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PCNA levels in neuroblastoma are increased in tumors with an amplified N-*myc* gene and in metastatic stage tumors

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N-*myc* oncogene amplification in neuroblastoma has been found to be significantly associated with advanced stage disease and tumor progression. However, there is a lack of data on tumors, regarding the relationship between N-*myc* gene amplification and proliferation activity. Proliferating cell nuclear antigen (PCNA) is a proliferation-induced 36 kD nuclear protein that is the auxiliary component of DNA polymerase δ. PCNA levels in tissues have been found to correlate with proliferative activity. We have examined PCNA levels in neuroblastomas in relation to N-*myc* gene amplification and tumor stage. Statistically, significantly higher levels of PCNA were observed in tumors with an amplified N-*myc* gene relative to tumors with a single gene copy. The highest levels of PCNA were observed in advanced stage tumors with an amplified N-*myc* gene. Treatment of neuroblastoma cells in culture with retinoic acid, which induces differentiation, resulted in a substantial decrease in PCNA. Our results suggest that PCNA levels may reflect differences in proliferative activity between neuroblastomas, related to stage of the disease and to N-*myc* gene copy number.

Keywords: metastasis, neuroblastoma, oncogene

Introduction

A large number of molecular events and factors responsible for progression of cells during the cell cycle have been defined [1]. PCNA was originally described as a nuclear antigen to which some patients with systemic lupus erythematosis develop autoantibodies [2–4]. In contrast to resting cells that synthesize very little PCNA, transformed cells synthesize PCNA constitutively and in amounts proportional to the degree of transformation [5–7]. A polypeptide designated cyclin was independently found to be preferentially synthesized during the S phase and has been found to be identical to PCNA [5, 6, 8, 9]. In normal cells that have been induced to proliferate, for example following mitogen stimulation of resting peripheral blood lymphocytes, PCNA expression increases from the late G1 phase through the S phase of the cycle [5, 9].

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Exposure of exponentially growing cells to antisense oligodeoxynucleotides to PCNA resulted in complete suppression of DNA synthesis and mitosis, indicating an important role for PCNA in cell proliferation [10]. Expression of PCNA is elevated in a wide range of abnormally proliferating cells, including carcinomas of the breast, colon, lung, kidney, stomach, skin, ovaries and testes [11]. PCNA positivity has been found to correlate with mitotic activity and tumor grade [11]. In chronic myeloid leukemia, PCNA expression is elevated during blast crisis [12]. We have previously studied PCNA expression in childhood acute leukemia and have observed differences in PCNA levels between lineage-related subtypes that have a different prognosis [13].

Neuroblastoma is a solid tumor of childhood which has a variable outcome related to stage and amplification of the N-*myc* oncogene [14–22]. To date, few data are available regarding the relationship between N-*myc* gene amplification and *in vivo* proliferative activity of tumors. In this study, we have examined the variability of PCNA expression in neuroblastoma in relation to tumor stage and N-*myc* gene copy number. Our findings indicate that higher PCNA levels occur in tumors with an amplified N-*myc* gene relative to tumors with a single N-*myc* gene, and in metastatic relative to non-metastatic tumors.

Materials and methods

Tumors and cell lines

Tumors were obtained from 40 newly diagnosed pediatric patients at the time of surgery, prior to chemotherapy, and represented all clinical stages of the disease, from localized (stage I) to metastatic disease (stage IV). Informed consent was obtained in all cases. These tumors were analysed previously for the expression of two other proliferation-related proteins, namely Op18 and p19/nm23 [23, 24]. Of the 40 tumors studied for their content of PCNA, four belonged to the special group of infants with metastatic disease (stage IV–S), associated with a very good outcome, all of whom had a normal N-*myc* gene copy number. N-*myc* gene copy number was determined as described previously [14]. Fifteen neuroblastoma cell lines were also analysed for their PCNA content by two-dimensional (2-D) PAGE. Five cell lines had a non-amplified and 10 had an amplified N-*myc* gene (25–150 copies). All cell lines were derived from advanced stage tumors. The cell line KCNRP-9 was treated with retinoic acid as described previously [24–26].

2-D PAGE

Samples of approximately 200 μg of tumor tissue were cut from larger size pieces and solubilized by addition of (per liter) 8 M urea, 20 ml of Nonidet P-40 surfactant, 20 ml of ampholytes (pH 3.5–10), 20 ml of 2-mercaptoethanol, and 0.2 mM of phenylmethylsulfonyl fluoride in distilled deionized water. For neuroblastoma cell lines, 2×10^6 cells were similarly solubilized. Two-dimensional PAGE was performed as described previously [27]. In most cases 20–30 μl aliquots containing approximately 70 μg of protein were applied immediately onto isofocusing gels. First dimension gels contained 50 ml of ampholytes per liter (pH 3.5–9.0). Isofocusing was performed at 1200 V for 16 h and 1500 V for the last 2 h. Twenty gels were run simultaneously. For the second dimension separation, an acrylamide gradient of 11.4–14.0 g/dl was used. Protein spots in gels were visualized using the silver-staining technique [28].

Quantification of PCNA

Spot detection and quantitation were performed as described previously [29]. Each gel was scanned in a 1024×1024 pixel format, giving 160 μm as the pixel width. The digitized images were assigned coded numbers and were analysed for the presence and quantity of PCNA, without knowledge of the sample to which a gel image correspond. PCNA was identified, based on its characteristic migration and position in relation to neighboring landmark spots that were present in all gels included in this study and that were recognizable to the analyst. The PCNA spot, as well as 20 other reference spots, were quantified by placing a cursor on each spot, on the graphics monitor, and typing in the name for the spot. The reference spots correspond to cellular proteins, most of which were identified recently by our group on the basis of sequencing information (manuscript in preparation). The integrated intensity of each spot was measured in units of optical density $\times$ mm^2. Data on these spots were analysed using the Michigan Interactive Data Analysis System, a statistical software package. The 20 reference spots were used to adjust the PCNA spot-integrated intensity as described previously [29], to compensate for any variability in protein loading or staining between gels. For comparison of spot intensities between groups, ANOVAs, *t*-tests and calculation of correlation coefficients were used where appropriate.

Results

We previously identified PCNA in 2-D gels on the basis of its molecular weight, pI, and its characteristic location relative to other landmark proteins (Figure 1). The polypeptide spot that was presumed to be PCNA increased substantially following phytohemagglutinin stimulation of peripheral blood lymphocytes. Its identity was further confirmed by N-terminal sequencing which yielded the sequence K,F,E,A,R,L,V,Q,G,S,I,L,K that uniquely corresponded to PCNA [13]. In this study, the identity of PCNA in neuroblastoma-derived 2-D PAGE patterns was confirmed by Western blotting [30]. An antibody preparation to PCNA (19F4) reacted strongly and exclusively in 2-D gels of neuroblastoma tumors with the polypeptide presumed to be PCNA.

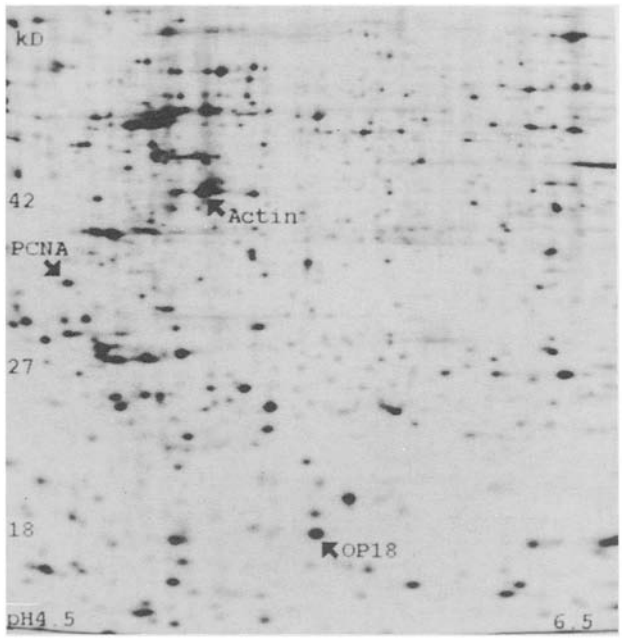

Figure 1. Two-dimensional polypeptide pattern of a neuroblastoma tumor. PCNA is indicated along with two reference proteins (actin and Op18).

Differential expression of PCNA in relation to tumor stage and N-myc gene copy number

Twenty-three neuroblastomas in which the N-*myc* gene occurred as a single copy were compared with 17 tumors in which the N-*myc* gene was amplified. A highly significant difference in PCNA levels was observed between tumors containing one N-*myc* gene copy and tumors with an amplified N-*myc* gene (Figure 2; Table 1; mean integrated intensities = 0.73 and 1.46, respectively, $P = 0.001$). This difference in mean integrated intensity corresponds to a three-fold difference in protein amount based on previous quantitative

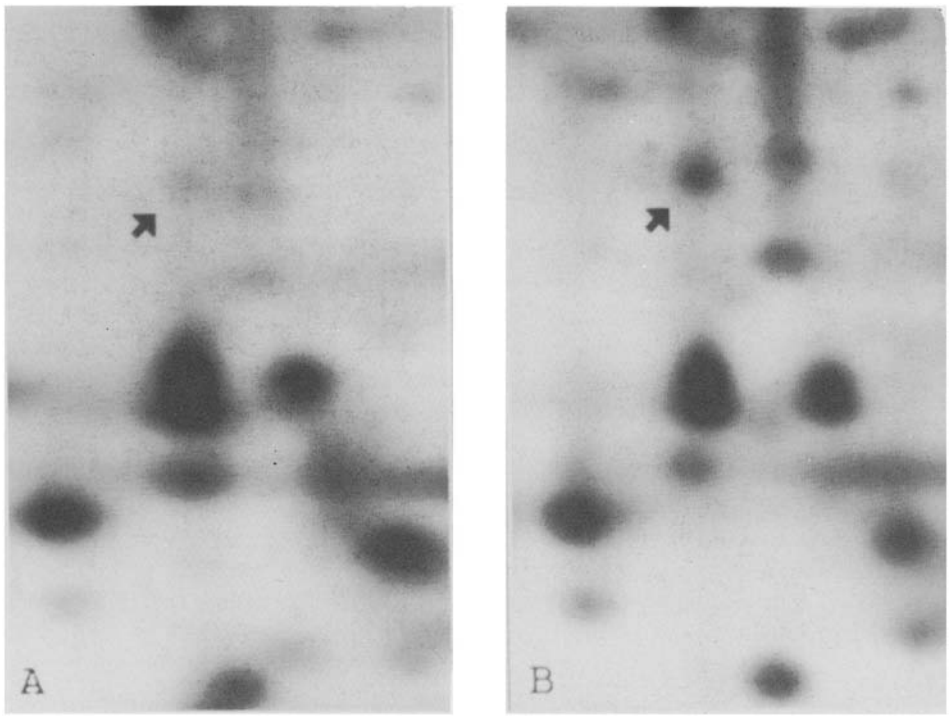

Figure 2. Enlarged 2-D PAGE pattern in the vicinity of PCNA in a neuroblastoma tumor tissue with one N-*myc* gene copy (A) and in tissue from a tumor with amplified N-*myc* (B). (Patients 7 and 28, respectively.)

Table 1. Patients with neuroblastomas as listed by N-*myc* gene copy number and stage. Relative PCNA values are provided for tumor tissue from each patient

Patient	Stage	N-*myc* copy number	PCNA
1	1	1	0.64
2	1	1	0.26
3	1	1	0.11
4	1	1	0.51
5	2	1	0.34
6	2	1	2.10
7	2	1	0.68
8	3	1	0.83
9	3	1	0.28
10	3	1	0.28
11	3	1	0.47
12	3	1	0.23
13	3	1	0.64
14	3	1	0.63
15	4	1	0.92
16	4	1	1.65
17	4	1	0.80
18	4	1	0.57
19	4	1	1.06
20	4S	1	0.46
21	4S	1	1.70
22	4S	1	0.90
23	4S	1	0.80
24	4	6–8	1.30
25	3	100	2.83
26	2	20	1.05
27	3	50	0.86
28	3	50	1.44
29	4	60	0.51
30	2	100	0.70
31	2	125	0.46
32	3	150	1.11
33	3	150	1.70
34	4	150	1.86
35	3	200	1.52
36	3	200	3.14
37	4	200	2.69
38	4	200	1.73
39	3	200	0.63
40	4	300	1.33

analysis of proteins using 2-D PAGE [29]. Most polypeptides observed in 2-D patterns of tumor tissue were also present in patterns of neuroblastoma cell lines. However, some polypeptides in the tissue patterns were absent from patterns of cultured neuroblastoma cells and were clearly derived from stromal tissue. They corresponded to major polypeptides observed in plasma and erythrocyte 2-D gel patterns. However, there was no significant relationship between the amount of PCNA and relative abundance of non-tumor cell proteins. Thus, the amount of PCNA observed in tumor tissue did not appear to vary in a manner related to variable amounts of stromal tissue.

Significant quantitative differences in PCNA levels were observed between tumors, based on stage, without categorization based on N-*myc* gene copy number. The means for stages I–IV were 0.38, 0.89, 1.11 and 1.31, respectively ($n = 4$, 6, 15 and 11; correlation coefficient, $r = 0.36$, $P = 0.01$; Spearman's rho = 0.42, $P = 0.012$). As these results are confounded by variation in N-*myc* gene copy number, variation in PCNA expression with stage was examined separately in the group of tumors with a single N-*myc* gene copy and in the group with an amplified N-*myc* gene. In the former, a significant difference in PCNA mean-integrated intensity was observed between non-metastatic (stages I, II and III) and metastatic tumors (stages IV and IV–S; mean intensities 0.57, $n = 14$, vs 0.98, $n = 9$, $P = 0.05$ by t-test). Interestingly in this group of N-*myc* = 1 there was no difference between stages IV and IV–S (respectively 1.00 and 0.97, $n = 5$ and 4). Among tumors with amplified N-*myc* gene, there was no difference in mean PCNA-integrated intensity between non-metastatic and metastatic stage tumors. For stage IV tumors, a higher mean level of PCNA was observed with N-*myc* gene amplification than with single gene copy tumors (mean = 1.00 for single gene copy and 1.57 for amplified gene copy). However, the difference did not reach statistical significance ($P = 0.15$).

PCNA levels in neuroblastoma cell lines

Fifteen neuroblastoma cell lines were analysed for their PCNA content. All neuroblastoma cell lines exhibited high levels of PCNA (Table 2). There was no significant difference between five cell lines derived from tumors with a single N-*myc* gene copy (mean = 2.54) and 10 cell lines derived from tumors with an amplified N-*myc* gene (mean = 2.78). It should be noted, however, that all cell lines were derived from advanced stage tumors.

Neuroblastoma cell lines can be induced to differentiate with retinoic acid (RA) [24–26]. The steady-state level of N-*myc* protein has been shown to decrease following RA treatment of cell lines in which the N-*myc* gene is amplified [25]. An increase in the number of receptors for the nerve growth factor (NGF) was reported to be associated with neuroblastoma differentiation

Table 2. Neuroblastoma cell lines with N-*myc* gene copy number and PCNA-integrated intensity values listed for each. All cell lines were from advanced study tumors. No difference in PCNA mean intensity was noted between non-amplified and amplified tumor cell lines

Cell line	N-*myc* copy number	PCNA-integrated intensity
SK-N-AS	1	3.76
SK-N-FI	1	3.59
SK-N-RA	1	2.08
LHN	1	2.39
LA-N-6	1	0.908
LAN-5	25	3.85
SMS-KANR	25	1.95
LA-N-1	50	3.97
KCNR	75	1.04
SK-N-BE1	100	2.44
CHP-134	100	1.69
MSN	100	3.99
SK-N-BE2	150	2.64
SMS-SAN	150	3.35
SMS-KAN	150	2.84

following RA treatment [31]. We therefore examined the effect of RA treatment on PCNA expression using the neuroblastoma cell line KCNRP-9 which contains 75 copies of the N-*myc* gene. There was a slight drop in PCNA mean integrated intensity by day 2 relative to control cells treated with ethanol alone (mean intensity 1.74 vs 2.28, $P = 0.10$). By day 10 the difference between retinoic-treated and control cells (Figure 3) was significant (0.86 and 2.29, $P < 0.001$).

Discussion

Neuroblastoma is a common childhood tumor which originates in neural crest derived cells. Children with the tumor present with local, regional or metastatic disease. In studies of neuroblastoma tumors, amplification of the N-*myc* oncogene has been found to be significantly associated with advanced stage disease and rapid tumor progression [14–18, 22]. In this study, we have examined the expression of PCNA in untreated neuroblastoma tumors, in relation to N-*myc* gene copy number, to determine the relationship between N-*myc* amplification and tumor proliferative activity. Our findings indicate that expression of PCNA in neuroblastoma is elevated in tumors with an amplified N-*myc* gene, and that in the group with a single copy N-*myc* gene, tumors that belong to the metastatic stages IV and IV–S exhibited higher levels than non-metastatic stage tumors. PCNA levels ranged from a mean of 0.38 for non-amplified N-*myc* gene stage I tumors, to 1.57 for stage IV tumors with an amplified N-*myc* gene. The mean level of PCNA in non-amplified stage I tumors approximates the level we have observed previously in normal resting lymphocytes [13], suggestive of low proliferative activity in these tumors. The mean PCNA level of 1.57 for amplified N-*myc* gene stage IV tumors approximates the mean observed for pre-B acute leukemia [13]. Established neuroblastoma cell lines, which are derived from advanced stage tumors and are believed to undergo selection as a result of culture conditions [14], display a markedly elevated PCNA level of 2.70 (S.D. 1.03), which approaches the

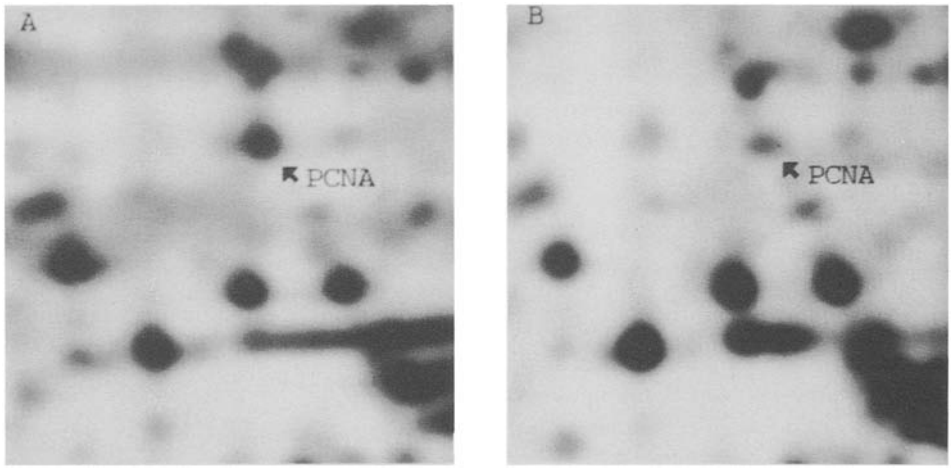

Figure 3. Two-dimensional PAGE peptide patterns for the KCNRP-9 neuroblastoma cell line. (A) PCNA for cells treated with ethanol alone as a control for 10 days; (B) PCNA at a reduced level for cells treated with retinoic acid for 10 days. Relative PCNA spot intensity values for (A) and (B) are, respectively, 2.37 and 0.81.

level observed in T-ALL, a subtype usually associated with high proliferative activity and a high tumor load. While direct comparisons between PCNA levels in lymphoid cells and in neuroblastoma are of limited use, the wide range of PCNA expression in neuroblastoma, approximating the range observed between resting lymphocytes and T-ALL, suggests a wide range of proliferative activity in different tumors.

To date, there have been limited studies of proliferation kinetics in neuroblastoma [32, 33]. Mean generation times for undifferentiated tumors range from less than 13 h to 260 h, with potential tumor doubling time ranging from 20 to 415 h. [^{3}H]Thymidine labeling indexes have been reported to vary between 3 and 28%, and growth fractions between 0.48 and 0.58. In neuroblastoma, the duration of the cell cycle has been found to vary by a factor of up to 20, from 0.5 to 11 days. The duration of DNA synthesis, the G2 phase and mitosis were rather constant in a particular case, whereas the G1 phase is a variable parameter [32, 34]. In these studies the relationship between proliferation kinetics and N-*myc* gene copy number was not determined. In another study, tumors with an amplified N-*myc* gene were found to have a higher percentage of cells in S phase, though the relationship was not statistically significant and no definitive conclusions could be made ($P = 0.14$) [35]. The PCNA data we have obtained, with statistically highly significant differences between tumors with a single N-*myc* gene copy and tumors with an amplified N-*myc* oncogene, suggest differences in proliferative activity in neuroblastoma related to the status of the N-*myc* gene.

Cell cycle differences between neuroblastoma tumors could be related to differences in rate limiting factors that control progression of tumor cells through the cell cycle. Such differences have been implicated for certain tumors [34]. Control of the rate of proliferation of tumors may occur at the G1/S interface, a phase during which PCNA synthesis peaks. Therefore, among neuroblastoma tumors, those exhibiting relatively high PCNA level may contain a greater number of cells that can progress through the cell cycle. It is possible that increased N-*myc* protein level resulting from N-*myc* gene amplification or from prolonged N-*myc* mRNA or protein half-life [35, 36] may either increase expression of the PCNA gene directly or indirectly through intermediate factors that in turn regulate the expression of the PCNA gene. Retinoic acid or phorbol ester-induced maturation of neuroblastoma cells in culture substantially decreases N-*myc* mRNA [37] and proliferative activity. The decrease in PCNA levels which we have observed with RA treatment of KCNR neuroblastoma cells further suggests a link between N-*myc*, PCNA protein levels and proliferation.

It is of interest that among tumors with a single N-*myc* gene copy, tumors that belong to stage IV and IV–S display equivalent PCNA levels. While these tumors have a markedly different outcome, they share a high tumor load. It is likely therefore that PCNA levels directly reflect tumor proliferative activity which in certain cases as in IV–S tumors may not be related to prognosis. We have previously reported that two other cellular polypeptides, Op18 and p19/nm23, that are induced in proliferating cells, displayed differences in their levels between advanced stages III and IV and stages with a good prognosis including stage IV–S [23, 24]. Several other polypeptides observed in 2-D gels which have yet to be identified have exhibited variable expression in neuroblastoma. Sequencing studies of these polypeptides are currently being undertaken. Detailed analysis of the extent of differential expression between neuroblastoma tumors of polypeptides that play a role in cell proliferation is likely to improve our understanding of the molecular basis of tumor heterogeneity that may be responsible for a varied outcome in neuroblastoma.

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