Neuroblastoma is the most common extracranial solid tumor of childhood and accounts for 10% of all cancers in children. The development of neuroblastoma involves both embryonic and tumorigenic factors, making the tumor very heterogeneous both biologically and clinically. Neuroblastoma tumors originate from embryonic neural crest cells committed to the development of the sympathetic nervous system, and their malignancy varies from totally benign with spontaneous regression to highly malignant (1). Prognostic factors include age of the patient at diagnosis, INSS (International Neuroblastoma Staging System) stage, tumor histopathology, and ploidy status of DNA content (2). Despite the availability of many clinical and biologic markers proposed as predictive of disease outcome and the use of advanced multimodal therapy, treatment results are still suboptimal for patients with these tumors. Thus, how to best estimate patient prognosis and choose optimal individual therapy remain fundamental challenges.

Since the discovery by Moss et al. (3) 20 years ago of neuroblastoma cells in the blood of a majority of patients with known disseminated disease, much work has been devoted to the development and improvement of methods for detection of minimal residual disease (MRD).2

In this issue of Clinical Chemistry Stutterheim et al. (4) address the question of how to best identify the presence of neuroblastoma cells in blood and bone marrow by use of reverse transcription quantitative PCR (RQ-PCR). The authors recently investigated the use of paired-like homeobox 2b (PHOX2B)3 as a marker for MRD in neuroblastoma and claimed reported that this transcript was the most specific single marker for this purpose (5). In the current study they selected 28 genes from SAGE libraries that showed high expression in neuroblastoma tumors and little or no expression in normal tissues. Preliminary studies of these 28 genes and 5 previously described markers (identified by using RQ-PCR and SYBR Green for analysis) showed that 10 markers had high expression in neuroblastoma tumors and little or no expression in control bone marrow. These 10 markers were selected for further study, together with GD2 synthase (β-1,4-N-acetyl-galactosaminyl transferase 1) for comparison. Investigation of this 11-marker panel with RQ-PCR and the use of specific probes demonstrated that the various transcripts were all positive but highly variable in 56 neuroblastoma tumors. Based on the results from control blood (n = 37) and bone marrow (n = 51) samples, 5 candidate genes were excluded because they were expressed in both the tumor and control tissues; 6 candidate genes remained for further studies. Using 222 blood and bone marrow samples from neuroblastoma patients, Stutterheim et al. identified 2 panels as most suitable for bone marrow and blood, respectively. Both panels contained PHOX2B, tyrosine hydroxylase (TH), dopa decarboxylase (aromatic L-amino acid decarboxylase) (DDC), and cholinergic receptor, nicotinic, alpha 3 (CHRNA3). Growth associated protein 43 (GAP43) was included in the bone marrow panel, and dopamine beta-hydroxylase (dopamine beta-monoxygenase) (DBH) was included in the blood panel.

The DNA-associated protein encoded by the PHOX2B gene is a member of the paired family of homeobox proteins localized to the nucleus. This protein is a transcription factor involved in the development of several major noradrenergic neuron populations and plays a key role in the early differentiation of the sympathico-adrenal lineage from neural crest cells. This protein also is a well-established intrinsic factor in developing autonomic ganglia, where its expression is triggered by the bone morphogenic proteins secreted by the dorsal aorta (6). How PHOX2B exerts its pleiotropic functions, both as a proneural gene and a neuronal subtype determinant remains unknown. In PHOX2B-null mutant mice, insufficient proliferation and increased cell death of sympathico-adrenal pro-

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2 Nonstandard abbreviations: MRD, minimal residual disease; RQ-PCR, reverse transcription quantitative PCR; TH, tyrosine hydroxylase.

3 Human genes: PHOX2B, paired-like homeobox 2b; TH, tyrosine hydroxylase; DDC, dopa decarboxylase (aromatic L-amino acid decarboxylase); CHRNA3, cholinergic receptor, nicotinic, alpha 3; GAP43, growth associated protein 43; DBH, dopamine beta-hydroxylase (dopamine beta-monoxygenase); DCK, doxicleorticin; CCNDI, cyclin D1; GABRR3, gamma-aminobutyric acid (GABA) A receptor, beta 3; DL1, ISL LIN homeobox 1; KIF1A, kinesin family member 1A; GUSB, glucuronidase, beta; HPRRT, hypoxanthine phosphoribosyltransferase 1; SDHA, succinate dehydrogenase complex, subunit A, flavoprotein (Fp); B2M, beta-2-microglobulin.
genitor cells leads to failure to form autonomic ganglia or functional adrenal medullas and produce noradrenalin. Also, expression of tyrosine hydroxylase (TH) and dopamine-β-hydroxylase, key enzymes of the noradrenalin synthesis route, is largely absent in PHOX2B-null mutant mice. A close signal connection seems to exist between PHOX2B, TH, DDC, and DBH, all of them being well expressed in neuroblastoma cells. It therefore makes sense that these transcripts would be well suited for the purpose of identification of MRD in neuroblastoma. The biology behind the suitability of CHRNA3 and GAP43 as targets for use in detection of MRD of neuroblastoma is less obvious and requires further investigation. One of the great merits of the study reported by Stutterheim et al. is that several transcripts, such as GD2 synthase, have been identified as less suitable for MRD detection of neuroblastoma, mainly because of nonspecific reaction with other cells in blood and bone marrow, a finding that is in accord with other reported results.

Two other recently reported studies were performed to identify suitable targets for inclusion in a panel for neuroblastoma MRD detection. In both of these investigations gene expression studies were performed by use of neuroblastoma tumors for selection of potential targets. Viprey et al. initially identified 240 potential targets by microarray analysis of 32 primary neuroblastomas and then selected 12 markers that were suitable for comparison with the gold standard TH. Nine of the 12 markers were found to be expressed in a pool of blood from healthy individuals, leaving the 3 markers TH, PHOX2B, and doublecortin (DCX) as suggested best transcripts. Cheung et al. applied genome-wide expression array analyses to 48 stage 4 neuroblastoma tumors. Excluding genes of ubiquitous nature and using 3 criteria as a filter, Cheung et al. selected 34 genes with tumor-to-marrow expression ratios better than TH to be of further interest. They performed sensitivity studies with use of 2 neuroblastoma cell lines and then studied the top 8 genes in a panel of normal blood and marrow samples to evaluate specificity. These genes were highly expressed in stage 4 tumors with little to no expression detected in normal samples. Six of the 8 markers [cyclic D1 (CCND1), DDC, gamma-aminobutyric acid (GABA) A receptor, beta 3 (GABRB3), ISL LIM homeobox 1 (ISL1), kinesin family member 1A (KIF1A), and PHOX2B] were highly prognostic in testing progression-free survival of neuroblastoma patients.

What can be learned from these studies? It is obvious that the 3 studies, all directed to finding the best panel for detection of MRD in neuroblastoma, have arrived at very different results. Only PHOX2B was found suitable for inclusion in the panel in all 3 studies. This gene is not expressed in blood or bone marrow of healthy control individuals, results that give this analyte high specificity for indicating neuroblastoma MRD. Furthermore, PHOX2B is thought to be an independent intrinsic factor, the expression of which is required for the formation of sympathetic and parasympathetic ganglia, and most PHOX2B transcriptional activity is sustained and maintained by an autoregulatory mechanism in which PHOX2B binds and transactivates its own promoter.

Why do the results differ so much between the studies? First, the results of the measurements cannot be considered quantitative. In each selection step the transcript concentrations have been normalized to the results of 1 or 2 reference (housekeeping) genes. The transcript concentration of these genes, however, may vary by several orders of magnitude between cells and tissues. The studies used different reference genes for this purpose: glucuronidase, beta (GUSB); hypoxanthine phosphoribosyltransferase 1 (HPRT1); xanthine phosphoribosyltransferase 1 (HPRT1); succinate dehydrogenase complex, subunit A, flavoprotein (Fp) (SDHA); and beta-2-microglobulin (B2M). The use of different reference genes introduces large variance between samples in each study as well as large interstudy variance. A second reason for differing results is that the procedures for selection of candidate genes varied greatly, as seen in these studies. Third, LAN1 and NMB7 cell lines were used in the study of Cheung et al. and the IMR 32 cell line was used in the study of Viprey et al. in the evaluation of sensitivity of detection by various transcripts. Differences in the cell lines used may account for some of the differences in results, because in various neuroblastoma cell lines the mean number of specific target transcripts per cell may vary widely. Recently Nezos et al. reviewed methodological pitfalls that may explain differences in results between studies using RQ-PCR in investigations of circulating tumor cells.

Has the time come to introduce detection of MRD by RQ-PCR into the clinical evaluation of neuroblastoma, particularly high-risk neuroblastoma? RQ-PCR is a technique of great analytical potential and wide utility in molecular medicine. The widespread use of RQ-PCR analysis for detection of disease-specific prognostic markers in leukemia patients provides an excellent example of the usefulness of this technique in diagnostics and treatment control. The rationale for use of RQ-PCR is far less apparent in investigations of disease-associated mRNA expressed by circulating tumor cells in patients with solid malignancies such as colorectal cancer, lung cancer, and melanoma, and at the present time RQ-PCR detection of MRD in neuroblastoma appears to fall in this category. However, further studies using this technology invariably may lead to new findings, as seen in the work of Stutterheim et al., who for the first time have reported
the expression of MRD transcript markers in CD34+ cells. The findings of Stutterheim et al. (4) are important because these investigators found all markers except PHOX2B to show at least some expression in CD34+ samples. Further studies may improve our options for characterizing MRD cells and enable us to explain why some seem to be more aggressive than others.

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