DNA proliferative index as a marker in Iraqi aneuploid mammary carcinoma

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مَنْسَب الدنا التكاثري كمؤشّر على اختلال الصيغة الصبغية لسرطانات الثدي في العراق

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خلاصة: في هذه الدراسة تم تقدير محتويات الصيغة الصبغية الدناوية للنويات ومَنْاسِب الدنا التكاثرية في سرطانات القنوات اللبنية لدى 120 مريضة عراقية. ومن بين العينات المفحوصة وُجد اختلال الصيغة الصبغية في 82.7%. وقد ترابطت الصيغة الصبغية الدناوية على نحو يُعتد به إحصائياً مع الدرجة النسيجية للورم الأوّلي ومحتواه من مستقبلات الاستروجين. وفي السرطانات المصحوبة باختلال الصيغة الصبغية، فإن مَنْسَب الدنا التكاثري أبدى ترابطاً أكثر وضوحاً من اختلال الصيغة الصبغية مع حالة الإياس ومحتوى مستقبلات البروجسترون في الورم. وكان مَنْسَب الدنا التكاثري والنسبة المئوية لاختلال الصيغة الصبغية أكبر في الأورام الكبيرة، كما أن حالة العقد لم تبد أي ارتباط مع نتائج تعداد الكريات الخلوية. ومع استعمال مَنْسَب الدنا التكاثري فإن المريضات المصنفات بأنهن مصابات بسرطانات آوير المصحوبة باختلال الصيغة الصبغية يمكن أن يقسَّمن إلى مجموعات ثانوية تختلف في مواصفات الأورام. الأمر الذي يساعد على تحسين مستوى اختيار المريضات المعرَّضات لخطر مرتفع واللاتي لم ينتشر المرض إلى عقدهن اللمفية، ومن ثَمَّ يصبحن مؤهلات لتناول العلاج المجموعي المساعد.

ABSTRACT This study estimated nuclear DNA ploidy and DNA proliferative indices (PI) in mammary ductal carcinomas from 120 Iraqi female patients. Of the examined specimens, 82.7% were aneuploid. DNA ploidy correlated significantly with histological grade and estrogen receptor content of the primary neoplasm. In aneuploid carcinomas, high PI showed a clearer association than aneuploidy with menopausal status and progesterone receptor content of the tumour. PI and percentage aneuploidy were higher in larger tumours; nodal status showed no association with these cytometric findings. Using PI, patients classified as having Auer aneuploid carcinomas can be divided into subsets with different tumour characteristics, thus improving the selection of those whose high risk, node-negative presentation makes them candidates for adjuvant systemic therapy.

L'index prolifératif de l'ADN comme marqueur du carcinome mammaire aneuploïde en Iraq
RESUME Cette étude visait à estimer l'index de ploïdie de l'ADN nucléaire et l'index prolifératif de l'ADN (PI) dans les épithéliums des canaux galactophores chez 120 patientes iraquiennes. Sur les échantillons examinés, 82,7% étaient aneuploïdes. La ploïdie de l'ADN avait une corrélation significative avec le degré histologique et le niveau des récepteurs des œstrogènes du néoplasme primaire. Dans les carcinomes aneuploïdes, le PI élevé montrait une association plus claire que l'aneuploïdie avec l'état ménopausique et le niveau des récepteurs de la progestérone de la tumeur. Le PI et le pourcentage d'aneuploïdie étaient plus élevés dans les grandes tumeurs ; l'état ganglionnaire ne montrait aucune association avec ces résultats cytométriques. En utilisant le PI, les patients classés comme ayant des carcinomes aneuploïdes selon la classification d'Auer peuvent être subdivisés en sous-ensembles avec différentes caractéristiques de tumeurs, améliorant ainsi la sélection des patients qui ont un risque élevé; l'absence d'envahissement ganglionnaire en fait des candidats pour une thérapie systémique adjuvante.

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Introduction

It is well established that complex forms of DNA damage are involved in tumour development and progression. Hence, quantitative measurement of nuclear DNA ploidy has acquired an important role in the assessment of the proliferative potential of various human neoplasms [*1,2*]. The first retrospective study of the value of DNA ploidy in the progression of mammary carcinoma was presented by Auer et al. [*3*], who classified breast tumours into four major groups according to the DNA distribution pattern given by image cytometry (Figure 1).

As a rule, patients with genetically stable tumours, i.e. where tumour cell nuclei have a diploid or tetraploid profile (types I and II histograms), have a better prognosis and a more favourable clinical course than those presenting with genetically unstable tumours (aneuploid histograms types III and IV) [*4*]. The image cytometry findings were later confirmed by flow cytometry, together yielding information about ploidy and proliferative activity [*5–7*]. Auer histograms types I and II are termed *euploid,* corresponding to a low rate of proliferative activity (i.e. low fraction of cycling cells within the $S+G_2/M$ regions of the histogram), while types III and IV Auer histograms are designated *aneuploid* with high proliferative activity [*8,9*].

Cytometrically, one of the systems most frequently used for presenting data on cell

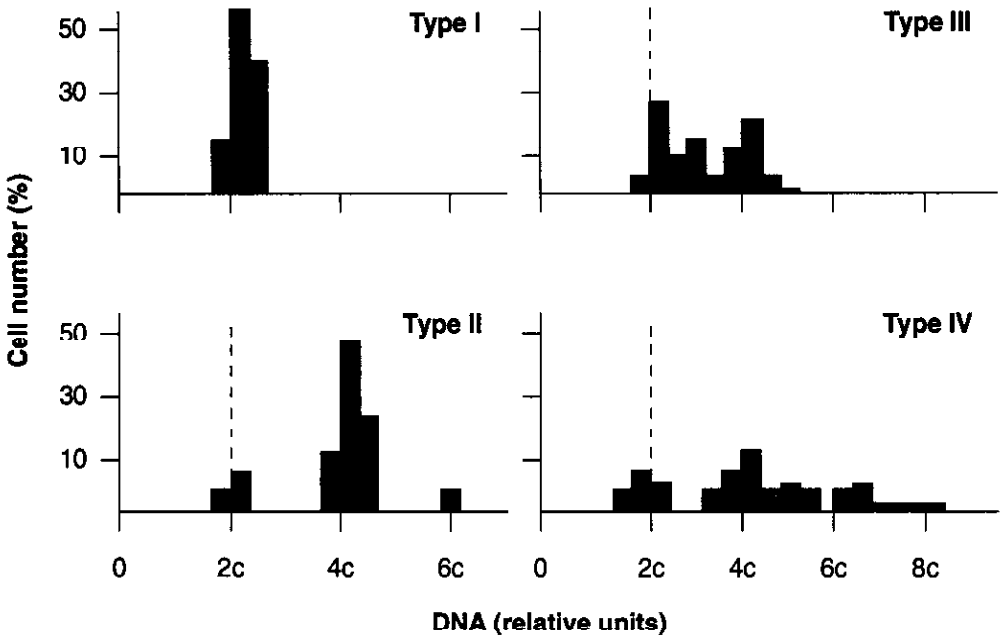

Figure 1 **Classification of DNA histogram types in mammary carcinoma according to Auer et al.**

proliferation is measurement of the S-phase fraction (SPF) and the proliferative index (PI). The latter records DNA values corresponding to the $S+G_2/M$ regions as determined by image cytometry. In accordance with the convention on nomenclature for DNA cytometry, non-diploid samples with SPF ≥ 12% and diploid samples with SPF ≥ 7% are classified as having high SPF, while samples with values below these levels are considered to have low SPF [*10*]. Similarly, for image cytometry it has recently been shown that a cut-off level of 12% for the $S+G_2/M$ fraction gave the best prognostic information [*8*]. However, the literature on the correlation of DNA ploidy content and PI of the tumour with other clinicopathological variables known to have prognostic significance is still contradictory.

This pioneer Iraqi study, which was initiated at the Karolinska Hospital in Stockholm, was designed to:

- Estimate the nuclear DNA ploidy content of mammary carcinomas from Iraqi patients.
- Explore the relationship of nuclear aneuploidy and of high PI with the various clinicopathological parameters recorded in the study group.
- Investigate whether PI showed a more significant association with established prognostic factors in Auer III and IV aneuploid mammary carcinomas.

Patients and methods

Patients

The material for this prospective study was collected over a period of 18 months (January 1996 to July 1997) from the Outpatient Surgical and Oncological Departments of the Medical City Teaching Hospital in Baghdad. During that period, 120 female patients under 80 years of age presented with unilateral mammary ductal carcinoma and were considered eligible for the study (the diagnosis was obtained by fine-needle aspiration cytology and confirmed histopathologically). Recorded variables of clinical relevance included the patient's menopausal status at the time of diagnosis, while data on tumour size (largest diameter) and nodal status were obtained by examination of the tissue biopsies. Malignant tumours were graded following the recommendations of Scarff, Bloom and Richardson [*11*].

Fine-needle sampling

Fine-needle aspiration was performed using 23-gauge needles mounted on 10 mL disposable syringes. The needle was pushed in all directions throughout the lesion and the material thus obtained expelled onto 5 slides. Two were immediately fixed in absolute methanol, stained by Papanicolaou's stain [*12*] and used for routine diagnosis. Another 2 slides were frozen at –20 °C for later use with specific estrogen and progesterone receptor monoclonal antibodies. The fifth slide was air-dried to be stained later with Feulgen stain [*12*].

Determination of estrogen and progesterone receptors (ER and PR)

The frozen smears were immersed in 3.6% formol phosphate buffered saline (PBS) for 10 minutes. They were then placed in absolute methanol followed by acetone, each for 2 minutes, rewashed in PBS and finally stored in a cold storage medium (PBS/glycerol 1:1 v/v) at –20 °C. All the reagents for the ER and PR immunocytochemical assay (ERICA/PRICA staining) were supplied by Abbott (Stockholm, Sweden) and the manufacturer's suggested procedure was followed in detail [*13*].

At least 100 tumour cells were available on each slide. The receptors were located

inside the nuclei and stained brown when positive and light grey when negative. Numerical values (I–III) were assigned to the intensity of staining. The final receptor values were calculated by multiplying the staining intensity by the percentage of positive receptor cells. The threshold for this method should be 20 in each case [*14*]. Following these criteria strictly, receptor information was recorded in 91 out of 98 cases subjected to cytometry.

DNA cytometric study

Initial training in this technique was received at the Department of Tumour Pathology of the Karolinska Hospital in Stockholm, Sweden. Quantitative cytophotometric DNA analysis was performed using air-dried or alcohol-fixed smears. After Feulgen staining, all slides were reviewed to assess the suitability of the preparation. Emphasis was placed on the availability of an adequate number of tumour cells (at least 100) and the admixture of normal lymphocytes (at least 20) which were used as an internal diploid DNA standard.

Of the 120 histologically confirmed malignant cases, 22 were excluded for technical reasons, leaving 98 aspirates for the analysis. The Feulgen DNA content of individual cell nuclei was determined by absorption measurement in a rapid scanning and integrating microspectrophotometer at a wavelength of 546 nm. All tumour values were expressed in C-relative units in relation to the corresponding internal controls. The DNA histogram patterns were classified according to the criteria of Auer et al. [*3*] into diploid type I, tetraploid type II, aneuploid type III, and highly aneuploid type IV. The proliferative index (PI) was calculated automatically. A cut-off level of 12% was used to classify aneuploid samples (81 cases) as having high or low PI [*4*].

Statistical analysis

The chi-squared test [*15*] was used to test the degree of association of aneuploidy and of high PI with the clinicopathological study variables.

Results

The mean age of the patients was 48.4 years at the time of diagnosis. The majority of the tumours (76%) were at advanced stages when the patients first presented, i.e. stages III and IV according to the TNM (tumour, node, metastasis) classification adopted by both the International Union Against Cancer and the American Joint Commission on Cancer Staging [*16*].

Of the 120 ductal mammary carcinomas examined, DNA histograms were evaluated for ploidy in 98 cases (81.7%). Table 1 shows the DNA histogram classification (according to Auer et al.) and ploidy content distribution found in these patients. The frequencies of diploid type I, tetraploid type II, and aneuploid types III and IV histograms were 10.2%, 7.1%, 36.7% and 45.9% respectively. Hence, aneuploidy was present in 82.6% of the cases.

In Table 2, the relationship between nuclear aneuploidy and the recorded clinical

Table 1 **Histogram types and ploidy content distribution in 98 cases of mammary carcinoma**

Histogram type	No. of cases	%
(I) Diploid	10	10.2
(II) Tetraploid	7	7.1
(III) Aneuploid	36	36.7
(IV) Highly aneuploid	45	45.9
Total	98	

There were 17 cases (17.3%) of euploid (diploid + tetraploid) histogram type.

and histopathological characteristics of the study group is explored. No correlation could be found between nuclear DNA content and menopausal status of the patient at the time of diagnosis. Although aneuploid frequency and PI seemed to increase in tumours measuring > 5 cm in diameter, the relationship with tumour size was not statistically significant. Likewise, no clear correlation was demonstrated between ploidy content and histopathological nodal status.

On the other hand, a highly significant association ($P < 0.001$) was observed between aneuploidy and histopathological grading of ductal carcinomas, with poorly differentiated tumours more likely to be aneuploid (Figure 2). Table 2 also clearly shows that the proportion of aneuploid tumours was significantly higher in ER-negative (Figure 3) than in ER-positive cases ($P < 0.01$). This relationship was not observed for PR.

Using the defined cut-off level, 63 out of 81 (77.8%) of aneuploid DNA histograms were found to have high PI (Figures 4 and 5). Table 3 displays data concerning the relationship between high PI and the

Table 2 **Relationship between DNA aneuploidy and the study variables**

Variable	No. of patients	Aneuploidy No.	Aneuploidy %	Statistical values
Total patients	98	81	82.7	
Menopausal status				
Premenopause	51	45	88.2	$\chi^2 = 2.31$
Postmenopause	47	36	76.6	NS
Tumour size (cm)				
< 2	8	6	75.0	$\chi^2 = 3.48$
2–5	41	31	75.6	NS
> 5	49	44	89.8	
Nodal status				
N0	16	12	75.0	$\chi^2 = 0.78$
N+	82	69	84.1	NS
Tumour grade				
I	4	–		$\chi^2 = 10.22$
II	48	37	77.1	$P = 0.001$
III	46	44	95.6	
ER status [a]				
ER+	48	36	75.0	$\chi^2 = 7.21$
ER–	43	41	95.3	$P < 0.01$
PR status [a]				
PR+	32	24	75.0	$\chi^2 = 3.51$
PR–	59	53	89.8	NS

[a] *Receptor information was available in 91 out of 98 cases which were subjected to cytometric analysis.*
ER = estrogen receptor.
PR = progesterone receptor.
NS = not significant.

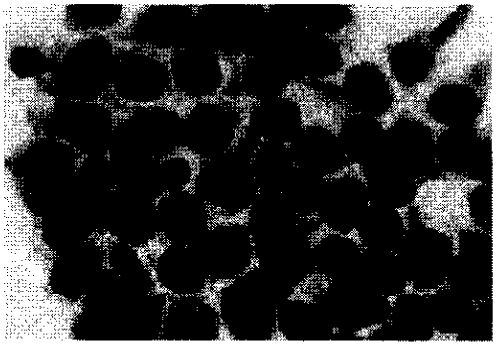

Figure 2 **Fine-needle aspiration cytology from a breast lump, showing anaplastic ductal cells with high nucleocytoplasmic ratio, hyperchromasia and prominent nucleoli (PAP 600)**

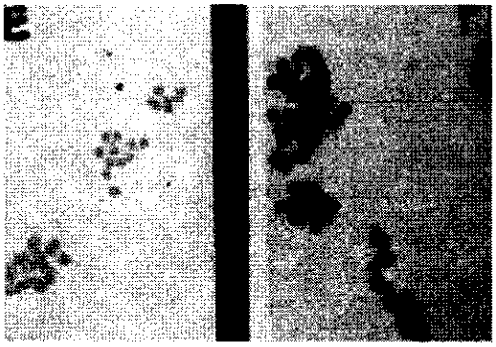

Figure 3 **Aspiration biopsy showing negative ERICA (left) and positive PRICA (right) staining reactions (ERICA/PRICA x 160)**

study variables in 81 aneuploid mammary carcinomas. In these aneuploid tumours, high PI correlated more significantly than percentage aneuploidy with the menopausal status of the patients and the PR content of the tumour ($P < 0.05$ and $P < 0.001$ respectively), in addition to its established relationship with tumour grade and ER content.

Discussion

In this study, DNA analysis of a random selection of primary mammary carcinomas from Iraqi patients revealed a ratio of euploid to aneuploid tumours equivalent to 17.3:82.7. The proportion of aneuploid tumours is clearly higher than the 41–68 range reported in most published studies [*3,4,7,17,18*].

In an earlier comparative clinicopathological study of mammary carcinoma in Iraq and Sweden, we showed a significantly lower frequency of aneuploid carcinomas among Swedish patients (58.5% from Sweden versus 80.9% from Iraq) [*19*]. The fact that this cancer tends to be diagnosed at more advanced stages in developing countries like Iraq cannot by itself explain the observed difference in ploidy pattern distribution, since earlier reports showed that it generally remains unchanged during tumour progression [*20*]. Therefore, possible variations in the biological behaviour of the tumour in the different populations studied (i.e. variation in genetic predisposition or in environmental, nutritional or lifestyle factors) should be considered.

It has been suggested that nuclear DNA content could provide independent prognostic information in addition to that given by the clinical and histopathological features of mammary carcinoma. Hence, an attempt was made to evaluate statistically the relationship between DNA ploidy and some of the clinicopathological parameters with documented prognostic significance.

Data demonstrating an enhanced frequency of aneuploidy within larger tumours have been presented by some inves-

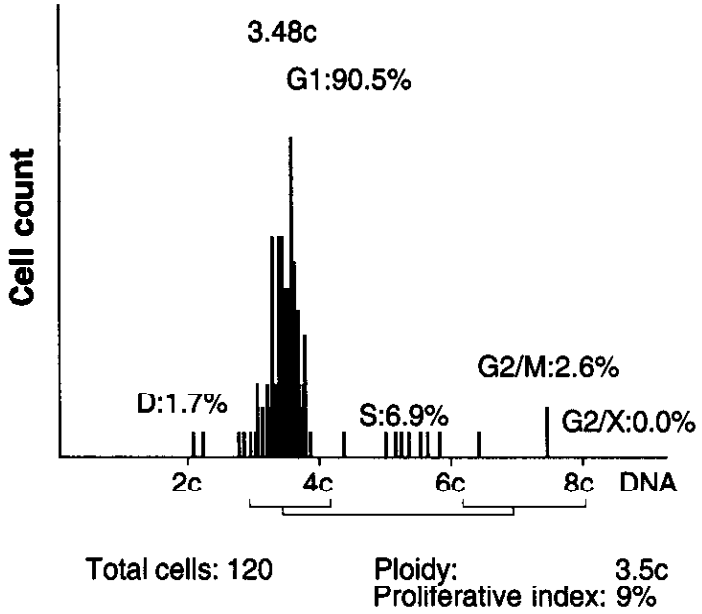

Figure 4 **Cytophotometric DNA analysis of a case of mammary ductal carcinoma showing aneuploid DNA histogram with low proliferative index**

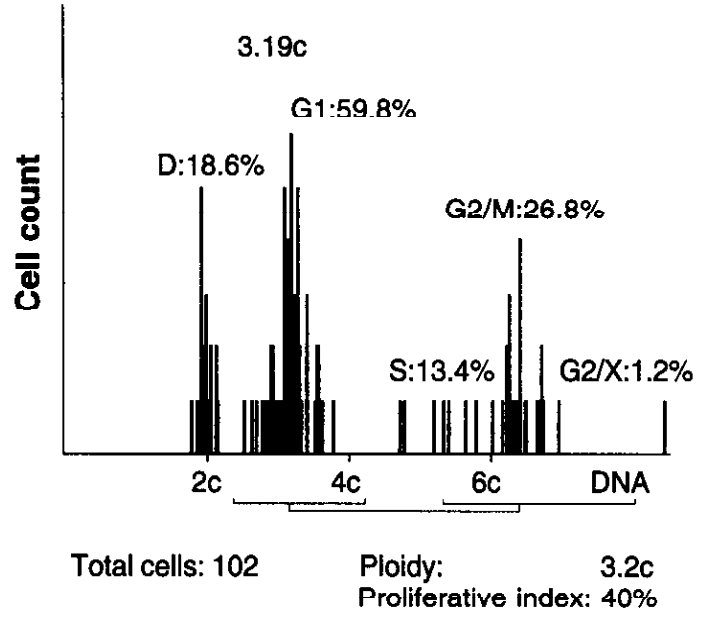

Figure 5 **Cytophotometric DNA analysis showing aneuploid histogram pattern with high proliferative index**

Table 3 **Relationship of high DNA proliferative index (PI) with the study variables**

Variable	No. of patients	High PI		Statistical values
		No.	%	
Total patients	81	63	77.8	
Menopausal status				
Premenopause	45	39	86.7	$\chi^2 = 4.63$
Postmenopause	36	24	66.7	$P < 0.05$
Tumour size (cm)				
< 2	6	3	50.0	$\chi^2 = 5.4$
2–5	31	22	70.9	NS
> 5	44	38	86.4	
Nodal status				
N0	12	8	66.7	$\chi^2 = 1.01$
N+	69	55	79.7	NS
Tumour grade				
I	–		–	$\chi^2 = 4.11$
II	37	25	67.6	$P < 0.05$
III	44	38	86.4	
ER status[a]				
ER+	36	22	61.1	$\chi^2 = 9.08$
ER–	41	37	90.2	$P < 0.01$
PR status[a]				
PR+	24	12	50.0	$\chi^2 = 13.8$
PR–	53	47	88.7	$P < 0.001$

[a]*Receptor information was available in 77 cases.*
ER = estrogen receptor.
PR = progesterone receptor.
NS = not significant.

tigators [*21–23*] but not others [*24*]. One proposed explanation is a possible progressive differentiation and increase in cancer malignancy during tumour development. In fact, in the current study, the nuclear DNA content, as a biological marker of tumour behaviour, was strongly correlated with histopathological grading of ductal carcinomas. This is in accordance with most previous reports [*7,18,21,23*]. In general, poorly differentiated tumours were characterized by a higher number of proliferating cells in the $S+G_2/M$ phases, corresponding to the common observation of histopathologists that more mitotic figures generally signify poorly differentiated malignant tumours and vice versa.

Our data also revealed a significantly increased frequency of aneuploid tumours with high PI associated with ER-negative phenotypes. Previous studies indicated that ER-positive diploid tumours have a much better prognosis and a lower risk of early recurrence than ER-negative aneuploid neoplasms [*3,5*]. Using the thymidine labelling index and flow cytometry, other investigators have documented a strong association between low ER content and high

SPF [*7,17,21*], the latter signifying rapid tumour growth.

Contrary to most previous reports, which failed to find any relationship with age at the time of diagnosis [*4,18*], our results showed that the high PI of aneuploid carcinomas had a clearer relationship than percentage aneuploidy with the patient's menopausal status. Premenopausal carcinomas gave higher PIs than those presenting postmenopausally. This finding, which is consistent with the results of a few other studies [*22,23,25*], may explain the aggressive behaviour of breast cancer encountered in younger women.

High PI also indirectly correlated with the hormone receptor content (ER and PR) of the primary tumour, while for aneuploidy this association was maintained for ER only. It has been proposed that PR is a gene product of estrogen induction and transcription [*26*]. Accordingly, PR might be a better marker of an intact ER pathway and could give better prognostic information since it is an end product of estrogen action [*27*]. Others have reported an absence of these receptors in mammary carcinomas exhibiting high thymidine labelling indices and in anaplastic tumours associated with the highest proliferative rates, indicating that the presence of hormone receptors might represent another aspect of tumour cell differentiation [*17,22,28*].

In conclusion, the DNA PI of the primary mammary carcinoma is closely related to its nuclear DNA ploidy content. These two parameters, which are also associated with established prognostic factors, can now be fully evaluated for their prognostic significance in broad patient populations. Using the DNA values corresponding to the $S+G_2/M$ fractions, patients presenting with aneuploid carcinomas can be divided into subgroups with different tumour characteristics and prognosis. The PI might be useful in defining subsets of patients with a high probability of short-term relapse, who are therefore good candidates for adjuvant cytotoxic treatment. Ploidy studies and high PI can be used together with other prognostic factors, such as histological grading and hormone receptor status, to improve the selection of high-risk, node-negative patients who might benefit from endocrine manipulation with systemic therapy.

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The main emphasis of regional cancer control continued to focus on the development and implementation of national cancer programmes, which allow the cancer burden to be assessed, priorities and achievable targets to be set, and appropriate strategies, approaches and measures to be formulated based on local conditions. Such programmes, with emphasis on primary prevention, detection and treatment, are the key to strengthening cancer control at country level.

Source: The work of WHO in the Eastern Mediterranean Region. Annual Report of the Regional Director. 1 January–31 December 2000. *Page 154.*