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Poly(A)polymerase Activity Levels in Breast Tumour Cytosols

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The enzyme poly(A) polymerase (PAP) catalyses the polyadenylation of mRNA and its activity levels vary within the cell cycle. The levels of activity of this enzyme were measured in the cytosol of breast tumours from 62 untreated patients and compared to clinical prognostic parameters as well as other biological markers. The enzyme levels measured ranged from 3 to 46 units/mg protein. A statistically significant association was observed between high PAP activity values and the TNM stage of the disease as well as node invasiveness. Furthermore, there was a positive correlation between PAP activity values and c-*erb*B-2 overexpression but not with its amplification. No significant correlation was observed with c-*myc* amplification or overexpression and cathepsin D levels. A direct relationship between steroid receptor content and PAP activity levels, which was more prominent in the case of the progesterone receptor, was observed. However, also on the basis of previous data PAP activity may prove to be indicative of aggressive disease. Furthermore, measurements of PAP activity may contribute to the definition of the biological profile of tumour cells.

Key Words: Poly(A)polymerase, C-erbB-2, C-myc, Breast cancer, Cathepsin D

Breast cancer is the most common malignant tumour among women and is responsible for an estimated 24% of all cancers and 18% of all cancer deaths (1). Ultimately, about one out of 13-14 women will develop breast cancer during their life time and at least half of these patients will die as a consequence of metastatic disease. One third of the node negative breast cancer patients are expected to have a distant recurrence within 10 years since occult disseminated disease at the time of diagnosis was difficult to detect. Furthermore, course of the disease and response to treatment varies greatly (1).

In view of the heterogeneity of breast carcinomas the need for parameters which define the biological profile of the tumour and, ultimately, disease progression is obvious. Classical prognostic factors in primary breast carcinoma are TNM status (tumour size, nodal status, distant metastases) and age. The histopathology and nuclear grading are standard variables (2). The presence of steroid receptors, apart from being markers of hormone responsiveness, are also indicators of a less aggressive behaviour(3). A number of cell biological parameters, such as oncogenes, growth factors and secretory proteins have been introduced and are considered as prognostic factors as well, because they strongly influence and characterise the behaviour of the tumour with respect to metastatic pattern, differentiation extent and growth rate. The expression of the protease cathepsin D has been considered as an adverse prognostic parameter (4) promoting invasive growth and metastasis of tumour cells (5). Ploidy (DNA content) (6, 7) and proliferative capacity (S-phase fraction) (8) are the best characterised prognostic factors, second only to hormone status (9). These measurements have been shown to predict disease-free survival and overall survival in node-negative and node-positive breast cancer.

As a whole, it proves necessary to identify as many cellular parameters as possible which will help to define the biological profile of the breast tumour cell. The levels of activity of the enzyme poly(A)polymerase (PAP) whose biological function is the polyadenylation of mRNAs vary greatly among tissues, normal and neoplastic, and within the cell cycle (10-12). The poly(A) tail present in most eukaryotic mRNAs has been involved in the initiation of translation as well as in the stability of mRNA. Such factors render the poly(A) synthesising factors important in gene expression. Poly(A) polymerase activity has been reported to reflect the proliferation status and the differentiation

status of the cell (11, 13). Most studies report that rapidly proliferating and actively metabolising lymphocytes have higher levels of poly(A)polymerase activity (14). In the case of chronic lymphocytic leukemias it has been suggested to be of prognostic value (15).

The possibility that varying levels of poly(A)polymerase activity could be detected in the cytosols of breast tumour samples and that these levels may be correlated to clinical and biological attributes of these tumours was tested. The values obtained were compared to clinical prognostic parameters such as tumour grade, steroid receptor status and node invasiveness as well as to the amplification and overexpression of the oncogenes c-*erb*B 2 and c-*myc* and the expression of cathepsin D. Our results indicate that poly(A) polymerase activity levels may define a subgroup of breast tumours and potentially be of prognostic value.

Materials and Methods

Tumour material. The study included 52 primary and 10 metastatic breast carcinomas collected at "St. Savvas" Hospital, Athens. None of the patients analysed had received hormonal therapy prior to surgery. All surgical biopsies were snap frozen within 2 hrs after surgery and stored at -80°C until processing.

Tumour processing. Frozen biopsies (1 g) were ground to a fine powder in liquid nitrogen and subsequently homogenized in 5 ml cytosol buffer (10 mM Tris, 1.5mM EDTA, 5mM NaMolybdate pH7.4, 5mM DTT) using a polytron (Kinematica RCV). The homogenates were centrifuged at 40,000 rpm for 1 hr at 4°C and the cytosols were kept at -80°C.

Poly(A) polymerase activity assay. The assay measures the incorporation of (5'-8-3H) ATP into acid insoluble material using poly(A) as initiator as described earlier (12). The standard assay mixture (100 µl) contains 200 mM Tris HCl (pH 8.3), 1mM $MnCl_2$, 1mM [^{3}H]ATP(20-30 cpm/pmol), 4mM 2-mercaptoethanol, 1mM (3'-OH) poly(A) and 20µl of cell extract, diluted to a concentration of 1 mg/ml. After incubation at 37°C, 20µl aliquots were spotted at at least three time intervals on GF/C discs and were processed and counted as described before (12). One unit of enzyme activity is defined as 1nmol of radioactive radionucleotide incorporated per hr. Specific activity is expressed as units of activity per mg of protein.

Nucleic acids isolation. DNA was isolated from 100mg of tumour tissue, which was finely minced using a pair of scalpels and dispersed in 1 ml of 2xTNE (20 mM Tris pH 8.0, 300 mM NaCl, 20 mM EDTA) containing 0.5% SDS and digested with proteinase K (100 µg/ml) at 37°C. After repeated phenol chloroform extraction intact DNA was pooled after ethanol precipitation. Equal amounts of DNA (10µg) were spotted on nylon membranes.

RNA was isolated from frozen samples, ground to a fine powder in liquid nitrogen and subsequently homogenized in acid guanidine-thiocyanate-phenol-chloroform solution according to Chomczynski et al.(16). RNA was slot blotted according to Thomas (17).

Detection of oncogenes. To determine c-*myc* overexpression or amplification, blots were hybridised overnight at room temperature to randomly primed (18), [α^{-32}P] dATP labelled c-*myc* probe (1,3 kb fragment Cla-I EcoRI from pHSR-1 plasmid containing a Hind III -EcoRI fragment from human genomic c-*myc* sequences (Exon 3) from Colo 320 cells (ATCC).

To determine c-*erb*B-2 overexpression or amplification blots were hybridised to c-*erb*B-2 oligonucleotide Pr 2 (Oncogene Sci., ON112) which was 5' end labelled . Hybridisation conditions were those suggested by the manufacturer.

Autoradiograms were scanned with a Helena Laboratories Cliniscan densitometer. The values obtained for c-*myc* and c-*erb*B-2 were normalised to values obtained for actin. The ratios obtained were compared to average values obtained for normal samples to determine amplification and overexpression.

Hormone receptors. Estrogen (ER) and progesterone receptors (PgR) were assayed by the charcoal method as described before(19). Results were expressed as specific binding sites per mg of cytosolic protein (fmol/mg protein). Tumours with ER and PgR concentrations below or equal to 10 fmol/mg protein were considered as receptor negative.

Cathepsin D assay. Cathepsin D was assayed by an immunoradiometric kit (Elisa Cath-D kit, CIS Bio International, Gif-Sur Yvette, France), according to the manufacturer in 1/40 and 1/80 dilution of the reconstituted cytosols both in duplicate. Tumours with cathepsin D concentrations of equal or below 80 fmol/mg protein were considered negative.

Statistical methods. Statistical differences within the population were determined by the Mann Whitney non-parametric tests for quantitative parameters. The χ^2 test with Yates correction when applicable was used for qualitative parameters. Linear regression was calculated by Pearson's least square method. Pearson's correlation coefficients were analysed by Student's *t* test.

Results

In 62 breast cancers the cytosolic poly(A)polymerase activity values were normally distributed and ranged from 3 to 46 units /mg protein. Axillary lymph nodes were invaded (N+) in 28 patients while 30 patients were node negative (N-) and the node status was not determined in 4 patients. The majority of the tumours were characterised as Grade II (43 out of 62). The c-*myc* and c-*erb*-B-2 amplification and overexpression were determined and steroid receptor content as well as cathepsin D levels were measured.

Relationship of poly(A)polymerase and other prognostic parameters. The distribution of poly(A)polymerase activity values within the three tumour grade groups is shown in Fig. 1 and Table I. The mean values obtained were 7.0, 9.7 and 12.4 units/mg for Grade I, II and III, respectively. If the value of 6 units/mg of cytosol protein-an average value obtained from 10 normal breast tissue specimens- was to be a cut-off point for defining normal and high levels of activity the distribution obtained would reveal that 33.3% of Grade I, 48.9% of Grade II and 77.8%of Grade III tumour samples had high PAP activity values (Fig. 1). When applying one-way analysis of variance, a statistically significant difference (P=0.05) was found between the mean PAP specific activity values in Grades I, II and III. Furthermore, a significant difference in PAP activity of tumour samples from node positive and node negative patients was observed as shown in Table I. As shown in Fig. 2 the majority of PAP values in node negative patients were clustered within the normal range and the mean value obtained was 7.2 units/mg. On the other hand, significantly higher values were obtained from tumour samples of node positive patients which exhibited a mean value of 12.8 units/mg. The

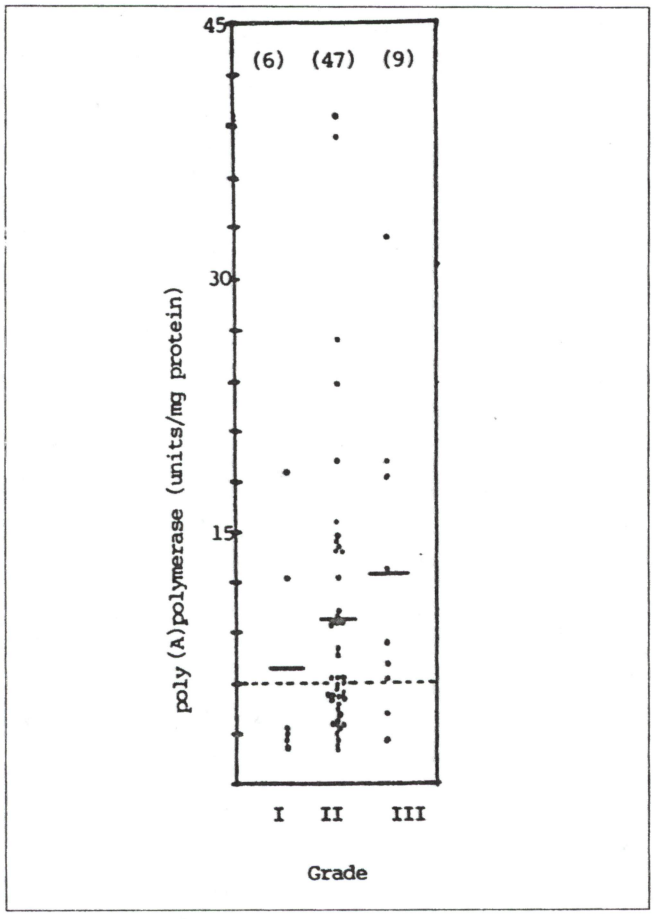

Fig. 1 - Distribution of PAP activity levels and tumour grade. The PAP activity levels were assayed in the cytosols of 58 tumours and plotted against pathological grade. (P=0.05 between the mean PAP specific activity values in Grades I, II and III.).

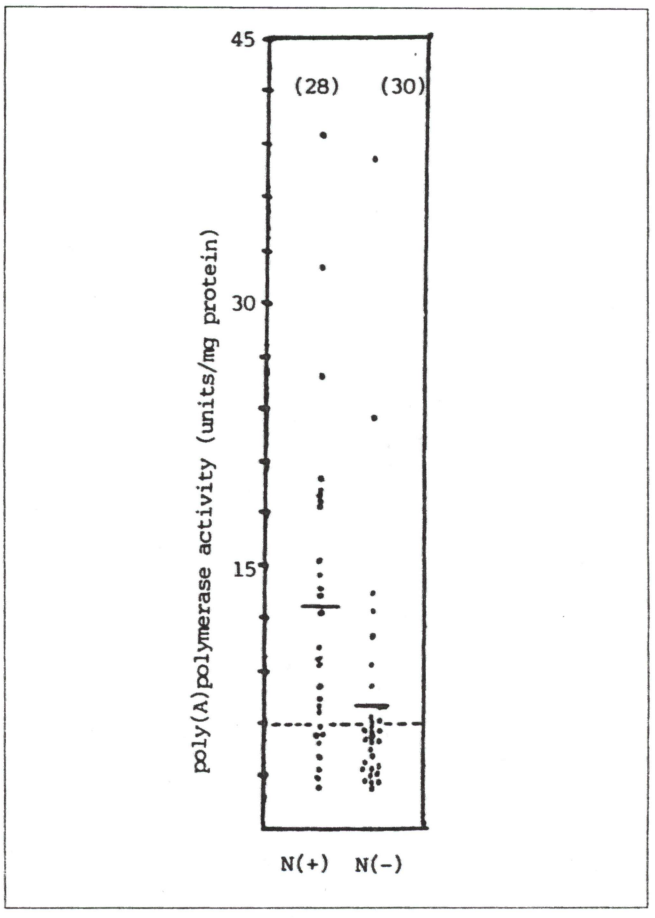

Fig. 2 - Relationship between PAP activity levels and node invasiveness. The PAP activity was assayed in the cytosols of 58 tumours and plotted according to the auxiliary lymph node status. (N+) invaded, (N-) non -invaded (P=0.002 Mann-Whitney non-parametric test).

TABLE I - Distribution of patients according to PAP activity levels (high and low) and other parameters

		No. of patients with high PAP activity levels (>6 units /mg)		No. of patients with low PAP activity levels (< 6 units/mg)		P value
		n	%	n	%	
Grade	I	2	(33.3)	4	(66.7)	
	II	23	(48.9)	24	(51.1)	N.S.[b]
	III	7	(77.8)	2	(22.2)	
Node invasiveness	+	21	(75.0)	7	(25.0)	
	-	10	(33.3)	20	(66.7)	0.0027[a]
	x	1	(25.0)	3	(75.0)	
Tumour status	T_1	6	(37.5)	10	(62.5)	
	T_2	22	(62.9)	13	(37.1)	N.S.[a]
	T_3	4	(36.4)	7	(63.6)	
TNM Stage	I	3	(23.1)	10	(76.9)	
	II	14	(45.2)	17	(54.8)	0.0069[b]
	III	12	(85.7)	2	(14.3)	
	IV	3	(75.0)	1	(25.0)	
c-*erb*B-2 overexpression	+	14	(77.8)	4	(22.2)	
	-	18	(40.9)	26	(59.1)	0.018[a]
c-*erb*B-2 amplification	+	8	(61.5)	5	(38.5)	
	-	24	(49.0)	25	(51.0)	N.S.[a]
c-*myc* overexpression	+	12	(63.2)	7	(36.8)	
	-	20	(46.5)	23	(53.5)	N.S.[a]
c-*myc* amplification	+	11	(64.7)	6	(35.3)	
	-	21	(46.7)	24	(53.3)	N.S.[a]
Cathepsin D	+	18	(64.3)	10	(35.7)	
	-	14	(41.2)	20	(58.8)	N.S.[a]
Estrogen Receptor	+	20	(50.0)	20	(50.0)	
	-	12	(54.5)	10	(45.5)	N.S.[a]
Progesterone Receptor	+	24	(52.5)	22	(47.5)	
	-	8	(50.0)	8	(50.0)	N.S.[a]

x status not determined

a Chi square test with Yates correction

b Chi square test

N.S. not significant

difference in the mean was found at a level of significance of P=0.002 using the Man-Whitney non-parametric test. Tumour size did not appear to have any relationship with PAP activity values. Nevertheless, significant differences were obtained in the levels of PAP activity within TNM stage groups (Table I). The percentage of the patients exhibiting high PAP activity values was 23.1, 45.2, 85.7 and 75.0 for stages I, II, III and IV respectively (P=0.0069).

The association between high PAP activity levels and node invasiveness led us to compare PAP levels with cathepsin D levels which is considered as a marker for relapse and metastasis. No correlation could be identified (Table I).

Comparison of PAP activity levels and steroid receptor status. The above data may suggest that high PAP activity levels may be associated with factors of adverse prognosis. However, when considering steroid receptor status a favourable prognostic parameter when present (1), high and low PAP activity values were evenly distributed among estrogen and progesterone receptor positive and negative tumour groups (Table I). On the other hand, the data, when plotted, revealed that at low and moderate PAP values there was no strong correlation with receptor concentration i.e. high PAP activity values were associated with high estrogen receptor content (Fig. 3) and an even stronger association was observed between high poly(A) polymerase activity values and high progesterone receptor content (Fig 4, r_p=0.512, P<0.001).

Poly(A)polymerase and oncogene amplification and/or overexpression. Comparison of poly(A)polymerase values and oncogene expression and amplification did not yield any significant association. Fig. 5 and Fig. 6 illustrate the distribution of PAP activity values from tumours which had the oncogenes *c-erbB*-2 and *c-myc* either overexpressed or amplified. The mean poly(A)polymerase activity value was higher in tumours overexpressing c-erbB-2 mRNA compared to those which did not (14.3 vs. 7.6 units /mg protein, P=0.01). A weak association was also detected between *c-myc* mRNA overexpression and PAP activity.

Discussion

Poly(A)metabolising enzymes mediate adenylation of eukaryotic heterogeneous RNA and mRNA and thus participate in the regulation of gene expression (10, 15) The fact that poly(A)polymerase has been reported to have some prognostic attributes, at least in chronic lymphocytic leukaemia (15), led us to determine poly(A)polymerase activity levels in the cytosol of breast cancer tumours which is the same used for steroid receptor content measurements and correlate the values obtained with other prognostic parameters

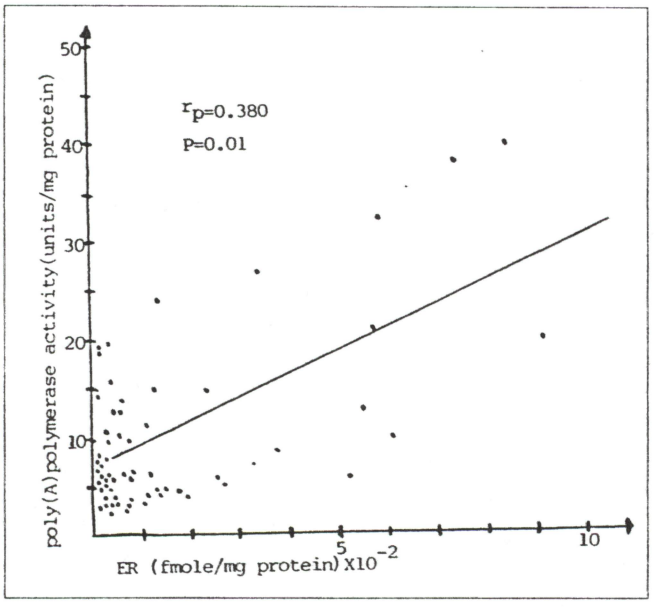

Fig. 3 - Correlation between PAP activity levels and estrogen receptor (ER) content in the cytosols of breast tumours. The correlation rate was significant (P<0.01) according to Student's T test.

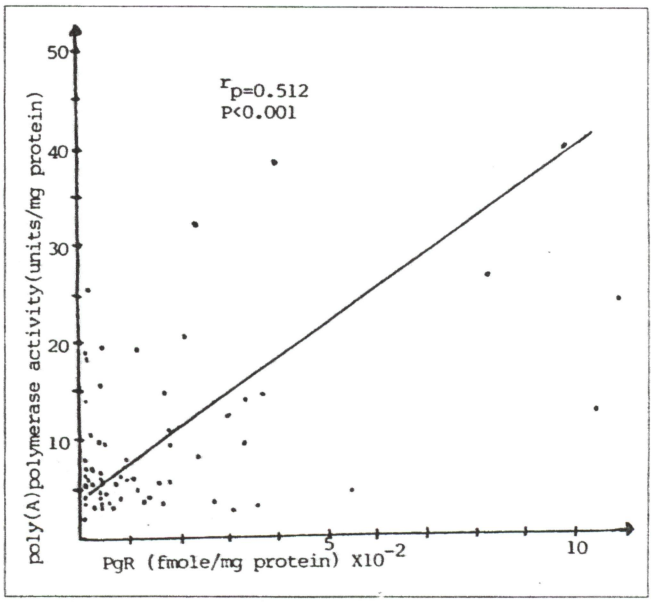

Fig. 4 - Correlation between PAP activity levels and progesterone receptor (PgR) content in the cytosols of breast tumours. The correlation rate was highly significant (P<0.001) according to Student's T test.

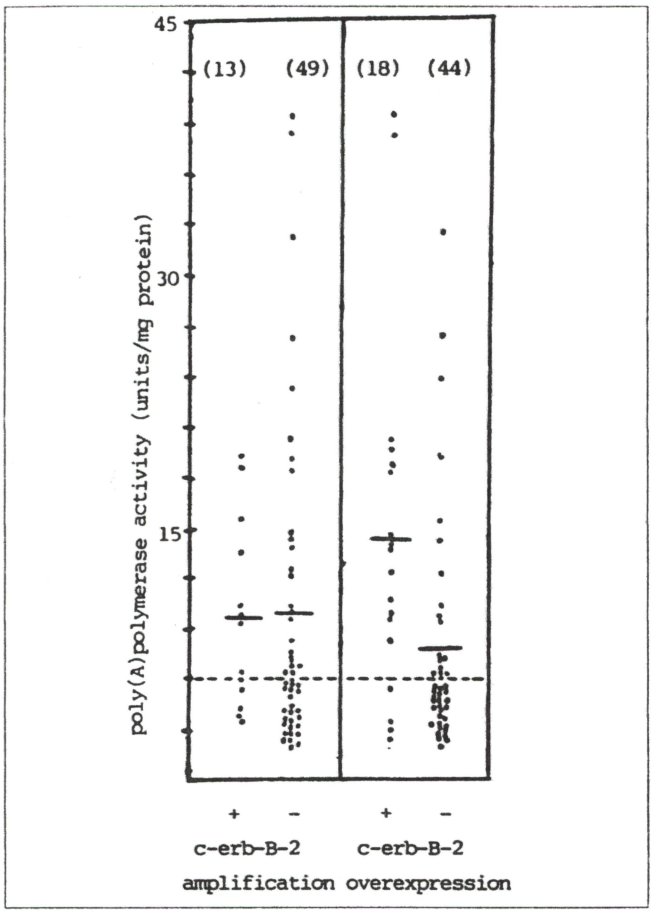

Fig. 5 - Relationship between PAP activity levels and the presence (+) or absence (-) of *c-erbB*-2 amplification or overexpression. The difference in the mean PAP activity values between the groups overexpressing or not *c-erbB*-2 was found statistically significant (P=0.01, Mann-Whitney non-parametric test).

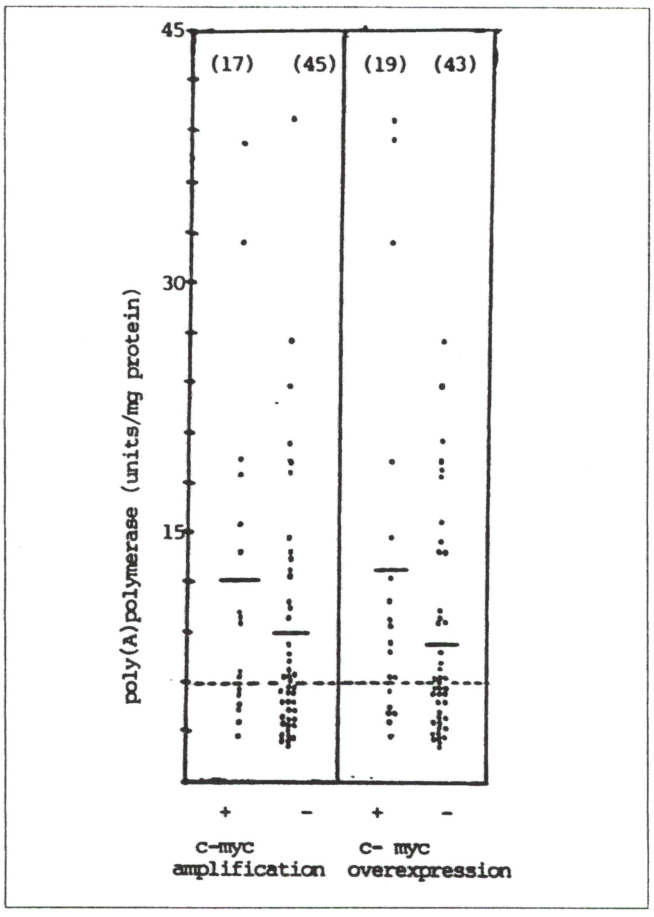

Fig. 6 - Relationship between PAP activity levels and the presence (+) or absence (-) of *c-myc* amplification or overexpression. No statistically significant difference of the mean PAP values in the presence or absence of *c-myc* overexpression or amplification was found. (Mann-Whitney non-parametric test).

used in breast cancer. The overall pattern of distribution of poly(A)polymerase activity among the clinically defined groups indicated that the activity of this enzyme was elevated in the groups with adverse prognosis. The values obtained increased along with tumour grade, were significantly higher in node positive patients and in advanced TNM stages.

Much attention has been paid to cellular oncogenes whose activation has been proven to be at least partly responsible for malignant transformation. The estimation of copy number and, more important, the expression status of oncogenes has been suggested to be of valuable diagnostic relevance (20, 21). We compared PAP activity values with the above parameters. Our data indicate an association of PAP activity with *c-erbB-2* overexpression, whereas no correlation with amplification was observed. Apart from the observation that *c-erbB2* overexpression is indicative of adverse prognosis (22, 23) several studies have been concerned with a possible correlation between *c-erbB-2* amplification and/or overexpression and tumour cell kinetics (24-27). In primary tumours there is an association between rapid cell proliferation and phenotypic *c-erbB-2* alterations but not genotypic (28). Furthermore, higher values of PAP activity were also associated with *c-myc* mRNA overexpression which is related to transcriptional activation and with cell proliferation (21). Lectin stimulation of lymphocytes results in an increase of the activity levels of PAP (29). The rapidly proliferating lymphoblasts of patients with acute leukaemia have several-fold higher PAP activity levels compared to the lymphocytes of patients with chronic leukaemia and to those of healthy donors (13). Furthermore, cell cultures have higher PAP activity levels under conditions of

growth as compared to stationary phase cultures (30). PAP activity levels are expected to reflect cellular transcriptional activity and may or may not be related to cellular proliferation. It has been demonstrated that steroid receptor content and proliferative activity have a reverse relationship in breast tumours (31) and that their presence is indicative of favourable prognosis. There was no overall significant difference between high and low PAP activity groups and hormone receptor status. This finding does not contradict the possibility that PAP levels reflect aggressive disease since when compared with TNM and histological grading, ER and PgR status have a low prognostic value, their major interest remaining solely in the domain of therapeutic decision (32).

On the other hand, there was a direct relationship between high steroid receptor content, particularly that of progesterone receptor and PAP activity levels. This is in agreement with findings reporting that patients with the highest estrogen receptor content have as poor prognosis as ER negative patients(33). Thus, the level of PAP expression may or may not be a parameter related to proliferation activity, reflecting transcriptional activation of the cell and it may prove to be an independent indicator of aggressive disease.

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