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Attempts to Detect Virus-Specific DNA Sequences in Human Tumors

III. Epstein-Barr Viral DNA in Non-Lymphoid Nasopharyngeal Carcinoma Cells

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Abstract. Fourteen tumors of the nasopharyngeal region were analyzed for the presence of Epstein-Barr virus-specific DNA by DNA-cRNA hybridization. These data were compared to the histology of the respective tumors and the seroreactivity of the tumor-bearing patient against EBV-related antigens. With one exception, all tumor pieces containing nasopharyngeal carcinoma cells hybridized significantly with EBV-cRNA. Tumors of predominantly epithelial morphology annealed in the highest range. *In situ*-hybridizations of freeze sections from a tumor containing almost equal amounts of tumor cells and lymphocytes revealed hybridizing DNA within nuclei of non-lymphoid cells. Although these data do not exclude the presence of EBV-DNA within lymphoid cells, they clearly demonstrate that in nasopharyngeal carcinomas the vast majority of EBV-specific DNA rests within non-lymphoid cells.

Introduction

The regular association of Epstein-Barr virus (EBV) with nasopharyngeal carcinoma (NPC) has been demonstrated repeatedly. Patients with nasopharyngeal carcinomas reveal high antibody titers against EBV-specific antigens (Old *et al.*, 1966; de Schryver *et al.*, 1969; Henle *et al.*, 1970; de Thé *et al.*, 1973) and EBV-specific nucleic acids have been observed in most biopsies examined by nucleic acid hybridizations (zur Hausen *et al.*, 1970; Nonoyama and Pagano, 1973; Wolf *et al.*, 1973; zur Hausen *et al.*, 1974b).

Since nasopharyngeal carcinomas represent tumors containing varying admixtures of lymphocytes and connective tissue (reviewed by Shanmugaratnam, 1972) it is of obvious interest to localize and to characterize those cells which harbor EBV-genetic information. The presence of EBV-DNA in a variety of lymphoblastoid cells (zur Hausen and Schulte-Holthausen, 1970; Nonoyama and Pagano, 1971; zur Hausen *et al.*, 1972) suggested to some investigators a passenger role of EBV in NPC by assuming the presence of viral DNA within infiltrating lymphocytes rather than within the epithelial tumor cells (McAllister, 1973).

In a previous report (Wolf *et al.*, 1973) we reported the presence of apparently EBV-specific DNA in epithelial cells of two tumors as demonstrated by *in situ*-hybridizations and anti-complementary immunofluorescence. The following is an

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extension of these studies. Additional tumor biopsies were analyzed histologically and their histology was compared to filter hybridizations with EBV-specific complementary RNA (cRNA). In addition, some of them were subjected to *in situ*-hybridizations. The results confirm the interpretation of previous data: EB viral DNA rests at least predominantly within the nuclei of non-lymphoid nasopharyngeal tumor cells.

Materials and Methods

The preparation of DNA from tumor biopsies has been described (zur Hausen *et al.*, 1970; Wolf *et al.*, 1973). Nucleic acid hybridizations were performed as reported elsewhere (zur Hausen *et al.*, 1974a).

In situ-Hybridization

The procedure of Gall and Pardue (1971) was followed with minor modifications. Freeze sections (10 μm) of biopsy material were air dried and fixed for 30 min in a solution of 25% acetic acid and 75% methanol. After fixation the slides were transferred for 2 min into 45% acetic acid and kept twice for 10 min each in ethanol. After air drying the sections were covered by RNase solution (100 μg/ml in 2× SSC) and kept for 30 min at 34°C, then washed four times in 2× SSC for 10 min each and in addition for 10 min in 70% ethanol and then in 100% ethanol. After additional air drying they were treated with 0.07N KOH for 3.5 min, washed twice for 5 min each with phosphate-citrate (PC)-buffer (pH 5.75) and then again for 5 min in a 1:1 mixture of ethanol-PC-buffer. Thereafter they were transferred into 100% ethanol for 5 min and then placed into a fresh solution of ethanol for additional 10 min.

Hybridization with EBV-specific cRNA (zur Hausen *et al.*, 1974b) was performed with 50000 cpm of cRNA in 50 μl of a solution containing 2.5× SSC, 50% formamide, and 0.05% sodium-dodecylsulfate under a coverslip (24×36 mm) for 4 days at 45°C. Thereafter the coverslip was washed off with 2× SSC and the slide then washed 4 times with 2× SSC (5 min each), treated with RNase (30 μg/ml in 2× SSC) at 34°C for 30 min, and washed again 5 times with 2× SSC. After 2 consecutive washings with destilled water for 1 min, the slides were kept in 70% ethanol (5 min), 100% ethanol (5 min), and again in 100% ethanol (10 min).

After air drying the slides were exposed to autoradiography by using an Ilford G 5 emulsion. The procedure has been described before (Wolf *et al.*, 1973). After 2 weeks of exposure the slides were developed and stained as described before.

Indirect Immunofluorescence

Methods to determine EBV-specific antibodies against viral capsid antigens (VCA) and R- and D-antigens of the early antigen (EA)-complex have been described (Henle *et al.*, 1971).

Results

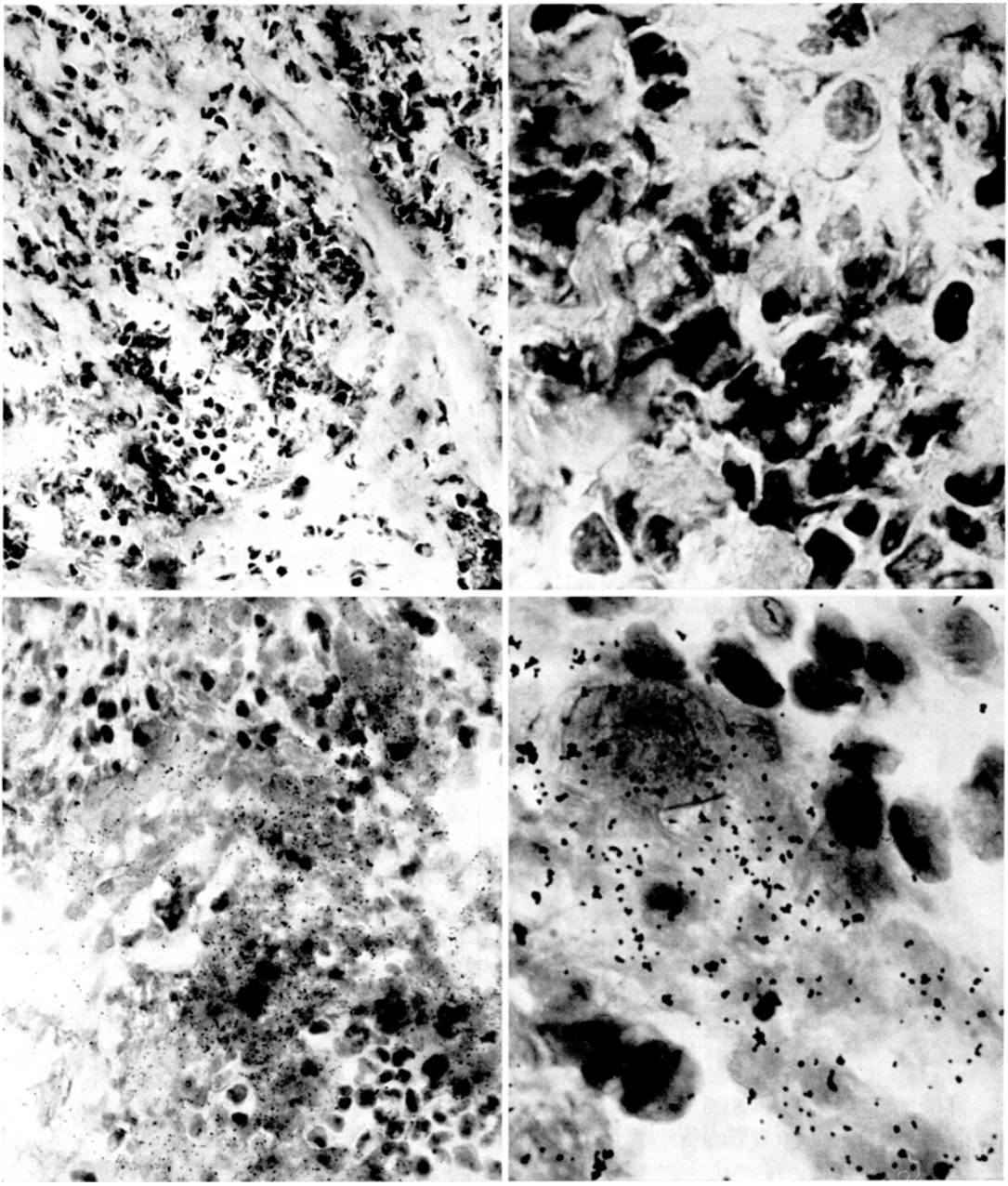
Table 1 summarizes the histological characteristics of 14 African tumors which were derived from the nasopharyngeal region and compares the nucleic acid hybridization data obtained by filter hybridization of 50 μg of tumor DNA with 50000 cpm of EBV-cRNA with the histology of the respective tumors and the seroreactivity of the tumor-bearing patients against EBV-specific antigens. The tumors represent unselected biopsies of which pieces were hybridized before histological examination of the remaining part.

All histologically typical nasopharyngeal carcinomas (tumors No. 1, 3, 5, 10, 13) hybridize clearly above the range of non-NPC-tumors (tumors No. 2, 6—8, 14) with the exception of tumor No. 11. In this case the hybridization was rather low despite the predominance of non-lymphoid carcinoma cells. All other highly hybridizing nasopharyngeal carcinomas represent in their majority epithelial

Table 1. Comparison of tumor histology with hybridization data of tumor DNA with EBV-cRNA and the serology of the tumor-bearing patient against EBV-specific antigens

Tumor No.	Histological diagnosis	cpm hybridized	EBV-serology of patient		
			VCA	EA	
				D	R
1 KCC 1496	Nasopharyngeal carcinoma, approx. 60% of epithelial morphology, remaining cells mainly lymphoc.	1740	480[a]	60[a]	N.T.
2 KCC 1499	Squameous epithelium, rather ca. *in situ*, with some infiltrations into deeper layers.	62	160	0	0
3 KCC 1497	NPC, considerable content of connective tissue, few lymphocytes only.	830	1280	320	N.T.
4 KCC 1497	Same patient as No. 3. This piece contains exclusively salivary gland tissue. Apparently paratumorous tissue of post-nasal space.	84	1280	320	N.T.
5 KCC 1377	NPC. Predominantly epithelial, about 20% lymphocytes. Some salivary gland tissue.	940	320	160	N.T.
6 40316	Squameous cell carcinoma No keratinization. No lymph. infiltration	69 80	1070	20	210
7 43632	Lymphatic tissue without ca.—cells. Numerous giant cells at the periphery.	68	N.T.	N.T.	N.T.
8 KCC 1519	Squameous cell carcinoma. Some keratinization, no lymph. infiltrat.	55	40	0	0
9 36187	Only necrotic and connect. tissue. No tumor cells detectable	77	640	40	160
10 46306	NPC. Approx. equal amounts of epithel. and lymph. cells. Some giant cell formation.	374 460	640	320	N.T.
11 KCC 845	NPC. Predominantly epithelial. Lymphatic infiltration only at the periphery.	101	640	20	N.T.
12 KCC 845	Same patient as No. 11. This piece contains almost no tumor tissue. Extensive scarification. Probably previous irradiation.	74	640	20	N.T.
13 KCC 1528	Predominantly epith. tumor with almost no lymph. infiltration. Almost pure carcinoma.	273 273	1280	640	N.T.
14 KCC 1535	No NPC. Contains some glandular tissue. Reticulosis, lymphoma.	55	80	0	0
Human embryonic lung fibroblasts	normal tissue, control	50 57	—	—	—

[a] Geometric mean of three tests performed with different sera of the same patient.



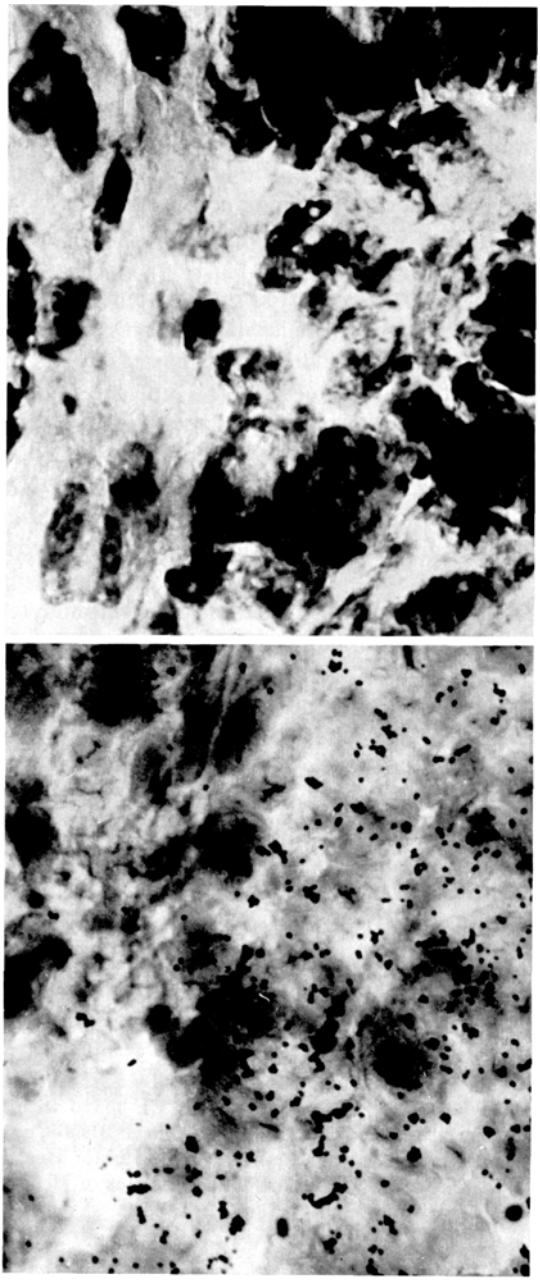


Fig. 1. Serial sections of a nasopharyngeal carcinoma biopsy. The right picture represents a freeze section without denaturation, the left figure the corresponding area after denaturation and *in situ*-hybridization with EBV-cRNA. Giemsa staining

tumor cells. The sera of all patients with tumors which hybridized with EBV-DNA reacted at high titers against EBV-specific antigens.

For *in situ*-hybridizations a tumor was selected which contained almost equal amounts of epithelial and lymphatic cells (Tumors No. 10). A non-hybridizing tumor (tumor No. 14) was treated identically and served as control.

The result is shown in Fig. 1. This figure shows the same histological region in consecutive sections before and after denaturation and hybridization with cRNA. It is obvious that regions showing cells with large pale nuclei are preferentially labelled which correspond to non-lymphoid cells in the non-denatured section. No labelling was observed in sections of the non-hybridizing control tumor.

Discussion

In a previous publication (Wolf *et al.*, 1973) we reported results which showed the specific association of EB viral genomes with epithelial nasopharyngeal carcinoma cells. The present study confirms and further clarifies this association. The labelling of non-lymphoid cells appears to be confined to the nuclear region, although the denaturation procedure affects the morphology of the cells. The data do not exclude that some of the lymphoid cells in nasopharyngeal tumor material also carry viral genomes. They show, however, that most of the virus-specific DNA which has been revealed by filter hybridizations is located within non-lymphoid cells. These results are also supported by filter hybridization experiments with DNA from Ficoll-separated lymphocytes and epithelial cells from NPC-materials (Desgranges *et al.*, 1974) in which the preferential annealing of lymphocyte-depleted cells was obvious, whereas isolated lymphocyte DNA did not anneal significantly.

The presence of EBV-DNA within the non-lymphoid carcinoma cells of NPC tumors substantially supports the specific role of EBV in this malignancy (zur Hausen, 1975). The regular presence of viral DNA within this tumor has been confirmed repeatedly (zur Hausen *et al.*, 1970; Nonoyama and Pagano, 1973; zur Hausen *et al.*, 1974b; Wolf *et al.*, 1975).

The presence of viral DNA in epithelial tumor cells raises questions on the mode of infection of these cells. Until now EBV had only been observed to infect B-type lymphocytes. Besides the possibility that specialized epithelial cells of the nasopharynx which cannot be grown *in vitro* up to now, bear receptors for EBV, fusion of epithelial cells with EBV-genome carrying lymphoblasts (Glaser and Rapp, 1972) still remains a possible mechanism.

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