Epstein-Barr Virus & Nasopharyngeal Carcinoma – Future Diagnosis & Prognostication

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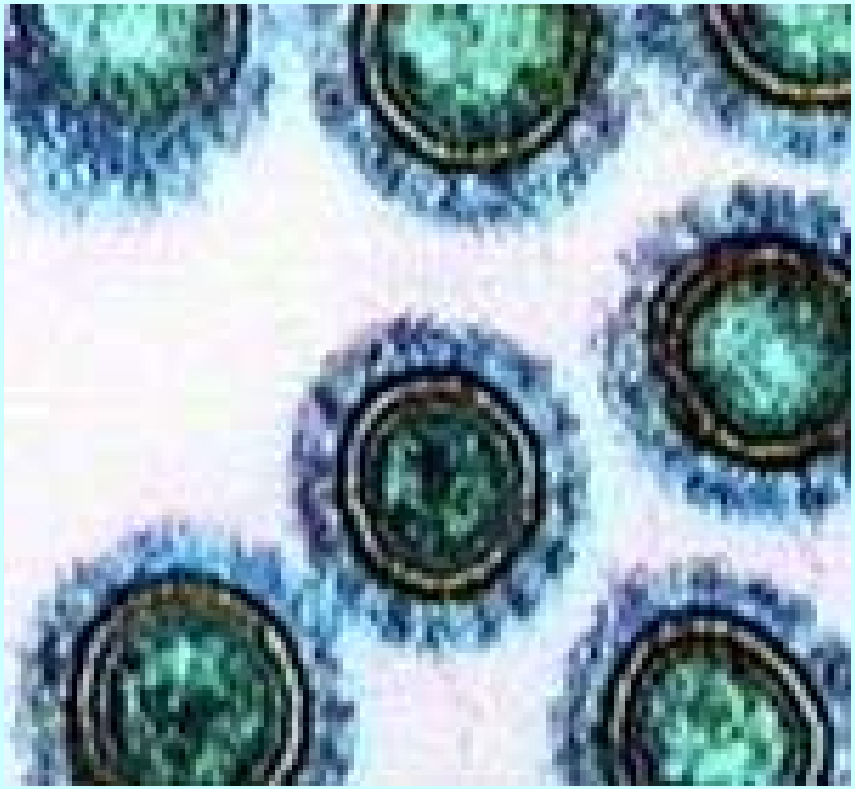
Epstein-Barr virus – a member of gamma herpes virus subfamily



EBNA1	EA & VCA
EBNA2	ZEBRA
EBNA3	LMP1
EBNA4	TP1
EBNA5	MA
EBNA6	EBER1 & 2
Bam-H1-W	

Epstein-Barr Virus (EBV)

Various EBV Genes & Antigens

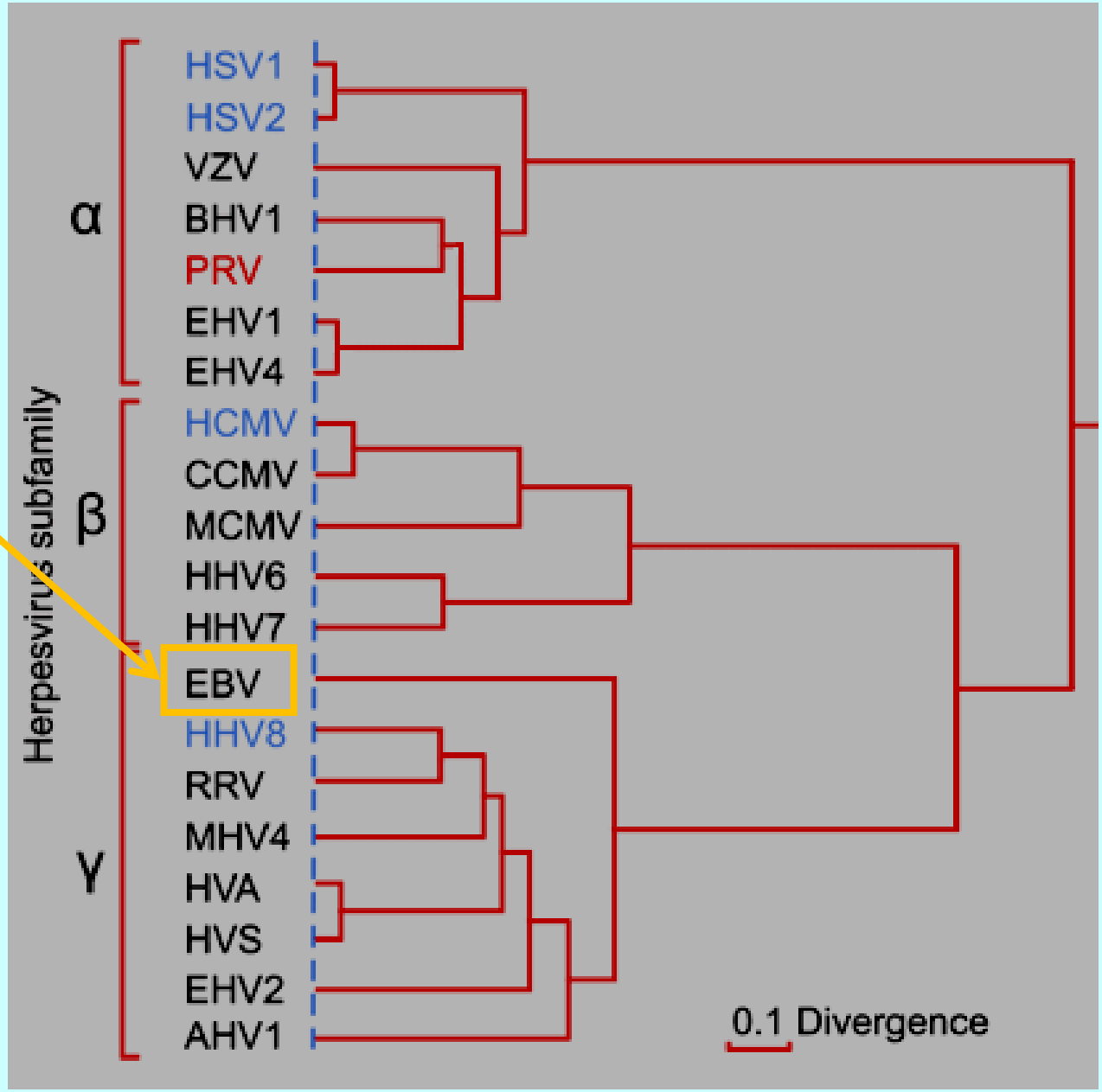
Phylogenetic tree of Epstein-Barr Virus

EBV

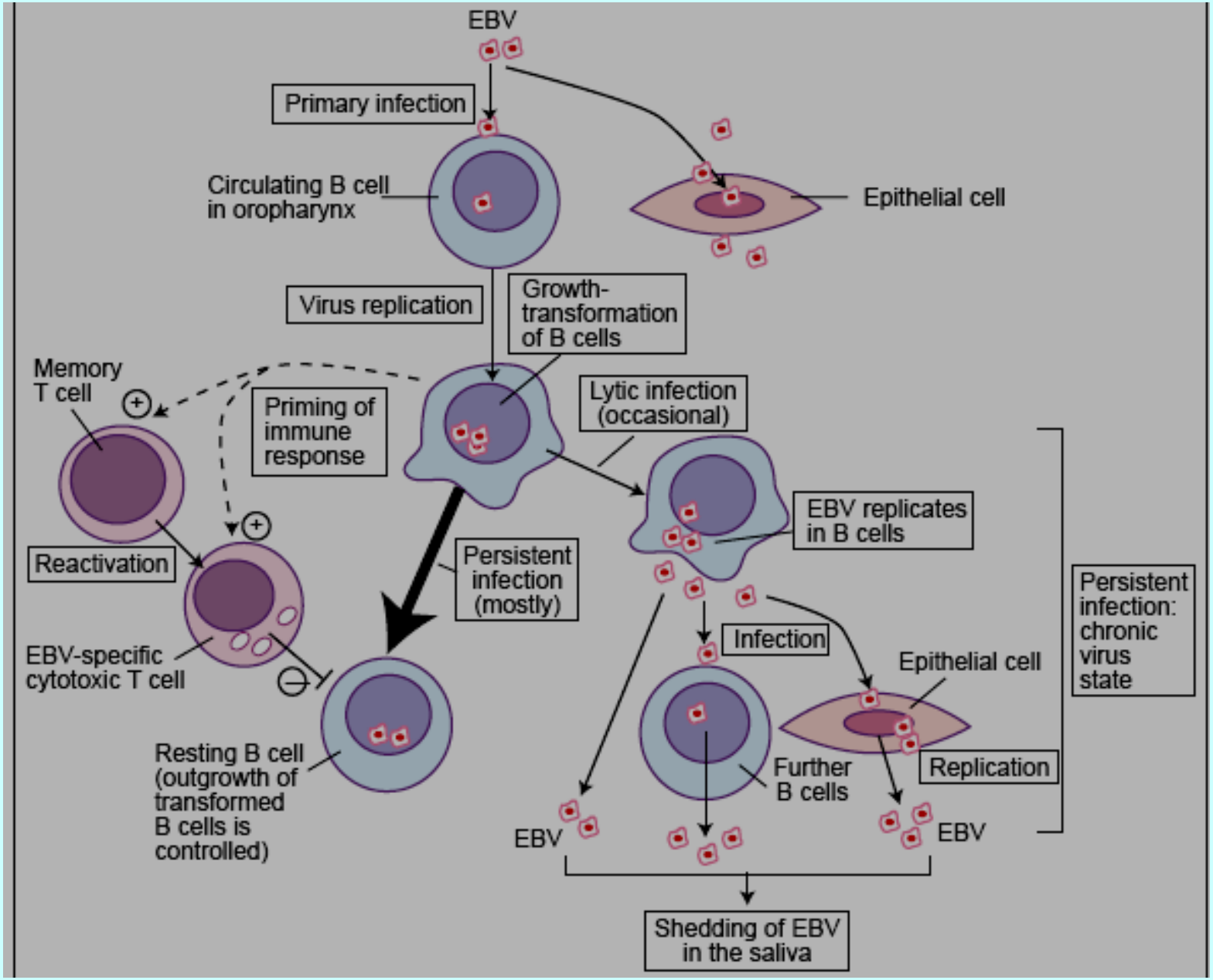
Subfamily-
Gammaherpesvirinae

Genus -
Lymphocryptovirus

Also classified as Human Herpes Virus-4 (HHV4)

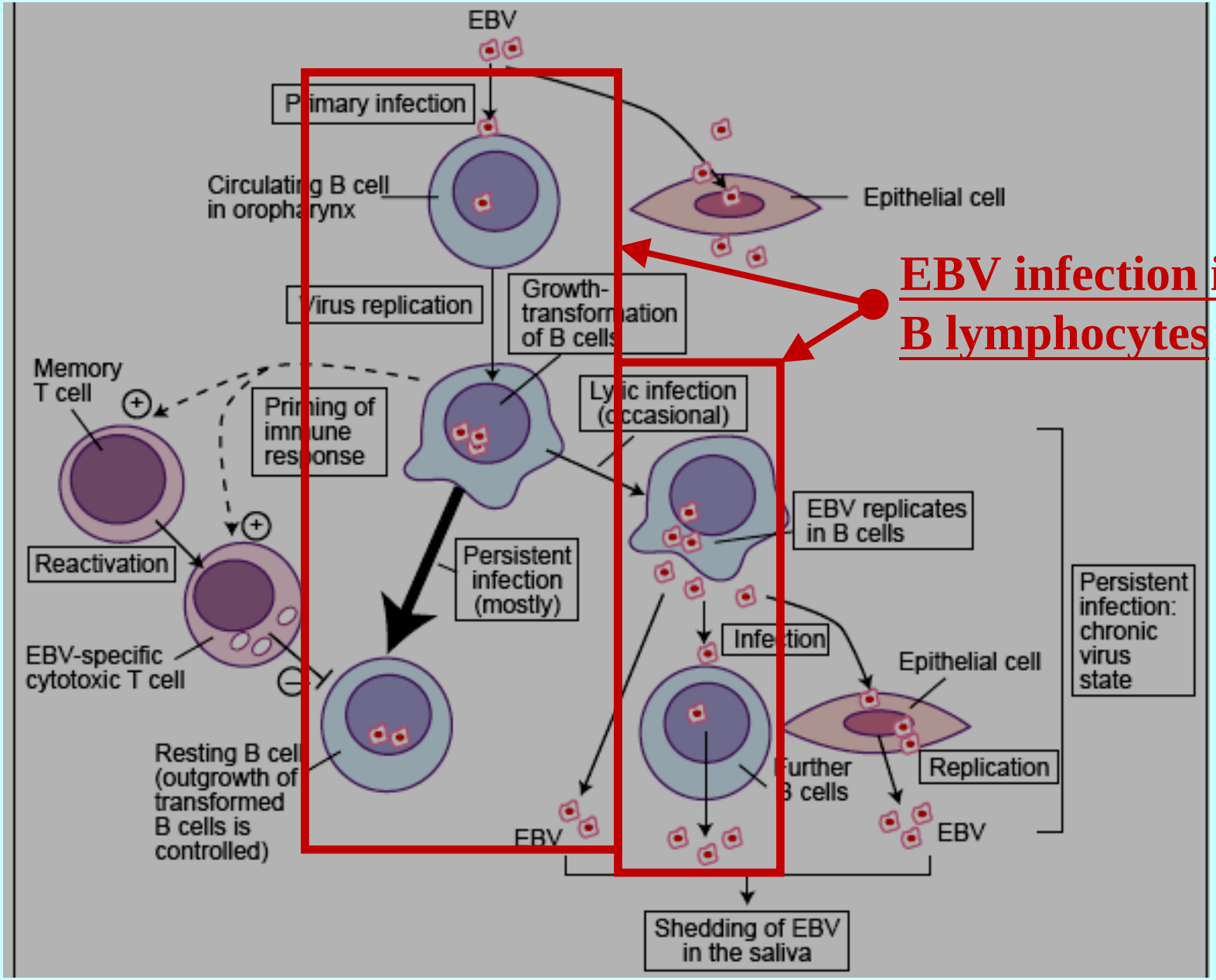


EBV infects B lymphocytes in healthy virus carrier



Murray & Young. Expert review in Molecular Medicine 2001.

EBV infects B lymphocytes in healthy virus carrier



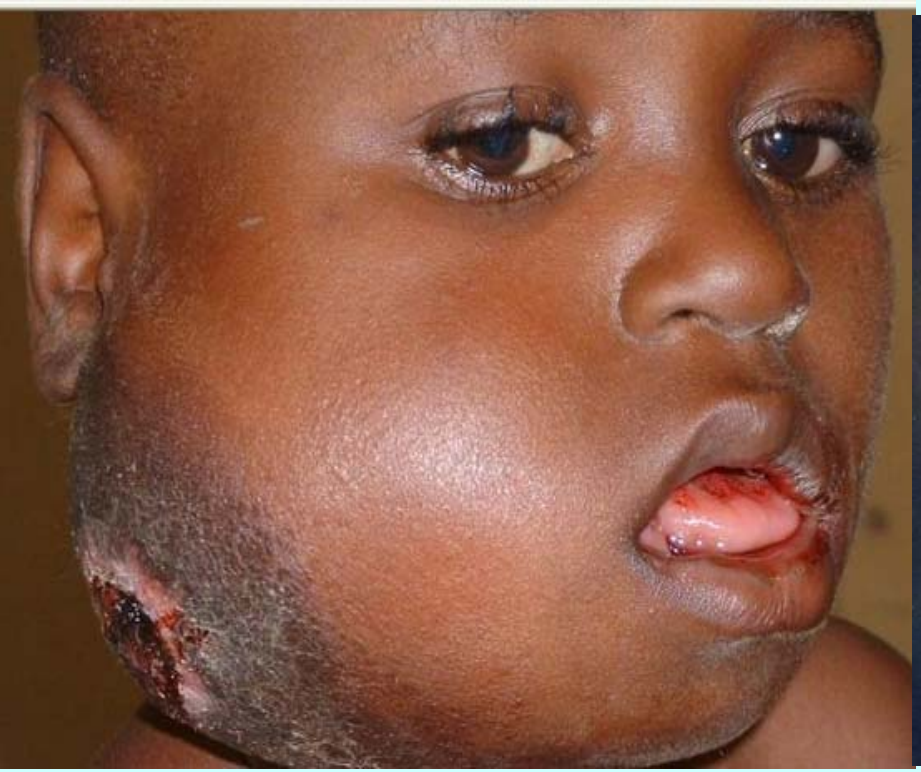
Murray & Young. Expert review in Molecular Medicine 2001.

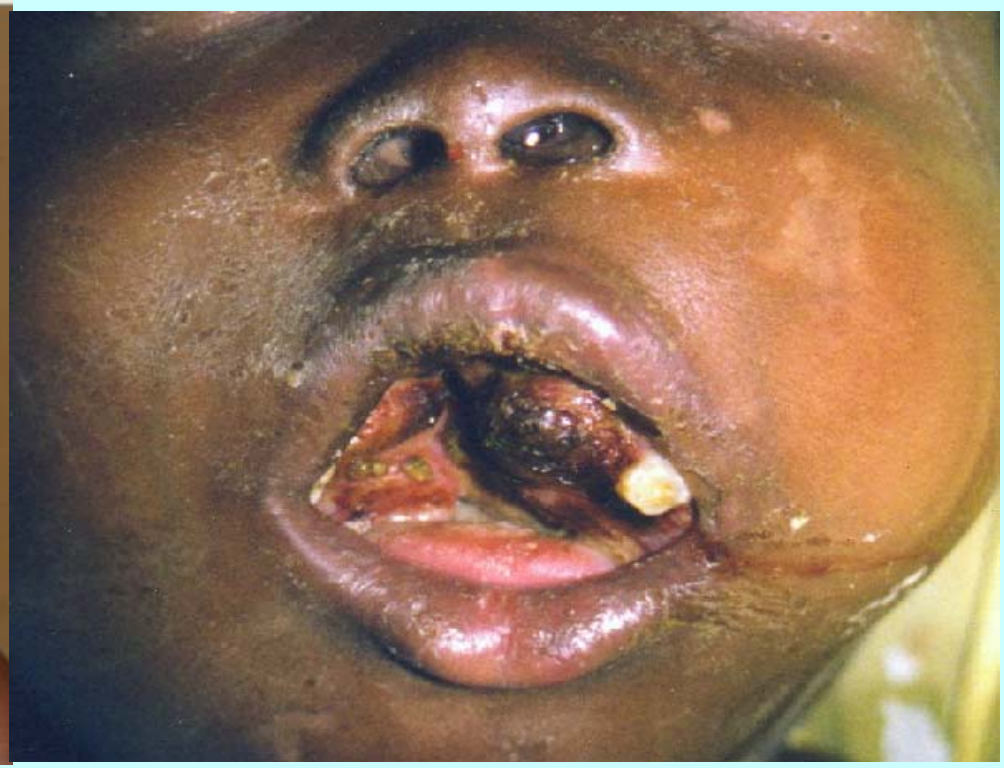
Epstein-Barr Virus Associated Tumors

Lymphoid cells in origin:-

- **Burkitt's Lymphoma (BL)**
- **Hodgkin's Lymphoma (HL)**
- **Post-Transplant Lymphoid-Proliferative Disease (PTLD)**
- **AIDS Associated Lymphoma (AIDS-L)**
- **NK/T cell Lymphoma (NK/TL)**
- **EBV associated Haemophagocytic lymphohistiocytosis (HLH)**

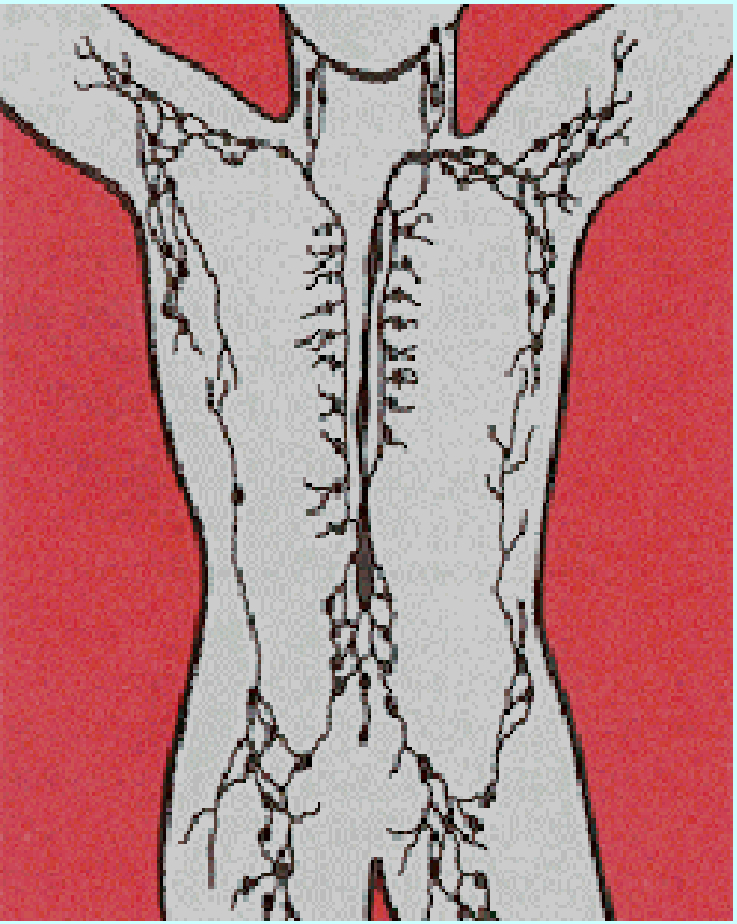
EBV associated with Burkitt's Lymphoma (BL) – a B lymphocyte malignancy



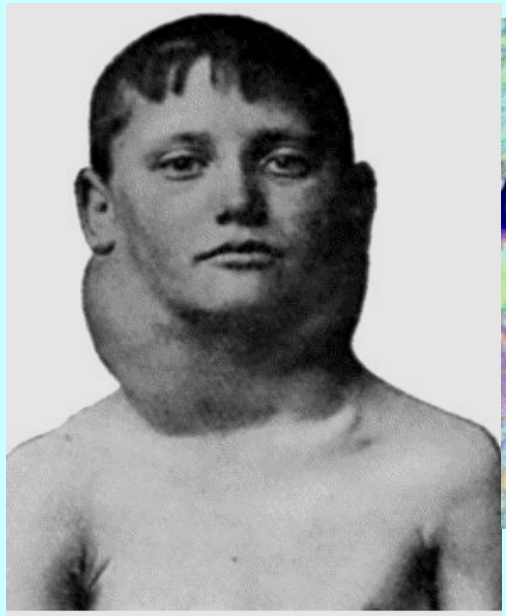


Burkitt's Lymphoma – a B lymphoid cell tumor grown in jaw of 2 African children

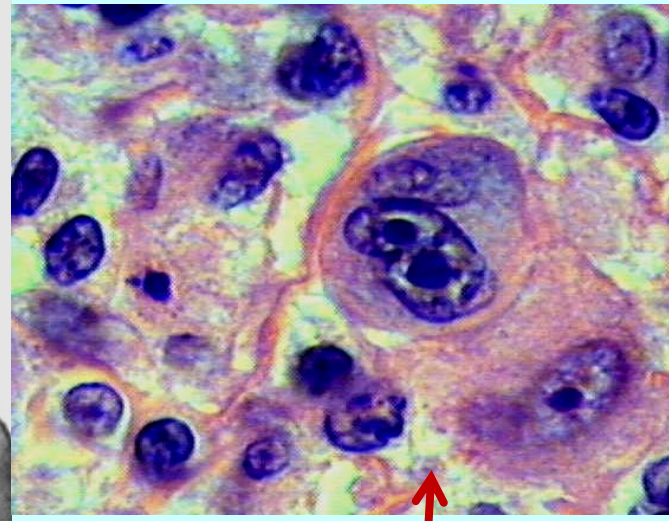
EBV associated with Hodgkin's Lymphoma (HL) – another lymphoid malignancy



Human lymphoid node distribution

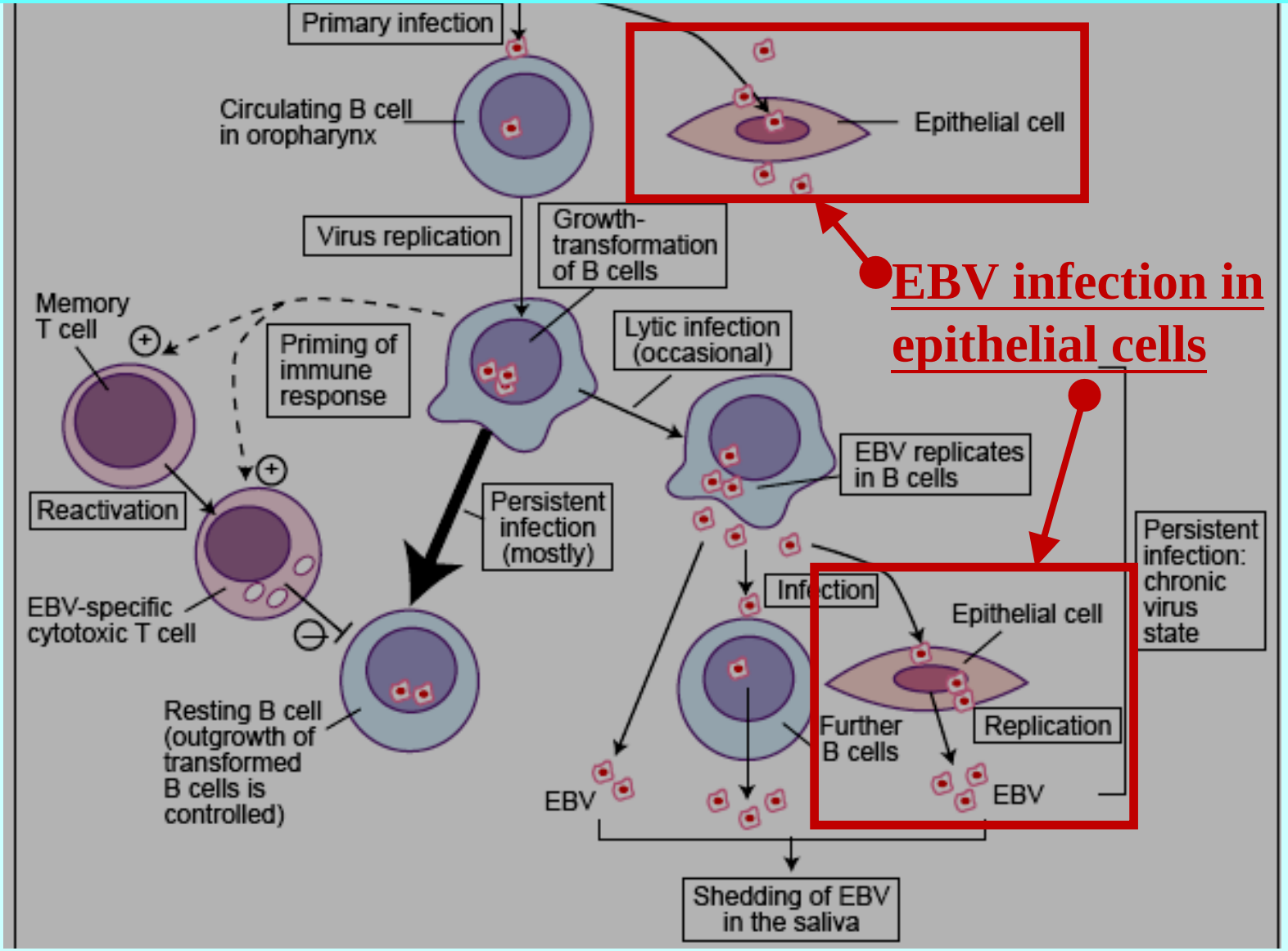


Lymphadenopathy



Enlarged Reed-Sternberg cells

EBV can also infect epithelial cells e.g. in nasopharynx or oropharynx



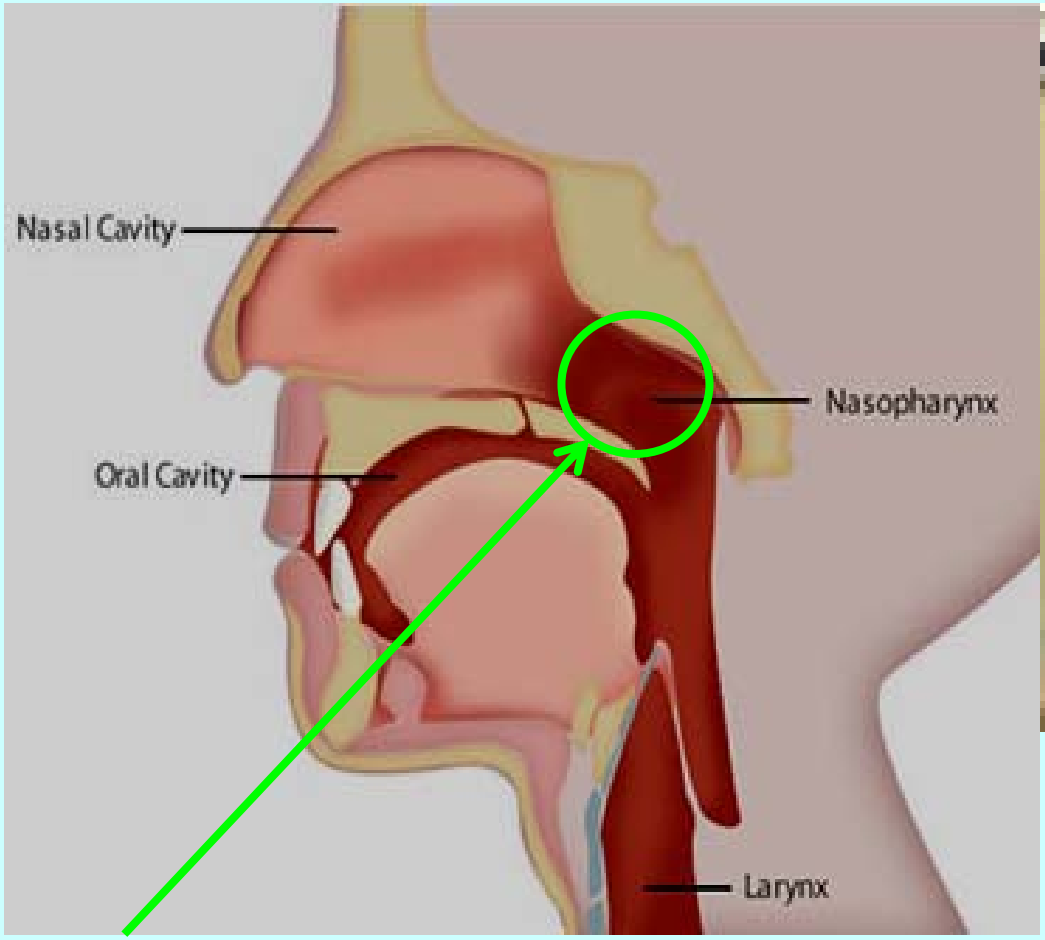
Murray & Young. Expert review in Molecular Medicine 2001.

Epstein-Barr Virus Associated Tumors

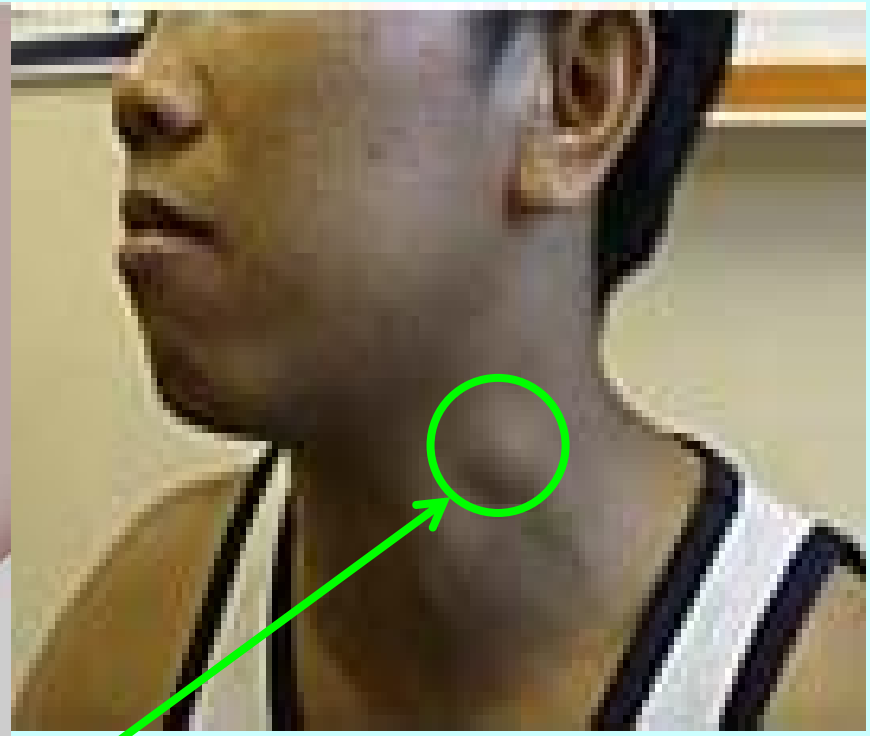
Epithelial cells in origin:-

- **Nasopharyngeal Carcinoma (NPC)**
- **Lymphoepithelioma of Lung Cancer (LELC)**
- **Salivary Gland Lymphoepithelioma (SGCa)**
- **Undifferentiated Carcinoma in Gastric Gland**
- **Breast cancer (??)**

EBV associated with Nasopharyngeal Cancer (NPC) – an epithelial cell tumor

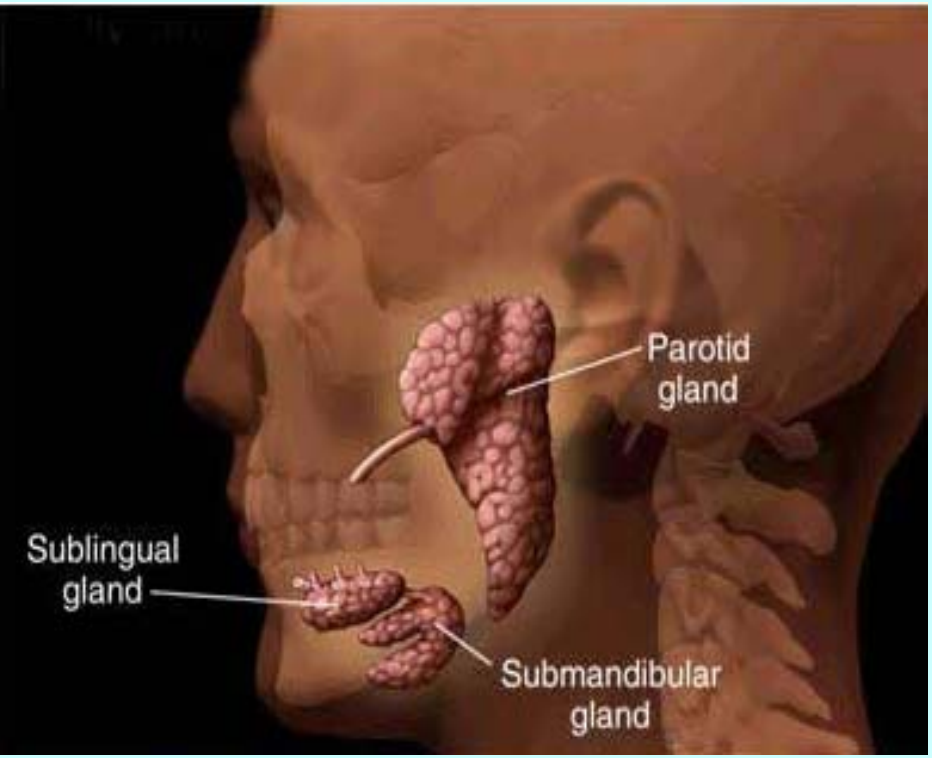


Tumor location in nasopharynx

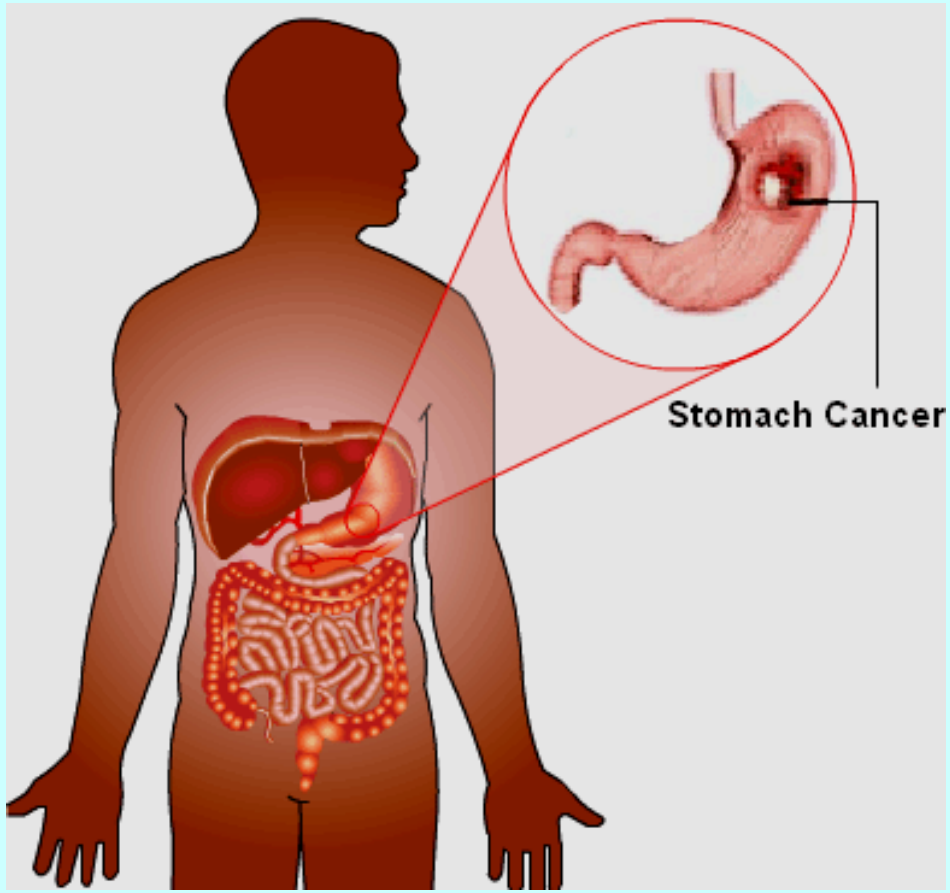


Tumor spread to lymph nodes in neck - lymphadenopathy

EBV associated with Salivary Gland & Gastric Cancers (undifferentiated form - rare)



EBV associated cancer - grows in salivary gland



EBV associated undifferentiated cancer - grows in stomach

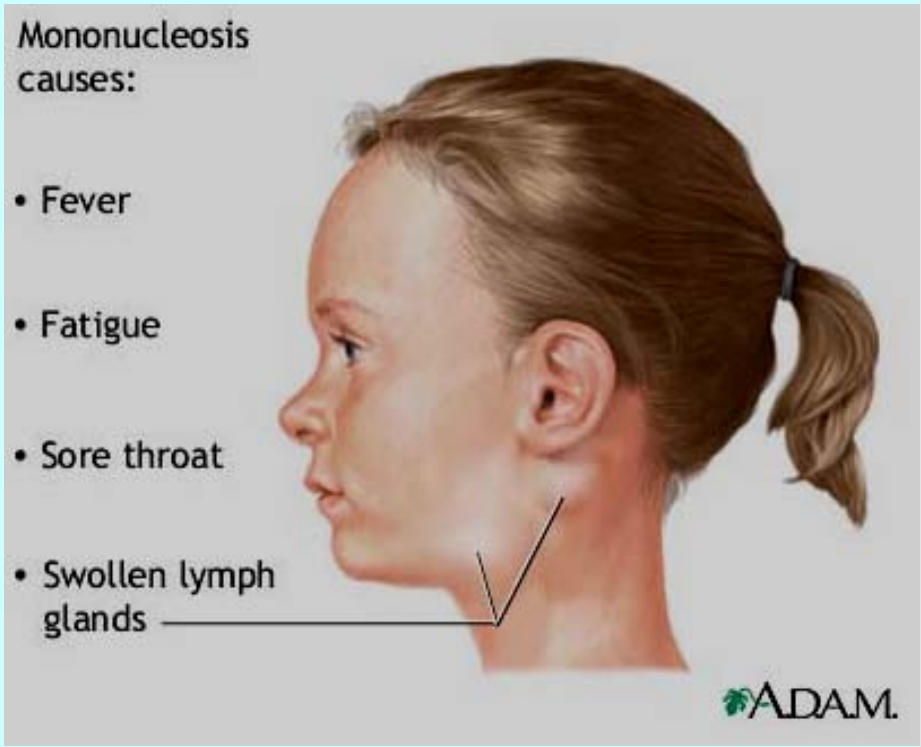
Other EBV Associated Diseases

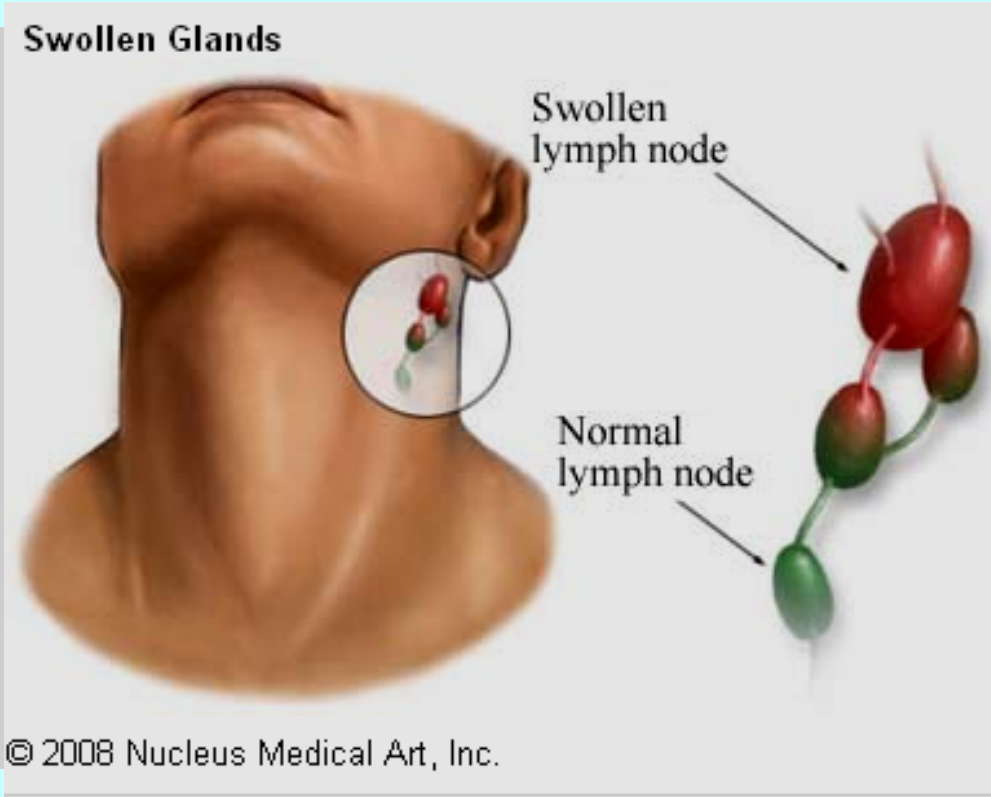
- Infectious mononucleosis (IM)
- Rheuatoid Arthritis (RA)
- Malaria infection
- Herpes Viral Infection in immuno-compromised patients

EBV associated Infectious Mononucleosis (IM)

- **IM is caused by EBV infection (another nick name for IM is <u>Kissing Disease</u> – common in college's adolescent .**

- **Clinical symptoms – abnormal increase of small B lymphocytes, fever, sore throat, swollen lymph nodes & fatigue.**

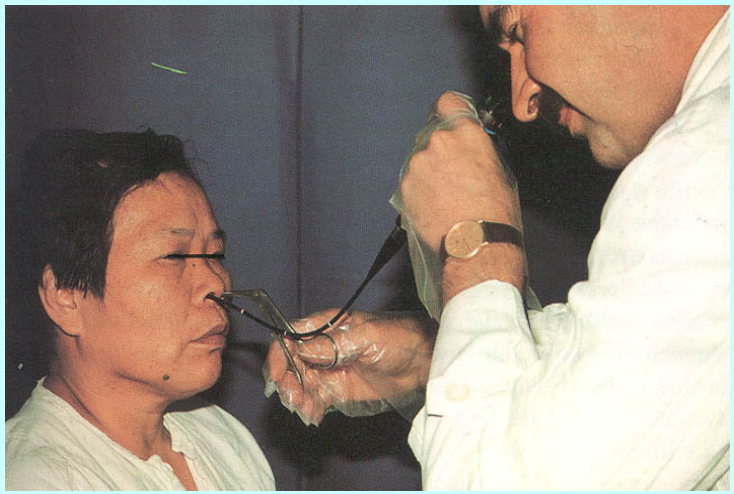


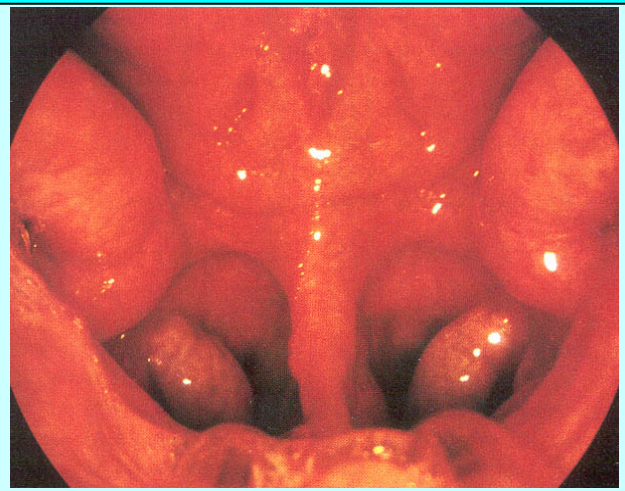


Our oncology center in Queen Elizabeth Hospital is the largest in Hong Kong in terms of cancer patients' intake.

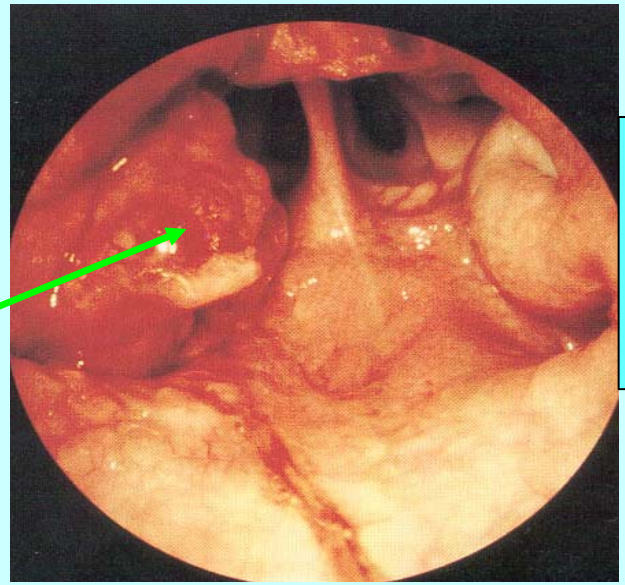
Intake of nasopharyngeal cancer (NPC) patients is also the highest.

Endoscopic examination for nasopharyngeal cancer





Normal nasopharynx



Nasopharynx with tumor

Tumor on the right eustachian cushion

From Van Hasselt & Gibb, 1991

Prevalence of nasopharyngeal cancer (NPC)

- **6th most prevalent cancer among male in Hong Kong.***

- **In male with age 20-44, it is the top most prevalent cancer (~27% of all cancer) .***

*** Hong Kong Cancer Registry Statistical Report, 2004**

Treatment of NPC

- **Radiotherapy +/- chemotherapy is the main treatment modality.**

- **Overall 5 years' survival rate – 75% (1996-2000).***

*** Lee AWM et al. Int J Radiat Oncol Biol Phys (2005)**

Problems in clinical management of NPC

- **(1). Diagnosis usually at late stages of disease (stage 3 or 4); prognosis in late stage usually poorer.**
- **(2). Frequent relapse (>50% patients in remission later still develop relapse).**
- **(3). For patients who develop distant relapses (in lung, liver or bone), >98% die despite chemotherapy implemented.**

Improvement to be made in

- Earlier diagnosis.
- Better relapse monitoring.
- Better prognostication.
- Better predicting chemotherapy & radiotherapy response.

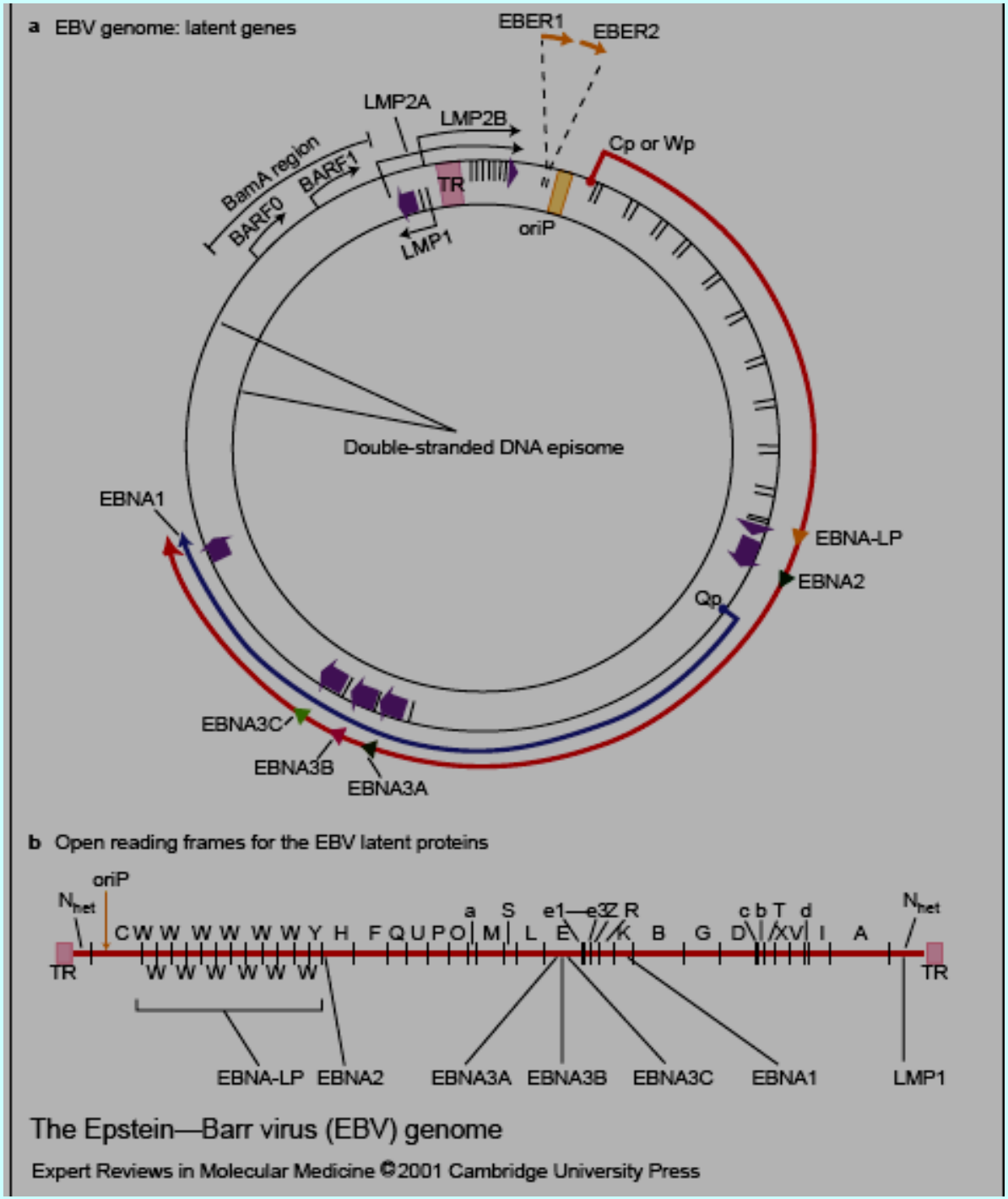
Diagnosis of NPC - EBV as target?

Which gene/antigen is a specific marker for detection in NPC tissues?

EBV Genome in episomal form

Little or no EBV genome is incorporated into human chromosomes.

Viral genome exists mainly as circular episome (like plasmid in bacteria) in human cells.



EBV latent cycle genes/antigens

- Epstein-Barr nuclear antigen (EBNA-1, -3A, -3B, -3C, & -LP; EBNA-LP which contains Bam-H1-W fragment).
- Latent membrane proteins (LMP-1, -2A & -2B).
- EBV encoded small RNAs (EBER-1 & -2).

Some of these viral RNA/proteins are expressed during viral latency.

EBV lytic cycle genes/antigens

- Bam-H1-Z fragment EBV transactivator protein (ZEBRA).
- Early antigen (EA).
- Viral capsid antigen (VCA).
- Membrane antigen (gp350 - MA).

These viral RNA/proteins are rarely expressed during latency.

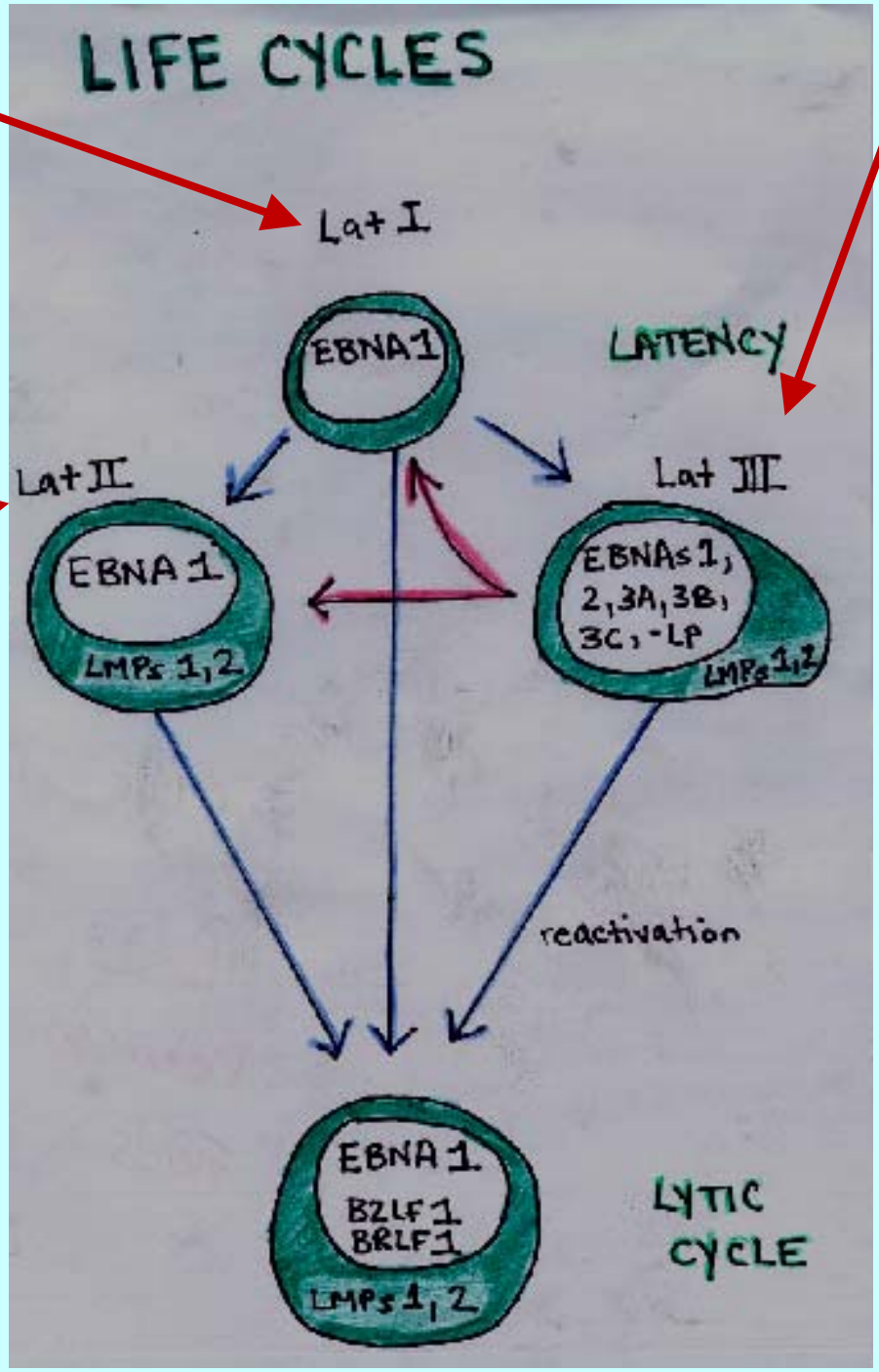
In immuno-competent subjects, EBV infected cells are under surveillance by T cell immunity & prevented from propagating.

- EBV is clever - down-regulating many immuno-competent viral proteins (some by DNA methylation) to escape from immuno-surveillance of human body.

- This enables the virus to be propagated in the human body.

Latency I- Burkitt's lymphoma (only EBNA1 & EBERs)

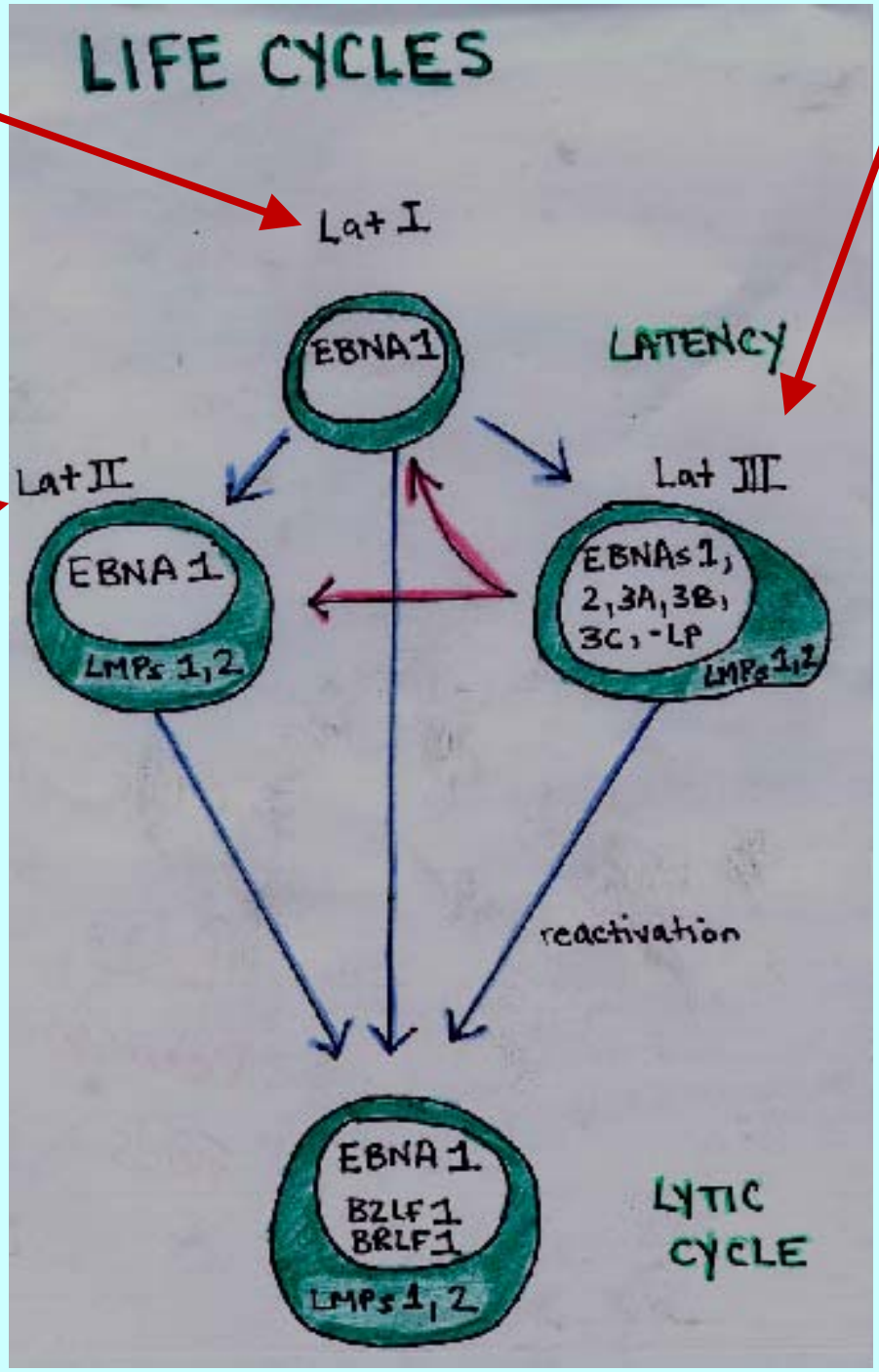
Latency II- NPC & Hodgkin's lymphoma (EBNA1, EBERs, LMP1, 2 & BamA)



Latency III- EBV-transformed cell lines (LCL) & post-transplant lymphoma (EBNA1, 2, 3A, 3B, LP, EBERs, LMP1, 2 & BamA)

Latency I- Burkitt's lymphoma (only EBNA1 & EBERs)

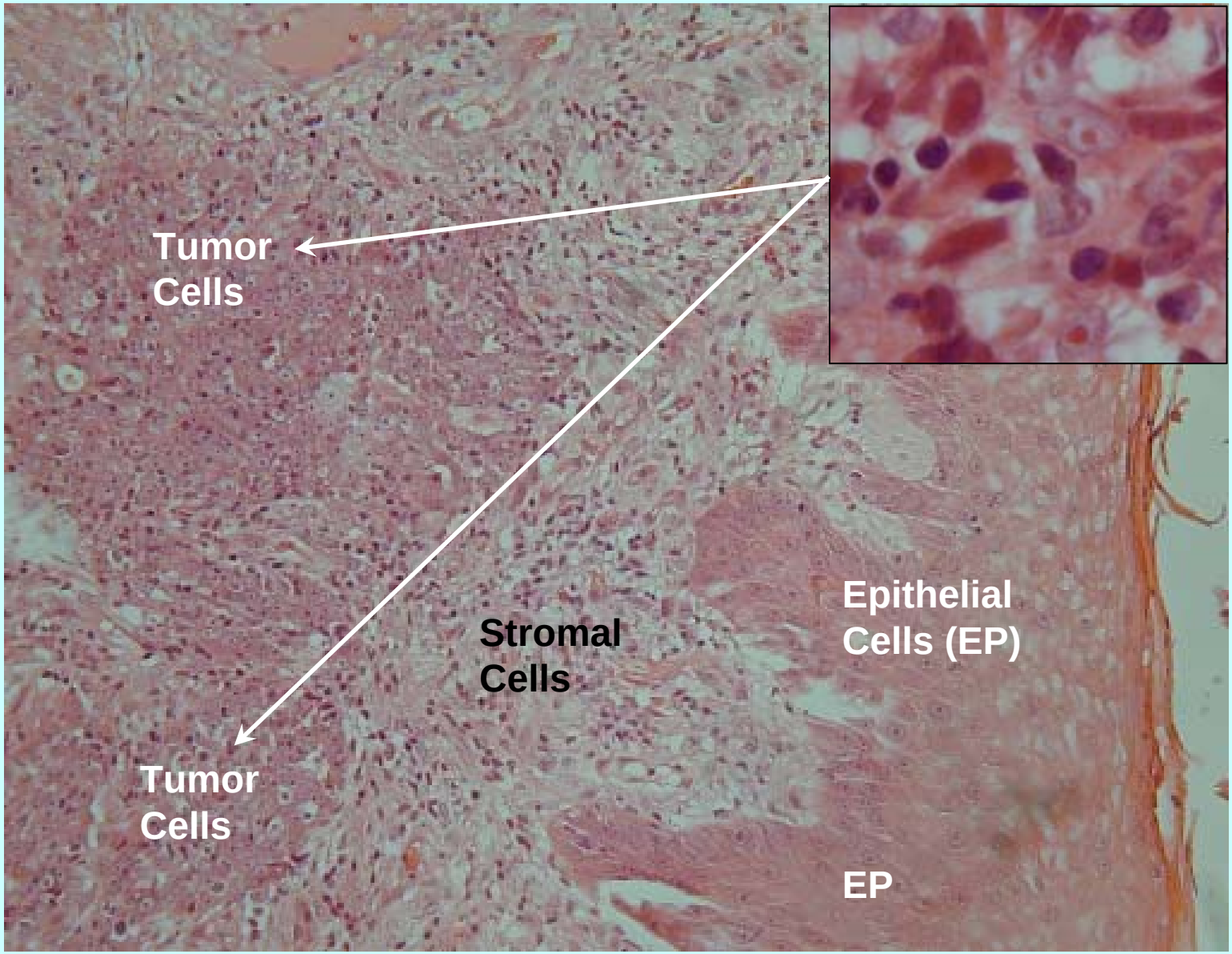
Latency II- NPC & Hodgkin's lymphoma (EBNA1, EBERs, LMP1, 2 & BamA)



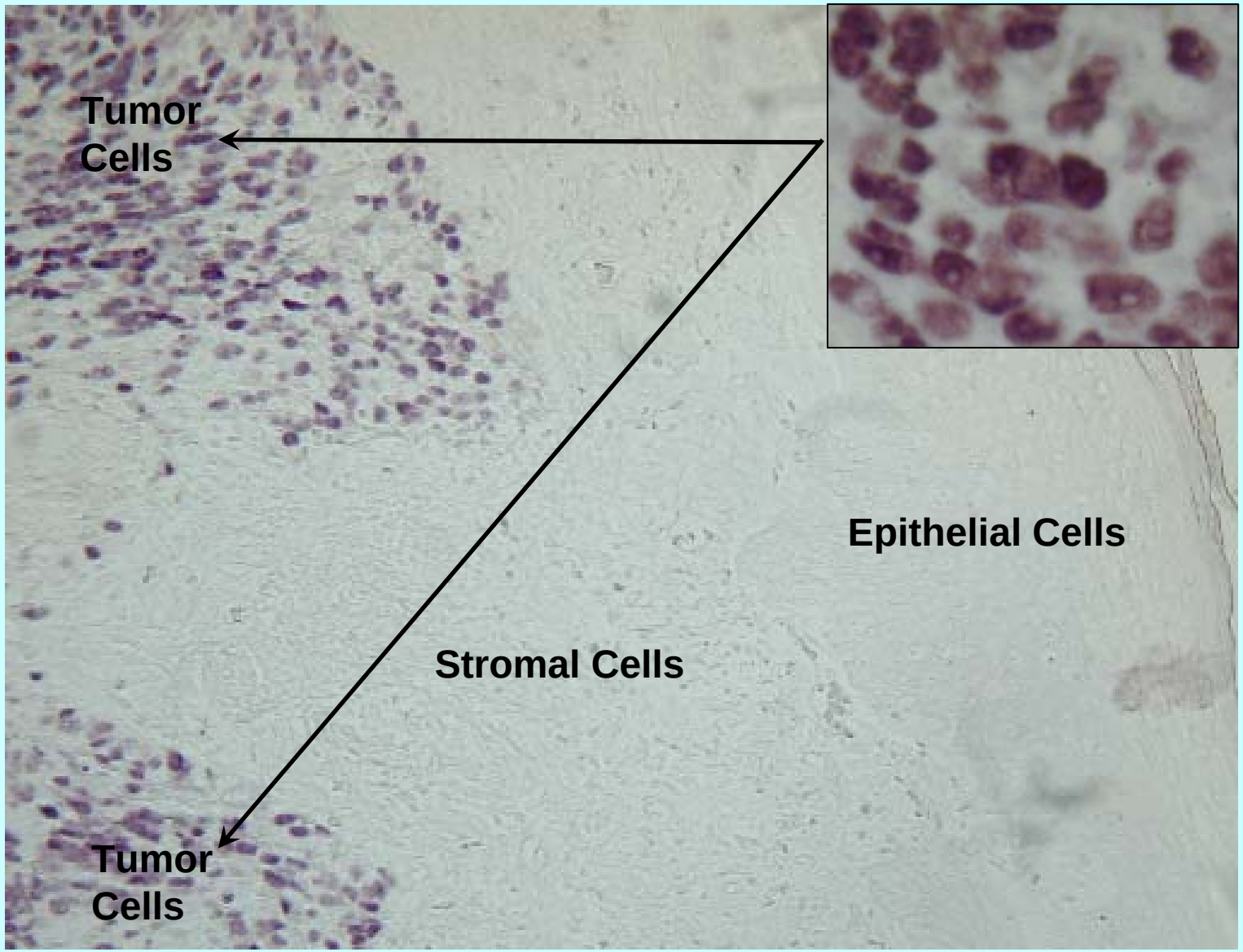
Latency III- EBV-transformed cell lines (LCL) & post-transplant lymphoma (EBNA1, 2, 3A, 3B, LP, EBERs, LMP1, 2 & BamA)

In situ hybridization (ISH) was used to test the tumor specificity of EBER RNA in NPC biopsies.

H&E staining of NPC biopsy tissues



In situ hybridization (ISH) for EBER (EBV small RNA) in NPC biopsy tissues



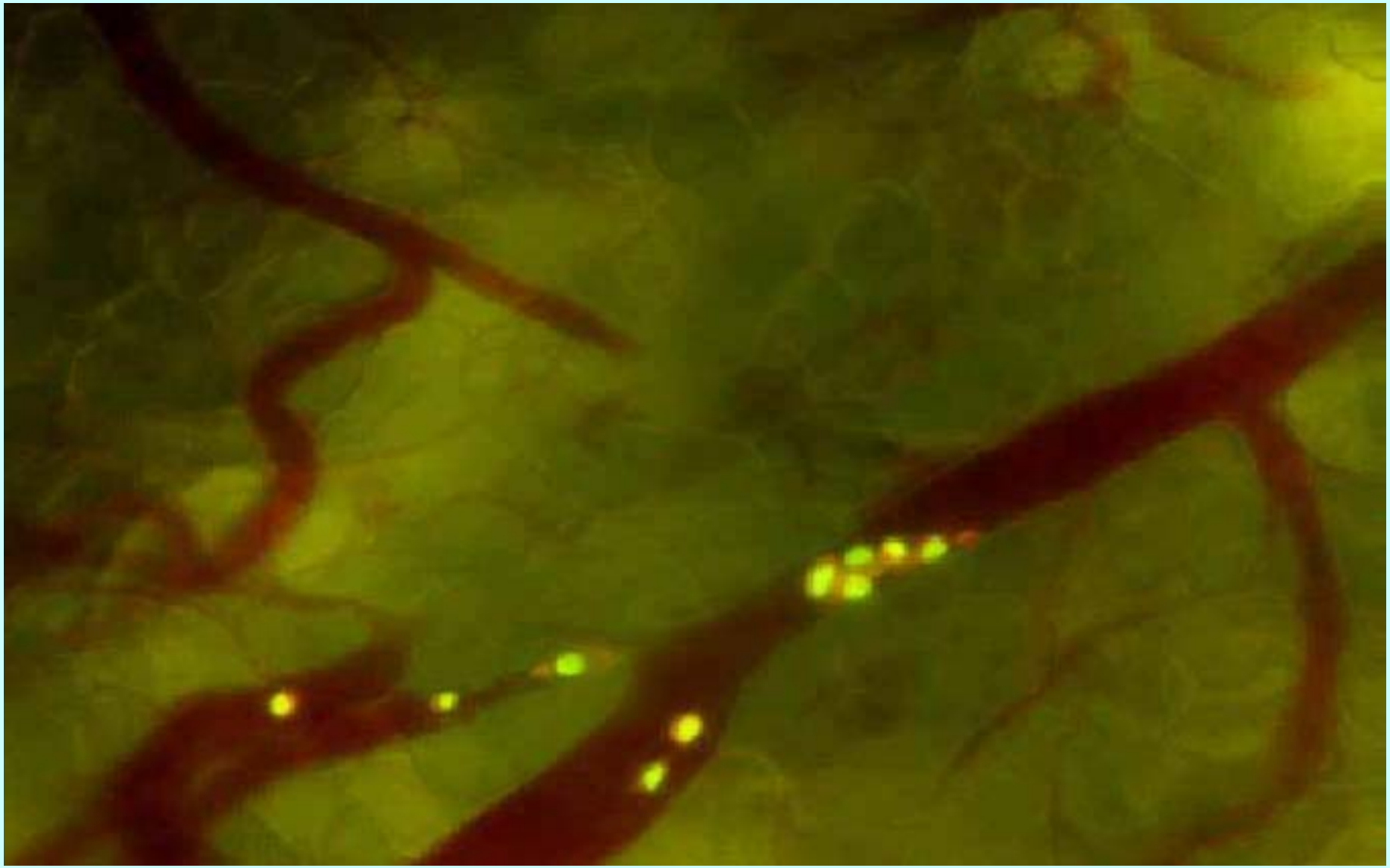
EBER is specifically expressed in NPC tumor cells

(a few hundreds of NPC biopsies tested)

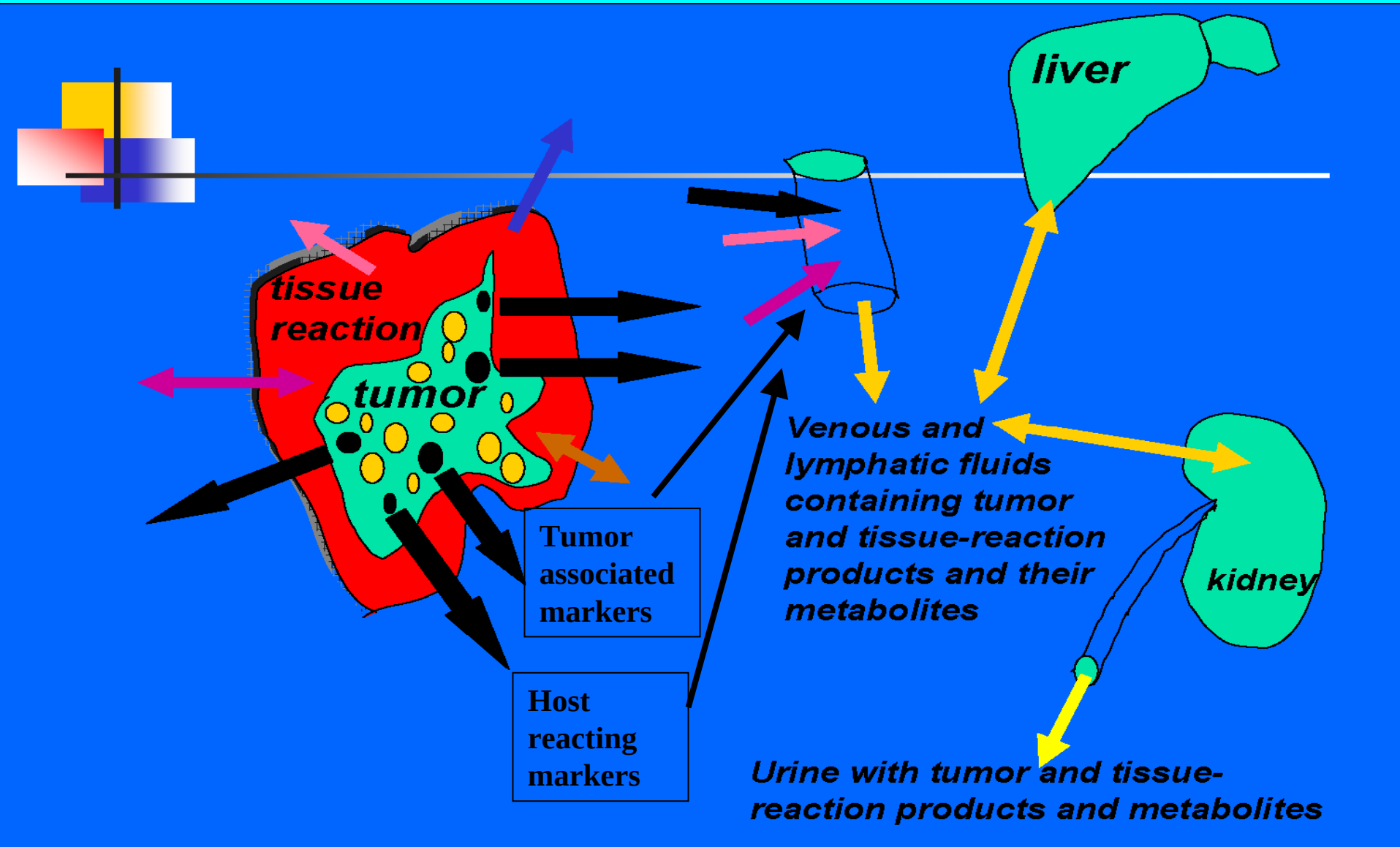
By the time a NP biopsy is taken, too late for early diagnosis of primary disease or local relapse

Development of non-invasive blood test

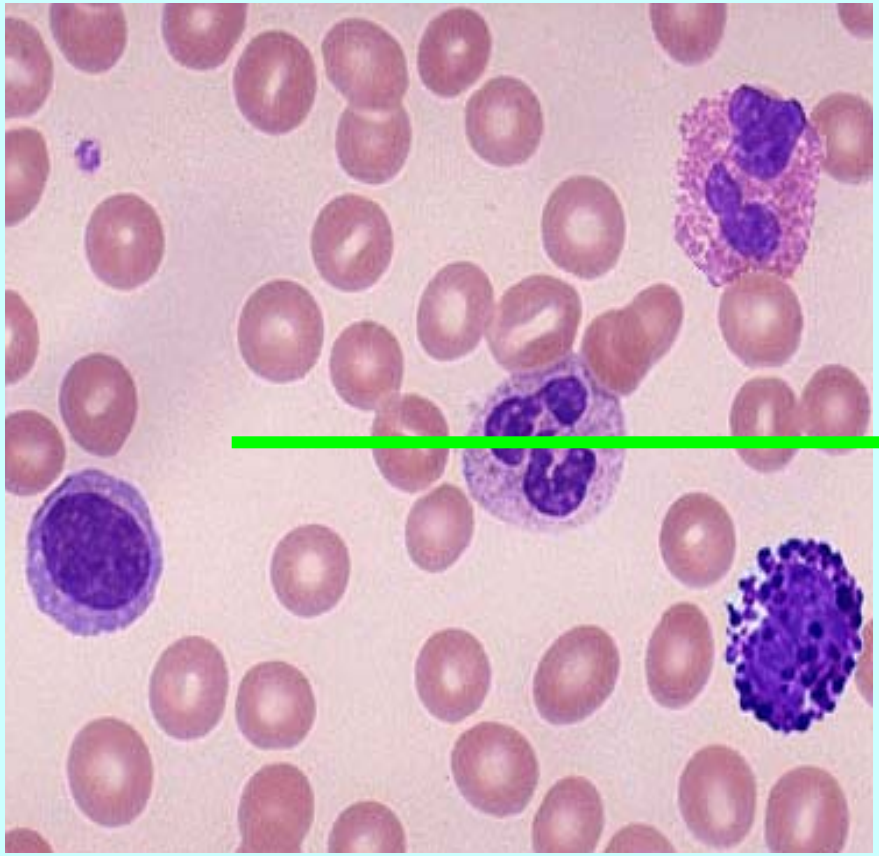
Tumor cells can enter the blood stream



Tumor DNA can also be shed Into blood



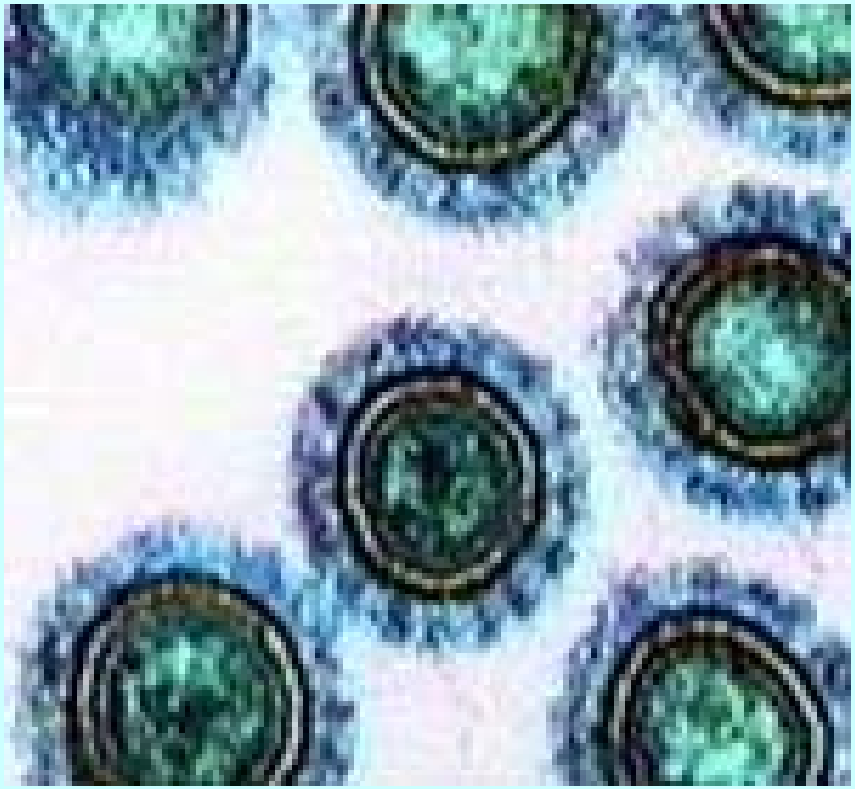
Tumor Associated DNA Circulating In Serum / Plasma



Serum fraction

Circulating free DNA

4 EBV DNA are tested in NPC patients' sera or plasma



EBNA1	EA & VCA
EBNA2	ZEBRA
EBNA3	LMP1
EBNA4	TP1
EBNA5	MA
EBNA6	EBER1 & 2
Bam-H1-W	

Epstein-Barr Virus (EBV)

4 EBV DNA tested

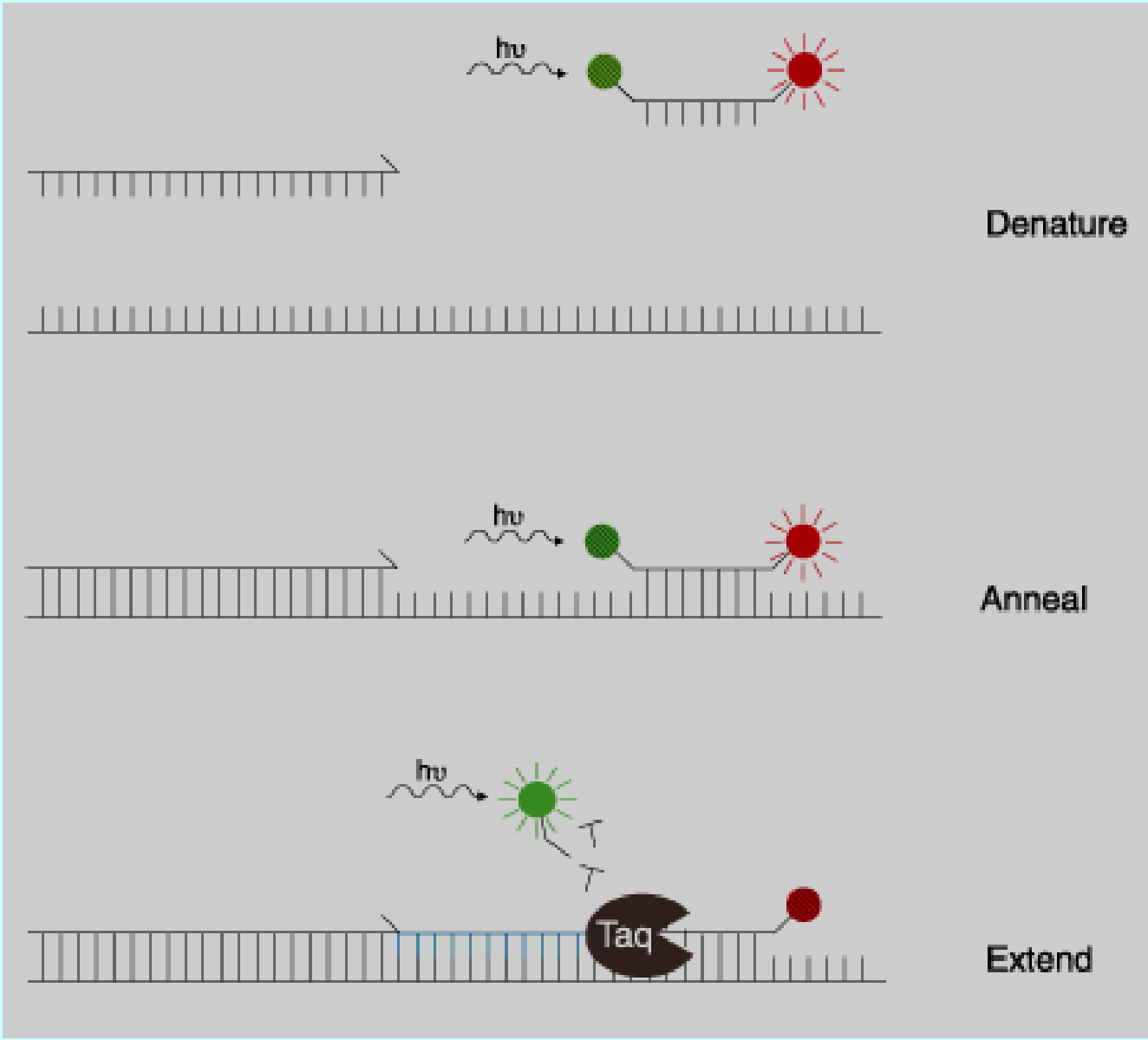
By conventional & real time PCR, Prof. Dennis Lo's group, a Thai group & our research unit showed –

circulating EBV DNA levels in the serum/plasma was substantially elevated in NPC patients versus normal controls.

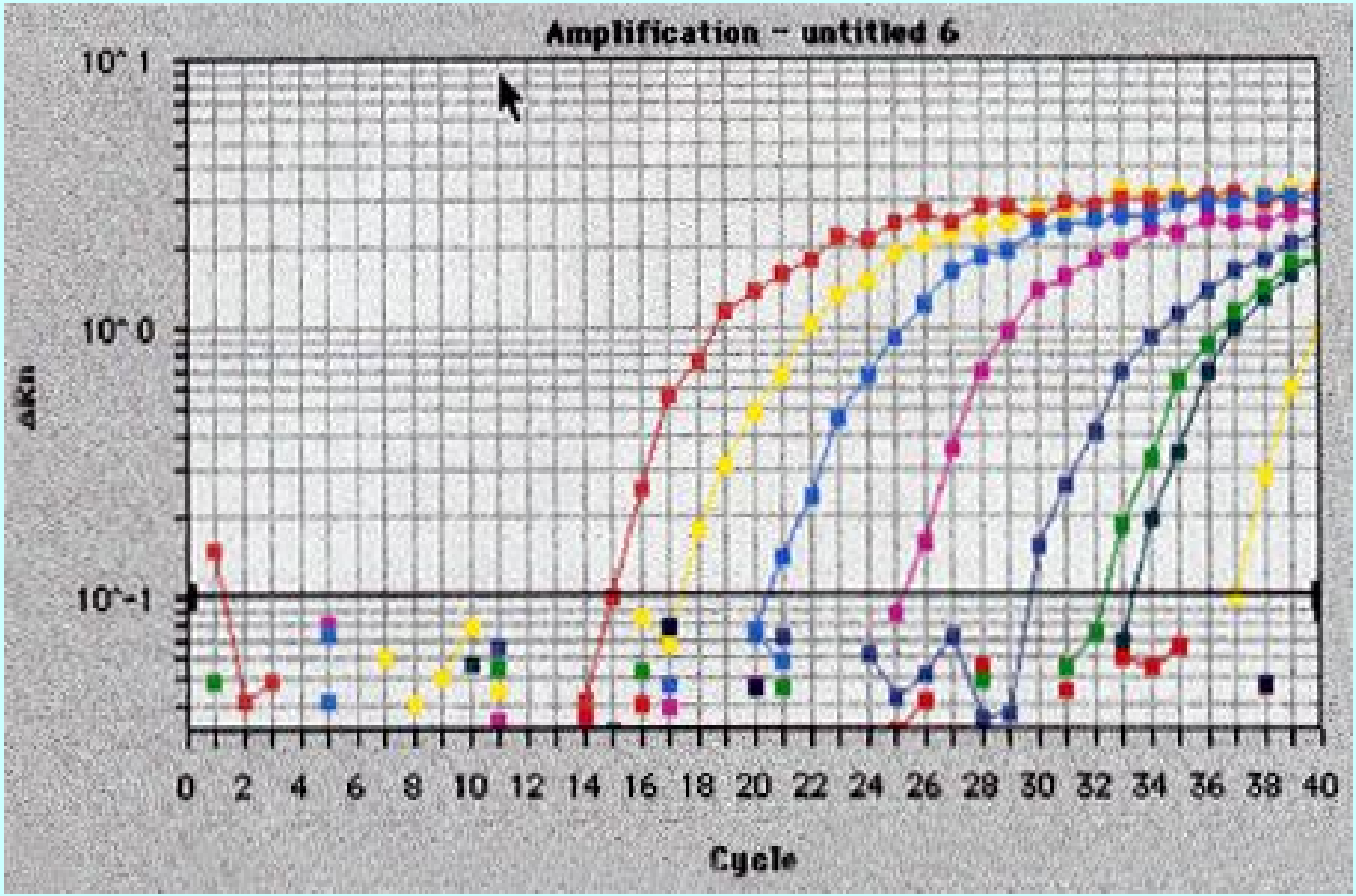
- Mutirangura A et al. Clin Cancer Res 1998.
- Yip TTC et al. Proc. AACR Annual Meeting 1998.
- Lo YM et al. Cancer Res. 1999a & b,

DNA in plasma or serum is purified by silica column method (such as Qiagene Mini-DNA purification column) or magnetic bead purification method.

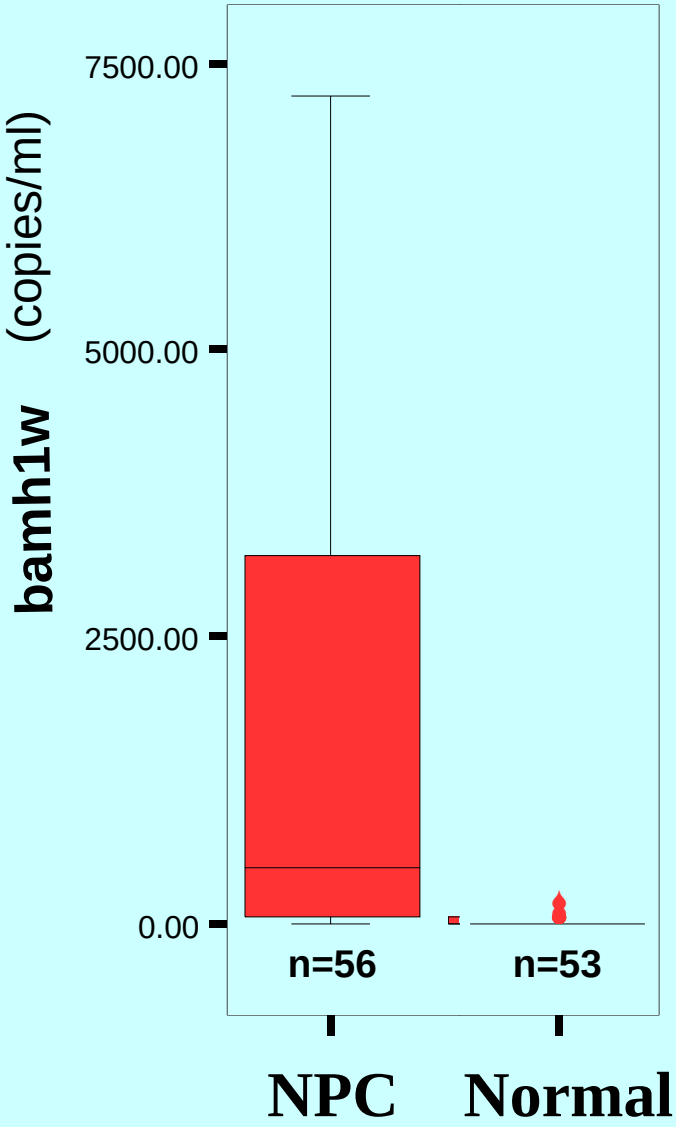
Principle of TaqMan quantitative real time PCR

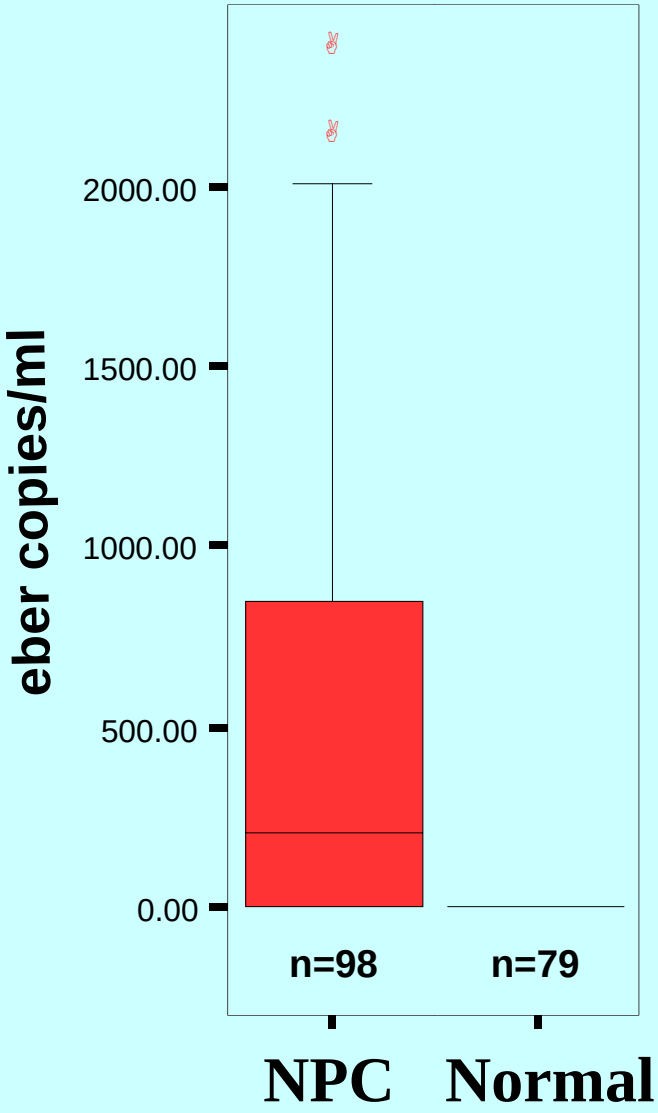


Adding known EBV standard DNA – determine EBV DNA quantity in NPC patients' plasma or sera

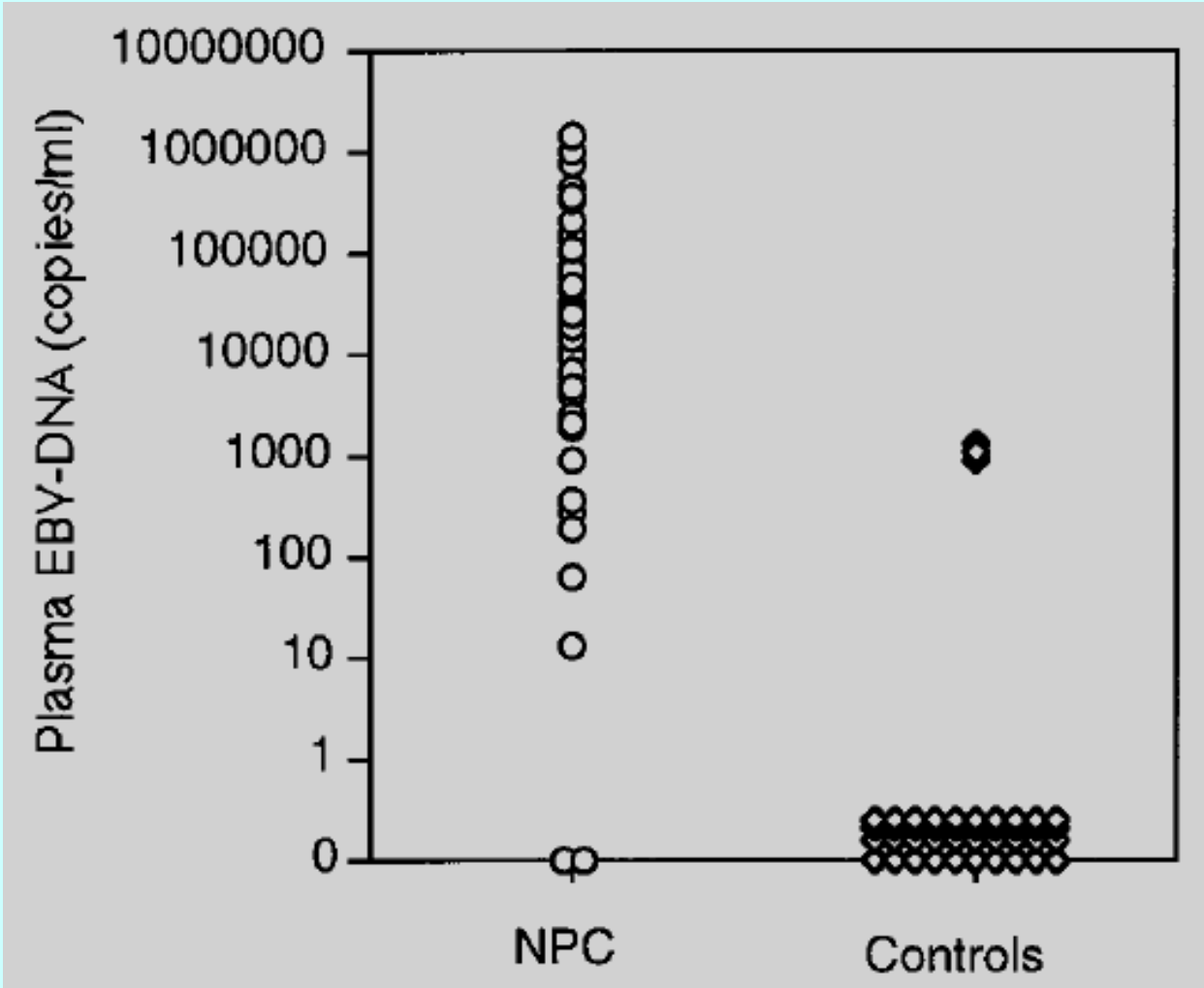


NPC patients had elevated serum circulating EBV DNA versus normals





Circulating plasma EBV DNA in NPC patient vs normal individuals



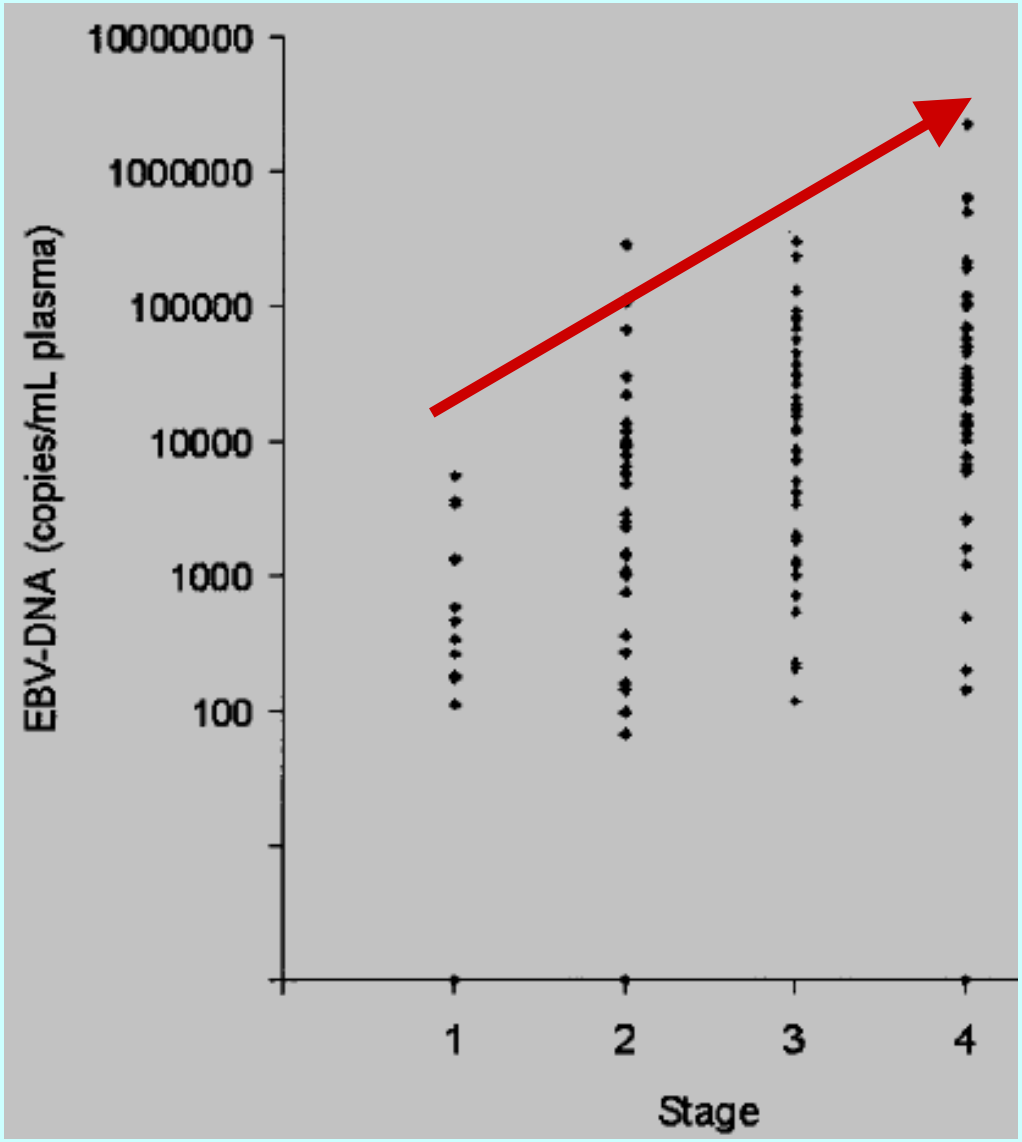
Lo DYM et al. Cancer Res (1999)

Comparison with conventional antibody based serological tests in primary diagnosis

Markers	EBV VCA/IgA	EBV ZEBRA/IgG	EBV DNA
Sensitivity	81%	97%	95%
Specificity	96%	98%	98%
References	Leung SF et al. Clin Chem (2004)	Yip TTC et al. Cancer (1994)	Leung SF et al. Clin Chem (2004)

Cut off titers for EBV DNA is 60 gene copies/ml & for VCA/IgA is 1/10 +ve.

Plasma/serum EBV DNA correlates with clinical stages of NPC



Leung SF et al. Clin Chem (2004)

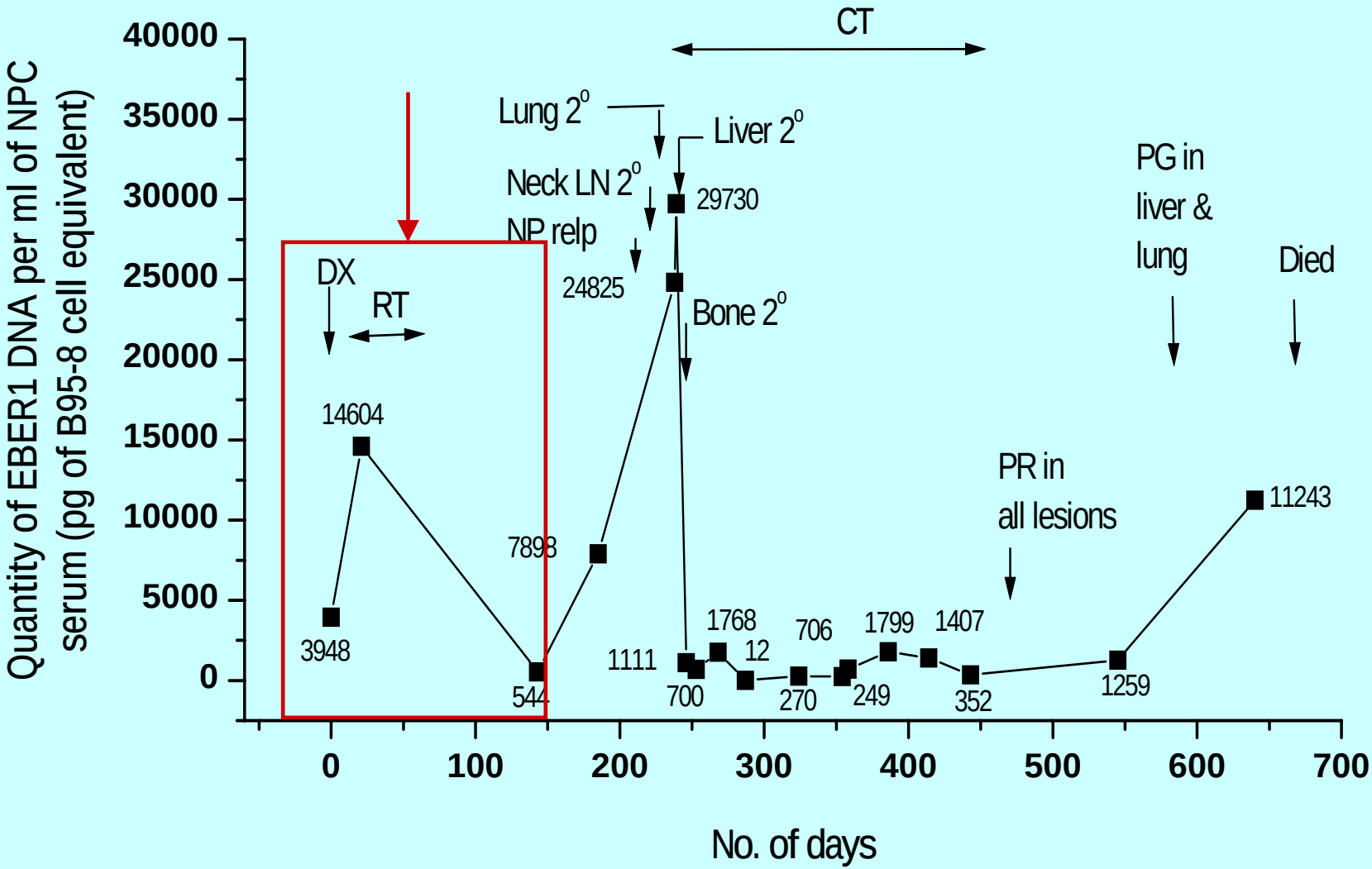
Does the plasma/serum EBV DNA come from the tumor or other cells?

Is the DNA level correlated with tumor load?

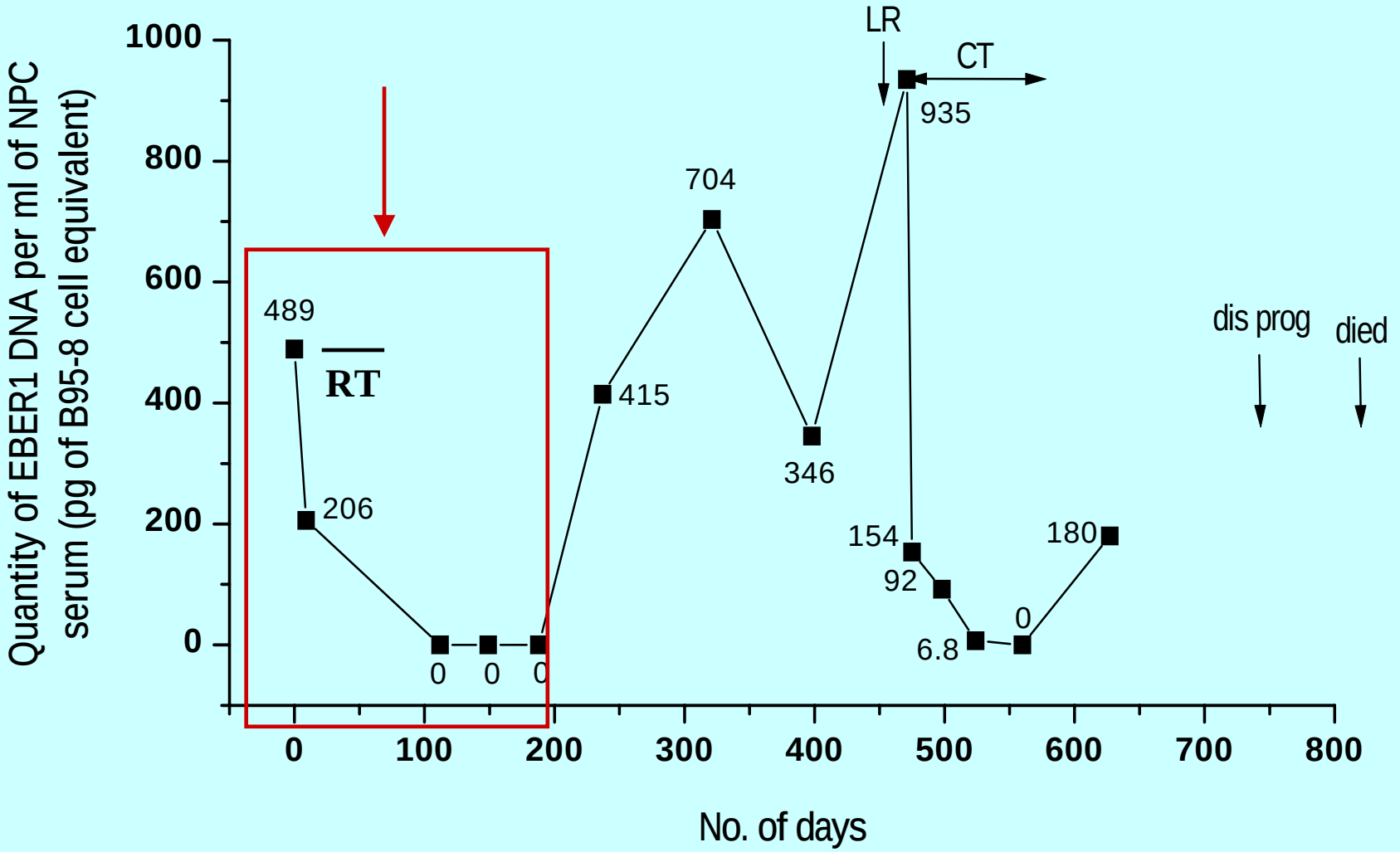
Circulating EBV DNA is generated from NPC tumor & correlated with tumor load:-

(1). A dramatic drop of plasma/serum EBV DNA level when tumor is eradicated by radiation therapy (RT)

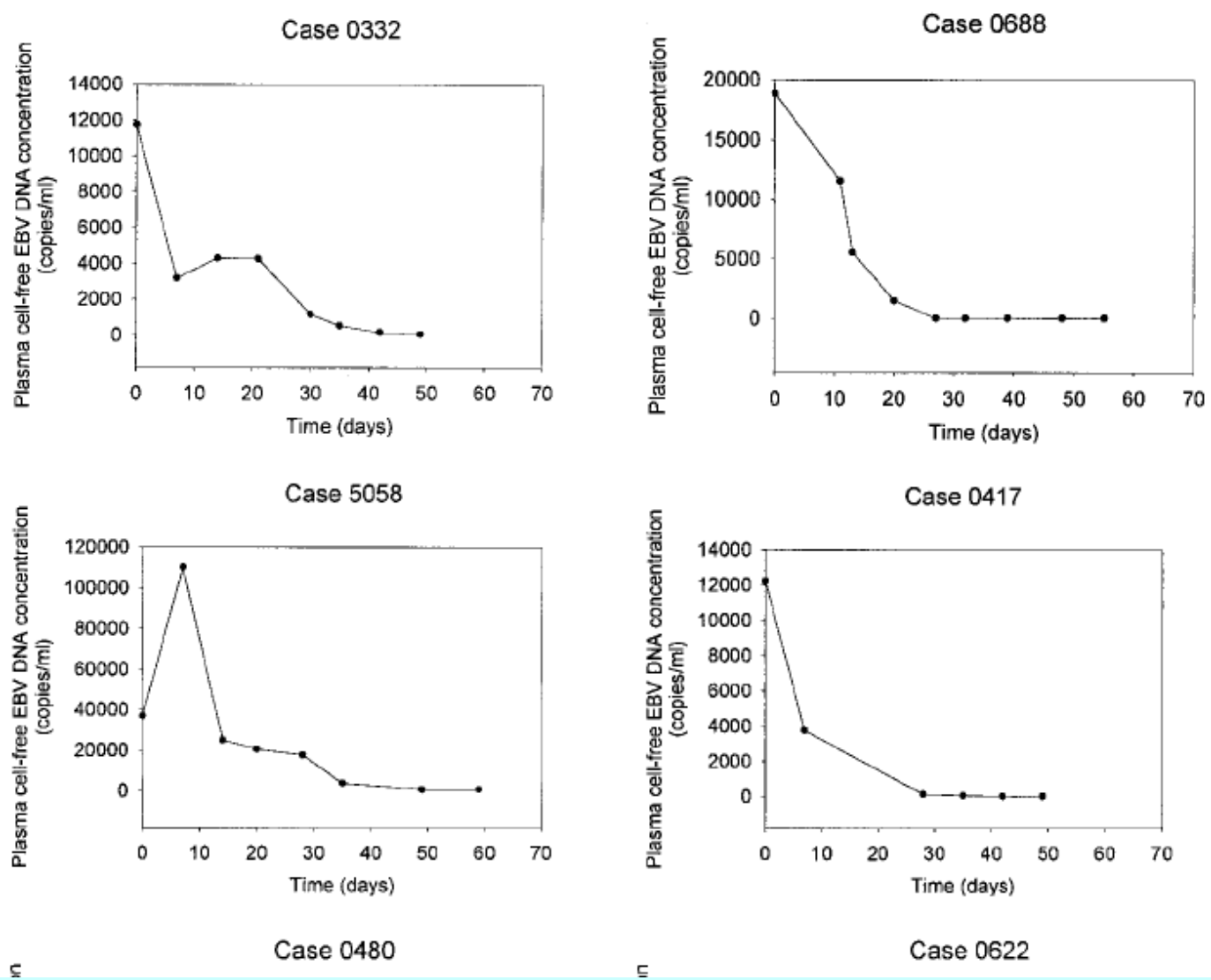
Drastic drop of circulating EBV DNA level post radiotherapy



Drastic drop of circulating EBV DNA level post radiotherapy



Drastic drop of circulating EBV DNA level post radiotherapy



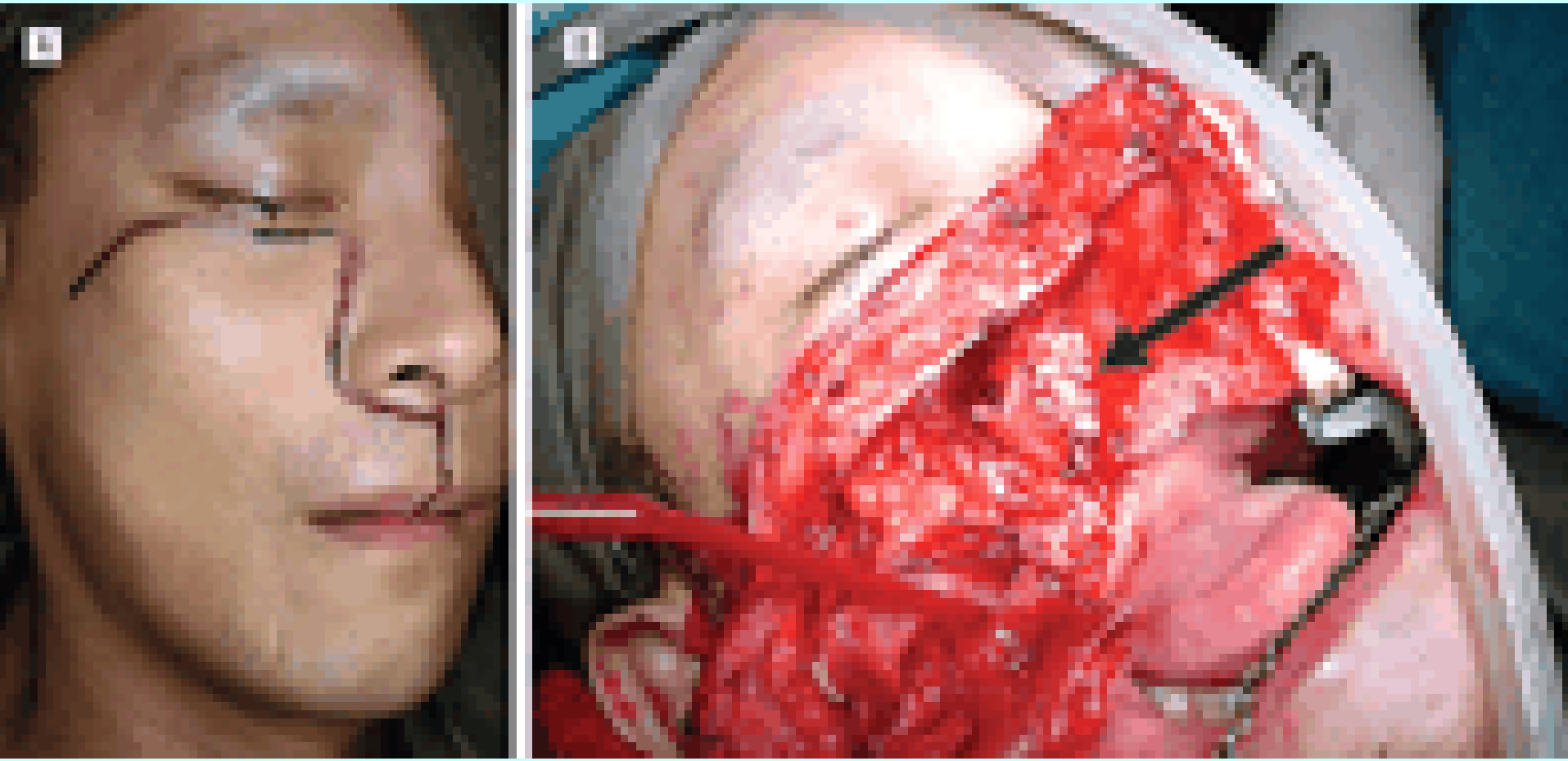
Lo et al. Cancer Res (1999)

Circulating EBV DNA is generated from NPC tumor & correlated with tumor load:-

(1). A dramatic drop of plasma/serum EBV DNA level after surgery (nasopharyngectomy).

Ref -

Drastic drop of circulating EBV DNA level after surgery (nasopharyngectomy)

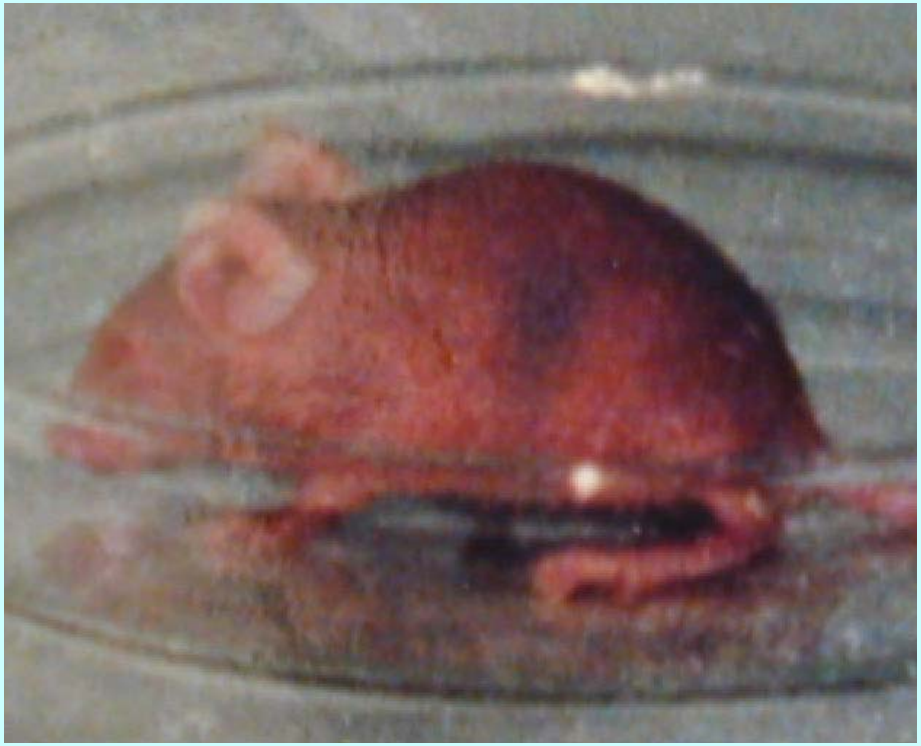


William Wei et al. Head & Neck 2004.

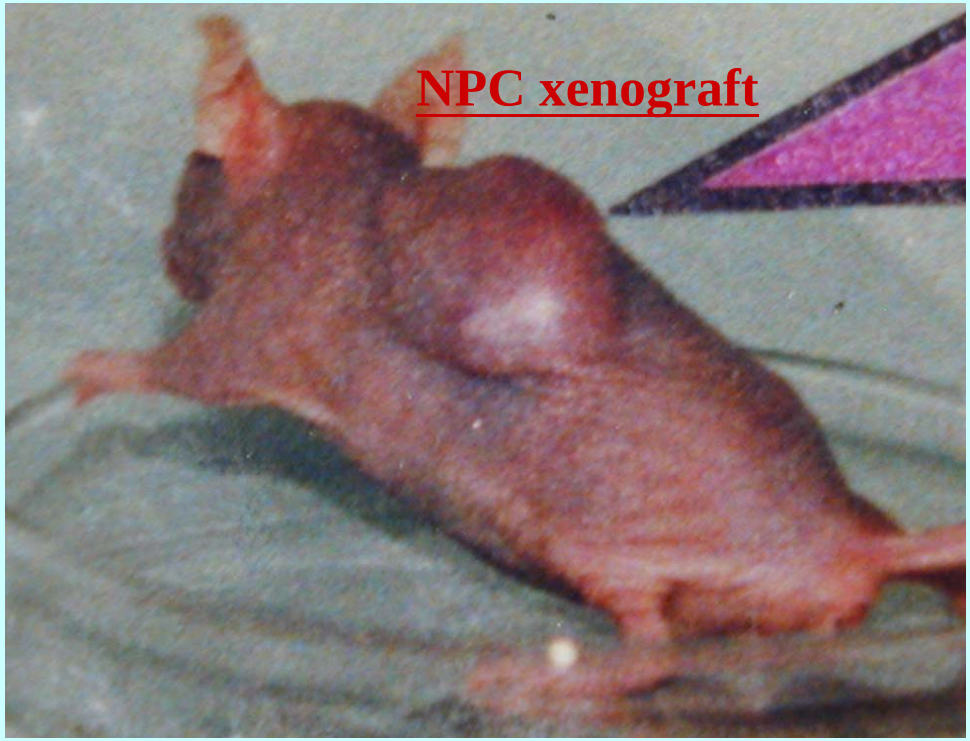
Circulating EBV DNA is generated from NPC tumor & correlated with tumor load:-

(1). A dramatic drop of mouse plasma/serum EBV DNA level NPC tumor xenograft dissected from nude mice.

After NPC tumor xenograft dissected from nude mice – mouse serum/plasma EBV DNA drops



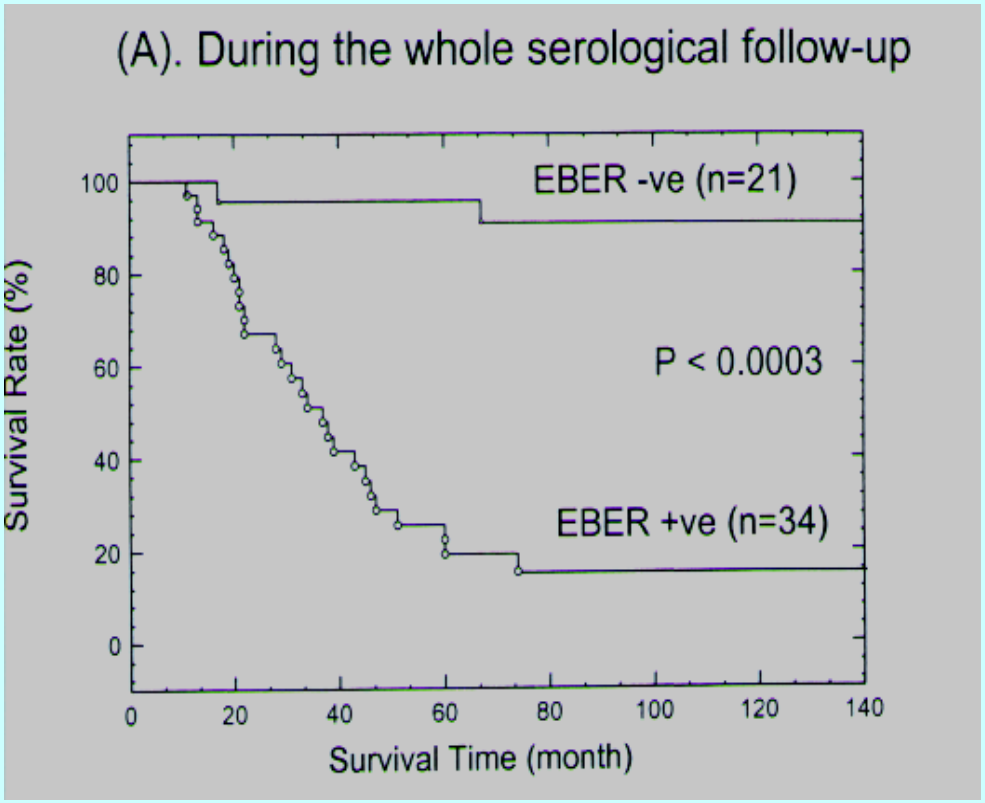
Nude mice without xenograft transplant

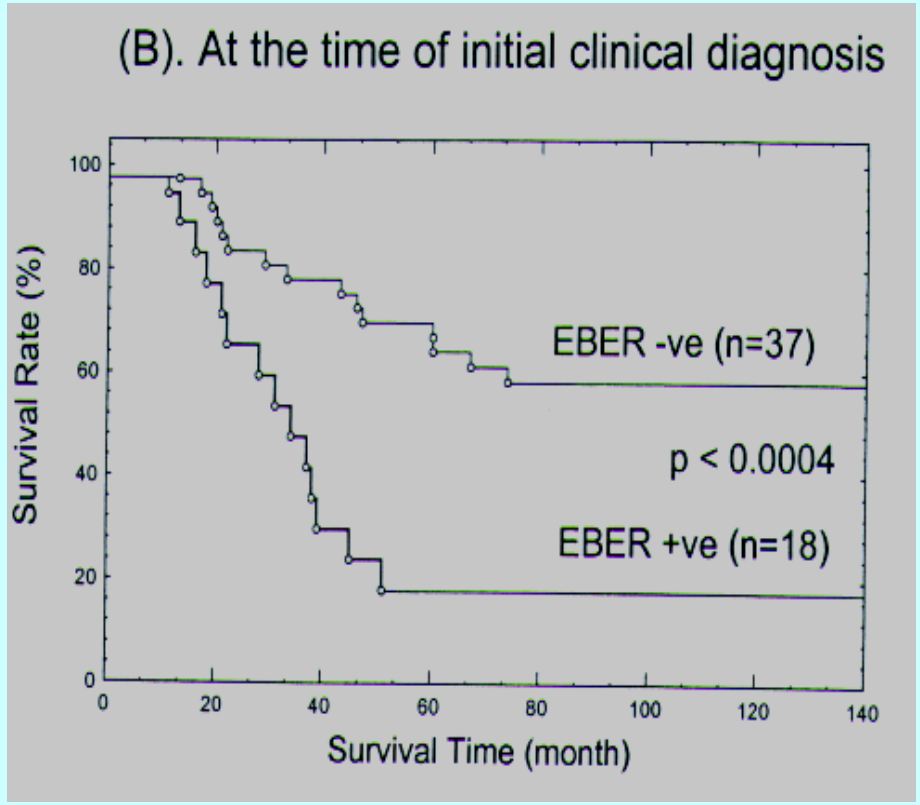


Nude mice with NPC xenograft

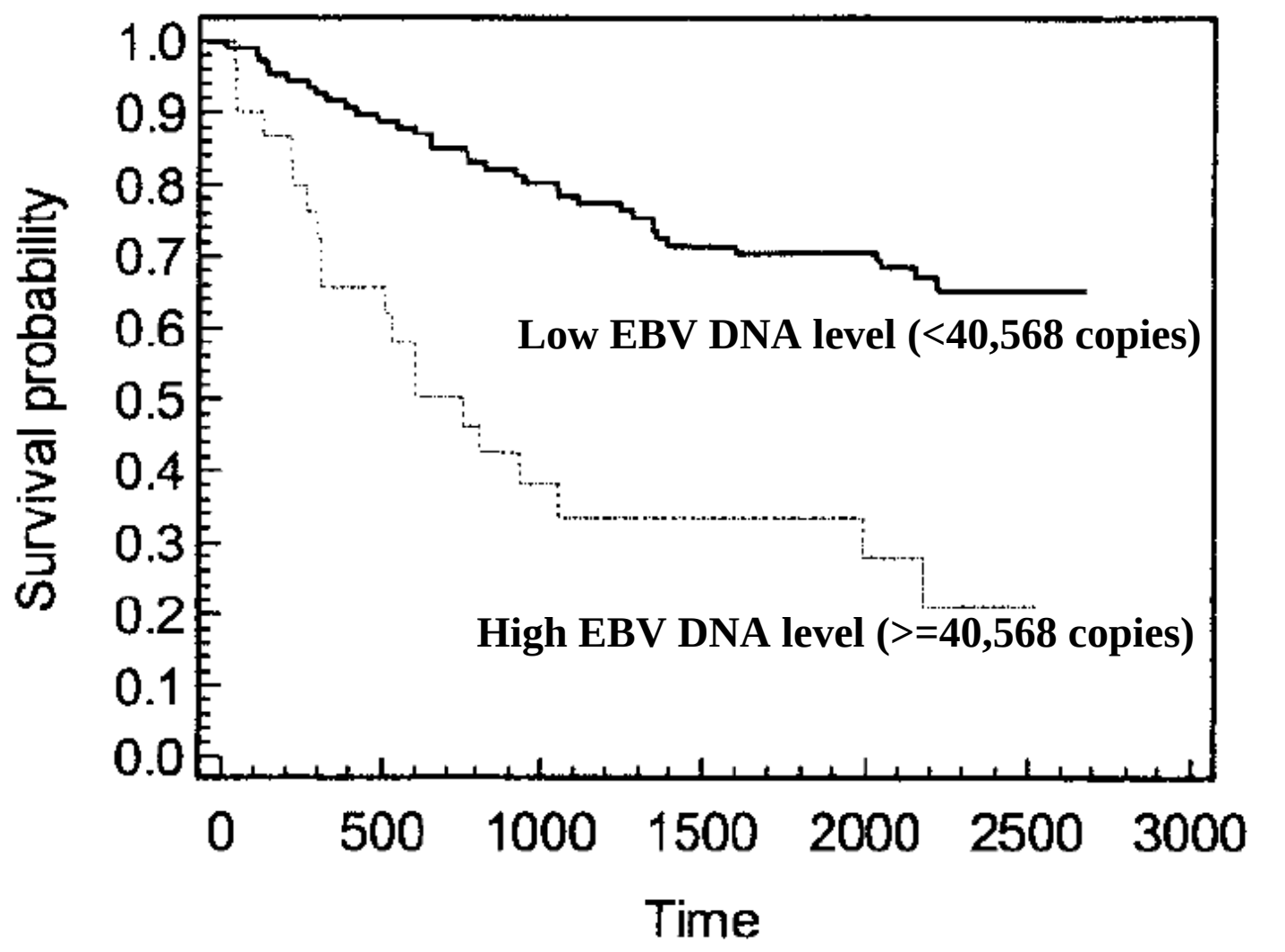
Circulating plasma/serumEBV DNA level was shown to be correlated with survival of NPC patients.

Survival relationship in circulating EBV DNA





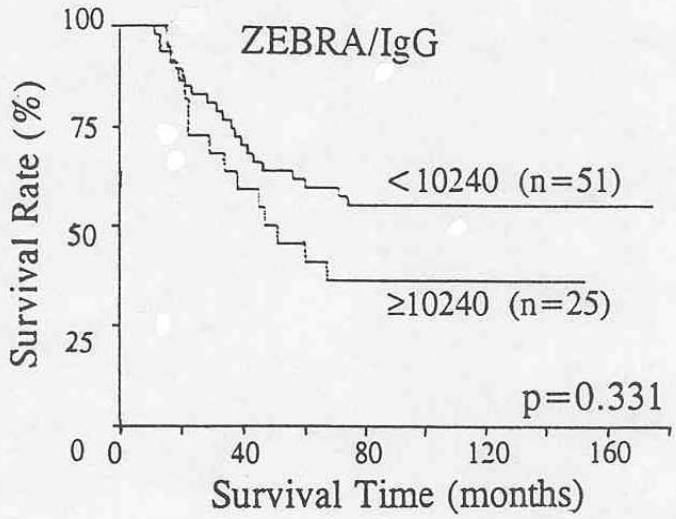
Circulating EBV DNA level correlated with survival in all UICC stages

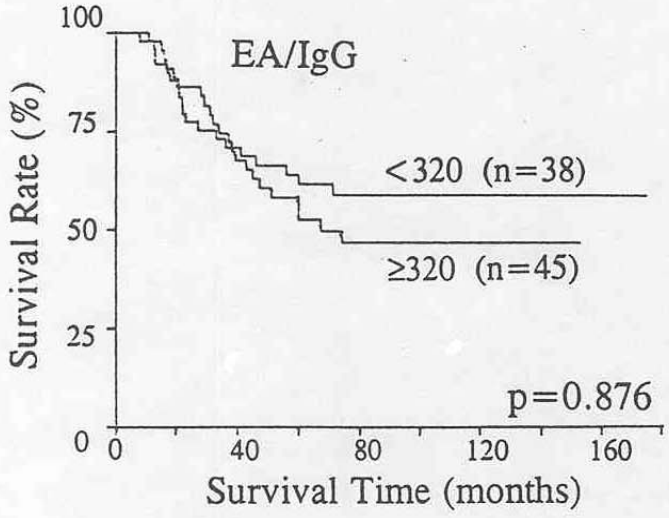


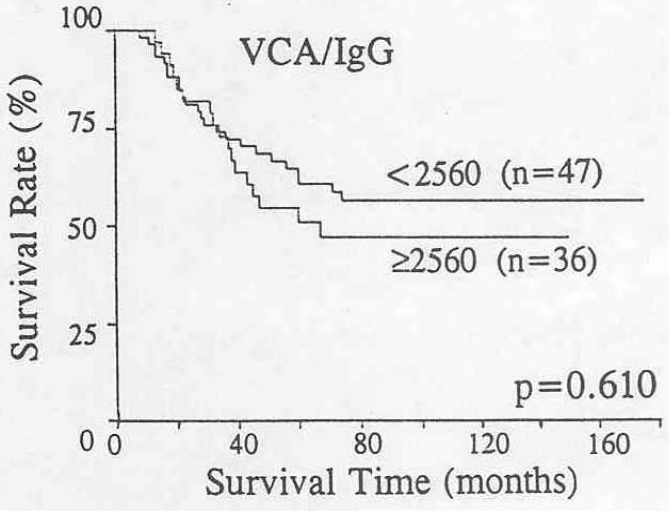
Lo DYM. Cancer Res (2000)

This is important because conventional EBV antibody serology test carried out at disease onset did not show any significant survival relationship.

Serum antibodies to EBV EA & VCA antigens tested at onset did not significantly correlate with survival

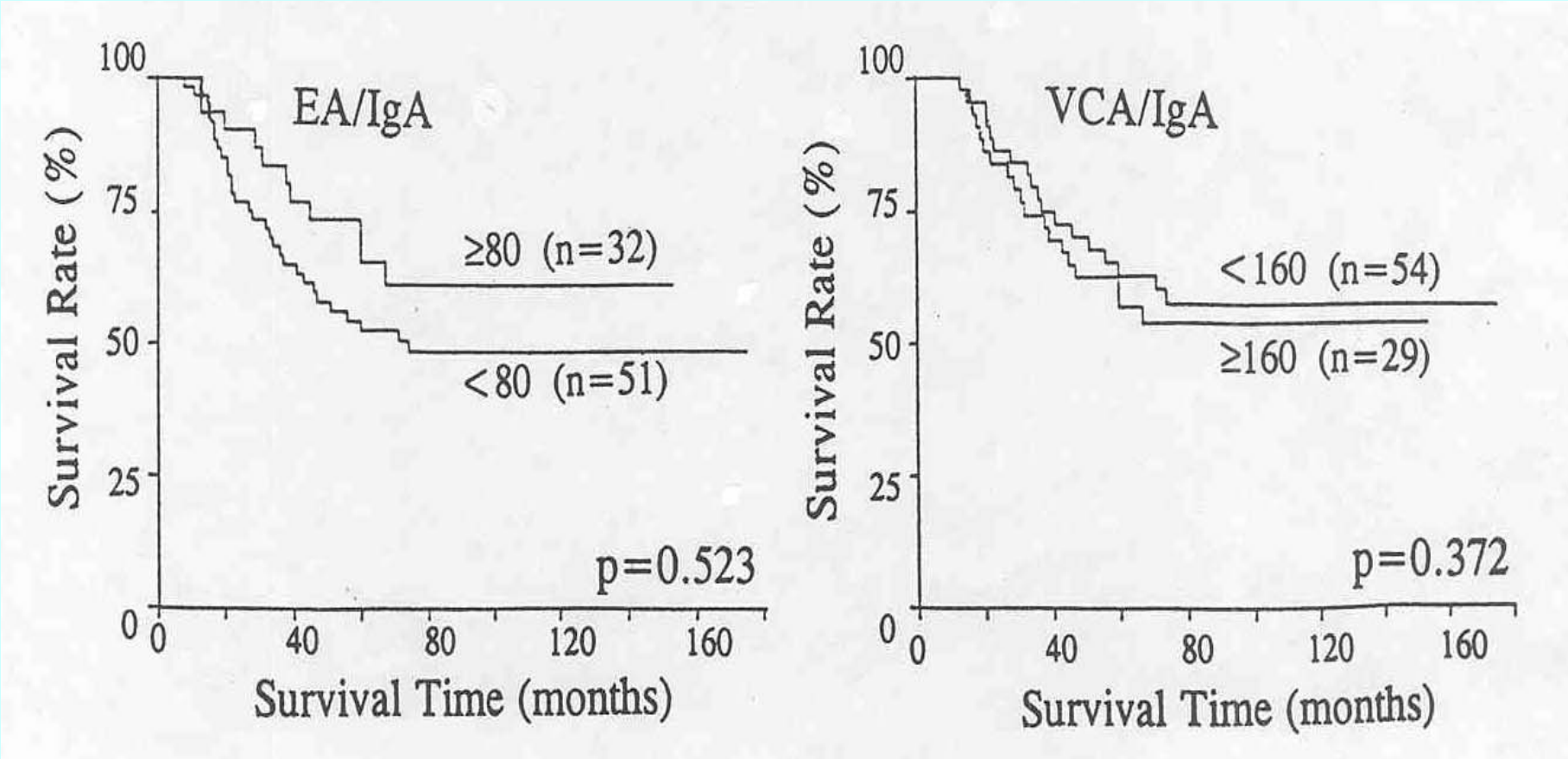






Yip TTC et al. EBV Report – Review 1992.

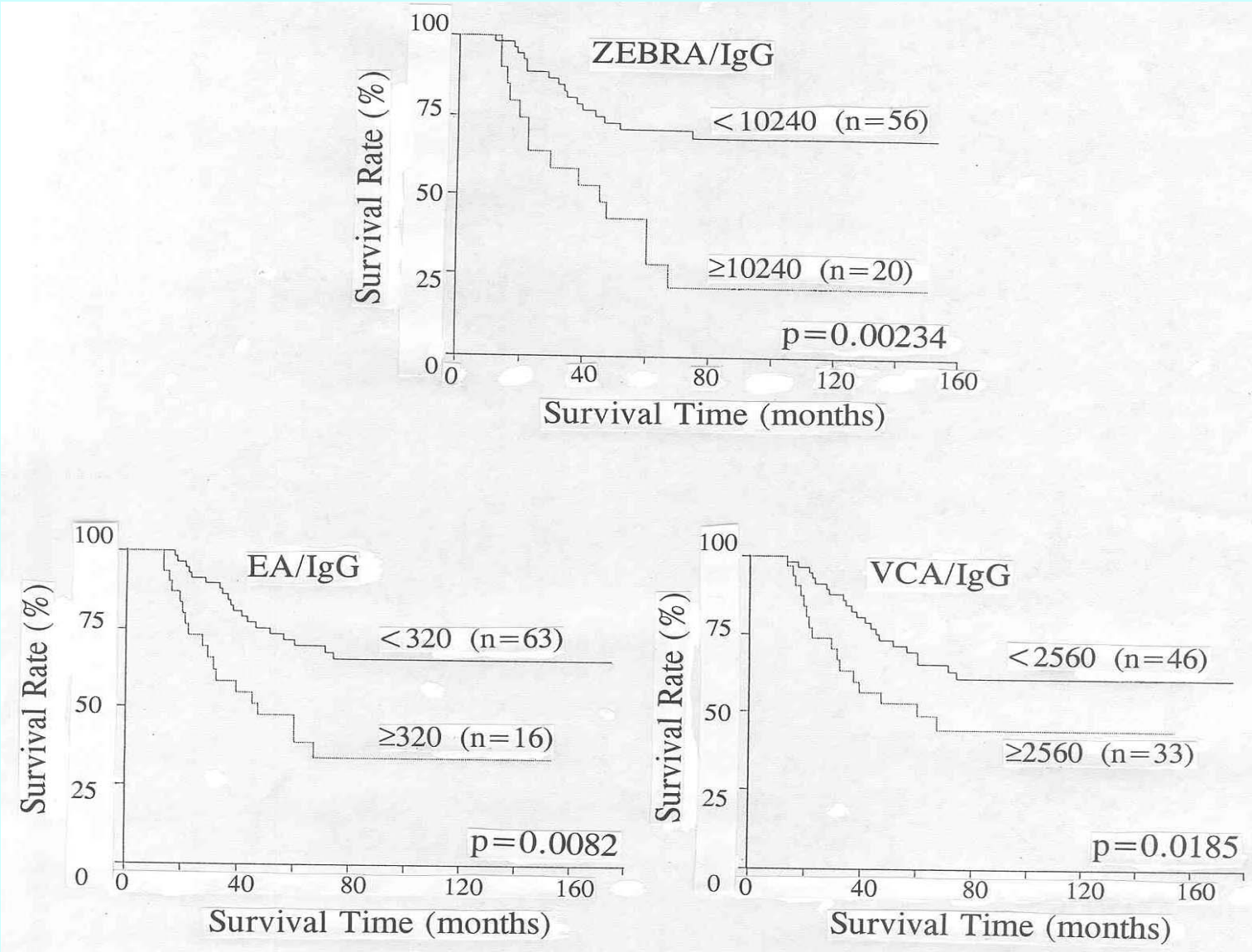
Serum antibodies to EBV EA & VCA antigens tested at onset did not significantly correlate with survival



Yip TTC et al. EBV Report – Review 1992.

Only post-radiotherapy tested EBV antibody titers demonstrated survival relationship

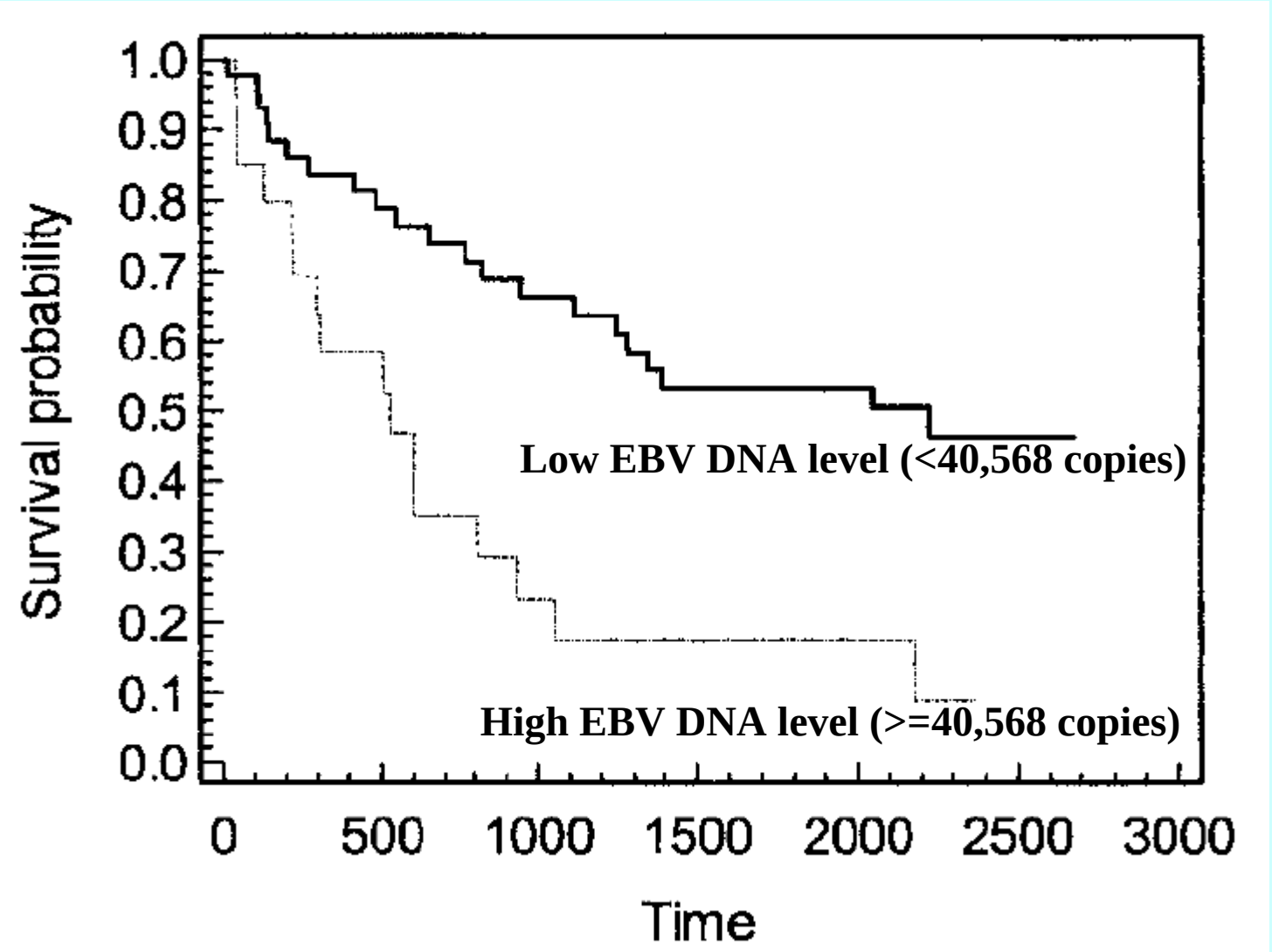
Serum antibodies to EBV EA & VCA antigens tested post RT significantly correlated with survival



Yip TTC et al. EBV Report – Review 1992.

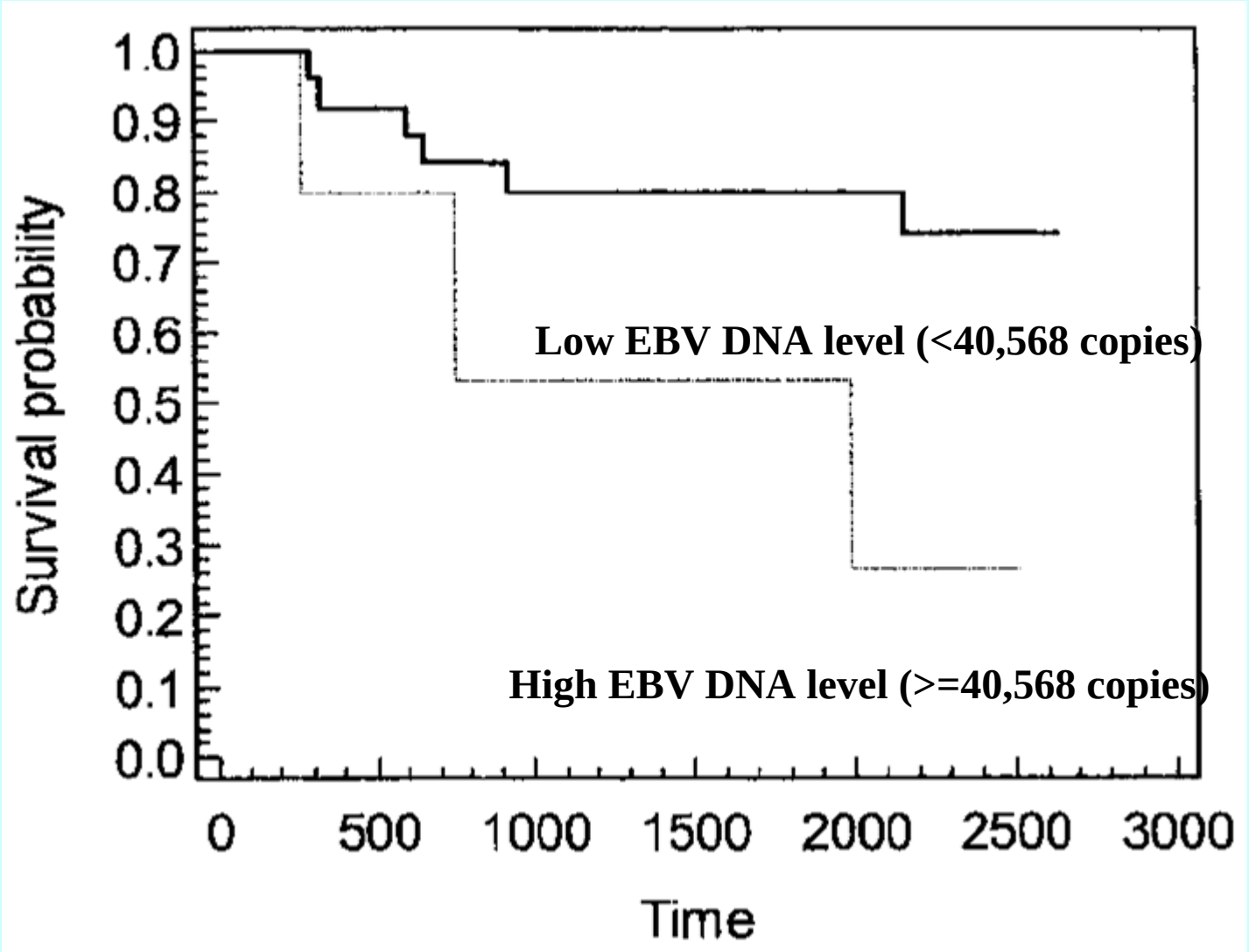
Survival relationship with EBV DNA is maintained when stratified to stages 3 & 4

Circulating high EBV DNA level correlated with poor survival when stratified at stage 4



Lo DYM. Cancer Res (2000)

Circulating high EBV DNA level correlated with poor survival when stratified at stage 3



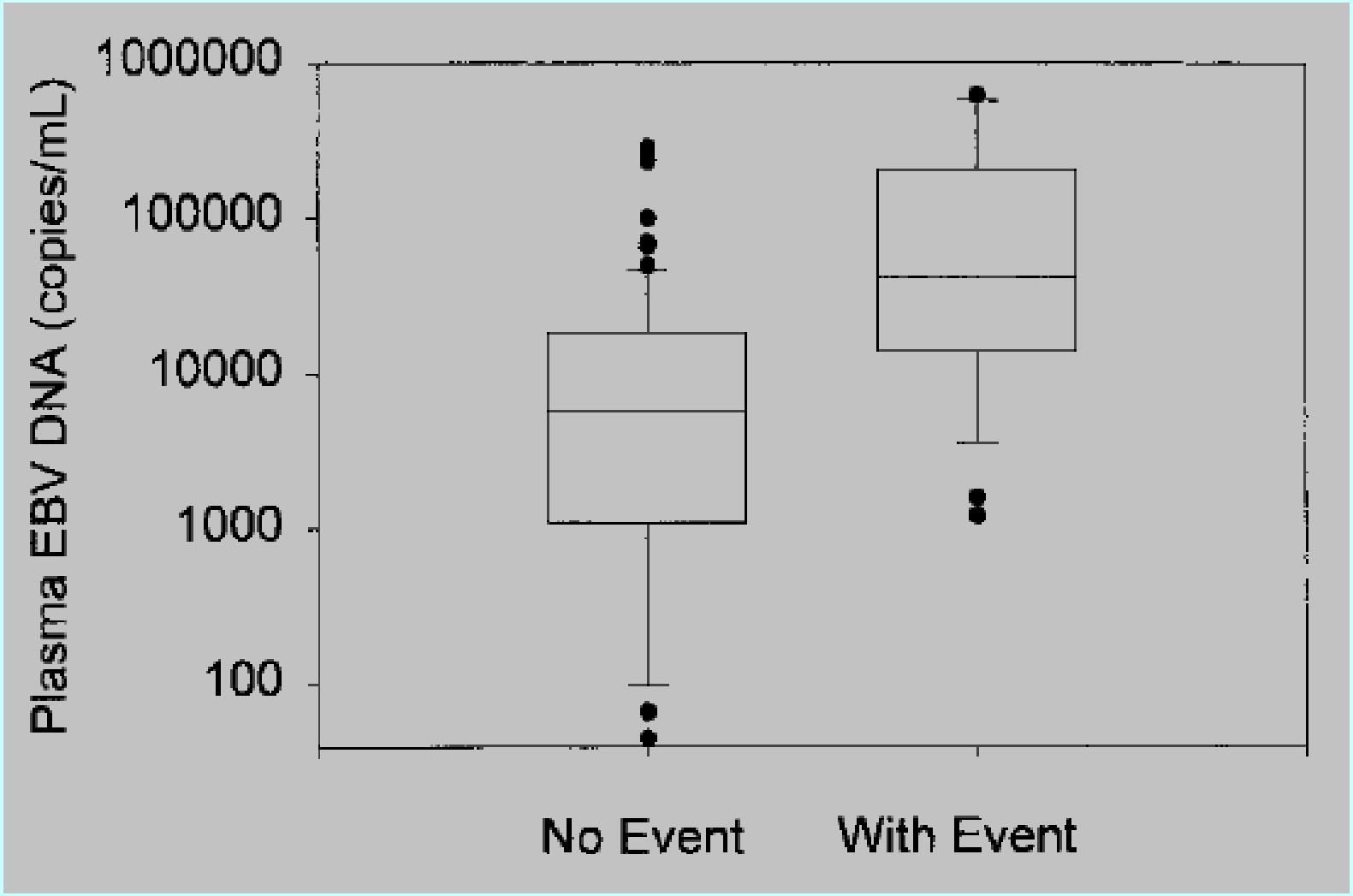
Lo DYM. Cancer Res (2000)

Plasma EBV DNA can also separate early stage (I & II) patients into poor & good prognostic groups

(S F Leung et al. Cancer 2003)

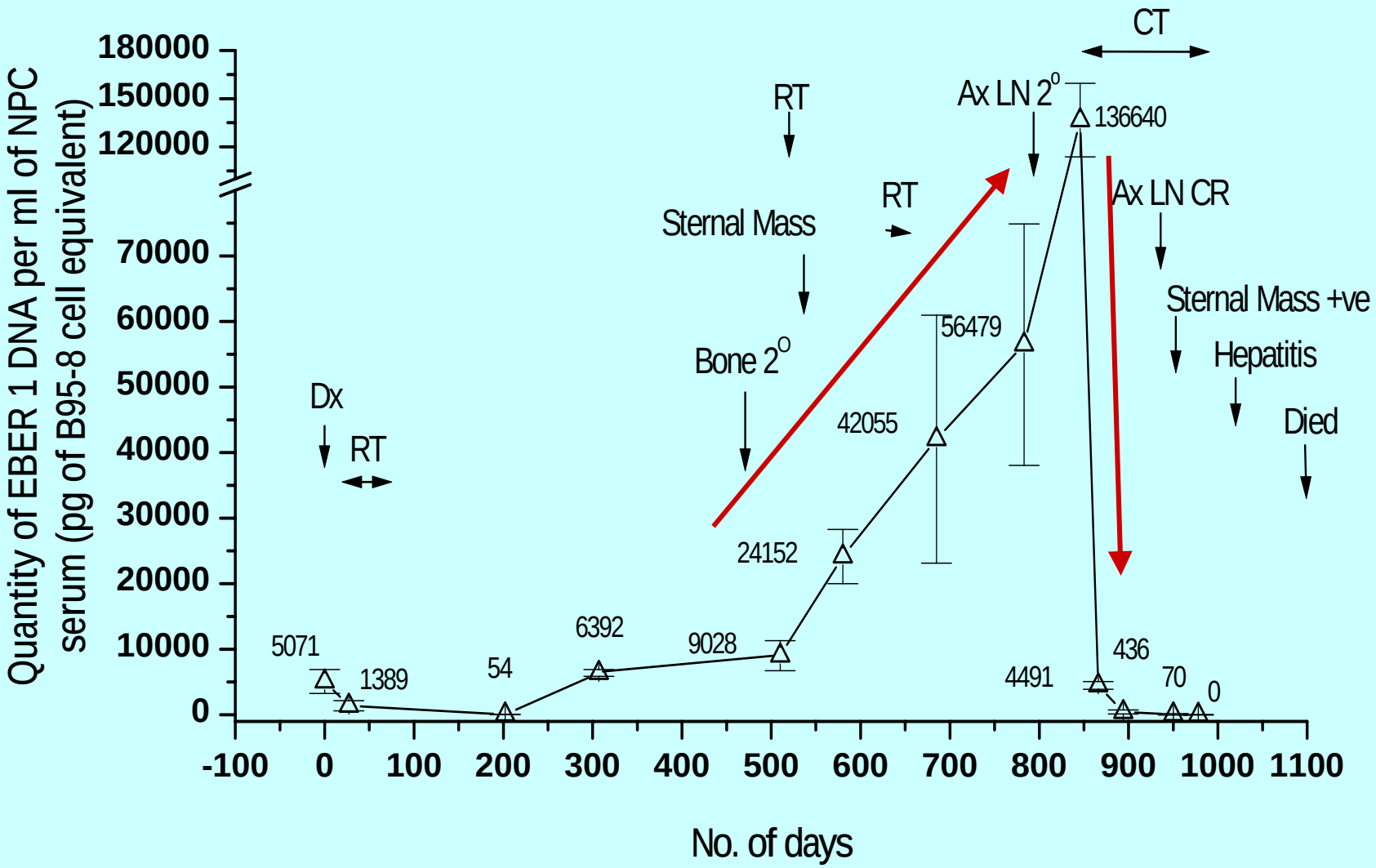
Circulating EBV DNA can be useful to monitor relapses chemotherapy response

Patients with clinical events within the first year after RT had higher circulating EBV DNA level



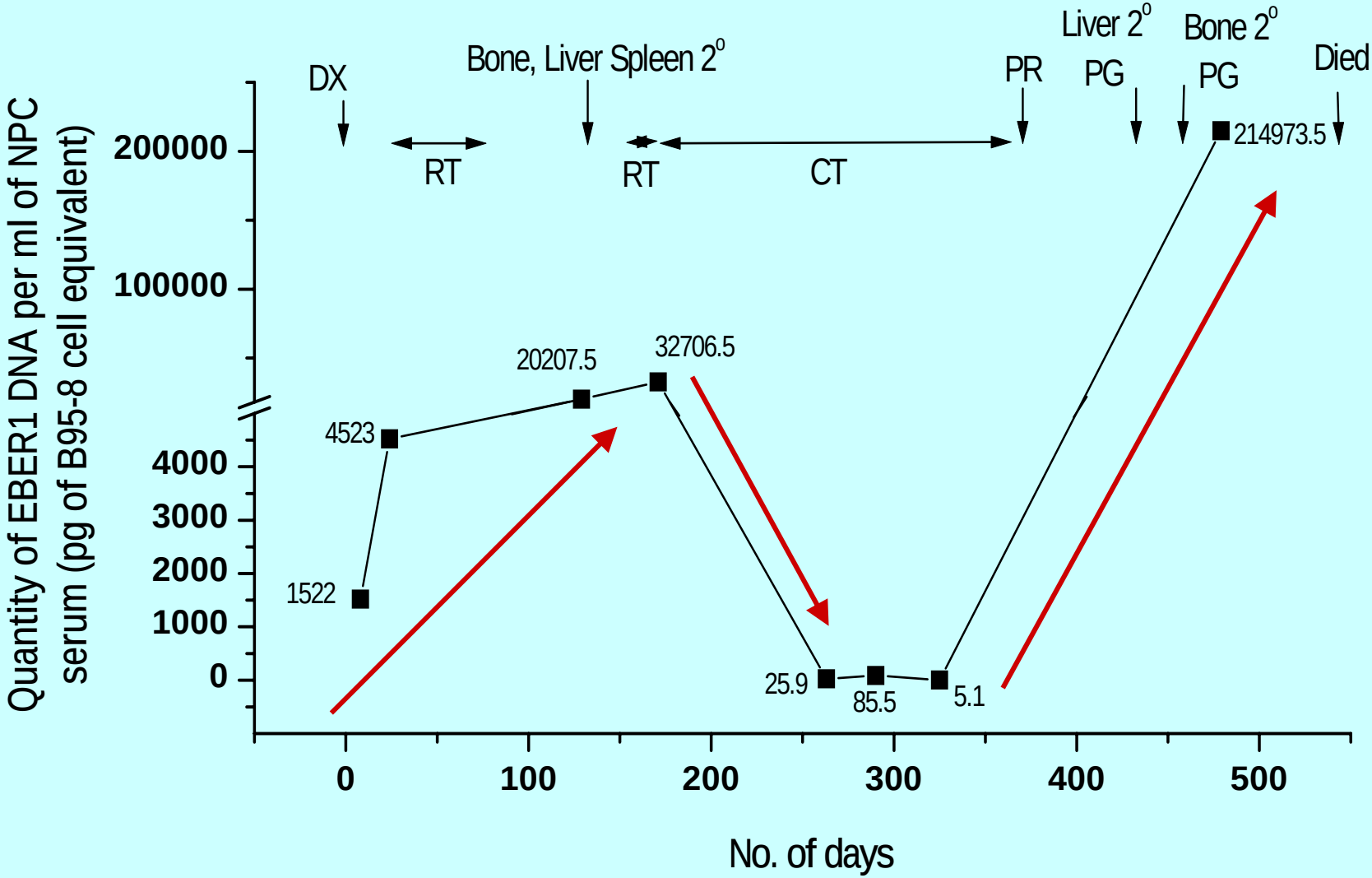
Lo DYM. Cancer Res (2000)

Elevations of circulating EBV DNA level on relapse & level drop on chemotherapy – patient 1

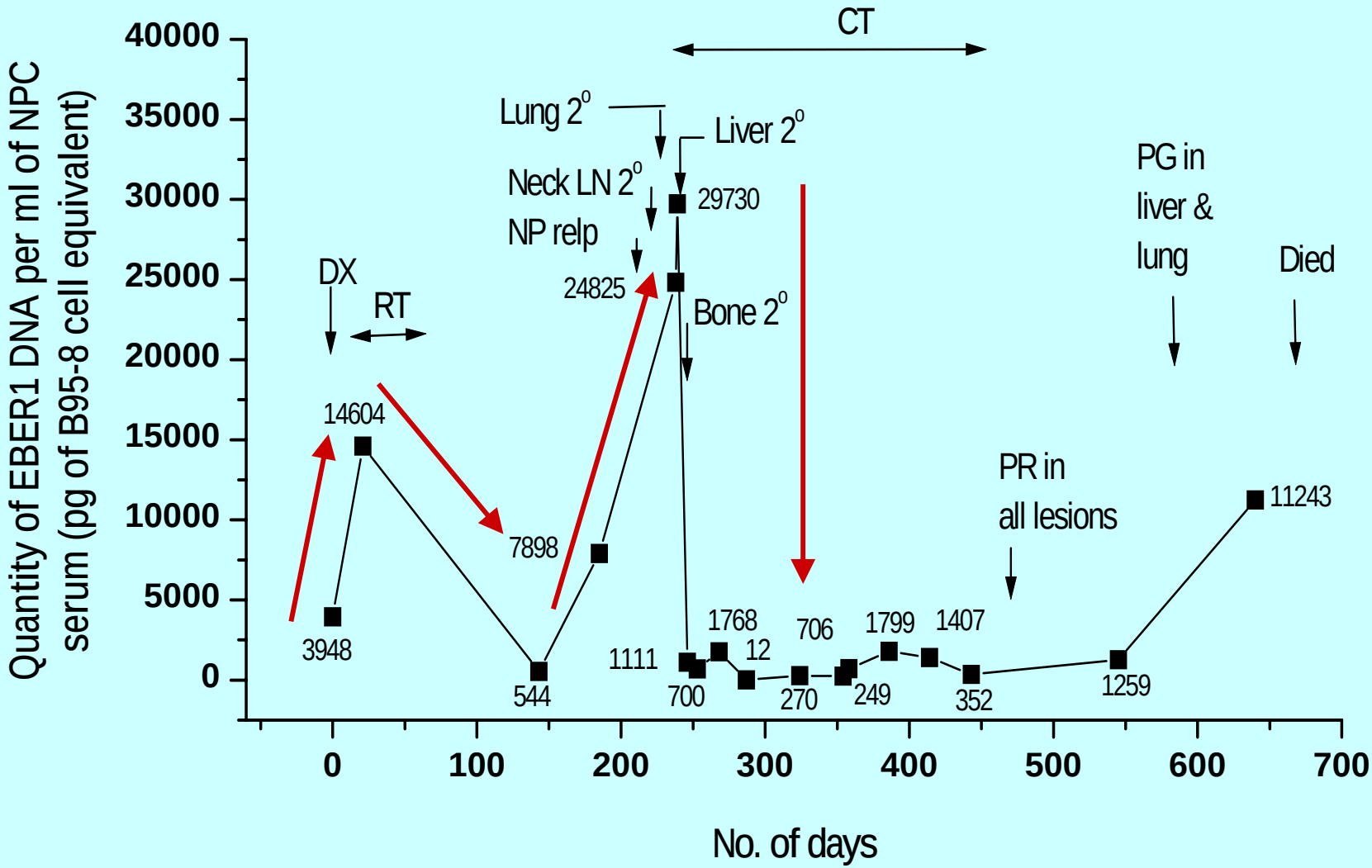


Ngan RKC, Lau WH, Yip TTC et al. Ann NY Acad Sci 2001

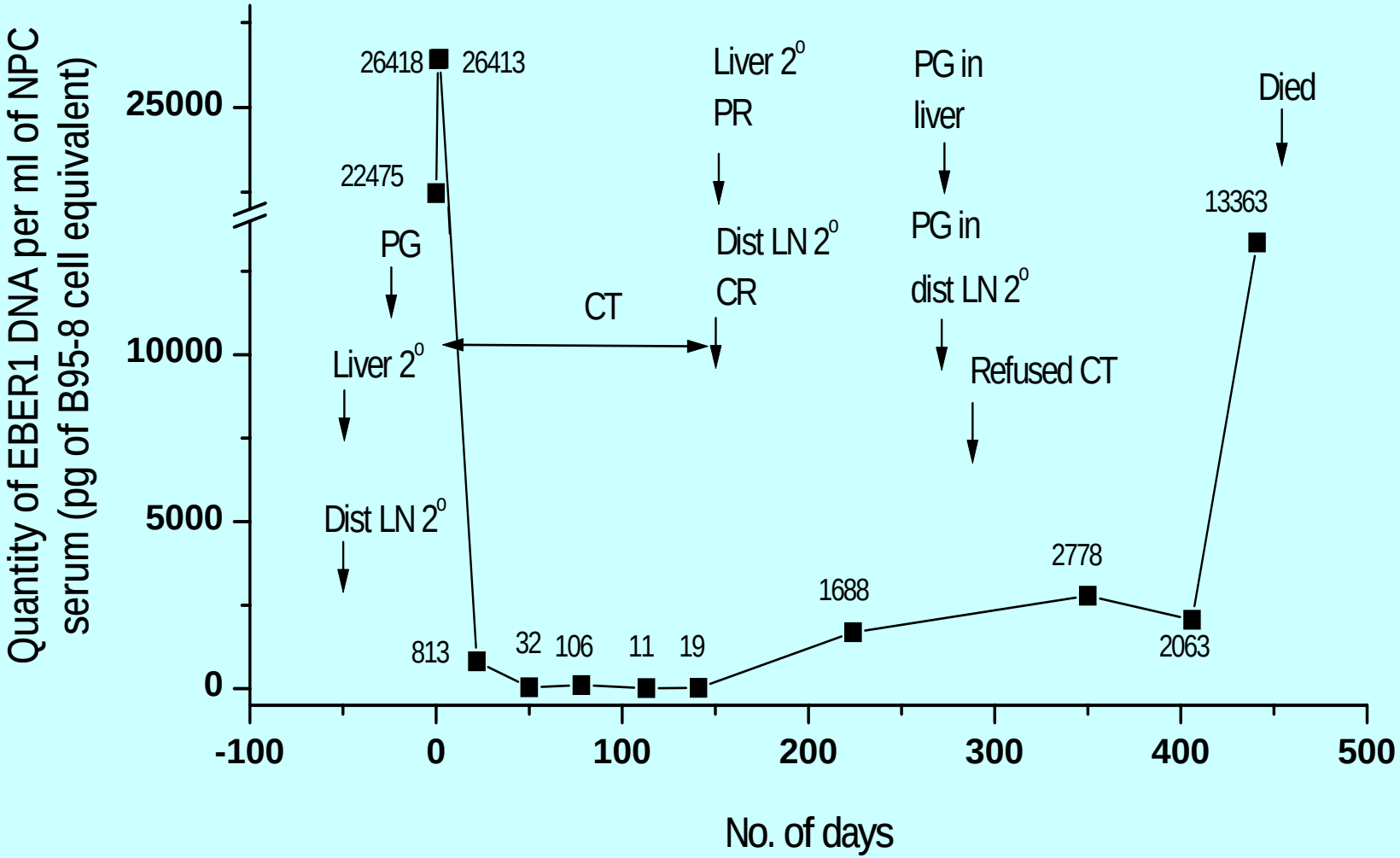
Elevations of circulating EBV DNA level on relapse & level drop on chemotherapy – patient 2



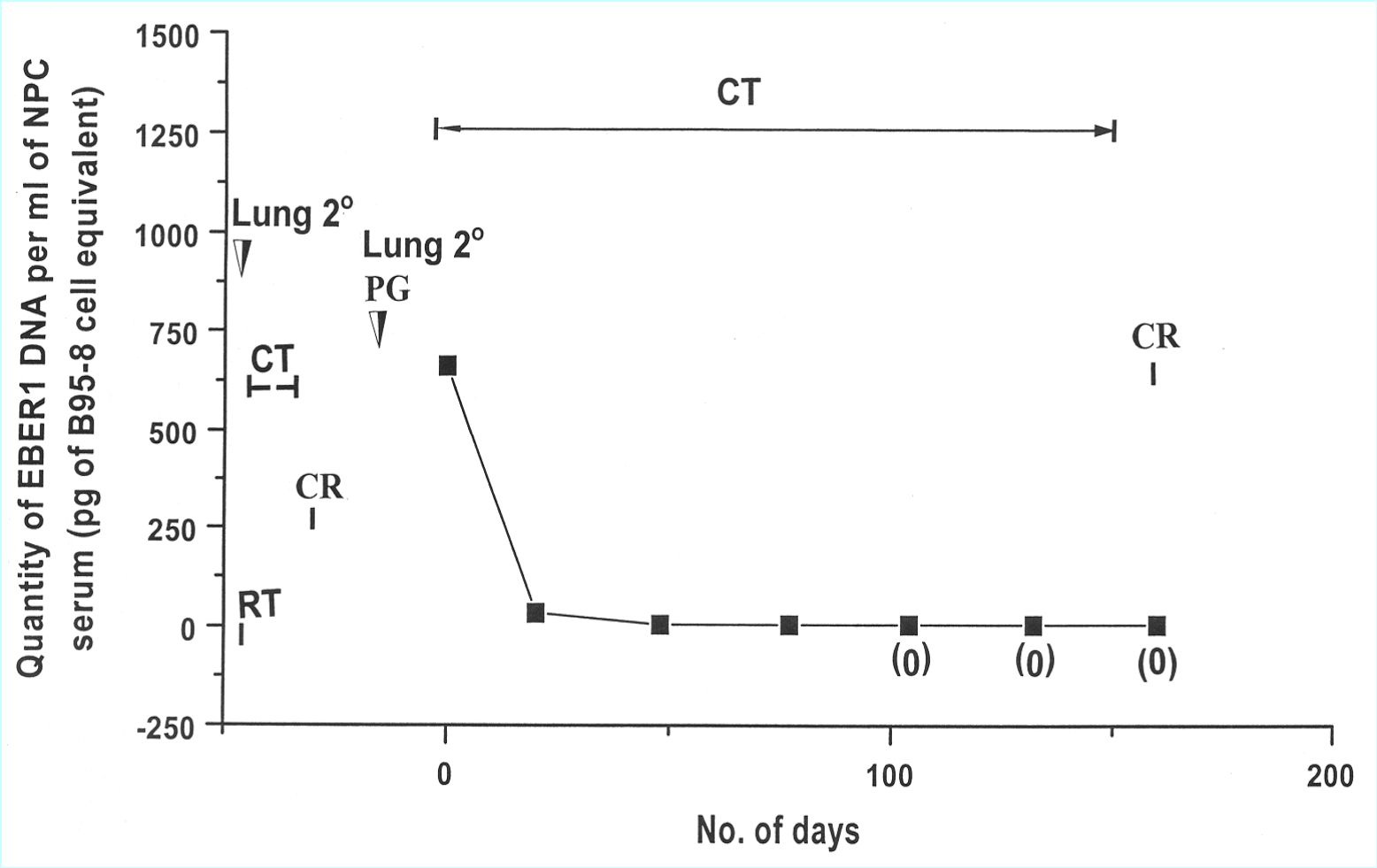
Elevations of circulating EBV DNA level on relapse & level drop on chemotherapy – patient 3



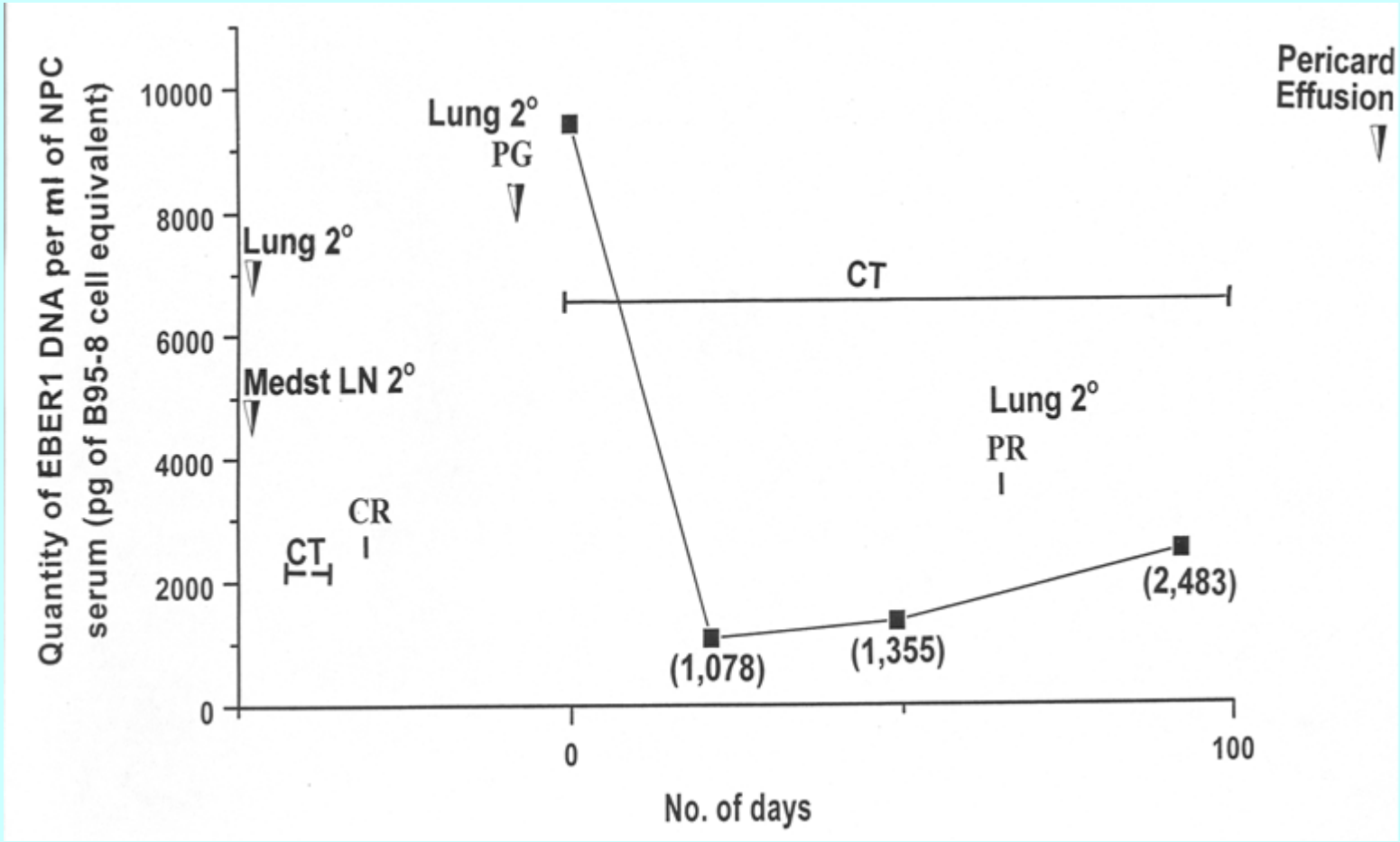
Elevations of circulating EBV DNA level on relapse & level drop on chemotherapy – patient 4



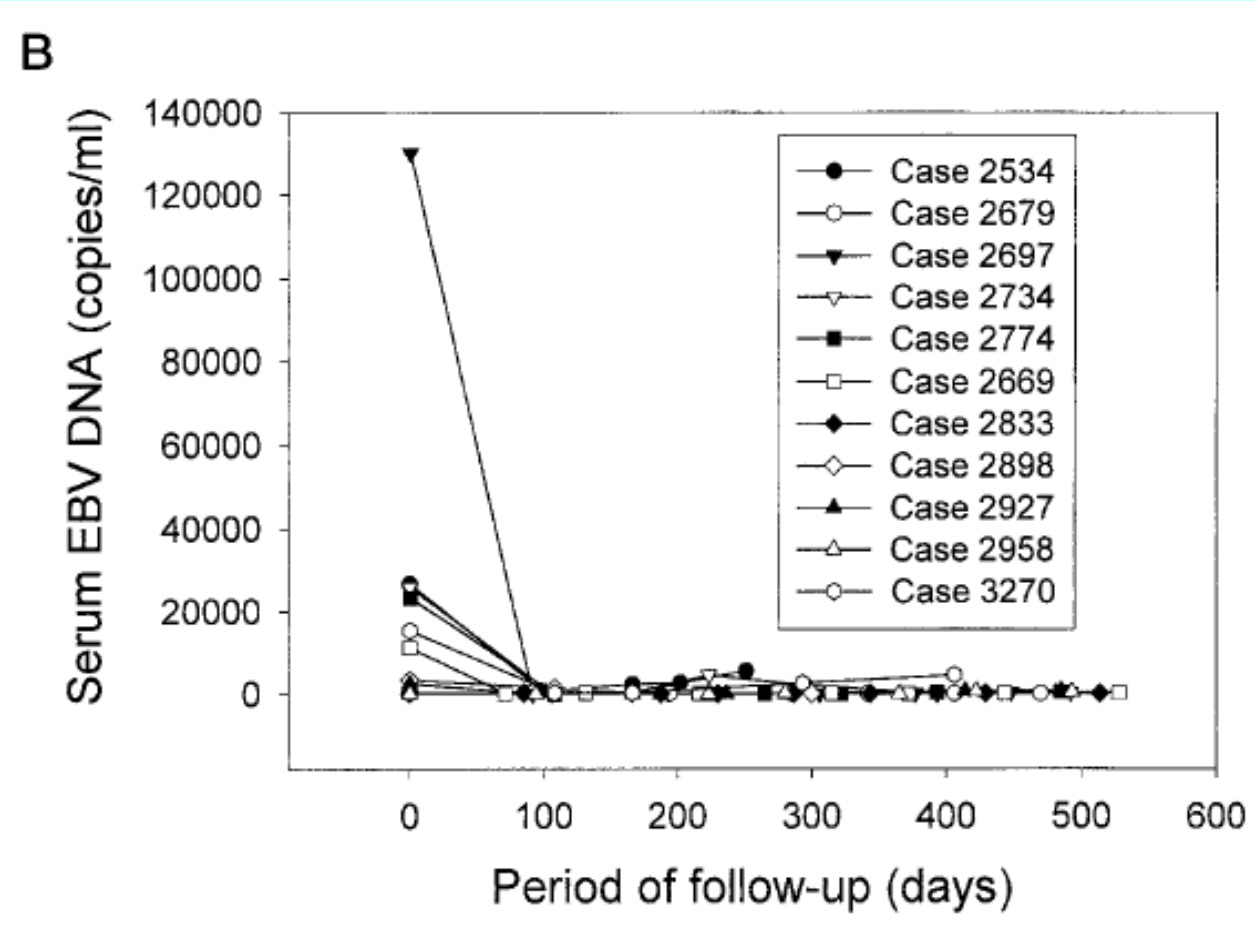
Elevations of circulating EBV DNA level on relapse & level drop on chemotherapy – patient 6



Elevations of circulating EBV DNA level on relapse & level drop on chemotherapy – patient 5



Circulating EBV DNA levels dropped to background on complete remission

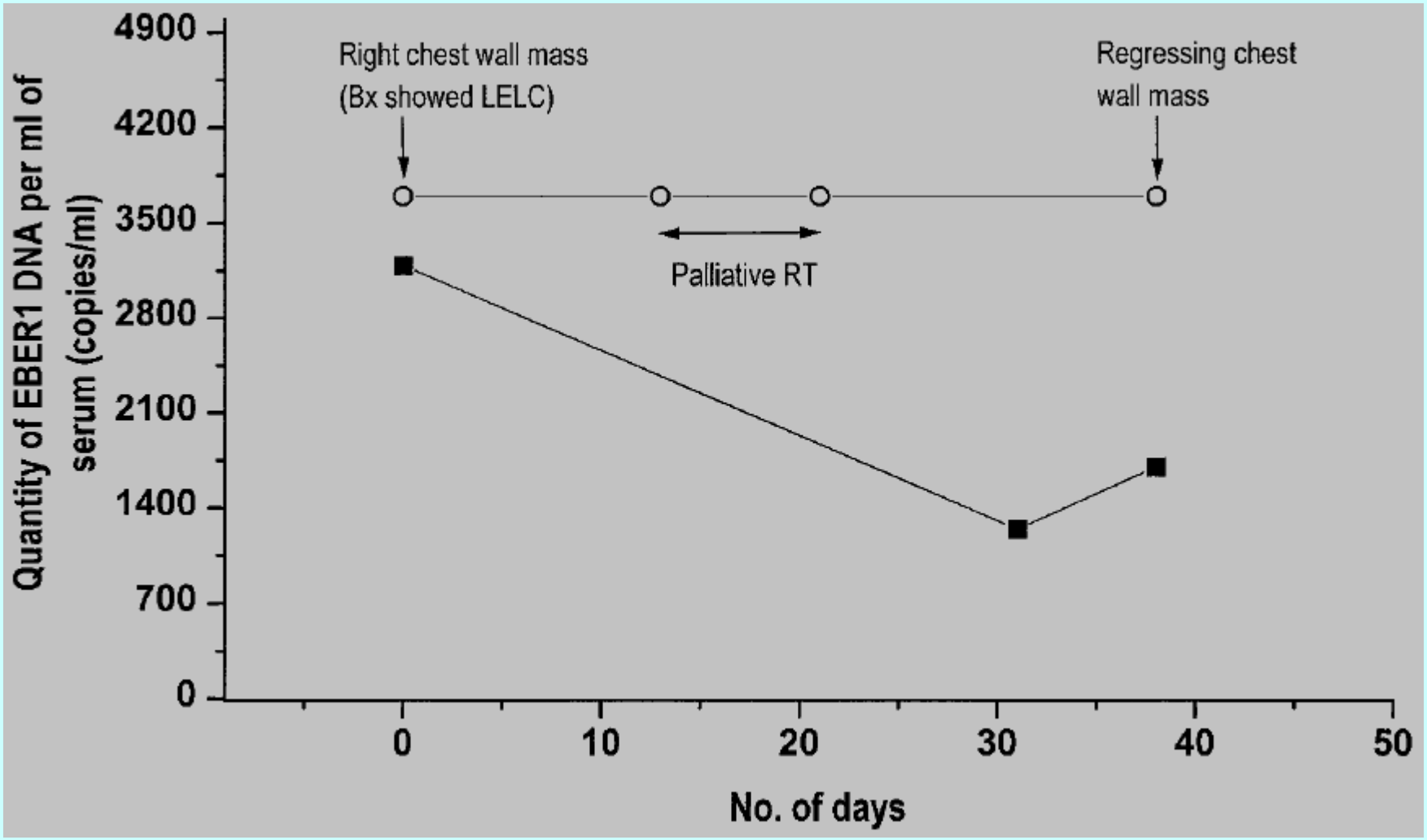


Lo DYM. Cancer Res (___)

With this high level of detection sensitivity & specificity, this test nowadays rapidly becomes a potentially useful test in primary diagnosis of NPC, monitoring relapse, prognostication & determining chemotherapy response.

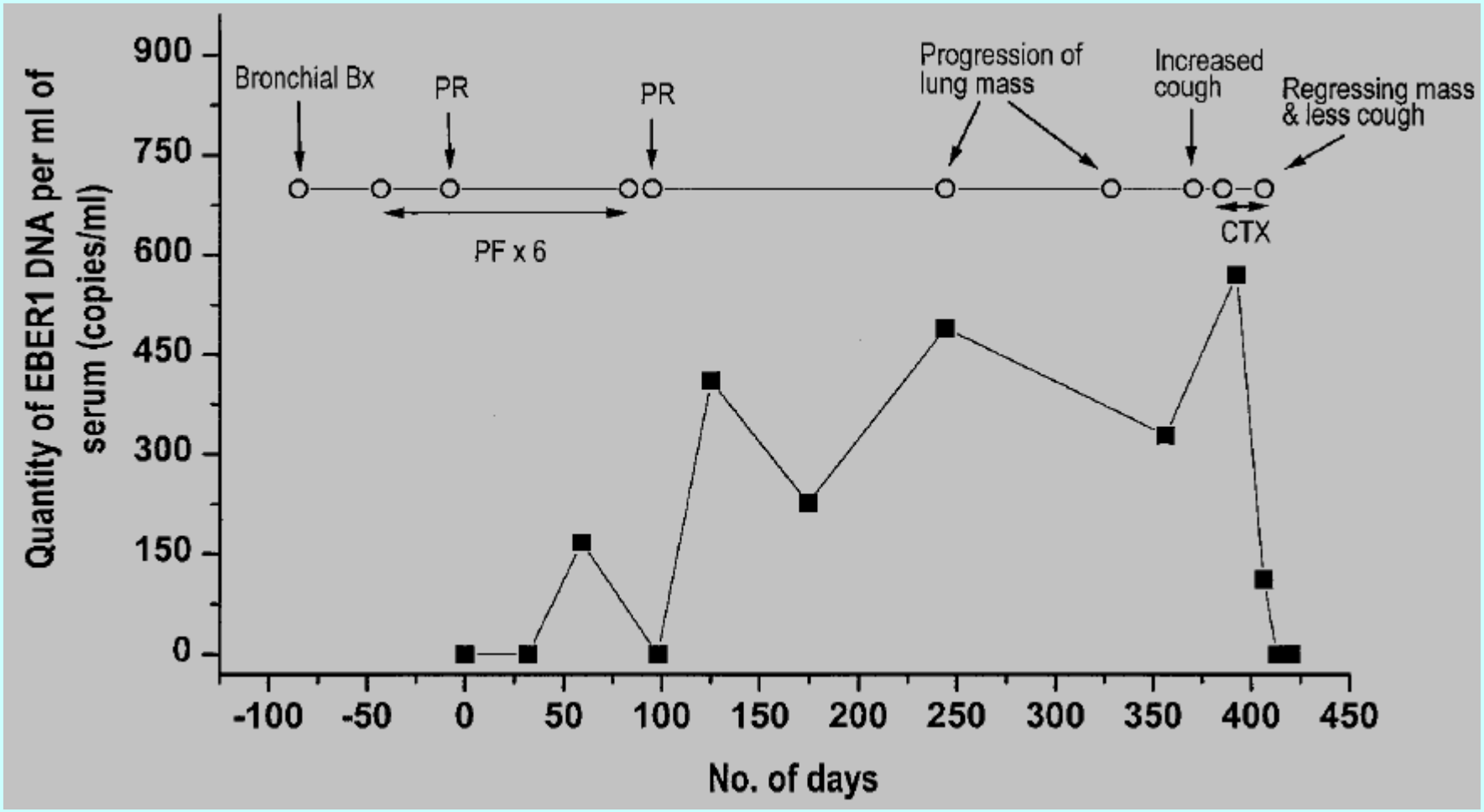
Monitoring role also shown in EBV +ve lymphoepithgelioma of lung (LELC)

Monitoring of circulating EBV DNA in 2nd LELC patient



Ngan RKC, Yip TTC et al. Clin Cancer Res 2002

Monitoring of circulating EBV DNA in 3rd LELC patient



Ngan RKC, Yip TTC et al. Clin Cancer Res 2002

Is shown to be useful in monitoring EBV +ve:-

(1). AIDS associated lymphoma.
(2). Hodgkin lymphoma.
(3). NK/T cell nasal lymphoma.
(4). Burkitt's lymphoma.

Could be useful in:-

(1). Gastric cancer.
(2). Salivary gland tumor.

But reported detection sensitivities from different groups in recent 10 years vary substantially from 50-60% to 95%

main reason – EBV DNA in plasma & serum exists as short fragments (87% <181 bp) (Chan et al. Cancer Research 2003).

(1). Many investigators design PCR primers to EBV DNA amplicon of too large in size (>200 bp).

(2). They used inappropriate DNA purification method for large DNA size.

Very important:-

(1). To design PCR primers for shortest amplicon length (<80 bp).

(2). Use appropriate DNA purification methods for short pieces of DNA.

Sensitivity will be dramatically increased.

Which marker is most sensitive?

(1). Bam-H1-W fragment have many repeat in the virla episome.

(2). EBNA1 gene also have repeated sequences.

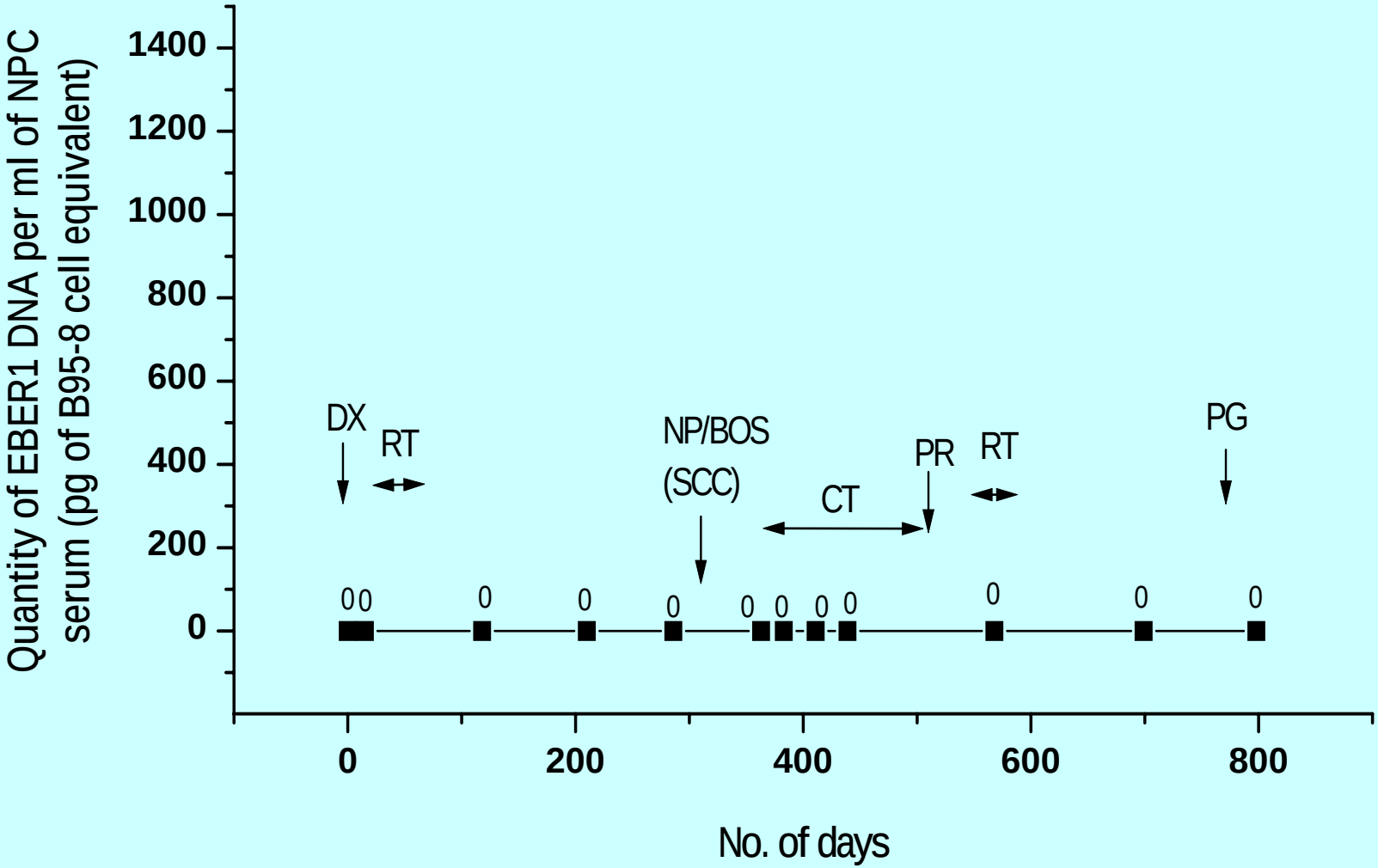
(3). Large quantity EBER DNA.

(4). LMP1, BZLF1 & VCA DNA - less sensitive.

- However, most of the NPC encountered in South East Asia are WHO types 2 & 3 (either undifferentiated or poorly differentiated squamous cell carcinoma) which are EBV +ve.

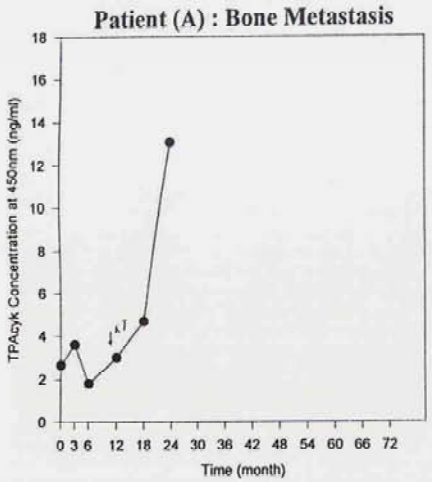
- Circulating EBV DNA test is not useful in EBV –ve WHO type 1 keratinized SCC – more prevalent in Caucasians than in Chinese.

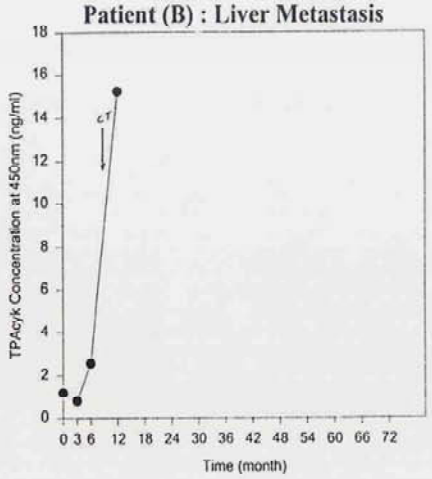
No elevation of circulating EBV DNA level at diagnosis & on relapse in an EBV –ve WHO type 1 NPC

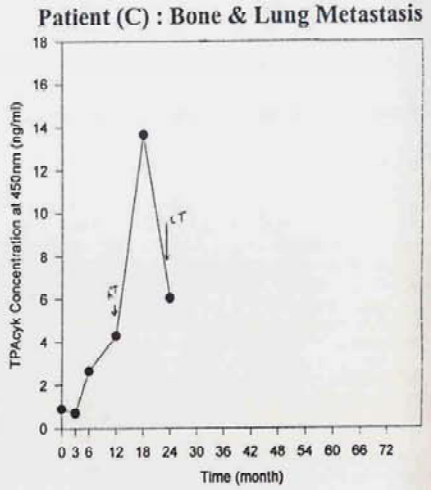


Serum cytokeratin test can detect relapse in both EBV+ve & -ve NPC patients

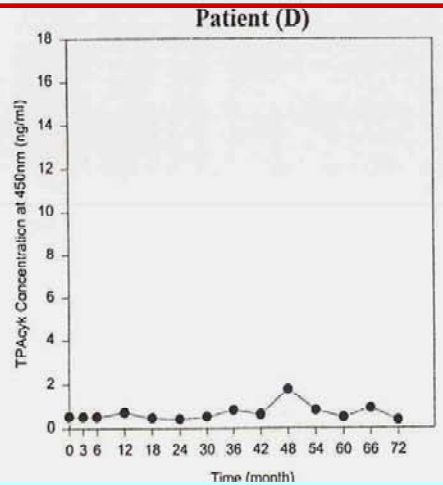
(I) Patients developed relapse after treatment

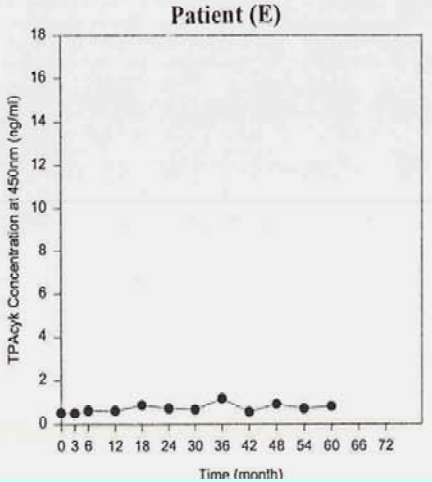


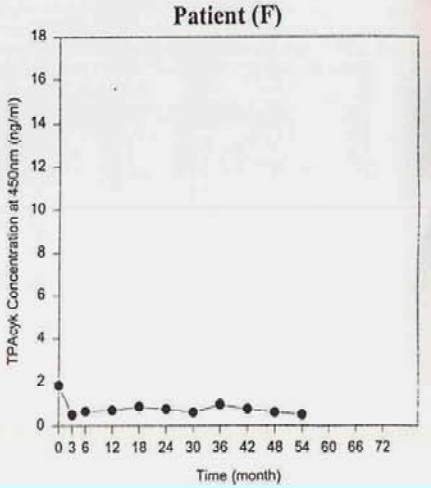




(II) Patients in remission after treatment

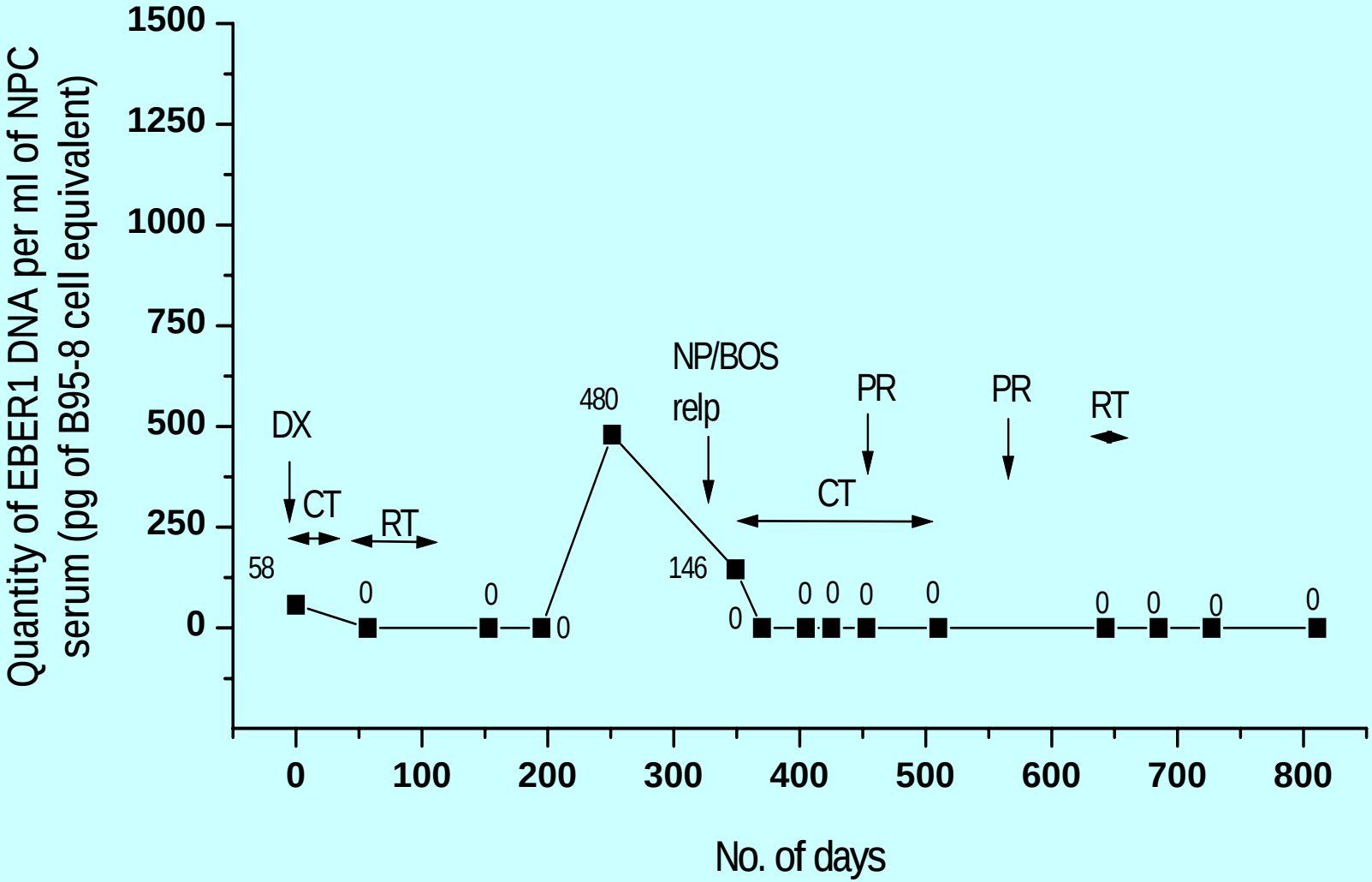




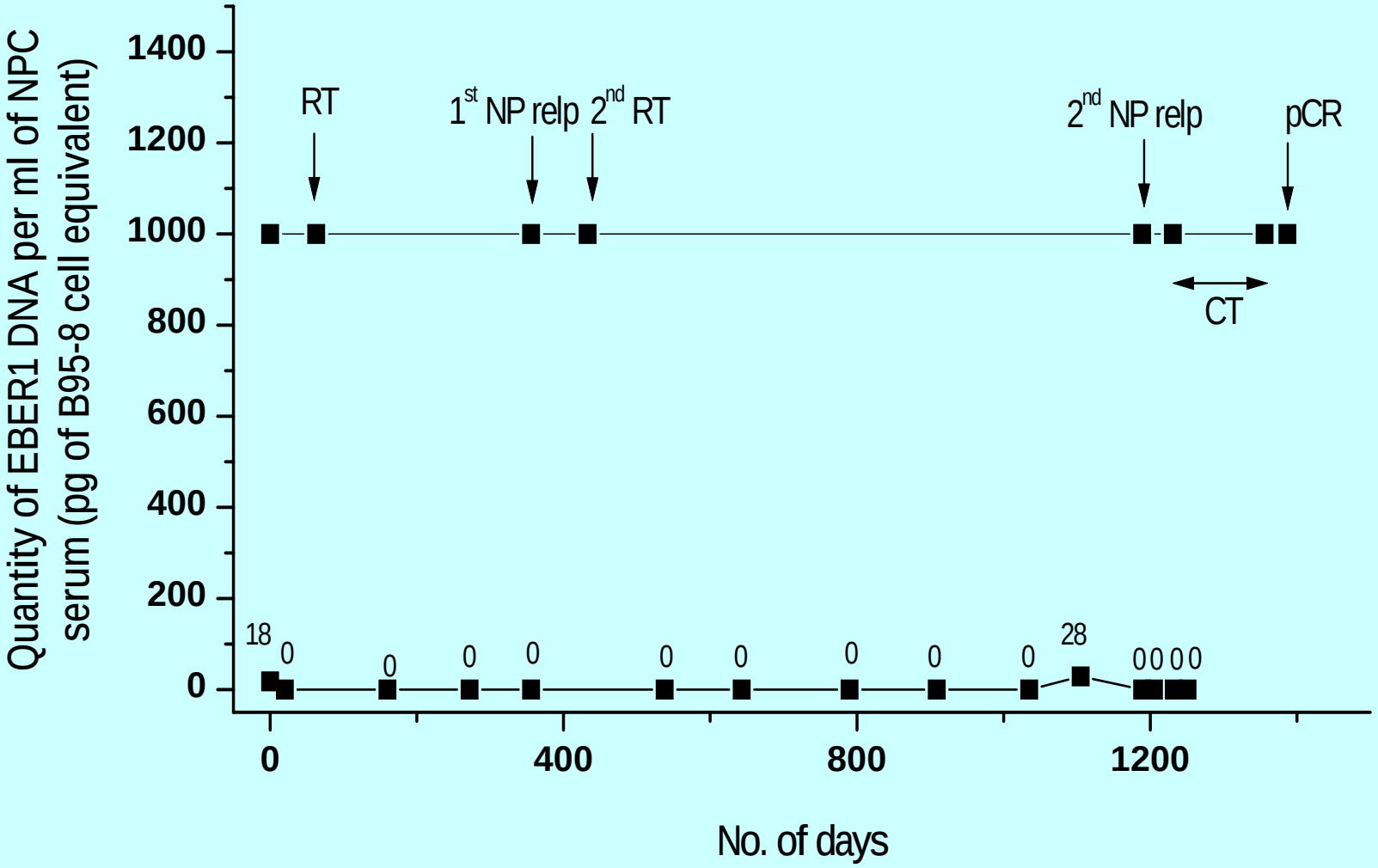


EBV DNA is less sensitive in picking up local recurrence in nasopharynx.

Modest level of elevation of circulating EBV DNA level on local recurrence in NP/BOS in 1st patient



No elevation of circulating EBV DNA level on local recurrence in NP in 3rd patient



Lower local recurrence detection by circulating EBV DNA level

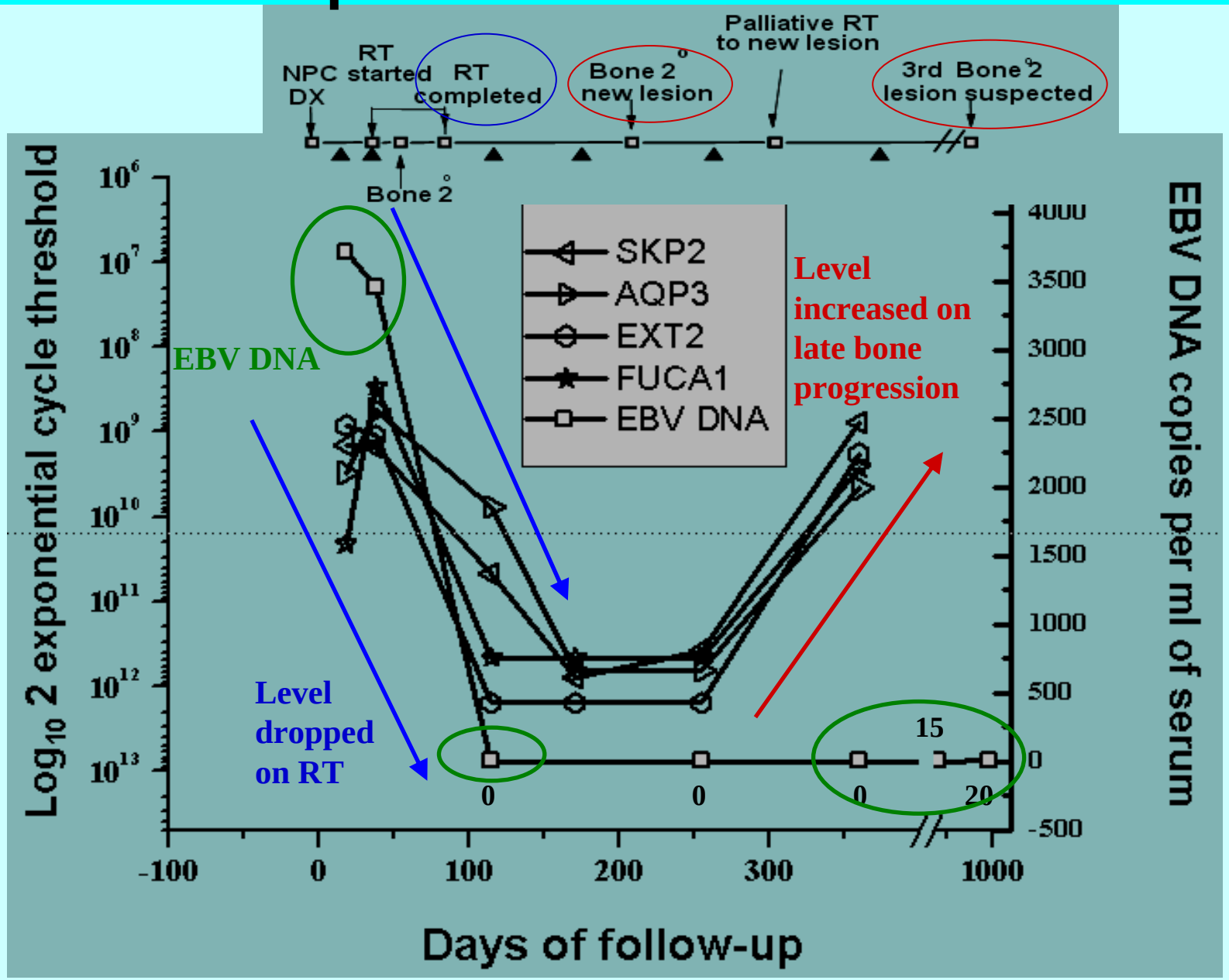
Table 1 Plasma EBV DNA positivity rate according to tumor stage (6) in radiation-naive tumor group and postirradiation locally recurrent tumor group

Tumor stage	Newly-diagnosed NPC		Locally recurrent NPC	
	Total no. of patients	No. with detectable EBV DNA	Total no. of patients	No. with detectable EBV DNA
Stage I	14	12 (86%)	8	3 (38%)
Stage II	37	35 (95%)	4	2 (50%)
Stage III	40	40 (100%)	7	5 (71%)
Stage IV	49	48 (98%)	5	5 (100%)
Stage I–IV	140	135 (96%)	24	15 (63%)

Leung SF et al. Clin Cancer Res (2003)

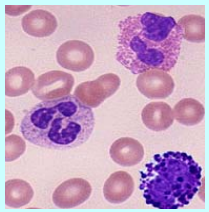
One should be cautious in the diagnosis of bone metastasis alone using circulating EBV DNA test.

Only minimal increase of circulating EBV DNA in 1 NPC patient with Bone Metastasis



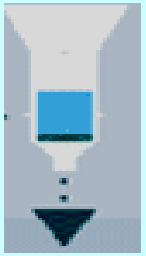
Serum RNA Microarray Analysis Strategy

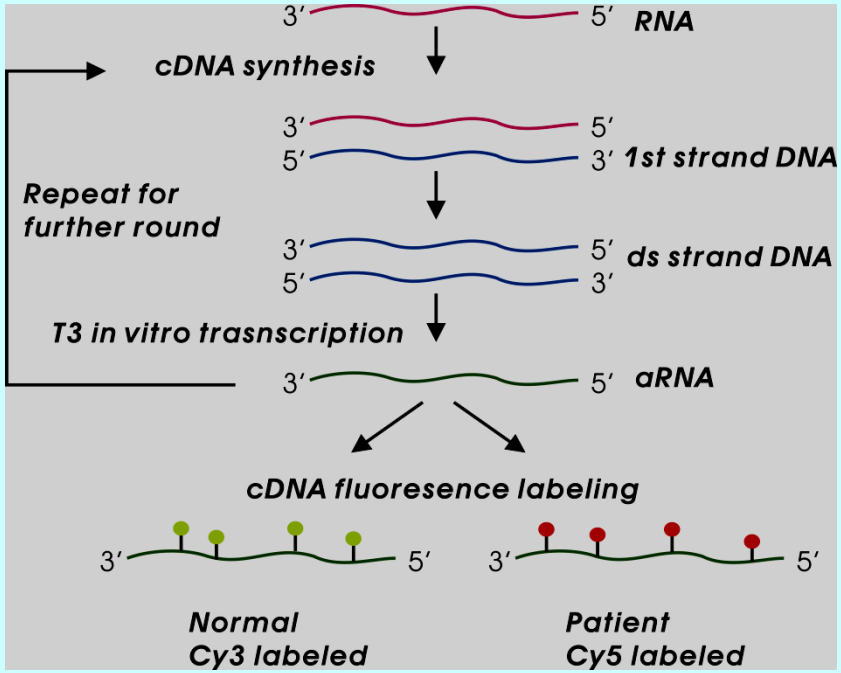
Blood from NPC patients & normals



40 ml of sera pooled

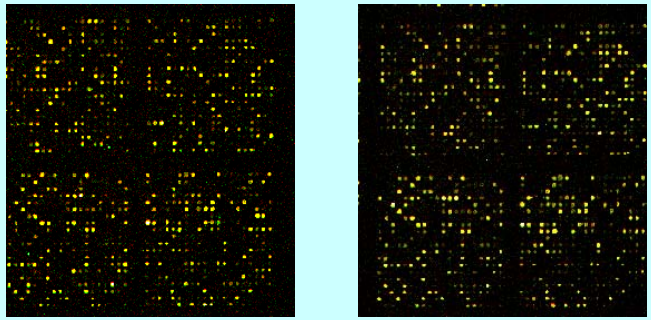
Total RNA extracted from serum



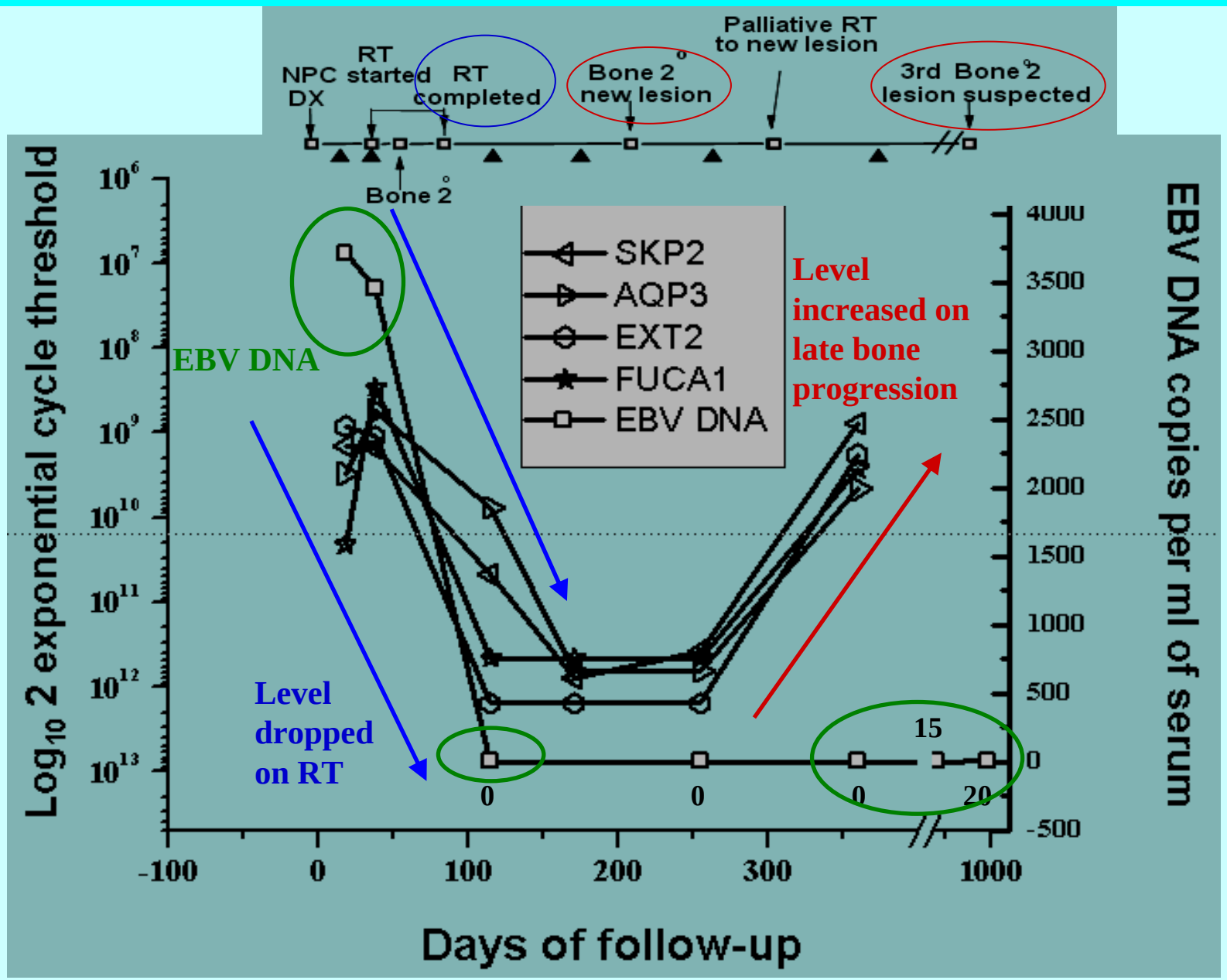


Minute quantity of RNA in serum – so 2 rounds of RNA amplification are performed

Hybridization onto microarray chips with dye swapping



Serum transcript microarray study found serum RNA markers elevated on bone metastasis



In conclusion, test for circulating EBV DNA is certainly useful for cancer diagnosis, prognostication and disease monitoring.

But one should be aware of their pros & cons before they can be of good bedside use.

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(4). Hong Kong Jockey Club


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Serum cytokeratin elevated in a secondary tongue cancer developed after RT