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Failure to Confirm Presence of SV40 Sequences in Human Tumours

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THE MONKEY polyomavirus, simian virus 40 (SV40), is a potent tumour-inducing virus in laboratory animals. Although SV40 has not been associated with human disease [1, 2], DNA sequences similar to those of SV40 have been found recently in ependymomas and choroid plexus tumours of chidren using a polymerase chain reaction (PCR) technique [3]. Our interest in the possibility that these sequences may be involved in the oncogenesis of other malignancies prompted us to reproduce and further investigate these data.

DNA from ependymomas was provided by H. Budka (Institute of Neuropathology) and an SV40-transformed rat fibroblast cell line by Ch. Czerni (Institute for Cancer Research). DNA was isolated from paraffin-embedded tissue and cell homogenates using standard methods. As DNA in paraffin-embedded tissue can be degraded, isolation of intact DNA was proven by amplification of β-globin sequences. To achieve high sensitivity for the amplification of SV40 DNA, we used a nested PCR protocol with outer primers: SVO for 5'TGATGAATGGGAGCAGTGGTGGAA 3'; SVO.rev 5' CCCACCTGGCAAACTTTCCTCAAT 3', amplifying a 490 bp fragment, followed by a second PCR with inner primers SV.for3 and SV.rev, as used by Bergsagel and associates [3]. The products were analysed on agarose gels and stained with ethidium bromide. Figure 1 shows dilution experiments of DNA isolated from the SV40-positive rat cell line, indicating a detection limit of our assay system of <10 copies of SV40.

In contrast to the findings of Bergsagel and associates [3], SV40-like DNA sequences could not be found in any of the 10 ependymomas investigated. The discrepancy of 10 out of 11 ependymomas positive for SV40-like sequences in the study by Bergsagel versus none in our series could have two possible explanations: firstly, the fact that in our system, the outer 3' primer (SVO.rev) was positioned 307 base pairs downstream of SV.rev, so that if only a shorter sequence was present, there would be no amplification. However, the 490 bp fragment we amplified is situated in a highly conserved region, well within the early region of SV40, which encodes for the T-antigen required for the initiation and presumably maintenance of transformation [4].

Secondly, SV40 might not be found with the same incidence in different areas. SV40 does not naturally infect humans, but it did contaminate polio vaccine in the 1950s and 1960s [1, 2]. If this were the ultimate source of the SV40-like DNA, a different prevalence of SV40 sequences can be expected in various countries. However, since all our ependymoma cases were negative, there is a lack of evidence that the described SV40 sequences [3] play a key role in the pathogenesis of this rare disease.

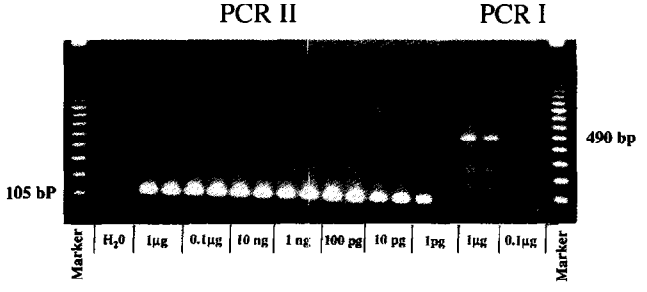

Figure 1. After the first polymerase chain reaction (PCR I) the SV40 DNA (490 bp) was hardly visible when 0.1 µg template was used; after PCR II with the nested primer, the inner product (105 bp) could be adequately detected in 10 pg of total cellular DNA; at 1 pg one of the two reactions was negative.

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Flow Cytometric Gating on Cytokeratin-containing DNA Aneuploid Breast Cancer Cells Improves the Prediction of Recurrence

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INCREASED S-PHASE fraction (SPF), as estimated by flow cytometry, is associated with an increased risk of recurrence for breast cancer patients [1, 2]. However, the analysis with single parameter flow cytometry is influenced by the presence of

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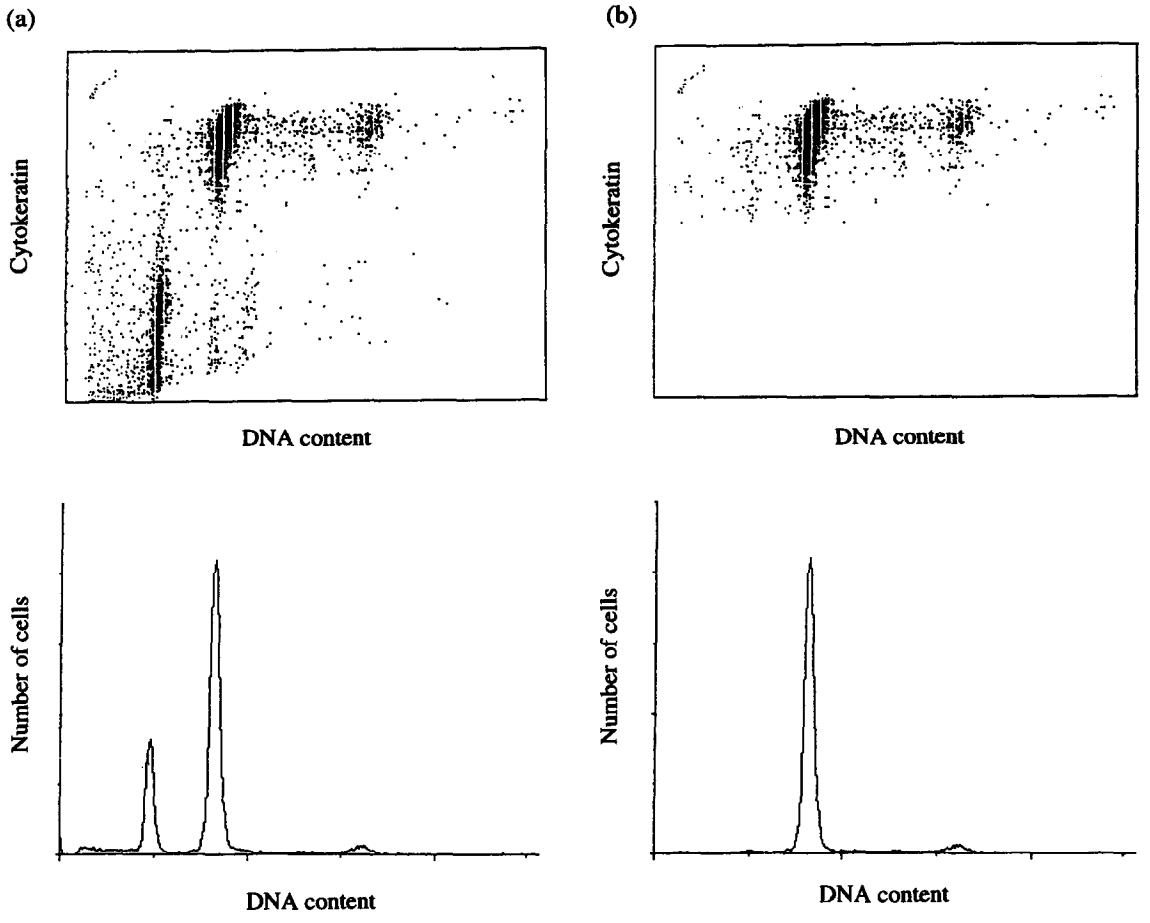

Figure 1. A DNA aneuploid tumour before (a) and after (b) gating on epithelial cells was performed.

inflammatory and stromal cells as well as by residual, non-neoplastic epithelial mammary tissue. These contaminating cells overlap with tumour cells in the DNA histogram, and may introduce artifacts in the calculation of SPF. The use of antibodies specific to cytokeratins, a component of the cytoskeleton of epithelial cells, enables flow cytometric selection of the tumour population, and thereby determination of the SPF without influence from inflammatory and stromal cells [3, 4]. Furthermore, SPF of the cytokeratin-gated cell population, compared to that of the unselected cell population, improves the identification of patients with different risks of recurrence [5–7]. However, the impact of gating in DNA aneuploid tumours has not been evaluated due to the small number of patients under investigation. Thus, the aim of the present extended study was to compare the ability of SPF, determined by a detergent–trypsin method and a cytokeratin method, to predict recurrence in DNA aneuploid breast cancer.

Table 1. *Univariate and multivariate Cox's regression analysis using the upper tertile (in parentheses) as a cut-off value for ungated and cytokeratin gated cell population*

	Number of recurrences*	Univariate *P*	Multivariate *P*
Detergent–trypsin (8.2)	17	0.058	0.69
Cytokeratin (7.3)	20	0.0064	0.046

*Number of recurrences with a SPF above the upper tertile.

The 198 patients with a DNA aneuploid primary breast cancer in pathological stage I–II (UICC) orginated from two cohorts [6, 7]. Median tumour size and median age was 20 mm and 60 years, respectively. 41 patients developed recurrent disease during a median follow-up time of 64 months.

Frozen tissue was cut with scissors in citrate buffer before filtration through a nylon mesh. Cell suspensions were then divided for preparation into single and dual flow cytometric analysis. The samples, used for flow cytometric gating on cytokeratin-containing epithelial cells, were incubated with the primary mouse monoclonal antibody, CAM 5.2 (Becton Dickinson No. 7650) and a secondary fluorescein isothiocyanate-conjugated antibody $F(ab)_2$ (Dakopatts No. F313) [6]. Figure 1 illustrates a DNA aneuploid tumour with and without selection of epithelial cells. The cell suspensions for single parameter analysis were prepared as described by Vindelöv and associates [8]. DNA was stained with propidium iodide in both methods before analysis with a FACscan flow cytometer. Histograms including more than one G0/G1 peak were classified as DNA aneuploid.

When SPF was tested as a continuous variable in Cox's regression analysis, both the detergent–trypsin method ($P = 0.0022$) and the cytokeratin method ($P < 0.0001$) significantly predicted recurrence; however, using multivariate analysis, only the cytokeratin method contributed significantly ($P = 0.0003$). SPF estimated using the cytokeratin method was

also better associated with recurrence when the upper tertile was used as cut-off point (Table 1). In the multivariate Cox's regression analysis, SPF of the gated population contributed prognostic information in addition to SPF of the ungated population when both variables were included in the analysis.

The strengthened correlation with recurrence in the DNA aneuploid breast carcinomas, when using the cytokeratin method, is in concordance with earlier findings in DNA-euploid breast carcinomas [5]. This is probably due to the flow cytometric exclusion of contaminating cells, which may overlap in the DNA aneuploid region and falsely enhances the SPF. The exclusion is indicated by the decreased SPF found with the cytokeratin method in the present study ($P = 0.0002$) as well as by Kimmig and associates [9]. To facilitate comparison between methods, SPF was divided either by the upper tertile, or treated as a continuous variable in the logistic regression analysis and, furthermore, cells were taken from the same suspension for both methods. Our conclusion is that SPF calculated from cytokeratin-positive cells provides prognostic information in addition to ungated S-phase values in DNA aneuploid breast carcinomas.

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Reversible Neurotoxicity During Interleukin-2 Therapy for Metastatic Renal Cell Carcinoma

J.M. van Laar, M.A. van Buchem, N. Weyl and F.J. Cleton

IMMUNOTHERAPY WITH interleukin-2 (IL-2) is commonly employed to treat metastatic renal cell carcinoma (RCC). However, the occurrence of systemic side-effects may lead to discontinuation of therapy. These include fever, hypotension, oliguria and oedema due to a vascular leak syndrome [1]. It is thought that the adverse effects are mediated by the interplay of activated endothelial cells, lymphocytes and natural killer cells as well as cytokines, such as TNFα (tumour necrosis factor). Since the severity of side-effects depends on dosage and mode of administration, current practice favours low dose, subcutaneous (s.c.) administration of IL-2. The present case report demonstrates that even this may lead to (reversible) neurotoxicity.

A 45-year-old female was referred to our hospital in November 1991 because of metastatic RCC. In 1984, she had undergone unilateral nephrectomy and retroperitoneal lymph node dissection for RCC (T3N0M0). After a disease-free interval of 7 years, she presented elsewhere with abdominal discomfort and vaginal bleeding due to an ovarian tumour. Because of this, hysterectomy and ovariectomy were performed. Microscopical examination of the ovarian tumour revealed the presence of metastatic RCC. During follow-up, metastatic RCC was also detected in the left adrenal gland. Therefore, on 4 December 1991, cyclic treatment with OKT3 monoclonal antibody and low-dose s.c. IL-2 (twice daily 3.6×10^6 IU/m^2) was initiated. Although mild fever and anorexia were present initially, the patient did not develop neurological symptoms in this period. The treatment resulted in stable disease until November 1993, when a CT (computed tomography) scan demonstrated an increase of the adrenal metastatis. Consequently a rechallenge with low dose s.c. IL-2 (twice daily 3.6×10^6 IU/m^2 was initiated on 11 November 1993. On day 14 after initiation of therapy, however, the patient complained of headache, disturbed vision, slowness of thought, nausea and vomiting. On day 22, she developed paresis of her left arm, dysarthria and incontinence. On both occasions, physical examination did not reveal additional abnormalities such as hypotension or meningism. Cortisol deficiency was excluded. A CT scan of the brain before and after intravenous administration of contrast medium failed to show any

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