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Detection of Epstein-Barr virus DNA in nasopharyngeal carcinoma using a non-radioactive digoxigenin-labelled probe

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Summary

The presence of Epstein Barr virus (EBV) DNA in biopsies from the post-nasal space (PNS) of patients suspected of nasopharyngeal carcinoma (NPC) was detected by in situ cytohybridisation with an EBV DNA probe labelled with the novel labelling compound digoxigenin. The digoxigenin probe was hybridised to cryostat sections of NPC biopsies and subsequently detected by an enzyme immunoassay procedure. It was found that in situ cytohybridisation using the digoxigenin probe was much more rapid and sensitive (96 h compared to five weeks) than the current method of using ³H-labelled probe. Using the digoxigenin EBV probe, it was found that in all the eighteen NPC biopsies tested, EBV DNA was detected in malignant epithelial cells and infiltrating lymphocytes. EBV DNA was also detected in some normal epithelial cells in these NPC biopsies. EBV DNA was not detected in epithelial cells of non-malignant biopsies.

Digoxigenin; Epstein-Barr virus; Nasopharyngeal carcinoma

Introduction

Nasopharyngeal carcinoma (NPC) is closely associated with the Epstein-Barr virus (EBV). The association was first realised when the majority of NPC patients

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were found to have elevated antibodies to EBV antigens (Old et al., 1966; Henle et al., 1970). Direct evidence of the presence of EBV in NPC was demonstrated by the presence of DNA in the malignant epithelial cells with the use of radioactive EBV probe (zur Hausen et al., 1970; Nonoyama et al., 1973; Wolf et al., 1973; Klein et al., 1974). Recently, there has been much interest in developing non-radioactive labels for probes, since the use of radioactivity carries inherent dangers and inconvenience. A non-radioactive, digoxigenin probe was used successfully for the detection of DNA binding factors using protein-blotting technique (Dooley et al., 1988). We report a study using digoxigenin-labelled EBV DNA in in situ cytohybridisation of NPC biopsies.

Digoxigenin is a hapten steroid, classed under the Cardenolides (C_{23}) group. The deoxyuridinetriphosphate (dUTP) molecule is linked via a space-arm to the digoxigenin at the C_3 position using the random primed DNA labelling method (Feinberg and Vogelstein, 1983). The digoxigenin is labelled to all the *Bam*HI fragments of EBV (Skare and Strominger, 1980) except the O and K fragments due to crossreactions with cellular DNA. The ^3H -labelled EBV probe used in this study was labelled only to the W fragments of EBV.

Materials and Methods

Biopsies

Tissue biopsies from 24 patients with clinical symptoms of NPC from University Hospital Kuala Lumpur were used for this study. Biopsies were taken from the post-nasal space (PNS), snap frozen and stored in liquid nitrogen until use. Snap frozen tonsil specimens were used as the negative tissue controls.

Sections

Sections used in the in situ cytohybridisation were cryostat sections cut at 5 μm thickness using a Reichert Histostat Cryostatmicrotome knife (185 mm).

DNA probes

Probes used in this study were the ^3H -labelled EBV DNA (specific activity: 1.5×10^7 cpm/ μg) and the digoxigenin labelled EBV DNA (20 ng/ μl).

Cell lines

The EBV positive lymphoblastoid lines P3HR-1, Raji and B95-8 were used as positive cell controls and EBV negative lymphoblastoid line BJAB was used as the negative cell control.

Chemicals

All chemical used were of Analar grade or of the highest grade available commercially. Buffers and all solutions used were steam sterilized at 121°C (15 psi) for 20 min. Solutions which were heat labile were sterilized by filtration through sterile membrane filters (0.22 μm pore size).

Pretreatment of slides and coverslips

Slides used in the in situ cytohybridisation technique had to be made certain of their cleanliness and their adhesive surface for tissue sections to adhere securely. 1. Slides were soaked in ethanol:acetone (1:1 v/v) overnight. 2. The following day the slides were baked at 100°C for 1 h. 3. Incubation in a mixture of 3 \times SSC (0.45 M NaCl, 0.045 M tri-sodium citrate dihydrate buffer), 0.02% Ficoll, 0.02% polyvinyl pyrrolidone (PVP), 0.02% bovine serum albumin (BSA) for 3 h at 65°C. 4. Slides were dipped briefly in bidistilled water. 5. Fixation with ethanol:acetic acid (3:1 v/v) for 20 min. 6. Slides were air dried and stored at -70°C. 7. The coverslips were dipped in siliconizing solution, air dried and baked at 100°C for 2 h. 8. Coverslips were stored at room temperature (RT). 9. Tissue sections and cell suspensions were placed on the pretreated slides, air dried, wrapped with aluminium foil and stored at -70°C.

In situ cytohybridisation using the digoxigenin-labelled probe was as follows:

Slides with sections or cells are brought to RT and unwrapped followed by: 1. Fixing in 4% paraformaldehyde for 2 $\frac{1}{2}$ min. 2. Washing twice in PBS/5 mM MgCl₂ for 10 min at RT. 3. Washing once in 2 \times SSC/5 mM EDTA for 30 min at 50°C. 4. Incubation with 1 $\mu\text{g}/\text{ml}$ proteinase K in PBS for 15 min at 37°C. 5. Washing in PBS/0.2% glycine for 10 min at RT. 6. Post-fixation with 4% paraformaldehyde for 10–30 min at RT. 7. Washing twice in PBS/5 mM MgCl₂ for 10 min. 8. Incubation with 50 μl of prehybridisation mixture (6 \times SSC, 45% formamide, 5 \times Denhardt solution and 100 $\mu\text{g}/\text{ml}$ heat denatured salmon testes DNA) for 15 min at RT. 9. The prehybridisation mixture was removed and 20 μl of the hybridisation solution was added on each slide, which consisted of 6 \times SSC, 45% formamide, 5 \times Denhardt solution, 10% Dextran sulphate and 20 ng/slide of the digoxigenin labelled EBV DNA probe. 10. Siliconized coverslips were placed on the slides and sealed with rubber sealant. 11. Slides were placed on a heating block (95°C) for 3–5 min. 12. Immediately the slides were chilled on ice. 13. Slides were placed in a moist chamber and placed on a waterbath of 42°C for 24 h.

Immunological detection

The detection was performed according to the Boehringer method 'DNA labelling and detection kit, nonradioactive' with modifications.

After 24 h the coverslips were removed and: 1. Washed twice for 10 min in 6 \times SSC, 45% formamide at 42°C. 2. Washed twice for 5 min in 2 \times SSC at RT. 3. Washed twice for 10 min in 0.2 \times SSC at RT. 4. Washed in 100 mM Tris-HCl; 150

mM NaCl; pH 7.5 (buffer 1) for 1 min at RT. 5. Slides were incubated with the blocking reagent 0.5% (w/v) in buffer 1 for 30 min at RT. 6. Briefly washed in buffer 1. 7. Antibody-conjugate (vial 8) was diluted to 150 mU/ml (1:5000) in buffer 1. 8. Slides were incubated with 20 ml of the diluted antibody-conjugated solution for 30 min at RT. 9. Slides were washed twice for 15 min in buffer 1. 10. Slides were equilibrated for 2 min with 100 mM Tris-HCl; 100 mM NaCl; 50 mM MgCl₂, pH 9.5 (buffer 3). 11. Colour solution was freshly prepared: 45 µl nitroblue tetrazolium salt (NBT) solution (vial 9) and 35 µl X-phosphate solution (vial 10) were added to 10 ml of buffer 3. 12. Slides were incubated with the colour solution in a narrow plastic container in order to save the solution. The container was sealed and placed in a dark plastic box and left in a dark corner. 13. The colour precipitate started to form within a few minutes and the reaction was completed after one day. 14. The reaction was stopped by washing the slides for 5 min with buffer

TABLE 1

Presence of EBV nucleic acids in NPC biopsies

Cases	Histopathology	EBV genomic status as determined by	
		Digoxigenin probe	³ H probe
P 1	WHO I NPC	++	+
P 2	WHO II NPC	+	-
P 4	WHO II NPC	+++	Not done
P 10	WHO II NPC	+	Not done
P 14	WHO II NPC	+	Not done
P 21	WHO II NPC	+	-
P 9	WHO II & III (mix)	++	+
P 13	WHO II & III (mix)	++	Not done
P 3	WHO III NPC	++	-
P 5	WHO III NPC	++	-
P 6	WHO III NPC	+	-
P 8	WHO III NPC	++	-
P 11	WHO III NPC	++	Not done
P 12	WHO III NPC	+	Not done
P 19	WHO III NPC	Not done	-
P 20	WHO III NPC	+	-
P 24	WHO III NPC	++	++
P 25	WHO III NPC	++	+
P 7	Non-malignant	-	-
P 15	Non-malignant	-	-
P 16	Non-malignant	-	-
P 17	Non-malignant	-	-
P 22	Non-malignant	-	-
P 23	Non-malignant	-	-
P 18	Tonsil	-	-
P3HR1-K	EBV +ve line	+++	++
B95-8	EBV +ve line	++	+
Raji	EBV +ve line	++	+
BJAB	EBV -ve line	-	-

-, Negative; +, slightly positive; ++, moderately positive; + + +, strongly positive.

4 (10 mM Tris-HCl; 1 mM EDTA, pH 8.0). 15. Slides were finally counter stained with aqueous eosin solution and mounted with aqueous mount.

Results

Table 1 shows the results of in situ cytohybridisation using EBV probes labelled with either digoxigenin or ^3H on sections of 24 biopsies from the post-nasal space of patients suspected of NPC. Of the 24 biopsies, 17 were read as NPC on parallel sections stained with haematoxylin and eosin.

Using digoxigenin EBV probe, in all 17 cases of NPC, including the one case of WHO I NPC, EBV positive malignant epithelial cells were detected (Fig. 1). EBV DNA was also detected in infiltrating lymphocytes and in some morphologically normal epithelial cells in the NPC biopsies (Fig. 2). It was observed that the frequency of such cells was low in the NPC biopsies studied. Only sections from one biopsy (out of 17) had consistently distinct EBV DNA staining in the normal epithelial cells. EBV positive cells were randomly found among the cells above the basal layer, with no identifiable areas of concentration of EBV positive cells. None of the six biopsies read non-malignant contained any EBV positive epithelial cells.

A comparative study was carried out on the relative merit of digoxigenin-la-

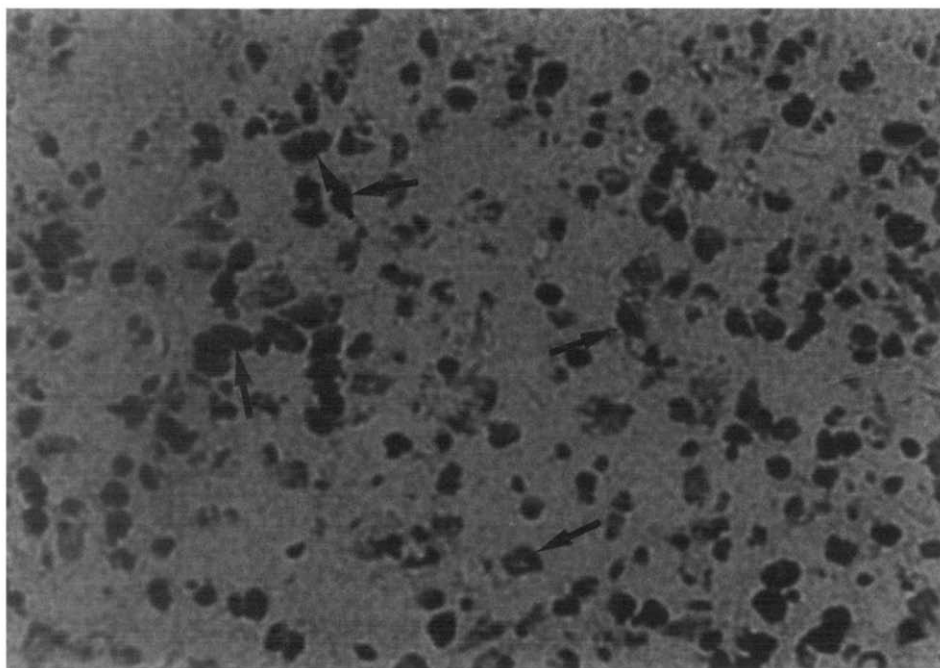


Fig. 1. In situ cytohybridisation using digoxigenin labelled DNA probe on a cross section of a NPC biopsy. (→ Tumour cells containing EBV DNA).

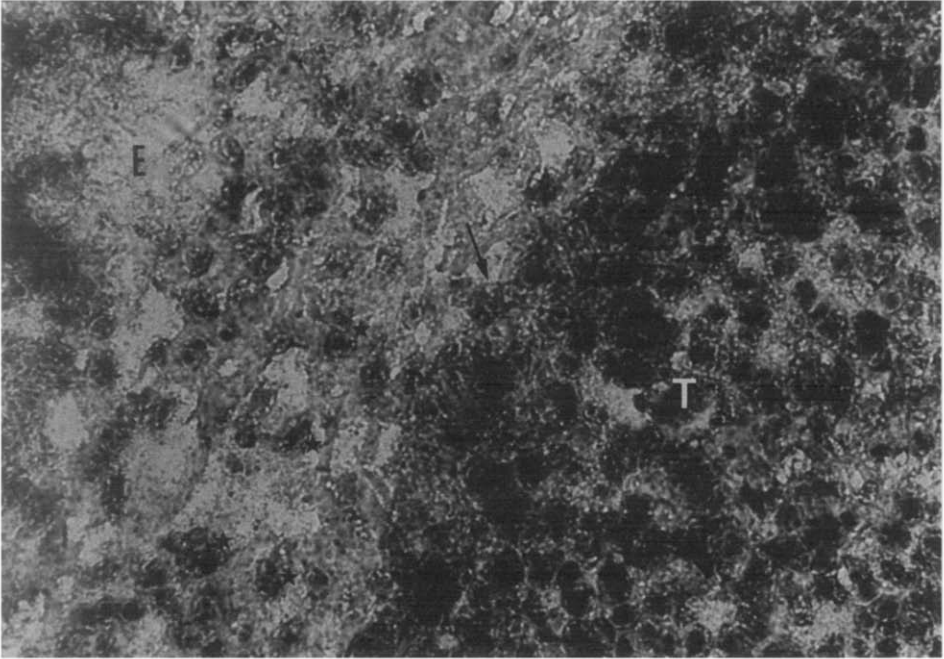


Fig. 2. In situ cytohybridisation demonstrating the presence of EBV DNA in morphologically normal epithelial cells in a cross-section of a NPC biopsy. T, tumour cells containing EBV DNA; →, interface between epithelial basement membrane and stroma; E, epithelium. Note positive cells on basal layer (*).

belled total EBV DNA probe and ^3H -labelled *Bam*HI W EBV probe in in situ cytohybridisation of 11 NPC biopsies. Positive hybridisation was detected in every case when digoxigenin EBV probe was used, but only four stained positive with ^3H probe. All six non-malignant biopsies tested negative for EBV DNA in the epithelial cells by both digoxigenin and ^3H probes.

There were no false positive reactions with either probe, since negative controls (tonsil and BJAB) which were always included in the test consistently tested negative. There was background staining in the ^3H technique but this could be identified as such staining was not confined to intracellular spaces. There was no background staining in the digoxigenin technique.

Discussion

Results of in situ cytohybridisation using digoxigenin-labelled EBV probe demonstrated that it is a sensitive and specific method for the detection of EBV positive cells in NPC biopsies. The method reduced the experimental time of 5 weeks required for in situ cytohybridisation using ^3H -labelled EBV probe to 96 h. It is a much more sensitive method, detecting EBV DNA positive cells in 100% (17/17)

compared to 36% (4/11) of NPC biopsies tested. It is specific since negative controls of tonsil and EBV negative lymphoblastoid cells were consistently negative. Being a nonradioactive method, it eliminates the risk of radioactivity. Since all the seventeen NPC biopsies tested were positive for EBV DNA and all the six non-malignant biopsies were negative for EBV DNA, *in situ* cytohybridisation by digoxigenin-labelled EBV probe is of diagnostic potential for NPC.

Morphologically epithelial cells positive for EBV DNA were detected in one of the NPC biopsies. No such cells were detected in non-malignant biopsies from the post-nasal space. Whether the observation has any significance with respect to the etiopathogenesis of NPC remains to be elucidated.

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