Level of seven neuroblastoma-associated mRNAs detected by droplet digital PCR is associated with tumor relapse/regrowth of high-risk neuroblastoma patients

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学 位 論 文 の 内 容 要 旨

Level of seven neuroblastoma-associated mRNAs detected by droplet digital PCR is associated with tumor relapse/regrowth of high-risk neuroblastoma patients

デジタル PCR で測定した 7 種の神経芽細胞腫関連 mRNA の発現量は

高リスク神経芽腫患者の再発/再増大と関連する

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【Background】

Neuroblastoma (NB) is the most common extracranial solid tumor in children and characterized by its extreme heterogeneity ranging from spontaneous regression to malignant progression. Approximately half of patients have high-risk disease, whose long-term survival remains as low as 40%. Although intensive multimodal treatment has been introduced over the past decades, 50% of high-risk patients still experience tumor relapse/regrowth due to chemoresistant minimal residual disease (MRD). MRD is conceptually defined as residual tumor cells that persistently reside in cancer patients following local and systemic cancer therapies. It persists as cancer stem cells (CSCs) in primary tumor, circulating tumor cells (CTCs) in peripheral blood (PB), and disseminated tumor cells (DTCs) in bone marrow (BM), lymph node, and micrometastasis in other tissues. These CSC, CTC, and DTC represent the dynamics of MRD in cancer patients. Due to the invasive nature of surgical biopsies, the detection and analysis of CTC and DTC by less invasive sampling of BM and PB has been shown to be of clinical relevance in many cancer types. To improve the outcome of high-risk NB patients, the accurate and sensitive detection of MRD is essential to monitor the disease burden and treatment response. Although several quantitative PCR (qPCR)-based MRD assays detecting a set of neuroblastoma-associated mRNAs (NB-mRNAs) are reported to have some prognostic values for NB patients, their clinical significance is not yet clear.

【Purpose】

To develop the MRD assay that has a significant prognostic value for predicting relapse/regrowth of high-risk NB patients.

【Patients and methods】

Non-NB control samples: 103 BM samples from non-NB controls were purchased from commercial sources. 107 PB samples from non-NB controls were obtained from Japanese Red Cross Society (JRCS).
NB patients and samples: 208 bone marrow (BM) and 67 peripheral blood (PB) samples from twenty high-risk NB patients treated at Kobe Children Hospital or Kobe University Hospital between June, 2011 and January, 2018.
Disease evaluation: Response was graded at every BM and PB sampling time point for computed tomography (CT)/magnetic resonance imaging (MRI), MIBG (metaiodobenzylguanidine), and BM assessments based on the available medical records, and these were combined into an overall response. Evaluation was conducted in accordance with the International neuroblastoma response criteria (INRC). Disease status for all BM and PB samples were then assigned to remission, stable, or progression according to BM and overall response.
RNA isolation and cDNA synthesis: Total RNA was extracted from mononucleated cells and cDNA was synthesized according to manufacturer's instructions. All total RNA and cDNA samples were stored at -80°C (for a median of 1 year, range 0–7 years).
Droplet digital PCR (ddPCR): ddPCR was performed using a QX200 ddPCR system (Bio-Rad Laboratories, Hercules, CA). To correct for differences in the amount of total RNA and efficiency of cDNA synthesis, target copy number was normalized using hypoxanthine phosphoribosyl transferase 1(HPRT1) as an endogenous reference. ddPCR analysis was performed in accordance with the digital MIQE guidelines.
Real-time PCR (qPCR): qPCR was performed using an ABI 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA). To correct for differences in the amount of total RNA and in the efficiency of cDNA synthesis, β2-microglobulin (*B2M*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and *PGK1* (phosphoglycerate kinase 1) were used as

endogenous references for normalization. The qPCR analysis was performed in accordance with the MIQE guidelines.
Level of each NB-mRNA and 7NB-mRNAs in ddPCR: Level of each NB-mRNA (each signature) was defined as the relative copy number of each NB-mRNA (each NB-mRNA copy number divided by HPRT1 mRNA copy number and multiplied by 10,000). Level of 7NB-mRNAs (combined signature) was defined as the weighted sum of 7 relative copy numbers (level of each NB-mRNA). The reciprocal of 90 percentile in non-NB control samples was used for the weighting for each NB-mRNA. For a direct comparison between ddPCR and qPCR, level of 7NB-mRNAs was defined as the geometric mean of 7 relative copy numbers (level of each NB-mRNA).
Statistical analysis: Differentiation of level of NB-mRNAs between two sample groups were evaluated using Mann-whitney U test. The diagnostic and prognostic values of level of each NB-mRNA and 7NB-mRNAs was assessed using receiver operator characteristic (ROC) analysis. $p < 0.05$ was considered statistically significant.

【Results】

Level of each NB-mRNA (CRMP1, DBH, DDC, GAP43, ISL1, PHOX2B, and TH mRNA) in BM and PB

In order to achieve the more robust detection of low-level of NB-mRNA than qPCR-based MRD monitoring, we used CRMP1, DBH, DDC, GAP43, ISL1, PHOX2B, and TH mRNA for ddPCR-based MRD monitoring in the present study. Although these NB-mRNAs were selected for no or little expression in normal cells by qPCR, ddPCR detected them in 45.6-93.2% and 10.3-78.5% of non-NB control BM and PB samples, respectively. Level of each NB-mRNA in BM and PB varied greatly between individuals and collection time points, it was all significantly higher in diagnosis (24 BM and 10 PB samples collected from NB patients at diagnosis) than control (103 BM and 107 PB samples obtained from non-NB controls).

Level of 7NB-mRNAs in BM and PB

In BM, its significant difference was detected in every combination of different collection time points (control, diagnosis, treatment, post-treatment, and relapse) except for the control-post-treatment combination. In PB, it only showed a significant difference in the combinations containing control or diagnosis other than the control-post-treatment combination. Level of 7NB-mRNAs in BM and PB samples also changed with disease status. In BM, it was significantly differed in every combination of disease status (control, remission, stable, or progression) except for the control-remission combination. In PB, it was also significantly differed in every combination other than the control-remission and remission-stable combinations.

Association between level of 7NB-mRNAs in BM and tumor Relapse/regrowth

In 73 post-treatment samples, 17 relapse samples expressed significantly higher level of 7NB-mRNAs than 56 non-relapse samples, suggesting a significant association between level of 7NB-mRNAs in BM and tumor relapse/regrowth.

Prognostic values for qPCR and ddPCR detection of 7NB-mRNAs

Although ddPCR potentially provides more simple and reproducible detection of low-level of NB-mRNAs than qPCR, their prognostic values have never been compared in the same samples from NB patients. Level of each NB-mRNA (CRMP1, DBH, DDC, GAP43, ISL1, PHOX2B, and TH mRNA) in the same 73 (56 non-relapse and 17 relapse) post-treatment BM samples was determined by qPCR. ROC curve was then plotted for level of qPCR 7NB-mRNAs (geometric mean) and estimated threshold value (TV) of 16.23 and AUC of 0.594. To make a direct comparison between qPCR and ddPCR, ROC curve was also plotted for level of ddPCR 7NB-mRNAs (geometric mean) and estimated TV of 3.31 and AUC of 0.706 with the significant accuracy (AUC > 0.7). Level of ddPCR 7NB-mRNAs (weighted sum) resulted in the further improved estimation of TV of 3.15 and AUC of 0.723 with the significant accuracy (AUC > 0.7).

【Discussion】

In the present study, we determined the level of CRMP1, DBH, DDC, GAP43, ISL1, PHOX2B, and TH mRNA in BM and PB by ddPCR for evaluating MRD in high-risk NB patients. To our knowledge, this is the first study of ddPCR-based MRD monitoring in NB patients and reveals that ddPCR detection of 7NB-mRNAs in BM has a significant prognostic value in predicting tumor relapse/regrowth of high-risk NB patients. ddPCR is the most sensitive method to measure the abundance of specific nucleic acids and potentially provides a more accuracy and reproducibility in quantitating NB-mRNAs compared to qPCR. To effectively make the most of the advantage of ddPCR over qPCR, we carefully validated HPRT1 mRNA as an endogenous reference for NB-mRNA and prepared the qualified cDNA template. As a result, the present results clearly demonstrated for the first time that ddPCR detection of 7NB-mRNAs estimated a higher AUC (> 0.7) than qPCR detection of 7NB-mRNAs.

【Conclusion】

In conclusion, ddPCR is a simple and reproducible method to detect low-level of NB-mRNAs in BM and PB samples collected from high-risk NB patients. High level of 7NB-mRNAs in BM detected by ddPCR predicts tumor relapse/regrowth in high-risk NB patients. ddPCR-based MRD monitoring would improve detection of MRD, evaluation of disease status and treatment response, and prediction of prognosis in high-risk NB patients.

神戸大学大学院医学(系)研究科（博士課程）

論文審査の結果の要旨			
受付番号	甲 第2928号	氏 名	KHIN KYAEMON THWIN
論文題目 Title of Dissertation	デジタルPCRで測定した7種の神経芽細胞種関連mRNAの発現量は高リスク神経芽細胞種の再発/再増大と関連する Level of seven neuroblastoma-associated mRNAs detected by droplet digital PCR is associated with tumor relapse/regrowth of high-risk neuroblastoma patients		
審査委員 Examiner	主 査 Chief Examiner 菊 榊信 副 査 Vice-examiner 井生康一 副 査 Vice-examiner 鴨本秀樹		

（要旨は１，０００字～２，０００字程度）

Introduction

Chemoresistant minimal residual disease (MRD) leads to relapse/regrowth in 50% of patients with high-risk neuroblastoma (NB). The accurate and sensitive detection of MRD is essential to monitor the disease burden and treatment response. Predictability of recurrence by droplet digital PCR (ddPCR)-based MRD monitoring was investigated in the present study.

Methods

From 20 patients with high-risk NB, 208 bone marrow (BM) and 67 peripheral blood (PB) samples were obtained, and RNA was extracted from mononucleated cells for cDNA synthesis. Response was evaluated according to the International neuroblastoma response criteria (INRC) at every BM and PB sampling time point. Disease status for all BM and PB samples were assigned to remission, stable, or progression. Among NB-related mRNA, 7 mRNAs (CRMP1, DBH, DDC, GAP43, ISL1, PHOX2B and TH) were selected and tested for MRD using ddPCR (Bio-Rad Laboratories, Hercules, CA) where target copy number was normalized using hypoxanthine phosphoribosyl transferase 1 (HPRT1) as an endogenous reference. Real-time PCR (qPCR) was performed in accordance with the MIQE guidelines using an ABI 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA). β2-microglobulin (B2M), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase 1 (PGK1) were used as endogenous references for normalization. Difference in NB-mRNA levels between two sample groups were evaluated using Mann-Whitney U test. The diagnostic and prognostic values of level of each NB-mRNA and 7NB-mRNAs were assessed using receiver operator characteristic (ROC) analysis.

Results

Although qPCR detected no or little expression of CRMP1, DBH, DDC, GAP43, ISL1, PHOX2B, and TH mRNA in normal cells, they were detected in 45.6-93.2% and 10.3-78.5% of non-NB control BM and PB samples, respectively, by ddPCR. They were all significantly higher at diagnosis than control. Level of 7NB-mRNAs was decreased by treatment in both BM and PB, and it was increased at relapse in BM. In 73 post-treatment samples, 17 relapse samples expressed significantly higher level of 7NB-mRNAs than 56 non-relapse samples. Furthermore, it had a better prognostic value than qPCR in the same 73 post-treatment BM samples.

Conclusion

Present study suggests that ddPCR-based MRD monitoring is significantly associated with tumor relapse/regrowth of high-risk NB, and advanced the field of knowledge in the area of MRD in NB. Therefore, the candidate is certainly recognized as having qualified for the degree of Ph.D.(Medicine).