PCR-based amplification of circulating RNAs as prognostic and predictive biomarkers – Focus on neuroblastoma ☆

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ABSTRACT

Metastatic disease is a major challenge for cancer cure, haematogenous spread and subsequent growth of tumour cells at distant sites being the cause of most cancer deaths. Molecular characterization and detection of the tumour cells responsible for haematogenous spread may increase understanding of the biology of metastasis, help improve patient management and allow evaluation of novel treatments to prevent and eradicate this disease. The bone marrow is a common site to which tumour cells metastasize, from which they may re-circulate to other organs with a favourable microenvironment for growth. The detection of tumour cells in blood suggests one route for metastasis, and provides an accessible, minimally invasive liquid sample through which it may be possible to monitor and detect minimal disease and early signs of metastasis. Significant improvements in the sensitivity and specificity of tumour cell detection have been made, such that it is now possible to unambiguously detect a single tumour cell in over 10 million normal cells. However, the clinical impact of such low level disease and how to interpret the natural variation that can arise from sequential sampling of bone marrow aspirates and blood is currently largely unknown. This commentary will focus on the technical advancements and application of reverse transcriptase polymerase chain reaction to detect cancer mRNAs in bone marrow and blood, and discuss the potential clinical impact of this test in neuroblastoma.

1. Background

Real-time quantitative reverse transcriptase polymerase chain reaction (RTqPCR) has revolutionized many molecular diagnostic tests by adding reliability, speed, high throughput, reduced frequency of contamination and a broad quantitative dynamic range for many RNA-based tests. This highly sensitive technique has successfully been used to detect disseminating or circulating tumour cells in many different cancer types with objective high precision in this commentary, tumour cells in the bone marrow are referred to as disseminating tumour cells and in the blood as circulating tumour cells. To reliably detect tumour cells using this approach, suitable mRNAs that can be specifically amplified in tumour cells but which have little or no expression in the normal cells of blood or bone marrow are needed. Since only viable circulating tumour cells produce mRNA, and mRNA from apoptotic or dying cells is rapidly degraded in blood products, RTqPCR for tumour mRNAs has become one of the most widely used methods for detecting circulating and disseminating tumour cells. However the amount of mRNA within a cancer cell can vary and,

consequently, it is not possible to precisely enumerate circulating tumour cells using RTqPCR. Nevertheless, with a robust cancer mRNA target, specific RTqPCR assay and precautions to prevent contamination of samples with PCR products, RTqPCR can objectively detect a single tumour cell in over ten million normal cells. Therefore the quantitative detection of minimal disease, and circulating or disseminating tumour cells over a wide dynamic range is possible.

Tumour specific mRNAs are ideal targets for the detection of circulating tumour cells since they are expressed solely by the tumour, and therefore minimise any risk of false positive results. This is the case for cancers with identified gene rearrangements such as the haematological cancers (e.g. myeloid leukaemia, in which the first tumour specific translocation BCR-ABL was described [1,2]). Tumour specific translocations have also been described in some solid malignancies, including many sarcomas. Some of these translocations have been used to detect circulating and disseminating tumour cells, for example EWS-ETS gene transcripts in tumours of the Ewing's sarcoma family [3–6], SYT-SSX in synovial sarcoma [7] and PAX3-/7-FOXO1 fusions in alveolar rhabdomyosarcoma [8,9]. In contrast, there is a large group of translocation-dependent sarcomas that have unexplored tumour-specific candidates for molecular surveillance of disseminated and circulating disease using RTqPCR [10]. However, for most solid cancers, tumour-specific mRNA targets have not been identified, driving the search to identify and validate tissue-of-origin specific mRNA targets. RTqPCR for tissue-of-origin specific mRNAs has successfully been applied for the detection of clinically significant circulating and disseminating tumour cells in a wide range of solid cancer types [11–15], including the childhood cancer neuroblastoma.

2. Neuroblastoma

Neuroblastoma is the most common extra-cranial solid cancer of childhood and accounts for approximately 40% of malignancy diagnosed in the first 4 years of life and 15% of cancer deaths in children. The disease has a heterogeneous clinical course ranging from spontaneous regression to incurable high-risk disease. One of the most informative predictors of poor outcome in children over the age of 6 months is the presence of metastatic disease at diagnosis [16], which informs initial staging and subsequent treatment. The majority of children present with high-risk unresectable metastatic disease. Furthermore, neuroblastoma cells within the bone marrow can directly contribute to disease recurrence and relapse [17]. While a number of multimodal treatments have anti-tumour activity in these children, survival rates for those with high-risk metastatic disease remain low with 15–20% of children having tumours that are resistant to induction therapy. Furthermore, 40% of those who attain complete or partial remission will relapse and die within 5 years of diagnosis. Clearly, new treatment approaches are needed to improve outcome, though progress has been slow in part reflecting a paucity of information on drug-resistant disease in the bone marrow and lack of clinically informative methods to monitor and assess this disease to enable a better choice of risk-adapted therapy.

The current gold standards for assessment of metastatic disease in children with neuroblastoma are imaging using metaiodobenzylguanidine (mIBG) [18] and examination of bone marrow aspirates or biopsies using cytology or immunohistochemistry respectively [19,20]. For initial staging at diagnosis these methods are recommended, although they have limited sensitivity and do not reliably quantify the minimal disease that can lead to progression and relapse. Consequently, there has been much work to develop and improve PCR-based assays to increase the sensitivity and specificity of neuroblastoma cell detection in bone marrow and blood, with the expectation that detection of minimal disease prior to overt clinical symptoms will provide an opportunity for earlier clinical intervention and improve outcome for some children.

3. Clinical application of PCR-based amplification of neuroblastoma mRNAs

In the absence of a tumour-specific mRNA and since neuroblastomas produce catecholamines, the rate-limiting enzyme in catecholamine biosynthesis (tyrosine hydroxylase) was the first mRNA amplified to detect neuroblastoma cells in blood and bone marrow [21–23]. Amplification of tyrosine hydroxylase mRNA was also shown to detect neuroblastoma cells in bone marrow aspirates [21,23], although low levels of the mRNA in normal bone marrow cells initially limited the specificity of this approach. The application of quantitative RT-PCR (RTqPCR), made it possible to quantify the number of mRNA transcripts and consequently allow an accurate relative measure of tumour content within and between clinical samples. This has the added advantage that if an mRNA is expressed at low levels in the sample that is being analysed it is possible to define a normal expression range, and subsequently identify a threshold above which the level of mRNA can be used to reliably identify tumour cells.

In a proof-of-principle trial, tyrosine hydroxylase mRNA detected in blood taken at diagnosis from children with high-risk disease was shown prospectively to identify children who had a three-fold increased risk of an event, compared to children in which tyrosine hydroxylase mRNA was not detected [24]. High levels of tyrosine hydroxylase mRNA in bone marrow from children with metastatic disease have also been shown to predict for worse outcome [25–27]. Additional neuroblastoma mRNAs have prognostic and predictive value in blood and bone marrow, the most promising emerging single marker, in our opinion, being paired-like homeobox 2b (PHOX2B) [28]. Given the heterogeneity of neuroblastoma, several groups have reasoned that multiple neuroblastoma mRNAs may be more informative than a single mRNA [29,30]. This is, however, target mRNA [31,29,26] and compartment [32] dependent.

Since the bone marrow is a frequent reservoir for drug refractory disease and site of neuroblastoma relapse, we and others have hypothesised that RTqPCR for neuroblastoma mRNAs in bone marrow aspirates may provide an informative quantitative tool to monitor the level of disseminated disease and the impact of treatment on that disease. Consistent with this hypothesis, high levels of neuroblastoma mRNAs (tyrosine hydroxylase and PHOX2B) in bone marrow at diagnosis and at the end of induction therapy prospectively predict poor outcome [27], paving the way for their introduction into bone marrow response criteria. A rapid decrease in the level of 1 of 5 neuroblastoma mRNAs (PHOX2B, tyrosine hydroxylase, DDC, GAP43, CHRNA3) in bone marrow during induction therapy is also associated with a better outcome [33]. Similarly, in the minimal disease setting, a reduction in neuroblastoma mRNAs (PHOX2B, B4GALNT1, CCND1, ISL1) after two cycles of im-munotherapy is reported to be an early response marker [34]. If validated, changes in the level of neuroblastoma mRNAs within the bone marrow during therapy might be used to guide treatment decisions for children with high-risk neuro-blastoma in real-time, which may mean children that do not respond sufficiently well may be offered an alternative (po-tentially more effective) treatment, or at least may be spared unnecessary treatment-related toxicity. If such assays reliably predict relapse, it may be possible to use neuroblastoma mRNAs as surrogate end-points to evaluate the activity of novel agents or treatment protocols to eradicate bone marrow disease, speeding the anticipated improvements in outcome from the use of new effective agents introduced into the treatment pathway.

Although neuroblastoma mRNAs in blood are detected over a narrower dynamic range than in bone marrow aspirates [27], blood remains a more convenient and potentially cost efficient compartment for repeated sampling. This may be particularly attractive for monitoring low level disease, and potentially follow up of children with neuroblastoma after treatment. Preliminary studies suggest that the early detection of the neuroblastoma mRNA tyrosine hydroxylase in blood can predict overt clinical disease and relapse [24,35], although this requires validation. Whether this translates into patient benefit will depend on the association between the detection or the level of neuroblastoma mRNAs in blood and outcome, and if more effective treatments to eradicate this disease are available. The predictive power of low level neuroblastoma mRNAs in blood is currently unclear, and is challenged by recent studies in children with localised disease, where the detection of selected neuroblastoma mRNAs did not consistently reflect the presence of active systemic disease or predict outcome [36,37]. Whether this reflects non-specificity of some of the neuroblastoma mRNAs in the panel, or low-level expression in normal cells that could be overcome by establishing predictive cut-points remains to be seen. Consistent with the hypothesis that high levels of neuroblastoma mRNAs in blood are prognostic [24], children with levels of the neuro-blastoma mRNAs tyrosine hydroxylase or PHOX2B which are above respective derived cut-points in blood at diagnosis have an exceptionally poor event-free survival, identifying 1 in 5 children that succumb to their disease within five-years of diagnosis [27]. This compares to a five-year event-free survival of 25% (95% CI 16–36%) for the remaining children. This observation suggests that children with drug-refractory disease might be identified using RTqPCR for tyrosine hydroxylase or PHOX2B mRNA in blood at diagnosis, and offered potentially more effective treatment from the start.

4. Conclusions and future perspectives

There is substantial evidence that RTqPCR for tumour- or tissue-of-origin specific mRNAs detect clinically relevant tu-mour cells in many haematological and solid cancer types, demonstrating the suitability of this approach to measure dis-seminated or circulating disease. PCR-based methods are already a part of disease assessment and monitoring in some paediatric and adult leukaemias [38]. However, the introduction of these assays into management of patients with solid cancers appears, in comparison, to be lagging behind. This may be a consequence of uncertainty on how to interpret the natural variation in results that can arise from sequential sampling of bone marrow aspirates and blood, and the challenge of validating the conditions under which specific target mRNAs (alone or in combination) could confidently be used to assign patients to different treatment groups. It certainly reflects the limited number of prospective quality-controlled studies evaluating the clinical significance of disseminated and circulating tumour cells detected by RTqPCR, and the consequent lack of a robust assessment of the independent and comparative value of these mRNAs compared to current clinical bio-markers for disease assessment and prognostication. For children with neuroblastoma there is an urgent requirement for a robust comparison of the clinical validity of RTqPCR for neuroblastoma mRNAs with mIBG imaging and cytology and im-munohistochemistry for bone marrow disease [27]. New genetic and epigenetic profiles of circulating and disseminated tumour cells might add additional insight into the metastatic process and effectiveness of therapy, identifying additional candidate circulating biomarkers that may increase the prognostic and predictive power of current mRNA targets for clinical outcome. The results from well powered quality-controlled prospective clinical outcome studies are now needed to assess the independent and concordant prognostic and predictive value of these circulating biomarkers.

In our opinion, the use of quantitative assessment of mRNAs in blood as a minimally invasive compartment for as-sessment of disease status and response in real-time is particularly appealing, with attractive logistical, financial and clinical advantages. The immediate challenge is to ensure that the methods for measuring and reporting of circulating tumour mRNAs in samples are standardised to allow comparison of results across trials [39,20], with the expectation that this will inform evidence-based adoption of standardised tests into appropriate pathways of care, contributing to prognostic and predictive algorithms of risk, and evaluation of novel therapeutic approaches, with the anticipated improvement of outcome.

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