Materials And Methods

Patients

Febrile neutropaenia (FN) is defined as an oral temperature of >38.3°C or two consecutive readings of >38.0°C for 2 h and an absolute neutrophil count (ANC) of <0.5×109/L or expected to fall below 0.5×109/L. All these patients were treated according to the international guidelines for (FN) [1,11]. Patients who fevered up to three days, given mNGS and traditional pathogen detection tests (mNGS and TT group) were eligible from July 2018 to December 2019. Patiants were not adopted if mNGS were given on the day more than 3 days later; Death cases during treatment were also excluded. As a comparison, patients who fevered up to three days with only traditional methods(TT group) were also collected from January 2018 to June 2018. The research program was approved by the institutional ethics committee.

Blood sampling

In the trial group, another 5ml blood sample was taken for mNGS analysis following published procedure[12]. Blood sample was placed in EDTA tubes and stored at room temperature for 3-5 minutes for plasma separation. The samples were centrifuged at 1,600 g for 10 min at 4℃ within 8 h of collection and the plasma were transferred to new sterile tubes. TIANamp Micro DNA Kit (DP316, TIANGEN BIOTECH ,Beijing, China) was used for DNA extraction from 300 uL of plasma using the following the manufacturer's manual. QIAamp ViralRNA Mini Kit Qiagen, Germany was used for extraction of RNA before cDNA was generated from the extracted RNA template via reverse transcription. The extracted DNA or cDNA was used for DNA libraries construction through DNA-fragmentation, end-repair, adapter-ligation and PCR amplification and quality control procedure according to published study[13]. Quality qualified libraries were sequenced on BGISEQ-50 platform.

Microbiological analyses

Sequencing data analysis followed the published methods[12,14], which included removal of low-quality, and short (length < 35bp) reads, computational substraction of human host sequences mapped to the

human reference genome (hg19), removal of low-complexity reads and alignment to four Microbial Genome Databases, consisting of viruses, bacteria, fungi, and parasites. The reference databases contained high quality whole genome or scaffolds sequence of 4,945 viral taxa, 6,213 bacteria (including 174 *Mycobacterium* spp.), 1,064 fungi, 234 parasites and 137 mycoplasma /chlamydia related to human diseases. All the sequences were downloaded from NCBI (ftp://ftp.ncbi.nlm.nih.gov/genomes/).

To determine infection pathogens , discriminating from colonization and contamination, we quote Li H's standards: (I) > 30% relative abundance at the genus level, regardless of culture or smear result; (II) culture and mNGS identified same microbe, and number of unique reads was ≥ 50 from a single species. (III) for unique readings less than 50 Pathongen, which can still be diagnosed as infectious pathogens with consistent clinical conditions, based on strict clinical criteria, combined with multiple-clinician adjudication [5,15].

Data and statistical analyses

The statistical analysis of our study data was performed using SPSS for Windows version 19.0 software (IBM SPSS Inc., Armonk, NY, USA). For comparison between groups, independent-samples T test was used,. $p<0.001$ was considered statistically significant.

Results

Characteristics of FN cases in mNGS and TT group

In mNGS and TT group, a total of 32 children with FN who fevered up to three days in our department were collected. The characteristics of these cases are described in Supplementary Table 1. According to the statistics of pathogen detection rate of the 37 children, the positive rate of mNGS figured out was 75.6%(28/37 cases), while the positive rate of the TT was 43.24%(16/37 cases). The sensitivity after the combination of the two method reached up to 86.4% (32/37 cases).

The detection of pathogens in mNGS and TT group

Simultaneously received with this two methods, totally 39 pathogens in 37 children were detected (Supplementary Table 2). 2 cases were found infected with two different pathogens. As for mNGS, the positivity for bacteria was 48.1% (13/37 cases). While the positive rate of TT , which basically means blood culture, was only 8% (3/37 cases). The positive rate of mNGS for fungus was on par with bacteria, also 48.1% (13/37 cases). The positive rate of TT for fungus was 24.3%(9/37 cases). As for virus, the initial reports of mNGS showed high expression of several viruses. However, with further verification and discussion, only 6 cases of viral infections were confirmed. Mycoplasma was detected in 2 cases by both methods. Furthermore, only mNGS found Orientia tsutsugamushi in a blood sample of a three-line descending boy with unidentified infected boy.

mNGS changed clinical treatment strategies

Among the 37 cases, clinical treatment strategies were changed in 9 cases due to the pathogen re-detected by mNGS (Supplementary Table 3). Adenovirus infection was confirrmed in 18B33176 and 18B40912. Antibacterial treatment was discontinued presently after the temperature droped in 18B33176, and symptomatic supportive treatment was strengthened, like Intravenous gamma and Ankylosaurus in both cases. Fungal infection was detected in 18B43375 and 19B00104, and anti-fungal was added to the treatment immediately. Fungal infections were also detected in 18B40094 and 18B33235 which had already been treated with the original anti-fungal treatment. Considering not effective, we did some adjustment, changing voriconazole to carbophenazine and amphotericin for Aspergillus oryzae pneumonia in 18B40094, and adding amphotericin to control the tropical candida sepsis in18B33235. 18B43375 was a case of Enterococcus faecium enteritis, with no anaerobic infection detected, metronidazole was discontinued. 19B00104 received azithromycin, which was sensitive to Orientia tsutsugamushi detected. 19B00502 also received azithromycin to treat of Mycoplasma pneumonia. Statistically, treatment plan was immediately adjusted in 9 cases (24.3%) due to the clear pathogen by mNGS. Others (75.7%) did not change the treatment strategy although some of them helped confirm the diagnosis.

Table 1. Characteristics of 37 FN cases with hematological and malignant tumor in mNGS and TT group

Characteristic		Patient, no,(%)
age		6.81±3.74
sex		
	male	21 (56.8)
	female	16 (43.2)
Immunodeficiency		
	Acute leukemia	29
	Solid tumor	3
	Aplastic anemia	2
	Subacute necrotizing lymphadenitis	1
	Hemophilic syndrome	1
	Unexplained decline in the third line	1
Site		
	Respiratory system	23
	Blood system	12
	Digestive system	2
Pathogen detection rate		
	mNGS	28（75.6%）
	TT	16（43.24%）
	mNGS and TT	32（86.4%）

FN,Febrile neutropaenia; mNGS,Metagenomic next-generation sequencing; TT,traditional pathogen detection tests.

Table 2 The detection of bacteria, fungi, viruses, mycoplasma and rickettsiain in 37 cases in mNGS and TT group

pathogen		mNGS	TT	mNGS and TT
Gram-positive bacteria				
	Human staphylococcus	2	0	2
	Staphylococcus	1	1	1
	Enterococcus faecium	1	1	1
	Enterococcus faecalis	1	0	1
	Cook Swamp	1	0	1
	Isoprene gordonia	1	0	1
		7	2	7
Gram-negative bacteria				
	Acinetobacter baumannii	3	0	3
	Haemophilus parainfluenzae	1	0	1
	Neisseria	1	0	1
	Lauterops kiwi	1	0	1
	Enterobacter cloacae	0	1	1
		6	1	7
		13(35.1%)	3(8.1%)	14(37.8%)
Fungus				
	Aspergillus oryzae	4	4	4
	Aspergillus niger	1	0	1
	Aspergillus fumigatus	1	1	1
	Penicillium citrinum	1	0	1
	Candida tropicalis	2	1	2
	Candida nearly smooth	2	1	2
	Fusarium	1	0	1
	Saccharomyces cerevisiae	1	0	1
	Aspergillus sp(Species unknown)	0	2	2
		13(35.1%)	9(24.3%)	15

				(40.5%)
virus				
	Adenovirus type 7	2	0	2
	Adenovirus type 4	2	0	2
	CMV	1	1	1
	Human boca virus	1	0	1
	Parainfluenza virus type III	0	1	1
		6	2	7
Mycoplasma	Mycoplasma pneumoniae	2	2	2
Rickettsia	Orientia tsutsugamushi	1	0	1

mNGS,Metagenomic next-generation sequencing; TT,traditional pathogen detection tests.

Table3. 9 cases changed the diagnostic investigations and/or treatments after detection of mNGS.

Patient no	mNGS-based diagnosis	Changes of diagnostic investigations and treatment
18B33176	Adenoviral pneumonia	Discontinued vancomycin, added Intravenous gamma
18B43375	Aspergillus fumigatus pneumonia	Discontinued imipenem cilastatin, added galinazolamide and voriconazole
18B40094	Aspergillus oryzae pneumonia	Discontinued galinazolamide, voriconazole added carbophenazine, and amphotericin
18B40912	Adenoviral pneumonia	added Intravenous gamma and ankylosaurus
18B33235	Tropical Yeastemia	Added amphotericin based on carbophenazine
19B00104	Fusarium pneumonia	Added voriconazole
18B43375	Enterococcus faecium enteritis	Discontinued metronidazole
19B00104	Orientia tsutsugamushi	Added azithromycin
19B00502	Mycoplasma pneumonia	Added azithromycin

Compare the fever time between mNGS+ TT group and TT group

The duration of fever of the mNGS and TT group and TT group were recorded respectively and compared (Supplementary Table 4). The duration of fever was 7.84 ± 0.17 in mNGS and TTgroup and 8.39 ± 0.25 in

TT group. The duration of fever in mNGS and TT group was shorter than that in TT group. The difference is significant(P < 0.001%).

Table 4. The duration of fever of the two groups with different methods.

	mNGS + TT group	TT group
Duration of fever（d）	7.84±0.17	8.39±0.25
t	13.74	
p	<0.001	

Abbreviations: mNGS, Metagenomic next-generation sequencing; TT,traditional pathogen detection tests.

P > 0.001, no statistical difference; P < 0.001, statistical difference

Discussion

Despite the great progress in prevention and treatment with prophylactic antibacterial and colony-stimulating factors, FN is still one of the most common and serious complications in Hematological and malignant diseases, which may lead to treatment delay, poor consequence and even death[16]. 20%-30% of patients need hospitalization, and the overall hospitalization mortality is about 10%. For 24-72 hours without relief in fever, reevaluation is needed to adjust the treatment plan according to European guidelines[1,17]. Bacteremia can be detected in 72 hours by traditional blood culture, but its sensitivity is low, often affected by the technician's technology, physical and chemical environment and contamination[18]. The instability and incompleteness drive us to find a better detection method. In recent years, new detection methods, such as polymerase chain reaction (PCR), not relying on culture, was adopted by some microbial technician. However it can only detect common pathogens qualitatively[19]. Since 2008, a large number of studies from more than twenty countries have shown that mNGS, directly detecting plasma free cells, has high practicability in the diagnosis of undiagnosed infections, and it performs well in the identification of rare, novel, occult and mixed infection [20].This makes the new technology suitable for detection of fever pathogens in children with hematological and malignant tumors.

According to the analysis of 37 samples in mNGS and TT group, we eventually detected 39 pathogens in 32 cases. The positive rate of mNGS for bacteria was 35.1% higher than the TT group，Mainly through blood culture，which was only 8.1%. That’s not surprisingly, as blood culture was always known with low sensitivity. In the past ten years, the common bacteria reported by different research centers are different. Gram-negative bacteria is slightly higher than Gram-positive bacteria[21]. In our study, Gram-positive and negative bacteria were found equal in 14 cases, different from Yan Chenhua's multicenter and prospective epidemiological study on FN in China in 2016[22]. Considering the regular use of carbapenem antibiotics treatment, many cases of Gram-negative bacteria infection might cure within 3 days, and not

incorporated in the group. With further analysis, despite of the high positivity for bacteria, the re-detection did not promote much adjustment of antibacterials. That may because of the sufficient experience in the use of antibacterial drugs in Hematological and malignant diseases, which the clinicians had.

15 fungal infections were detected in 37 cases, accounting for a large proportion. Histopathological diagnosis is the gold standard for IFD, but it is time consuming and invasive. We make clinical diagnosis by G test, galactomannan test and chest CT.[25,26,27]. G test and galactomannan test are somehow lack of classification of different phylum of fungi. Early during the collection phase in our study we found a case of which the early G test was highly positive. Antifungal (Voriconazole) was added to combine with the initial empiric antibacterial therapy, meanwhile mNGS were given. However the disease still worsened and could not be reversed. Two days later, the patient died, with the blood samples of mNGS reported as tropical candidiasis. The precise selection of antifungal drugs is directly related to the therapeutic effect in the treatment of fungal bacteremia which is more dangerous. mNGS, which provided more specific species of fungal, could have helped if given earlier. In our study, totally 4 cases got more active and effective antifungal treatment due to mNGS. Here we suggest that mNGS accurately did provide positive improvements to treatment.

In addition, it is more sensitive to virus with the unbiased detection, and is also simpler operating with multiple viruses. However, the presence of contamination in DNA extraction is obvious in virus detection. We received the reports of multiple viruses in some cases with various levels of sequence expression although the technicians had already performed the comparison and selection according to the most advanced approach[28,29]. Although with higher sequence expressions of virus in some cases, they were still confirmed as previous infection in combination with subsequent pathogen antibody detection. For the confirmed virus infection, we reduced the use of antibacterial drugs and adopted targeted treatment. In addition, for rare infections like parasitic, without giving a clear indication in the previous clinical examination, it is difficult to diagnose through routine laboratory tests. At this moment, mNGS absolutely provided help.

In summary, a total of 9 cases (24.3%) got a positive effect on the treatment strategy, especially for fungal infection. In the remaining cases, with most of the pathogens ware found, although there was no change in treatment, the diagnosis was clear and it was still helpful for the clinician to keep track of the diseases.

To learn more about the effect, we also compared fever duration time in children who received mNGS and TT with those only had traditional ones before. The results showed that the duration of fever in mNGS and TT group was shorter than that of the TT group. For children of FN with hourly mortality, the fever duration is closely related to the survival rate. With the high positive rate, mNGS contributed to precise anti-infective treatment, and this finally resulted in earlier control of infection and for some less experience burden. In addition, we want to clarify a situation in this retrospective study. Children with more severe symptoms (like higher temperature, worse cough, poor mental state, et) are more likely to agree with the new detection. For those relatively mild ones, parents balanced interests, and disagree with

this check finally. Therefore, in fact, the condition of children in mNGS and TT group is somehow more serious than that in the TT group. We believe If given more random and equal trial conditions, duration of fever of mNGS and TT group could be shorter than the actual value.

Theoretically, given enough sequencing length, almost all microorganisms can be uniquely identified through mNGS by multiple matching with microbial genome and reference database [30]. With the comprehensive and multi-pathogen coverage, positivity of pathogens in FN which with complex pathogens and atypical manifestations is high. However, the specificity of this method is opposite. This is also a challenge for technician at present. The accurate interpretation of pollution and pathogens not only requires a more scientific sequence analysis method, but also emphasizes the clinical thinking of clinicians. Diagnosis requires clinicians to combine with the patient's clinical manifestations. In our study, pathogens was selected by discussion with multiple clinicians and sometimes should be verified by other experimental methods, so as to minimize false positive rates. Imitation of our study was the small sample size. The price was an important factor affecting its large-scale promotion. However, with the maturity of the test technology and the standardization of the process, the turnaround time can be constantly shortened, and definitely the cost will constantly be reduced. Then mNGS will become a widely accepted method, not just a last resort[20].

mNGS was found to have practical clinical value for FN in our research. However, to ensure the correct use of the new model of microbiology test, clinician and microbiologists should fully understand the function and limitations of this method.

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