Short Communication

Use of Tumor-Specific Gene Expression for the Differential Diagnosis of Neuroblastoma from Other Pediatric Small Round-Cell Malignancies

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The differential diagnosis of neuroblastoma from other small round-cell tumors of childhood, although clinically of great importance, is sometimes difficult due to the almost indistinguishable appearance of such tumors by conventional microscopy. Because neuroblastomas are characterized by the synthesis of catecholamines, we investigated the possibility that expression of genes involved in this pathway could serve as a molecular marker for this disease. A reverse transcriptase polymerase chain reaction assay was used to analyze expression of tyrosine hydroxylase and dopa decarboxylase in 84 pediatric malignancies including 55 neuroblastomas, 6 Ewing's sarcomas/primitive neuroectodermal tumors, 7 lymphomas, 6 leukemias, 2 rhabdomyosarcomas, 6 osteosarcomas, and 2 pheochromocytomas. Of the 55 neuroblastoma samples analyzed, 54 expressed clearly detectable levels of both genes. The one sample that did not express either of the genes was rediagnosed both clinically and by molecular genetic analysis as a Ewing's sarcoma. Of the 29 non-neuroblastoma tumor samples examined, the only tumor samples that expressed clearly detectable levels of both tyrosine hydroxylase and dopa decarboxylase were pheochromocytomas. Like neuroblastomas, these tumors are characterized by high levels of catecholamines. These findings suggest that expression of genes involved in catecholamine biosynthesis may be useful for differentiating neuroblastoma from other small round-cell tumors of childhood. (*Am J Pathol* 1999, 155:17–21)

Neuroblastoma is the most common solid tumor of early childhood. Although patients with localized disease have a favorable prognosis, the majority of children with neu-

roblastoma present with metastases and have a poor outcome despite intensive multimodal therapy.¹ The accurate diagnosis of this disease and other pediatric malignancies has become increasingly important with the continued development of treatments tailored to specific tumor types, and the resultant improvement in survival rates.² Neuroblastoma, together with lymphoma, osteosarcoma, Ewing's family of tumors, rhabdomyosarcoma, and lymphoblastic leukemia, all belong to a group of undifferentiated pediatric malignancies known as the small round-cell tumors of childhood. In some instances, the differential diagnosis of this group of tumors can prove difficult,² due to the fact that they share morphological similarities that can make them indistinguishable by conventional light microscopy.

The accurate diagnosis of small round-cell tumors can in some cases be facilitated by cytogenetic and, more recently, by molecular biological analysis. Thus, for example, the Ewing's family of tumors, consisting of Ewing's sarcoma and primitive neuroectodermal tumors (PNET), is characterized by the genetic abnormality of a chromosomal translocation at t(11;22) in the majority of cases and the less common t(21;22) in a small number of cases.^{3,4} Recent molecular advances have allowed for the PCR-based detection of such translocations.^{3,5} However, many of the small round-cell tumors of childhood, including neuroblastoma, do not have consistent molecular genetic abnormalities amenable to either cytogenetic or DNA analysis. Because neuroblastomas are characterized by the secretion of catecholamines, we have investigated the possibility of employing expression of genes involved in the catecholamine biosynthetic pathway as

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potential molecular markers for this disease. The results demonstrated that coexpression of two genes, tyrosine hydroxylase and dopa decarboxylase, appears to be highly specific for neuroblastoma and suggest that these markers may aid in distinguishing neuroblastoma from other small round-cell tumors of childhood.

Materials and Methods

Tumor Samples

Samples of 55 primary neuroblastoma tumors from untreated patients, obtained either from the Neuroblastoma Tumor Bank of the U. S. Pediatric Oncology Group (Memphis, TN), or from the Sydney Children's Hospital, Sydney, Australia, and representing all clinical stages, have been described previously.⁶ The 29 non-neuroblastoma tumor samples were obtained at diagnosis from patients presenting at the Sydney Children's Hospital included 2 pheochromocytomas, 6 Ewing's sarcomas/PNETs, 7 lymphomas, 6 leukemias, 2 rhabdomyosarcomas, and 6 osteosarcomas. All samples were taken during the course of the patients' routine management.

Analysis of Gene Expression by Polymerase Chain Reaction

Total cellular RNA was isolated from frozen tumor tissue as previously described.⁷ High quality intact RNA was routinely obtained from over 95% of tumors processed. Complementary DNA (cDNA) was synthesized from 2- μ g aliquots of RNA with random hexanucleotide primers and Moloney murine virus reverse transcriptase.⁸ Aliquots of cDNA corresponding to 50 ng of RNA were amplified in a well-established reverse transcriptase polymerase chain reaction (RT-PCR) assay,⁹ which involved co-amplification of the target gene sequence (tyrosine hydroxylase or dopa decarboxylase, respectively) with a control sequence (β_2 -microglobulin), for 30 cycles using gene-specific oligonucleotide primers. For amplification of dopa decarboxylase, the forward and reverse primers, respectively, were 5'-GGGGACCACAACATGCTGCTC-3' and 5'-CCACTCCATTGAGAAGGTGCC-3'. The primers for β_2 -microglobulin have previously been described.⁸ Following an initial denaturation step of 3 minutes at 94°C, each cycle consisted of denaturation at 94°C for 45 seconds, primer annealing at 55°C for 45 seconds, and primer extension at 72°C for 90 seconds. Where indicated, PCR amplification of dopa decarboxylase was performed in the absence of β_2 -microglobulin primers for 35 cycles under the same PCR conditions. Due to the high guanine and cytosine content of the tyrosine hydroxylase gene, an annealing temperature of 72°C was used for PCR reactions amplifying this gene. The forward and reverse primers for tyrosine hydroxylase were 5'-CCCTGACCTGGACT-TGGACCACCC-3' and 5'-TCTCCTCGGCGGTGTACTC-CACAC-3', respectively. β_2 -microglobulin gene-specific primers suitable for amplification at this elevated annealing temperature were 5'-ATGTCTCGCTCCGTGGCCTTA-GCTG-3' and 5'-TCCATTCTCTGCTGGATGACGTGAG-3'

Figure 1. Expression of tyrosine hydroxylase (TH) in pediatric tumors following competitive RT-PCR analysis as described in the Materials and Methods. **A:** Tyrosine hydroxylase gene expression in primary neuroblastoma tumors of stage I (lanes 1–2), stage II (lanes 3–5), stage III (lanes 6–9), stage IV (lanes 10–13), stage IVs (lane 14) and tumor no. AG (lane 15). **B:** Tyrosine hydroxylase gene expression in pheochromocytomas (lanes 1–2), Ewing's family of tumors (lanes 3–8), lymphomas (lanes 9–15), leukemias (lanes 16–21), osteosarcomas (lanes 22–27) and rhabdomyosarcomas (lanes 28–29). W, water control; β_2 M, β_2 -microglobulin.

for the forward and reverse primers, respectively. Following electrophoresis on 12% polyacrylamide gels and staining with ethidium bromide, PCR products were visualized and photographed under ultraviolet transillumination. In all cases, analyses were performed in triplicate.

RT-PCR analysis for the t(11;22) translocation specific to the Ewing's family of tumors was performed essentially as described.¹⁰ Briefly, cDNA was synthesized from 1 μ g of total RNA using random hexanucleotide primers and the entire mixture was subjected to PCR amplification with t(11;22) translocation-specific primers.³ Following an initial denaturation step of 10 minutes at 96°C, each cycle consisted of denaturation at 96°C for 30 seconds, primer annealing at 65°C for 1 minute, and primer extension at 75°C for 1 minute for a total of 40 cycles. PCR products were subjected to agarose gel electrophoresis and photographed.

Results

To determine the frequency and specificity of tyrosine hydroxylase and dopa decarboxylase gene transcripts in neuroblastoma and other pediatric malignancies, expression of these target genes was evaluated in 84 tumor samples using a competitive RT-PCR assay. Included were 55 neuroblastomas, 6 Ewing's sarcomas/PNETs, 7 lymphomas, 6 leukemias, 6 osteosarcomas, 2 rhabdomyosarcomas, and 2 pheochromocytomas. The pheochromocytomas were included as positive controls, because this tumor type is also characterized by high levels of catecholamine secretion.¹¹ After RT-PCR, clearly detectable tyrosine hydroxylase gene expression was evident in 54 of 55 neuroblastoma specimens, as shown by a representative gel in Figure 1A. For the remaining tumor sample (Panel A, lane 15; tumor no. AG), however, no expression of this gene could be discerned. In contrast to

Figure 2. Expression of dopa decarboxylase (DDC) in pediatric tumors following competitive RT-PCR analysis. The legend to this figure is the same as Figure 1.

this result, tyrosine hydroxylase gene expression was undetectable in 22 of the 29 non-neuroblastoma tumor specimens (Figure 1B). Of the seven specimens in which any tyrosine hydroxylase expression was evident, only the pheochromocytoma positive controls displayed levels of expression comparable to those of neuroblastoma. The remaining five specimens, comprising one Ewing's tumor, one leukemia, two osteosarcomas, and one rhabdomyosarcoma, displayed very low levels of tyrosine hydroxylase gene expression, as evidenced by the tyrosine hydroxylase PCR product being considerably weaker than the control β_2 -microglobulin product in each case (Figure 1B). No tyrosine hydroxylase expression could be detected in any of the lymphomas.

Variable but clearly detectable expression of the dopa decarboxylase gene was similarly evident in the 54 neuroblastoma specimens which had been positive for tyrosine hydroxylase expression (Figure 2A). The one sample which had failed to express tyrosine hydroxylase (tumor no. AG) also showed no dopa decarboxylase expression (Panel A, lane 15). The complete absence of dopa decarboxylase expression from this sample was confirmed by conducting PCR amplification of the target gene in the absence of competing β_2 -microglobulin gene primers. Even under these more stringent conditions, no PCR product for dopa decarboxylase was evident (data not shown). In contrast, apart from the two pheochromocytoma controls, expression of dopa decarboxylase was not observed in any of the other 27 non-neuroblastoma tumor samples examined (Figure 2B).

Further investigation of tumor no. AG, which had failed to express either tyrosine hydroxylase or dopa decarboxylase, revealed that the patient had independently been clinically re-diagnosed as having Ewing's sarcoma. To confirm this clinical diagnosis, RT-PCR analysis was performed using PCR primers which amplify the t(11;22) hybrid transcript believed to be specific for Ewing's family of tumors.³ Following amplification, a positive PCR product was obtained from this tumor sample (Figure 3, lane 6), consistent with the positive result obtained from

Figure 3. RT-PCR analysis of the Ewing's t(11;22) transcript. Lanes 1–5: Five cytogenetically confirmed Ewing's sarcoma specimens. Lane 6: Tumor no. AG. W, water control.

the other Ewing's sarcoma samples included in this study. The differing sizes of the products reflect differences in the length of the hybrid transcripts, which can vary depending on the location of the translocation breakpoints.^{3,5} Sequencing of the PCR product from tumor no. AG confirmed a Ewing's-specific transcript (data not shown).

Discussion

The increased survival rates observed over recent decades in pediatric malignancies have resulted largely from the development and streamlining of specific therapies for specific tumor types.² Accordingly, accurate diagnosis has become increasingly important in order to achieve optimal treatment. Despite improvements in molecular genetic techniques, differential diagnosis can still present a difficulty, particularly among the small round-cell tumors of childhood. For neuroblastoma, there have been reports of this disease being misdiagnosed as acute leukemia; often the correct diagnosis is made only postmortem.^{12,13} Similarly, Kessler et al¹⁴ reported 5 cases of intrarenal neuroblastoma that were originally misdiagnosed as Wilms' tumor, despite the fact that Wilms' tumor is not included in the small round-cell tumors of childhood. Thus, there is a continuing need for the development of methods to improve the differential diagnosis of these malignancies. The present findings suggest that among the small round-cell tumors of childhood, coexpression of genes involved in the catecholamine biosynthetic pathway is a molecular characteristic specific to neuroblastoma.

Tyrosine hydroxylase is the first and rate-limiting enzyme in the catecholamine biosynthetic pathway, thus suggesting a requirement for tight regulatory control of the gene encoding this enzyme. This indeed appears to be the case, since the use of tyrosine hydroxylase expression as a specific marker of residual neuroblastoma cells has been demonstrated in numerous studies involving the detection of circulating tumor cells in patient's peripheral blood and bone marrow samples.^{15–19} In the present study, the expression of tyrosine hydroxylase in the non-neuroblastoma tumor specimens was at very low levels, suggesting that it is unlikely to play any significant biological role. Such low-level expression might be due either to contamination of the tumor sample by surrounding tissue or to the phenomenon known as illegitimate transcription.²⁰ Illegitimate transcription is the expression of otherwise tissue-specific genes in any cell type, with the detection of such expression resulting from the in-

creased sensitivity afforded by PCR.^{20,21} Interestingly, of the two genes investigated in the present study, expression of dopa decarboxylase demonstrated greater specificity for neuroblastoma than did expression of tyrosine hydroxylase. Using coexpression of two target genes as a specific marker of neuroblastoma affords increased confidence in the accuracy of the differential diagnosis. In this regard, Hoon et al²² have recently reported that RT-PCR analysis performed with more than one target gene provides a more sensitive and reliable means of detecting residual melanoma than assays using only a single target gene.

A number of chromosomal abnormalities have proven useful in the characterization of individual pediatric disease types, as has been demonstrated in the present context by the use of the t(11;22) translocation for the diagnosis of Ewing's sarcoma. Similarly, for rhabdomyosarcoma, recent studies have shown that the t(2;13) and t(1;13) translocations are valuable in the diagnosis of alveolar rhabdomyosarcoma,^{23,24} whereas a number of genetic rearrangements occurring in leukemia have also proven useful as markers for the detection of that disease.^{25,26} For neuroblastoma, although aggressive tumors are often characterized by deletions of chromosome 1p and amplification of the N-myc oncogene, these abnormalities occur, on average, in 30% or less of tumors.^{27,28} In contrast, the present results suggest that coexpression of tyrosine hydroxylase and dopa decarboxylase is ubiquitous in neuroblastoma and hence a useful molecular diagnostic marker for this malignancy.

As molecular techniques become increasingly valuable in the diagnosis and detection of childhood malignancies, their limitations are also becoming evident. For example, recent reports have identified the t(11;22) translocation in confirmed cases of rhabdomyosarcoma²⁹ as well as neuroblastoma,¹⁰ indicating that it is possible for tumor markers to be present in more than one disease. Such findings highlight the need for a panel of molecular markers to maximize the likelihood of making the correct differential diagnosis. The results presented here suggest that expression of the tyrosine hydroxylase and dopa decarboxylase genes may prove a valuable aid in the differential diagnosis of neuroblastoma from other small round-cell tumors of childhood.

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