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Genetic Alterations Associated with Metastatic Dissemination and Chemoresistance in Neuroblastoma

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Knowledge about genetic alterations specific to the metastatic process and chemoresistance in neuroblastoma is progressing steadily. Low or no *CD44* expression, increased *NM23* expression and specific mutations of the 5′ coding regions of *NM23* are distinct features of aggressive, metastatic neuroblastoma. *MYCN* down-regulates Class I HLA antigen expression in many neuroblastoma cell lines and, in turn, may be regulated by a suppressor gene. The *MYCN* amplified human neuroblastoma cell line, IGR-N-91, established *in vitro*, metastasises in the nude mouse and has exhibited co-activation of *MYCN* and *PGY1*, resulting from direct activation of the oncoprotein on the *PGY1* promoter. In this model, the *MYCN* product activates angiogenesis, the dissemination process and chemoresistance via specific genes (*PGY1* and *GST3*). *MYCN*, like the *BCL-2* and *TP53* products, may also play a key role in apoptosis. The implication of these genes in the potential for metastasis and chemoresistance in neuroblastoma is discussed.

Key words: neuroblastoma, metastatic dissemination, genetic alterations, *MYCN* activation, chemoresistance, *in vivo* models

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INTRODUCTION

THE GENETIC analysis of tumour tissues has offered considerable insights into the tumour heterogeneity of neuroblastoma. Indeed, cytogenetic and molecular biology studies, currently in progress, have identified recurrent genetic alterations which, when combined, appear to denote the existence of neuroblastoma subtypes [1, 2]. In the clinic, knowledge of such genetic anomalies has provided immediate applications for the treatment and outcome of patients with localised forms and stage IV-S [1, 3, 4]. So far, however, there has been no impact on the management of clinically unfavourable forms (disseminated stage IV) of neuroblastoma at diagnosis, in children older than a year. It is possible that information on genetic alterations specific to metastatic cells could help to improve the prognosis and to tailor therapy to these aggressive neuroblastomas.

Cancer invasion and dissemination is characterised by a long

series of sequential, inter-related steps [5] consisting, at a physiopathological level, of angiogenesis, invasion, intravasion, transport, arrest, extravasation, and proliferation to metastatic sites. [6] Metastatic dissemination is generally regarded as a late event in tumour progression [7] which, at the molecular level, is the gradual accumulation of multiple genetic changes affecting cell growth and neoplastic behaviour. There is considerable evidence to support the notion that tumour progression may be due to the activation, mutation or loss of different genes which belong to three major functional groups [8], i.e., oncogenes, suppressor genes and modulator genes. As proposed by Klein and Klein [8], the modulators seem to influence the neoplastic behaviour of tumour cells during their dissemination and interaction with host tissues. These genes form a large heterogeneous group, and include the genes of the major histocompatibility complex, those expressed in the control of proteolytic and homing mechanisms, genes involved in cellular resistance to immune rejection as well as those activated during resistance to treatment.

This review will present and discuss (i) the genetic alterations which could be specifically associated with metastasis in neuroblastoma; (ii) experimental models used to detect these genetic abnormalities; and (iii) the role of genes involved in the response of neuroblastoma cells to chemotherapy.

INTRINSIC GENETIC AND MOLECULAR DETERMINANTS RELATED TO TUMOUR PROGRESSION AND THE METASTATIC PROCESS IN NEUROBLASTOMA

Acquired recurrent genetic alterations characterise primary neuroblastoma. Mostly, these include loss of heterozygosity (LOH) on the short arm of chromosome 1, at band 1p36-2 [9, 10], *MYCN* proto-oncogene amplification [11, 12], hyperdiploidy or near diploidy [3] and defects in the expression or function of the nerve growth factor receptor [13]. Hyperdiploidy, i.e., increased DNA content, is associated with early stages of the disease and with a favourable outcome in infants. LOH for chromosome 1, and *MYCN* amplification are more common in children older than 1 year of age with advanced stages of disease: these two latter genetic abnormalities appear to be related [14]. LOH for 1p36 may precede *MYCN* gene amplification. By combining the DNA content, 1p LOH and *MYCN* amplification, 3 distinct genetic subtypes of neuroblastoma can be defined. The first group has a hyperdiploid modal karyotype, the second group, a near-diploid mode karyotype and the third, a diploid mode, 1p deletions or LOH for 1p36 and *MYCN* amplification. These three groups correspond to the different levels of prognosis (good, intermediate and very poor, respectively) and typify three distinct subtypes of neuroblastoma [1]. Of great importance, no connection exists between the 3 groups and molecular proof of a stepwise transition from one neuroblastoma type to another is lacking. Regarding the third group, which corresponds to advanced aggressive neuroblastoma, it is noteworthy that 1p deletion and *MYCN* amplification have been found in the primary tumour prior to metastatic dissemination [2]. If amplification of the *MYCN* gene is correlated with increased metastatic potential [12], the mechanism enabling *MYCN* to increase neuroblastoma malignancy is poorly understood. However, evidence has recently been presented that *MYCN* disrupts protein kinase C-mediated signal transduction in neuroblastoma [15]. Other alterations of oncogenes and growth factors, such as nerve growth factor (NGF) receptor [13, 16] and *HA-RAS* [17] expression have recently been reported and are additional elements regarding disease characterisation but so far their association with a potential for metastasis has not been determined.

More extensive research has been conducted for the heterogeneous class of modulator genes, possibly involved in the neuroblastoma metastatic process, and results obtained invariably refer to *MYCN* expression. The human CD44 cell surface integral glycoprotein, involved in a variety of functions, including lymphocyte homing, extracellular cell matrix attachment, and tumour metastasis, and subjected to alternative splicing, has been described as overexpressed in metastatic colon cancer [18]. In neuroblastoma, low expression or the absence of CD44 in cell lines strongly suggested that, unlike that found in other tumours, repressed CD44 expression could be a marker of aggression [19]. Two recent studies using immunohistochemical [20] and molecular [21] approaches confirmed this seminal work. In human tumours, CD44 is highly expressed in its standard isoform in 100% of stage I–III, stage IV-S neuroblastomas and ganglioneuromas, but only in a subset of stage IV tumours [21]. In contrast, no CD44 expression was detected on *MYCN* amplified stage IV tumours, indicating a highly negative relationship between *MYCN* amplification and CD44 expression in neuroblastoma [21]. In a recent multivariate analysis, CD44 expression appeared to be a marker of good prognosis [20].

Reduced expression of the *NM23-H* gene, which encodes the nucleoside diphosphate kinase, is associated with a high potential for metastasis in some tumour types, but this expression is increased in aggressive neuroblastoma [22]. Recent studies on mutations in primary tumours at different stages showed that specific mutations in the *NM23-H* gene 5′ coding regions, i.e., leu 48→val [23] and ser 120→Gly [24] were frequently and specifically present in advanced tumours, but not in any early stage tumours. Altogether, these data indicate that molecular alterations to *NM23*, other than reduced expression, can be associated with tumour aggressiveness, and imply that the mutation could be a feature of advanced neuroblastomas.

The expression of Class I HLA antigens, membrane proteins that play an critical role in the recognition of tumour cells by cytotoxic T cells and NK cells, is down-regulated in many neuroblastoma lines. Class I HLA genes appear to be governed by the *MYCN* gene [25, 26] which, in turn, could be regulated by a suppressor gene [27].

IN VITRO ESTABLISHED *MYCN* AMPLIFIED HUMAN NEUROBLASTOMA CELL LINES METASTASISE IN THE NUDE MOUSE

Many neuroblastoma cell lines have been established *in vitro* from tumour tissue or involved bone marrow [28], the most frequent site of metastases in patients. In our laboratory, two *MYCN*-amplified neuroblastoma lines were both derived *in vitro* from stage IV disease, the former from a human primary tumour, IGR-N-835 [29], the latter from bone marrow metastases, IGR-N-91 [30], from another patient. Both IGR-N-91 and IGR-N-835 lines, which generated large tumours after subcutaneous (s.c.) xenografting on to nude mice, were able to disseminate and induce macroscopic metastases in the kidneys, adrenals and in lymphatic tissue of the mouse. In addition, occult neuroblastoma cells were present in the blood, the bone marrow and in the myocardium of animals, and could be characterised through *in vitro* organ culture. Subculturing of

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these neuroblastoma cells gave rise to established metastatic sublines. Non-vascularised, vascularised and haemorrhagic areas from the primary tumour xenografts of both models were subcultured, and preliminary results suggest that the haemorrhagic area is the initial seat of metastatic dissemination (Cappellen & Bénard, Institut Gustave Roussy, France). Preliminary data indicate that human neuroblastoma cells are able to metastasise in the nude mouse *via* the lymphatic route as well as the haematogenous route. These murine models of metastasis originating from human cells may be adequately mimicking human metastatic disease and, thus, be a useful means of studying the genetics of tumour progression and metastases in human neuroblastoma.

Most neuroblastoma lines established *in vitro*, including IGR-N-835 and 91 lines, show *MYCN* amplification. Given the pivotal role played by the *MYCN* oncogene in the expression of the neuroblastoma malignant phenotype, this oncoprotein may be a determinant, not only for oncogenesis, but also for metastasis. There is evidence to support this assumption. Firstly, *in vitro* transfection of stable *MYCN* to SH-EP neuroblasts not expressing *MYCN*, generated advanced malignancy in human neuroblastoma cells, characterised by the expression of an autocrine basic FGF (fibroblast growth factor) loop and angiogenesis [31, 32]. Secondly, IGR-N-91 neuroblasts, cultured *in vitro* from blood cells, bone marrow and the myocardium of mice bearing a subcutaneous tumour IGR-N-91 xenograft, elicited, with consistent *MYCN* amplification, a significant increase in *MYCN* gene transcript levels [30].

GENETIC ALTERATIONS SPECIFICALLY RELATED TO A LACK OF RESPONSE TO CHEMOTHERAPY

If metastatic cells are the essential targets of chemotherapy, then a lack of response to such treatment could signify that the metastatic cancer cell has acquired additional genetic alterations leading to the chemoresistance phenotype. Among the genes possibly activated in chemoresistant cells is the *PGY1* (previously termed the *MDR1*) gene, which encodes the 170 kDa P-glycoprotein (P-gp) [33], a plasma membrane energy-dependent multidrug efflux "pump", which expels many hydrophobic chemotherapeutic agents from the target cancer cell (anthracyclines, vinca alkaloids). Neuroblastoma is a chemosensitive neoplasm which can become refractory to many drugs able to select or to induce a multidrug resistant phenotype in many patients and, as such, is no longer chemocurable [34]. This is consistent with the frequent overexpression of the *PGY1* gene in this tumour, and the fact that approximately 40% of neuroblastoma derive from the medulla adrenal which overexpresses the *PGY1* gene at a high level. The clinical significance of *PGY1* gene activation has been extensively investigated [35, 36]. In our laboratory, the prognostic value of *PGY1* gene expression in neuroblastoma was assessed on a series of 84 patients, taking into account the main known clinical and biological factors of the disease. In the multivariate analysis, only *MYCN* amplification and *PGY1* overexpression remained significantly associated with an increased risk of death. In agreement with a previous report [37], our data strongly suggested that *PGY1* gene overexpression is an independent prognostic factor in neuroblastoma [38]. In this series, a subset of very aggressive metastatic neuroblastomas with *MYCN* amplification, which were refractory to treatment, raised the question of whether *PGY1* gene overexpression was due to treatment itself or due to spontaneous tumour progression and metastatic dissemination. These questions were tested experimentally using *in vivo* models. We, therefore, decided to study the possible relationship between *MYCN* and *PGY1* gene expression in the nude mouse xenograft model, IGR-N-91, during metastatic dissemination.

All IGR-N-91 cells, either from primary subcutaneous tumour, IGR-N-91, xenografts or metastases, consistently exhibited 60 copies of *MYCN* per haploid genome. A significant increase in *MYCN* gene transcript levels was observed in neuroblastoma metastatic cells compared to those of the primary. *MYCN* overexpression was associated with a significant rise in *PGY1* gene mRNA levels leading to a functional P-glycoprotein [30]. This study showed that the *MYCN* oncogene and *PGY1* gene can both be activated during the metastatic process in the absence of any chemotherapy. Increased *MYCN* expression has also been demonstrated after progression in human neuroblastoma using paired lines derived from patients at diagnosis and during progression [39]. The extra amount of the *MYCN* transcript present during the migration of metastatic neuroblastoma cells probably activates genes biologically involved in the so-called "super decathlon", a term used to describe the complex pathway used by metastatic cells (traversing the membranes, adhesion, etc).

The *PGY1* gene is not the only potential determinant of chemoresistance in neuroblastoma. Resistance to drugs, such as cisplatinum and cyclophosphamide, also used in the treatment of this cancer, can be mediated by increasing the cytoplasmic thiol detoxification pathways *via* key enzymes, such as glutathione-S-transferases (GST). In our search for *GST3* gene activation in advanced neuroblastomas, we found that increased *GST3* expression was not an indicator of tumour response to chemotherapy [40]. In contrast, the combined overexpression of *GST3* and *PGY1* genes appeared to be significantly related to a poor response of the primary tumour [40], a relationship which remains to be demonstrated in metastases. However, given the results obtained with the IGR-N-91 model and patient tumours, pleiotropic activation of various genes responsible for chemoresistance would appear to occur in advanced neuroblastomas. In *MYCN* activated neuroblastomas, one possibility is that the nuclear oncoprotein, operating as a transcription factor, activates target genes involved in chemoresistance. Consistent with this assumption is our recent finding that the proximal promoter of the *PGY1* gene was activated by *MYCN* in neuroblastoma cell lines (Ferrandis and Bénard, Institut Gustave Roussy, France), thus indicating that an oncogene can trigger *PGY1* gene activity.

BASIC AND INTRINSIC MECHANISMS RELATED TO CELL RESPONSE TO CHEMOTHERAPY IN NEUROBLASTOMA

A possible option in a cell's resistance to treatment is its refusal to undergo apoptosis. In some cancer models, a mass of evidence indicates that the relative expressions of *TP53* tumour suppressor gene, *C-MYC* protoncogene and *BCL-2* products are intrinsic genetic factors which determine whether cancer cells proliferate or undergo apoptosis, according to external signals. *TP53*-dependent apoptosis has been shown to modulate the cytotoxicity of anticancer drugs [41] and most of these drugs are able to induce apoptosis [42]. Thus, the idea that interaction between the drug and its target *per se* is the sole determinant of cell sensitivity to cytotoxic drugs merits re-appraisal. Tumour response to drugs and therapeutic indexes of treatments may be contingent on the thresholding of the expression of proteins involved in apoptosis, i.e., p53, bcl-2 and c-myc [43]. Furthermore, it is suggested that the intrinsic killing power of the drugs may not be as critical as their ability to activate self-destruction

in appropriate tumours [44]. Although anticancer drugs can activate late events of apoptosis, there are essential differences in signalling pathways between phamacological cell death and the physiological induction of an active suicide programme [45]. Differences may also stem from the nature of the tumour tissue [46]. Thus, genes implicated in apoptosis must be scrupulously analysed, taking into account each tumour type and the corresponding treatment.

What, in fact, do we know about the status of these genes in neuroblastoma? Clearly, *TP53* gene mutations are rare in neuroblastoma tumours [47–49]; and yet *TP53* overexpression has been measured in cell lines [50]. Many authors refute or are sceptical about *TP53* gene alterations in the aetiology and progression of neuroblastoma because of the absence of *TP53* mutations. It is likely that *TP53* may, however, be inactivated in a different manner in this tumour (nuclear *MDM2* p53-inactivating protein [51, 52], or cytoplasmic localisation of the p53 protein, as seen in breast cancer [53]. Very recent results indicate that, in the absence of *MDM2* amplification, most undifferentiated neuroblastomas exhibit increased p53 cytoplasmic content, as assesed by immunohistochemistry. This suggests a defect in the transport of p53 from the cytoplasm to the nucleus [54].

Activation of the *BCL-2* oncogene in neuroblastoma in culture is mostly found in immature neuroblasts, while differentiated cells do not express the anti-apoptotic oncogene [55]. Whether this differentiation-dependent *BCL-2* expression operates in patients' tumours and metastases remains to be established.

The last key gene involved in the ability of neuroblastoma cells to undergo apoptosis is, of course, *MYCN*. In our IGR-N-91 cell model, *MYCN* overexpression associated with the chemoresistant phenotype suggests a possible role of this oncoprotein in the apoptosis of neuroblastoma.

In brief, the status of the *TP53*, *BCL-2* and *MYCN* genes needs to be thoroughly investigated in neuroblastoma cell lines derived from tumours and metastasis to evaluate how apoptosis is involved in the response of cancer cells to chemotherapy.

CONCLUSION

Our knowledge about the genetic alterations involved in the metastatic process of neuroblastoma is still limited. Major anomalies, including DNA content, *MYCN* amplification and 1p deletion(s), seem to occur prior to metastatic dissemination, and the expression of the so-called modulator genes seems to be more specifically involved in this process.

In the *MYCN* activated neuroblastoma IGR-N-91 experimental model, data strongly suggest that the oncogene drives neuroblastoma cells to metastatic dissemination as well as emergence of chemoresistance phenotype. This finding throws light on and supports previous observations reported in human lung carcinoma [56] and in metastatic metastatic rhabdomyosarcoma [57]: *C-JUN*, *C-FOS* and *C-MYC*, all oncogenic transcriptional nuclear factors, are activated in parallel with the *PGY1* gene and the potential for metastasis. A direct link between drug resistance and metastatic dissemination is thus currently sought [58].

Neuroblastoma cells appear to resist chemotherapy via two types of mechanisms, a general one causing cells to resist apoptosis and specific mechanisms of detoxification. A neuroblastoma cell's ability to undergo apoptosis appears to be dependent on the levels of expression of some key genes, particularly *MYCN*. Importantly, results obtained from *in vivo* metastatic models [30] suggest that the *MYCN* product activates not only angiogenesis and the dissemination process [31, 32], but also chemoresistance *via* chemoresistant specific genes, i.e., *PGY1* and *GST3*. As the *MYCN* product plays a crucial role in progression to aggressive neuroblastoma, we are currently testing the hypothesis that the expression of this oncoprotein and MAX associated partner [59] attains a certain threshold level which determines proliferation, apoptosis, and/or tumour progression and, perhaps also, resistance to treatment.

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