Flow cytometric DNA analysis of oral squamous cell carcinoma in Iraqi patients

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تحليل التدفُّق DNA cytometric لسرطانة فموية حرشفية الخلايا في المرضى العراقيين – دراسة سريرية باثولوجية

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الخلاصة: في هذا التقرير، تمت دراسة محتوى الدي إن إي ومَنْسب الدي إن إي ومتثابتات الدورة الخلوية والتي تعتبر علامات موثوقة في تقييم النشاط التكاثري والعدوانية الحيوية للخباثة. وقد تم تحليل سيتوميتريك الدي إن إي على شرائح مسجاة بالبرافين ومثبتة بالفورمالين من 36 مريض عراقي مصاب بسرطانة فموية حرشفية الخلايا. على الفورمالين ثُبّت نفط أبيض حصلت عليها من 36 مريض بسرطان خلية squamous فموي. وقد تم تحضير العينات وتلوينها طبقاً لتعديل هيدلي وزملاؤه لتحليل التدفّق cytometric. أوضحت تلك النتائج أن 20 من 36 حالة (55.5٪) من السرطانة الفموية الحرشفية الخلايا في المرضى العراقيين كانت ضعفانية، بينما ظهرت الأورام المختلة الصيغة الصبغية في 15 حالة (41.7٪). وقد تميّزت الأورام المختلة الصيغة الصبغية بأجزاء عالية بوضوح من الطور S ومناسب عالية من الدي إن إي. وقد لوحظت اختلافات وارتباطات هامّة فيما يتعلق بالمتثابتات السريرية والهستولوجية والتي تشير إلى أهمية تحليل الدي إن إي النووي في التقييم والتنبؤ بالمآل في السرطانة الفموية الحرشفية الخلايا. وهذا الأخير تباعاً يؤثّر على اختيار استراتيجيات المعالجة الكافية.

ABSTRACT We studied the DNA content, DNA index and cell cycle parameters that are reliable markers for assessing the proliferative activity and aggressiveness of malignancies. Cytometric DNA analysis was performed on formalin-fixed paraffin embedded sections from 36 Iraqi patients with oral squamous cell carcinoma. The results showed that 20 of 36 cases (55.5%) were diploid, while 15 cases (41.7%) were aneuploid. Significantly higher S-phase fractions and higher DNA indices characterized aneuploid tumours. Nuclear DNA analysis as part of the evaluation of oral squamous cell carcinoma will influence the selection of appropriate treatment strategies.

Analyse de l'ADN des carcinomes épidermoïdes oraux par cytométrie en flux chez des patients iraquiens

RESUME Nous avons étudié le contenu en ADN, l'indice d'ADN et les paramètres du cycle cellulaire qui sont des marqueurs fiables pour évaluer l'activité proliférante et l'agressivité des tumeurs malignes. L'analyse cytométrique de l'ADN a été réalisée sur des sections fixées au formol et incluses dans la paraffine chez 36 patients iraquiens atteints de carcinomes épidermoïdes oraux. Les résultats ont montré que 20 des 36 cas (55,5 %) étaient diploïdes tandis que 15 cas (41,7%) étaient aneuploïdes. Des fractions de la phase S significativement plus élevées et des indices d'ADN plus importants caractérisaient les tumeurs aneuploïdes. L'analyse nucléaire de l'ADN dans le cadre de l'évaluation des carcinomes épidermoïdes oraux pourrait influencer le choix des stratégies de traitement appropriées.

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Received: 16/08/01; accepted: 04/10/01

Introduction

The quantitative evaluation of the growth potential of oral squamous cell carcinoma (SCC) by DNA measurement shows promise in providing objective parameters for assessing the behaviour of neoplastic cells during development and progression [*1–3*].

Numerous researchers have indicated that genetically stable tumours, with a diploid or tetraploid DNA profile, generally have a better prognosis than genetically unstable tumours with aneuploid DNA content [*4–6*]. Non-diploid oral carcinomas thus have a worse prognosis and a higher recurrence rate than diploid tumours [*7*].

To the best of our knowledge, this is the first study from Iraq to correlate the ploidy nuclear DNA content and cell cycle kinetics of oral SCC with their clinicopathological parameters using flow cytometric (FCM) analysis.

Methods

The study included 36 patients with oral SCC who had surgery between October 1997 and January 1999 in the Al-Shaheed Adnan Hospital, Baghdad, Iraq. Twenty-five non-malignant tissue samples were used as controls. There were 21 males and 15 female patients with ages ranging between 14 and 92 years. All tumours were classified according to the American Joint Committee against Cancer (AJCC) [*8*] and graded according to WHO criteria [*9*].

Tissue preparation and staining for FCM

Nuclear DNA was prepared in the Al-Amal Centre laboratories, Jordan. Two 50 mm sections were cut from each representative tumour area (using formalin-fixed, paraffin-embedded blocks), together with a 5 mm section before and after each series of 50 mm sections. The 5 mm sections were stained with haematoxylin and eosin to confirm tissue homogeneity and to guide the removal of necrotic and excess normal surrounding tissues from the sections.

The nuclear preparation was carried out following the modification of Hedley et al. [*10*]. Briefly, the tissue was deparaffinized with xylene and hydrated in a series of 99%, 95%, 70%, and 50% ethanol washes, followed by a wash in distilled water, which was then kept overnight. The following day the sample was washed with phosphate buffered saline (PBS). The tissue was then digested with 0.5% pepsin (Sigma) in PBS at pH 1.5 for 30 minutes at 37 °C to produce a suspension of bare nuclei. This suspension was re-washed with PBS, filtered through 35 mm nylon mesh, pelleted (at 400 g for 10 minutes) and re-suspended in PBS to dissociate aggregates. The cell concentration was adjusted to a maximum of 5×10^5 cells/mL. The nuclei were pelleted and resuspended in 250 mL trypsin buffer (10 minutes), followed by trypsin inhibitor and RNase (10 minutes) and 200 mL propidium iodide (PI) for 10 minutes in the dark at 2–8 °C. The cell suspensions were refiltered and analysed by FCM within one hour of staining.

The stained nuclear suspension was analysed by FACS Calibur™ flow cytometry (Becton Dickinson, California) using an air-cooled argon laser. The PI-stained nuclei were excited at 488 nm and fluorescence was detected with a 585/42 pass-band filter. The instrument was adjusted using DNA quality control particles following the manufacturer's recommendations. A total of 10 000 events per sample were recorded at a low flow rate. The DNA content was measured, and data recorded and analysed with a Macintosh computer connected to the apparatus. The acquisition and analysis of the data were performed

using Cell Quest and Modfit software respectively.

Criteria for DNA histogram interpretation

Histograms were classified as diploid, aneuploid or tetraploid. Diploid tumours were defined as those having a single symmetric G_0G_1 peak with G_2M prevalence < 20%. If there was evidence of one asymmetric wide G_0G_1 peak (peridiploid), or an additional G_0G_1 peak, the tumours were classified as aneuploid [*11*]. A tetraploid tumour cell population was defined as one having cells in the region corresponding to diploid G_2M cells constituting > 20% of the total analysed cell population [*11*].

The coefficient of variation (CV) was determined and used as a parameter for the quality of the measurement. Only histograms where the CV value of the diploid peak was = 8% were accepted.

DNA Index (DI)

This is defined as the ratio of the modal channel of the peak in question to the modal channel of the diploid standard [*12*]. The DI for a non-diploid population was calculated by dividing the channel number of the non-diploid peak by that of the diploid peak. Diploid specimens were defined as having DI = 1.1 and aneuploid specimens as having DI > 1.1. Tumours with DI values between 1.9 and 2.1 were classified as tetraploid [*12*].

Statistical analysis

The variable means between subgroups were tested using a two-tailed *t*-test, while the chi-squared distribution test was used when proportions were compared. The linear correlation coefficient of biparametric variables was estimated using the Pearson Product Moment Test. Significance was defined as $P < 0.05$.

Results

A typical diploid tumour (Figure 1) was seen in 20 of 36 cases (55%). Tetraploidy was observed in one case (2.7%) (Figure 2), and aneuploid tumours were observed in 15 cases (41.7%) (Figure 3, Table 1).

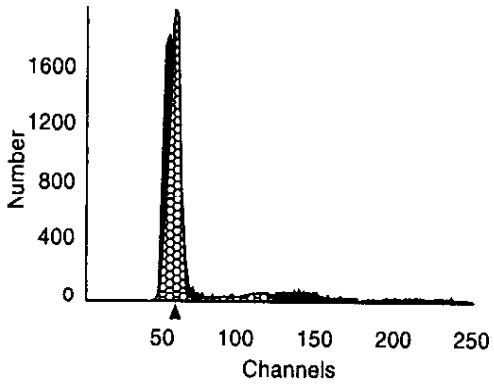

Figure 1 **DNA histogram of a diploid oral squamous cell carcinoma**

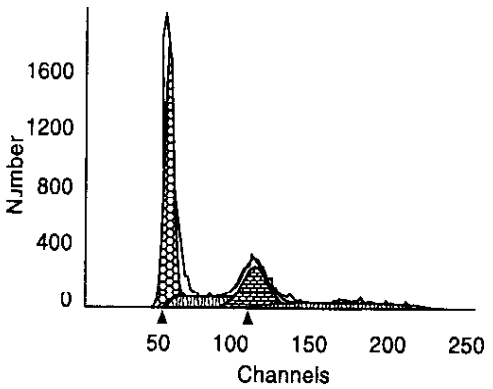

Figure 2 **DNA histogram of a tetraploid oral squamous cell carcinoma**

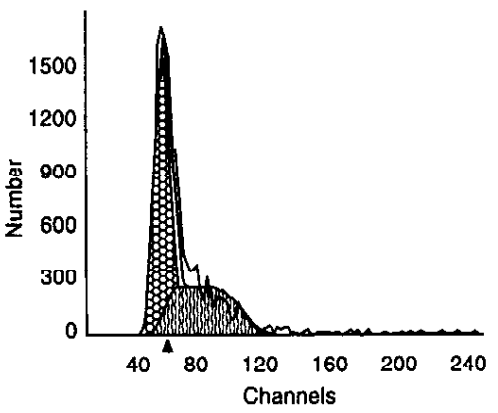

Figure 3-A

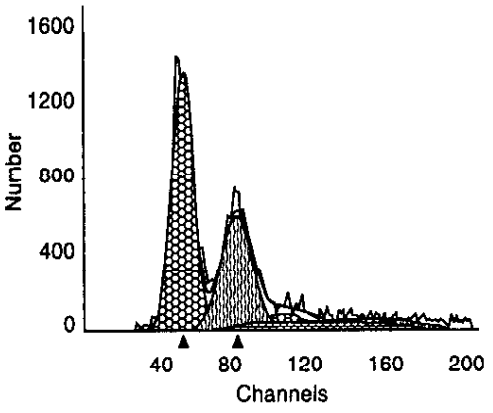

Figure 3-B

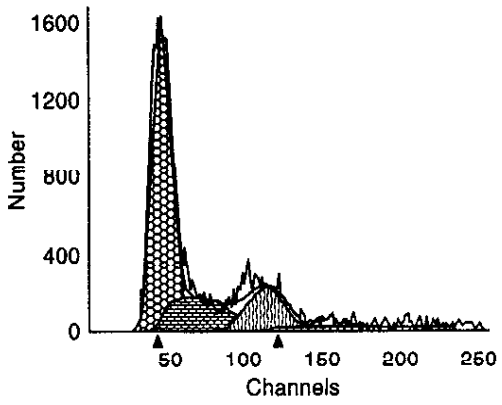

Figure 3-C

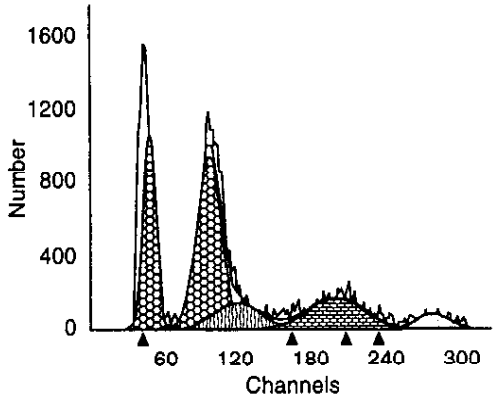

Figure 3-D

Figure 3 **DNA histogram pattern in FCM measurements of oral squamous cell carcinoma A: peridiploid; B: hyperdiploid; C: hypertetraploid; D: polyploid**

The mean value of S-phase fraction (SPF) and DNA index (DI) for diploid carcinoma was 11.59 and 1.03 respectively, and 25.45 and 1.6 in 15 aneuploid carcinomas. Correlation of ploidy type with SPF cells and DI were both statistically significant ($r = 0.49$, $r = 0.41$) (Table 2).

The tumour size and lymph node status did not correlate with either DNA ploidy or DI. There was a tendency for large tumours (T3 T4) to be aneuploid with high SPF, which was statisically significant. Lymph node-negative cases were predominantly diploid (71%) with low SPF, which

Table 1 **Distribution of DNA histogram types and nuclear DNA ploidy content in 36 Iraqi patients with oral squamous cell carcinoma**

Histogram	No. (%)	Histogram sub-type	No. (%)
Euploid	21 (58.3)	Diploid	20 (55.55)
		Tetraploid	1 (2.8)
Aneuploid	15 (41.7)	Peridiploid	8 (22.2)
		Hyperdiploid	4 (11.1)
		Hypertetraploid	2 (5.55)
		Polyploid	1 (2.8)

was also statistically significant. Aneuploidy was seen in seven cases out of twelve, with lymph node metastasis. There was a significant correlation between clinical staging on the one hand, and DNA ploidy and SPF on the other. Neither DNA content nor cell cycle parameters correlated significantly with the sex or the age of the patient, or the duration of the symptoms before seeking medical care. However, an exception was observed in patients with a longer disease duration who had high SPF (50% versus 24%) (Table 3).

Aneuploidy increased with loss of differentiation and increased significantly with the invasive front malignancy scoring system ($r = 0.38$) (Table 3). On the other hand, SPF showed a significant correlation with both the histological grading and malignancy scoring systems ($r = 0.41$, $r = 0.56$). Thus SCC that were poorly differentiated and had high malignancy scores expressed significantly higher SPF and lower G_0G_1 values.

The cell mitosis score did not correlate significantly with DNA ploidy content. However, high mitotic scores had higher G_2M percentages (33% versus 10%, $r = 0.3$) and lower occurrence of SPF > 20 (17% versus 37%) (Table 3). In addition, histological parameters such as nuclear pleomorphism were correlated with ploidy

Table 2 **Mean values of DNA index and cell cycle parameters by nuclear DNA ploidy content**

Variable	No.	DNA index fraction	S-phase	G_2M	G_0G_1
Diploid	20	1.03 ± 0.06	11.59 ± 5.84	5.67 ± 4.04	53.59 ± 16.7
Aneuploid	15	1.6 ± 0.87	25.45 ± 16.6	6.16 ± 12.53	53.59 ± 16.7
t-test		$P < 0.05$	$P < 0.05$	NS	$P < 0.05$
Correlation test		$r = 0.41$	$r = 0.49$	NS	$r = -0.76$

NS = not significant.

Table 3 **Nuclear aneuploidy, DNA index and cell cycle parameters by clinicopathological parameters**

Variable	Total No.	Aneuploidy No. (%)	DNA index Mean ± *s*	S-phase fraction <10 No. (%)	S-phase fraction >20 No. (%)	G_2M ≤ 20 No. (%)	G_2M >20 No. (%)	G_0G_1 < 80 No. (%)	G_0G_1 ≥ 80 No. (%)
Sex									
Male	21	8 (38)	1.23 ± 0.7	8 (38)	7 (33)	17 (81)	4 (19)	13 (62)	8 (38)
Female	15	7 (47)	1.33 ± 0.52	5 (33)	5 (33)	14 (93)	1 (7)	8 (53)	7 (47)
Age (years)									
< 60	19	8 (42)	1.17 ± 0.37	8 (42)	6 (32)	17 (90)	2 (10)	10 (53)	9 (47)
≥ 60	16	7 (44)	1.41 ± 0.83	4 (25)	5 (31)	13 (81)	3 (19)	10 (63)	6 (38)
Duration (months)					*				
≤ 6	21	9 (43)	1.29 ± 0.72	10 (48)	5 (24)	18 (86)	3 (14)	9 (43)	12 (57)
> 6		5 (50)	1.26 ± 0.49	1 (10)	5 (50)	9 (90)	1 (10)	7 (70)	3 (30)
Tumour size				*r* = 0.3					
T1 + T2	18	7 (39)	1.33 ± 0.78	9 (50)	3 (17)	16 (89)	2 (11)	9 (50)	9 (50)
T3 + T4	12	6 (50)	1.16 ± 0.34	4 (33)	6 (50)	10 (83)	2 (17)	8 (67)	4 (33)
Nodal status				*r* = 0.58					
N0	17	5 (29)	1.45 ± 0.82	10 (59)	2 (12)	14 (82)	3 (18)	7 (41)	10 (59)
N+	12	7 (58)	1.04 ± 0.16	3 (25)	10 (83)	11 (92)	1 (8)	7 (75)	3 (25)
TNM stage		*r* = 0.4*		*r* = 0.59		*r* = −0.31		*	
I + II	13	3 (23)	1.42 ± 0.9	8 (62)	0 (0)	12 (92)	1 (8)	4 (31)	9 (69)
III + IV	17	10 (59)	1.14 ± 0.3	5 (29)	9 (53)	14 (82)	3 (18)	13 (76)	4 (24)
Histological grading				*r* = 0.41					
Well	16	7 (44)	1.38 ± 0.5	9 (56)	3 (19)	15 (94)	1 (6)	7 (44)	9 (56)
Moderate	14	5 (36)	1.33 ± 0.84	3 (21)	5 (36)	11 (79)	3 (21)	10 (71)	4 (29)
Poor	6	4 (67)	0.99 ± 0.07	1 (16.7)	4 (67)	5 (83)	1 (17)	5 (83)	1 (17)
Invasive front malignancy score grading		*r* = 0.38	*	*r* = 0.56				*r* = 0.33*	
1–2.5	19	6 (32)	1.47 ± 0.8	9 (47)	3 (16)	17 (89)	2 (11)	7 (37)	12 (63)
2.6–4	17	10 (59)	1.09 ± 0.26	4 (24)	9 (53)	14 (82)	318)	13 (76)	4 (24)

Table 3 Nuclear aneuploidy, DNA index and cell cycle parameters by clinicopathological parameters *(concluded)*

Variable	Total No.	Aneuploidy	DNA index	S-phase fraction		G_2M		G_0G_1	
		No. (%)	Mean ± *s*	<10 No. (%)	>20 No. (%)	≤ 20 No. (%)	>20 No. (%)	<80 No. (%)	≥ 80 No. (%)
Keratinization			*r* = –0.3*	*r* = 0.5					
1–2	15	6 (40)	1.59 ± 0.8	8 (53)	2 (13)	13 (87)	2 (13)	5 (33)	10 (67)
3–4	21	9 (43)	1.09 ± 0.24	5 (24)	10 (48)	18 (86)	3 (14)	15 (71)	6 (29)
Nuclear pleomorphism		*r* = 0.3		*r* = 0.4*				*r* = 0.4	
1–2	13	4 (31)	1.55 ± 0.53	8 (62)	1 (8)	13 (100)	0 (0)	5 (38)	8 (62)
3–4	23	11 (48)	1.26 ± 0.69	5 (22)	11 (48)	18 (78)	5 (22)	16 (70)	7 (30)
Mitotic index						*r* = 0.3			
1–2	30	16 (53)	1.25 ± 0.43	11 (37)	11 (37)	27 (90)	3 (10)	18 (60)	12 (40)
3–4	6	4 (67)	1.53 ± 1.27	2 (33)	1 (17)	4 (67)	2 (33)	4 (67)	2 (33)

**Significant difference at P < 0.05 (chi-squared or t-test).*
r = significant correlation (P < 0.05)

status and SPF ($r = 0.3$, $r = 0.4$ respectively). It is noteworthy that the degree of keratinization was significantly correlated with both DI and SPF ($r = -0.3$, $r = 0.5$ respectively).

Discussion

There is convincing evidence that complex nuclear DNA alterations, shown in DNA aneuploidy, are of decisive importance in various human malignant tumours [*13,14*]. To the best of our knowledge this is the first study in which flow cytometric nuclear DNA analysis has been carried out on oral SCC in Iraqi patients. The study revealed that the frequency of aneuploid DNA content was 41.7%. Although this is in accordance with the results of some other studies [*15–17*], reviews of the published literature show conflicting results with a wide range of aneuploidy both in oral cavity and head and neck SCC (Table 4). This discrepancy might be attributed to the differences in the cytometric methods applied to assess nuclear DNA ploidy content (i.e. image or flow), the type of tissue assessed (tongue, oral cavity, head and neck region), the methods of tissue preparations (fresh/fixed) or to the various methods used to define ploidy. Other reports showed that no one method can provide the best result for DNA ploidy content in a variety of tumours from various sites [*18*].

The lack of association between DNA ploidy content and tumour size and lymph node status observed in this study is in agreement with other studies [*7,16,19,20*], which emphasize the existence of chromosomal alterations at early stages of tumour growth. However, it contradicts some others [*1,12,21–*

Table 4 **Comparison of DNA ploidy content in oral and head & neck squamous cell carcinoma**

Study	Material	No. of cases	Method	Percentage of non-diploid[a]
Current study	Oral	36	FCM	44.4
Halvorson et al. [3]	Maxillary sinus	16	FCM	25
Schimming et al. [23]	Oral	52	ICM	60
Rubio-Bueno et al. [25]	Oral	93	FCM	55
Melchiorri et al. [20]	Oral	25	FCM	32
Baretton et al. [13]	Oral	106	FCM/ICM	68
Suzuki et al. [24]	Head & neck	96	FCM	26
Saito et al. [18]	Tongue	36	FCM	42
Chen et al. [26]	Oral	40	FCM	58

[a] *Comprising DNA tetraploid (1) and aneuploid (15) cases.*
FCM = flow cytometry.
CM = image cytometry.

25]. On the other hand, lower values of SPF were observed in smaller lesions as noted in previous reports [19,20,22, 23,26]. The major observation of our study was the significant association between lymph node involvement and alterations in the percentage of cells at SPF and G_2M, which has not been recorded as significant in previous studies [16,17,18,19,23].

Although a few studies have demonstrated an association between clinical staging and patient's age or sex and either DNA ploidy or SPF [12,27], no such association was observed here, in line with the results of most studies [1].

Interestingly, a clear parallel existed between microscopic malignancy grading on the one hand and SPF and DNA ploidy on the other. The data suggest that an increased frequency of aneuploidy and higher SPF cell populations occur in poorly differentiated specimens with highly invasive front malignancy scores. This is in accordance with some studies [20,25,28], but not with others [19,22,23, 28,29]. A direct relationship between conventional histological grading and ploidy status was not observed in this study. This is in line with some reports [12,22,30], but again contradicts some others [19,23,24]. Aneuploid tumours and those expressing high SPF had a significant tendency to express nuclear pleomorphism, less keratinization and a higher grading invasiveness. These findings are in line with previous results [14,16,20,23, 26,31]. Thus DNA ploidy content and SPF in our report indicated both the degree of differentiation and of proliferation.

Our study implies that the increase in the second peak of DNA histogram (G_2M region) is directly related to a higher number of cycling cells at the mitotic stage observed by light microscopy during histomorphological assessment. This variation in the proportion of cells in the different cycle phases has also been reported by Nervi et al. [32]. The number of inflammatory cell infiltrates surrounding neoplastic cells correlated significantly with the per-

centage in S-phase. This has not been presented previously [2].

The results of our study thus strongly indicate that DNA cytometric analysis could serve as a useful independent tool for evaluating the aggressiveness and progression of oral SCC.

Acknowledgement

The authors wish to thank Mr Muhammed N. Asfour (Al-Amel Centre, Amman, Jordan) for his technical help.

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National cancer control programmes: policies and managerial guidelines

The second edition of *National cancer control programmes: policies and managerial guidelines* was produced following a meeting on national cancer control programmes in developing countries, held in Geneva in December 2000. This monograph aims to provide a framework for the development of national cancer control programmes. Its underlying approach is the application of science to public health practice, providing a concise statement of what is feasible and desirable in cancer prevention and control, with the ultimate goal of reducing cancer morbidity and mortality, and improving quality of life in the targeted population. It is intended primarily for policy-makers in health and related fields, but will also be of interest to health ministries and academic institutions and, more generally, to oncologists and other health professionals who need to be aware of developments in cancer control. This publication is available free on line at: http://whqlibdoc.who.int/hq/2002/9241545577.pdf