Pergamon

Oral Oncol, Eur J Cancer, Vol. 31B, No. 4, pp. 222–226, 1995
Elsevier Science Ltd
Printed in Great Britain
0964–1955/95 $9.50+0.00

0964–1955(95)00025–9

Feulgen Hydrolysis Profiles and Acid-labile DNA in Oral Squamous Cell Carcinoma

J.V. Soames, R.I. Macleod and P.J. Kelly

The full Feulgen hydrolysis profiles of healthy and malignant oral epithelial cells were compared by measuring the staining density of nuclei using microdensitometry after varying hydrolysis times. Malignant nuclei contained significantly increased levels of acid-labile DNA. The relative amounts of the rapidly hydrolysable fraction were compared after 5 min hydrolysis for exfoliating epithelial cells from healthy oral mucosa, healing chronic ulcers and squamous cell carcinomas. Although the latter exhibited a wide range of values, analysis of variance showed significant differences ($P<0.05$) between healthy control and ulcer groups compared to oral carcinoma. The relative proportion of highly acid-labile DNA in malignant nuclei showed a significant positive correlation with mitotic score ($P<0.01$) but no significant correlation with nuclear area. It was concluded that since the susceptibility of DNA to acid hydrolysis probably reflects functional differences in nuclear activity between cells, estimation of the highly acid-labile fraction may have diagnostic and/or prognostic value.

Keywords: oral cancer, cytology, Feulgen hydrolysis, Feulgen-DNA

Oral Oncol, Eur J Cancer, Vol. 31B, No. 4, pp. 222–226, 1995.

INTRODUCTION

ESTIMATION OF the DNA content of tumour cells by Feulgen cytophotometry or flow cytometric analysis has been widely used in tumour pathology and several studies have suggested that DNA ploidy may have prognostic implications. For squamous cell carcinoma of the head and neck, for example, aneuploidy is associated with an unfavourable prognosis [1–6]. However, up to about half of oral squamous cell carcinomas may be diploid [1–3, 6, 7] and the emergence of aneuploid cell lines appears to be a relatively late event in the natural history of oral squamous cell carcinoma [1–3]. The DNA ploidy level may, therefore, merely reflect the clinical staging rather than having intrinsic prognostic significance [8]. In addition, abnormalities of nuclear DNA content do not appear to correlate well with dysplasia in the oral mucosa [7, 9–11] or with the detection of field changes in oral cancer patients [12]. For these reasons estimation of nuclear DNA content has limitations as a prognostic indicator in oral cancer and precancer.

In contrast to quantifying DNA, other studies have investigated the dynamics of the Feulgen hydrolysis reaction [13, 14]. The physical parameters can be controlled but the rate at which the reaction proceeds depends on the degree of binding and condensation of the DNA within the chromatin. This is a function of cell biology and may reflect the biological state of the cell. In particular, a more acid-labile form of DNA has been identified in some malignant cells [15–18]. However, we have been unable to find reference to the Feulgen hydrolysis profiles of oral squamous cell carcinoma. The aims of this study were, therefore, to determine full hydrolysis profiles for normal and malignant oral epithelial cells and to assess their clinical application by testing whether, after only brief hydrolysis, it was possible to differentiate between chronic inflammatory oral ulcers and oral squamous cell carcinoma in cytological material.

Correspondence to J.V. Soames.
J.V. Soames is at the Department of Oral Pathology, The Dental School, University of Newcastle upon Tyne, Framlington Place, Newcastle upon Tyne NE2 4BW; R.I. Macleod is at the Department of Oral Medicine, Edinburgh Dental Hospital, Chambers Street, Edinburgh EH1 1JA; P.J. Kelly is at the Department of Medical Statistics, The Medical School, University of Newcastle upon Tyne, Framlington Place, Newcastle upon Tyne NE2 4HH, U.K.
Received 3 Nov. 1994; provisionally accepted 21 Nov. 1994; revised manuscript received 16 May 1995.

MATERIALS AND METHODS

Full Feulgen hydrolysis profiles

To determine the full hydrolysis profiles of normal and malignant oral epithelium, samples of exfoliating cells were obtained using a metal spatula from 10 subjects with clinically healthy buccal mucosa and from the margins of the malignant ulcer for 10 patients with biopsy-proven oral squamous cell carcinoma.

Slides were prepared using the techniques described by Millett *et al.* [16] and Mays [17]. Briefly, each sample was fixed as a suspension in acetic alcohol (1:3). After centrifuging, a drop of cell suspension was placed on each of nine glass slides and allowed to dry in air. One slide from each series was stained routinely by the Papanicolaou method for morphological assessment. The other slides were hydrolysed at room temperature in 5 M hydrochloric acid for various times (5 min intervals up to 20 min then 10 min intervals up to 60 min) for

plotting the hydrolysis curves. After hydrolysis the slides were rinsed in 1 M hydrochloric acid and immersed, in the dark, into freshly prepared Schiff reagent for 1 h.

For each slide the optical density of 20 randomly selected nuclei was measured by a microdensitometer and the results expressed as the relative absorbance in machine units. The data were analysed using the summary measures approach as recommended by Matthews *et al.* [19] for the analysis of serial measurements. The total and mean amounts of absorbance per subject were compared between the cancer and control groups.

Short Feulgen hydrolysis profiles

To determine whether differences in the amounts of highly acid-labile DNA could be detected between healthy and diseased mucosa, samples of exfoliating oral epithelial cells were obtained from the right and left buccal mucosae of 6 subjects with clinically healthy mucosa and from the ulcer margin and a clinically healthy contralateral site for 10 patients with chronic non-specific oral ulcers (i.e. present longer than 10 days). Samples of exfoliating cells from oral squamous cell carcinomas were obtained from the 10 patients used for the full hydrolysis profiles and from a further 6 patients. From each of the 16 patients an additional smear from a clinically healthy site was taken as a control for comparison.

Paired samples from the lesion and from the healthy control site (or from right and left buccal mucosae for the clinically healthy group) were processed at the same time using the same batch of reagents. They were subjected to 5 min hydrolysis at room temperature in 5 M hydrochloric acid and analysed as before. Since the paired samples were collected over a period of time the ratio of the relative absorbance of lesional and control nuclei (or between right and/or left buccal mucosae for the clinically healthy group) was used for comparative purposes. This minimised any proportionality error that might be introduced by variation in hydrolysis kinetics or dye–substrate affinity associated with different batches of reagents.

Correlation of absorbance ratio with nuclear area and mitotic score

Absorbance ratios after short hydrolysis were compared with nuclear areas and mitotic scores for the oral carcinoma group. The nuclear areas of 20 randomly selected cells were measured from a Papanicolaou-stained smear and the mean nuclear area calculated. The mitotic score was evaluated from biopsy material and expressed as the total number of mitotic figures in ten high power fields.

RESULTS

Full Feulgen hydrolysis profiles

The full Feulgen hydrolysis profiles for each subject in the oral cancer (subjects 1–10) and control groups (subjects 11–20) are shown in Figs 1 and 2, respectively. Both the total amount of absorbance per subject, measured by the area under the curve, and the mean amount of absorbance per subject, measured by averaging each subject over time, were significantly higher in the cancer group compared to the control group (Table 1). The means and standard deviation of absorbance over time for all subjects in the cancer and control groups are presented in Figs 3 and 4 for visual comparison with data from other studies.

Short Feulgen hydrolysis profiles

The ratios of relative absorption of control and test nuclei for epithelial cells from the margins of chronic inflammatory and malignant ulcers are presented in Fig. 5. The ratio for the clinically healthy control group was derived by dividing the relative absorbance values of paired samples from right and left buccal mucosae using the lower of the two figures as the

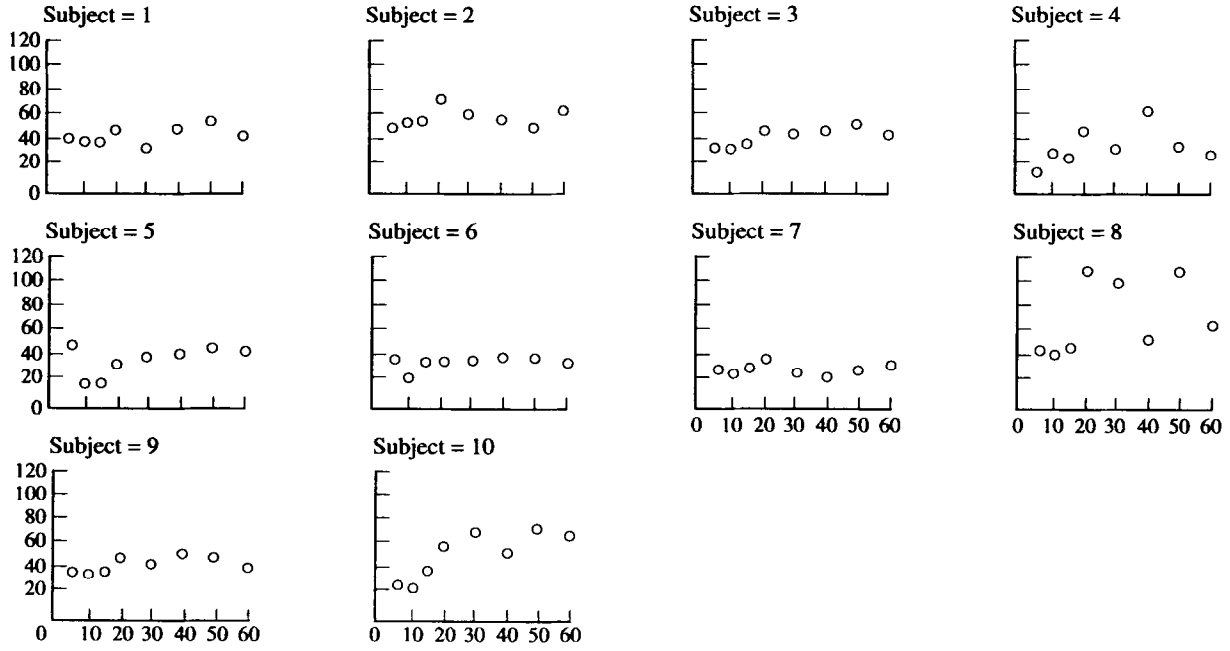

Fig. 1. Feulgen hydrolysis curves using individual plots of absorbance (*y*-axis, machine units) over time (*x*-axis) for cells from 10 patients with oral squamous cell carcinoma.

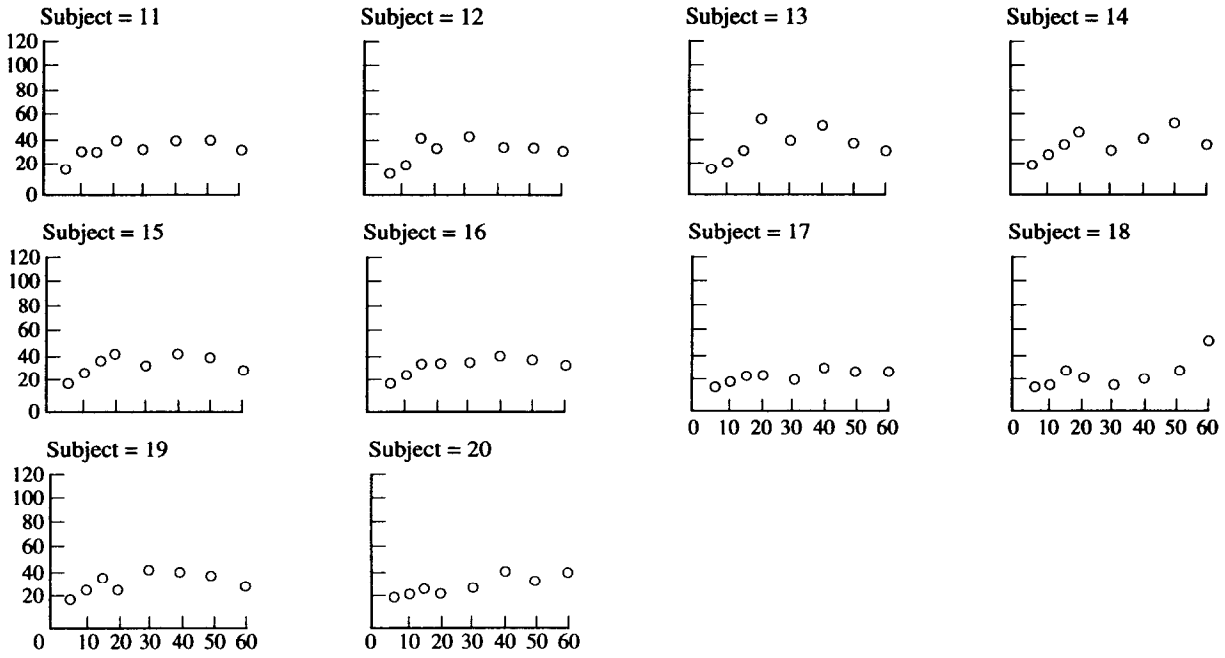

Fig. 2. Feulgen hydrolysis curves using individual plots of absorbance (*y*-axis, machine units) over time (*x*-axis) for cells from 10 patients with clinically healthy buccal mucosa.

Table 1. *Comparison of the total (area under the curve) and mean amounts of absorbance per subject for cancer and control groups*

	Mean (S.D.)	*P* value	95% C.I. for difference
Total absorbance			
Cancer group	2.46 (0.80)	0.024	0.10–1.24
Control group	1.79 (0.29)		
Mean absorbance			
Cancer group	0.042 (0.013)	0.005	0.0047–0.023
Control group	0.029 (0.0046)		

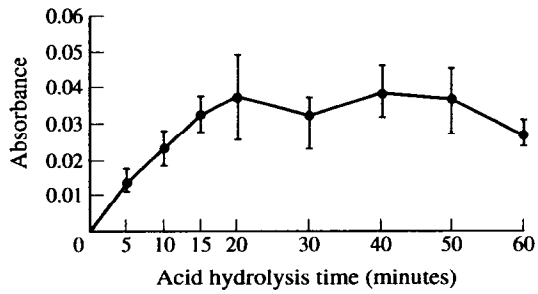

Fig. 3. Feulgen hydrolysis profile using pooled data for normal buccal epithelial cells. Means and standard deviations of absorbance (machine units) plotted against hydrolysis time.

denominator. The data were compared using an analysis of variance (ANOVA). No significant differences were detected between control and chronic inflammatory ulcer groups but both were significantly different ($P < 0.05$) when compared to the oral carcinoma group. The relationship of absorbance ratios to mitotic score and nuclear areas are shown in Figs 6 and 7, respectively. Statistical analysis showed a significant

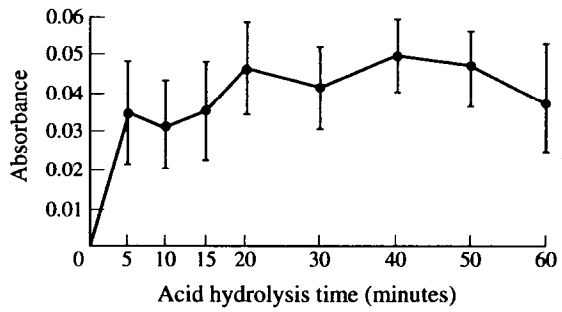

Fig. 4. Feulgen hydrolysis profile using pooled data for cells from oral squamous cell carcinomas. Means and standard deviations of absorbance (machine units) plotted against hydrolysis time.

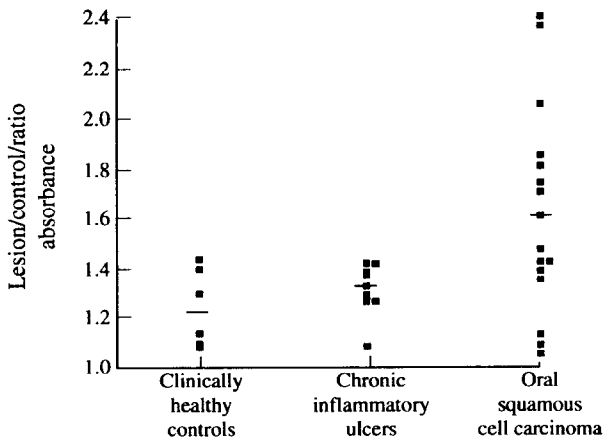

Fig. 5. Relative absorbance of epithelial nuclei from normal buccal mucosa, healing chronic ulcers and squamous cell carcinomas after 5 min hydrolysis corresponding to highly acid-labile DNA.

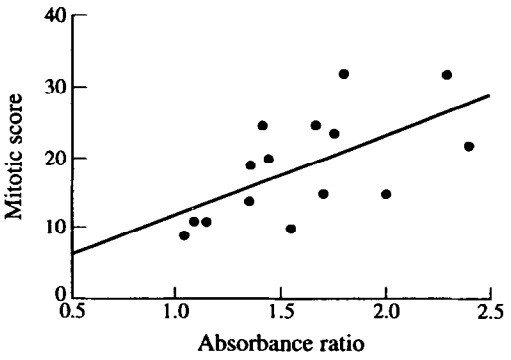

Fig. 6. Correlation between mitotic score and absorbance ratio for cells from squamous cell carcinomas after 5 min hydrolysis.

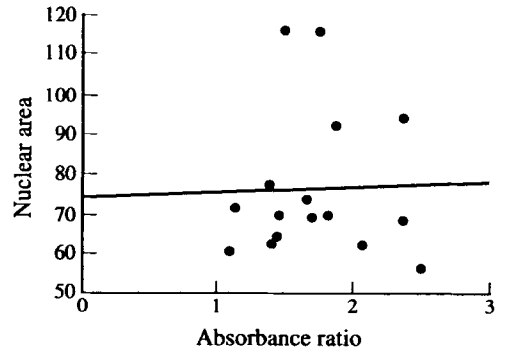

Fig. 7. Correlation between nuclear area and absorbance ratio for cells from squamous cell carcinomas after 5 min hydrolysis.

positive correlation with mitotic score ($r=0.62$, $P=0.01$) but no significant correlation was demonstrated with nuclear area ($r=0.01$, $P=0.95$).

DISCUSSION

Acid hydrolysis of DNA in the standard Feulgen reaction continuously generates aldehyde molecules on the DNA, increasing the intensity of the staining reaction with Schiff reagent, but also depolymerises the DNA leading to diffusion of stainable product from the nucleus [14]. However, by using 5N HCl at room temperature the DNA backbone is cleaved more slowly than in the standard reaction [16] allowing subfractions of DNA of differing susceptibilities to acid hydrolysis to be identified.

The profiles of the full Feulgen hydrolysis curves showed variation between subjects both in the oral cancer and control groups but statistical analysis demonstrated that exfoliating cells from oral squamous cell carcinomas contain significantly increased amounts of acid-labile DNA compared to controls. Visual examination of the curves for pooled data from healthy and malignant cells showed profiles similar to those obtained for carcinomas at other sites [15–18]. However, comparison of such data by analysis of mean absorbance at each time interval is open to statistical criticism. The curve joining the means may hide variations in the profile for different subjects and does not take into account the fact that since measurements at different times are from the same individual, successive observations are likely to be correlated: the use of summary measures to analyse serial measurements avoids these problems [19].

Significantly elevated levels of rapidly hydrolysed DNA in exfoliating cells from oral carcinomas compared to either normal controls or cells from the margins of chronic ulcers were also demonstrated using short hydrolysis only, similar to the raised levels of acid-labile DNA reported in invasive cervical cancer and cervical intra-epithelial neoplasia [15, 20–22]. The variation of absorbance levels with time in the Feulgen hydrolysis profiles represent differences in the sensitivity of DNA to acid hydrolysis related to the degree of condensation of the nuclear chromatin. The latter probably reflects functional differences in nuclear activity between individual cells [14, 23]. In particular, it has been suggested that enhanced susceptibility of DNA to acid hydrolysis is a characteristic of newly synthesised DNA [24, 25] and increased content of acid-labile DNA appears to be a feature of rapidly proliferating nuclei [26, 27]. The positive correlation between mitotic score and absorbance after short hydrolysis for cells from oral squamous cell carcinomas supports the latter. However, no such increase was found in cells from the margins of chronic ulcers where active DNA synthesis and cell division might be expected. This may be due to sampling error in that whilst cell proliferation is largely confined to basal and suprabasal cells it is likely that mainly intermediate and superficial cells were harvested. Transcriptionally-active DNA in the form of decondensed euchromatin may also be more susceptible to acid hydrolysis in comparison to heterochromatin, but nuclear size, as in the present study, does not appear to be a limiting factor in the Feulgen hydrolysis [27].

Failure to hydrolyse all of the highly acid-labile DNA in the present study may account, in part, for the spread of absorbance ratios of the samples from squamous cell carcinomas after short hydrolysis and might be overcome by increasing the hydrolysis time from 5 to 10 min. In addition, sampling error could be a factor if mainly degenerating cells with pyknotic nuclei were harvested or if different areas of a tumour comprised clones of cells with varying functional activities. Sampling from several areas would help to overcome these problems. In addition, whether or not the ploidy state of the tumour influences the amount of dye product produced by short hydrolysis is unresolved. It has been suggested that the proportion of rapidly hydrolysable acid-labile DNA in malignant cells does not equate with the total DNA, as estimated in ploidy studies [22], but others have found that the amount of reaction product is proportional to DNA content despite differences in DNA concentration [27].

In conclusion, this study has demonstrated increased amounts of acid-labile DNA in exfoliating cells from oral squamous cell carcinomas compared to cells from healthy and inflamed mucosa. Further studies are required to determine whether the information has prognostic value or if similar DNA can be detected in premalignant lesions. The susceptibility of DNA to hydrolysis could have a role in screening and cytodiagnosis and in monitoring the response of tumours to treatment.

1. Holm LE. Cellular DNA amounts of squamous cell carcinomas of the head and neck region in relation to prognosis. *Laryngoscope* 1982, **92**, 1064–1069.

2. Kokal WA, Gardine RL, Sheibani K, *et al.* Tumour DNA content as a prognostic indicator in squamous cell carcinoma of the head and neck region. *Am J Surg* 1988, **156**, 276–280.
3. Hemmer J, Kreidler J. Flow cytometric DNA ploidy analysis of squamous cell carcinoma of the oral cavity. Comparison with clinical staging and histologic grading. *Cancer* 1990, **66**, 317–320.
4. Burgio DL, Jacobs JR, Maciorowski Z, Alonso MDM, Pietraszkiewicz H, Ensley JF. DNA ploidy of primary and metastatic squamous cell head and neck cancers. *Arch Otolaryngol Head Neck Surg* 1992, **118**, 185–187.
5. Chen RB, Suzuki K, Nomura T, Nakajima T. Flow cytometric analysis of squamous cell carcinomas of the oral cavity in relation to lymph node metastasis. *J Oral Maxillofac Surg* 1993, **51**, 397–401.
6. Balsara BR, Borges AM, Pradham SA, Rajpal RM, Bhisey AN. Flow cytometric DNA analysis of squamous cell carcinomas of the oral cavity: correlation with clinical and histopathological features. *Oral Oncol, Eur J Cancer* 1994, **30B**, 98–101.
7. Doyle JL, Manhold JH. Feulgen microspectrophotometry of oral cancer and leukoplakia. *J Dent Res* 1975, **54**, 1196–1199.
8. Bundgaard T, Sorensen FB, Gaihede M, Sogaard H, Overgaard J. Stereologic, histopathologic, flow cytometric and clinical parameters in the prognostic evaluation of 74 patients with intraoral squamous cell carcinomas. *Cancer* 1992, **70**, 1–13.
9. Saito T, Notani K, Miura H, *et al.* DNA analysis of oral leukoplakia by flow cytometry. *Int J Oral Maxillofac Surg* 1991, **20**, 259–263.
10. Schulte EKW, Joos U, Kasper M, Eckert HM. Cytological detection of epithelial dysplasia in the oral mucosa using Feulgen DNA image cytometry. *Diagn Cytopathol* 1991, 7, 436–441.
11. Grassel-Pietrusky R, Deinlein E, Hornstein OP. DNA-ploidy rates in oral leukoplakias determined by flow cytometry. *J Oral Pathol* 1982, **11**, 434–438.
12. Ogden DR, Cowpe JG, Green MW. Detection of field change in oral cancer using oral exfoliative cytologic study. *Cancer* 1991, **68**, 1611–1615.
13. Kjellstrand P. The influence of natural and induced changes in chromatin on the Feulgen hydrolysis. *Acta Histochemica Suppl B* 1977, **18**, 141–152.
14. Schulte EKW, Wittekind DH. Standardization of the Feulgen reaction: the influence of chromatin condensation on the kinetics of acid hydrolysis. *Anal Cell Pathol* 1990, **2**, 149–157.
15. Zelenin AV, Kushch AA, Cherbance TA. Peculiarities of cytochemical properties of cancer cells as revealed by study of deoxyribonucleoprotein susceptibility to Feulgen hydrolysis. *J Histochem Cytochem* 1977, **25**, 580–584.
16. Millet JA, Husain OAN, Bitensky L, Chayen J. Feulgen hydrolysis profiles in cells exfoliated from the cervix uteri: a potential aid in the diagnosis of malignancy. *J Clin Pathol* 1982, **35**, 345–349.
17. Mays RGW. The Feulgen hydrolysis profiles of a metastatic lung tumour in lymph node and a primary breast carcinoma. *Med Lab Sci* 1987, **44**, 307–311.
18. Mays RG, Elston CW, Ellis IO, Blamey RW, Dowe CS. The Feulgen hydrolysis profile and breast carcinoma: a preliminary study. *Med Lab Sci* 1988, **45**, 40–44.
19. Matthews JNS, Altman DG, Campbell MJ, Royston P. Analysis of serial measurements in medical research. *Br Med J* 1990, **300**, 230–235.
20. Sincock AM, Middleton J. A semi-automated procedure for aiding the diagnosis of cervical neoplasms based on the measurement of acid labile DNA in exfoliated cells. *Int J Cancer* 1983, **31**, 733–736.
21. Sincock AM. Semi-automated diagnosis of cervical intra-epithelial neoplasia grade 2 by the measurement of acid labile DNA in cytologically normal nuclei. *Cancer* 1986, **58**, 83–86.
22. Partington CK, Sincock AM, Steele SJ. Quantitative determination of acid-labile DNA in cervical intraepithelial neoplasia. *Cancer* 1991, **67**, 3104–3109.
23. Rasch RW, Rasch AM. Kinetics of hydrolysis during the Feulgen reaction for deoxyribonucleic acid. A re-evaluation. *J Histochem Cytochem* 1973, **21**, 1053–1065.
24. Trusal LR, Anthony A, Docherty JJ. Differential Feulgen-deoxyribonucleic acid hydrolysis patterns of Herpes simplex virus type 1 and type 2 infected cells. *J Histochem Cytochem* 1975, **23**, 283–288.
25. Salomons JR. Cellular biological studies of human breast tumours and the possible influence of steroid hormones. PhD Thesis, University of London, 1978.
26. Agrell I, Bergqvist H-A. Cytochemical evidence for varied DNA complexes in the nuclei of undifferentiated cells. *J Cell Biol* 1962, **15**, 604–606.
27. Böhm N, Sandritter W. Feulgen hydrolysis of normal cells and mouse ascites tumor cells. *J Cell Biol* 1966, **28**, 1–7.