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Genetic Basis of Neuroblastoma, A Model for Embryonal Tumours

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ABSTRACT

Cancer is the first cause of childhood death by disease in Canada. Pediatric embryonal tumours such as neuroblastoma, medulloblastoma and Wilms' tumour have their peak incidence in the first four years of life. Neuroblastoma is a heterogeneous disease, both at the genetic and clinical levels, but little is known about its molecular pathogenesis. In order to better define the molecular and cellular mechanisms of neuroblastoma development, we have initiated the characterization of the transcription of this disease.

We have used two complementary approaches to analyze large-scale expression patterns in neuroblastoma. A cDNA microarray approach revealed the expression of 173 genes, including 49 that showed differential expression, whereas the application of the differential display PCR approach led to the identification of 20 genes that are differentially expressed. This information can be combined to provide insight about the pathways important in neuroblastoma carcinogenesis and progression. In addition, the data regarding differential expression within neuroblastoma cell lines may give clues as to the molecular basis for the heterogeneity of this disease.

Among the genes revealed by differential display is Glypican 3 (*GPC3*), the expression of which is usually limited to fetal mesodermal tissue. The inactivation of *GPC3* has been found to be responsible for the X-linked Simpson-Golabi-Behmel overgrowth syndrome and it may be considered a candidate in the development of embryonal tumours. We have found that *GPC3* mRNA was present in neuroblastoma and Wilms' tumour, but not in medulloblastoma or normal kidney tissue from Wilms' tumour patients. In addition, all samples examined that express *GPC3* also express *IGF2*, coding for a growth factor important in the survival and growth of many cancer types including neuroblastoma. Cell transfection studies showed that GPC3 may influence the growth capacity of neuroblastoma cells, as well as alter the reaction to serum deprivation in a cervical carcinoma cell line. In order to determine the effect of *GPC3* expression on these cells, we performed cDNA microarray analysis of a neuroblastoma cell line that does not have endogenous expression of this gene, before and after transfection with *GPC3*. Fourteen genes were found to be upregulated or downregulated following *GPC3* transfection and expression.

In addition to revealing the possible role of Glypican 3 in the development of neuroblastoma and other embryonal tumours, this project has supplied information regarding the expression of almost 200 genes in neuroblastoma. This will provide researchers with clues as to which genes and pathways may be important in the development of this disease, and possibly also with information about the molecular basis of the observed heterogeneity.

RÉSUMÉ

Le cancer est la cause principale de décès, par maladie, chez les enfants de moins de seize ans au Canada. Les cancers pédiatriques sont différents des cancers chez les adultes, surtout en ce qui concerne la distribution des différents types de néoplasies, ainsi que l'étiologie des tumeurs. Les tumeurs dites embryonnaires sont un groupe de tumeurs pédiatriques qui dérivent de cellules en développement. Pour cette raison, les tumeurs embryonnaires se manifestent en général dans les premières années de la vie.

Une des tumeurs embryonnaires la plus fréquente est le neuroblastome. Cette néoplasie dérive de cellules de la crête neurale, une structure embryonnaire transitoire. Ces cellules migrent dans l'embryon pour participer à la formation d'un grand nombre de tissus et cellules différents, entre autre au sein du système nerveux périphérique sympathique. C'est pour cette raison que le neuroblastome se manifeste au niveau de ce système, en particulier dans les surrénales et les ganglions sympathiques paravertébraux. Le neuroblastome est un cancer hétérogène, tant au niveau clinique que génétique. Ainsi, des cas classifiés comme étant peu avancés peuvent progresser rapidement et résister aux traitements, tandis que d'autres cas qui se présentent à un niveau avancé peuvent dans certains cas suivre une évolution favorable même sans traitement. Des méthodes de classification sont aujourd'hui disponibles pour aider les cliniciens à déterminer le pronostic et le traitement pour chaque patient. Cependant, ces méthodes n'ont pas permis pour l'instant de hausser le taux de survie à cette maladie, il reste autour de 65%.

Un des problèmes qui limitent la possibilité de guérir plus d'enfants atteints de cette tumeur est le manque de connaissances concernant le développement du neuroblastome au niveau moléculaire et cellulaire. Les rôles d'un facteur de transcription, MYCN, et de plusieurs facteurs de croissance tels IGF-II, sont connus mais ne peuvent pas pour l'instant expliquer la carcinogenèse du neuroblastome. En plus, les gènes dits suppresseurs de tumeurs sont, jusqu'à présent, absents du tableau des évènements moléculaires qui pourraient mener au développement de cette maladie. Sachant que les sites génomiques présentant une perte d'hétérozygosité sont fréquents dans ce type de néoplasie, les gènes suppresseurs de tumeurs pourraient êtres importants dans l'étiologie du neuroblastome. Il est donc important et pertinent de procéder à une étude d'expression génétique de cette tumeur, dans le but de découvrir quels gènes

pourraient participer à son développement, ainsi que pour déterminer les caractéristiques moléculaires et cellulaires menant à son hétérogénéité clinique et génétique.

Pour accomplir l'étude de l'expression génétique de ce type de tumeur, nous avons choisi deux méthodes complémentaires. La première méthode est celle de l'affichage différentiel de l'ARNm qui permet de voir les différences d'expression entre plusieurs échantillons. Nous avons modifié cette méthode afin de la rendre plus reproductible et informative. Cette approche nous a permis d'identifier vingt ADNc qui montrent une expression différentielle dans les cellules de neuroblastome étudiées. Parmi ces gènes, on note la présence de *MYCN*, *IGF2* et *BDNF*, présence qui indique la puissance de l'analyse, ces gènes étant déjà connus dans le neuroblastome.

Nous avons ensuite procédé à une analyse d'expression par "microarray" d'ADNc. Nous avons utilisé un "microarray" commercial, (Atlas™ Human 1.2 cDNA expression array), avec lequel nous avons analysé l'expression de deux lignées cellulaires de neuroblastome. Dans cette analyse, 173 gènes au total ont montré une expression dans au moins une des lignées cellulaires. L'analyse quantitative a permis de comparer les deux lignées entre elles pour déterminer s'il y a une surexpression ou sousexpression des gènes analysés.

Ces analyses permettent de brosser un tableau de l'expression générale (transcription) dans les neuroblastomes pour montrer les fonctions cellulaires affectées dans la carcinogenèse. Parmi ces gènes, glypican 3 (*GPC3*) a montré une expression différentielle et fut plus amplement étudié à cause de son potentiel comme gène candidat. L'absence de *GPC3* a été impliquée dans l'étiologie du syndrome de surcroissance de Simpson-Golabi-Behmel, une condition liée au chromosome X. *GPC3* est exprimé surtout pendant le développement fœtal au niveau du mésoderme, dans les mêmes tissus que l'*IGF2*. Les connaissances actuelles indiquent que *GPC3* coderait pour une protéoglycane de la membrane cellulaire pouvant jouer un rôle comme co-récepteur pour un ou plusieurs facteurs de croissance. Ces informations suggèrent que *GPC3* serait un candidat intéressant dans l'étiologie ou dans la progression du neuroblastome et possiblement aussi dans d'autres tumeurs embryonnaires.

Nous avons donc amorcé une étude de l'expression de *GPC3* dans les tumeurs embryonnaires, particulièrement dans le neuroblastome, la tumeur de Wilms (ou néphroblastome), et le médulloblastome. Un grand nombre de tumeurs et de lignées

cellulaires de neuroblastome expriment *GPC3*, mais aucun échantillon de médulloblastome ne l'exprime. Tous nos échantillons de tumeurs de Wilms expriment ce gène, mais au niveau du tissu rénal normal disponible chez ces patients nous retrouvons très peu ou pas d'expression, ce qui indique une surexpression ou une absence de répression dans les cellules cancéreuses. Nous avons également étudié l'expression des gènes *MYCN* et *IGF2*. Nos résultats montrent une corrélation entre l'expression de *GPC3* et celle d'*IGF2*, mais pas avec celle de *MYCN*. Pour expliquer ce résultat dans le contexte des connaissances actuelles, nous avançons l'hypothèse que les cellules possédant des molécules de glypican 3 à leur surface sont plus sensibles à la mort cellulaire, mais sont sauvées par IGF-II.

Pour mieux étudier le rôle de glypican 3, nous avons entamé des expériences de transfection cellulaire dans plusieurs lignées cellulaires. L'expression exogène de *GPC3* dans une lignée de neuroblastome accroît sa capacité de croissance. Par contre, dans une lignée de carcinome cervical (HeLa), l'expression exogène de *GPC3* ne change pas la capacité de croissance, mais modifie plutôt la réponse à une privation en sérum. Le rôle putatif de GPC3 comme co-récepteur pour des facteurs de croissance pourrait expliquer ces effets différents sur des cellules différentes, surtout connaissant la capacité de cette protéoglycane d'interagir avec plusieurs facteurs différents.

Dans le but de découvrir l'effet que peut avoir GPC3 sur les cellules de neuroblastome, l'analyse d'expression par microarray d'ADNc a été utilisée pour analyser des cellules de neuroblastome avec et sans l'expression de ce gène. Nos résultats montrent que quinze gènes subissent un changement significatif dans leur niveau d'expression après transfection avec *GPC3*. L'expression de deux de ces gènes, *NPY* et *IL-8*, n'est pas détectable dans la lignée non-transfectée, tandis que les transfectants n'expriment plus le gène *PCP4*.

En résumé, nous avons identifié plus de 190 gènes pouvant agir dans la carcinogenèse et la progression du neuroblastome. L'étude de ces gènes, dans le contexte du neuroblastome, peut être bénéfique pour la compréhension de l'étiologie et des bases moléculaires de l'hétérogénéité de cette tumeur. L'étude de l'effet de GPC3 sur les cellules de neuroblastome peut aussi possiblement mettre en évidence des mécanismes cellulaires agissant au niveau de la croissance, de la différenciation et/ou de la mort cellulaire.

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List of Abbreviations

Abbreviation	Name
BDNF	brain-derived neurotrophic factor
BWS	Beckwith-Wiedemann Syndrome
CGH	Comparative genomic hybridization
CNS	central nervous system
DD-PCR	differential display PCR
DM	double minutes
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
dpp	decapentaplegic
EWS	Ewing's sarcoma
FBS	fetal bovine serum
GAPDH	glyceraldehyde phosphate dehydrogenase
GPC3	glypican 3
GPI	glycosylphosphatidylinositol
GTF3A	general transcription factor 3A
HBSS	Hank's balanced salt solution
HLA	human leukocyte antigen
HSPG	heparan sulfate proteoglycan
HSR	homogeneously staining region
IGF	insulin-like growth factor
IGF1R	IGF receptor type 1
IGF2R	IGF receptor type 2
IL-8	interleukin 8
LOH	loss of heterozygosity
MB	medulloblastoma
MCAF	monocyte chemotactic and activating factor
MCP1	monocyte chemoattractant protein 1
MEM	minimum essential medium
NB	neuroblastoma
NGF	nerve growth factor

List of Abbreviations (continued)

Abbreviation	Name
NPY	neuropeptide Y
NT	neurotrophin
PCR	polymerase chain reaction
PNET	primitive neuroectodermal tumour
RA	retinoic acid
RB	retinoblastoma
RMS	rhabdomyosarcoma
RNA	ribonucleic acid
rpm	rotations per minute
RPS6	ribosomal protein S6
RT	reverse transcription
SGBS	Simpson-Golabi-Behmel syndrome
WT	Wilms' tumour
XPR1	xenotropic and polytropic murine leukemia virus receptor 1

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ORIGINAL CONTRIBUTIONS

All the results are original contributions except section C.3, which was published as an article in the *International Journal of Cancer* (see Annex I).

All the results were produced solely by the candidate with the exception of certain northern blots in section C.1.3. and the mapping of Unigene Hs. 142856 to chromosome 11 (Table VIII).

"Reflecting on the prospects for further advance, one may be tempted to take an attitude of romantic pessimism: all that remains is either applications or epistemological disquisition. Such was the mood in physics around 1900 – after Maxwell and Boltzmann, and just before Curie, Planck, Rutherford, Einstein and Bohr entered the picture. And so there is hope for the young biologists who dream of discovery."

Salvador Luria, 1986

I would like to dedicate this thesis

to my parents

to Pierre

to Iskandar, Youmna, Dalal, Marie-Lyne, Dimitri, Nour, Mom and Dad

SECTION A. INTRODUCTION AND LITERATURE REVIEW

A.1. Cancer

Cancer is a major disease of the modern world in terms of incidence, morbidity and mortality, in particular in industrialized countries. However, cancer has been known as a deadly disease since early human civilizations, including the Egyptians, Greeks and Romans [NIH publication No. 98-2955, 1998]. At the time, many factors were thought to be causative, in particular psychological and behavioural factors derived from mythology and folklore. The evolution of scientific understanding relating to the causes and treatment of cancer accompanied that of modern medicine. A number of key discoveries nurtured this evolution such as that of the microscope, epidemiological approaches, diagnostic imagery, and molecular and cellular biology techniques. Using these methods many risk factors have been revealed, the most important being: air and water pollution (including cigarette smoke), alcohol, diet, drugs (including anticancer and immunosuppressive drugs), familial factors, hormones, occupation, pesticides, radiation (in particular ionizing and solar) and viruses [Harras *et al.*, 1996]. The involvement of chromosomes in the process of tumorigenesis has been recognized for almost a century [Boveri, 1914]. Thus, most cancers are caused by a combination of environmental and genetic factors. This interaction forms a continuum of effects, such that in certain cancers an inherited mutation in a gene is a major trigger of carcinogenesis whereas in others strong external factors are the primary cause. However, in every case, a cancer cell is mutated at the genetic level, making cancer a genetic disease. In addition, different groups of genes are affected in different cancer types. Hence, the identification of genes affected in particular cancers is fundamental to the understanding of the development and progression of that cancer type.

A.2. Genes and Cancer

A.2.1. Genetic Mutations Found in Cancer Cells

Genes involved in oncogenesis may be mutated in different ways. Some mutations in key genes are inherited in the form of familial predispositions, responsible for an estimated 10% of cancer cases [Knudson, 1993a]. However, the majority of cancers

involve somatic mutations due to either spontaneous DNA lesions or those induced by physical or chemical factors. These mutations may be in various forms, some of which are listed in Table I.

TABLE I. TYPES OF MUTATIONS FOUND IN CANCER CELLS

Mutation	Example	Reference
deletion	*RB-1*	Venter *et al.*, 1991
point mutation	*ras* genes	Bos, 1989
gene amplification	*MYCN*	Schwab, 1983
chromosomal aberration, including translocations	Philadelphia chromosome (translocation 9:22) involving *bcr* and *c-abl* genes	Rowley, 1973
methylation defects leading to regulatory changes	*VHL*	Baylin *et al.*, 1998

These various mutations have the capacity to alter gene expression or structure in different ways, leading to upregulation or downregulation of genes, as well as alteration or absence of protein function. The genes affected by these mutations are traditionally divided into two groups: oncogenes and tumour suppressor genes. The first to be discovered were oncogenes, revealed by work on tumour-inducing viruses in animals [Bishop, 1991]. This group consists of genes whose activation contributes to the development or progression of cancer. The second group consists of genes whose inactivation or downregulation, usually at the level of both alleles, contributes to tumour development [Knudson, 1993b]. They were initially referred to as antioncogenes, and are now known as tumour suppressor genes. Genes from these two groups constitute the bulk of those involved in cancer, but some genes having an effect on cancer development may not be classified in either group. Most notable among these are DNA mismatch repair genes [Fishel and Kolodner, 1995] and genes involved in apoptosis, angiogenesis, mutagenic processes, etc. In addition, many genes that act as tumour suppressors (by a dosage effect or haploinsufficiency) are found mutated or deleted only in a heterozygous manner and hence do not fit the formal definition of this group [Bishop, 1991].

A.2.2. Identity and Function of Genes Involved in Cancer

Different strategies have been used to reveal the identity of genes involved in cancer. Among these is the documentation of chromosomal alterations in various malignancies and their use to identify the disrupted genes targeted by these alterations [Trent and Meltzer, 1993]. Other approaches have become practical with the advent of large-scale molecular biology techniques, including the use of genetic mapping in familial cancers [Knudson, 1993b] and genome-wide analysis techniques such as loss of heterozygosity (LOH) studies [Dracopoli and Fogh, 1983; reviewed in Gray and Collins, 2000].

The cellular processes affected by these genes and their mutations in tumours may be diverse. Some of these genes are involved in the deregulation of the cell cycle and programmed cell death (or apoptosis). For example, the *RB-1* gene that was initially discovered in familial retinoblastoma codes for a nuclear protein functioning in the G1 cell cycle checkpoint and in the cell's decision to enter a differentiation pathway [Wiman, 1993]. Similarly, another tumour suppressor gene, *TP53*, plays an important role in cell cycle arrest and can induce apoptosis [Lee and Bernstein, 1995]. Indeed, the two processes of cell cycle regulation and apoptosis seem to be intrinsically linked [Meikrantz and Schlegel, 1995], and many genes involved in cancer will have an effect on them. However, this does not mean that all genes involved in cancer have the same basic cellular function. On the contrary, these genes appear to encompass a host of different functions at every level of the cell-cycle decision-making process. These include genes that code for growth factors, hormones and their receptors, membrane-associated and intracellular signalling proteins, cytoplasmic regulators and nuclear transcription factors [Hunter, 1991].

The classical example of a growth factor with oncogenic properties is *sis*, a gene encoding the Platelet-Derived Growth Factor B-chain. This oncoprotein, like many others, acts via an autocrine loop to maintain cell growth stimulation [Heldin and Westermark, 1999]. The activation of receptor (ex. *ErbB*) and non-receptor (ex. *src*) tyrosine kinases may also participate in the transformation process [Cantley *et al.*, 1991]. The mode of

action of these oncogenes, as well as transcription factors such as *rel*, *jun*, *fos* and *erbA*, is the delivery of a continuous, ligand-independent mitogenic signal [Lewin, 1991].

Many of these genes in the growth-factor-to-transcription-factor cascades were first isolated from retroviruses and considered dominant oncogenes [Cantley *et al.*, 1991]. However, non-viral mechanisms have since been observed for activation of these genes. For example, there have been many discoveries showing the potential role in cancer of genes that code for proteins involved in cellular adhesion or in defining or interacting with the extracellular matrix, such as E-cadherin [Becker *et al.*, 1994]. There are many other cellular mechanisms affected over the course of carcinogenesis and tumoural progression. There is increasing evidence that a multitude of cellular processes will be affected in every tumour type, even though the genes that lead to aberrant cellular function are not the same in different tumours. What is similar in every cancer, however, is the need for several aberrations or losses of function, in a multistep or cooperative manner [Hunter, 1991].

A.3. The Multistep Nature of Cancer

The need for more than one factor in cancer development has long been suspected in an empirical manner, suggested by the increasing cancer incidence with age. This idea started to be defined when statistical analysis of cancer frequency over time showed that five or six steps are necessary for cancer development in humans [Peto *et al.*, 1975]. In parallel, it has been shown that cancer is a monoclonally-derived disease [Fialkow, 1974; Minden *et al.*, 1985]. These characteristics lead to the hypothesis that cancer is the result of multiple events (or 'hits') occurring in the same cell and probably in a sequential manner [Vogelstein and Kinzler, 1993].

A.3.1. Characteristics of Transformed Cells *in vitro*

Experiments in cell biology have also contributed to the notion of such a multistep process of tumorigenesis. It had been known for some time that normal cells have a limited lifespan in cell culture [Hayflick, 1965]. Although the number of divisions that they can undergo *in vitro* may be variable, normal cells will inevitably reach a state of senescence and, eventually, cell death [Hayflick and Moorhead, 1961]. This is considered a crisis period for the culture, and cells that pass this crisis point and continue to

proliferate become 'immortalized'. However, these cells generally are not considered 'transformed'. This description is reserved for cells or cell lines that are estimated to have characteristics of tumours. In culture, there are four characteristics that lend themselves to straightforward testing and are widely accepted as defining a transformed state [Buick and Tannock, 1992]:

1. Anchorage independence: normal and immortalized cells generally need to be attached to a solid substrate such as plastic or glass in order to grow (with the exception of most cells of hematopoietic origin). Transformed cells become anchorage independent and develop the ability of growth in suspension or on semisolid media such as agar.
2. Contact inhibition: normal and immortalized cells are usually growth-inhibited by contact with another cell. This property leads to the formation of cell monolayers when normal cells are cultured. In transformed cells, the inhibition does not take place and cells will continue to grow past and over each other, leading to the formation of colonies or foci.
3. Serum independence: normal and immortalized cells need a continuous influx of growth-promoting signals to be maintained in culture, usually in the form of serum-supplying growth factors. Transformed cells have bypassed the need for such signals and are able to grow in medium without serum.
4. Tumour-forming in an animal model: transformed cells have the capacity to produce tumours when injected or grafted into immune-defective laboratory mice. This is not true of normal or immortalized cells. This property is considered one of the most definitive in determining tumourigenic potential of a cell.

Transformed cells may or may not have all of these characteristics, however it is generally true that most will have more than two of these properties. These characteristics, therefore, give scientists the means necessary to label cells as normal, immortalized or transformed, and allow them to study the multistep nature of cancer with gene transfections in cell culture.

A.3.2 Multiple Genetic Events are Required for Cancer Development

Transfection of normal rat cells with a single oncogene was quickly understood to be insufficient for transformation and thus at least two oncogenes were found to be required [Rassoulzadegan *et al.*, 1982; Land *et al.*, 1983; Ruley, 1983]. It was also

realized that cells that were already immortalized could be transformed with one oncogene, which suggested that the order of transfection was important [Land *et al.*, 1983]. In addition, these experiments tended to use one human oncogene (often *ras*) with one gene for a viral antigen that did not have a human equivalent, such as polyoma large T antigen. This observation is important since we know that the large T antigen perturbs at least two separate cellular pathways [Zalvide *et al.*, 1998; Damania *et al.*, 1998], which means that at least three events are needed for transformation of a normal rodent cell. Similar experiments were tried in normal human cells, but it appeared that transfection of two oncogenes was insufficient to transform these cells [O'Brien *et al.*, 1986; Stevenson and Volsky, 1986]. Recent experiments have shown that human cells require the transfection of at least three oncogenes to be transformed *in vitro*, with a minimum of four to five events taking place [Hahn, Counter *et al.*, 1999]. All these are *in vitro* observations, and the complexity of *in vivo* tumour biology implies that direct studies using tumour samples are irreplaceable and must be used in conjunction with cell culture and other approaches to understand the biological mechanisms of tumourigenesis and cancer progression.

Two other approaches have contributed to the advances in this field: experiments using transgenic mice and studies of familial cancers. In the first approach, mutated genes known to play a role in cancer development are introduced or knocked out from mouse embryos, depending on the gene. One example is the transfer of the *myc* oncogene under control of a breast-specific promoter. After six months, breast tumours were observed in less than 20% of the mice produced, as well as other malignancies [Pattengale *et al.*, 1986]. When double knockouts were produced, by breeding these transgenics with *ras* knockouts, 100% of the animals developed breast tumours in the same period of time [Sinn *et al.*, 1987]. However, even in the case of the double knockouts, the stochastic appearance and monoclonal origin of the observed malignancies indicates that additional events were required for tumourigenesis. This type of information from transgenic mice can explain and complement observations made regarding families with germline mutations in genes that lead to higher frequencies of cancers, such as Li-Fraumeni syndrome, an autosomal dominant disease. In individuals with this syndrome, one copy of the *TP53* gene is inherited in a mutated form [Malkin *et al.*, 1990]. Individuals carrying

the mutation develop a wide range of malignancies at variable ages [Birch, 1994], not only because the other copy of the *TP53* gene needs to undergo a somatic mutation, but also because other genes need to be mutated before a given malignancy can develop.

A.3.3. Sequence and Order of the Genetic Events Occurring in Cancer Cells

The order of genetic events leading to cancer may also be important. This has been highlighted by the fact that mutations in particular genes often tend to occur either early or later in the course of carcinogenesis. It is suspected that carcinogenic mutations often need to have a 'terrain', meaning a cell context of particular mutations that occur and particular pathways that have been damaged, in order for the full oncogenic effect of those mutations to be seen. Important advances in the understanding of this phenomenon have been made from research on colorectal carcinogenesis. In particular, the study of two inherited conditions leading to colorectal carcinoma, familial adenomatous polyposis and hereditary non-polyposis colorectal cancer, has led to the establishment of a genetic model for this disease [Vogelstein and Kinzler, 1993]. Figure 1 schematizes such a model, showing genetic events that are largely known to occur at a certain point and in a certain order in carcinogenesis of this tissue.

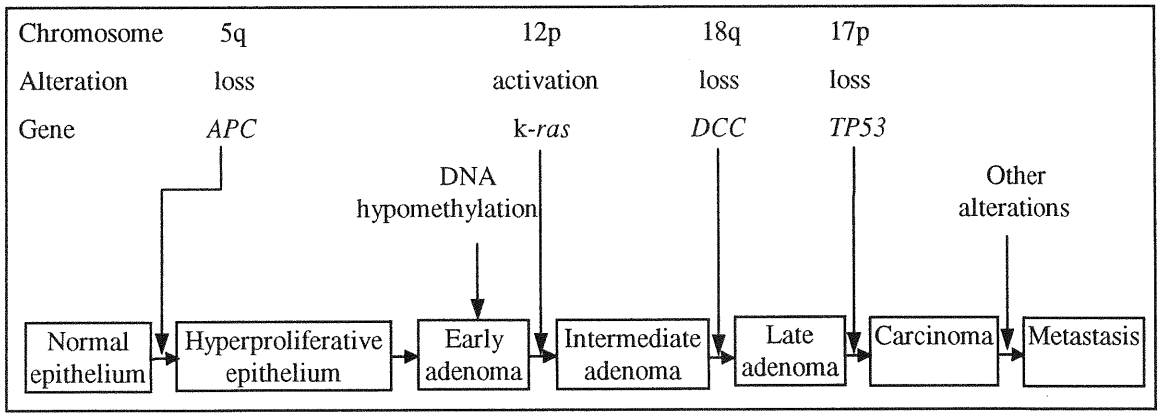


Figure 1. A genetic model for colorectal tumorigenesis. Modified from Vogelstein and Kinzler, 1993.

A.4. Pediatric Cancer

Cancer in childhood, although sharing many fundamental aspects of malignancies with cancer in adults, is different in a number of ways including type of the most frequent tumours and population incidence. Pediatric cancers account for only 1% of the total number of tumours [Ries *et al.*, 1983], yet the relative impact on children's health is nonetheless important. This is due to the fact that in industrialized countries like Canada, children's health has improved dramatically in the twentieth century, likely due to advances in prevention and treatment of congenital and microbial diseases. This places cancer in the unenviable position of first cause of children's death by disease (16.3% of deaths in the 1-14 year age group) and second only to accidents [Statistics Canada, 1999]. There are approximately 900 cases of pediatric cancers diagnosed in Canada every year, with an incidence of 150 cases per million children aged zero to fourteen per year [National Cancer Institute of Canada, 1999]. This means that approximately one out of every 400 children will develop a malignancy by the age of 18, and this figure is comparable to those available from most countries around the world. The patterns of incidence for different tumour types with respect to country and race are relatively similar, although there are some reports of differences. For example, there is a lower rate of Ewing's sarcoma for black children in the US and in Africa [Fraumeni and Glass, 1970; Jensen and Drake, 1970]. Investigation into the causes of such differences has the potential to reveal susceptibility factors. One striking difference with adult cancers is the low level of carcinomas in children, whereas in adults carcinomas of different types are know to be highly prevalent, estimated at higher than 80% of all adult cancers [Triche, 1993].

Statistical data about cancer incidence in children with respect to age and histologic type is important, as it may be informative regarding the etiology of the different types of tumours. For example, tumour types found frequently in the first age group and quickly declining in frequency in the other age groups often belong, as can be expected, to the classification known as embryonal tumours (Figure 2).

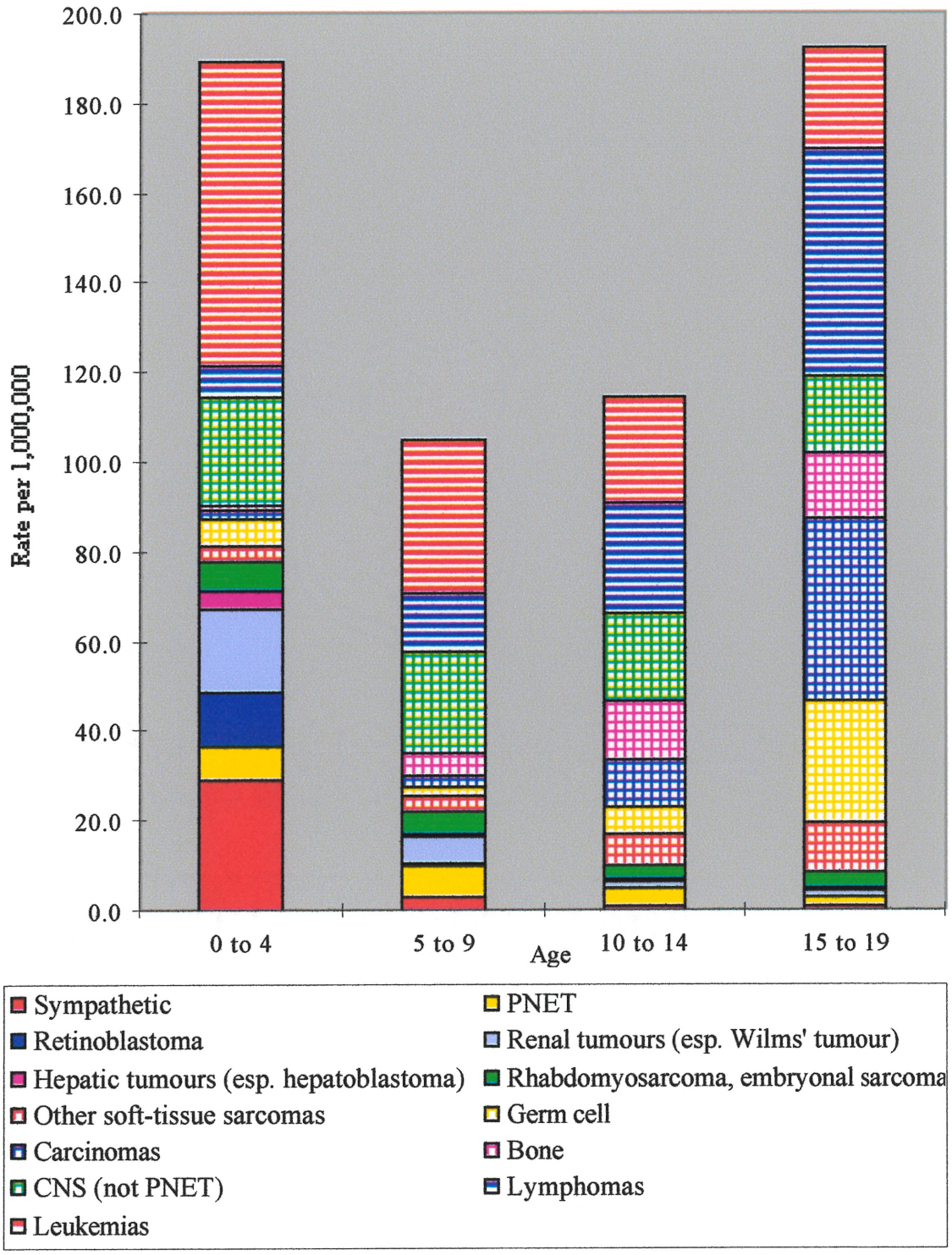


Figure 2. Contribution of various tumour types to the total rate of childhood cancer, classified by age. Each tumour type shows its contribution to the total rate of cancer for that age group. Solid colours represent embryonal tumours, bars represent leukemia/lymphoma and chequered colours represent other types of cancers. Based on incidence rates for 1973-1996, obtained from the US SEER program. PNET: primitive neuroectodermal tumour; CNS: central nervous system.

The participation of this group of tumours to the high incidence rate of the first four years of life is quite significant (calculated to 41%), and this figure declines abruptly in the next age group, where the leukemias, lymphomas and brain tumours represent around two-thirds of all cancers. This dichotomy is even more pronounced when age groups are further broken down, showing that neuroblastoma is the most frequent tumour of the first year (over a quarter) and that embryonal tumours represent over half of the tumours found in this age group [Triche, 1993]. This propensity for embryonal tumours seems to be an important factor in the high rate of tumours for the first year, the highest annual rate in the first 16 years of life [Triche, 1993].

As for survival rates in children, they exhibit fluctuations according to histological type, similarly to those in adults. The overall survival rates for children's cancers have improved dramatically in the last forty years. The five-year relative survival rate for acute lymphocytic leukemia, one of the most frequent malignancies of childhood, was only 4% in children under the age of 15 in the US for 1960-1963, rising to 75% for 1983-1990 [Harras *et al.*, 1996], largely due to multimodal chemotherapy. In some specialized centres across the world, the survival rate for this form of leukemia is even higher. However, other forms of pediatric malignancies still have a very low 5-year survival rate, such as acute myeloid leukemia with 29.4%, neuroblastoma with 56.4%, bone and joint tumours with 59.4% and brain and nervous system tumours with 62.2%, according to US data for 1983-1990 [Harras *et al.*, 1996]. These lower rates are undoubtedly important contributors to the role of cancer as a major cause of children's death. In addition, even when multimodal chemotherapy is successful, its side effects may be severe in children. These include growth retardation, negative effects on intellectual development, secondary cancers, etc.

A.5. Embryonal Tumours

These tumours are a loosely-defined subset of pediatric cancers. They generally include any malignant neoplasms in children showing pathological characteristics with similarities to those of developing organs or tissue [Dehner, 1998]. Of course, in children, this is a large definition encompassing many malignancies, estimated at 30% of pediatric tumours [Dehner, 1998]. This classification generally includes Wilms' tumour (WT),

neuroblastoma (NB), the embryonal subset of rhabdomyosarcoma (RMS), Ewing's sarcoma (EWS), primitive neuroectodermal tumours (PNET), retinoblastoma (RB) [Dehner, 1998; Brodeur, 1995a] and teratomas [Azizkhan and Caty, 1996]. The most well-studied of these, probably due to frequency of incidence and sample availability, are NB, PNET, RB and WT.

A.5.1. Primitive Neuroectodermal Tumours and Medulloblastoma

PNETs should not be confused with peripheral neuroectodermal tumours. The latter are usually located in the peripheral nervous system, derive from neural crest cells and may include NB, EWS and primitive peripheral neuroepithelioma [Thiele, 1991]. Both are of neuroectodermal origin and the designation PNET previously included RB and tumours in the peripheral nervous system such as NB and peripheral neuroepithelioma [Becker and Hinton, 1983; Dehner, 1986]. Today, PNETs are usually classified as neuroectodermal tumours limited to the CNS and the terminology of medulloblastoma (MB) may be retained for PNETs limited to the cerebellum [Rorke, 1983; Kleihues et al., 1993]. MB is the most common malignant brain tumour of the first year of childhood [Rickert, 1998]. The rate of survival from this type of cancer is generally low (averaging between 25% and 50%), and varies widely according to the type of therapy and the center where it is administered [Gaffney *et al.*, 1985; Packer and Finlay, 1988]. The most recent treatment protocols do seem to be improving the survival rate [Zeltzer *et al.*, 1999], which is still far below the general average for childhood cancer. One of the factors that may explain the relatively low survival rate of MB is the suspected biological and molecular heterogeneity of this type of tumour [Adesina, 1999]. A subtyping may be both necessary for more accurate diagnosis and instrumental in adapting treatment protocols. One of the strategies that may be successful in the elucidation of the biological properties of MB is the examination of its neuroblast potential. It appears that MB cancer cells may contain or be entirely composed of normal human neuroblasts that are defective in their ability to accurately differentiate, due to molecular defects in the sequence of events that induces neuronal precursors to exit the cell cycle and commit to terminal differentiation [Trojanowski *et al.*, 1992]. The potential for functional, if not histogenetic, similarity with NB should prompt more comparative molecular studies of these two cancers.

A.5.2. Wilms' Tumour

Renal neoplasms in children are most often WTs (also known as nephroblastomas) [D'Angio and Green, 1993]. This tumour originates in the mesodermal stem cells that normally form the renal parenchyma [Sharpe and Franco, 1995]. As with many other pediatric tumours of the embryonal type, WT is usually formed of cells that present abnormalities in their capacity to differentiate to more specialized structures, in this case metanephric tissue, and cells within the tumour present markers of a large variety of stages of normal kidney development [Droz *et al.*, 1990]. Precursor lesions of the kidney, known as a nephrogenic rests, are found in about one-third of kidneys resected for WTs, and they are often correlated with overgrowth syndromes [Beckwith, 1998]. Because of the young age at which patients develop this tumour, a number of studies have turned to environmental effects on the parents as a likely etiology. Many different risk factors have thus been identified, however only a few such as maternal exposure to pesticides have been strongly demonstrated [Sharpe and Franco, 1995]. A number of prognostic factors have also been identified for WT, such as tumour size, age of the patient, histological and pathological features, and metastases to the lymph nodes [D'Angio and Green, 1993]. However, the high efficiency of treatment for WT has made a number of these factors irrelevant, with the possible exception of age, where younger patients still have a significantly higher survival rate [D'Angio and Green, 1993]. This has led to WT being one of the most-often cured tumours of childhood, with a survival rate of over 90%, similarly to Hodgkin's disease [Lukens, 1994].

A.5.3. Neuroblastoma

A.5.3.1. Statistics and Epidemiology

About half of NBs are found in the adrenal gland and the rest are localized in the abdomen, thorax and, rarely, the neck or pelvis [Gao *et al.*, 1997]. The incidence of NB is the third highest of cancers in children up to age 14 in Canada, comprising 7% of new cases, but 11% of mortalities [Gao *et al.*, 1997]. In addition, as described above, NB is the most frequent tumour in the first year of life. This is partly due to the early occurrence

of the disease, since a majority of NBs (50% to 60%) occur before the age of 2, and 97% before the age of 10 [Seeger and Reynolds, 1993; Matthay, 1997]. As for survival rates, NB has not enjoyed the large increase in survival in the last 30 years that other pediatric cancers did: pediatric cancers in general show a 5-year survival rate of 69.7% for 1983-1990, compared to 56.4% in NB for the same time period [Harras *et al.*, 1996]. One of the factors influencing the high rate of mortality from this malignancy may be the advanced stage at presentation of many patients: 60% of these have high-risk tumours that are very resistant to therapy [Seeger and Reynolds, 1993].

Despite being a relatively common, relatively high-risk and well-studied tumour, the etiology of NB is not well-understood. Hereditary transmission is only rarely observed, unlike other embryonal tumours such as WT [Kushner *et al.*, 1986]. Neither have environmental risk factors been identified and verified, although initial reports suggest a role for maternal exposure to cetain drugs, alcohol and hair dyes, and paternal exposure to electromagnetic fields [Kramer *et al.*, 1987]. Until now, cell biology and molecular genetic studies have contributed more to the understanding of this disease than any other approach.

A.5.3.2. Biology and Pathology

NB arises from neural crest cells normally fated to participate in the formation of various structures of the peripheral sympathetic nervous system, such as the adrenal medulla, paravertebral sympathetic ganglia and sympathetic paraganglia [Shimada *et al.*, 1999a]. These tumours are highly heterogeneous in their biological behaviour, and can demonstrate one of three major progressions [Shimada *et al.*, 1999a]:

1. Involution/spontaneous regression, where massive cell death occurs in immature neuroblasts before they achieve terminal differentiation.
2. Maturation, where immature NB develops into more differentiated tumours known as ganglioneuromas.
3. Aggressive proliferation, which is the major form observed in most NBs that present as clinically advanced tumours.

All three possible behaviours can be understood as either a slowing-down or aberration of normal development of the neuroblasts of origin. This idea is supported by autopsy

observations in infants aged three months or younger that died of non-tumour related causes, showing that as many as one adrenal gland out of 220 has neuroblastic nodules consisting of poorly-differentiated neural crest cells [Beckwith and Perrin, 1963]. This supposes that this tissue may be somewhat prone to slower development; some of the cells undergoing this slower or arrested development acquire tumourigenic capacities, whereas others will eventually differentiate despite having shown rapid proliferation.

A.5.3.3 Classification, Prognostic Factors and Staging

In order to establish a classification system that would reconcile the observed biological and pathological features of NB with its clinical presentation, an International Neuroblastoma Pathology Committee was formed. In 1999, this committee proposed a classification [Shimada *et al.*, 1999a and 1999b] based on a review of cases and existing classifications [Shimada *et al.*, 1984; Joshi *et al.*, 1992; Joshi *et al.*, 1996]. It is based on the morphology that accompanies the maturation sequence and assigns NBs to one of four categories: NB (stroma-poor), intermixed ganglioneuroblastoma (stroma-rich), nodular ganglioneuroblastoma (composite stroma-rich/stroma dominant and stroma-poor) and ganglioneuroma (stroma-dominant) [Shimada *et al.*, 1999a]. In addition, certain indicators among those known to influence the prognosis of NB were incorporated into the classification system in order to make a better assessment of the favourable or unfavourable character of each patient's tumour: clinical stage of the tumour, age of the patient (more than one year, or less) and amplification of the *MYCN* gene [Shimada et al., 1999b].

Analysis of prognostic factors is important at the time of diagnosis of all tumours, but particularly when this will highly influence the therapeutic approach, as in the case of NB. Because of the heterogeneity of this malignancy, ranging from rapid progression and resistance to therapy, to spontaneous regression, much effort has been deployed to identify such prognostic factors [Matthay, 1995]. Table II illustrates some of the accepted prognostic factors known for NB.

TABLE II. INDICATORS OF PROGNOSIS IN NEUROBLASTOMA

Factor	Prognosis		Reference
	Favourable	Unfavourable	
Clinical stage	1, 2a, 2b and 4S	3 and 4	Evans et al., 1987
Age	< 1 year	≥ 1 year	Breslow and McCann, 1971
Histopathology	Shimada favourable	Shimada unfavourable	Shimada et al., 1984; Shimada et al., 1999b
Serum ferritin levels		elevated in stages 3 and 4	Hann et al., 1985
DNA content (ploidy)	hyperdiploidy	diploidy	Look et al., 1991
Status of *MYCN* gene	single copy	amplified	Brodeur et al., 1984; Seeger et al., 1985; Nakagawara et al., 1987
Chromosome 1		deletion at 1p	Maris et al., 1995 Caron et al., 1996
Expression of neuronal growth factor receptors	TrkA TrkC	TrkB	Nakagawara et al., 1993 Nakagawara et al., 1994 Yamashiro et al., 1996

The stage of the tumour at the time of diagnosis is of primary importance in determining the prognosis and therapy of each patient. Several staging systems have been developed for this purpose, the most used and well-known in North America being the Evans/Children's Cancer Group System [Evans *et al.*, 1971] and the St. Jude/Pediatric Oncology Group System [Hayes *et al.*, 1983]. In order to integrate available systems, a new classification shown in Table III was proposed [Brodeur *et al.*, 1988; Brodeur *et al.*, 1993].

Stage 4S (with 'S' indicating 'special') is a small subset of NB accounting for only 5% of all cases [van Noesel *et al.*, 1997]. Despite the apparent advanced stage of disease in patients with a 4S tumour, the prognosis is excellent. These patients have a histopathological presentation that almost always places them in the favourable category of the Shimada classification, allowing a majority to go untreated with observation until full regression of the tumour [Nickerson *et al.*, 2000]. Their 3-year survival rate averages around 90%, whereas for stage 4 the rate is around 44% [Nickerson *et al.*, 1985]. The 4S subset is considered in the low-risk group, along with stages 1, 2a and 2b, with a combined survival rate of higher than 85% [Evans *et al.*, 1996]. This survival rate seems to conflict with the clinical presentation, and authors have suggested that it could be due to spontaneous tumour regression [Evans et al., 1971; van Noesel et al., 1997]. However, even in this small and highly defined subset, a number of patients will progress to Stage 4 disease and unfavourable outcome, usually patients younger than 1 or 2 months old at presentation [van Noesel *et al.*, 1997; Nickerson *et al.*, 2000].

The case of stage 4S is a very illustrative example of the heterogeneity of NB, where staging allows for only a tentative idea of the progression of patients' disease since it cannot alone determine the outcome or even the treatment, as is often the case with other types of tumours. Thus, prognostic factors are of utmost importance, and molecular genetic studies delineating the rationale for the clinical heterogeneity are of clinical as well as biological interest [Brodeur and Nakagawara, 1992].

TABLE III. INTERNATIONAL NEUROBLASTOMA STAGING SYSTEM

Stage	Criteria
1	Localized tumour confined to area of origin. Complete resection. Negative lymph nodes.
2a	Unilateral tumour with incomplete resection. Negative lymph nodes.
2b	Unilateral tumour with complete or incomplete resection. Negative contralateral lymph nodes. Positive ipsilateral lymph nodes.
3	Tumour past midline, regardless of regional lymph node involvement. OR Unilateral tumour with positive contralateral regional lymph nodes. OR Midline tumour with positive bilateral regional lymph nodes.
4	Tumour with dissemination to distant lymph nodes, bone, bone marrow, liver, and/or other organs. Exclusion of tumours falling under criteria of 4S.
4S	Primary tumour fitting criteria for stage 1 or 2. Dissemination limited to liver, skin, and/or bone marrow.

A.5.3.4. Screening Trials

Mass screening trials for NB started in the 1970s, and are based on the observation that over 85% of these tumours secrete high levels of the urinary catecholamine metabolites homovanillic acid or vanillylmandelic acid [Tuchman *et al.*, 1987]. The initial purpose of the screening trials was to facilitate early detection of NBs and improve the prognosis of the patients [Sawada *et al.*, 1984]. Thus, trials were initiated in Japan, Québec and the Greater Delaware Valley [Sawada *et al.*, 1984; Lemieux *et al.*, 1988; Tuchman *et al.*, 1989], and later on in other regions. However, after over ten years of implementation, all indications point to the fact that the screening trials were not able to detect high-risk NBs. Thus, the overall incidence of NB rose after the implementation of the trials, in the case of infants to almost double its normal rate in certain regions, but with no corresponding lower incidence in older children and no substantial decrease in mortality [Yamamoto *et al.*, 1995; Woods *et al.*, 1997]. In addition, NBs found by screening are cured at a much higher rate than those presenting clinically, for example over 95% in Japan [Bessho, 1998]. This is likely due to the fact that NBs found by mass screening were predominantly lower-stage tumours (stages 1, 2a, 2b and 4S) with favourable prognosis, whereas those presenting clinically were very often advanced-stage tumours with unfavourable prognosis [Takeuchi *et al.*, 1995; Bessho, 1998]. Therefore, mass screening trials appear to be unable to detect NBs with unfavourable prognosis and are likely raising the incidence of NB by detecting low-grade tumours that may otherwise have gone unnoticed due to spontaneous regression [Carlsen, 1992; Takeuchi *et al.*, 1995]. All evidence so far indicates that NBs likely do not evolve from tumours with favourable prognosis and slow progression to tumours with unfavourable prognosis and rapid progression [Bessho, 1998], once again demonstrating the biological heterogeneity of this malignancy.

A.6. Molecular Genetics of Neuroblastoma

Because of the frustrating nature of NBs, in particular the difficulties in prognosis prediction, many studies have been carried out to analyze the molecular genetics of NB cells. However, most of the molecular events leading to NB still need to be elucidated. The most significant advances are summarized in this section.

A.6.1. Chromosomal Aberrations

It has long been known that NB is characterized cytogenetically by the presence of homogeneously staining regions (HSRs) and double minutes (DMs) [Balaban-Malenbaum and Gilbert, 1977; Biedler *et al.*, 1980]. These are usually phenomena that occur due to the amplification of large portions of DNA, the former as separate chromosome-like entities, and the latter as inclusions into existing chromosomes. The nature of these amplified sequences was revealed by a study identifying an oncogene called *MYCN* that was amplified in NB cell lines with double minutes or homogeneously staining regions, but not without [Schwab *et al.*, 1983]. This gene has since been studied intensively, particularly in the context of NB, and is discussed below.

Another cytogenetic phenomenon that has been found to be of particular interest in NB is aberrations of DNA content. Although this occurs in other cancers, it is of special interest in NB because of its predictive prognostic abilities. DNA content of NB was first investigated by flow cytometry using NB tumour material from infants [Look *et al.*, 1984]. This study showed that abnormally high DNA content correlated with low stage of disease and response to chemotherapy, whereas near diploid content correlated with advanced stage of disease and poor response to chemotherapy. This finding has been confirmed by later studies, using both flow cytometric analysis [Taylor *et al.*, 1988] and karyotyping [Christiansen and Lampert, 1988], although the relevance of this conclusion appears to be somewhat limited to infants up to one year [Look *et al.*, 1991].

The most frequent cytogenetically-detectable abnormality, however, is deletion of the short arm of chromosome 1, usually leading to partial 1p monosomy due to the loss of 1p32 to 1pter [Brodeur *et al.*, 1981]. This finding suggests the presence of a yet unknown tumour suppressor gene in the deleted region, and its deletion may be important for other cancer types that also show a chromosomal loss in this area, such as colorectal cancer [Leister *et al.*, 1990]. Deletion of 1p in NB has been found to correlate with *MYCN* amplification [Fong *et al.*, 1992] and adverse clinical and biological characteristics leading to unfavourable outcome regardless of age or stage [Maris *et al.*, 1995; Caron *et al.*, 1996a]. Surprisingly, deletion of this region is not implicated in the few known cases of familial NB [Maris *et al.*, 1996], despite the fact that constitutional deletion of this region may predispose to NB [Biegel *et al.*, 1993].

More recently, comparative genomic hybridization (CGH) studies performed on NB revealed loss of chromosomal regions that were previously known in this tumour [Lastowska *et al.*, 1997; Vandesompele *et al.*, 1998]. However, they find that the most frequent chromosomal aberration is neither 1p deletion nor *MYCN* amplification, but rather gain of 17q, occurring in 75% to 81% of tumours [Lastowska *et al.*, 1997; Vandesompele *et al.*, 1998], and this gain is associated with bad prognostic factors [Plantaz *et al.*, 1997]. Gain of 17q in NB had been known for some time [Gilbert *et al.*, 1984], but it had not been evident that trisomy of this region was so frequent. The disparity between evidence from cytogenetic studies and that from CGH may be explained by the finding that portions of 1p are often replaced by 17q material in unbalanced 1;17 translocations [Van Roy *et al.*, 1994; Caron *et al.*, 1994]. This may very well mask the frequency of 17q gain and not that of 1p loss, due to a difference in size of the chromosomal regions involved.

With techniques such as karyotyping and CGH, aberrations in large areas of chromosomes are detectable, but small deletions often escape detection. These smaller deletions may be of significance because of their potential to harbour tumour suppressor genes. This loss of genetic material can be revealed by loss of heterozygosity (LOH) studies. LOH analysis of chromosome 1p has allowed a finer definition of the area of loss (1p36.1-pter) [Fong *et al.*, 1992; Takayama *et al.*, 1992; Takita *et al.*, 1995]. Other chromosomal regions now found to undergo deletions in a significant proportion of NBs are: 14q [Suzuki *et al.*, 1989; Fong *et al.*, 1992], 9p [Takita *et al.*, 1995], 3p [Ejeskar *et al.*, 1998], 4p [Caron *et al.*, 1996b], 2q [Takita *et al.*, 1995] and 11q [Takita *et al.*, 1995; Takeda *et al.*, 1996]. The deletion of the chromosomal region of 14q32-qter [Takayama *et al.*, 1992] has drawn more attention because of its predictive prognostic value [Fong *et al.*, 1992].

A.6.2. Genes Involved in Neuroblastoma

The most studied gene implicated in NB is *MYCN*. This gene is often found amplified up to several hundred copies in NB [Schwab *et al.*, 1983] and the amplification is preferentially of the paternal allele [Cheng *et al.*, 1993], indicating a possible role of imprinting processes in the amplification. The amplification of *MYCN* in primary NB

tumours from untreated patients was found to correlate with advanced stage of the disease [Brodeur *et al.*, 1984]. In a large study, the amplification of *MYCN* was found to be a prognostic factor that could help predict which patients with advanced stage disease would not respond to current treatment regimens [Look *et al.*, 1991]. However, in patients with localized NB (usually stage 1 or 2), *MYCN* amplification does not appear to predict adverse outcome [Cohn *et al.*, 1995], and in these other prognostic factors are needed (see Table II). A number of NB cell lines express *MYCN* at relatively high levels despite a lack of gene amplification [Wada *et al.*, 1993]. It was also found that *MYCN* protein expression without amplification may predict an adverse outcome [Chan *et al.*, 1997], although this may not be the case for infants [Bordow *et al.*, 1998].

Interestingly, the amplification of *MYCN* may correlate with LOH of chromosome 1p, another prognostic factor in NB, and these two genetic events may characterize a subset of more aggressive NBs [Fong *et al.*, 1989]. In addition, it appears that the relationship with a candidate gene on 1p is not limited to *MYCN* but may also exist with other members of the *myc* family. This was demonstrated with a study showing that amplification of *MYC* (also known as *c-myc*) is linked to LOH on 1p32-pter in primary breast tumours [Bieche *et al.*, 1994]. Thus, the distal part of 1p appears to harbour a gene that affects the amplification of *myc* family genes, possibly through a mechanism of instability.

A number of other genes are being identified as altered or mutated in NB, although none have been implicated as strongly as *MYCN* so far. Among these is *nm23H1*, a gene coding for nucleoside diphosphate kinase A. This gene has been found to be mutated and/or amplified in 6 of 28 NBs, all advanced tumours [Chang *et al.*, 1994]. Another study showed that *nm23H1* is amplified in 13 of 95 NBs and may predict poor prognosis independently of 1p LOH and *MYCN* amplification [Takeda *et al.*, 1996]. *MDM2* is also a gene that appears to be amplified in NB, where certain NB cell lines containing DMs and HSRs carry amplified DNA not identical to *MYCN*, but rather to *MDM2* [Corvi *et al.*, 1995]. This is particularly interesting because of the known role of MDM2 in the binding and sequestration of p53. The latter is encoded by the tumour suppressor gene most often found mutated in different types of cancer, but not in NB [Vogan *et al.*, 1993; Castresana *et al.*, 1994]. Thus, the p53 pathway could be targeted

for inactivation in NB not through *TP53* mutations but rather via the inhibitory binding of MDM2 to p53, possibly through a mechanism of cytoplasmic sequestration [Moll *et al.*, 1995]. Whatever the mechanism of action of MDM2 in NB, it is becoming increasingly clear that amplification is a relatively frequent method of activation of genes in this malignancy. Other genes that may be involved will be discussed in the next section and in relation to their effect on NB cell biology.

A.6.3. Candidate Genes of Potential Interest in Neuroblastoma

Probably the most sought-after genes in the field of NB are tumour suppressors, likely because many known tumour suppressor genes have not been strongly implicated in NB carcinogenesis. This is the case for *TP53*, as previously mentioned, but also for other tumour suppressor genes known to be involved in a variety of cancer types. Among these is *CDKN2A*, a gene that had been an interesting candidate because of its location in a region of frequent chromosomal loss (9p21) in NB and other cancers. This gene, coding for p16, a cyclin dependent kinase inhibitor, was subsequently found not to be mutated in primary NB samples [Takita *et al.*, 1997]. The same study, however, found that 13 of 19 cell lines did not express *CDKN2A*, and that five of these had been transcriptionally silenced by methylation of the CpG island surrounding the first exon of the gene. Immunohistochemical analysis of 74 paraffin sections of primary NBs by the same group later showed that 61% of NBs do not have p16 protein, and this absence correlates with poor prognosis and advanced stage of the disease [Takita *et al.*, 1998]. In addition, *CDKN2A* expression could be re-induced by 5-aza-2-deoxycytidine in cell lines with methylation of *CDKN2A* [Takita *et al.*, 1998]. This information taken together indicates that p16 may play a role in NB pathogenesis. However, this remains to be proved knowing that *CDKN2A* itself does not seem to be mutated in NB [Takita *et al.*, 1997; Diccianni *et al.*, 1996], but especially knowing that NB cell lines continue to proliferate in the presence of elevated levels of p16 [Diccianni *et al.*, 1999]. Thus, it is quite possible that *CDKN2A* aberrant expression is a consequence rather than a cause of mutations leading to NB pathogenesis, not mutated but nevertheless effectively silenced by methylation.

For these reasons and the fact that LOH of the short arm of chromosome 1 is indicative of poor prognosis, the putative tumour suppressor gene in this region has been actively sought. Studies showed that there was in fact more than one distinct chromosomal area subject to loss on 1p, roughly around 1p32 and 1p36, and that these two regions were associated with different subsets of the disease [Takeda *et al.*, 1994; Schleiermacher *et al.*, 1994]. The 1p36 region was then further divided into two regions, one of which is associated with amplification of *MYCN*, the other possibly imprinted [Caron *et al.*, 1995; Cheng *et al.*, 1995]. This information identifies at least three regions of loss and confirms the finding of genomic imprinting in the region [Caron *et al.*, 1993]. It may also explain why some studies looking at large areas have not found a prognostic value for 1p deletions without *MYCN* amplification [Gehring *et al.*, 1995].

Given the size of the regions of 1p affected by deletions, there are many candidate genes that have been mapped to this area. The *p73* gene, closely related to *TP53*, was hailed as the long-awaited tumour suppressor on 1p36 [Kaghad *et al.*, 1997]. Its profile was very much in line with that expected for such a gene: at least one allele is deleted in NB cell lines, expression is absent or low and the gene is imprinted [Kaghad *et al.*, 1997]. It was also found to behave as transcriptional activator for many of the same genes activated by p53, and to induce apoptosis in the same manner [Kaghad *et al.*, 1997; Jost *et al.*, 1997]. Subsequent studies showed that in both primary NB and cell lines, mutations of *p73* are not as frequent as initially thought and this gene is often biallelically expressed [Kovalev *et al.*, 1998; Kong *et al.*, 1999]. Lack of imprinting of *p73* in NB was later confirmed, and a correlation between aberrant expression of *p73* and advanced stage of the disease was discovered [Yang *et al.*, 2000]. This suggests an involvement in progression rather than development and implies that although *p73* may not be a classical tumour suppressor gene in NB, it could still play a role in advanced-stage malignancies of this type.

Two other examples of candidates in the 1p36 region are the *PITSLRE* protein kinase gene complex [Lahti *et al.*, 1994] and *MEMO-1*, a human modifier of methylation for class I HLA genes [Cheng *et al.*, 1996a]. The former was found to be deleted on one allele in all 18 cell lines studied, but no subsequent work was published showing deletions in primary tumours, or demonstrating aberrations in expression levels. As for the latter, it

is in fact a minimal region of deletion that identifies a locus involved in methylation of class I HLA genes, and the gene itself has not yet been identified. These are two examples that illustrate the repeated problems that researchers in this field have encountered, in particular the lack of inactivating mutations found in the second allele of the putative tumour suppressor gene.

As for tumour suppressor genes on other chromosomal regions, they appear to be similarly elusive. The LOH on chromosome 18q was thought to possibly involve one or more of three candidate tumour suppressor genes in this area: *DCC* (deleted in colorectal carcinomas), *DPC4* (deleted in pancreatic carcinomas, locus 4) and *MADR2* (MAD-related gene 2). But a subsequent study found few or no mutations in these genes in NB tumours and cell lines [Kong *et al.*, 1997]. As previously mentioned, loss of the *DCC* gene is associated with stage 4 NB and likely with disease dissemination [Reale *et al.*, 1996]. In addition, a recent study showed that Netrin-1, a protein that participates in a complex formation that includes DCC, is not produced in about half of brain tumours and NBs [Meyerhardt *et al.*, 1999]. This combined information may implicate this pathway in NB pathogenesis, but much work is still needed before the possibility is confirmed.

A.7. Cell Biology of Neuroblastoma

In order to fully understand the biology of a cancer cell, a complex group of interactions needs to be considered and studied. Of particular interest are: the nature of the cell of origin, its growth potential, usually with the help of stimuli from growth factors, escape from differentiation and apoptosis, low immune recognition, telomerase activity enabling long-term cellular multiplication, and angiogenesis leading to enlargement of the tumour and its metastatic dissemination. Chemoresistance is also important especially with regard to antitumour therapy. Most of these topics have been addressed in NB.

A.7.1. MYCN

MYCN is a nuclear phosphoprotein that acts as a transcription factor like other members of the myc family [Ryan and Birnie, 1996]. The members of this family, including c-myc and L-myc, appear to have a strong transforming capability [Ryan and Birnie, 1996]. These proteins usually need to form a heterodimeric complex with a

partner, most often Max, in order to become active [Mukherjee *et al.*, 1992]. MYCN normally contributes to embryonal development of epithelial tissues, in particular those of the nervous system and organs characterized by interactions between epithelial and mesenchymal components [Stanton *et al.*, 1992]. Attesting to this is the lethality of mice homozygously mutated for this gene due to effects on the central and peripheral nervous systems, mesonephros, lung and gut [Stanton *et al.*, 1992]. Thus, the involvement of MYCN in NB is in line with its expression in the developing embryo.

However this information does not provide us with any clear biological roles for this protein in NB oncogenesis, but some clues have started to emerge from studies in NB cell lines. One theory advances that Max homodimers may act to repress the effect of MYCN-Max heterodimers, and in NB the high levels of available MYCN protein lead to the perturbation of this control mechanism and abnormally high levels of target gene transcription [Wenzel and Schwab, 1995]. These targets are still not well-identified, but many effects of MYCN on NB cell lines have been documented. For example, expression of *MYCN* in NB cell lines appears to upregulate the expression of ornithine decarboxylase and alpha-prothymosin, and increase the rate of cellular proliferation [Lutz *et al.*, 1996]. In addition, MYCN may cooperate with other proteins such as Interferon Gamma to induce apoptosis [Lutz *et al.*, 1998]. Finally, clues to the unfavourable outcome of patients that have NB with *MYCN* gene amplification and expression come from studies showing that this gene may contribute to the invasive, and hence metastatic, potential of cells [Zaizen *et al.*, 1993; Taguchi *et al.*, 1997].

A.7.2. Growth Factors and Morphogens

The importance of growth factors and morphogens in cancer is especially true for NB. Three groups of factors are known to be important, the Insulin-like Growth Factors (IGFs), the neurotrophic growth factors and retinoic acid (RA).

A.7.2.1. Insulin-Like Growth Factors

IGF-II is particularly important in maintaining NB growth. This factor was found to be expressed in two of eight NBs, and to be an autocrine growth factor responsible for mediating autonomous growth in a NB cell line [El-Badry *et al.*, 1989]. In addition, all of 21 NB tumours were later found to produce IGF-II, either by the tumour cells themselves, or from nonmalignant adjacent tissue [El-Badry *et al.*, 1991]. Hence, IGF-II is either an autocrine or a paracrine growth factor for NB. However, its production by NB cells does not appear to be associated with any tumour prognostic features and may have a broad general role in NB pathogenesis [Sullivan *et al.*, 1995].

It has become evident that the effect of IGF-II in NB is mediated by the IGF type 1 receptor (IGF-1R) [Singleton *et al.*, 1996]. This tyrosine kinase receptor is activated by insulin, IGF-I and IGF-II, and is known to play a role in cell growth, malignant transformation and blockage of apoptosis in many cell systems [Baserga *et al.*, 1997]. It likely has both a cell growth effect and an important antiapoptotic effect in NB cells [Zumkeller and Schwab, 1998]. Antisense strategy directed against IGF-1R was successful in obtaining the regression of NB tumours growing in mice, in an immune-system dependent manner [Liu *et al.*, 1998]. *IGF1R* expression has been found to be in part regulated by *MYCN*, providing a link between these important elements of NB pathogenesis [Chambery *et al.*, 1999]. Thus, the use of IGF-II rather than IGF-I by NB tumours may be simply due to the cellular origin of this system. IGF-II is an important growth factor in fetal development [Cohick and Clemmons, 1993] and it is entirely possible that it is already expressed in the cells of origin of NB. The *IGF2* gene is imprinted in humans and certain other mammals and its overexpression in carcinogenesis is often due to loss of imprinting [Issa *et al.*, 1996; Yaginuma *et al.*, 1997]. In an NB cell line, *IGF2* expression is known to be induced by retinoic acid, an important morphogen in embryonal and fetal development [Ueno et al., 1993]. Thus, there may be more than one way to activate *IGF2* expression in NB cells. It is also possible that the effect of IGF-II is not necessarily limited by IGF-1R action, for instance IGF-II could stimulate cell proliferation through the insulin receptor [Morrione *et al.*, 1997; Louvi *et al.*, 1997]. Recent evidence shows that a new isoform of the insulin receptor is bound by IGF-II with high affinity and may be particularly important in fetal and cancer cells [Frasca *et al.*,

1999]. The importance of IGF-II in certain cancers is demonstrated by the downregulation of the IGF type 2 receptor (IGF2R), for example in liver cancer [Yamada *et al.*, 1997]. This receptor is identical to the cation-independent mannose 6-phosphate receptor, is not bound by insulin or IGF-I and is responsible for clearance of IGF-II from the circulation via internalization and lysosomal degradation [Kornfeld, 1992]. Thus, the action of IGF-II in NB cells may be mediated by more than one receptor and its importance in NB (rather than that of IGF-I) may be due to more than pre-existing expression in the cells of origin.

A.7.2.2. Neurotrophic Growth Factors

The neurotrophin family of peptide growth factors includes Nerve Growth Factor (NGF), Brain-Derived Neurotrophic Factor (BDNF), Neurotrophin-3 (NT-3) and Neurotrophin-4/5 (NT-4/5) [Klein, 1994]. Three high-affinity receptors are known for this family, all tyrosine kinases: TrkA which is primarily the receptor for NGF, TrkB which is primarily the receptor for BDNF and NT-4, and TrkC which is the NT-3 receptor [Conover and Yancopoulos, 1997]. In addition, a member of the TNF/Fas superfamily, p75NTR, binds all neurotrophins with low affinity. Its known relationship with NGF is particularly interesting: p75NTR induces neuronal apoptosis in the presence of NGF and the absence of TrkA, while in the presence of TrkA it enhances the response of the latter, that of inducing survival and differentiation of neurons [Kaplan and Miller, 1997]. NB cell lines and tumour cells activated by NGF undergo growth inhibition and show signs of differentiation when they express *TrkA* [Lucarelli *et al.*, 1997; Nakagawara and Brodeur, 1997]. In line with these results, *TrkA* expression has been shown to correlate with favourable outcome in patients with NB [Nakagawara *et al.*, 1993], to be absent from aggressive NB [Suzuki *et al.*, 1993] and to be inversely correlated with amplification of *MYCN* [Nakagawara *et al.*, 1992]. The expression of another member of the family, *TrkC*, was also associated with favourable NB [Yamashiro *et al.*, 1996].

However, the same cannot be said for the third neurotrophin receptor, TrkB. Full-length expression of this receptor correlates with advanced stage NB, whereas expression of a naturally-occuring truncated form lacking the kinase domain correlates with more differentiated tumours [Nakagawara *et al.*, 1994]. This difference may be explained by the fact that truncated TrkB receptors appear to bind and internalize BDNF and NT-4/5,

effectively removing them from the extracellular environment [Fryer *et al.*, 1997], although a ligand-independent effect may also be considered [Haapasalo *et al.*, 1999]. On the other hand, the correlation between full-length *TrkB* expression and advanced stage NB is being elucidated. BDNF acting through TrkB elicits signs of differentiation in a NB cell line, but unlike the response to NGF, proliferation is not inhibited [Lucarelli *et al.*, 1997]. A NB cell line differentiated with retinoic acid and expressing *TrkB* is induced by BDNF to increase survival and invasiveness [Matsumoto *et al.*, 1995]. In addition, it appears that BDNF, acting through TrkB, may protect NB cells from a variety of chemotherapeutic agents including vinblastine [Scala *et al.*, 1996] and cisplatin [Middlemas *et al.*, 1999], both used in the treatment of NB. Knowing that BDNF is probably not a limiting factor *in vivo* since it is highly expressed in NBs with unfavourable biologies and NB cell lines [Aoyama *et al.*, 2001], TrkB may be promoting survival of NB cells both from differentiating effects and those induced by chemotherapeutic agents.

A.7.2.3. Retinoic Acid

Among the most well-known differentiating agents for NB cells is RA. It has a highly diverse effect on different NB cell lines, and the study of its effects may be rewarding both for the understanding of NB cell biology and for therapeutic design. Human NB cells in culture exhibit distinct morphologies. The most well-studied are the neuroblastic or N type having neurites and other characteristics of neuronal cells, and the substrate-adherent or S type with properties resembling those of epithelial cells [Melino *et al.*, 1997]. *In vitro*, RA has the potential to differentiate some NB cells to the N type or increase existing N type differentiation [Melino *et al.*, 1997]. In so doing, it inhibits proliferation in some but not all NB cells, possibly by a mechanism of G1 arrest [Cheung *et al.*, 1998]. This differentiation may be mediated in several ways, such as by the induction of TGF-β and Protein Kinase C pathways [Cohen *et al.*, 1995; Fagerstrom *et al.*, 1996; Scarpa *et al.*, 1996], as well as TrkB [Kaplan *et al.*, 1993] or IGF-II [Matsumoto *et al*, 1992; Ueno *et al.*, 1993]. In addition, RA induces a transcriptional down-regulation of *MYCN*, a phenomenon amplified in a synergistic manner by the action of interferon gamma [Wada *et al.*, 1997]. It is possible to hypothesize that the induction of TrkB and/or IGF-II in different NB cell lines allows cells to exhibit resistance to the

growth inhibitory effect of RA. Another possibility is the alteration of RA receptor levels. Given the differentiating effect of this morphogen and its capacity to down-regulate *MYCN*, a known prognostic factor, RA is a candidate for therapeutic differentiation and growth arrest of NB tumours. Trials using RA therapeutically in NB and other cancers are presently under way [Niles, 2000].

A.7.3. Apoptosis

The effect of mitogens and growth factors on NB cells would not necessarily lead to a transformed phenotype if these cells still had intrinsic safeguards against uncontrolled growth, in the form of apoptosis. But apoptosis is blocked in malignant NB cells as it is in most other cancers. The potential role of proteins known to inhibit apoptosis, in particular bcl-2, have been examined in NB. Some groups have detected bcl-2 in most of their tumour samples with no discrimination for stage or other prognostic factors [Ramani and Lu, 1994; Ikegaki *et al.*, 1995]. Other studies show significant association between *bcl-2* expression and unfavourable histology and amplification of *MYCN* [Castle *et al.*, 1993; Mejia *et al.*, 1998]. In both cases, it is quite clear that this protein is present in enough tumours to contribute significantly to the blockage of apoptosis. In addition, *in vitro* experiments show that a variety of factors have the ability to block apoptosis in NB cells, including IGF1R and bcl-2 [van Golen *et al.*, 2000], and that this block may contribute to the resistance to chemotherapeutic agents [Dole *et al.*, 1994].

A.7.4. Drug Resistance and Other Pertinent Mechanisms

Other drug resistance factors are also present in NB cells. The *MDR1* gene product P-glycoprotein, known to confer a multidrug-resistant phenotype, is often present in human NB cells, but its prognostic value is still unclear [Dhooge *et al.*, 1997; Kurowski and Berthold, 1998]. However, the presence of the multidrug-resistance-associated protein (MRP) in NB tumours has been correlated with *MYCN* amplification and reduced survival [Norris *et al.*, 1996].

Other general biological or genetic processes associated with cancer can be found in NB. Telomerase is expressed in a majority of NB tumours [Hiyama *et al.*, 1995], thereby preventing the shortening of telomeres to a degree that would elicit cellular

senescence. In addition, angiogenesis is present in many NB tumours, although its correlation with prognosis is as of yet unclear. Initial studies had indicated that a higher proportion of blood vessels in the tumour correlates significantly with dissemination and poor survival, and possibly with *MYCN* amplification [Meitar *et al*, 1996]. However, recent studies in large cohorts show no association between angiogenic markers and prognostic factors except advanced stage disease [Canete *et al*., 2000; Eggert *et al*., 2000]. Such a conclusion can be understood as the anatomical dependence of tumours on an adequate blood supply in order to grow beyond a certain critical size.

A.7.5. Immune Recognition

Many of the biological features described above are common to most malignancies. However, NB tumours share a unique feature with very few other tumour types, that of immune escape by generalized very low or absent expression of Class I Human Leukocyte Antigens (HLA) [Lampson *et al*., 1983]. Studies show that the lack of another component of this system, beta 2-microglobulin, is characteristic of neuroendocrine tumours including NB and small cell lung cancer [Funa *et al*., 1986]. Cell fusion experiments have shown that suppression of *MYCN* expression leads to re-expression of HLA class I antigens (also known as the major histocompatibility complex class I), indicating that MYCN may lower the immunogenicity of NB and enhance its ability to bypass the body's immune surveillance [Versteeg *et al*., 1990]. The possible functional correlation between *MYCN* expression and lack of HLA class I antigens has been addressed in other studies. That lead to the identification of a locus linking the two events at a genetic level: a modifier of methylation for HLAs on chromosome 1p36 [Cheng *et al*., 1996b]. These observations suggest that low immune recognition may contribute significantly to the aggressive and resistant nature of NB.

A.7.6. Integration of Biological Factors and Classification Systems in NB to Form Risk Groups

Even a decade ago, enough was known about prognostic factors to divide NB patients into three risk groups with favourable, intermediate and unfavourable prognosis [Brodeur *et al*., 1992]. In the first group, tumours are localized, exhibit hyperdiploidy or

near-triploidy, with few cytogenetic rearrangements and usually occur in children younger than 1 year of age. In the second group, some but not all of the negative prognostic factors are found: near diploid tumours with few cytogenetic aberrations in children that are generally older. These tumours, despite a slow rate of progression, are often fatal. The third group of tumours has cytogenetic aberrations such as *MYCN* amplification and 1p deletions or LOH, a near-diploid or even tetraploid karyotype, and rapid, aggressive progression.

Since then, other molecular and cellular information and improved pathological classification has contributed to the refining of these subgroup definitions. The intermediate outcome group is found to sometimes contain 1p allelic loss [Brodeur *et al.*, 1997]. Given that different patterns of LOH on 1p appear to be associated with different clinical/biological subtypes [Mora *et al.*, 2000], the future identification of genes at this locus involved in NB may further clarify the classification. In addition, the correlation between TrkA and the cell membrane protein CD44, and between either of them and favourable outcome [Kramer *et al.*, 1997], now contributes to the definition of the categories [Brodeur *et al.*, 1997], as does the correlation between TrkB and unfavourable outcome [Nakagawara *et al.*, 1994].

A.8. Goal

It is clear that we do not fully understand the molecular and cellular mechanisms of the different forms of NB. We propose that NB is not a single heterogeneous disease, but rather a group of distinct diseases. NB derives from neural crest cells, and it is already well-known that the pluripotential character of these cells becomes more restricted and their level of differentiation more defined as they migrate [Le Douarin, 1988]. Thus, proliferative capacity may be stronger in certain cells, as it is for different subtypes of NB. Some evidence to support these ideas exists in the knowledge that growth factor requirement, in particular of neurotrophins, varies in different neural crest lineages [Sieber-Blum, 1998], as it does in different subtypes of NB. These concepts from developmental biology contribute to the idea that NB is more than one disease, a... clinical evidence from screening trials also. As we have seen, the evidence from these trials is not in support of a good-prognosis NB being able to develop into a tumour with

bad prognosis. Knowing that pathological classification is insufficient to differentiate between the subtypes and unable to define their etiology, more genetic and cellular information is needed about NB.

Strictly speaking, the biology of a cell is the complex execution of its genetic programming. Theoretically, this implies that information about a cell's genetic mutations and expression can lead to the elucidation of biological processes that lend the cell its characteristics and identity. However, in practice, punctual genetic information does not necessarily lead us to the understanding of biological function because of its isolation from a complex scheme of genetic directives. Many examples attest to this in NB cells, such as *MYCN* amplification and expression which, although serving as a marker for prognosis, does not provide us with much knowledge regarding final biological effect in these cells. Conversely, knowledge about biological processes that occur in cancer cells is not always informative regarding the genetic etiology of the tumour, for example in the case of growth factor involvement in NB cells. One way to link genetics and cell biology in the study of cancer and other cells is to look at genetic expression. The presence of a certain mRNA in a cell may be influenced by both upstream mutations (in the gene coding for this mRNA) and 'parallel' mutations (those affecting other genes), but in either case some effect on cellular function may be postulated. For this reason, techniques aiming at revealing differentially-expressed genes and large-scale genetic expression patterns have become increasingly popular in the study of malignancies [Gray and Collins, 2000]. The goal of this project is to use such gene expression profiling techniques to identify genes and pathways that could participate in the development of NB and discriminate between the different types of this tumour.

SECTION B. MATERIAL AND METHODS

B.1. Biological Material

B.1.1. Cell Lines

Twenty-three human cell lines were used for various analyses. Their type and source are listed in Table IV. Primary MB cell lines are derived from both classical and desmoplastic variants. No existing classification of NB cell lines according to aggressive nature is known. However, the amplification and expression status of *MYCN* is known to indicate prognosis in patients, as discussed above. Therefore, the status of this gene was here used to loosely classify the aggressive nature of each cell line, where lines with known *MYCN* amplification such as SK-N-DZ, IMR-32, SJNB-7 and SJNB-10 [Reynolds *et al.*, 1988; Shapiro *et al.*, 1993] are considered to represent aggressive forms of the disease, those with expression without amplification such as NBL-S [Cohn *et al.*, 1990] are considered intermediate and those without *MYCN* expression are considered mild forms. This classification cannot be understood, however, as an exact representation of the tumourigenic potential of each cell line.

B.1.2. Tissue Specimens

Sixteen primary tumour specimens were obtained from patients treated at Sainte-Justine Hospital (Montreal, Canada), including: four NBs, one neurofibrosarcoma, one adrenal carcinoma and one rhabdomyosarcoma as well as nine WTs and in three cases the corresponding normal kidney tissue. Other biological specimens (preterm placenta, placenta at term, fetal kidney, fetal lung and adult liver) were also obtained from Hôpital Sainte-Justine. The Institutional Review Board approved the research protocol and informed consent was obtained from all participating individuals or their parents.

B.1.3. Antibodies

Polyclonal anti-GPC3 antibodies were obtained from two sources: K. Tamai, MBL Co., Japan, and D. Schlessinger, National Institute of Aging, National Institutes of Health, Maryland.

TABLE IV. LIST OF CELL LINES USED

Name	Type	Type of Analysis	Reference	Source
SK-N-MC	NB	DD	Biedler *et al.*, 1973	ATCC
SK-N-SH	NB	DD	Biedler *et al.*, 1973	ATCC
SK-N-FI	NB	Microarray, gene expression, analysis of *GPC3* function		ATCC
SK-N-AS	NB	Microarray, gene expression, analysis of *GPC3* function		ATCC
SK-N-DZ	NB	Gene expression		ATCC
NBL-S	NB	DD	Cohn *et al.*, 1990	G.M. Brodeur
SJNB-1	NB	DD	Lahti *et al.*, 1994	T. Look
SJNB-7	NB	DD	Lahti *et al.*, 1994	T. Look
SJNB-10	NB	DD	Lahti *et al.*, 1994	T. Look
IMR-32	NB	Gene expression	Biedler and Spengler, 1976	ATCC
Ntera2	embryonal teratocarcinoma	Gene expression		ATCC
143N2	osteosarcoma	Gene expression	Morais *et al.*, 1994	R. Morais
MB1	primary MB	Gene expression	Kenigsberg *et al.*, 1997	Y. Théorêt
MB3	primary MB	Gene expression		Y. Théorêt
MB4	primary MB	Gene expression		Y. Théorêt
MB5	primary MB	Gene expression		Y. Théorêt
MB7	primary MB	Gene expression		Y. Théorêt
HeLa	cervical carcinoma	Gene expression, analysis of *GPC3* function		ATCC
HepG2	liver carcinoma	Gene expression		ATCC
R-	Immortalized mouse	Analysis of *GPC3* function	Rubini *et al.*, 1997	R. Baserga
R+	Immortalized mouse	Analysis of *GPC3* function	Rubini *et al.*, 1997	R. Baserga

ATCC: American Type Culture Collection, cell repository in Manassas, Virginia.

B.2. Isolation, Treatment and Analysis of Nucleic Acids

B.2.1. Isolation of DNA and RNA

RNA was isolated by a variety of methods. Total cellular RNA was isolated by the guanidium isothiocyanate method [Chirgwin *et al.*, 1979] or using the RNAqueous™ kits from Ambion Inc. (Austin, Texas). The latter was used according to the manufacturer's instructions. In the former, cell samples are resuspended, homogenized in 9ml of 4M guanidine isothiocyanate, then layered onto 3ml of a 5.7M cesium chloride solution and centrifuged at 32,000 rpm and 20^0C for 18 hours in a Beckman LE-80 ultracentrifuge with a rotor model SW 41 Ti. The resulting RNA pellets were then resuspended in sterile water and precipitated with 0.3M sodium acetate and 2.5 volumes of ethanol. DNA was obtained simultaneously by dialysing the corresponding band twice against 1X TE in a 1:25 ratio at room temperature, then precipitating in 0.3M sodium acetate and two volumes of ethanol. Isolation of mRNA was performed using the Oligotex Direct mRNA Mini kit (QIAGEN, Mississauga, Ontario), as per the manufacturer's instructions.

B.2.2. DNase Digestion

DNaseI digestions were performed on all RNA samples used for RT-PCR and Differential Display experiments: 25µg RNA was incubated with 10U DNaseI (Amersham Pharmacia Biotech, Baie d'Urfé, Québec) in a reaction volume of 25µL containing 40mM Tris-Cl pH 7.5 and 6mM $MgCl_2$ in the presence of 1U PRIME RNase Inhibitor (5 Prime → 3 Prime, Inc., Boulder, Colorado). RNA samples were then extracted with a standard phenol/chloroform method and precipitated in 0.3M sodium acetate, 2.5 volumes ethanol and 40ng/µL glycogen (Boehringer Mannheim, Laval, Quebec).

B.2.3. Northern Blot Analysis

For northern blot analysis, 7.5µg of total RNA was fractionated on a 0.66M formaldehyde/1.0% agarose gel and transferred onto nylon membranes (GeneScreenPlus, NEN-Dupont, Guelph, Ontario) with 20X SSC overnight. Membranes were UV crosslinked, then pre-hybridized with 40% formamide, 5X SSPE, 5X Denhardt's solution, 10% dextran sulfate, 1% SDS and 0.1mg/ml sonicated fish sperm DNA (Boehringer

Mannheim, Laval, Quebec) for at least 2h at 42°C. Hybridization was carried out by adding the randomly-labeled, Sephadex G-50-purified cDNA probe at 10^5-10^6cpm/ml hybridization solution and incubating in the same conditions for 16h. Washes were performed once at room temperature with 2X SSC/0.1% SDS for 15 min, twice at 42°C with 2X SSC/0.1% SDS for 30 min, once at 55°C with 0.5X SSC/0.1% SDS, and when needed once at 65°C with 0.5X SSC/0.1% SDS. Membranes were exposed to either BIOMAX MR or X-OMAT AR photographic film (Eastman Kodak Company, Rochester, New York). Relative expression levels were evaluated by comparison with the signal intensity of the internal control, *β-actin*.

B.2.4. Southern Blot Analysis

For Southern blot analysis, 5µg of restriction-digested DNA was fractionated on a 0.8% agarose gel and transferred onto nylon membranes (GeneScreenPlus, NEN-Dupont, Guelph, Ontario) with 0.4N NaOH overnight. Membranes were UV crosslinked, then pre-hybridized with 10% dextran sulfate, 1M NaCl, 1% SDS and 50µg/ml sonicated fish sperm DNA (Boehringer Mannheim, Laval, Quebec) for at least 2h at 65°C. Hybridization was carried out by adding the randomly-labeled, Sephadex G-50-purified cDNA probe at 10^6cpm/ml hybridization solution and incubating in the same conditions for 16h. Washes were performed once at room temperature with 2X SSC/1% SDS for 15 min, once at 42°C with 2X SSC/1% SDS for 30 min and, when needed, once at 65°C with 2X SSC/1% SDS or 0.5X SSC/0.1% SDS for 30 min. Membranes were exposed to either BIOMAX MR or X-OMAT AR photographic film (Eastman Kodak Company, Rochester, New York).

B.2.5. Gene-Specific RT-PCR

cDNA was synthesized from 0.5-2µg DNase-treated total RNA in a 20µL reaction containing 20mM Tris-HCl (pH8.4), 50mM KCl, 5mM $MgCl_2$, 1mM of each dNTP, 1U PRIME RNase Inhibitor (5 Prime → 3 Prime, Inc., Boulder, Colorado), 2.5µM random hexamers (Amersham Pharmacia Biotech, Baie d'Urfé, Québec) and 500U MMLV reverse transcriptase (Gibco BRL Life Technologies, Burlington, Ontario). Reactions are first incubated at room temperature for ten minutes to allow annealing of the random

hexamers, then allowed to proceed at 37^{0}C for one hour before inactivating the enzyme for 10 min. at 95^{0}C. One reaction without reverse transcriptase was included for each RNA sample, to control for the presence of DNA.

PCR amplification was carried out in a total volume of 12.5μL containing 2.5μL of the cDNA products, 0.48μM of each primer and 0.5U Taq DNA polymerase (Gibco BRL Life Technologies, Burlington, Ontario) in a final concentration of 20mM Tris-HCl pH 8.4, 50mM KCl and 2mM $MgCl_2$, and in some cases 1.5μCi [α-^{32}P]dCTP (3000 Ci/mmole, ICN, Mississauga, Ontario). dNTPs carried over from the RT reaction were sufficient for the PCR that was performed for 35 cycles of denaturation at 94^{0}C for 30s; annealing for 30s; elongation at 72^{0}C for 30s; completed by a final extension cycle at 72^{0}C for 5 min. The number of cycles varied between 22 and 28, depending on the primer pair, when radioactive nucleotides were included in the reaction. The radiolabelled PCR products were fractionated on a 7% non-denaturing polyacrylamide (29:1, acrylamide : bis acrylamide) gel in 1X TBE buffer and then transferred to 3M paper, dried, and exposed to X-OMAT film (Eastman Kodak Company, Rochester, New York). Non-radiolabelled PCR products were fractionated on a 1% agarose gel in 1X TBE buffer in the presence of ethidium bromide and photographed under UV light.

B.3. Gene Expression Profiling of Neuroblastoma

B.3.1. Differential Display

B.3.1.1. Reverse Transcription

DNase-treated RNA was reverse transcribed using each of the 3' anchored oligo(dT) primers listed in Table V. Each 25μl reaction contained 2μg DNAse-treated total RNA, 2μM of each oligo(dT) primer, 25μM of each dNTPs, 3mM $MgCl_2$, 50mM Tris-HCl (pH8.3), 75mM KCl, 1U Prime RNase Inhibitor (5-prime, 3-prime, Inc, Boulder, Colorado) and 600U MMLV reverse transcriptase (Gibco BRL Life Technologies, Burlington, Ontario). The reaction was carried out at 37^{0}C for 70 min.

TABLE V. LIST OF PRIMERS USED IN DIFFERENTIAL DISPLAY EXPERIMENTS

Name	Type	Sequence (5'-3')	Reference
E	3' oligo d(T)	gcgcaagcttT$_{10}$GA	Linskens *et al.*, 1995
F	3' oligo d(T)	gcgcaagcttT$_{10}$GA	Linskens *et al.*, 1995
K	3' oligo d(T)	gcgcaagcttT$_{10}$AA	Linskens *et al.*, 1995
M	3' oligo d(T)	gcgcaagcttT$_{10}$GC	Linskens *et al.*, 1995
00	5' arbitrary	cgggaagcttATCGACTCCAAG	Linskens *et al.*, 1995
01	5' arbitrary	cgggaagcttTAGCTAGCATGG	Linskens *et al.*, 1995
02	5' arbitrary	cgggaagcttGCTAAGACTAGC	Linskens *et al.*, 1995
03	5' arbitrary	cgggaagcttTGCAGTGTGTGA	Linskens *et al.*, 1995
04	5' arbitrary	cgggaagcttGTGACCATTGCA	Linskens *et al.*, 1995
05	5' arbitrary	cgggaagcttGTCTGCTAGGTA	Linskens *et al.*, 1995
E2AP2	5' arbitrary	cgtgaattcgGACCGCTTGT	Zhao *et al.*, 1995
Nmyc3'	5' arbitrary	cgggaagcttTGCGGCCAGTAT	Saikali *et al.*, 2001

B.3.1.2. PCR

The cDNA fragments were amplified in a 20μl reaction containing 1μl of the reverse transcribed mixture, 2U Taq DNA polymerase (Gibco BRL Life Technologies, Burlington, Ontario), 7.5μM of each dNTPs, 2μCi α^{33}P-dCTP (2000Ci/mmole, NEN-Dupont, Guelph, Ontario), 2mM $MgCl_2$, 20mM Tris-HCl (pH8.4), 50mM KCl, and 1.25μM of both 3' and 5' amplimers (see above). Amplification was performed for 4 cycles at low stringency (94^0C, 45s; 41^0C, 60s; 72^0C, 60s), followed by 18 cycles at high stringency (94^0C, 45s; 60^0C, 45s; 72^0C, 120s). The PCR products were fractionated on both denaturing (6% polyacrylamide [19:1, acrylamide : bis acrylamide], 8.3M urea) and non-denaturing (6% polyacrylamide [29:1, acrylamide : bis acrylamide], 10% glycerol) gels in 1X TBE. Fragments that appear on the display of cDNAs (cDNA fingerprint) from at least one cell line but not the others were considered differentially-expressed mRNAs. Twenty-five primer combinations were used, generating twenty-five differential display experiments. To control the reproducibility, each reverse transcription and PCR reaction was performed in duplicate, leading to four cDNA fingerprints for each RNA sample.

B.3.1.3. Reamplification and Confirmation of Differential Display

The bands of interest were cut out from the gel, isolated as described in Zietkiewicz *et al.* [1992] and re-amplified by 25 cycles of PCR using the high-stringency conditions described above with the corresponding primer pair combinations, using 50 μM dNTPs instead of 7.5 μM. Re-amplified cDNAs were purified using the QIAquick PCR purification kit (QIAGEN, Mississauga, Ontario) from an agarose gel after electrophoresis. Differential display was confirmed by Northern blot and/or RT-PCR analysis of a panel of neuroblastoma cell lines. When needed, the purified PCR products were directly sequenced using the ThermoSequenase kit (Amersham Life Science, Oakville, Ontario), according to the manufacturer's instructions, and analyzed for homology with sequences in the Genbank database.

B.3.2. Hybridization to Atlas™ cDNA Expression Arrays

We used the Atlas™ Human 1.2 Arrays from CLONTECH Laboratories, Inc. (Palo Alto, California). The probe preparation and hybridization were performed as per the manufacturer's specifications, with mRNA isolated using the Oligotex Direct mRNA Mini kit (QIAGEN, Mississauga, Ontario). Briefly, each cell line-specific probe was synthesized using an array-specific primer mix and the supplied reagents, in the presence of α^{32}P-dATP (3000Ci/mmole, NEN-Dupont, Guelph, Ontario). The probe pool was purified on Nucleospin extraction columns (CLONTECH Laboratories, Inc., Palo Alto, California). Hybridization took place in the presence of ExpressHyb and C_ot-1 DNA, both from CLONTECH Laboratories, Inc. (Palo Alto, California), 0.1 mg/ml sonicated salmon sperm DNA and 0.4 X 10^6 – 1.5 X 10^6 cpm of probe per ml of hybridization solution at 68^{0}C overnight. Membranes were exposed to either BIOMAX MR or X-OMAT AR photographic film (Eastman Kodak Company, Rochester, New York) for a variable amount of time to get an optimal signal to background ratio. Spot intensity was quantified from the autoradiograms using the Un-Scan-It™ software (Silk Scientific, Orem, Utah). Only unambiguous signals having at least 5% of the intensity of a control spot (we chose to use GAPDH) were retained.

B.4. Analysis of *GPC3* Expression in Embryonal Tumours

B.4.1. RNA Isolation and Analysis

Total RNA was isolated as described in section B.2.1. The expression of the genes *GPC3*, *IGF2*, *MYCN* and *ß-actin* was analysed by northern analysis and/or RT-PCR, as described in sections B.2.3 and B.2.5. Primers used for RT-PCR are listed in Table VI.

TABLE VI. LIST OF PRIMER PAIRS USED IN GENE-SPECIFIC RT-PCR

Name	Sequence (5'-3')	Target	Annealing Temp.	Product Length	Reference
GPC35	GATACAGCCAAAAGGCAG	GPC3 exon 5	55°C	250 bp	modified from Pilia *et al.*, 1996
GPC36	ATCATTCCATCACCAGAG	GPC3 exon 6			
ACTB2	ACCATGGATGATGATATCGC	β-actin exon 2	60°C	398 bp	Saikali and Sinnett, 2000
ACTB4	ACATGGCTGGGGTGTTGAAG	β-actin exon 4			
APA-P2	CTTGGACTTTGAGTCAAATTGG	IGF2 exon 9	55°C	292 bp	modified from Vu and Hoffman, 1994
APA-P3	GGTCGTGCCAATTACATTTCA				
NMYC2C	AGACCAGCGGCGGCGACCA	MYCN exon 2	62°C	360 bp	Saikali and Sinnett, 2000
NMYC3D	ATGACACTCTTGAGCGGACG	MYCN exon 3			

Temp.; temperature

B.4.2. Statistical Analysis

The relative expression of *GPC3, IGF2* and *MYCN* was analyzed by comparison with expression of *ß-actin* from at least three independent RT-PCRs and/or northern blots. Statistical analysis of the expression of these genes was performed using two models: i) by assigning the value 0 to samples with no expression and the value 1 to samples with expression at any level; ii) by assigning the value 2 to samples with expression, the value 1 to samples with trace levels of expression and the value 0 to samples with no expression. Spearman's rank correlation coefficient was used to analyse the data according to both models.

B. 5. Analysis of Function of GPC3

B.5.1. Cell Culture

B.5.1.1. Cell Culture Products and General Conditions

Cell culture products were from Gibco BRL Life Technologies (Burlington, Ontario), unless otherwise specified. Fetal bovine serum (FBS) was inactivated at 56^0C for 30 min before use. Cells were passaged using 0.05% Trypsin / 0.53mM EDTA.4Na, after rinsing with Hanks' Balanced Salt Solution (HBSS). All cells were maintained at 37^0C with 5% CO_2. Cryopreservation of cell cultures was performed in the appropriate medium supplemented with 20% FBS and 5% DMSO. Cells were counted using a Neubauer Brightline Hemacytometer (Hausser Scientific Company, Horsham, Pennsylvania) after staining with Trypan Blue. Isolation of clones from single cells was accomplished using cloning rings (Scienceware®, Pequannock, New Jersey) after seeding serial dilutions of cells.

B.5.1.2. Maintenance of Cell Lines

All NB cell lines as well as HeLa, Ntera2 and 143N2 were maintained in Minimum Essential Medium (MEM) with Earle's salts and non-essential amino acids. Unless otherwise specified, culture medium for these cells was supplemented with 10% FBS, 110 mg/L sodium pyruvate (1mM), 292 mg/L L-Glutamine (2mM), 100U/ml Penicillin G

sodium, 100μg/ml Streptomycin sulfate and 1μg/ml Fungizone® (amphotericin B).

R cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with high glucose (4500 mg/L), 584 mg/L L-glutamine, 110 mg/L sodium pyruvate, 100U/ml Penicillin G sodium, 100μg/ml Streptomycin sulfate, 1μg/ml Fungizone® (amphotericin B) and 10% FBS. In addition, R- cells were cultured in the presence of 50 μg/ml geneticin and R+ cells in the presence of 50 μg/ml Hygromycin B.

B.5.2. Expression Systems and Vector Preparation

B.5.2.1. pcDNA3.1

Expression vector pcDNA3.1 was obtained from Invitrogen Corp. (Carlsbad, California). This vector contains a multiple cloning site that allows insertion of a desired sequence under the control of the cytomegalovirus (CMV) constitutive promoter (Figure 3). The full-length human *GPC3* cDNA was obtained from H.C. Hsu at National Taiwan University Hospital, where it had been subcloned (from base 10 to base 2180 of Genbank entry Z37987) into pBluescript using EcoR I at the 5' end and Xho I at the 3' end. Two vectors were prepared: one with the cDNA in the sense orientation, produced by inserting an EcoR I / Xho I fragment of the *GPC3* cDNA into pcDNA3.1, and another with the cDNA in the antisense orientation, produced by inserting an EcoR I / Kpn I fragment of the *GPC3* cDNA into pcDNA3.1.

B.5.2.2. Ecdysone-Inducible Mammalian Expression System

The Ecdysone-Inducible Mammalian Expression System was purchased from Invitrogen Corp. (Carlsbad, California). This system uses a steroid hormone analog, ponasterone A, to induce expression of the cloned gene of interest. Two vectors are required for this system. The first, pVgRXR, encodes two subunits of a heterodimer of the ecdysone receptor. This heterodimer binds the inducer, in this case ponasterone A, and activates transcription of the target gene cloned downstream of a hybrid ecdysone response element on the second vector, pIND (Figure 4). This allows transcription of the gene of interest only in the presence of the inducer in cells that have been cotransfected

with both vectors. pVgRXR utilizes the Zeocin™ antibiotic resistance gene for selection in both bacteria and eukaryotic cells. pIND contains resistance genes to both ampicillin and neomycin. The *GPC3* cDNA was subcloned into pIND with the same strategy and restriction sites as with pcDNA3.1, also producing sense and antisense constructs. Zeocin™ and Ponasterone A were both obtained from Invitrogen Corp. Induction experiments were performed in the presence of 5μM Ponasterone A, with antibiotic levels at 25 μg/ml Zeocin™ and 50 μg/ml geneticin, for 20 hrs or 24 hrs. Ethanol was used to control for the inducer, since Ponasterone A is solubilized in 95% ethanol.

B.5.2.3. Vector Preparation

For plasmid propagation, calcium chloride-competent *Escherichia coli* (*E. coli*) DH5α strain from Gibco BRL Life Technologies (Burlington, Ontario) was transformed with the appropriate plasmid by heat shock [Sambrook *et al.*, 1989]. Transformed bacteria were cultured in LB supplemented with 100μg/ml ampicillin, except in the case of pVgRXR, cultured in low-salt LB and 50μg/ml Zeocin™. For cloning purposes, extraction of the plasmid from *E. coli* was performed with a standard SDS-alkaline lysis method [Sambrook *et al.*, 1989]. For transfection purposes, plasmid was isolated with the CONCERT™ High Purity Plasmid Midiprep system (Gibco BRL Life Technologies, Burlington, Ontario), as per the manufacturer's instructions.

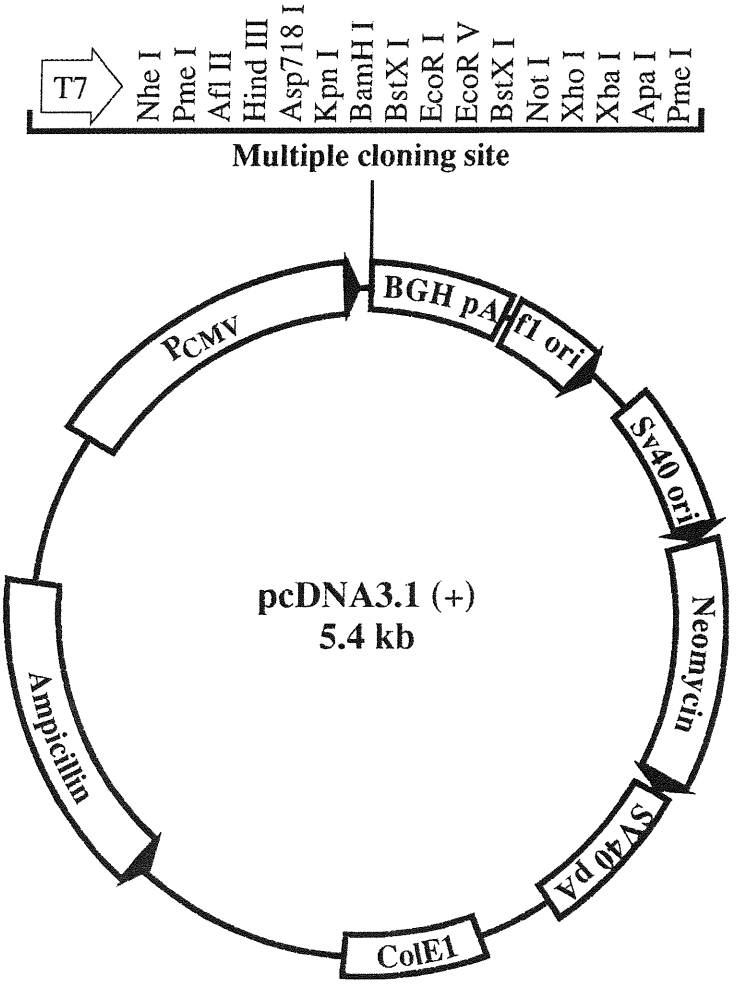


Figure 3. Map of expression vector pcDNA3.1. Features highlighted include the cytomegalovirus (CMV) promoter that induces constitutive expression of the inserted DNA, an ampicillin resistance gene for selection in bacteria, a neomycin resistance gene for selection in eukaryotic cells and two origins of replication, one for eukaryotic cells and another for prokaryotic cells.

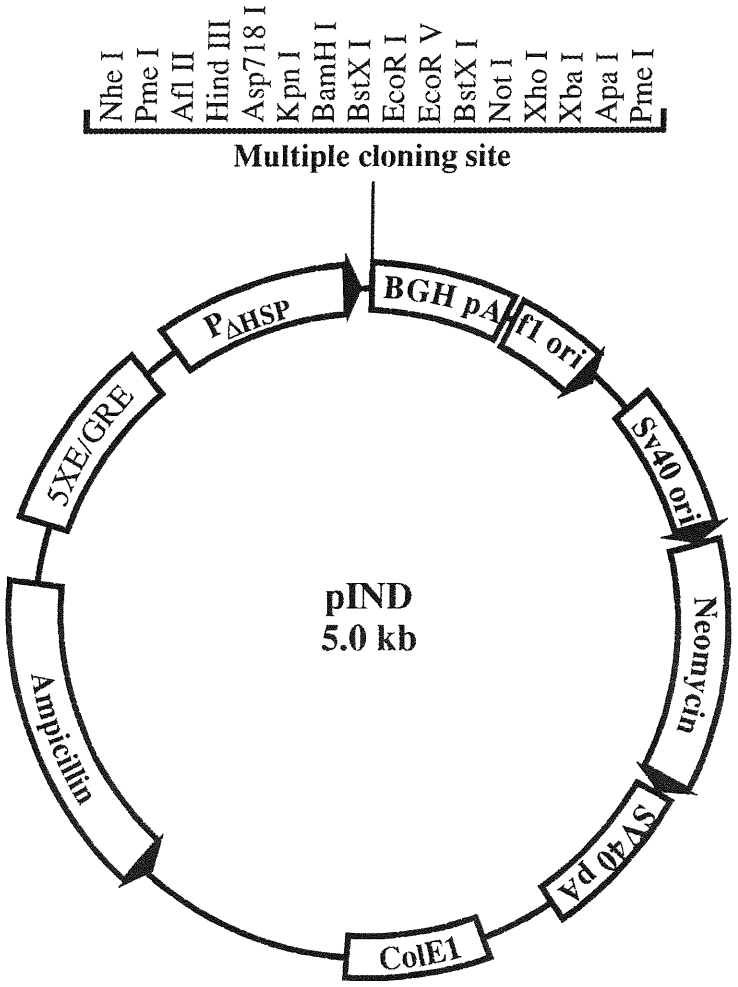


Figure 4. Map of expression vector pIND. Features highlighted include the minimal heat shock promoter ($P_{\Delta HSP}$), the ecdysone/glucocorticoid response elements (5XE/GRE), an ampicillin resistance gene for selection in bacteria, a neomycin resistance gene for selection in eukaryotic cells and two origins of replication, one for eukaryotic cells and another for prokaryotic cells.

B.5.3. Cell Transfection

All eukaryotic cell transfections were performed with a cationic lipid reagent, LIPOFECTAMINE PLUS™ from Gibco BRL Life Technologies, as per the manufacturer's instructions. Typical conditions for transfection in a well of a 6-well plate (an area of approximately 950 mm^2) were: 1 to 2 μg of DNA, 6μl PLUS reagent (4μl for SK-N-FI cells) and 4μl LIPOFECTAMINE™ (2μl or 4μl for R cells). In every case, medium was replaced with fresh medium without any antibiotics or antimycotics several hours before the transfection, and these agents were not present during transfection. In general, cells were 40%-60% confluent at the time of transfection. SK-N-AS, SK-N-FI and all R cells were transfected in medium without FBS, while HeLa cells were transfected in medium with 8-10% FBS. In the case of stable transfection with pcDNA3.1, 750 μg/ml neomycin (in the form of Geneticin® from Gibco BRL Life Technologies) was used for selection and 200-400 μg/ml for maintenance of transfected cells. In the case of stable transfection with pVgRXR, 300 μg/ml Zeocin™ was used for selection and 100 μg/ml for maintenance of transfected cells. When pVgRXR-transfected cells were stably transfected with pIND, 400 μg/ml and 100 μg/ml Geneticin® were used for selection and maintenance, respectively, in the presence of 50 μg/ml Zeocin™.

SECTION C. RESULTS

In this project, we applied two complementary approaches to analyze at a large-scale level the expression patterns in NB cells: the differential display technique, pioneered in 1992 by Liang and Pardee, and cDNA microarray analysis, described by a number of groups [Chalifour *et al.*, 1994; Schena *et al.*, 1995]. These methods led to the identification of candidate genes involved in the development of NB, one of which, Glypican 3 (*GPC3*), was further investigated.

C.1. Differential Display

C.1.1. Optimization of Differential Display

The PCR approach of differential display (DD-PCR) consists of using anchored oligo(dT) primers to reverse transcribe a subpopulation of mRNAs that was then amplified by PCR with arbitrary amplimers, to generate a cDNA fingerprint of the corresponding cells (Figure 5). This enables a comparison between the expression patterns of a variety of samples. Using the original experimental conditions [Liang and Pardee, 1992], we observed inconsistencies in the cDNA fingerprints generated from duplicated experiments. To circumvent this problem, we made the following modifications (details in Table VII): 1) The combination of longer anchored oligo(dT) and arbitrary amplimers and a two-step PCR protocol increased the reproducibility [Linskens *et al.*, 1995; Zhao *et al.*, 1995]; 2) The use of ^{33}P instead of ^{32}P or ^{35}S resulted in greater sensitivity and specificity [Tokuyama and Takeda, 1995]; and 3) The electrophoresis on a non-denaturing polyacrylamide gel reduced the complexity of the cDNA patterns observed in denaturing gels [Bauer *et al.*, 1993]. To verify the reproducibility of the modified DD-PCR, each reverse transcription and PCR reaction was done in duplicate leading to four cDNA fingerprints for each RNA sample. A representative result using RNA from 6 NB cell lines is shown in Figure 6. The presence of contaminating chromosomal DNA was controlled by the PCR amplification of each RNA sample without reverse transcription. The results between duplicates were consistent confirming the reproducibility of our version of DD-PCR.

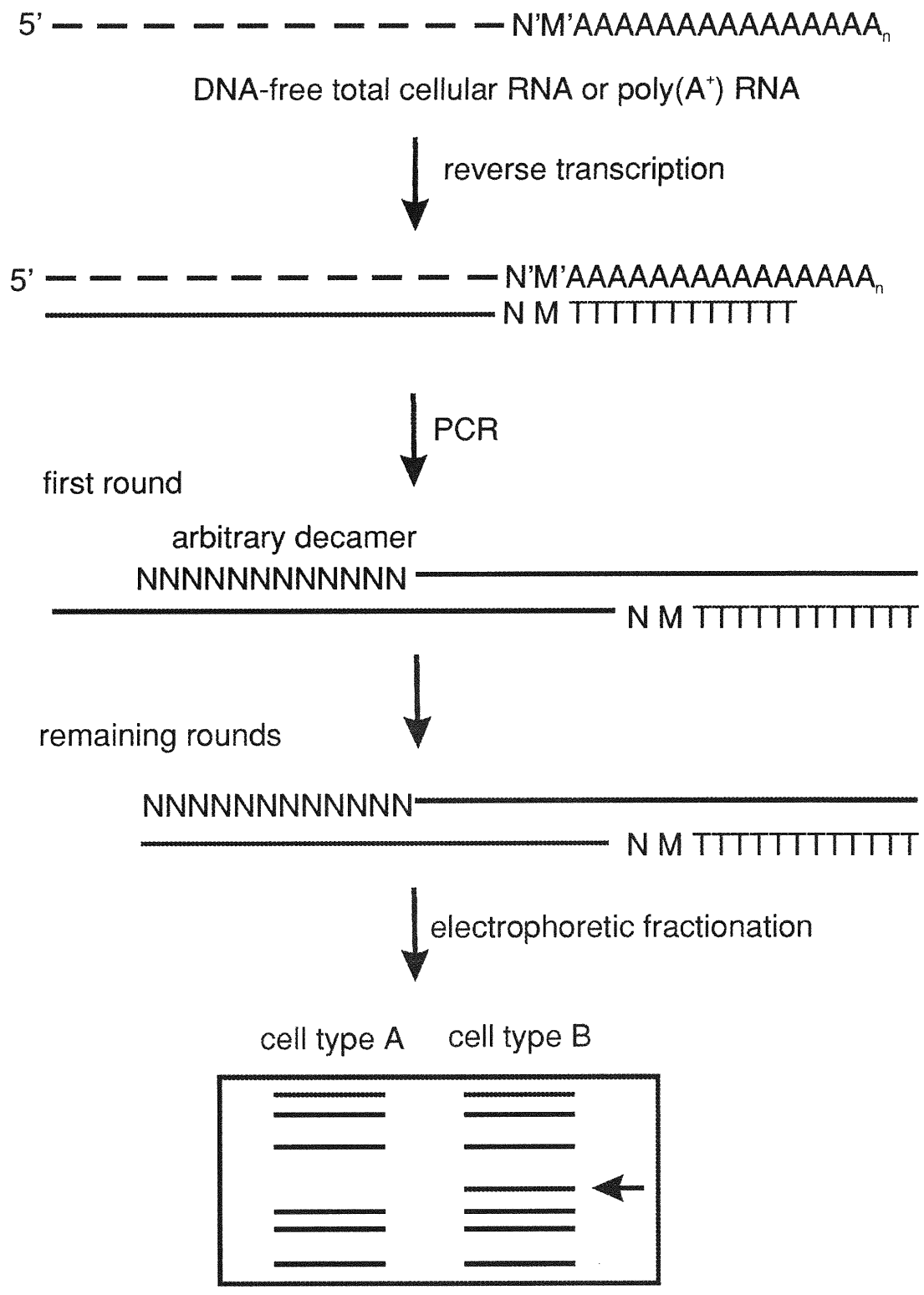


Figure 5. Schematic diagram of the differential display technique. The arrow indicates a cDNA fragment that is differentially expressed.

C.1.2. Use of *MYCN* Expression Levels as Control

To verify whether or not this modified version of DD-PCR was capable of detecting changes in mRNA levels in NB cell lines, we designed an arbitrary primer, nmyc3' (see Table V), that would allow the RT-PCR amplification of *MYCN* at the least. Two of the 6 cell lines tested, SJNB-7 and SJNB-10, are known to have amplification of the *MYCN* gene leading to an increased mRNA level. The cDNA fingerprint of these cell lines generated with the combination of the nmyc3' amplimer and the anchored oligo(dT) K included a band, denoted KNMYC33/04, corresponding to the predicted *MYCN* transcriptional product (Figure 6). The identity of this cDNA was examined by Northern and Southern hybridizations: KNMYC33/04 was amplified in SJNB-7 and SJNB-10 (Figure 7a), and overexpressed in SJNB-7, SJNB-10 and NBL-S compared to other NB cell lines (Figure 7b). DNA sequencing confirmed that this cDNA corresponds to a fragment in the 3' end of the *MYCN* gene (Figure 8). Noteworthy, the NBL-S cell line that is not associated with *MYCN* amplification also showed an increased expression of *MYCN*, suggesting other mechanisms of activation [Cohn *et al.,* 1990]. These experiments show the ability of DD-PCR to detect the differential expression of a known gene in NB cell lines.

C.1.3. Differential Display of mRNA in Neuroblastoma Cell Lines

RNA isolated from NB tumours is most often of poor quality and quantity, thus we chose to perform our DD-PCR analysis with cell lines. RNA species from two human NB cell lines with *MYCN* amplification (SJNB-7 and SJNB-10) and four without *MYCN* amplification (SJNB-1, SK-N-MC, SK-N-SH and NBL-S) were analyzed with 25 combinations of the 4 anchored oligo(dT) primers and the 8 arbitrary amplimers listed in Table V. In general, each combination of amplimers generated 50-100 cDNA fragments (Figure 6), resulting in a total production of approximately 1900 cDNA species. We identified 137 cDNA fragments that displayed a putative differential expression in the six NB cell lines. These bands were extracted from the gel and re-amplified with the appropriate primers. 118 of the resulting fragments were purified on agarose gels and used as probes for Northern hybridizations on membranes containing RNA from ten NB cell lines. Out of these 118 fragments, 41 were confirmed for differential expression. Twenty of these fragments generated unambiguous sequence information (Table VIII).

TABLE VII. DIFFERENCES BETWEEN STANDARD CONDITIONS AND MODIFIED CONDITIONS FOR EACH PARAMETER IN THE DIFFERENTIAL DISPLAY METHOD

Parameter	Standard conditions[1]	Improved conditions
Oligo dT amplimer 5'	$T_{11}MN$	GCGCAAGCTT-$T_{10}MN$[2]
Arbitrary amplimer 3'	decamer	CGGGAAGCTT-dodecamer[2]
PCR conditions	40 cycles at 42°C annealing	4 cycles at 41°C annealing 18 cycles at 60°C annealing[2]
Labelling	S^{35} or P^{32}	P^{33} [3]
PAGE	6% denaturing	6% non-denaturing[4]

1. Liang, P. and A.B. Pardee (1992) *Science* **257:**967-971.
2. Linskens, M.H.K., *et al.* (1995) *Nucleic Acids Research* **23:**3244-3251.
3. Tokuyama, Y. and Takeda J. (1995) *BioTechniques* **18:**424-425.
4. Bauer D., et al. (1993) *Nucleic Acids Research* **21:**4272-4280.

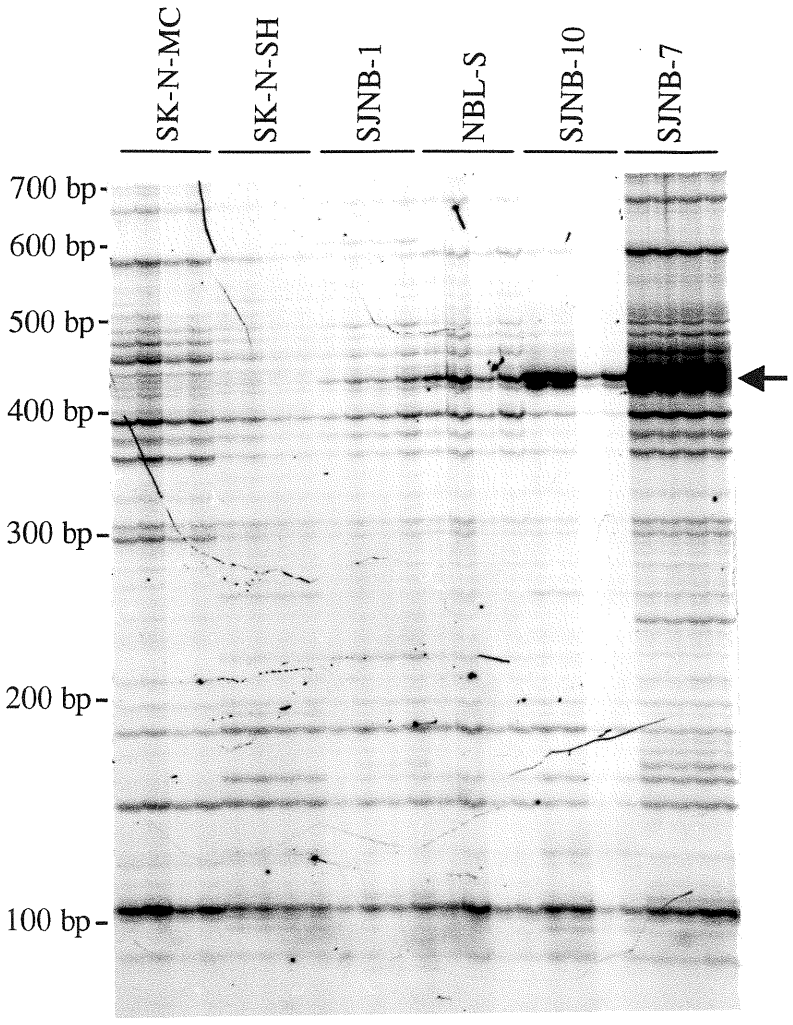
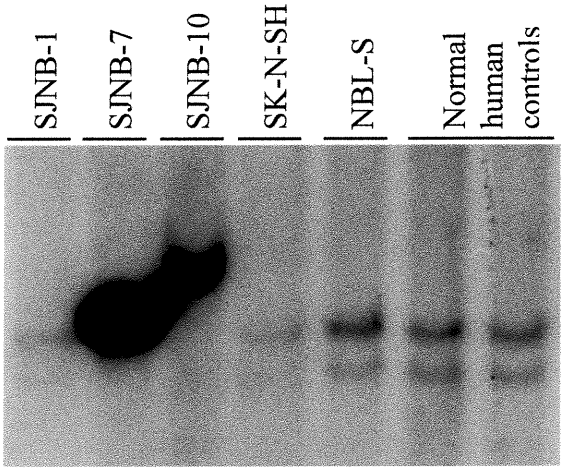


Figure 6. A representative result of a differential display experiment. This cDNA fingerprint was generated with a combination of the primers nmyc3' and K. Every four lanes represent four distinct RT-PCR reactions of RNA from a single cell line. This enables to control for reproducibility and verify the stringency of observed differences between cell lines. The arrow indicates a band suspected to correspond to *MYCN* (fragment denoted KNMYC33/04).

a)



b)

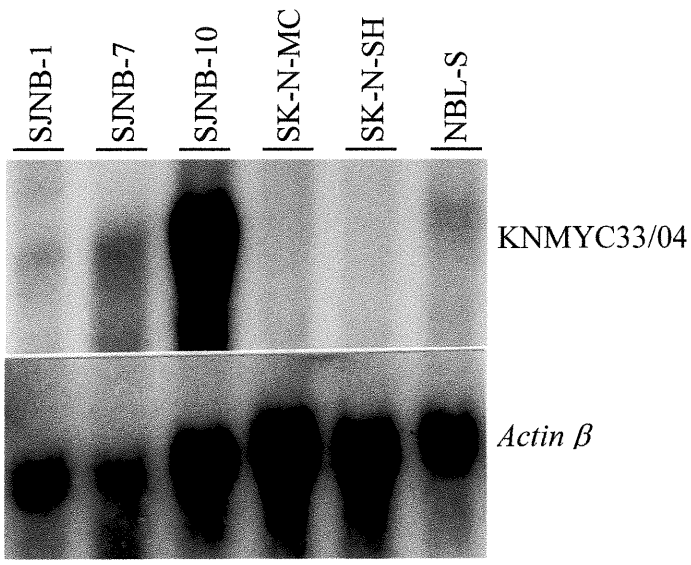


Figure 7. Analysis of the identity of the fragment KNMYC33/04.
a) Southern blot of KNMYC33/04 probe hybridized with genomic DNA from cell lines used in the differential display and normal controls. Amplification of the DNA corresponding to this fragment is visible in cell lines SJNB-7 and SJNB-10.
b) Northern blot of KNMYC33/04 probe hybridized with RNA from the cell lines used in the differential display. Blot shows higher expression of the mRNA corresponding to this fragment in cell lines SJNB-7, SJNB-10 and NBL-S. The lower panel shows the same blot hybridized with an *actin β* probe to control for quality and quantity of RNA.

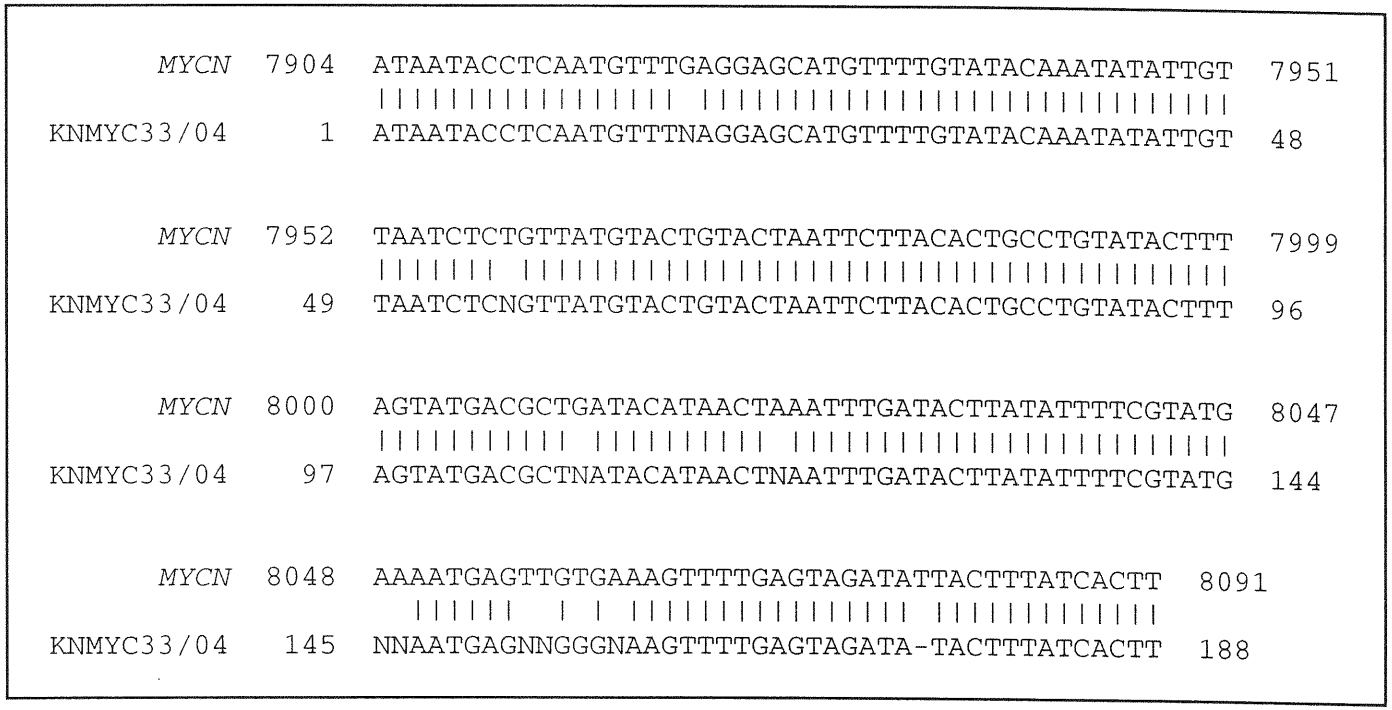


Figure 8. Confirmation of the identity of fragment KNMYC33/04. Alignment of 188 bp sequenced from the KNMYC33/04 fragment with the DNA sequence of the human germ line *MYCN* gene obtained from Genbank showing a match between *MYCN* nucleotides 7904 and 8091.

Seven of these differentially expressed cDNA species corresponded to known genes: *MYCN*, *IGF2*, *GPC3*, Ribosomal Protein S6 (*RPS6*), General Transcription Factor 3A (*GTF3A*), *BDNF*, as well as a xenotropic and polytropic murine leukemia virus receptor 1 (*XPR1*) (Table VIII). One cDNA fragment was highly similar to two genes encoding Eukaryotic Elongation Beta Factors, *eEF1B1* and *eEF1B2* (87% sequence identity in each case), although it remains unclear whether this corresponds to a known form of Elongation Factor Beta or represents a new gene. Four groups of ESTs corresponding to Unigene entries were also detected, two of which have predicted protein sequences. Finally, three fragments did not correspond to any sequence in Genbank.

Three genes were identified more than once independently: the *MYCN* gene was isolated four times, *RPS6* twice, and expressed sequence corresponding to Unigene Hs.28890 twice (Table VIII). In the case of two fragments, chromosomal localization was not previously available, but the fragments were placed either by sequence similarity to a genomic clone (fragments 56 and 57), or by using a radiation hybrid panel (fragment 54).

TABLE VIII. REPERTOIRE OF TWENTY cDNA FRAGMENTS ISOLATED BY DIFFERENTIAL DISPLAY

ID	Fragment size	Sequence Identity	Accession number	Chromosomal localization	Relative expression in NB cell lines									
					SJNB-7	SJNB-10	SK-N-DZ	IMR-32	NBL-S	SK-N-FI	SJNB-1	SK-N-MC	SK-N-SH	SK-N-AS
29	450 bp	*MYCN* (99%)	Y00664	2p24.1	++	++	++	++	+	+	tr	-	-	-
30	450 bp													
31	450 bp													
59	450 bp													
27	150 bp	*IGF2* (100%)	J03242	11p15.5	+	++	tr	tr	++	+	++	tr	+	++
18	450 bp	*GPC3* (100%)	L47125	Xq26.1	++	++	-	+	+	-	+	-	+	++
32	350 bp	*RPS6* (95%)	M20020	9p21	++	+	++	++	++	+	+	++	+	++
53	350 bp													
28	150 bp	*eEF1B1* (87%) *eEF1B2* (87%)	X60656 X60489	15 2	+	+	++	++	+	+	+	++	tr	++
16	350 bp	ESTs for *GTF3A* (100%)	Unigene Hs.75113	13q12.3-q13.1	++	+	++	+	+	tr	+	+	+	+
127	280 bp	*BDNF* (98%)	M61176	11p13	+	+	+	++	+	+	+	+	tr	++
98	150 bp	*XPR1* (98%)	AF089744	1q25.1	+	+	+	+	+	+	+	+	+	++
103	300 bp	hypothetical protein (95%)	NM_015679 Unigene Hs.98541	9	+	+	++	++	+	tr	+	+	tr	++
113	380 bp	hypothetical protein TOMM70A (98%)	NM_014820 Unigene Hs.21198	3	ND	+	+	+	tr	+	tr	tr	tr	+
54	350 bp	ESTs (95%)	Unigene Hs.142856	11q22-q23*	ND	+	+	tr	-	+	+	tr	-	+
56	260 bp	ESTs (99%)	Unigene Hs.28890	16*	++	+	ND	ND	+	ND	+	+	++	ND
57	260 bp													
21	150 bp	unknown	unknown	unknown	+	+	ND	ND	+	ND	+	+	++	ND
106	150 bp	unknown	unknown	unknown	+	+	++	++	+	tr	++	+	tr	++
129	120 bp	unknown	unknown	unknown	+	+	+	+	++	+	tr	++	tr	++

Shown for each is fragment size, sequence similarity, chromosomal localization and pattern of expression in up to 10 NB cell lines. Percentage similarity given is with the sequence corresponding to the indicated accession number.
tr, trace levels of expression; +, expression; ++, strong expression; ND, not done
* gene mapped in our study

C.2. Hybridization to Atlas™ cDNA expression arrays

The Atlas™ Human 1.2 cDNA expression array (CLONTECH Laboratories, Inc.) contains cDNAs representing 1176 genes, in addition to positive and negative controls, immobilized on nylon membranes. The identity of the genes is given on the company internet site (http://www.clontech.com/atlas/genelists/index.shtml). Two NB cell lines, SK-N-AS and SK-N-FI, were chosen for analysis using this method since the former expresses *MYCN* and the latter does not. mRNA from these cell lines was used to prepare probes for hybridization to the arrays. The methodology is schematized in Figure 9. Several exposures were obtained for each membrane in order to optimize the signal to background ratio and evaluate the relative intensity of each spot using the Un-Scan-It program. Only unambiguous signals having at least 5% of the intensity of the control spot corresponding to the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene were retained. Thus, 147 of the 1176 genes were found to be expressed in both cell lines (Table IX), 16 in only SK-N-AS (Table X) and 10 in only SK-N-FI (Table XI). In order to highlight the quantitative differences in expression, the ratio between the relative expression of the two cell lines is indicated (Table IX).

Of the 6 housekeeping genes serving as positive controls on the array, all show differences in relative expression between cell lines of less than 1.38, except *HLA-C*. As discussed in section A.7.5., *HLA-C* is known in NBs to be weakly and aberrantly expressed [Lampson *et al.*, 1983], and hence not suitable for use as a control with these samples. The low values for differences in expression in the other positive controls shows the ability of the technique and results to be used for quantitative analysis. We chose an arbitrary value of 2.5-fold as an indicator of quantitative differences in expression between cell lines. Twenty-six of the 147 genes (18%) expressed in both cell lines show differences in their relative expression levels (Table IX). The largest difference was observed for the expression of cyclin K (ratio of 4.69).

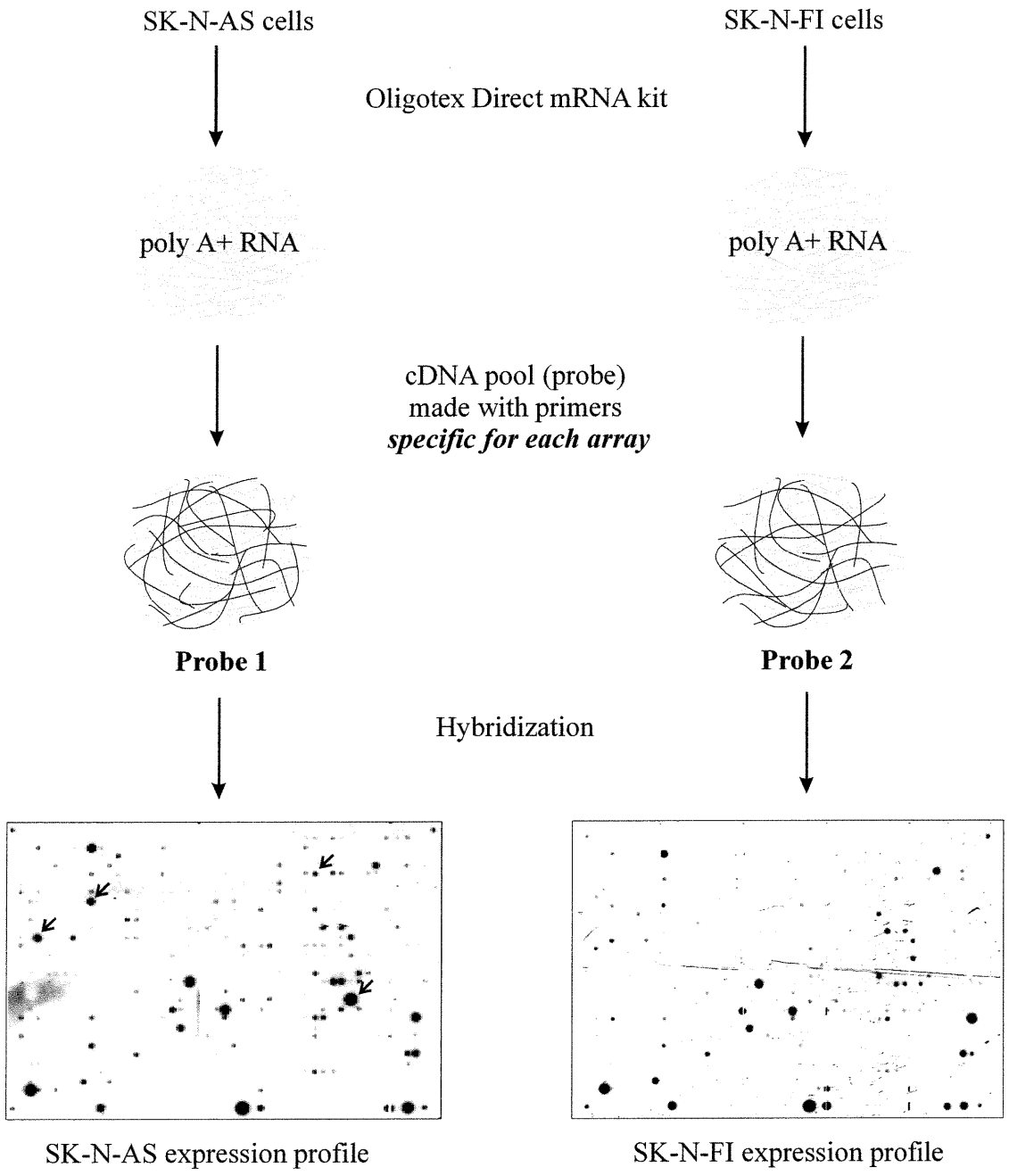


Figure 9. Scheme of the methodology for Atlas™ cDNA arrays. The arrows indicate overexpressed mRNA species in SK-N-AS.

TABLE IX. LIST OF GENES SHOWN BY cDNA MICROARRAY ANALYSIS TO BE EXPRESSED IN NB CELL LINES SK-N-AS AND SK-N-FI

Gene Name	Coordinate	SK-N-AS	SK-N-FI	ratio
tumour suppressors and related proteins				
EB1 protein	A03b	11.23	10.13	1.11
c-myc purine-binding transcription factor puf; nucleoside diphosphate kinase B (NDP kinase B; NDKB) + nm23-H2S	A09b	61.47	47.41	1.30
nucleoside diphosphate kinase A (NDKA); NDP kinase A; tumor metastatic process-associated protein; metastasis inhibition factor NM23 (NM23-H1)	A10b	9.98	5.86	1.70
transcription factor-related oncogenes				
c-jun proto-oncogene; transcription factor AP-1	A10c	5.30	4.36	1.22
B-myb	A05d	7.40	5.44	1.36
tyrosine kinase receptors				
papillary thyroid carcinoma-encoded protein + ret proto-oncogene	A09e	11.76	12.00	1.02
vascular endothelial growth factor receptor 1 (VEGFR1); tyrosine-protein kinase receptor flt + soluble VEGFR; tyrosine-protein kinase receptor SFLT	A01f	5.32	3.16	1.68
intracellular signal transduction-related oncogenes				
transforming protein rhoA H12 (RHO12; ARH12; ARHA)	A01g	10.93	5.21	2.10
N-ras; transforming p21 protein	A03g	7.15	3.60	1.99
cyclins				
cyclin K	A13g	13.92	2.97	4.69
G2/mitotic-specific cyclin B1 (CCNB1)	A02h	8.44	5.60	1.51
G1/S-specific cyclin D1 (CCND1); cyclin PRAD1; bcl-1 oncogene	A03h	5.88	10.29	1.75
fte-1; yeast mitochondrial protein import homolog; 40S ribosomal protein S3A (RPS3A)	A09h	54.96	18.98	2.90
cell cycle regulating kinases				
cell division protein kinase 5 (CDK5); tau protein kinase II catalytic subunit (TPKII catalytic subunit); serine/threonine protein kinase PSSALRE	A06i	3.14	12.41	3.95
CDC-like kinase 1 (CLK1)	A14i	5.12	4.21	1.22
serine/threonine-protein kinase PLK1 (STPK13)	A02j	6.39	2.37	2.70

Gene Name	Coordinate	SK-N-AS	SK-N-FI	ratio
cyclin-dependent kinase regulatory subunit 1 (CKS1)	A13j	17.17	9.07	1.89
cyclin-dependent kinase regulatory subunit (CKS2)	A14j	11.33	5.86	1.93
other cell cycle regulators				
CDC25B; CDC25HU2; M-phase inducer phosphatase 2	A01l	5.59	2.75	2.03
prothymosin alpha (ProT-alpha; PTMA)	A03l	53.89	13.16	4.09
40S ribosomal protein S19 (RPS19)	A07l	25.29	13.83	1.83
p55CDC	A13l	5.82	4.28	1.36
RCL growth-related c-myc-responsive gene	A14l	6.85	3.30	2.08
transmembrane 4 superfamily protein; SAS	A01m	12.72	4.81	2.64
signal transduction receptors and cell surface proteins				
ephrin type-A receptor 1 precursor; tyrosine-protein kinase receptor eph	B14d	12.45	10.75	1.16
receptor-associated proteins and adapters				
growth factor receptor-bound protein 2 (GRB2) isoform; GRB3-3; SH2/SH3 adaptor GRB2; ASH protein + epidermal growth factor receptor-bound protein 2 (EGFRBP-GRB2)	B14f	16.51	6.47	2.55
tyrosine-protein kinase lyn	B02g	7.38	5.23	1.41
intracellular kinase network members				
cAMP-dependent protein kinase I alpha regulatory subunit; tissue-specific extinguisher 1 (TSE1)	B14g	8.87	4.41	2.01
mitogen-activated protein kinase p38 (MAP kinase p38); cytokine suppressive anti-inflammatory drug binding protein (CSAID binding protein; CSBP); MAX-interacting protein 2 (MXI2)	B07h	15.39	6.96	2.21
MAP kinase-activated protein kinase 2 (MAPKAP kinase 2; MAPKAPK-2)	B09h	9.90	12.39	1.25
protein kinase C alpha polypeptide (PKC-alpha; PKCA)	B03i	5.09	3.01	1.69
protein kinase MLK-3; sprk	B04k	7.34	5.60	1.31
serine kinase	B06k	10.39	4.24	2.45
phospholipases and phosphoinositol kinases				
phosphatidylinositol-4-phosphate 5-kinase II beta; 1-phosphatidylinositol-4-phosphate kinase (PTDINS(4)P-5-kinase); diphosphoinositide kinase	B08l	5.46	4.94	1.11

Gene Name	Coordinate	SK-N-AS	SK-N-FI	ratio
G proteins				
ras-related protein RAB-7	B10m	14.03	13.71	1.02
guanine nucleotide-binding protein G(I)/G(S)/G(T) beta subunit 1 (GNB1); transducin beta-1 subunit	B12m	8.83	6.40	1.38
ras-related C3 botulinum toxin substrate 1; p21-rac1; ras-like protein TC25	B13m	18.02	8.44	2.14
ras-related protein RAP-1B; GTP-binding protein SMG p21B	B02n	5.09	9.89	1.94
ras-related protein RAB2	B03n	3.40	5.77	1.70
guanine nucleotide-binding protein G-i/G-s/G-t beta subunit 2; transducin beta 2 subunit 2	B10n	5.19	2.58	2.01
tyrosine phosphatases				
protein-tyrosine phosphatase 2C (PTP-2C); SH-PTP2	C04a	8.01	3.47	2.31
serine/threonine protein phosphatase 2B catalytic subunit alpha isoform; calmodulin-dependent calcineurin A subunit alpha isoform; CAM-PRP catalytic subunit	C10a	11.02	4.49	2.45
protein phosphatase 2C alpha isoform (PP2C-alpha)	C11a	5.93	2.56	2.32
serine/threonine protein phosphatase PP1-alpha 1 catalytic subunit (PP-1A)	C12a	8.90	7.60	1.17
PTPCAAX1 nuclear tyrosine phosphatase (PRL-1)	C01b	9.40	3.19	2.95
GDP/GTP exchangers and GTPase stimulators/inhibitors				
rho GDP dissociation inihibitor 1 (RHO-GDI 1); RHO-GDI alpha (GDIA1); ARHGDIA	C09d	40.45	39.55	1.02
kinase substrates and inhibitors				
cortactin; amplaxin; ems-1 oncogene	C12d	10.01	12.19	1.22
14-3-3 protein beta/alpha; protein kinase C inhibitor protein-1 (KCIP-1); protein 1054	C01e	10.10	8.29	1.22
protein kinase C substrate 80-kDa protein heavy chain (PKCSH); 80K-H protein	C02e	22.33	6.97	3.20
hint protein; protein kinase C inhibitor 1 (PKCI1)	C04e	17.40	15.22	1.14
macMARCKS; MARCKS-related protein (MRP); MLP	C05e	11.13	6.34	1.76
other intracellular signal transduction modulators and effectors				
tuberin; tuberous sclerosis 2 protein (TSC2)	C08e	8.49	8.88	1.05
TRRAP protein	C09e	6.11	4.18	1.46

Gene Name	Coordinate	SK-N-AS	SK-N-FI	ratio
caspases				
caspase-3 (CASP3); apopain precursor; cysteine protease CPP32; YAMA protein; SREBP cleavage activity 1; SCA-1	C05h	4.29	5.49	1.28
calpains				
calcium-dependent protease small (regulatory) subunit; calpain; calcium-activated neutral proteinase (CANP)	C02i	16.70	23.21	1.39
Bcl family				
induced myeloid leukemia cell differentiation protein MCL-1	C10i	8.43	3.67	2.30
other apoptosis-associated proteins				
growth arrest & DNA-damage-inducible protein 153 (GADD153); DNA-damage-inducible transcript 3 (DDIT3); C/EBP homologous protein (CHOP)	C12j	2.56	5.36	2.09
defender against cell death 1 (DAD1)	C03k	28.92	31.09	1.08
cytoplasmic dynein light chain 1 (HDLC1); protein inihibitor of neuronal nitric oxide synthase (PIN)	C05k	32.33	25.89	1.25
ALG-2 calcium-binding protein	C11k	9.63	8.21	1.17
DNA polymerases, replication factors and recombination proteins				
proliferating cyclic nuclear antigen (PCNA); cyclin	C06l	32.85	22.11	1.49
MCM2 DNA replication licensing factor; nuclear protein BM28; KIAA0030	C01m	5.77	3.25	1.78
MCM4 DNA replication licensing factor; CDC21 homolog	C02m	6.77	3.73	1.82
MCM5 DNA replication licensing factor; CDC46 homolog	C03m	6.75	5.04	1.34
MCM7 DNA replication licensing factor; CDC47 homolog; p1.1-MCM3	C05m	6.32	5.71	1.11
DNA damage repair proteins, ligases and helicases				
mutL protein homolog; DNA mismatch repair protein MLH1; COCA2	C06n	10.73	28.38	2.64
HHR23A; UV excision repair protein protein RAD23A	D04a	12.57	5.84	2.15
ubiquitin-conjugating enzyme E2 17-kDa (UBE2A); ubiquitin-protein ligase; ubiquitin carrier protein; HR6A	D05a	10.98	8.93	1.23
other DNA synthesis, repair and recombination proteins				
translin; recombination hotspot binding protein	D06a	7.82	6.10	1.28

Gene Name	Coordinate	SK-N-AS	SK-N-FI	ratio
recA-like protein HsRad51; DNA repair protein RAD51 homolog	D07a	3.50	5.91	1.69
ligand-gated ion channel receptors				
neuronal acetylcholine receptor protein alpha-3 subunit precursor (NACHRA3); cholinergic receptor nicotinic alpha polipeptide 3 (CHRNA3)	D14c	9.42	12.15	1.29
neurotransmitter synthesizing and degrading enzymes				
aromatic-L-amino-acid decarboxylase; DOPA decarboxylase (DDC)	D02e	10.87	6.43	1.69
dopamine beta-hydroxylase (DBH); dopamine-beta-monooxygenase precursor	D01f	24.15	7.09	3.41
neuromediators				
secretogranin II precursor (SGII); chromogranin C	D03f	8.08	19.35	2.39
acyl-CoA-binding protein (ACBP); diazepam binding inhibitor (DBI); endozepine (EP)	D09f	19.34	14.26	1.36
neuronal development, plasticity, degeneration and cell adhesion				
achaete-scute homolog 1 (ASH1)	D01h	13.05	9.18	1.42
disease-related proteins				
Alzheimer's disease amyloid A4 protein precursor; protease nexin-II (PN-II); APPI	D09i	33.42	18.54	1.80
nervous system-related transcription factors				
histone acetyltransferase B subunit 2; retinoblastoma-binding protein p46; retinoblastoma-binding protein 7	D11j	3.02	5.84	1.93
activated RNA pol II transcriptional coactivator p15; PC4	D14j	20.16	28.80	1.43
YL-1 protein	D03k	4.32	5.36	1.24
glucocorticoid receptor repression factor 1	D07l	2.15	6.26	2.91
interleukin enhancer-binding factor (ILF) ILF + interleukin enhancer binding factor 2 (ILF2) + interleukin enhancer binding factor 3 (ILF3)	D10l	5.89	3.11	1.89
E2F dimerization partner 1; DRTF1-polypeptide 1 (DP1)	D02m	7.08	10.39	1.47
endothelial transcription factor GATA2	D08m	28.74	48.50	1.69
helix-loop-helix protein; DNA-binding protein inhibitor Id-2	D11m	9.93	7.33	1.35
60S ribosomal protein L6 (RPL6); TAX-responsive enhancer element binding protein 107 (TAXREB107); neoplasm-related protein C140	D02n	84.89	86.08	1.01

Gene Name	Coordinate	SK-N-AS	SK-N-FI	ratio
cellular nucleic acid binding protein (CNBP); sterol regulatory element-binding protein	D03n	15.68	9.27	1.69
estrogen receptor hSNF2b; global transcription activator SNF2L4; brg-1 protein; mitotic growth and transcription activator; brahma protein homolog 1	D05n	9.55	4.52	2.11
early growth response protein 1 (hEGR1); transcription factor ETR103; KROX24; zinc finger protein 225; AT225	E10a	3.30	13.82	4.19
transcription factor ETR101	E11a	2.66	9.11	3.42
nuclease-sensitive element DNA-binding protein (NSEP)	E04b	86.57	70.21	1.23
paired box protein PAX-5; B-cell specific transcription factor; BSAP	E08b	2.42	5.57	2.32
R kappa B DNA-binding protein	E02c	3.15	7.23	2.30
trans-acting T-cell specific transcription factor GATA3	E08c	9.88	9.85	1.00
transcription initiation factor TFIID 31-kDa subunit; TAFII31; TAF2G	E03d	7.72	8.10	1.05
26S protease regulatory subunit 6A; TAT-binding protein 1 (TBP1); proteasome subunit p50	E10d	11.93	15.37	1.29
CCAAT-binding transcription factor subunit B (CBF-B); NF-Y protein subunit A (NF-YA); Hap2; CAAT-box DNA-binding protein subunit A	E13d	12.71	17.54	1.38
cAMP-dependent transcription factor ATF-4; DNA-binding protein TAXREB67; cAMP-response element binding protein (CREB2)	E02e	39.09	51.78	1.32
heat shock factor protein 1 (HSF1); heat shock transcription factor 1 (HSTF1); TCF5	E03e	9.77	6.07	1.61
putative transcription activator DB1	E05e	7.20	6.36	1.13
zinc finger protein 91 (ZNF92); HPF7; HTF10	E07e	11.21	8.92	1.26
guanine nucleotide-binding protein G-s alpha subunit (GNAS); adenylate cyclase-stimulating G alpha protein	E08e	78.59	59.17	1.33
nucleobindin precursor (NUC)	E12e	7.07	13.26	1.88
transcription factor LSF	E14e	3.49	14.24	4.08
Ini1	E01f	5.99	7.17	1.20

DNA-binding and chromatin proteins

high mobility group protein (HMG-I)	E08f	14.60	10.74	1.36

Gene Name	Coordinate	SK-N-AS	SK-N-FI	ratio
cell surface antigens and adhesion proteins				
cadherin 5 (CDH5); vascular endothelial cadherin precursor (VE-cadherin); 7B4 antigen; CD144 antigen	E03g	48.40	51.48	1.06
NADH-ubiquinone oxidoreductase B18 subunit; complex I-B18 (CI-B18); cell adhesion protein SQM1	E05h	9.11	11.48	1.26
neural-cadherin precursor (N-cadherin; NCAD); cadherin 2 (CDH2)	E06h	6.24	6.50	1.04
fibronectin receptor beta subunit (FNRB); integrin beta 1 (ITGB1); VLA4 beta subunit; CD29 antigen	E09i	11.09	11.72	1.06
growth factor and chemokine receptors				
neuromedin B receptor (NMBR); neuromedin-B-preferring bombesin receptor	E03k	2.42	6.91	2.86
homeostasis and detoxification				
microsomal glutathione S-transferase 12 (GST12; MGST1)	E11n	3.03	11.74	3.87
glutathione S-transferase pi (GSTP1; GST3)	E12n	9.34	37.48	4.01
heat shock cognate 71-kDa protein	F02a	21.18	31.59	1.49
natural killer cell enhancing factor (NKEFB) + thiol-specific antioxidant protein (TSA); thioredoxin peroxidase 1 (TDPX1); thioredoxin-dependent peroxide reductase 1	F07a	26.90	8.49	3.17
thioredoxin peroxidase 2 (TDPX2); thioredoxin-dependent peroxide reductase 2; proliferation-associated gene (PAG); natural killer cell enhancing factor A (NKEFA)	F08a	10.33	12.57	1.22
heat-shock protein 40 (HSP40)	F02b	3.31	9.16	2.77
mitochondrial matrix protein P1 precursor; p60 lymphocyte protein; chaperonin homolog; HUCHA60; heat shock protein 60 (HSP-60); HSPD1	F03b	7.14	13.26	1.86
heat shock 90-kDa protein A (HSP90A; HSPCA); HSP86	F04b	48.67	32.16	1.51
27-kDa heat-shock protein (HSP27); stress-responsive protein 27 (SRP27); estrogen-regulated 24-kDa protein; HSPB1	F05b	42.20	24.51	1.72
70-kDa heat shock protein 1 (HSP70.1; HSPA1)	F06b	5.96	7.52	1.26
cytosolic superoxide dismutase 1 (SOD1)	F07b	14.76	22.53	1.53
glutathione synthetase (GSH synthetase; GSH-S); glutathione synthase	F12b	2.40	9.27	3.86

Gene Name	Coordinate	SK-N-AS	SK-N-FI	ratio
glutathione S-transferase mu1 (GSTM1; GST1); HB subunit 4; GTH4	F13b	2.72	9.38	3.45
glutathione S-transferase A1 (GTH1; GSTA1); HA subunit 1; GST-epsilon	F14b	5.29	12.87	2.43
growth factors, cytokines and chemokines				
macrophage-specific colony-stimulating factor (CSF-1; MCSF)	F03e	8.02	9.70	1.21
hepatoma-derived growth factor (HDGF)	F05e	22.00	11.52	1.91
neuroleukin (NLK); glucose-6-phosphate isomerase (GPI); phosphoglucose isomerase (PGI); phosphohexose isomerase (PHI)	F07e	11.78	11.09	1.06
monocyte chemotactic protein 1 precursor (MCP1); monocyte chemotactic and activating factor (MCAF); monocyte secretory protein JE; monocyte chemoattractant protein 1; HC11; small inducible cytokine A2 (SCYA2)	F12e	3.23	11.29	3.50
insulin-like growth factor binding protein 1 (IGFBP1); placental protein 12 (PP12)	F01f	4.98	5.69	1.14
vascular endothelial growth factor precursor (VEGF); vascular permeability factor (VPF)	F02f	23.60	11.59	2.04
pleiotrophin precursor (PTN) + osteoblast specific factor 1 (OSF-1) + heparin-binding neurite outgrowth promoting factor 1 (HBNF-1) ; heparin-binding growth-associated molecule (HB-GAM); heparin-binding growth factor 8 (HBGF-8)	F03f	24.74	24.43	1.01
thymosin beta-10 (TMSB10; THYB10); PTMB10	F13f	63.69	82.53	1.30
interleukins				
interleukin-1 beta precursor (IL-1 ; IL1B); catabolin	F10i	3.41	5.07	1.49
other extracellular communication proteins				
parathymosin	F12j	31.79	49.87	1.57
thymosin beta 4; FX	F13j	46.95	19.24	2.44
protein turnover				
methionine aminopeptidase 2 (METAP2); peptidase M2; initiation factor 2-associated 67-kDa glycoprotein	F12k	7.83	9.87	1.26
proteasome component C2; macropain subunit C2; proteasome nu subunit; multicatalytic endopeptidase complex subunit C2; 30-kDa prosomal protein (PROS30)	F011	3.99	6.01	1.51

Gene Name	Coordinate	SK-N-AS	SK-N-FI	ratio
proteasome component C3; macropain subunit C3; multicatalytic endopeptidase complex subunit C3	F02l	18.75	10.66	1.76
proteasome component C5; macropain subunit C5; proteasome gamma subunit; multicatalytic endopeptidase complex subunitC5	F03l	7.13	14.82	2.08
proteasome component C8; macropain subunit C8; multicatalytic endopeptidase complex subunit C8	F04l	7.42	11.87	1.60
housekeeping genes (controls)				
liver glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	G27	100.00	100.00	1.00
brain-specific tubulin alpha 1 subunit (TUBA1)	G29	40.08	55.42	1.38
HLA class I histocompatibility antigen C-4 alpha subunit (HLAC)	G31	3.12	6.01	1.93
cytoplasmic beta-actin (ACTB)	G43	41.78	46.77	1.12
23-kDa highly basic protein; 60S ribosomal protein L13A (RPL13A)	G45	82.27	60.23	1.37
40S ribosomal protein S9	G47	12.67	15.51	1.22

Values are relative to GAPDH levels. *Coordinate*, position on the microarray grid; *Gene name* and categories, according to manufacturer's list (accessible on manufacturer's internet site); *ratio*, higher level relative to lower level. Ratios highlighted indicate values above the 2.5-fold threshold.

TABLE X. LIST OF GENES SHOWN BY cDNA MICROARRAY ANALYSIS TO BE EXPRESSED ONLY IN NB CELL LINE SK-N-AS

Gene Name	Coordinate	SK-N-AS
transcription factor-related oncogenes		
c-myc oncogene	A12c	18.03
v-erbA related protein (EAR3); COUP transcription factor (COUP-TF)	A11d	10.94
tyrosine kinase receptors		
ERBB-3 receptor protein-tyrosine kinase precursor; epidermal growth factor receptor	A12e	7.28
other oncogenes		
insulin-like growth factor binding protein 2 (IGFBP2)	A09g	17.60
cyclins		
G2/mitotic-specific cyclin A (CCNA; CCN1)	A01h	5.28
signal transduction receptors and cell surface proteins		
neurotrophic tyrosine kinase receptor-related 3; TKT precursor	B03f	5.23
receptor-associated proteins and adapters		
c-src kinase (CSK); protein-tyrosine kinase cyl	B10f	5.26
lnk adaptor protein	B07g	5.40
intracellular kinase network members		
glycogen synthase kinase 3 beta (GSK3 beta); tau kinase subunit; factor A	B01h	6.63
calcium/calmodulin-dependent protein kinase type IV catalytic subunit (CAMK IV); CAM kinase-GR	B12i	14.15
focal adhesion kinase (FADK); proline-rich tyrosine kinase 2 (PYK2)	B07j	6.03
tyrosine phosphatases		
leukocyte common antigen precursor (L-CA); CD45 antigen; PTPRC	C02a	6.54
transcription factors		
cAMP-response element binding protein (CREB)	C03d	5.47
growth factors, cytokines and chemokines		
insulin-like growth factor II (IGF2); somatomedin A	F06d	93.91
delta-like protein precursor (DLK)	F02h	10.38
hormones		
cellular retinoic acid-binding protein II (CRABP2)	F13h	5.93

Values are relative to GAPDH levels. *Coordinate*, position on the microarray grid; *Gene name* and categories, according to manufacturer's list (accessible on manufacturer's internet site).

TABLE XI. LIST OF GENES SHOWN BY cDNA MICROARRAY ANALYSIS TO BE EXPRESSED ONLY IN NB CELL LINE SK-N-FI

Gene Name	Coordinate	SK-N-FI
tyrosine phosphatases		
protein phosphatase PP2A 55-kDa regulatory subunit neuronal isoform; protein phosphatase PP2A B subunit beta; beta-PR55	C07a	6.65
other intracellular signal transduction modulators and effectors		
zyxin + zyxin-2	C12e	11.49
other apoptosis-associated proteins		
clusterin precursor (CLU); complement-associated protein SP-40,40; complement cytolysis inhibitor (CLI); apolipoprotein J (APO-J); TRPM-2; sulfated glycoprotein 2	C13j	7.31
neuronal development, plasticity, degeneration and cell adhesion		
brain-specific polypeptide PEP-19; brain-specific antigen PCP4	D02h	6.68
disease-related proteins		
paraneoplastic encephalomyelitis antigen HUD; HU-antigen D	D11i	5.10
nervous system-related transcription factors		
transcriptional regulator interferon-stimulated gene factor 3 gamma subunit (ISGF3G); interferon-alpha (IFN-alpha) responsive transcription factor subunit	E05b	5.77
MSX-1 homeobox protein; HOX7	E10b	9.09
cell surface antigens and adhesion proteins		
CD44 antigen hematopoietic form precursor (CD44H); phagocytic glycoprotein I (PGP-1); HUTCH-I; extracellular matrix receptor-III (ECMR-III); GP90 lymphocyte homing/adhesion receptor (LHR); hermes antigen	E08h	6.25
integrin alpha 6 precursor (ITGA6); VLA6; CD49F antigen	E10i	5.05
homeostasis and detoxification		
thioredoxin reductase	F09b	5.27

Values are relative to GAPDH levels. *Coordinate*, position on the microarray grid; *Gene name* and categories, according to manufacturer's list (accessible on manufacturer's internet site).

C.3. Analysis of GPC3 Expression in Embryonal Tumours

The expression of *GPC3* in NB was detected by DD-PCR (see section C.1.3.). A DD-PCR experiment using the primers F and 01 identified a 550 bp cDNA fragment that was present only in two cell lines, SJNB-7 and SJNB-10 (Figure 10). The band of interest (F0126/08) was cut from the gel, re-amplified, isolated and used to probe a northern blot containing RNA from an extended panel of NB cell lines as well as an embryonal carcinoma cell line (Ntera2) and a liver carcinoma cell line (HepG2) (Figure 11). The presence of a 2.5 kb mRNA species was detected in the SJNB-7, SJNB-10, SK-N-SH and SK-N-AS neuroblastoma cell lines, as well as in Ntera2 and HepG2, but not in normal liver. Direct DNA sequencing of the PCR product yielded a 120 bp sequence that showed a perfect match with the 3'-end region of the *GPC3* gene [Pilia *et al.*, 1996] (Figure 12), which is identical to the Genbank sequences *MXR7* [Lage and Dietel, 1997] and *GTR2-2* [Hsu *et al.*, 1997]. Of note, the predicted size of the *GPC3* gene product, 2.5 kb, [Pilia *et al.*, 1996], is similar to the one in Figure 11.

To determine whether *GPC3* is expressed in the same cell lines as *MYCN*, the same RNA panel was rehybridized with a probe derived from the 3'UTR of *MYCN* (Figure 11). Only SJNB-7 and SJNB-10 displayed co-expression of both *GPC3* and *MYCN*, whereas *GPC3* and *MYCN* were expressed independently in two (SK-N-SH and SK-N-AS) and four (NBL-S, SK-N-FI, SK-N-DZ and IMR-32) cell lines, respectively. This suggests that these two genes are not necessarily expressed in the same cell, at least not in the context of NB. To the best of our knowledge, this is the first time that *MYCN* was shown to be expressed in the cell line SK-N-FI carrying a non-amplified *MYCN* locus (Figure 11).

GPC3 encodes glypican 3, a putative extracellular membrane proteoglycan that is expressed especially in embryonic and fetal tissues [Pilia *et al.*, 1996]. In light of this, we sought to evaluate its expression, as well as that of *MYCN* and *IGF2*, in a wider range of embryonal tumors including NBs, WTs and MBs. In order to detect even small quantities of mRNA, northern analyses were complemented by the use of gene-specific RT-PCR (see section B.4.1. for details). Representative results of the RT-PCR are shown in Figure 13. The expression patterns for *GPC3*, *IGF2* and *MYCN* are summarized in Table XII and are based on comparisons with *ß-actin* expression levels for each sample. *GPC3* mRNA was detected, although at various levels, in placenta, fetal kidney, fetal

lung and in adult liver. Most NBs, one neurofibrosarcoma, and all WTs examined express *GPC3*. However, *GPC3* was not, or barely, expressed in the normal kidney tissues obtained from 3 of the WT patients. None of the 5 MB cell lines as well as the osteosarcoma, the adrenal carcinoma and the rhabdomyosarcoma show expression. Similarly, the expression levels of *IGF2* and *MYCN* were determined in the same panel (Table XII).

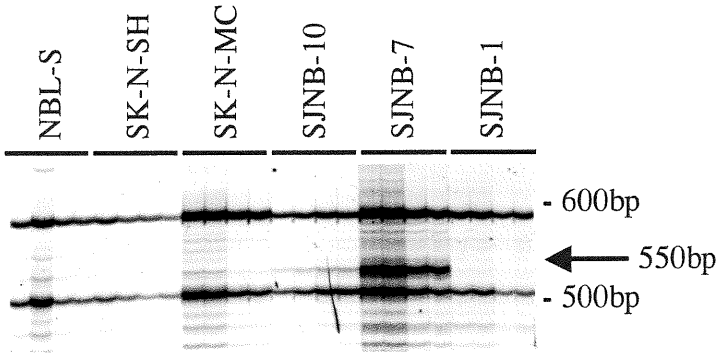


Figure 10. mRNA differential display of human neuroblastoma cell lines. Each sample was reverse transcribed and amplified in duplicate (giving four lanes each). The arrow indicates the differentially displayed band F0126/08.

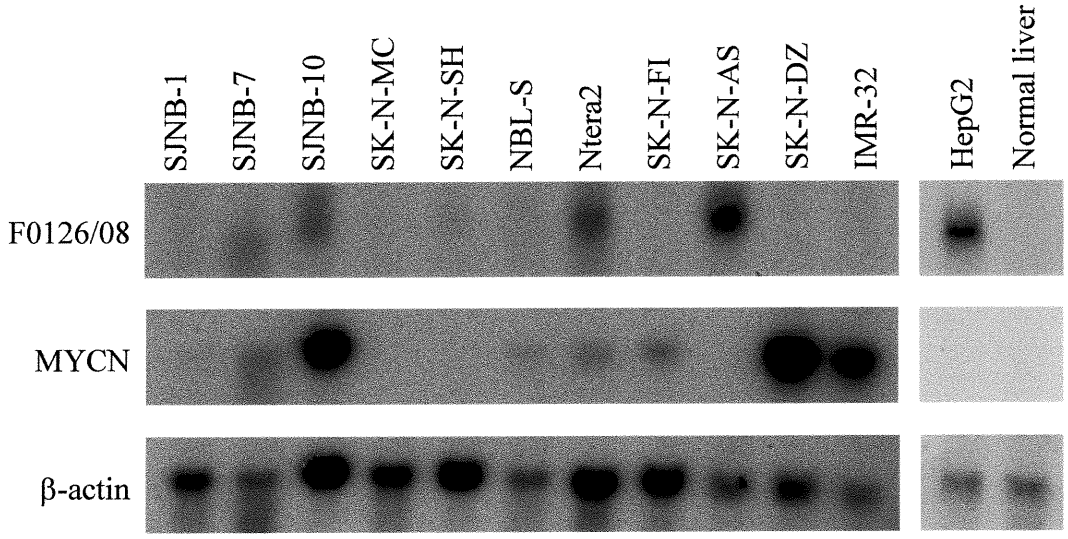


Figure 11. Analysis of expression of band F0126/08 and *MYCN* in cancer cell lines. The northern blot was hybridized with the F0126/08 cDNA fragment (upper panel) and a 450-bp sequence from the *MYCN* 3' UTR (middle panel) as probes. *ß-actin* hybridization (lower panel) was used to control the quantity and the integrity of RNA samples.

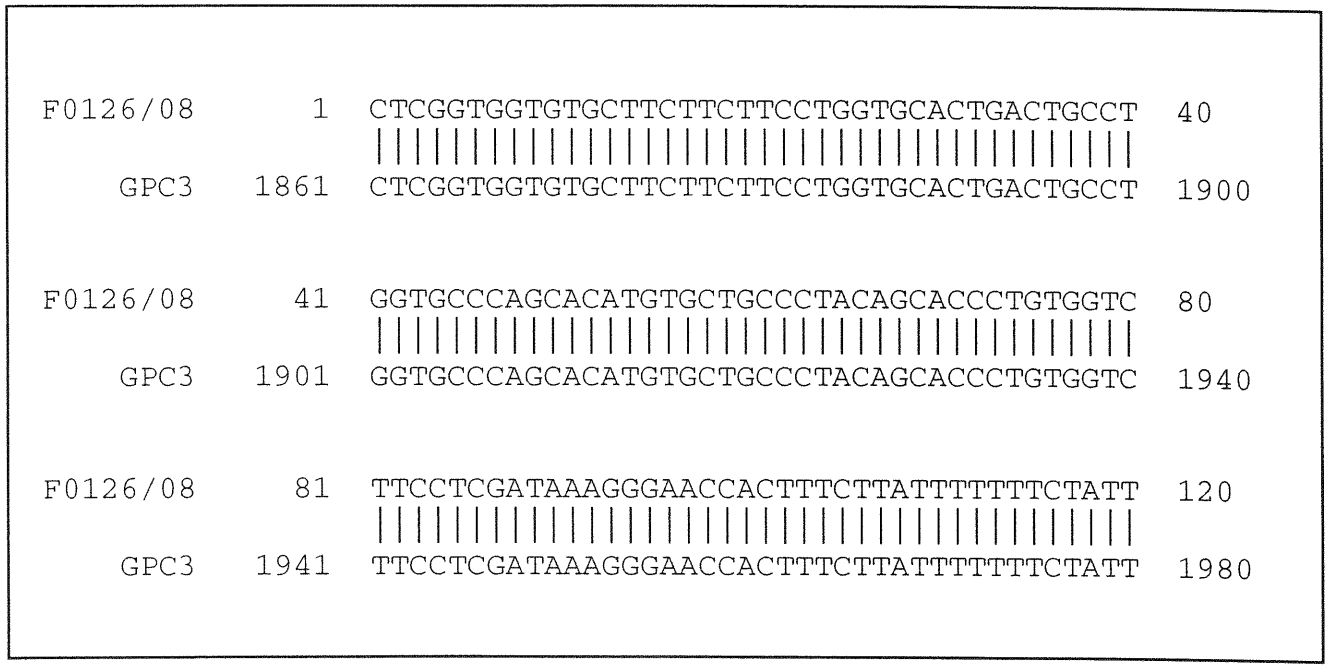


Figure 12. Alignment of differentially-displayed band F0126/08 with the partial sequence of glypican 3 (*GPC3*). The nucleotide sequence of F0126/08 cDNA (upper lane), obtained from direct sequencing, was found to be identical to the *GPC3* cDNA sequence (lower lane) from base 1861 to base 1980 (Genbank accession number L47125).

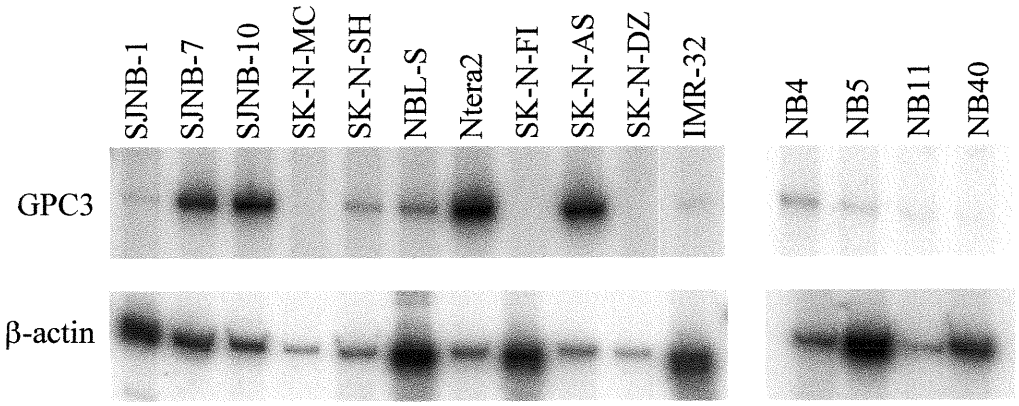


Figure 13. RT-PCR analysis of *GPC3* mRNA in neuroblastomas. Exons 5 and 6 of *GPC3* mRNA (top) were amplified from neuroblastoma cell lines (left panel) and primary tumors (right panel). The quantity and integrity of RNA was verified by RT-PCR amplification of *β-actin* mRNA (bottom).

TABLE XII. SUMMARY OF EXPRESSION DATA OF *GPC3*, *IGF2* AND *MYCN* IN EMBRYONAL TUMOURS

Sample		Relative level of expression		
		GPC3	***IGF2***	***MYCN***
Neuroblastoma cell line	SJNB-1	+	+	+
	SJNB-7	+	+	+
	SJNB-10	+	+	+
	SK-N-MC	-	tr	-
	SK-N-SH	+	+	-
	NBL-S	+	+	+
	SK-N-FI	-	+	+
	SK-N-AS	+	+	tr
	SK-N-DZ	-	tr	+
	IMR-32	+	tr	+
Embryonal carcinoma cell line Ntera2		+	tr	+
Osteosarcoma cell line 143N2		-	+	-
Medulloblastoma cell line	MB1	-	-	tr
	MB3	-	-	tr
	MB5	-	-	+
	MB7	-	-	-
	MB4	-	-	tr
Wilms' tumor	Normal kidney 177	tr	ND	ND
	WT177	+	+	tr
	Normal kidney 040	tr	ND	ND
	WT40	+	+	+
	Normal kidney 130	-	tr	tr
	WT130	+	+	+
	WT42	tr	+	+
	WT51	+	+	tr
	WT101	+	+	tr
	WT106	+	+	tr
	WT116	+	+	tr
Neurofibrosarcoma 005		+	+	-
Adrenal carcinoma 007		-	+	-
Rhabdomyosarcoma 013		-	+	-
Neuroblastoma	NB4	+	+	+
	NB5	+	+	+
	NB11	tr	+	+
	NB40	tr	+	+
Preterm placenta		+	+	+
Placenta at term		+	+	+
Fetal kidney		+	+	+
Fetal lung		+	+	+
Adult liver		tr	+	tr

+, expression; -, no expression; tr, trace amounts of mRNA detected; ND, not done. Data based on 3 to 5 independent experiments.

The correlation between the expression of *GPC3*, *IGF2*, and *MYCN* was evaluated using Spearman's rank correlation coefficient, as described in section B.4.2. A significant correlation was observed between the expression of *GPC3* and that of *IGF2* (Table XIII, $p<0.01$). No such correlation was found at this significance level between *IGF2* and *MYCN* or between *GPC3* and *MYCN* ($p>0.01$ in each case). Similar results were obtained regardless of the model used to analyze the data. This supports the observed co-expression of *GPC3* and *IGF2* in the same cells.

TABLE XIII. RESULTS OF STATISTICAL TESTS USED TO ANALYZE CORRELATION BETWEEN EXPRESSION OF *GPC3*, *IGF2* AND *MYCN*

		GPC3/IGF2	IGF2/MYCN	GPC3/MYCN
Model 1	Correlation coefficient	0.5752	0.0205	0.4120
	Probability (two-tailed)	0.0064*	0.5341	0.0960
	Probability (one-tailed)	0.0032*	0.2670	0.0480
Model 2	Correlation coefficient	0.6377	0.1959	0.3680
	Probability (two-tailed)	0.0038*	0.0851	0.5857
	Probability (one-tailed)	0.0019*	0.0425	0.2929

*considered significant

C.4. Analysis of Function of *GPC3*

In light of the experiments showing selective expression of *GPC3* in certain NB cells, as well as reduction or lack of expression of this gene in normal kidney tissue from patients with WT, a possible role in tumorigenicity should be examined. Cell transfection experiments were initiated, using a NB cell line (SK-N-FI) and a cervical carcinoma cell line (HeLa) that do not express *GPC3*, and a NB cell line that expresses *GPC3* (SK-N-AS).

C.4.1. Constitutive Exogenous Expression of *GPC3* in Various Cell Lines

The expression system used in these experiments was based on the pcDNA3.1 vector that allows constitutive expression of a transgene (see Figure 3). The full-length human *GPC3* cDNA was cloned into this vector at the appropriate site in both the sense and antisense orientations, as described in Section B.5.2.1. The cell lines were transfected with each of the resulting vectors, pcDNA/GPC3S (sense) and pcDNA/GPC3A (antisense), in addition to the empty pcDNA3.1 vector as a control. Initial selection led to a heterogeneous population of transfectants in which the expression of the transgene was verified by RT-PCR and northern analyses (Figure 14). In order to determine a possible effect on GPC3 protein levels, western blotting was attempted on these cells and others. However, the available anti-GPC3 antibodies failed to detect this protein in our samples. The difficulty in obtaining good antibodies for GPC3 has unfortunately hampered the efforts of several research groups looking into the function of this protein [D. Schlessinger, personal communication].

Clones from single cells were obtained for each cell line and with each construct. *GPC3*-expressing HeLa and SK-N-FI clones were selected by RT-PCR. *GPC3* mRNA-producing HeLa or SK-N-FI cells did not undergo any changes in their inability to grow on soft agar. Additionally, the growth of substrate-adherent HeLa cells was not affected by *GPC3* expression. On the other hand, a significant difference in growth was observed with SK-N-FI cells producing *GPC3* mRNA. A standard growth curve was established for two SK-N-FI/pcDNA3.1 clones, one SK-N-FI/pcDNA/GPC3A clone (used as an additional control) and two SK-N-FI/pcDNA/GPC3S clones, with duplicates for each time-point (Figure 15). A significant increase in average growth of clones expressing

GPC3 can be observed (2.5-fold at 10-12 days post-seeding) compared to those without *GPC3* mRNA.

Susceptibility to serum withdrawal was investigated, in particular because of the tight association between the expression of *GPC3* and that of *IGF2*. In the transfected SK-N-FI cells, the effect of serum withdrawal did not appear to differ according to vector identity (data not shown). This was true regardless of whether these cells were plated in the presence of serum and later submitted to deprivation, or deprived of serum at the time of seeding. However, this was not the case for the HeLa transfectants. Two HeLa/pcDNA3.1 clones and three HeLa/pcDNA/GPC3S clones were each plated in complete medium without serum and harvested in duplicate three and six days after seeding (Figure 16). The variation in cell numbers (calculated in terms of standard deviation) is quite high for the *GPC3*-producing clones, but it is possible to see that these cells exhibit a significantly increased initial growth spurt and a subsequent decrease in growth compared to the controls. In order to better examine this effect, we performed northern analyses to search for HeLa clones producing high levels of *GPC3* mRNA. *GPC3* expression could not be detected in any of the available clones by this technique. Knowing that in another study, *GPC3* had been observed to induce apoptosis in two of four cell lines [Gonzalez *et al.*, 1998], it was possible that high levels of *GPC3* mRNA were inhibiting the long-term growth of HeLa clones bearing the transgene. Therefore, we decided to use an inducible system of expression with this cell line to better examine the effect of exogenous *GPC3* expression (see next section).

As for the cell line producing endogenous *GPC3* mRNA, SK-N-AS, it did not show any alteration of behaviour when transfected with the antisense transgene, despite testing for speed of growth, response to serum withdrawal and growth on soft agar. This could be due to a lack of effectiveness of the antisense transcript in reducing the amount of endogenous sense transcripts.

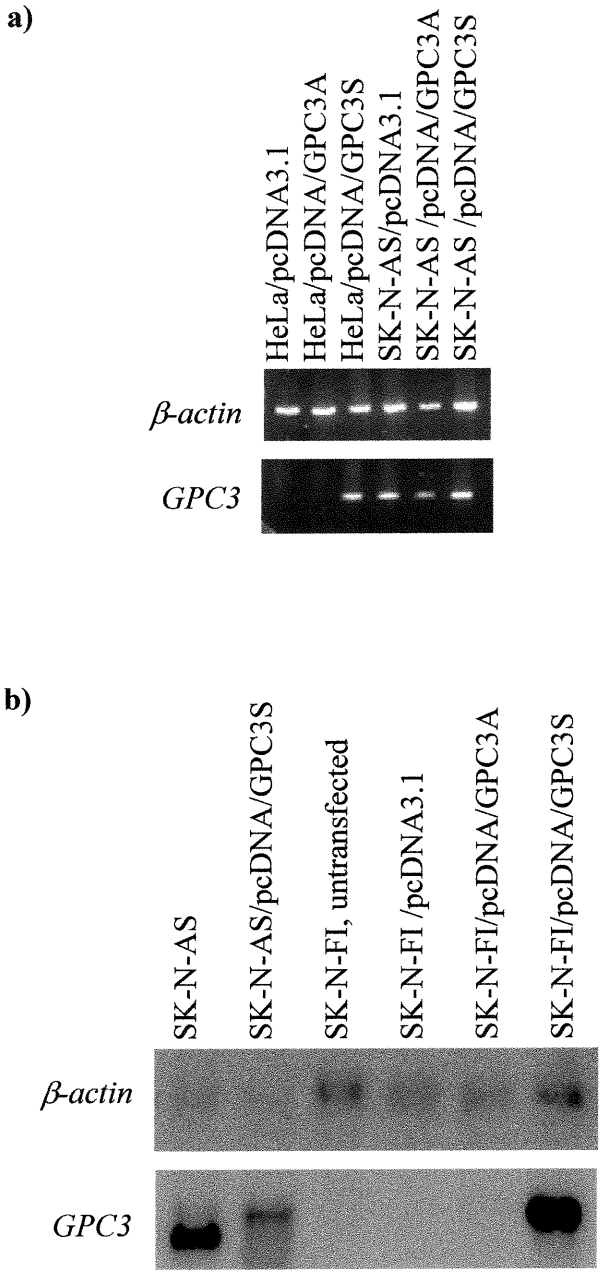


Figure 14. Analysis of expression of transfected GPC3 in three cell lines: HeLa, SK-N-AS and SK-N-FI by RT-PCR (a) or Northern blot hybridization (b). The endogenous *GPC3* message (in SK-N-AS) has a distinct size compared to the transgene. Expression of *β-actin* was used to control for integrity of the RNA.

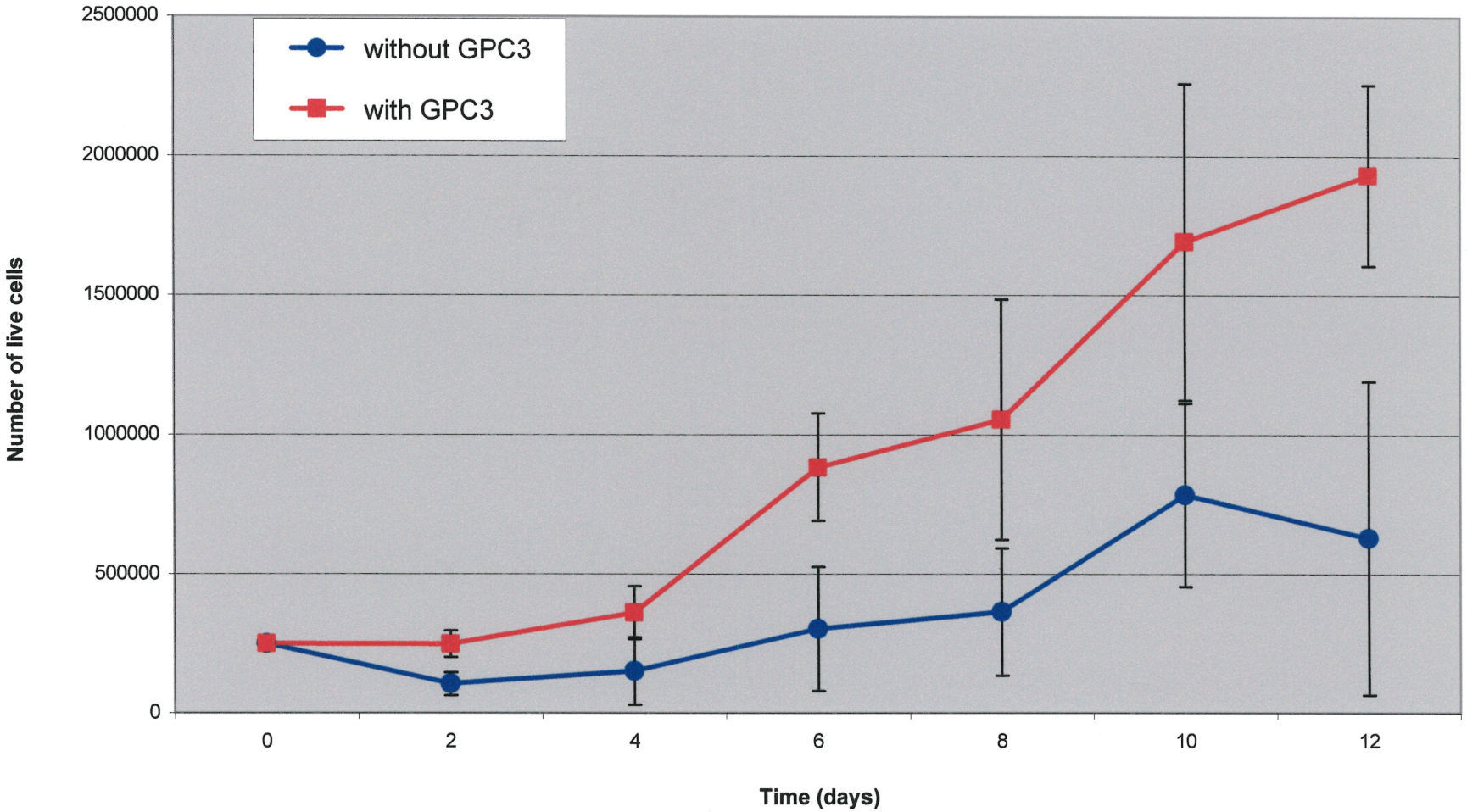


Figure 15. Growth of SK-N-FI cells transfected with *GPC3*. The curve 'without GPC3' is the average of two duplicates for each of two SK-N-FI/pcDNA3.1 clones and an SK-N-FI/pcDNA/GPC3A clone, while that 'with GPC3' is the average of two duplicates for each of two SK-N-FI/pcDNA/GPC3S clones. Bars represent standard deviation for each timepoint.

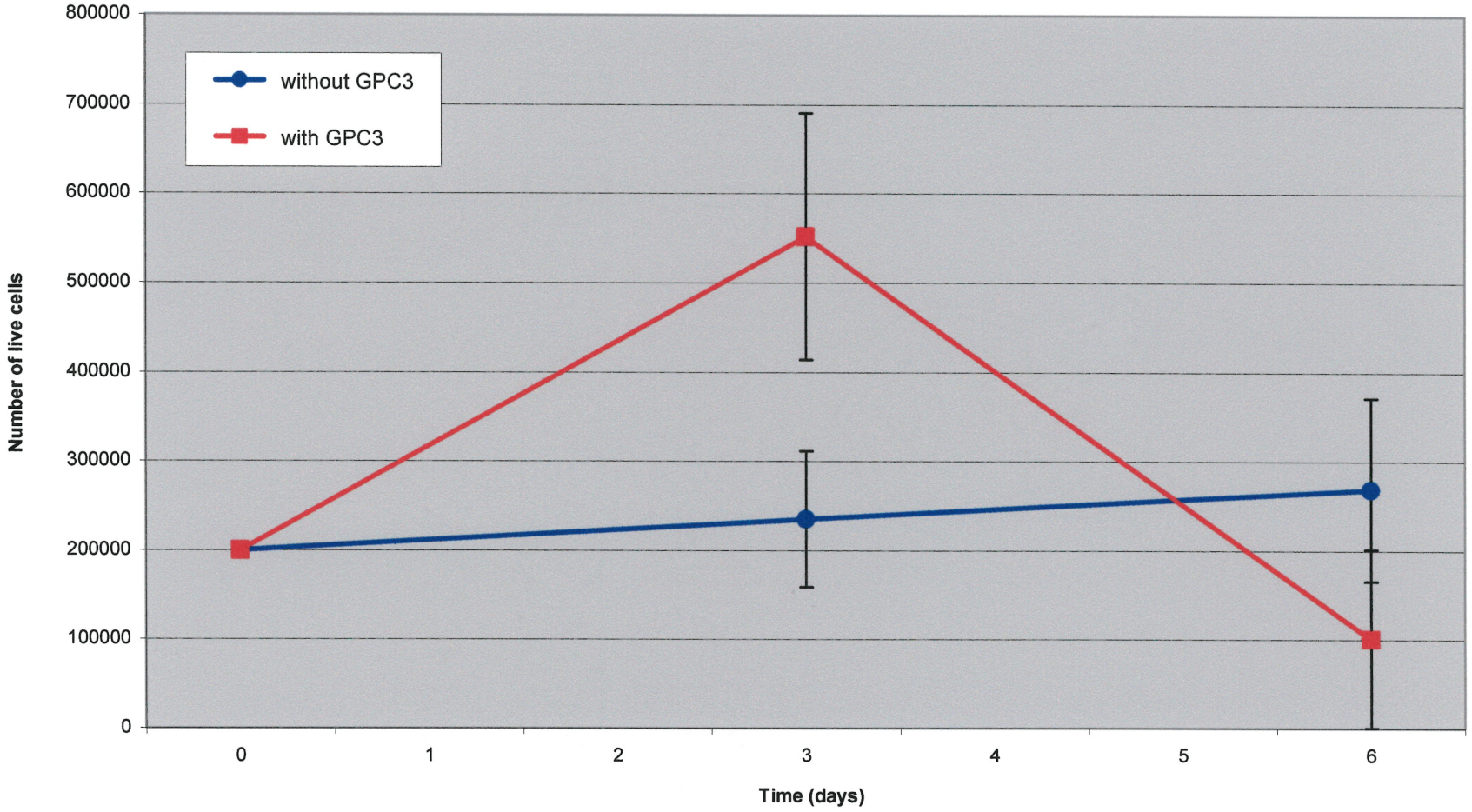


Figure 16. Growth of HeLa cells transfected with *GPC3* after serum withdrawal. The curve 'without GPC3' is the average of two duplicates for each of two HeLa/pcDNA3.1 clones, while that 'with GPC3' is the average of two duplicates for each of three HeLa/pcDNA/GPC3S clones. Bars represent standard deviation for each timepoint.

C.4.2. Induced Expression of *GPC3* in HeLa Cells

Use of this inducible system, described in section B.5.2.2., was attempted in two NB cell lines that do not express GPC3, SK-N-FI and SK-N-DZ. However, the first-step transfections with pVgRXR were not successful due to the very high sensitivity to Zeocin^{TM} of these cell lines (threshold < 25 μg/ml).

HeLa cells were transfected with pVgRXR, as described in section B.5.3. A stable, non-clonal pool of HeLa/pVgRXR was then transfected with either pIND, pIND/GPC3A or pIND/GPC3S. HeLa/pVgRXR/pIND, HeLa/pVgRXR/pIND/GPC3A and HeLa/pVgRXR/ pIND/GPC3S cells were selected and inductions performed using Ponasterone A, with ethanol as a control, for 20 and 24 hours. Induced and control cells were harvested and submitted to RT-PCR analysis for expression of *GPC3*. This analysis showed expression of *GPC3* in even mock-induced HeLa/pVgRXR/pIND/ GPC3S cells (data not shown), probably due to leakiness of the expression system. Because of this, experiments were not pursued with this expression system.

C.4.3. Transient Expression of *GPC3* in R cells

R- cells are immortalized mouse fibroblasts derived in a 3T3-like manner from embryos with null mutations for the *IGF-1R* gene [reviewed in Baserga, 1999]. Knowing the relationship between *IGF2* expression and that of *GPC3*, and the fact that IGF-II acts primarily through the IGF-1R, these cells provided an opportunity to examine the effect of *GPC3* expression in relationship to the presence or absence of a signal from this receptor. Parental R- cells have been transfected with *IGF-1R*, producing a variety of different lines with variable numbers of the receptor at the cell surface. R+ cells contain 900 x 10^3 receptors [Rubini *et al.*, 1997], allowing them to respond mitogenically to IGF-I alone.

Since R- cells are already resistant to geneticin, stable transfectants of these cells cannot be obtained with the pcDNA3.1 system. Therefore, R- and R+ cells were transiently transfected with pcDNA3.1 and pcDNA/GPC3S. The cells were harvested and counted two and three days after transfection (Figure 17). No significant differences could be observed between growth of cells transfected with pcDNA3.1 and those transfected with pcDNA/GPC3S.

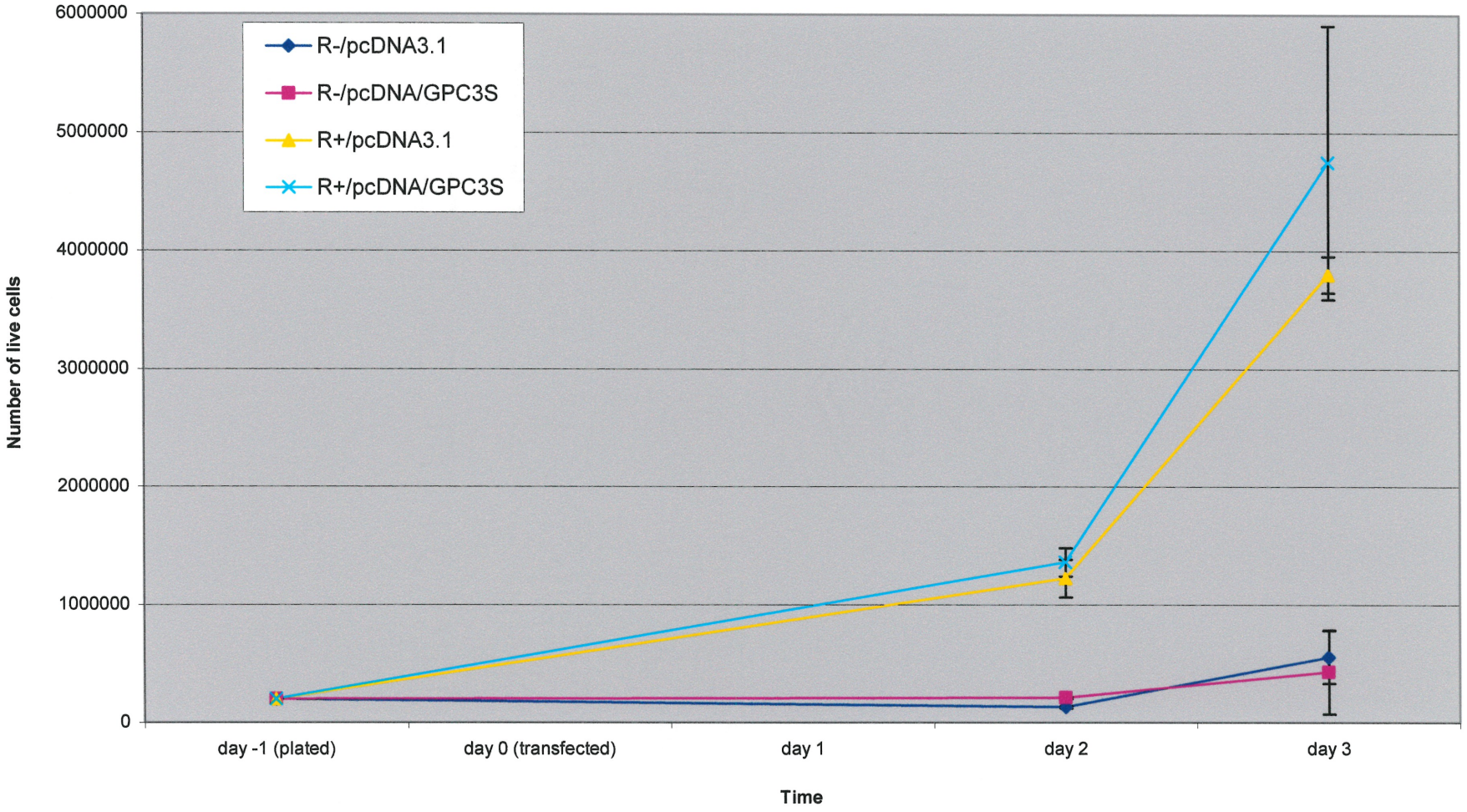


Figure 17. Growth of R- and R+ cell lines after transient transfection with GPC3. These results represent the average of duplicates (day 3) or triplicates (day 2), and bars represent standard deviation for each timepoint.

C.4.4. Analysis of Genetic Expression in GPC3-Expressing SK-N-FI Cells Using cDNA Microarrays

The Atlas™ Human 1.2 cDNA expression array (CLONTECH Laboratories, Inc.), described in section C.2. and Figure 9, was used to identify differences in expression induced by the presence of GPC3 in the cell. In order to reduce the amount of background differences from cell line variation, a *GPC3*-transfected NB cell line without endogenous *GPC3* expression, SK-N-FI, was used in this experiment. Untransfected SK-N-FI mRNA was analyzed using this technique in section C.2. *GPC3*-transfected SK-N-FI mRNA was analyzed in the same manner and spot intensity was quantified from the film. Unambiguous signals having at least 5% of the intensity of the *GAPDH* control spot were retained and compared with those obtained for the parental line. As in the previous analysis with these microarrays, we chose an arbitrary value of 2.5-fold to be an indicator of quantitative differences in expression between cell lines. Ratios found to be significant by these criteria are listed in Table XIV, along with the values for the control spots. Of the seven housekeeping genes serving as positive controls on the array, all show differences in relative expression between cell lines of less than 1.36, except *HLA-C* which, as discussed in section A.7.5., is not suitable for use as a control with these samples. The low values for differences in expression in the other positive controls are an indication of the strength of the quantitative analysis.

As listed in Table XIV, two genes were found to be expressed only in the *GPC3*-transfected SK-N-FI cells and not in the parental line (*NPY* and *IL-8*), and one gene in the parental but not the transfected line (*PCP-4*). The analysis of another cell line with this method, SK-N-AS, reported in section C.2., is of use here since this cell line expresses endogenous *GPC3* and may serve to further indicate genes with expression correlating with that of *GPC3*. In particular, the finding of lack of expression of *PCP-4* in SK-N-AS strengthens the possibility of an inverse correlation of expression patterns between this gene and *GPC3*. In addition, SK-N-AS was found to express low levels of NPY, although this was not reported in section C.2. because the relative expression in this sample of 3.22% of the control fell below the selection criteria. However, it may still serve to support a possible correlation between the expression of *GPC3* and that of *NPY*. Twelve other genes show significant differences in expression and are listed in

decreasing order of the ratio of expression between samples (Table XIV). It is interesting to note the number of genes known to be involved in neuronal and hematopoietic processes. In order to verify the differences in expression, other analyses are needed, such as northern analysis or RT-PCR. However, the potential role of such differences in expression may be speculated and will be addressed in the discussion.

TABLE XIV. DIFFERENCES IN GENE EXPRESSION BETWEEN *GPC3*-TRANSFECTED AND NON-TRANSFECTED SK-N-FI CELLS, REVEALED BY cDNA MICROARRAY ANALYSIS

Gene Name	Coordinate	SK-N-FI	SK-N-FI /*GPC3*	ratio
DNA polymerases, replication factors and recombination proteins				
MCM7 DNA replication licensing factor; CDC47 homolog; p1.1-MCM3	C05m	5.71	2.05	2.79
neuromediators				
neuropeptide Y precursor (NPY)	D12f	0.00	8.37	N/A
neuronal development, plasticity, degeneration and cell adhesion				
achaete-scute homolog 1 (ASH1)	D01h	9.18	2.83	3.24
brain-specific polypeptide PEP-19; brain-specific antigen PCP4	D02h	6.68	0.00	N/A
nervous system-related transcription factors				
E2F dimerization partner 1; DRTF1-polypeptide 1 (DP1)	D02m	10.39	2.59	4.01
CCAAT-binding transcription factor subunit B (CBF-B); NF-Y protein subunit A (NF-YA); Hap2; CAAT-box DNA-binding protein subunit A	E13d	17.54	2.46	7.13
cell-surface antigens and adhesion proteins				
CD44 antigen hematopoietic form precursor (CD44H); phagocytic glycoprotein I (PGP-1); HUTCH-I; extracellular matrix receptor-III (ECMR-III); GP90 lymphocyte homing/adhesion receptor (LHR); hermes antigen	E08h	6.25	2.08	3.00
fibronectin receptor beta subunit (FNRB); integrin beta 1 (ITGB1); VLA4 beta subunit; CD29 antigen	E09i	11.72	4.06	2.89
growth factor and chemokine receptors				
neuromedin B receptor (NMBR); neuromedin-B-preferring bombesin receptor	E03k	6.91	2.25	3.07
homeostasis and detoxification				
thioredoxin peroxidase 2 (TDPX2); thioredoxin-dependent peroxide reductase 2; proliferation-associated gene (PAG); natural killer cell enhancing factor A (NKEFA)	F08a	12.57	3.71	3.39
growth factors, cytokines and chemokines				
neuroleukin (NLK); glucose-6-phosphate isomerase (GPI); phosphoglucose isomerase (PGI); phosphohexose isomerase (PHI)	F07e	11.09	4.12	2.69

Gene Name	Coordinate	SK-N-FI	SK-N-FI /*GPC3*	ratio
monocyte chemotactic protein 1 precursor (MCP1); monocyte chemotactic and activating factor (MCAF); monocyte secretory protein JE; monocyte chemoattractant protein 1; HC11; small inducible cytokine A2 (SCYA2)	F12e	11.29	72.71	6.44
interleukin-8 precursor (IL-8); monocyte-derived neutrophil chemotactic factor (MDNCF); T-cell chemotactic factor; neutrophil-activating protein 1 (NAP1); lymphocyte-derived neutrophil-activating factor (LYNAP); protein 3-10C	F14g	0.00	7.37	N/A
other extracellular communication proteins				
thymosin beta 4; FX	F13j	19.24	7.50	2.57
protein turnover				
proteasome component C5; macropain subunit C5; proteasome gamma subunit; multicatalytic endopeptidase complex subunitC5	F031	14.82	4.61	3.21
housekeeping genes (controls)				
ubiquitin	G11	62.90	53.38	1.18
liver glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	G27	100.00	100.00	1.00
brain-specific tubulin alpha 1 subunit (TUBA1)	G29	55.42	40.84	1.36
HLA class I histocompatibility antigen C-4 alpha subunit (HLAC)	G31	6.01	12.98	2.16
cytoplasmic beta-actin (ACTB)	G43	46.77	37.05	1.26
23-kDa highly basic protein; 60S ribosomal protein L13A (RPL13A)	G45	60.23	44.46	1.35
40S ribosomal protein S9	G47	15.51	11.93	1.30

Values are relative to GAPDH levels. *Coordinate*, position on the microarray grid; *Gene name* and categories, according to manufacturer's list (accessible on manufacturer's internet site); *ratio*, higher level relative to lower level; *N/A*, not applicable.

SECTION D. DISCUSSION

D.1. Expression Patterns in Neuroblastoma Cells

The differential display PCR (DD-PCR) technique has been in use since 1992 and has been modified and adapted in various ways. In this project, a number of modifications have been combined to optimize its reproducibility, specificity and sensitivity, and to reduce the complexity of the cDNA patterns fractionated by polyacrylamide gel electrophoresis. This modified protocol allowed the identification of a limited number of differentially expressed sequences in a large number of NB cell lines. In a complementary manner, we used cDNA microarrays to generate a large amount of expressed sequence information in a more limited number of cell lines. These approaches allowed the identification of expressed genes that could uncover new molecular pathways and/or biological processes involved in NB.

DD-PCR and cDNA microarray analysis have both been used with a variety of different objectives and on a variety of different samples, and in many cases they present overlapping potential for discovery. For example, DD-PCR has been used to identify genes induced or activated by the transcription factor p53 [Lo *et al.*, 2001; Okamura *et al.*, 2001], as has cDNA microarray analysis [Kannan *et al.*, 2001], and they have both been used extensively for studying tumour tissue expression profiles [Dhanasekaran *et al.*, 2001; Ying *et al.*, 2001]. However, different advantages exist for each technique. In particular, DD-PCR has the advantage of being able to identify novel genes more easily, such as in the case of the discovery of a putative tumour suppressor gene for breast cancer [Chen *et al.*, 2000]. On the other hand, cDNA microarray analysis is a comprehensive method for looking at the expression of a large amount of genes simultaneously, and this has the potential for example of revealing biological pathways involved in the cells or tissue studied [Lapteva *et al.*, 2001]. Because of the different advantages and disadvantages of each technique, cDNA microarray analysis and DD-PCR may be used in a complementary manner for the purpose of gene expression profiling, as has been done with metastatic murine squamous carcinoma cells [Dong *et al.*, 2001] and as we have done with NB cells here.

D.1.1. cDNA Microarray Analysis

We performed hybridization experiments to cDNA microarrays to determine the expression profile of NB cells. In our hands, the microarrays provided us with both qualitative and quantitative information regarding a large amount of genes expressed in two NB cell lines, SK-N-FI and SK-N-AS. The quantitative aspect was demonstrated by the similarities between cell lines regarding expression levels obtained from housekeeping genes used as positive controls (Table IX). However, and as could be predicted because of the threshold of sensitivity of this technique, certain transcripts seem to elude detection. This is demonstrated by the failure to detect the presence of *MYCN* or *IGF2* transcripts in SK-N-FI, the expression of which we have reported in Saikali and Sinnett [2000].

Microarray analysis allowed for the detection of a large number of transcripts, some of which were known to be present in NB cells, while others were not known to be expressed in these cells. These transcripts can be classified according to function, and their presence may reveal the activity of certain biological pathways. We have chosen to represent them according to the classification scheme utilized by the manufacturer (Tables IX, X and XI). We find that genes from many, but not all, the classification groups are expressed in our samples. Many basic cellular functions and some specialized aspects of neuroendocrine cells may be revealed by the expression of certain genes in this microarray. For example, cell cycle control is represented by cyclin D1, cyclin K and cyclin-dependent kinases, cell division by PCNA and DNA repair proteins, differentiation along the neuroendocrine path by amyloid A4, dopamine β hydroxylase, DOPA decarboxylase and chromogranin C, etc. Genes that may be interesting in the context of cancer studies are also frequently found: transcription factors, G-proteins, thymosins, growth factors, proteins involved in apoptosis and others in angiogenesis, etc. Examples are GATA-2, a transcription factor important for certain hematopoietic processes and recently implicated in neurogenesis [Ikonomi *et al.*, 2000; Zhou *et al.*, 2000] and DAD1, an anti-apoptotic protein that may regulate N-linked glycosylation and leads to lethality when homozygously inactivated in mice [Hong *et al.*, 2000]. Both genes are expressed at relatively high levels in both NB cell lines studied.

Among these genes and/or pathways, several need to be discussed further because of their relevance to NB:

1) The thymosin family. For over thirty years, hormones from this family have been known to be produced by the thymus [Goldstein *et al.*, 1970]. It is now clear that these peptides may have roles in hematopoiesis, carcinogenesis, angiogenesis and wound healing [Bonnet *et al.*, 1996; Malinda *et al.*, 1998; Santelli *et al.*, 1999]. In our study, thymosin beta 4, thymosin beta 10, prothymosin alpha and parathymosin are all expressed in the cell lines tested at levels ranging from 13% to 82% of GAPDH expression. Studies have shown that thymosin beta 10, but not thymosin beta 4, is regulated by retinoic acid in human and rat NB cell lines [Hall *et al.*, 1990; Hall *et al.*, 1991]. In addition, the gene encoding prothymosin alpha is a target for the transcriptional activity of MYCN [Ben-Yosef *et al.*, 1998]. This makes these peptides worth studying in NB tumours, especially knowing that beta thymosins have been implicated in the development of neural tissue in at least two animal models [Yamamoto *et al.*, 1994; Roth *et al.*, 1999]. On the other hand, parathymosin has not yet been linked with NB, but may be equally interesting to study. Recent evidence indicates that parathymosin may function in early DNA replication [Vareli *et al.*, 2000] and is present in significantly higher levels in malignant versus benign breast lesions [Tsitsilonis *et al.*, 1998]. Given that the gene for parathymosin maps to the area of chromosome 17 that is known to be amplified in a subset of NBs [Szabo *et al.*, 1989], this peptide may be considered an interesting candidate for proteins involved in NB.

2) Genes in development. Genes normally involved in development were frequently encountered in this study. This may be expected due to the embryonal character of NB and NB-derived cells, although it may also indicate pathways that are active in developmental processes that contribute to the maintained lack of differentiation of NB cells, as is the case for some other cancers. One of these genes, encoding the transcription factor MSX-1, was found to be expressed only in SK-N-FI (Table XI). Gene disruption experiments in mice have shown that Msx-1 is implicated in a variety of developmental processes, including that of the neural tube [Foerst-Potts and Sadler, 1997], and this protein may be involved in mediating the effects of retinoids in certain contexts [Brown *et al.*, 1997]. Given the importance of retinoic acid in the differentiation of NB cells and developing cells, this protein would also be interesting to

study in the context of NB research, in particular if it is limited to certain subtypes of NB, here represented by the cell line SK-N-FI.

3) Myc family transcription factors. Among the genes expressed in SK-N-AS and not in SK-N-FI we noted the presence of the *c-myc* oncogene (Table X). As we have shown, SK-N-AS expresses only trace amounts of *MYCN* (Table XII). Suppression of *c-myc* transcripts by high levels of *MYCN* has been suggested in NB cells [Breit and Schwab, 1989]. It is therefore possible that the expression of *c-myc* is due to the low level of *MYCN* transcripts. In addition, knowing that these two members of the same family share a number of transcriptional targets, it is possible to postulate that *c-myc* has a role in this cell line that is similar to that of *MYCN* in other NB cell lines.

D.1.2. Differential Display PCR (DD-PCR)

The screening of six NB cell lines using DD-PCR led to the identification of twenty confirmed differentially-expressed cDNAs, including known genes. The *MYCN* gene, isolated four times independently, encodes a transcription factor known to be associated with unfavourable prognosis in NB patients when amplified or overexpressed [Brodeur *et al.* 1992; Chan *et al.*, 1997]. *BDNF* was found to be highly expressed in all NB cell lines tested. We observed higher expression levels of *BDNF* in NB cell lines than what has previously been reported in primary tumours. *BDNF* expression was recently shown to be significantly higher in NBs with unfavourable prognosis [Aoyama *et al.*, 2001], suggesting an *in vitro* selection against NB cell lines with characteristics of more favourable NB tumours. Another fragment isolated from the differential display experiments was found to correspond to the *IGF2* gene which was already known to encode a growth factor for many NB cell lines and tumours [Sullivan *et al.*, 1995].

The identification of genes known to be involved in NB indicates the usefulness of this modified DD-PCR approach in detecting genes that were not known to contribute to NB development. This includes the gene encoding the ribosomal protein S6 (*RPS6*), involved in cell signalling pathways that may be mitogen-activated [Seufferlein and Rozengurt, 1996; Parrizas *et al.,* 1997; Chung *et al.,* 1997] and *XPR1*, encoding a cell surface receptor for xenotropic and polytropic murine leukemia viruses. XPR1 has homology with yeast proteins that have transmembrane signaling components that interact with G proteins [Battini *et al.*, 1999]. G proteins are known to

be involved in cellular transformation [Bishop, 1991; McCormick, 1989] and the potential of XPR1 to interact with them makes it a relevant subject of study in the context of NB.

D.1.3. Comparison

cDNA microarray analysis is a robust and highly efficient method of obtaining expression profiles for any number of samples. It presents the advantage of revealing a large amount of information that may be both qualitative and quantitative in a single experiment. However, as with all techniques, it has its limitations and disadvantages. One issue of concern is the reported low level of sensitivity with this technique, thought to be lower than the sensitivity of a Northern blot analysis [Taniguchi *et al.*, 2001]. Another concern in the scientific community has been the difficulty in treating the large amount of data generated and the low level of accessibility due to the highly technological nature of the technique [Bowtell, 1999]. This issue is now partly resolved by the fact that manufacturers have made available microarrays that do not require special equipment, such as the one used in this project.

DD-PCR is a simple and versatile technique that does not require highly specialized equipment. Its merits include the ability to detect novel genes, a high level of sensitivity and the ability to analyze different samples in parallel in a single experiment [Sunday, 1995]. On the other hand, this technique is highly labour-intensive, requiring many primer combinations to adequately represent the expression profile. In addition, we and others have found that the large number of bands displayed implies a selection by the user that may on occasion lead to some bias in favour of more highly expressed and hence clearer bands.

In this study, the merits and disadvantages of both techniques may be observed. The difference in sensitivity is perhaps prevalent in the comparison. Only three cDNAs detected by DD-PCR were also present on the microarray used; none of the three were detected correctly by the latter in the two cell lines studied: expression of *IGF2* was detected in SK-N-AS but not SK-N-FI, that of *MYCN* was not detected in SK-N-FI and that of *BDNF* was not detected in either cell line. This clearly shows the superiority of DD-PCR over microarrays in terms of sensitivity. However, microarrays were able to detect a much larger amount of cDNAs than DD-PCR in a much shorter period of time

needed to complete the experiment. Many more combinations of primers would have had to be performed in the scope of an analysis by DD-PCR in order to reveal a much larger amount of cDNAs. In addition, microarray information appears to be quantitative or semi-quantitative, whereas in the case of DD-PCR the amplification cycles inherent to the technique render it relatively poor at quantification, implying that many cDNAs potentially revealable as differentially-expressed were missed by DD-PCR.

D.1.4. Relevance of Expression Profiling Studies in Neuroblastoma

The importance of expression studies in NB is multifold. On one hand, this type of tumour is known to be heterogeneous both at the genetic and the clinical levels [Brodeur, 1995b]. In this regard, the analysis of a large number of cell lines for similarities and differences in genetic expression may indicate genetic elements leading towards unfavourable prognosis and the distinction between different subtypes of NB. On the other hand, although several factors are now available for the determination of prognosis in a patient with NB, the basic genetic and biological pathways involved in NB carcinogenesis and progression are still relatively unknown. Indeed, many pathways leading to cell cycle regulation and others involved in apoptosis that are often defective in different cancers seem to remain untouched in NB [Beltinger *et al.*, 1995; Vogan *et al.*, 1993]. Our study allows the identification of new biological processes and pathways involved in NB carcinogenesis and progression by the profiling of expressed genes. Even if some of these genes are not key players in cancer initiation and progression, they may still point out the pathways involved. Thus, genes whose expression is revealed may either be key players in NB carcinogenesis, progression or metastasis, or reflect aberrations in particular pathways that play an important role. A number of examples to attest to this are available from the data generated by this study, in addition to confirmation for a number of genes previously known to play a role in cancer in general and NB in particular.

D.1.5. *GPC3* as a candidate gene

The gene encoding glypican 3 (*GPC3*, also known as *MXR7*) was also isolated during the course of our DD-PCR experiments and found to be expressed in some but not all NB cell lines. We decided to study this gene further because of its potential relevance as a NB-related gene. *GPC3* is highly expressed in embryonal tissues such as the developing intestine and the mesoderm-derived tissues, and its expression is downregulated in most adult tissue, implying a potential role in development [Filmus *et al.*, 1988; Pellegrini *et al.*, 1998]. *GPC3* encodes a putative extracellular membrane proteoglycan of the glypican family. Other members of the glypican family are also thought to play a role in embryonic development: *GPC1*/glypican [David *et al.*, 1990], *GPC4*/K-glypican [Watanabe *et al.*, 1995], *GPC5* [Veugelers *et al.*, 1997; Saunders *et al.*, 1997] and *GPC6* [Paine-Saunders *et al.*, 1999; Veugelers *et al.*, 1999]. One member of the glypican family, *GPC2*/cerebroglycan, is expressed transiently in rat brain immature neurons but not after axon outgrowth is completed, suggesting a potential role in proliferating neuroepithelial cells [Stipp *et al.*, 1994].

A variety of different mutations in *GPC3* cause the X-linked Simpson-Golabi-Behmel Syndrome (SGBS) [Pilia *et al.*, 1996]. This syndrome is characterized by a wide variety of clinical manifestations, including pre- and post-natal overgrowth, tissue dysplasia, in particular of the kidneys, and cardiac anomalies [reviewed in Neri *et al.*, 1998]. In addition, SGBS patients seem to be associated with a greater risk of developing embryonal cancers [Hughes-Benzie *et al.*, 1992]. However, this relationship between SGBS and tumours is still unclear because of the small number of identified SGBS families and the discovery of normal siblings of SGBS patients with embryonal tumours, indicating a possible maternal effect [Xuan *et al.*, 1999]. As a possible explanation for the phenotype of this syndrome, it has been suggested that GPC3 forms a complex with IGF2, thereby possibly modulating the action of this growth hormone [Pilia *et al.*, 1996]. *GPC3* has been shown to be aberrantly upregulated in hepatocellular carcinoma compared to normal liver [Hsu *et al.*, 1997] and in metastatic colorectal malignancies [Lage *et al.*, 1998]. On the other hand, *GPC3* is frequently silenced in mesotheliomas [Murthy *et al.*, 2000] and in ovarian cancer cell lines [Lin *et al.*, 1999], often due to hypermethylation of the *GPC3* promoter. Despite this conflicting evidence from studies of *GPC3* in cancer, it is clear that GPC3 may have a function in cancer

cells. This, taken with the involvement of this gene in SGBS and embryonal development, indicates that it is an interesting candidate for implication in NB and possibly other embryonal tumours as well.

D.2. Analysis of *GPC3* Expression in Embryonal Tumours

D.2.1. *GPC3* is Expressed in Neuroblastoma and Wilms' Tumour

In this study, we report for the first time the expression of *GPC3* in NB and WT, two embryonal tumours, but not in MB. No general conclusions may be made for the time being regarding the possible role of GPC3 in the other tumour types studied here, since not enough representatives of these other neoplasias were available. Of note, we showed that *GPC3* was expressed in the liver carcinoma cell line HepG2, but not in normal liver tissue. With regard to tumours with neuronal phenotype, *GPC3* was detected at variable levels in a neurofibrosarcoma and in most NBs, but was absent from all MB cell lines. These findings suggest that *GPC3* expression is differentially regulated in the various cell lineages giving rise to pediatric tumours of the peripheral and central nervous systems. Because *gpc3* mRNA has not been detected in central nervous tissue of mouse embryos or fetuses, its absence in MBs is not surprising. We also showed that the expression of *GPC3* was upregulated in all WTs tested as illustrated by its absence in the normal kidney tissues derived from some of the patients. This would indicate that the presence of GPC3 in WT does not have growth-inhibitory effect. This notion is in agreement with the study of hepatocellular carcinoma patients where the corresponding normal hepatic tissue did not express *GPC3* [Hsu *et al.*, 1997], suggesting a derepression (or activation) of this gene in cancer cells. However, this upregulation of *GPC3* expression seems to be cell-specific because of its absence in several other tumoural material: MBs (this study), mesotheliomas [Murthy *et al.*, 2000], and ovarian cancer cell lines while being expressed at relatively high levels in normal ovarian tissue [Lin *et al.*, 1999].

D.2.2. Correlation Between the Expression of *GPC3* and that of *IGF2*

In addition to the fact that these proteins may bind each other [Pilia *et al.*, 1996], two other lines of evidence prompted the simultaneous analysis of *GPC3* and *IGF2*

expression. First, *GPC3* expression was shown to induce apoptosis in two cell lines out of four tested, and this effect could be reversed by the addition of either IGF peptide [Gonzalez *et al.*, 1998]. Second, *in situ* hybridization studies on mouse fetuses reported co-expression of *gpc3* and *igf2* in the tissues tested [Pellegrini *et al.*, 1998]. In this study, we showed that this correlation was also true for certain embryonal tumours and cell lines in a strict manner. On the other hand, we did not observe any correlation with *MYCN* expression, a known prognostic factor in NB.

The link between GPC3 and IGF-II exists also in another context. *GPC3* mutations lead to SGBS, a syndrome that shares significant similarities with the Beckwith-Wiedemann syndrome (BWS). The latter is an overgrowth syndrome that is thought to be associated with increased expression of *IGF2* [reviewed in Li *et al.*, 1998]. In addition, mice with mutations causing very high levels of IGF-II exhibit features of both syndromes [Eggenschwiler *et al.*, 1997]. It may therefore be postulated that GPC3 has a downregulatory effect on IGF-II, leading to at least partially overlapping phenotypes when IGF-II is overabundant or when GPC3 is absent. This, however, is not supported by the correlation observed between the expression of these two genes. The possible functional aspects of this correlation will be discussed in the next section.

D.3. Analysis of Function of Glypican 3

D.3.1. Analysis of Cell Lines Transfected with *GPC3* cDNA

SK-N-FI cells without endogenous expression of *GPC3* exhibit an increase in growth capacity after stable transfection with *GPC3* cDNA (Figure 15). On the other hand, HeLa, another non-expressing cell line, does not show any changes in growth when transfected with GPC3, but exhibits an altered response to serum withdrawal (Figure 16). The difference in behaviours of the two cell lines after transfection with *GPC3* could be explained by a cell- or tissue-specific effect. This phenomenon was observed in other studies as well. For instance, Gonzalez and coworkers [1998] showed that *GPC3* exogenous expression could induce apoptosis in two cell lines, a mesothelioma cell line and a breast cancer cell line, but not in NIH 3T3 fibroblasts or in a colorectal cancer cell line. These results are supported by studies describing the

pattern of expression of *GPC3* in cancer cells: apoptosis was induced by GPC3 in a mesothelioma cell line and a study showed downregulation of *GPC3* expression in mesotheliomas [Murthy *et al.*, 2000]; apoptosis could not be induced in a colorectal cancer cell line while a study showed expression of *GPC3* in colorectal cancers [Lage *et al.*, 1998]; we have shown here that apoptosis cannot be induced in a NB cell line by exogenous expression of *GPC3* and our previous results showed that many NB cell lines and primary tumours express this gene. Hence, cell context will determine whether *GPC3* has an apoptotic effect and is often downregulated in certain cancer cells and cell lines, or whether it has positive or neutral effects and may therefore be expressed in other cancer cells and cell lines. In this regard, it is quite clear that neither SK-N-FI nor HeLa are in the category of cells that enter cell death pathways after *GPC3* expression. However, they can still be considered as exhibiting different cell contexts since they do not respond in the same manner to expression of this gene.

D.3.2. Possible Function of Glypican 3 in Neuroblastoma and Other Embryonal Tumour Cells

The biochemical function of GPC3 has yet to be established. However, information on the effect of this proteoglycan in various cells, its possible binding to certain factors and the pattern of expression of the gene encoding it, in addition to data compiled about other members of the family, allow us to speculate on the role GPC3 may be playing, in particular in embryonal tumours.

GPC3 has been shown to bind to IGF-II [Pilia *et al.*, 1996], Song *et al.* [1997] reported binding of GPC3 to Fibroblast Growth Factor 2 but not IGF-II, while Mast *et al.* [1997] observed binding with the tissue factor pathway inhibitor. These results have been difficult to reproduce, probably because of the lack of good antibodies. Despite these problems in determining which factors are bound by GPC3 and whether this occurs through the protein and/or the heparan sulfate residues, the fact that GPC3 is likely to bind growth factors is supported by evidence from other heparan sulfate proteoglycans, in particular members of the glypican family.

Heparan sulfate proteoglycans (HSPGs) of the cell surface are highly interactive macromolecules playing diverse roles in cell migration, proliferation, differentiation and adhesion, and participating in many developmental and pathological processes

[reviewed in Tumova *et al.*, 2000]. HSPGs consist of two major families: syndecans and glypicans. Syndecans are attached to the cell membrane by a transmembrane domain while glypicans are attached through a glycosylphosphatidylinositol (GPI) anchor [Tumova *et al.*, 2000]. The glypican family is represented by at least two members in *Drosophila*, dally [Nakato *et al.*, 1995] and dally-like [Khare and Baumgartner, 2000]. Dally is now known to act as a co-receptor that controls signalling by morphogens and growth factors such as decapentaplegic (dpp) [Jackson *et al.*, 1997] and wingless [Lin and Perrimon, 1999; Tsuda *et al.*, 1999]. Consistent with the cell- or tissue-specific effect of GPC3, dally mutations show tissue-specific effects, at the level of both dpp and wingless signalling [Jackson *et al.*, 1997; Lin and Perrimon, 1999; Tsuda *et al.*, 1999]. Although GPC3 cannot as yet be considered the orthologue of dally, this information strengthens the notion that it may have growth factor binding and regulatory properties.

Because of the cell-specific effect of GPC3, researchers are narrowing the scope of their analysis to one system or tissue at a time in order to understand its possible function(s). Two such studies have now been published. The first utilizes GPC3-deficient mice that were elsewhere shown to exhibit some, but not all, of the abnormalities characteristic of SGBS [Cano-Gauci *et al.*, 1999]. In particular, no GPC3-deficient mice were found to exhibit polydactyly, although this phenomenon occurs relatively frequently in SGBS patients. Although BMP4-deficient mice also do not exhibit polydactyly, this phenomenon was among the skeletal defects observed when GPC3-deficient mice were crossed with BMP4-deficient mice, indicating the synergistic effect of GPC3 and BMP4, and postulating that GPC3 controls cellular responses to this growth factor [Paine-Saunders *et al.*, 2000]. The interaction with BMP4 is not surprising since this factor is an orthologue of *Drosophila* dpp and is regulated in a similar manner during embryonic development [reviewed in Nakayama *et al.*, 2000]. Thus, the similarities between dally and GPC3 are reinforced.

In the second study, the renal dysplasia observed in both SGBS patients and GPC3-deficient mice was examined [Grisaru *et al.*, 2001]. Normal and GPC3-deficient embryonic kidney tissue was used to show that GPC3 participates in the control of renal branching morphogenesis by modulating the actions of several different growth factors, including BMP2, BMP7 and the keratinocyte growth factor (also known as fibroblast

growth factor 7) [Grisaru *et al.*, 2001]. Therefore, it appears likely that GPC3 is able not only to bind more than one growth factor, but also to functionally affect the signalling of different growth factors. The possible interaction of GPC3 with many growth factors may, in part, explain the observed cell- and tissue-specific effects.

Taken together, these observations allow us to speculate on the effect of GPC3 in embryonal tumours. The increased growth observed in *GPC3*-transfected SK-N-FI cells could be explained by an enhanced proliferative response to growth factors in the serum. Conversely, the inhibition or downregulation by GPC3 of mechanisms that normally slow down or limit the growth of these cells is also possible. At this stage, it is still unclear whether GPC3 is playing a role in carcinogenesis and/or progression in NB and WT, or whether the expression of this gene is a characteristic of undifferentiated cells with fetal expression patterns, specifically of neural crest cells and embryonic kidney cells. One possibility is a combined scenario, where normal expression in the cells of origin is never turned off, contributing to the tumourigenicity. This would mean that NB cells expressing *GPC3* come from different neural crest cells than NB cells not expressing *GPC3*, and that the difference could be either temporal, positional or both. This scenario implies that *GPC3* has the potential to participate in the determination of which neural crest cells differentiate into certain cells and tissues. In the case of HeLa cells transfected with *GPC3*, their response to serum withdrawal could be due to differences in interaction with members of the pool of growth factors in the serum. Knowing that HSPGs can 'trap' growth factors, i.e. affect their bioavailability [Bernfield *et al.*, 1999], it is entirely possible that HeLa cells with GPC3 molecules on their surface could be benefiting from a larger pool of remaining growth factors after serum withdrawal, leading to maintained proliferation for a short period of time until this pool is exhausted.

However, in the case of both HeLa cells and SK-N-FI cells, it is not possible at this moment to speculate about the identity of the growth factors involved. Although a correlation exists between expression of *GPC3* and that of *IGF2* [Saikali and Sinnett, 2000], and an interaction between these two proteins has been demonstrated before [Pilia *et al.*, 1996], a heightened response to IGF-II is not necessarily the cause of the observed *in vitro* behaviour. A possible explanation could be put forward by the study showing GPC3-induced apoptosis [Gonzalez *et al.*, 1998], where apoptosis was

prevented by the addition of either IGF-I or IGF-II to the culture medium. It has already been shown that IGF-II is a growth factor that can act as a survival factor in the early stages of tumourigenesis [Christofori *et al.*, 1994; Harrington *et al.*, 1994]. Therefore, IGFs could be needed to prevent GPC3-induced apoptosis in any cell, allowing cellular responses to other factors to take place and be mediated, enhanced or inhibited by GPC3. This scenario favours cells that produce IGF-I or IGF-II, since they would not have to rely on a paracrine or endocrine source of IGFs for survival if they express *GPC3*. In support of this possibility, our expression studies show clearly that although cells with *IGF2* expression can exist without *GPC3* expression, the opposite is not observed. Furthermore, in MBs, tumours that have not shown expression of *GPC3*, we and others failed to detect the presence of *IGF2* transcripts [Saikali and Sinnett, 2000; Ogino *et al.*, 2001]. On the other hand, WT samples expressed both *GPC3* and *IGF2* [Yun *et al.*, 1993; Saikali and Sinnett, 2000], to the point that it has been suggested that overproduction of IGF-II is a rate-limiting step in WT growth [Qing *et al.*, 1996]. This rate-limiting step could be necessary to uncouple the apoptotic effect of GPC3 from its potentially beneficial effects on a tumourigenic cell.

Finally, it is becoming clear that the roles GPC3 may be playing in cellular functions are very complex, involving an interaction with many factors. Thus, despite the ideas put forth above, GPC3 may still be a negative regulator of IGF2, involved in the control of cell proliferation and/or survival, as suggested to explain the possible mechanism of development of SGBS [Pilia *et al.*, 1996; Hughes-Benzie *et al.*, 1996]. In support of the suggested complexity, another glypican, GPC1, was recently shown to be overexpressed in human breast cancer where it modulates the effects of at least five different growth factors [Matsuda *et al.*, 2001]. Much more work is needed to further understand the functions of GPC3 and other glypicans in cells in general and embryonal tumours in particular, and to establish whether the presence of GPC3 in these tumours offers any potential for diagnostic or therapeutic tools.

D.3.3. Analysis of Genetic Expression in *GPC3*-Expressing SK-N-FI Cells Using cDNA Microarrays

We applied the cDNA microarray technique to compare the expression patterns of SK-N-FI cells expressing or not expressing *GPC3*. We identified a total of fifteen

genes that show differential expression after *GPC3*-transfection. Since GPC3 is a membrane protein, it is expected that none of these genes are affected directly at the transcriptional or translational level by GPC3, but the presence of this proteoglycan could lead to downstream effects that result in expression changes.

Three of these genes underwent radical changes in expression: neuropeptide Y (NPY) and interleukin-8 (IL-8) precursors had undetectable expression in the untransfected SK-N-FI cells but were expressed after transfection, whereas PCP4 shows significant expression in the untransfected cells that was not detectable in the transfected cells. NPY and PCP4 have functions at the level of the nervous system.

NPY is a neuropeptide involved in a variety of neuro-endocrine functions including stress responses, central autonomic functions and feeding behaviour [reviewed in Wahlestedt *et al.*, 1989]. Neuroendocrine cell lines, particularly NB, often express the *NPY* gene [O'Hare and Schwartz, 1989]. *NPY* transcription can be activated by NGF and negatively regulated by RA in NB cell lines [Wernersson *et al.*, 1998; Magni *et al.*, 2000], and activated by BDNF in cultured spinal neurons [Kim *et al.*, 2000]. Thus, its expression in *GPC3*-transfected SK-N-FI cells could be due to the modulation by GPC3 of the response to any of these factors or others. It is possible that a dosage effect is in play, where certain factors are below the effective threshold or not bioavailable for receptor activation and that GPC3 allows this activation and response to take place, as has been suggested for dally in a co-receptor scenario [Lin and Perrimon, 1999; Tsuda *et al.*, 1999]. Whatever the mechanism, if GPC3 is leading to *NPY* expression, the correlation between GPC3 and NB tumour prognostic factors may be worth studying because of information regarding this peptide in NB tumours and patients. Plasma NPY levels have been shown to be increased in patients with NB [Kogner *et al.*, 1990] and these levels also correlate directly with stage of the disease [Dotsch *et al.*, 1998]. More than three-quarters of NB primary tumours tested express the *NPY* gene [Cohen *et al.*, 1990]. In a recent study, a low level of processing of NPY from its prohormone precursor correlated with unfavourable metastatic NB [Bjellerup *et al.*, 2000]. Therefore, it appears likely that NPY production is regulated at more than one level in NB cells, one of which could be modulated by GPC3.

PCP4, also known as PEP-19, appears to be highly reduced or blocked in transfected cells, and no expression was detectable in SK-N-AS cells either (see section

C.2. and Tables X and XI). The function of this peptide is still unknown, although it has been shown to be a marker for Purkinje neurons [Mugnaini *et al.*, 1987] and to bind calmodulin [Slemmon *et al.*, 1996]. Its distribution is limited to the brain and levels of PCP4 increase during the late maturation of the rat nervous system [Sangameswaran *et al.*, 1989]. Recently, this peptide was shown to lower levels of cell death in a pheochromocytoma cell line [Erhardt *et al.*, 2000]. Thus, the inverse correlation between the expression of *GPC3* and that of *PCP4* is in agreement with the literature: *GPC3* is not expressed in the brain at any time of development whereas *PCP4* is expressed solely in the brain, and GPC3 may promote apoptosis whereas PCP4 appears to inhibit it. Such an inverse relationship may be part of a developmental process whereby pre-neuronal cells acquire their CNS-targeted neuronal character at a time when expression of a group of genes including *GPC3* is turned off, allowing *PCP4* and others to be expressed and/or activated. Such a scenario supports a role for PCP4 in blocking neuronal apoptosis. It would also imply that cells that maintain *GPC3* expression would be targeted towards other functions, would still need IGF-I or IGF-II stimuli to counter apoptosis, and may express a battery of genes such as *NPY* that are characteristic of differentiation along another pathway. In this regard, the regulation of expression of *GPC3* would be very interesting to study, given that the loss of expression may be at a crossroad for neuroendocrine differentiation. However, that issue is beyond the scope of this discussion.

An intriguing observation of this expression analysis is that most of the other genes that exhibit an alteration in their expression patterns after transfection with *GPC3* are downregulated, similarly to *PCP4* (Table XIV). This suggests an inhibitory action of GPC3 on some growth factor pathway, where such genes as the neuromedin B receptor gene and the neuroleukin gene are turned off at the same time as *PCP4*, in the type of scenario suggested above. An exception was the expression of the monocyte chemotactic protein 1 gene (*MCP1*) that is more than six-fold higher in *GPC3*-expressing cells. Relative activation of the *MCP1* gene (also known as *MCAF*, monocyte chemotactic and activating factor) is similar to what is observed for *NPY* and *IL-8*, which is interesting because of the known relationship between IL-8 and MCP-1.

IL-8 and MCP-1 are prototypes for CXC chemokines and chemotactic cytokines, respectively, the two main groups of chemokines that determine neutrophil-

and macrophage-mediated inflammation [reviewed in Mukaida *et al.*, 1998]. Particularly interesting is their involvement in neural trauma and inflammation, where they play an important role in leukocyte recruitment [reviewed in Ransohoff, 1997]. The fact that this analysis revealed the activation of two different genes involved in similar functions is an indication of the dependability of the method used in obtaining information about pertinent changes in cell behaviour. In addition, both of these chemokines are produced in response to interleukin 1 alpha in the SK-N-SH but not the SK-N-MC NB cell lines [Chuluyan *et al.*, 1998] and IL-8 production is induced by RA in SK-N-SH cells [Yang *et al.*, 1993]. Finally, IL-8 has been shown to be a mediator of angiogenesis [Koch *et al.*, 1992] and its production in NB primary tumours but not in benign adrenal tissue is thought to have some impact on tumour angiogenesis [Ferrer *et al.*, 2000]. Thus, the production of these chemokines in *GPC3*-transfected SK-N-FI cells could be due to activated, enhanced or inhibited response to growth factors, in a manner similar to that already suggested for NPY and PCP4.

D.4. Conclusion and Future Perspectives

In this project, we have performed large-scale expression profiling of NB cells, revealing the possible role of biological cellular pathways in NB pathogenesis by the expression of genes that are components of these pathways. This is a powerful analysis, since even mutated, deleted or downregulated tumour suppressors may be revealed by downstream effects of their transcript absence or mutation. Such an analysis is particularly important in the context of NB, because many sites of loss of heterozygosity are observed, but important tumour suppressors are still to be found, and because the molecular and biological bases of NB heterogeneity still need to be defined. This is a discovery-driven approach that will lead to more traditional hypothesis-driven projects, as was the case for the GPC3 project. We hope that our results will help others to select candidates for further study in NB research. Future perspectives that may be interesting include the possible role of certain transcription factors and thymosins in NB development, the identification of the unknown cDNAs revealed by DD-PCR, and the possible role of GPC3 in mechanisms such as tumour angiogenesis and recruitment of tumour-associated macrophages.

SECTION E. REFERENCES

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ANNEX I

Int. J. Cancer (Pred. Oncol.): **89,** 418–422 (2000)

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EXPRESSION OF GLYPICAN 3 *(GPC3)* IN EMBRYONAL TUMORS

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Embryonal tumors, such as neuroblastoma, medulloblastoma and Wilms' tumor, have their peak incidence in the first 4 years of life. These neoplasias exhibit genetic and clinical heterogeneity, but little is known about their molecular pathogenesis. Application of the differential-display PCR approach led to the identification of a gene, *glypican 3 (GPC3),* that is differentially expressed in cancer cells. Expression of this gene is usually limited to fetal mesodermal tissue, and its inactivation has been found to be responsible for the X-linked Simpson-Golabi-Behmel overgrowth syndrome. Here, we show that *GPC3* mRNA is present in several neuroblastomas and all Wilms' tumors tested to date but not in medulloblastoma. *GPC3* was not expressed in normal kidney tissues obtained from the corresponding Wilms' tumor patients, suggesting that in these cancer cells expression was not repressed (or was activated). No correlation was found between expression of *GPC3* and the known indicator of neuroblastoma prognosis *MYCN* mRNA. However, all samples that expressed *GPC3* also expressed *IGF-II,* coding for a growth factor important in the survival and growth of many cancer types. Although the biological significance of this relationship remains unclear, our results suggest that *GPC3* may be implicated in the development of embryonal tumors through a signaling pathway that appears to involve IGF-II.
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Embryonal tumors, such as neuroblastoma, medulloblastoma and Wilms' tumor, have their peak incidence in the first 4 years of life. These tumor types exhibit characteristics of undifferentiated tissue typical of developing cells. In addition, these neoplasias exhibit genetic and clinical heterogeneity, making prognosis prediction problematic, especially in the case of medulloblastoma (Adesina, 1999) and neuroblastoma (Brodeur, 1995). Molecular genetic studies have shown that embryonal tumors often contain a combination of fusion proteins, loss of tumor suppressors and/or activation of proto-oncogenes (Thorner and Squire, 1998). These genetic features include expression of *MYCN* oncogene in neuroblastoma (Schwab *et al.,* 1983; Brodeur *et al.,* 1984), medulloblastoma (Garson *et al.,* 1989) and Wilms' tumor (Nisen *et al.,* 1986) as well as that of IGF-II in Wilms' tumor (Reeve *et al.,* 1985) and neuroblastoma (El-Badry *et al.,* 1991; Sullivan *et al.,* 1995). In neuroblastomas, high expression of *MYCN,* which is often due to gene amplification, appears to be associated with rapid disease progression and, thus, a poor prognosis (Seeger *et al.,* 1985; Chan *et al.,* 1997).

Despite these results, little is known about the molecular pathogenesis of these pediatric malignancies. To address this question, we applied a modified differential-display approach to detect genes whose expression is altered in embryonal tumors. One of the genes determined using this technique to have altered expression patterns was *GPC3,* coding for the extracellular proteoglycan glypican 3. Here, we report expression of *GPC3* in neuroblastoma and Wilms' tumor cells but not in medulloblastoma. We also examined the correlation between expression of *GPC3* and other genes, such as *MYCN* and *IGF-II.*

MATERIAL AND METHODS

Patients and cell lines

Sixteen primary tumor specimens were obtained from patients treated at Sainte-Justine Hospital (Montreal, Canada), including 4 neuroblastomas, 1 neurofibrosarcoma, 1 adrenal carcinoma and 1 rhabdomyosarcoma as well as 9 Wilms' tumors and, in 3 cases, the corresponding normal kidney tissue. The Institutional Review Board approved the research protocol, and informed consent was obtained from all participating individuals or their parents. Eighteen human cell lines were studied: 10 neuroblastomas, SK-N-AS, SK-N-DZ, SK-N-MC, SK-N-FI, IMR-32, SK-N-SH (obtained from ATCC, Rockville, MD), NBL-S (obtained from G.M. Brodeur, St. Louis Children's Hospital, St. Louis, MO), SJNB-1, SJNB-7 and SJNB-10 (obtained from T. Look, St. Jude Children's Research Hospital, Memphis, TN); Ntera2 embryonal carcinoma (ATCC); an osteosarcoma line, 143N2 (obtained from R. Morais, University of Montreal, Montreal); 5 primary medulloblastoma cell lines, MB1, MB3, MB4, MB5 and MB7, derived from both classical and desmoplastic variants (obtained from Y. Théorêt, Hôpital Ste-Justine, Montreal); and HepG2 liver carcinoma (ATCC). Neuroblastoma cell lines SK-N-DZ, IMR-32, SJNB-7 and SJNB-10 have known *MYCN* amplification (Reynolds *et al.,* 1988; Shapiro *et al.,* 1993).

Differential-display PCR

Two micrograms of DNase-treated total RNA isolated from the cell lines were reverse-transcribed in a 25 μl reaction containing 2 μM of the anchored oligo(dT) primer gcgcaagcttT$_{10}$GA (3′ amplimer; Linskens *et al.,* 1995), 25 μM of each dNTP, 3 mM $MgCl_2$, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 1 U PRIME RNase Inhibitor (5-Prime, 3-Prime, Inc., Boulder, CO) and 600 U MMLV reverse transcriptase (GIBCO BRL, Burlington, Canada). The reaction was carried out at 37°C for 70 min. cDNA fragments were amplified in a 20 μl reaction containing 1 μl of the reverse-transcribed mixture, 2 U Taq DNA polymerase (GIBCO), 7.5 μM of each dNTP, 2 μCi [α-^{33}P]dCTP (2,000 Ci/mmole; NEN-Dupont, Guelph, Canada), 2 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.25 μM of the 3′ amplimer and 1.25 μM of the 5′ arbitrary primer, cgggaagcttTAGCTAGCATGG (Linskens *et al.,* 1995). Amplification was performed for 4 cycles at 94°C, 45 sec; 41°C, 60 sec; 72°C, 60 sec (low-specificity conditions), followed by 18 cycles at 94°C, 45 sec; 60°C, 45 sec; 72°C, 120 sec (high-specificity conditions). PCR products were fractionated on a 6% non-denaturing polyacrylamide gel. To control reproducibility, each reverse transcription and PCR was performed in duplicate.

Re-amplification and sequencing of cDNA fragments

Bands of interest were cut out from the gel and isolated (as in Zietkiewicz *et al.,* 1992), then re-amplified by 25 cycles of PCR using the high-specificity conditions described above and the same primer pair combination but with 50 μM dNTPs instead of 7.5 μM. Re-amplified cDNAs were purified using the QIAquick PCR purification kit (Qiagen, Mississauga, Canada) from an agarose gel after electrophoresis. These products were directly sequenced using the ThermoSequenase kit (Amersham Pharmacia Biotech, Inc,

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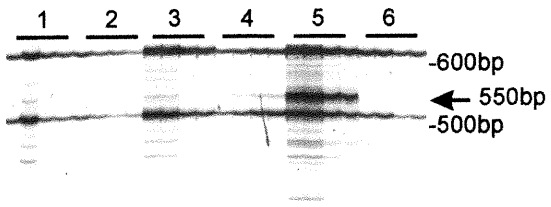

FIGURE 1 – mRNA differential display of human neuroblastoma cell lines. Total RNA was isolated from cell lines NBL-S (lane 1), SK-N-SH (lane 2), SK-N-MC (lane 3), SJNB-10 (lane 4), SJNB-7 (lane 5) and SJNB-1 (lane 6). Each sample was reverse-transcribed and amplified in duplicate (giving 4 lanes each). Arrow at right indicates differentially displayed band F0126/08.

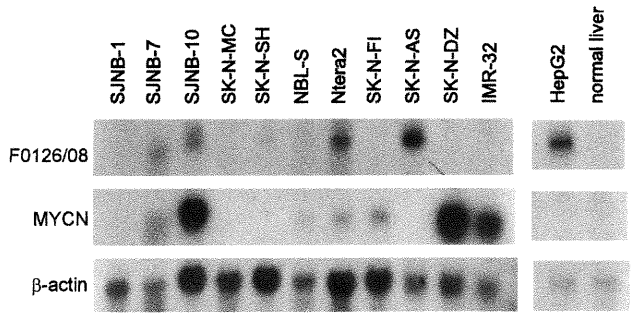

FIGURE 2 – Analysis of expression of band F0126/08 and *MYCN* in cancer cell lines. The Northern blot was hybridized with the F0126/08 cDNA fragment (upper panel) and a 450 bp sequence from the *MYCN* 3′ UTR (middle panel) as probes. *β-actin* hybridization (lower panel) was used to control the quantity and integrity of RNA samples.

```
F0126/08      1   CTCGGTGGTGTGCTTCTTCTTCCTGGTGCACTGACTGCCT   40
                  |||||||||||||||||||||||||||||||||||||||
GPC3        1861  CTCGGTGGTGTGCTTCTTCTTCCTGGTGCACTGACTGCCT 1900

F0126/08     41   GGTGCCCAGCACATGTGCTGCCCTACAGCACCCTGTGGTC   80
                  |||||||||||||||||||||||||||||||||||||||
GPC3        1901  GGTGCCCAGCACATGTGCTGCCCTACAGCACCCTGTGGTC 1940

F0126/08     81   TTCCTCGATAAAGGGAACCACTTTCTTATTTTTTTCTATT  120
                  ||||||||||||||||||||||||||||||||||||||||
GPC3        1941  TTCCTCGATAAAGGGAACCACTTTCTTATTTTTTTCTATT 1980
```

FIGURE 3 – Alignment of differentially displayed band F0126/08 with the partial sequence of glypican 3 *(GPC3).* The nucleotide sequence of F0126/08 cDNA (upper lane), obtained from direct sequencing, was identical to the *GPC3* cDNA sequence (lower lane) from base 1861 to base 1980 (Genbank accession number L47125).

Baie d' urfée, Canada) and analyzed for sequence identity in the Genbank database.

Northern blot analysis

For Northern blot analysis, 7.5 μg of total RNA were fractionated on a 0.66 M formaldehyde/1.0% agarose gel and transferred onto nylon membranes (GeneScreenPlus; NEN-Dupont). Membranes were pre-hybridized with 40% formamide, 5 × SSPE, 5 × Denhardt's solution, 1% SDS and 0.1 mg/ml sonicated fish sperm DNA (Boehringer-Mannheim, Laval, Canada) for at least 2 hr at 42°C. Hybridization was carried out by adding the randomly labeled cDNA probe and incubating in the same conditions for 16 hr. Washes were performed once at room temperature with 2 × SSC/0.5% SDS for 15 min, twice at 42°C with 2 × SSC/0.5% SDS for 30 min, once at 55°C with 0.5 × SSC/0.5% SDS and, when needed, once at 65°C with 0.5 × SSC/0.5% SDS. Relative expression levels were evaluated by comparison with the signal intensity of the internal control, *β-actin.*

RT-PCR

cDNA was synthesized from 0.5–2 μg DNase-treated total RNA in a 20 μl reaction containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 5 mM $MgCl_2$, 1 mM of each dNTP, 1 U PRIME RNase Inhibitor, 2.5 μM random hexamers and 500 U MMLV reverse transcriptase. PCR amplification was carried out in a total volume of 12.5 μl containing 2.5 μl of the cDNA product, 0.48 μM of each gene-specific primer pair (see below) and 0.5 U Taq DNA polymerase (GIBCO) in 20 mM Tris-HCl (pH 8.4), 50 mM KCl and 2 mM $MgCl_2$, 100 μM each dNTP and 1.5 μCi [α-^{32}P]dCTP (3,000 Ci/mmole; ICN, Mississauga, Canada) in some cases. The following primers were used: *GPC3* F, 5′-GATACAGCCAAAAGGCAG-3μ; R, 5μ-ATCATTCCATCACCAGAG-3μ (modified from Pilia *et al.,* 1996); *β-actin* F, 5μ-ACCATGGATGATGATATCGC-3μ; R, 5μ-ACATGGCTGGGGTGTTGAAG-3μ; *IGF-II* F, 5μ-CTTGGACTTTGAGTCAAATTGG-3μ; R, 5μ-GGTCGTGCCAATTACATTTCA-3μ (modified from Vu and Hoffman, 1994); *MYCN* F, 5μ-AGACCAGCGGCGGCGACCA-3μ; R, 5μ-ATGACACTCTTGAGCGGACG-3μ. PCR was performed for 35 cycles (25 cycles with radiolabeled dCTP) of denaturation at 94°C, 30 sec; annealing for 30 sec; elongation at 72°C, 30 sec; with a final extension cycle at 72°C, 5 min. Annealing temperatures were 55°C for *GPC3* and *IGF-II,* 60°C for *β-actin* and 62°C for *MYCN.* Radiolabeled PCR products were fractionated on a 7% non-denaturing polyacrylamide gel in 1 × TBE buffer, and non-radiolabeled PCR products were fractionated on a 1% agarose gel in 1 × TBE buffer.

Statistical analysis

Expression of *GPC3*, *IGF-II* and *MYCN* was analyzed by comparison with expression of *β-actin* from at least 3 independent RT-PCRs or Northern blots. Statistical analysis of expression of these genes was performed using 2 models: *(i)* by assigning the value 0 to samples with no expression and the value 1 to samples with expression at any level and *(ii)* by assigning the value 2 to samples with expression, the value 1 to samples with trace levels of expression and the value 0 to samples with no expression. Spearman's rank correlation coefficient was used to analyze the data according to both models.

RESULTS

We used a modified differential display of RNA approach to compare the gene-expression patterns generated from 6 human neuroblastoma cell lines with (n = 2) and without (n = 4) *MYCN* amplification. We identified a 550 bp cDNA fragment that was present only in 2 cell lines, SJNB-7 and SJNB-10 (Fig. 1). The band of interest (F0126/08) was cut from the gel, re-amplified, isolated and used to probe a Northern blot containing RNA from an extended panel of neuroblastoma cell lines as well as an embryonal carcinoma cell line (Ntera2) and a liver carcinoma cell line (HepG2) (Fig. 2). We detected a 2.5 kb mRNA species in the SJNB-7, SJNB-10, SK-N-SH and SK-N-AS neuroblastoma cell lines, as well as in Ntera2 and HepG2 but not in normal liver (Fig. 2). Direct DNA sequencing of the PCR product yielded a 120 bp sequence that showed a perfect match with the 3′-end region of the *GPC3* gene (Fig. 3), which is identical to *MXR7* (Lage and Dietel, 1997) and *GTR2-2* (Hsu *et al.,* 1997). The predicted size of the *GPC3* gene product, 2.5 kb (Pilia *et al.,* 1996), is similar to that in Figure 2.

To determine whether *GPC3* is expressed in the same cell lines as *MYCN,* a neuroblastoma prognostic biomarker, we rehybridized the RNA panel with a probe derived from the 3′UTR of *MYCN* (Fig. 2). Only SJNB-7 and SJNB-10 displayed co-expression of both *GPC3* and *MYCN,* whereas *GPC3* and *MYCN* were expressed independently in 2 and 4 cell lines, respectively. This suggests that these 2 genes are not necessarily expressed in the same cell (see below). Thus, *MYCN* was expressed in the cell line SK-N-FI carrying a non-amplified *MYCN* locus (Fig. 2).

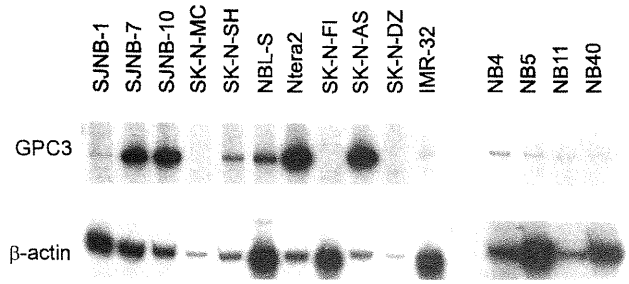

FIGURE 4 – RT-PCR analysis of *GPC3* mRNA in neuroblastomas. Exons 5 and 6 of *GPC3* mRNA (top) were amplified from neuroblastoma cell lines (left panel) and primary tumors (right panel). The quantity and integrity of RNA were verified by RT-PCR amplification of *β-actin* mRNA (bottom).

GPC3 encodes a putative extracellular membrane proteoglycan that is expressed particularly in embryonic and fetal tissues. In light of this, we sought to evaluate its expression as well as that of *MYCN* and *IGF-II* in a wider range of embryonal tumors, including neuroblastomas, Wilms' tumors and medulloblastomas. To detect even small quantities of mRNA, these analyses were done using an RT-PCR approach (representative results are shown in Fig. 4). Expression patterns for *GPC3, IGF-II* and *MYCN* are summarized in Table I and are based on comparisons with *β-actin* expression levels. *GPC3* mRNA was detected, although at various levels, in placenta, fetal kidney, fetal lung and adult liver. Most neuroblastomas, 1 neurofibrosarcoma and all Wilms' tumors examined expressed *GPC3*. However, *GPC3* was not (or barely) expressed in the normal kidney tissues obtained from 3 Wilms' tumor patients. None of the 5 medulloblastoma cell lines, the osteosarcoma, the adrenal carcinoma or the rhabdomyosarcoma showed expression. Similarly, expression levels of *IGF-II* and *MYCN* were determined in the same panel (Table I). The correlation between expression of *GPC3, IGF-II* and *MYCN* was evaluated as described in Material and Methods. We observed a significant correlation between expression of *GPC3* and that of *IGF-II* ($p < 0.001$), whereas no such correlation was found at this significance level between *IGF-II* and *MYCN* or *GPC3* and *MYCN* ($p > 0.01$ in each case). Similar results were obtained regardless of the model used to analyze the data. This supports the observed co-expression of *GPC3* and *IGF-II* in the same cells.

TABLE I – SUMMARY OF EXPRESSION DATA OF *GPC3, IGF-II* AND *MYCN* IN EMBRYONAL TUMORS

Sample	Relative level of expression*		
	GPC3	IGF-II	MYCN
Neuroblastoma cell line			
SJNB-1	+	+	+
SJNB-7	+	+	+
SJNB-10	+	+	+
SK-N-MC	−	tr	−
SK-N-SH	+	+	−
NBL-S	+	+	+
SK-N-FI	−	+	+
SK-N-AS	+	+	tr
SK-N-DZ	−	tr	+
IMR-32	+	tr	+
Embryonal carcinoma cell line Ntera2	+	tr	+
Osteosarcoma cell line 143N2	−	+	−
Medulloblastoma cell line			
MB1	−	−	tr
MB3	−	−	tr
MB5	−	−	+
MB7	−	−	−
MB4	−	−	tr
Wilms' tumor			
Normal kidney 177	tr	ND	ND
WT177	+	+	tr
Normal kidney 040	tr	ND	ND
WT40	+	+	+
Normal kidney 130	−	tr	tr
WT130	+	+	+
WT42	tr	+	+
WT51	+	+	tr
WT101	+	+	tr
WT106	+	+	tr
WT116	+	+	tr
Neurofibrosarcoma 005	+	+	−
Adrenal carcinoma 007	−	+	−
Rhabdomyosarcoma 013	−	+	−
Neuroblastoma			
NB4	+	+	+
NB5	+	+	+
NB11	tr	+	+
NB40	tr	+	+
Pre-term placenta	+	+	+
Placenta at term	+	+	+
Fetal kidney	+	+	+
Fetal lung	+	+	+
Adult liver	tr	+	tr

+, expression; −, no expression; tr, trace amounts of mRNA detected; ND, not done. *Based on 3 to 5 independent experiments.

DISCUSSION

We report expression of *GPC3,* encoding a putative extracellular membrane proteoglycan of the glypican family, in neuroblastoma, and Wilms' tumor 2 embryonal tumors but not in medulloblastoma. Expression of *GPC3* has also been observed in adult cancers, including colorectal malignancies (Lage *et al.,* 1998) and hepatocellular carcinomas (referred to as MXR7 in Hsu *et al.,* 1997). *GPC3* was expressed in the liver carcinoma cell line HepG2 but not in normal liver tissue. Regarding tumors with neuronal phenotype, *GPC3* was detected at variable levels in a neurofibrosarcoma and in most neuroblastomas but was absent from all medulloblastomas. These findings suggest that *GPC3* expression is differentially regulated in the various cell lineages giving rise to pediatric tumors of the peripheral and central nervous systems. Because *GPC3* mRNA has not been detected in CNS tissue of mouse embryos or fetuses, its absence in medulloblastomas is not surprising. Also, expression of *GPC3* was up-regulated in all Wilms' tumors tested, as illustrated by its absence in the normal kidney tissues derived from some of the patients. This indicates that GPC3 in Wilms' tumor does not have a growth-inhibitory effect. This is in agreement with results on hepatocellular carcinoma patients, where the corresponding normal hepatic tissue did not express *GPC3* (Hsu *et al.,* 1997), suggesting a derepression (or activation) of this gene in cancer cells. However, this up-regulation of GPC3 expression appears to be cell-specific because of its absence in medulloblastomas (this study) and in ovarian cancer cell lines, while being expressed at relatively high levels in normal ovarian tissue (Lin *et al.,* 1999).

GPC3 is highly expressed in embryonal tissues such as the developing intestine and the mesoderm-derived tissues, and its expression is down-regulated in most adult tissue, implying a potential role in development (Filmus *et al.,* 1988; Pellegrini *et al.,* 1998). Other members of the glypican family are also thought to play a role in embryonic development: *GPC1*/glypican (David *et al.,* 1990), *GPC4*/K-glypican (Watanabe *et al.,* 1995) and *GPC5* (Veugelers *et al.,* 1997; Saunders *et al.,* 1997). One member of the glypican family, *GPC2*/cerebroglycan, is expressed transiently in immature rat brain neurons but not after axon outgrowth is completed, suggesting a potential role in proliferating neuroepithelial cells (Stipp *et al.,* 1994). Support for a role for GPC3 in embryonic development came from Pilia *et al.* (1996), who showed that mutations in *GPC3* cause the X-linked Simpson-Golabi-Behmel syndrome (SGBS), which is characterized by pre- and post-natal overgrowth in affected hemizygous males. These patients often present with dysplastic kidneys and appear to be associated with a greater risk of developing embryonal cancers (Hughes-Benzie *et al.,* 1992), though this relationship remains unclear (Xuan *et al.,* 1999).

SGBS shares significant similarities with Beckwith-Wiedemann syndrome, another overgrowth disorder that is thought to be associated with over-expression of *IGF-II,* a growth factor that can also act as a survival factor in early stages of tumorigenesis (Christofori *et al.,* 1994; Harrington *et al.,* 1994). This led to the hypothesis that GPC3 is involved either directly (Pilia *et al.,* 1996) or indirectly (Song *et al.,* 1997) in the down-regulation of *IGF-II* activity. To examine the potential association between *GPC3* and *IGF-II* in embryonal tumors, we also determined the expression status of *IGF-II* in our samples, as well as that of an unrelated gene important in these cancers, *MYCN.* We observed a significant correlation between expression of *GPC3* and *IGF-II* but not between expression of *GPC3* and *MYCN* or between *IGF-II* and *MYCN.* This observation is supported by *in situ* hybridization results on mouse fetuses, also showing co-expression of *gpc3* and *igf-II* (Pellegrini *et al.,* 1998).

The biochemical function of GPC3, a highly glycosylated extracellular membrane protein, remains unclear. Proteoglycans are highly interactive macromolecules, playing diverse roles in the cell migration, growth and adhesion processes that are critical during tumorigenesis (Turley, 1984; Iida *et al.,* 1996). The SGBS phenotypic characteristics suggest that GPC3 is involved in the control of cell proliferation and/or survival (Hughes-Benzie *et al.,* 1996). Co-expression of *GPC3* and *IGF-II* in the same cells may provide insight into the putative role of GPC3 in cancer cells. This protein appears to bind growth factors such as IGF-II (Pilia *et al.,* 1996), bFGF (Song *et al.,* 1997) and tissue factor pathway inhibitor (Mast *et al.,* 1997). Such binding of GPC3 to growth factors available to different tumor cell types could influence the survival of these cells. In this regard, GPC3 can induce apoptosis or inhibit proliferation in a cell line-specific manner, and these cells can be rescued by IGF-II (Gonzalez *et al.,* 1998). Hence, the effect of *GPC3* expression may depend on the cell type and, in particular, on the presence of certain growth factors and the capacity to respond to these. However, this scenario does not exclude that GPC3 is a negative regulator of IGF-II, as suggested by Pilia *et al.* (1996) to explain the possible mechanism of development of SGBS.

In summary, expression of *GPC3* may be implicated in the development of certain embryonal tumors through a signaling pathway that appears to involve IGF-II. At this stage, it remains unclear whether GPC3 plays a role in carcinogenesis and/or progression or whether its expression is characteristic of undifferentiated cells with fetal expression patterns. Additional studies should increase our understanding of the genetic mechanism and biological significance underlying the increased *GPC3* expression as well as its potential association with tumorigenesis.

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