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NASOPHARYNGEAL CARCINOMA. X. PRESENCE OF EPSTEIN-BARR GENOMES IN SEPARATED EPITHELIAL CELLS OF TUMOURS IN PATIENTS FROM SINGAPORE, TUNISIA AND KENYA

by

C. DESGRANGES [1], H. WOLF [2], G. DE-THÉ [1], K. SHANMUGARATNAM [3], N. CAMMOUN [4], R. ELLOUZ [4], G. KLEIN [5], K. LENNERT [6], N. MUÑOZ [1] and H. ZUR HAUSEN [2]

[1] *International Agency for Research on Cancer (IARC), 150 Cours Albert Thomas, 69008 Lyons, France;* [2] *Institut für Klinische Virologie der Universität Erlangen, 852 Erlangen, Loschgestrasse 7, Federal Republic of Germany;* [3] *Department of Pathology, University of Singapore, Outram Road, Singapore 3;* [4] *Institut Salah Azaiz, Tunis, Tunisia;* [5] *Department of Tumour Biology, Karolinska Institutet, Stockholm 60, Sweden;* [6] *Institut für Pathologie, University of Kiel, Kiel, Federal Republic of Germany.*

Nasopharyngeal carcinoma (NPC) biopsies from Singapore, Tunisia and Kenya were compared, before and after separation of epithelial and lymphoid cells, for their EBV-DNA content, using the cellular DNA-EBV-cRNA hybridization test. In all instances where successful separation of the two cell types was achieved, epithelial tumour cells showed a higher EBV-DNA content than lymphoid cells or tumour before cell separation. It is, therefore, suggested that EBV-DNA is mostly limited to epithelial cells. No significant difference was observed between NPC tumours originating from various geographical areas.

The presence of Epstein-Barr virus (EBV) in nasopharyngeal carcinoma (NPC) has been repeatedly demonstrated by various nucleic-acid hybridization techniques (zur Hausen *et al.*, 1970; Nonoyama *et al.*, 1973; Wolf *et al.*, 1973; zur Hausen *et al.*, 1974; Wolf *et al.*, 1975). Recently, virus-specific nuclear antigens (EBNA) have also been detected by immunofluorescence in NPC biopsies and specifically in the epithelial tumour cells (Wolf *et al.*, 1973; de-Thé *et al.*, 1973*a*; Huang *et al.*, 1974; Klein *et al.*, 1974).

Taken together with the above, the specific serological response of NPC patients to EBV (reviewed by Klein, 1973 and de-Thé and Geser, 1974) points to a role of EBV in the development of this malignancy.

The aim of the present study was: (1) to compare the EBV DNA content in separated epithelial and lymphoid cell subpopulations from tumour pieces; and (2) to compare NPC biopsies from different geographical areas and ethnic groups for their EBV DNA content.

MATERIAL AND METHODS

Tumour material

Biopsies from tumours of the nasopharynx were obtained from three geographical areas as follows: (1) 28 tumours from the Cancer Institute Salah Azaiz, Tunis; (2) 17 tumours from Kenya; (3) 16 tumours from Singapore. Fifteen tumours other than NPC were obtained from these

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different areas and grouped as controls. We are indebted to Dr. Williams (Kuluva Hospital, Arua, Uganda) and Dr. Brubaker (Shirati Hospital, Musoma, Tanzania) for providing Burkitt lymphoma (BL) biopsies tested in the present study.

The histopathological diagnosis of all tumours was obtained from each institute. Upon receipt at IARC, a small piece of each biopsy was processed to evaluate histopathologically the tumour pieces used for DNA extraction.

Biopsies from Tunis arrived in the laboratory within 24 h, and had been kept in RPMI tissue culture medium at 10 to 12° C between the time of removal and arrival. Tumours from Kenya were sent in tissue-culture medium at +4° C and arrived usually between 48 and 72 h after departure. Tumours from Singapore were frozen after removal and sent to Lyons in dry ice.

Separation of epithelial and lymphoid cells from biopsies

After a small portion had been taken for histopathology as noted above, each tumour was divided into two parts: one was stored at −70° C until DNA extraction, the other was manipulated in tissue-culture medium (RPMI 1640 with 10% fetal calf serum) with mounted needles so that lymphoid and epithelial cells were liberated into the medium. The degree of dissociation was followed by phase-contrast microscopy and if this dissociation was not complete, separation of clumped cells was achieved by trypsin treatment (0.25% for 30 min at 37° C). After complete

TABLE I

HYBRIDIZATION OF DNA FROM TOTAL TUMOURS AND SEPARATED CELL PREPARATIONS FROM NPC BIOPSIES WITH EBV-cRNA

Tumours		Total tumour— CPM hybridized	Epithelial cells— CPM hybridized	Lymphoid cells— CPM hybridized [1]
Origin	Reference No.			
Singapore	S 19038	329.1	1,627.0	—
Kenya	K 74559	48.8	727.0	201.6
	K 75452 (LN) [2]	1,669.2	1,528.4	—
	K 81457	233.1	377.0	30.3 [3]
	K 85372 (LN)	648.4	2,159.3	183.85
	K 88527	24.9	315.7	30.3 [3]
	K 89017	1,074.9	3,900.4	114.8
	K 89017 (LN)	145.5	1,337.0	30.3 [3]
	K 93595	389.2	2,240.3	—
Tunisia	Tu 280	333.2	492.5	—
	Tu 333	76.7	187.2	69.2
	Tu 367	144.4	283.2	106.0
	Tu 369	93.2	293.2	—
	Tu 370	63.2	300.4	—
	Tu 397	30.8	106.8	42.4
	Tu 409	253.1	453.9	—
	Tu 411	222.4	497.7	—

Controls:	CPM hybridized [4]
HeLa	80.0
Cord blood lymphocytes	93.6
Raji	2,094.5
HR1K	9,192.2

[1] Tumours from which less than 50 μg of DNA were yielded by lymphoid cell sub-populations.
[2] LN = cervical lymph-node metastasis.
[3] These three preparations were mixed to obtain 50 μg of DNA.
[4] Mean of three experiments.

dissociation, the cell suspension was carefully layered on a Radioselectan Ficoll gradient (1 vol of 31% Radioselectan with 2.4 volumes of 9% Ficoll solution) as previously described (Yata *et al.*, 1973). After 30 min of centrifugation at $400 \times g$, the epithelial cells sedimented to the bottom, whereas the lymphocytes remained on top of the Radioselectan Ficoll layer. The lymphocyte-enriched layer was found to contain more than 90% lymphocytes whereas the pellet contained approximately 80% epithelial cells. After removal and washing with PBS, these cell subpopulations were stored at −70° C until DNA extraction.

DNA extraction

DNA extraction was performed from total tumours, and from each separated cell subpopulation, following techniques described previously (zur Hausen and Schulte-Holthausen, 1970; Wolf *et al.*, 1973).

Hybridization with EBV-specific cRNA

EBV-specific cRNA was prepared as previously described (zur Hausen *et al.*, 1972). Fifty μg of DNA from each cell sample were heat-denaturated, bound to nitrocellulose membrane filters and then kept for 4 h at 80° C. After addition of 57,000 CPM of denaturated cRNA in a total volume of 1 ml containing 48% formamide 2.5×SSC (sodium chloride, sodium citrate) and 0.05% SDS (sodium dodecylate sulphate) the filters were incubated for 6 days at 45° C. After washing with 2×SSC, filters were treated with RNase (20 μg/ml) for 60 min at 37° C, washed again and counted in a Packard liquid spectrometer type 3375.

EBV serology

VCA antibody determination was performed with the Jijoye cell line containing approximately 5% of immunofluorescent cells according to Henle and Henle (1966). Antibodies to EA were determined by using Raji cells 3 days after superinfection with P3HR-1 virus (Henle *et al.*, 1970). Complement-fixing (CF) antibodies were titrated with soluble antigen (S) extracts from the Raji cell line (de-Thé *et al.*, 1973*b*). Titration of EBNA antibodies on Raji cells followed the techniques described by Reedman and Klein (1973) with minor modifications.

RESULTS

Table I presents the hybridization data obtained by annealing DNA from separated epithelial and lymphoid cells with EBV-cRNA. On comparison of these data with hybridization rates obtained with DNA from cord blood lymphocytes or HeLa cells, nine out of 17 NPC tumour specimens showed high levels of hybridization with EBV-cRNA. DNA from the epithelial cell preparations annealed in 15 out of 17 specimens at significantly higher rates than the DNA from the same total tumour. For some lymphoid subpopulations insufficient DNA was available for hybridization tests. However, in all instances where lymphoid subpopulations were tested, the hybridization rate was always lower than that of corresponding epithelial subpopulations.

Tables II, III and IV show hybridization data obtained from total biopsy for all tumour specimens obtained from each area. In most of the cases, separation of epithelial and lymphoid cells was either not successful or not possible because of the small size of the original biopsy. In these Tables, arranged according to geographical areas, the histopathological characteristics, including the degree of differentiation, the amount of lymphoblastoid infiltration and of stroma, are given together with the EBV serology. The level of hybridization showed great variations within each series of tumours. Eleven out of the 28 specimens from Tunisia, 6 out of the 16 from Singapore and 12 out of the 17 from Kenya revealed significantly elevated annealing rates as compared to the controls.

Table V gives the results for tumours other than nasopharyngeal carcinoma, but collected from the same geographical areas. In this group of " other tumours " only 2 out of 16 showed significant levels of hybridization, both being from BL cases.

Tables II to V also reveal the EBV seroreactivity of the corresponding patients. EA antibody titres only appear to correspond to a certain extent to the hybridization data: in Kenyan patients high EA titres corresponded to high annealing rates, while low titres related to low rates; in Tunisian patients with high EA titres no such pattern emerged, whereas patients with low EA titres also had low hybridization rates; in Singapore patients no relationship was apparent.

TABLE II

NPC BIOPSIES FROM TUNISIA
HYBRIDIZATION OF NPC-DNA WITH EBV-cRNA

Tumour No.	Sex/Age	Histopathological characteristics		Proportion of various cell types [2]			CPM hybridized	EBV serology			
		Degree of differentiation [1]	Degree of lymphoid infiltration [1]	Ep.	Ly.	Str.		VCA	EA	CF/Raji	EBNA
Tu 152	F/16	Undiff. ca. [3]	Marked	10	40	50	35.8	— [4]	—	—	—
Tu 154	M/47	Undiff. ca.	Moderate	5	60	35	58.0	>2,560	2,560	128	>20,000
Tu 156	M/18	Well diff. ca.	Moderate	30	40	30	149.2	320	<10	—	1,280
Tu 185	F/43	Undiff. ca.	Moderate	5	45	50	677.2	—	—	—	—
Tu 187	F/47	Undiff. ca.	Mild	5	40	55	981.5	—	—	—	—
Tu 206	M/65	Fusif. undiff. ca.	None	0	0	98	140.5	⩾2,560	1,280	<4	40
Tu 221	F/60	Undiff. ca.	Moderate	0	40	60	77.7	—	—	—	—
Tu 226	M/50	Undiff. ca.	Moderate	30	20	50	1,499.4	>2,560	640-1,280	64	512
Tu 231	F/19	Undiff. ca.	Mild	ND [5]	ND	ND	256.1	>2,560	320-640	⩾2,560	2,560
Tu 279	F/48	Undiff. ca.	Mild	40	20	40	91.7	>2,560	40-80	128-255	2,560
Tu 280	M/15	Undiff. ca.	Moderate	40	10	50	333.2	1,280	160-320	128	2,560
Tu 282	M/30	Undiff. ca.	Mild	40	10	50	343.8	⩾2,560	320-640	<8	20
Tu 296	M/40	Undiff. ca.	Moderate	0	5	95	268.8	—	—	—	—
Tu 297	F/18	Undiff. ca.	Moderate	5	15	80	334.6	—	—	—	—
Tu 333	F/12	Fusif. undiff. ca.	Moderate	ND	ND	ND	76.7	—	—	—	—
Tu 334	M/50	Undiff. ca.	Moderate	ND	ND	ND	67.4	1,280	20	128	2,560
Tu 336	F/45	Undiff. ca.	Mild	ND	ND	ND	49.7	1,280	160	128	2,560
Tu 338	M/14	Undiff. ca.	Moderate	30	30	40	107.3	—	—	—	—
Tu 340	M/39	Undiff. ca.	Moderate	0	0	100	72.9	2,560	320	32-64	1,280
Tu 367	M/14	Undiff. ca.	Mild	—	—	—	144.4	640	<10	⩾256	>5,120
Tu 369	F/39	Undiff. ca.	Mild	—	—	—	93.2	640	160	⩾256	>5,120
Tu 370	M/48	Undiff. ca.	Mild	—	—	—	63.2	—	—	—	—
Tu 397	M/61	Undiff. ca.	Mild	80	5	15	30.8	—	—	—	—
Tu 404	M/55	Undiff. ca.	Marked	30	40	30	19.6	—	—	—	—
Tu 409	M/18	Undiff. ca.	Moderate	30	20	50	253.1	⩾2,560	640	64	640
Tu 411	F/41	Undiff. ca.	Mild	40	50	10	222.4	—	—	—	—
Tu 414	F/47	Undiff. ca.	Mild	No tumour tissue			105.2	—	—	—	—
Tu 416	M/26	Undiff. ca.	Mild	ND	ND	ND	309.1	—	—	—	—

[1] Obtained from the Pathology Dept., Tunis, from a biopsy different from that used for DNA extraction.
[2] Estimated from sections made from biopsy received in Lyons and used for DNA extraction. Proportional amount of epith. tumour cells (Ep.), of lymphoid cell (Ly) and of stroma (Str.).
[3] Undifferentiated carcinoma.
[4] — = no serum available.
[5] ND = not done.

TABLE III

NPC BIOPSIES FROM SINGAPORE
HYBRIDIZATION OF NPC-DNA WITH EBV-cRNA

Tumour No.	Sex/Age	Histopathological characteristics		Proportion of various cell types [2]			CPM hybridized	EBV serology			
		Degree of differentiation [1]	Degree of lymphoid infiltration [1]	Ep.	Ly.	Str.		VCA	EA	CF/Raji	EBNA
S 1242	M/27	Poor, basaloid features	Marked	ND [3]	ND	ND	186.6	>2,560	640	>320	>5,120
S 1479	M/25	Doubtful squam. ca.	Marked	30	—	70	268.9	1,280	20	<10	40
S 17633	M/65	Poor	Mild	40	10	50	52.4	— [4]	—	—	—
S 19038	M/38	Undiff. ca.	None	ND	ND	ND	329.1	⩾2,560	1,280	40	5,120
S 19208	M/67	Undiff. ca.	None	20	—	80	96.0	640	<10	10	80
S 20539	M/37	Undiff. ca.	Moderate	60	20	20	385.8	>2,560	640	40-80	1,280
S 20719	M/58	Undiff. ca.	Moderate	30	10	60	104.6	—	—	—	—
S 20720	F/27	Undiff. ca.	Moderate	50	40	10	88.0	2,560	640	320	2,560
S 21053	F/41	Undiff. ca.	Moderate	50	20	30	148.8	1,280	40	80-160	5,120
S 21229	F/61	Undiff. ca.	Marked	30	10	60	214.5	1,280	80	80	5,120
S 21564	M/42	Undiff. ca.	Mild	10	20	70	65.2	2,560	320	80	640
S 21684	M/?	Well-diff. extensive ca. *in situ*	None	10	40	50	70.0	—	—	—	—
S 22074	F/36	Doubtful	Moderate	60	5	35	379.4	—	—	—	—
S 22596	F/49	Doubtful	Marked	80	10	10	1,050.9	—	—	—	—
S 22598	M/25	Undiff. ca.	Moderate	30	30	40	149.2	—	—	—	—
S 22784	M/60	Undiff. ca.	Marked	20	10	70	135.0	640	320	320	>5,120

[1] Based on biopsy examination in Singapore on specimen different from that used for DNA extraction.

[2] Estimated sections made from tumour specimens received in Lyons and used for DNA extraction. Proportional amount of epith. tumour cells (Ep.), of lymphoid cell (Ly) and of stoma (Str.).

[3] ND = not done.

[4] — = no serum available.

TABLE IV

NPC BIOPSIES FROM KENYA
HYBRIDIZATION OF NPC-DNA WITH EBV-cRNA

Tumour No.	Sex/Age	Site of biopsy	Proportion of various cell types [1]			CPM hybridized	EBV serology			
			Ep.	Ly.	Str.		VCA	EA	CF/Raji	EBNA
K 15280	F/60	Nasopharynx	80	10	10	1,019.2	— [2]	—	—	—
K 34621	M/50	Cervical lymph-node	30	30	40	496.4	1,280	320	20	1,280
K 36140	M/18	Cervical lymph-node	Few malignant cells			383.4	640	160	20-40	640
K 55401	M/40	Cervical lymph-node	0	20	80	117.4	—	—	—	—
K 56816	M/50	Nasopharynx	0	0	100	123.0	640	640	40	1,280
K 57833	F/46	Cervical lymph-node	30	0	70	267.8	—	—	—	—
K 58045	M/29	Cervical lymph-node	20	10	70	491.1	>2,560	640	40-80	2,560
K 74559	M/73	Nasopharynx	ND [3]	ND	ND	48.8	>2,560	1,280	80-160	2,560
K 75452	M/16	Cervical lymph-node	ND	ND	ND	1,669.2	2,560	320	80-160	2,560
K 76128	F/45	Nasopharynx	ND	ND	ND	47.4	320-640	20	20	640
K 78638	F/22	Nasopharynx Cervical lymph-node	ND	ND	ND	302.0 435.8	>2,560	>2,560	⩾160	>5,120
K 81457	M/51	Nasopharynx	ND	ND	ND	233.1	1,280-2,560	320-640	20	640
K 85372	F/33	Cervical lymph-node	5	10	75	648.4	>2,560	>2,560	80-160	2,560
K 88527	M/44	Nasopharynx	5	0	95	24.9	320	80	10	80
K 89017	M/50	Nasopharynx Cervical lymph-node	40 60	20 20	40 20	1,074.9 145.5	—	—	—	—
K 93595	M/41	Nasopharynx	20	20	60	389.2	⩾2,560	⩾2,560	40	640
K 93596	M/50	Nasopharynx	20	—	80	83.1	160	<10	20	—

[1] Based on histopathological sections done with biopsy specimen used for DNA extraction.
[2] — = no serum available.
[3] ND = not done.

TABLE V

TUMOURS OTHER THAN NPC
HYBRIDIZATION OF DNA FROM TOTAL TUMOURS WITH EBV-cRNA

Origin	Reference number	Sex/Age	Histological characteristics	CPM hybridized	EBV serology			
					VCA	EA	CF/Raji	EBNA
Kenya	K 51321	M/35	Adenoid carcinoma	30.6	20-40	<10	10	160
	K 77905	M/63	Hypopharynx carcinoma	47.0	— [3]	—	—	—
	K 84825	M/45	Tumour of palate	78.6	640	<10	<5	320
	K 91339	F/28	Hypopharynx carcinoma	82.9	80	<10	10	160
Tanzania	T 754	M/12	Burkitt's lymphoma	2,194.1	—	—	—	—
	T 757	M/6	Neuroblastoma	72.3	—	—	—	—
Uganda	U 202	F/5	Burkitt's lymphoma	546.1	320	1,280	160	1,024
Tunisia	Tu 190	F/65	Nasopharyngeal lymphosarcoma	52.1	—	—	—	—
	Tu 227	M/25	Nasopharynx negative biopsy [1]	81.1	320	<10	AC	10
	Tu 229	M/71	Plasmocytoma of nasopharynx	158.7	320	<10	16	320
	Tu 230	M/57	Nasopharynx negative biopsy [1]	73.2	320	<10	64	640
	Tu 232	M/54	Nasopharynx negative biopsy [2]	68.2	320-640	<10	AC	80
	Tu 288	M/38	Larynx carcinoma	114.8	—	—	—	—
	Tu 368	M/53	Hodgkin's disease	55.8	1,280-2,560	<10	32	512
	Tu 403	M/15	Nasopharynx negative biopsy [2]	32.1	—	—	—	—

[1] Cervical lymph-node biopsy showed presence of tuberculosis.
[2] Cervical lymph-node biopsy showed malignant metastasis from unknown primary tumour.
[3] — = no serum available.

DISCUSSION

The above results confirm the presence of EBV DNA in epithelial tumour cells in nasopharyngeal carcinomas. They also show that the lymphoid cell preparations from NPC do not contain substantial amounts of EBV DNA.

In these experiments a relatively high proportion of NPC biopsies showed a low annealing rate. When we examined histopathologically a small piece of the tumour from which DNA was extracted, there existed a better correlation between the amount of carcinoma cells and the level of hybridization as compared to the histology of the same specimen used for diagnosis in the hospital. After separation, DNA from the epithelial cell preparation annealed at a significantly higher rate than DNA from the whole tumour. It is probable, therefore, that the stroma and lymphoid cells lowered the level of hybridization.

In NPC tumours, Yata *et al.* (1974) found regularly both B- and T-cells, but on the edge of the epithelial tumour cells mostly B-lymphocytes. In contrast, Jondal and Klein (1975) found that the vast majority of lymphocytes in NPC originate from the T-subpopulation. However, the markers for B- or T-cell populations were not the same in both studies and further clarification of the nature of the lymphoid cells close to the epithelial tumour cells is therefore needed.

The correlation between serological data and annealing rates of the corresponding tumour DNA did not reveal any outstanding association. Similarly, the comparison between hybridization level of undifferentiated and differentiated carci-

nomas did not permit any conclusion, the number of well-differentiated tumours being very small. The comparative analysis of annealing rates of NPC tumours from the different geographical areas did not show significant differences, although there were relatively more tumours with high EBV DNA content from Kenya and Singapore than from Tunis.

This study has thus further established that no significant difference existed between NPC originating from various geographical areas. The questions which now need to be urgently answered are whether normal nasopharyngeal epithelium is infected by EBV, and how the epithelial cells acquire the EBV genomes. Do epithelial cells have EBV receptors or are they infected by transfer of viral information from lymphoid cells? Electron microscopic evidence of loss of cell membrane continuity between epithelial and lymphoid cells in both normal mucosa and NPC (Gazzolo *et al.*, 1972; Vuillaume *et al.*, 1973) would favour such a possibility. The studies of Glaser and Rapp (1972) on somatic cell hybrids should enlight this question; and our present aim is to obtain hybrids between epithelial tumorous cells from NPC and cells susceptible to EBV infection and transformation.

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CANCER DU RHINOPHARYNX. X. PRÉSENCE DE GÉNOMES EB DANS DES CELLULES ÉPITHÉLIALES SÉPARÉES PROVENANT DE TUMEURS OBSERVÉES CHEZ DES MALADES DE SINGAPOUR, DE TUNISIE ET DU KENYA

Des biopsies de cancers du rhinopharynx en provenance de Singapour, de Tunisie et du Kenya ont été comparées, avant et après séparation des populations de cellules épithéliales tumorales et des cellules lymphocytaires, pour leur contenu en DNA viral EB, titré par hybridation du DNA cellulaire avec un RNA complémentaire viral. Dans tous les cas où la séparation des deux types cellulaires a été obtenue, les populations épithéliales contenaient plus de DNA viral que les populations lymphoïdes et que les tumeurs avant séparation. La présence de DNA viral semble donc être concentrée au niveau des cellules tumorales épithéliales. Par ailleurs, il n'a pas été observé de différences significatives entre les biopsies des différentes régions, quant à leur contenu en DNA viral.

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