High concentration of plasma cell free DNA alerting disease recurrence in high risk neuroblastoma children

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Abstract

Background Neuroblastoma is the third-most common cancer in children. The high rate of tumor recurrence accounts for a low survival rate in high risk neuroblastoma. Therefore it is clinically of extreme importance to find an effective biomarker for alerting disease recurrence.

Methods Total 116 high risk neuroblastoma patients were recruited in Beijing Children’s Hospital from February, 2015 to December, 2017. All patients had received multiple-disciplinary treatment, then went into maintenance treatment phase after evaluation. Blood samples were collected to quantify plasma cell-free DNA (cfDNA) at time points of the beginning of maintenance treatment, every three months afterwards, and diagnosis of recurrence.

Results Results showed that 36 high risk neuroblastoma patients developed recurrence during maintenance treatment. The plasma cfDNA concentration was significantly higher in recurrence than in event-free patients (29.34 ng/ml VS 10.32 ng/ml). The time span of cfDNA level higher than 29 ng/ml was consistently detected ahead of recurrence at mean of 0.55 months. The ROC analysis showed that AUC was 0.825, optimal sensitivity and specificity of 80.6% and 71.3% respectively, at cfDNA level of 12.93 ng/ml.

Conclusions We concluded that high level of plasma cfDNA could serve as a promising molecular marker to alert recurrence disease in high risk neuroblastoma children.

Background

Neuroblastoma (NB), originating from neural crest precursor cells of sympathetic nervous system, is one of the most common pediatric malignancy which accounts for approximately 10% of tumors in children.[1-3] It is reported that around 90% of NB cases occurred in children younger than 5 years old.[4, 5] Primary tumors usually arose in the abdomen, but might also develop in the neck, thorax, or pelvis. Symptoms and signs included various neoplasms in the abdomen, neck, or thorax, and bone pain.[6, 7]

Treatment of NB was based on risk stratification, typically including surgery, chemotherapy, radiation, immunotherapy, et cetera.[8-11] The toughest thing for physicians is tumor recurrence in high risk NB patients, as results in a survival rate less than 50%.[12-14] Currently, imagological and cytological
examinations could identify recurrent diseases and metastatic tumor sites.[15] But the recurrence could not be identified until tumor cells re-grew significantly. Consequently, patients missed out on an optimal treatment time window. Therefore it is of extreme importance to find out an effective biomarker for alerting recurrence.

Nowadays plasma cell free DNA (cfDNA) has become an active and promising biomarker in cancer research.[16-18] Generally, cfDNA is degraded DNA fragments which enter the bloodstream during tumor apoptosis or necrosis. Those DNA fragments are normally cleaned up by macrophages, but some cfDNA is left in blood because of overproduction of tumor cells. This gave a hint that cfDNA could be used to monitor cancer development in clinic.[3, 19, 20] Few researches had been carried out so far to identify cfDNA as a biomarker for NB recurrence. In this research, we monitored cfDNA levels in high risk NB patients during maintenance treatment to detect tumor burden and alert recurrence. We found that high level of plasma cfDNA was closely correlated with tumor recurrence, and it could potentially be a satisfying molecular marker in clinic.

Patients And Methods

Patients

A total of 116 high risk NB patients were recruited in the Hematology Oncology Center, Beijing Children’s Hospital from February 1, 2015 to December 31, 2017. Informed consent was obtained from parents or guardians of each patient according to the Declaration of Helsinki. High risk neuroblastoma was classified as age older than 18 months, and stage IV by International Neuroblastoma Staging System (INSS); or any age, and stage II, III or IV with MYCN gene amplification. All patients had received multiple-disciplinary treatment, then went into maintenance treatment after evaluation. All patients were monitored and evaluated during maintenance treatment. The whole follow-up ended at September 30, 2018. This research and the BCH-NB-2007-HR protocol were approved by the Beijing Children’s Hospital Institutional Ethics Committee (No. 2016-65). The BCH-NB-2007-HR protocol was based on the Hong Kong Pediatric Hematology and Oncology Study Group (HKPHOSG) [21] and a German report.[22]

Diagnostics and Evaluation
For initially diagnosed patients, microscopic examinations of bone marrow aspirates and biopsies were performed to identify the presence of neuroblastoma cells. Genetic abnormalities, i.e., amplification of the MYCN gene, deletion of the short arm of chromosome 1 (1p36) and the long arm of chromosome 11 (11q23), were detected by Fluorescence in situ hybridization. Serum tumor markers such as lactate dehydrogenase (LDH), neuron specific enolase (NSE), and cfDNA were quantified.

The evaluation of therapeutic response was performed, when multiple-disciplinary treatment finished, by serum tumor markers quantification, minimal residual disease (MRD) detection including $^{131}$I-metaiodobenzylguanidine ($^{131}$I-MIBG), ultrasound and computed tomography (CT). According to the standards of Response Evaluation Criteria in Solid Tumors (RECIST), therapeutic response was classified into four degrees, complete remission (CR), partial remission (PR), stable disease (SD), and progressive disease (PD). Patients of CR, PR and SD could step into maintenance treatment. Evaluation during maintenance treatment included serum tumor markers detection, microscopic examinations of bone marrow and imaging examination every three months, and $^{131}$I-MIBG every six months.

**Treatment**

According to the BCH-NB-2007-HR protocol, initially diagnosed high risk NB patients received multiple-disciplinary treatment including induction chemotherapy, surgery, consolidation therapy, and radiotherapy. Some patients received autologous stem cell transplantation. Regular regimens included chemotherapy with high dose cyclophosphamide, adriamycin and vincristine (CAV), chemotherapy with high dose cisplatinum and VP16 (CVP), surgery after 4-5 cycles of chemotherapy, and then harvest peripheral blood stem cell for possible autologous hematopoietic stem-cell rescue.

Maintenance treatment was with 13-cis-retinoic acid 160 mg/m$^2$/day, 14 days on alternative with 14 days off for 9-12 months; and 13-cis-retinoic acid stopped.

**Sample collecting**

Blood samples were collected to quantify cell-free DNA (cfDNA) at time points of the beginning of
maintenance treatment, every three months afterwards, and diagnosis of recurrence. Venous blood samples were collected in EDTA-coated tubes and plasma was separated by centrifugation at 1600g for 10 minutes. Supernatant was transferred into a new tube and centrifuged at 16000g for 10 minutes. Purified plasma was carefully removed from the tube and stored at -80°C for DNA extraction.

**Plasma cfDNA detection**

Before quantification, DNA was extracted from 200 μL plasma and eluted by 300 μL elution buffer by QIAmp DNA Blood Mini Kits (Qiagen, Valencia, CA, USA). cfDNA level detection was detailed in our previous paper.[23] Briefly, after preparation of extracted DNA, quantitative polymerase chain reaction (qPCR) was performed on LightCycler LC 480 PCR (Roche Molecular Systems, Inc, Pleasanton, CA, USA) to amplify the apoptotic and nonapoptotic LINE-1 79bp DNA fragments by specific primers. A reference standard curve was established by serially diluting standard solution of human genomic DNA (Thermo Fisher Scientific, Waltham, Ma, USA). The qPCR reaction mixture contained 2 μL of eluted DNA, 1 μL (final concentration 0.2 μm) of each forward and reverse primer of LINE-1 79bp, 5 μL of UltraSYBR Mixture (Cwbiotech, Beijing, China), and 1 μL of double-distilled water. Cycling conditions were 1 minute at 95°C and 35 cycles of 95°C for 8 seconds and 60°C for 15 seconds. The qPCR reaction was performed in triplicate and the mean of the triplicates were used for further analysis. Each plate contained as a plasma DNA sample, a water template (negative control) and 7 serially diluted standard DNA solutions. The cfDNA level of each sample was detected based on standard curve by using the $2^{\Delta\Delta Ct}$ method.

**Statistics analysis**

Statistical analysis was performed in R statistical environment (version 3.4.0), including Mann-Whitney U test, Chi-Square test, and ROC (receiver operating characteristic, Bioconductor ROC package). A $p$-value lower than 0.05 was considered statistically significant.

**Results**

**Demographic and clinical characteristics**

Eligible 116 high risk NB pediatric patients at maintenance treatment were enrolled (Table 1). All 7 high risk patients younger than 18 months were detected amplification of MYCN gene. Ninety-four
percent of patients were older than 18 months, one of those with amplification of MYCN gene was classified as stage III by INSS and others as stage IV. There was no significant difference of proportion between female and male. At initial diagnosis, 80.2% of the patients had primary tumor site in abdomen, 17.2% in thorax, and 2.6% in others. Around 75.9% of the patients had NSE level blow 370 ng/ml. while 75.9% of the patients were detected LDH concentration between 500 IU/L and 1500 IU/L, 12.1% lower than 500 IU/L, and others higher than 1500 IU/L. Remarkably, 50.9% of the patients had less than 3 organs with metastasis, 33.6% three organs, and 15.5% more than three organs. The most frequent metastatic sites were bone, bone marrow and distant lymph node, accounting for 72.4%, 62.1% and 65.5% respectively.

**Recurrence during maintenance treatment**

All 116 patients stepped into maintenance treatment after evaluation, when their multiple-disciplinary treatments finished, with results that two independent microscopic examinations confirmed no NB cells in bone marrow, independent radiological experts confirmed no progression via radiography, LDH and NSE were down-regulated, and $^{131}$I- MIBG scan negative.

During maintenance treatment, recurrence was diagnosed by positive microscopic examinations of bone marrow and tumor finding in situ or metastasis via radiography. A total of 36 patients developed recurrence during maintenance treatment (Table 2). Five cases occurred in the first-three months with 13-cis-retinoic acid, and 26 cases in the next 6~9 months. Other 5 cases happened after 13-cis-retinoic acid was stopped.

**Analysis of cfDNA level in recurrence and event-free patients**

As presented in Figure 1, plasma cfDNA concentration of recurrence patients at time point immediately ahead of diagnosis of recurrence was significantly higher than the average level of event-free patients during a whole maintenance treatment (median, 29.34 ng/ml VS 10.32 ng/ml, $p<0.001$). Whereas the baseline comparison showed that the average cfDNA level of recurrence patients pre-peak concentration (pre-recurrence) was not significantly different from the average level of events free patients during a whole maintenance treatment (median, 9.75 ng/ml VS 10.32 ng/ml, $p>0.05$, Figure S1).
Nineteen recurrence patients had cfDNA peak level higher than the median of 29 ng/ml (Table 3). Time span from time point of cfDNA peak level to diagnosis of recurrence ranged from 0 to 3 months, at mean of 0.55 months. This suggested that high level of plasma cfDNA could potentially be a timely molecular marker to alert recurrence.

The ROC analysis showed that AUC (area under ROC curve) was 0.825, optimal sensitivity and specificity of 80.6% and 71.3% respectively, at cfDNA level of 12.93 ng/ml (Figure 2). It suggested that high level of plasma cfDNA was significantly correlated with recurrence disease. The consistence between high level of cfDNA and NB recurrence elucidated the correlation between heavier tumor burden and high level of cfDNA.

**Recurrence among subgroups of NB patients during maintenance treatment**

All patients were classified into three subgroups (i.e., CR, PR, SD) based on the evaluation of therapeutic response to multiple-disciplinary treatment before they stepped into maintenance treatment. As shown in Table 4, 12% of CR patients developed recurrence during maintenance treatment, 45.5% in PR and 45.5% in SD subgroups. The recurrence rate of CR patients was significantly lower than in PR or SD. Meanwhile, the time span from beginning of maintenance to diagnosed recurrence in CR subgroup was significantly longer than it in PR or SD (mean, 17.52 months VS 10.97 and 6.64 months respectively).

**Discussion**

Neuroblastoma is one of the most common cancer in children, with 7% increment of incidence during 10 years.[22, 24] Although clinical therapy including radiological imaging, cytological, biochemical and molecular techniques, et cetera, progressed immensely, long-term survival rate of high risk NB patients was still below 50%.[8, 25, 26] Many factors contributed to this disappointed result, and the most notable one was chemo-resistant minimal residual disease which caused recurrence in more than half of high risk NB patients.[2, 14, 24, 27] Therefore, it is on top priority for physicians to identify minimal residual disease accurately, to evaluate therapeutic response reliably, and to establish alerting system effectively.

Nowadays many studies focused on finding promising biomarkers to evaluate prognosis or response
to treatments in cancer therapy. Plasma cfDNA as a potential biomarker in clinic was investigated profoundly recent years, especially in malignant metastatic cancers.[28] Compared with tissue-based histological or imaging examinations which were liable to sampling bias and low repeatability, plasma cfDNA enabled a minimally invasive monitoring of tumor burden at a molecular level, as well as a valuable biomarker for clinical oncology and molecular pathology.[29] It had been demonstrated that quantification of cfDNA could reflect tumor burden in a way that the level of plasma cfDNA was significantly higher in cancer patients than in patients with benign disease.[30] Moreover, the detection of plasma cfDNA could assist evaluating therapeutic response and prognosis of a wide range of cancers such as non-small cell lung cancer, gastrointestinal tract malignancy, advanced non-small cell lung cancer, lung cancer, et cetera.[16, 31-33]

Our previous study demonstrated that plasma cfDNA level was highly correlated with tumor burden in NB children, and could potentially serve as a more effective biomarker compared with LDH widely used in clinic.21 Furthermore, plasma cfDNA concentration was significantly down-regulated in partial remission patients than in stable disease, and dynamically associated with tumor burden in response to chemotherapy.[34] However, whether cfDNA could serve as an effective molecular marker for recurrence had yet to clarify.

Conclusions
In our current work, we found that plasma cfDNA level of recurrence patients at time point immediately ahead of diagnosis of recurrence was significantly higher than the average level of event-free patients during a whole maintenance treatment (median, 29.34 ng/ml VS 10.32 ng/ml, \(p<0.001\), Figure 1). While the average cfDNA level of recurrence patients before time point of peak level had no significant difference from the average level of event-free patients during a whole maintenance treatment (median, 9.75 ng/ml VS 10.32 ng/ml, \(p>0.05\), Figure S1). Then we investigated whether high level of cfDNA had the potential to serve as an effective molecular marker to alert recurrence in advance. As shown in Table 3, time span from time point of cfDNA peak level (more than 29 ng/ml) to diagnosis of recurrence ranged from 0 to 3 months, at mean of 0.55 months. This indicated that plasma cfDNA could serve as a promising molecular marker to alert recurrence
disease in NB patients. The performance of ROC analysis showed that AUC was 0.825, optimal sensitivity and specificity of 80.6% and 71.3% respectively, at cfDNA level of 12.93 ng/ml (Figure 2). It further suggested that high level of cfDNA was significantly correlated with heavier NB tumor burden, such as in recurrent disease.

In conclusion, high concentration of plasma cfDNA could be one promising timely molecular marker to alert recurrence disease for high risk NB patients during maintenance treatment, at least an effective assistance in clinic.

Abbreviations

cfDNA: cell free DNA
NB: neuroblastoma
ROC: receiver operating characteristic
AUC: area under ROC curve
LDH: lactate dehydrogenase
NSE: neuron specific enolase
MRD: minor residual disease
CR: complete remission
PR: partial remission
SD: stable disease
PD: progressive disease

Declarations

Acknowledgement

The authors thank the patients for their participation in this study.

Authors’ contributions

XLM and ZY designed the study. XLM, YS and LJW conceptualized the study. All authors contributed in executing collection, analysis of the data, and writing of the manuscript. All authors agreed to be responsible for all aspects of the study. All authors read and approved the final manuscript.

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Availability of data and materials

The raw data available upon reasonable request from the corresponding authors

Ethical approval and consent to participate

This research was approved by the Beijing Children’s Hospital Institutional Ethics Committee (No. 2016-65). It was conducted in accordance with the principles of the Declaration of Helsinki. All parents or guardians of each patient provided written informed consent.

Consent for publication

Written informed consent for publication of their clinical details was obtained from each patient’s parents or guardians.

Competing interests

The authors declare that there is no conflict of interest.

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Tables
Due to technical limitations the tables are available only as downloads in the Supplementary Files.

Additional File
**Figure S1.** The levels of cfDNA between pre-recurrence and event-free NB patients. The $p$ value was >0.05, analyzed by Man-Whiney U Test.

Figures
The level of cfDNA in NB patients

Figure 1

The level of cfDNA between NB patients in recurrent and event-free cases. The p value was <0.001, analyzed by Mann-Whiney U Test).
The performance of cfDNA to evaluate recurrent disease in NB patients.

Supplementary Files
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