

Hunting the Subset-Specific Genes of Neuroblastoma: Expression Profiling and Differential Screening of the Full-Length-Enriched Oligo-Capping cDNA Libraries

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Background. Neuroblastoma (NBL) has a distinct nature in different prognostic subgroups. **Procedure.** To understand the molecular mechanism of NBL's genesis and biology as well as that of the neural crest development, we constructed full-length-enriched cDNA libraries by an oligo-capping method from two different subsets of primary NBL, one with favorable biology and the other with MYCN amplification. **Results.** Sequencing analysis of

these libraries revealed that the expression profile was markedly different between both subsets. To identify the genes differentially expressed between the subsets, semi-quantitative RT-PCR analyses are proceeding. **Conclusion.** So far, 54 transcripts have been found to be expressed at high levels in favorable NBLs, and significantly at low levels in unfavorable NBLs. *Med. Pediatr. Oncol.* 35:547–549, 2000.
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Key words: neuroblastoma; oligo-capping; expression profile; subset-specific; cDNA library

INTRODUCTION

Neuroblastoma (NBL) is one of the most common pediatric tumors originates from the sympathoadrenal lineage derived from the neural crest [1]. It has a distinct biological nature in different prognostic subgroups. The tumors found in infants very frequently regress spontaneously by differentiation and/or undergoing programmed cell death, while the tumors in children over 1 year of age are very aggressive and eventually kill the patients [2]. Recent studies have revealed that growth, differentiation, and programmed cell death of the developing neural crest-derived cells are strongly regulated by neurotrophins and their receptors [3,4]. For example, TrkA, a high-affinity receptor for nerve growth factor, has been shown to be expressed in neuroblastomas with favorable prognoses, and it may regulate the differentiation and/or regression of the tumor [5]. On the other hand, TrkB, a receptor for brain-derived neurotrophic factor and neurotrophin 4, is frequently expressed in tumors with MYCN amplification, which may promote cell survival and increase the invasive activity [6].

To understand the molecular mechanism of NBL genesis and its biology, it is necessary to identify novel neuroblastoma related genes that affect growth, differentiation, and programmed cell death of the developing neural crest-derived cells. Recently, large-scale expression profiling has been widely performed using DNA chips, and comparison of gene expression between tu-

mors and normal tissues is progressing [7,8]. To introduce this system into the NBL study, large-scale collection of the NBL-specific genes from various subsets is necessary. Here, we describe the construction of the cDNA libraries from the different subsets of NBL, and differential screening of the genes from the libraries.

MATERIALS AND METHODS

Patients

Fresh, frozen tumorous tissue, as well as corresponding peripheral blood samples, were sent to the Division of Biochemistry, Chiba Cancer Center Research Institute, from various hospitals in Japan. Twenty-nine NBL cell lines and 32 primary NBL samples were used in this

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study. The tumors were staged according to the International Neuroblastoma Staging System [9].

cDNA Library Construction

Oligo-capping cDNA libraries [10] were constructed from the mixture of stage 1 and 2 NBLs with single-copy *MYCN* gene (favorable subset), and from the NBLs in stage 3 and 4 with *MYCN* amplification (unfavorable subset).

Semi-quantitative RT-PCR

mRNA Expression of the independent genes was examined in 16 primary NBLs with favorable or unfavorable prognosis by semi-quantitative RT-PCR. cDNAs synthesized from 16 favorable tumors and from 16 unfavorable tumors with *MYCN* amplification were used. Those cDNAs were at first amplified with Cy5-labeled *GAPDH* primers in 25 cycles, and the amounts of the PCR products were measured by ALF Express™ sequencer and normalized. The band intensities were measured by Intelligent Quantifier (Bio Image).

RESULTS

Two cDNA libraries, one derived from the mixture of favorable NBLs in stage 1 with single-copy *MYCN*, and the other from unfavorable NBLs in stages 3 and 4 with *MYCN* amplification, were constructed. For the library construction, the oligo-capping method, which enables full-length cDNA cloning with high-efficiency, was applied [10]. The average insert size was about 2.5 kb. We are planning to isolate more than 4,000 of clones from each library. We performed end sequencing, and surveyed homology against the public databases. At present, we have finished end sequencing of 2,410 clones from the favorable NBL library, and 2,244 clones from the unfavorable NBL library. About 39% of the former and 44% of the latter of clones were identical to known genes, while 53% and 50% had a possibility to be novel genes, including those hit to only human ESTs, genome sequences, and nothing. Table I shows the list of frequently appearing known genes in the favorable and unfavorable NBL libraries. In addition to the housekeeping genes such as the elongation factor, initiation factor, and ribosomal proteins, neuron-specific or brain-specific genes such as *14-3-3 epsilon*, *P311*, *calmodulin*, *nerve-terminal protein*, and so on, frequently appeared. After excluding the housekeeping genes, the most frequently expressed gene in the favorable library was *14-3-3 epsilon*. The expression profile was markedly different between both subsets. Compared to the favorable library, the unfavorable library seemed to contain more variety of genes, and genes related to protein synthesis like elongation factor and initiation factor were much more frequent (data not shown). Notable was the frequency of the

TABLE I. Ranking of Genes That Differentially Appeared Between Favorable and Unfavorable Neuroblastomas

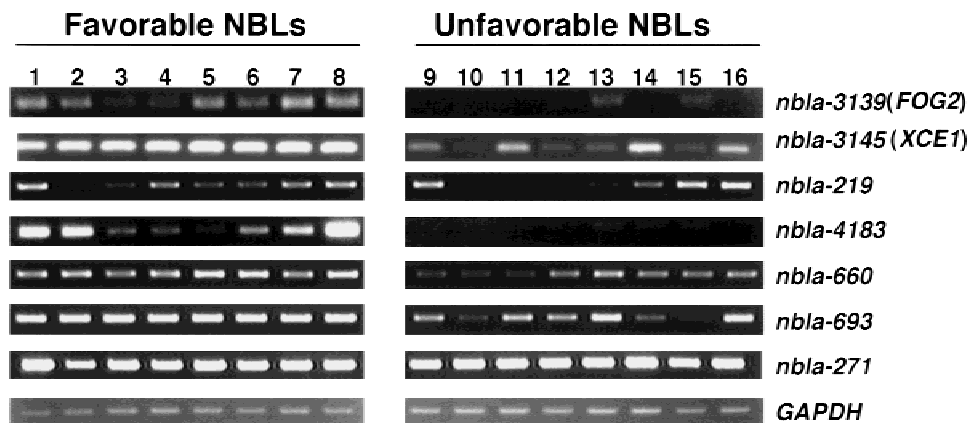
Gene name	No. of appearances	
	F ^a	UF ^a
Polyubiquitin	8	0
Dihydropyrimidinase related protein-2	6	0
Silencer element	5	0
PRAD1 (Cyclin D1)	4	0
DDX1	1	69
Alpha enolase like 1	0	12
AI568796	0	10
Lactate dehydrogenase-A	0	8
AA767051	0	5
T complex polypeptide 1, eta	0	5
PBK1	0	4
KIAA0002	0	4
KIAA0026	0	4
	2168	2066

^aF, Favorable NBL library; UF, Unfavorable cDNA library.

DDX1 gene, which appeared only once in the favorable library (Table I). Because the *DDX1* gene was closely linked to and coamplified with the *MYCN* gene [11,12], this expression pattern was reasonable.

To identify genes expressed differentially between the subsets, all independent clones were subjected to semi-quantitative RT-PCR analysis. Sixteen favorable NBLs and 16 unfavorable NBLs used as PCR templates after normalization by *GAPDH* and β -*actin* expression. We focused at first on the clones with novel sequences and/or known genes with interesting functions. So far, 847 independent genes from the favorable library have been surveyed and as a result, we found 54 genes were expressed at high levels in favorable NBLs and at low levels in unfavorable NBLs. In addition, nine genes were found to be expressed at high levels in unfavorable NBLs. These genes have been evaluated for clinical significance using primary neuroblastomas by quantitative real-time PCR [13] and Northern analysis. Figure 1 shows an example of semi-quantitative RT-PCR. Among these differentially expressed genes, *FOG2* (*nbla-3139*) and *XCE-1* (*nbla-3145*) genes were included. *FOG2*, a recently cloned member of friend of GATA, acts as an important cofactor for GATA-mediated transcriptional activation in cardiac and neural cell lineages [14]. By real-time RT-PCR, this gene was significantly highly expressed in the favorable neuroblastoma (H.I in preparation). The other gene was *nbla 3145*, which encoded a novel member of endothelin-converting enzyme-like metalloproteinase *XCE-1* [15]. By Northern analysis using 96 NBL samples, expression of this gene was associated with favorable prognosis and high levels of *TrkA* expression (T.K., submitted). Moreover, molecular functions of most of these genes in neuroblastoma,¹ including *FOG2* and *XCE-1*, were unclear.

Fig. 1. Semi-quantitative RT-PCR. To identify genes differentially expressed between the subsets, all independent nbla clones from the favorable NBL library were subjected to semi-quantitative RT-PCR analysis. **Lane 1 to 8:** favorable NBLs, **lane 9 to 16** unfavorable NBLs. These template DNAs were normalized in advance with *GAPDH* primers.



DISCUSSION

Our libraries provide many novel genes expressed in neuroblastoma with high efficiency of obtaining of the full-length cDNA clones. Nearly 7% of the genes from the favorable NBL library were differentially expressed between the subsets of neuroblastoma. We are also preparing a microarray for those NBL clones. Whole clone microarray may enable large-scale identification of differentially expressed genes at one time, and when such genes are accumulated and applied to a microarray it could be used for diagnosis. The project is still ongoing, and from this system, genes which determine the biological character of neuroblastoma might be detected. Novel prognostic factors, in addition to the *MYCN* and *TrkA*, may also be identified. Now, *in situ* hybridization using mouse whole embryo, multiple tissue RT-PCR for expression analysis, and physical localization by radiation hybrid mapping are ongoing for further characterization.

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