

## Association of Epstein-Barr Virus with Nasopharyngeal Carcinoma and Current Status of Development of Cancer-derived Cell Lines

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### Abstract

It is well known that the Epstein-Barr virus (EBV) contributes directly to tumourigenesis in nasopharyngeal carcinoma (NPC), primarily in the undifferentiated form of NPC (WHO type III; UNPC or UC), which is commonly found in South East Asia. Unfortunately, research in NPC has been severely hampered by the lack of authentic EBV-positive (EBV+) human NPC cell lines for study. Since 1975, there have been more than 20 reported NPC cell lines. However, many of these NPC-derived cell lines do not express EBV transcripts in long-term culture, and therefore that finding may dispute the fundamental theory of NPC carcinogenesis. In fact, currently only one EBV+ human NPC cell line (C-666) in long-term culture has been reported. Hence, most of the NPC cell lines may not be representative of the disease itself. In order to better understand and treat NPC, there is an urgent need to develop more EBV+ human NPC cell lines. In this review, we discuss the authenticity of existing NPC cell lines and the impact of our understanding of NPC biology on the treatment of the disease and the relationship of EBV to NPC in the context of cell lines.

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### Introduction

Although nasopharyngeal carcinoma (NPC) has been reported in almost all parts of the world, most cases of NPC are found in South East Asia, Southern China (including Hong Kong), North Africa and in the Eskimo population of Alaska, USA.<sup>1-3</sup> For reasons that still remain unclear, the Chinese are more susceptible to NPC than other races.<sup>4</sup> Earlier studies have suggested that dietary habits could be major contributors to the pathogenic process. These include the ingestion of salted fish and preserved food.<sup>5,6</sup> High-risk areas include southern China, Tunisia and Greenland, where the diet contain large amounts of N-nitrosodimethylamine (NDMA), N-nitrosopyrrolidene (NPYR) and N-nitrosopiperidine (NPIP),<sup>7</sup> common ingredients found in salted fish and preserved food. Moreover, environmental factors may also contribute to the development of NPC. For example, exposure to smoke or chemical pollutants, including trace elements (e.g. nickel) have been reported to be associated with the development

of NPC.<sup>8</sup> A recent study suggests that among occupational hazards such as exposure to smoke, construction, metal, wood dust, motor fuel and oil, paint and varnishes etc., only wood dust was statistically significant for the development of NPC.<sup>9</sup> Therefore, the development of NPC disease is multi-factorial with genetics, diet, and environmental exposure all playing large roles.

### *NPC Histological Subtypes*

The World Health Organization (WHO) classifies NPC into 3 varieties. Type I or squamous cell carcinoma (SCC) is seen in 25% of cases of NPC. These tumours produce keratin and demonstrate the presence of intracellular bridges when observed under the electron microscope. Approximately 20% of NPCs are of the Type II or non-keratinising carcinoma (NKC) variety. Tumour cells of the NKC phenotype demonstrate a range of cellular morphologies, from mature to anaplastic cells. Finally, Type III or undifferentiated carcinoma (UC) NPC constitutes

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the bulk (55%) of the tumours seen in patients with NPC.<sup>10</sup> Since there is no uniform morphological characteristic of NPC-affected tissues, diagnosis of UC NPC is usually based on the location of the tumour in the nasopharynx and the presence of EBV transcripts in the tumour cells. The EBV genome is found in transformed epithelia cells but not in lymphocytes. Clonal EBV genome is present in the early preinvasive dysplastic lesion or carcinoma in situ; indicating that infection of EBV precedes the development of malignant invasive tumour.<sup>11</sup>

#### *Geographical Distribution of NPC subtypes*

The distribution of NPC subtypes in certain geographical areas of the world does not necessarily conform to the overall worldwide distribution of NPC subtypes according to the WHO classification above. For example, in the regions where NPC is most prevalent, i.e. South East Asia, Southern China and Hong Kong, there is a predilection for UC NPCs. For example, most (85%) NPCs found in Singaporean Chinese are classified as UCs; and the incidence is greater among childhood and adolescent patients.<sup>12</sup> In contrast, most NPCs in Western countries (e.g. USA) are SCCs and thought to be related to the use of tobacco.<sup>13,14</sup> These differences contest the applicability of Western data and studies on Eastern populations, and *vice versa*. Derivations of cell lines from Eastern populations are limited and should be established based on the above findings.

#### **The role of EBV in the pathogenesis of NPC**

##### *EBV*

Epstein and his group discovered novel viral particles within cultured cells from patients with Burkitt's lymphoma in 1964,<sup>15</sup> and 4 years later; this new virus was named EBV. EBV is a relatively large gamma herpesvirus, and its DNA is double stranded and approximately 172 kilobases (kb) in length.<sup>16</sup> Approximately 90% of the adult population throughout the world are EBV-positive by serology. Elevated titers of IgA antibody to EBV viral capsid antigen (VCA) are usually found in patients with NPC, therefore this method of measuring patients' EBV-specific IgA antibodies is useful in screening for early detection of NPC.<sup>2</sup> EBNA1 and EBERS are expressed in all EBV-positive cases of NPC and LMP1 is present in up to approximately 65% of cases.<sup>17,18</sup> In almost all cases of EBV infection, the oropharynx is the primary site of infection, as well as the site of viral replication. Epstein-Barr virus is known to target primarily B-lymphocytes. *In vitro* studies demonstrate that EBV infects and potentially activates B cells by binding to the type 2 complement receptor (CR2, or CD21), the putative EBV receptor.<sup>16</sup> Hence, EBV appears to home to the oropharynx, and more specifically, the B cells within the oropharynx. The strain B95-8 can be found

in EBV-positive cell lines such as Raji, Namalwa, and CA 46.<sup>19</sup> These cell lines are all of B-lymphocyte lineage. This strain has been used as a benchmark in order to check for EBV positivity in NPC. The EBV latent proteins include the 6 nuclear antigens (EBNAs 1, 2, 3A, 3B and 3C, and EBNA-LP) and the 3 latent membrane proteins (LMPs 1, 2A, 2B). EBNA-LP is transcribed from variable numbers of repetitive exons. LMP2A and LMP2B are composed of multiple exons located on either side of the terminal repeats (TR) region, which is formed during the circularisation of the linear DNA to produce the viral episome. EBER1 and EBER2 are highly transcribed nonpolyadenylated RNAs and their transcription is a consistent feature of latent EBV infection.

##### *EBV and NPC*

It was in 1966 that Old et al first discovered the relationship between Epstein Barr virus and NPC, using *in situ* hybridisation and the anticomplement immunofluorescent (ACIF) assay.<sup>20</sup> Subsequent studies by others demonstrated the expression of EBV latent genes – Epstein-Barr virus nuclear antigen (EBNA), latent membrane protein-1 (LMP-1), LMP-2 and Epstein-Barr virus encoded small RNAs (EBER) – in NPC cells<sup>16</sup> confirming the infection of tumour cells by EBV. Intriguingly, expression of EBV early antigen (EA) is positively correlated with the consumption of salted and preserved food, suggesting that development of EBV-positive NPC could be related to dietary habits<sup>21</sup> and provides another link to the epidemiological studies with NPC. Future studies should consider the effects of dietary risk factors on the risk of specific histologic subsets of NPC, and not assume that the disease is aetiologically homogeneous. Since EBV infection indeed precedes clonal expansion of malignant cells,<sup>22</sup> EBV is thought to contribute, at least in part, to the overall pathogenesis of NPC. Many studies have shown that undifferentiated nasopharyngeal carcinomas (UNPC) are invariably EBV-positive, regardless of geographical origin.<sup>13,23,24</sup>

The process of EBV entry into keratinocytes and NPC cells is more complex, as both keratinocytes and NPC cells express only low levels of CR2 receptor.<sup>25</sup> In addition, the relevance of serological tests for EBV infection in predicting the occurrence of NPC is presently still unclear. Specifically, a positive serological test for EBV EA<sup>26</sup> may be found in greater than 80% of patients with NPC in Singapore,<sup>27</sup> yet many normal individuals also express a positive EBV EA serological test, and never develop NPC. The procedures that researchers have carried out, such as PCR, revealed expression of the LMP2A and LMP2B genes and of latent transcripts running through the BamHI A region of the EBV genome in the opposite direction to the conventional lytic cycle mRNAs transcribed over this region.<sup>28,29</sup> The

BamHI A transcripts have also been detected in other EBV associated tumours such as Burkitt's lymphoma and Hodgkin's lymphoma.<sup>30,31</sup> Analysis using western blot suggested a latent EBV infection in UNPC, and the expression of BZLF1 has been reported in some cases,<sup>32</sup> although the tumour cells do not seem to be fully permissive for virus replication. These findings are difficult to reconcile with the frequent detection of antibodies against structural viral proteins in the sera from patients with UNPC; especially patients with UNPC have raised IgA antibody titers to the VCA, EA, and MA complexes.<sup>2</sup> The rise in IgA titres to these antigens can be noticed before the development of UNPC and correlates with tumour burden, remission and recurrence.<sup>33,34</sup> Recently, a group in Taiwan used an animal model to carry out functional analysis of EBV in NPC cells. Their results showed that EBV-positive tumours grew faster than EBV-negative tumours, and also had clonal EBV terminal repeat sequences. Furthermore, EBV up regulates host genes only in cells that express those genes but not in cells that do not express them.<sup>35</sup> All these studies have unfortunately been greatly hampered by the lack of authentic EBV+ NPC cell lines, and little more is known about the mechanisms of entry of EBV, and the growth and survival NPC cells. In fact, numerous cell cultures that were initially positive for EBV eventually lost EBV expression after prolonged cell culture.

#### *EBV Activation of Anti-apoptotic and Cell Proliferation Pathways in NPC*

NPC is consistently associated with Epstein-Barr virus (EBV) infection. EBV-encoded LMP1, expressed in most of NPC, has been suggested to have an important role in the pathogenesis and development of NPC and its expression correlates with poor prognosis. LMP1 molecules aggregate in the cell membrane and through 2 C-terminal activating regions, interact with TNF receptor associated factors (TRAFs) and TNF receptor associated death domain protein (TRADD).<sup>36-38</sup> In vitro, LMP1 expression in epithelia cells can upregulate the expression of intercellular adhesion molecule 1 (ICAM-1), CD40, and cytokines such as interleukin 6 (IL-6) and IL-8.<sup>39,40</sup> LMP1 also can induce the expression of CD70 antigen, a member of the TNF family, in epithelial cells in vitro.<sup>17,41</sup> LMP1 can induce a matrix metalloproteinase, MM-9, through C-terminal activating regions 1 and 2 (CTAR-1 and CTAR-2), an effect blocked by overexpression of inhibitor of nuclear factor kappa B (I $\kappa$ B).<sup>42</sup> LMP1 encoded by EBV is a membrane protein that activates multiple signalling pathways and transcription factors, including Nuclear Factor-kappaB (NF- $\kappa$ B). NF- $\kappa$ B activation is necessary for Hodgkin/Reed-Sternberg (HRS) cells to proliferate and inhibit apoptosis.<sup>43</sup> Furthermore, activation of NF- $\kappa$ B is essential for B-cell immortalisation by EBV and LMP1-mediated

transformation of fibroblasts.<sup>44,45</sup> EBV-negative Hodgkin Lymphoma (HL) also displays constitutive NF- $\kappa$ B activation, indicating that without LMP1, other mechanisms exist to activate NF- $\kappa$ B. LMP1 has been shown to activate the mitogen activated protein kinase pathways and Janus kinase (JAK) signal transducers and activators (STATs) of transcription pathway in epithelia cells, as well as in B cells.<sup>46</sup> LMP1 and 2A also activate the phosphatidylinositol 3'-OH kinase (P13K)/Akt pathway, which is commonly activated inappropriately in malignancy.<sup>47</sup> A recent study showed that P13K/Akt pathway was of importance in NPC pathogenesis. P13K/Akt pathway activation with subsequent phosphorylation and inactivation of GSK-3 $\beta$  and nuclear  $\beta$ -catenin accumulation were characteristic of primary NPC specimens.<sup>47</sup> Thus it is clear that NPC uses its viral proteins to activate numerous cellular pathways in the NPC tumour that results in proliferation and prevents the transformed cells from dying.

#### *The B Specific Properties of the Viral Genes of EBV*

Primary EBV infections occur early in life and in most individuals it is asymptotic. *In vitro*, EBV readily infects resting peripheral blood B cells that express the EBV receptor, CD21, resulting in latent infection and proliferation. The Lymphoblastoid cell line (LCL) is an in vitro model of transformed B cells (B cells that are infected by EBV). The LCL carries many copies of the EBV viral genes in an episomal fashion and constantly expresses a limited set of viral gene products consisting of latent proteins, (6 nuclear antigens; EBNA1, 2, 3A, 3B, 3C and -LP and 3 latent membrane proteins; LMPs 1, 2A and 2B).<sup>45</sup> LCLs expressed high level of B cell activation markers CD23, CD39, and CD70, and of the cellular adhesion molecules LFA1 (CD11a/18), LFA3 (CD558) and ICAM1 (CD54).<sup>48</sup> These markers are induced to high levels when the B cells are activated by antigenic or mitogenic stimulation, suggesting that EBV-induced immortalisation can be brought out through the constitutive activation of the similar cellular pathways that result in physiological B-cell proliferation.<sup>49</sup> The EBNA1 protein binds to viral DNA and allows the EBV genome to be maintained in the B cells as a circular DNA episome.<sup>50</sup> EBNA-2 upregulates the expression of LMP 1 and LMP2, as well as cellular proteins that contribute to the growth and transformation of B cells.<sup>51</sup> LMP-1, which acts as an oncogene,<sup>52</sup> mimics the constitutively active form of the B-cell surface molecule CD40 inducing a CD40 signalling response.<sup>53</sup> It also upregulates the expression of the antiapoptotic proteins (e.g. Bcl-2 and A20)<sup>54</sup> and results in cytokine production (e.g. IL6 and IL8).<sup>40,55</sup> Thus, viral proteins confer strong pro-survival signal to EBV infected cells of the B cell lineage. LMP-1 also mimics the functions of the members of the tumour necrosis factor receptor

(TNFR) family, activating a number of signalling pathways in a ligand independent manner.<sup>56</sup> Many of these downstream pathways result from the ability of TNFR-associated factors (TRAFs) to interact either directly with CTAR1 or indirectly via the death domain protein TRADD to CTAR2.<sup>45</sup> The binding of TRAFs to the cytoplasmic tails of LMP1 provides a platform for the assembly and activation of upstream signalling molecules including non-receptor tyrosine kinases.<sup>57,58</sup> However, since NPC is a result of infection of the epithelia, EBV LCL biology may not be representative of the Biology of EBV in NPC itself.

Thus, Epstein-Barr virus (EBV) infection *in vitro* causes transformation of B cells and can result in the generation of B lymphoblastoid cell lines. These cells have been useful as tools for 1) raising cell-mediated immunity (both CD4+ and CD8+ T cells) against EBV, 2) diagnostics of genetic metabolic disorders, 3) understanding EBV pathogenesis and 4) interrogating B cell biology (e.g. CD40 and CD40L signalling). Although there are many similarities between EBV/LCL and EBV/NPC tissue or cell lines there are some notable differences. One important difference is that whereas LCLs constitutively express a small number of EBV-coded latent proteins, the nuclear antigens EBNA 1, EBNA 2, EBNA 3 and EBNA-LP and the latent membrane protein (LMP), tissue biopsies from EBV/NPC shows no expression of EBNA 2, EBNA 3 or EBNA-LP.<sup>18</sup> Furthermore, none of the biopsies studied expressed any of the EBV lytic cycle antigens. Another major difference is the cell of origin for EBV infection. The strongest links between the association of EBV and human cancer is the undifferentiated nasopharyngeal carcinoma which is of epithelial origin while LCLs are universally B cells. The tissue tropism is not defined by the receptor for EBV as CR2 is also believed to be the EBV receptor for B cells as well.<sup>59</sup> However, the immune properties such as CD40-CD40L interactions as well as the mode of EBV viral gene maintenance in the B cells are likely to play significant roles in the observed differences between epithelial cell EBV/NPC and B cells EBV/LCL EBV pathogenesis.

### **Authentic EBV+ Human NPC Cell Lines**

Today there are only limited numbers of authentic EBV-positive NPC cell lines in existence. HONE-1 C40 (subclone of HONE-1),<sup>60,61</sup> which has maintained EBV-positively up to 42 passages and contains 85% to 90% EBV nuclear antigen (EBNA)-positive cells may be an example of an authentic NPC cell line. Other examples include CG-1<sup>62</sup> and C-666<sup>63</sup> that have been shown to maintain EBV positively in long-term culture. The authenticity of these cell lines is complicated due to the fact that the EBV genome is lost in many cell lines after long-term culture.

### *Original Tissue Used for Cell Line Development*

In general, tissues from 2 sites from NPC patients have been used to establish long-term cell lines. Most of the cell lines have been derived from primary tumour tissue samples obtained from the nasopharynx, e.g. CNE-1,<sup>1</sup> CG-1,<sup>62</sup> and C15<sup>64</sup> (Table 1). A number of cell lines were also derived from metastatic NPC tissue from other sites. For example, the NPC-BM1 cell line was established from bone marrow biopsy sample in a patient with bony metastases and the C17 cell line was derived from a skin biopsy sample in a patient who had skin, bone and liver metastases and C18 cell line, from a cervical lymph node biopsy in a patient with locally advanced tumour with regional lymph node involvement.

### *Short-term EB positive NPC-derived Cell Lines*

Several cell lines have been derived from UC and poorly differentiated SCC NPC tissue (the most common subtypes of NPC tissue found in the South East Asia, South China and Hong Kong) that were initially EBV-positive (Table 1). These include: UC derived C-15, C-17, C-18, C666-1 (HK666), CAO, CG 1, CNE-2, CNE-2Z, CNE-3, HNE-1, HONE-1, NPC/HK2117 (Xeno-1), NPC/HK1915 (Xeno-2), NPC/HK1530 (Xeno-3), NPC-TW03, NPC-TW 06, NPC-TW07, SUNE and SUNE.<sup>60-76</sup> Other cell lines were also derived from the less common EBV+ SCC NPC tissue, including NPC-TW 01 (a.k.a. NPC-TW 039), NPC-TW 05, and NPC-TY861.<sup>70,71,77,78</sup> The limitation of these cell lines are that they have become EBV-negative after prolonged culture, thus their physiological relevance is uncertain. By 1989, only 3 permanent epithelioid NPC cell lines were established, CG1 and CNE2 (from China), and NPC/HK1 (from Hong Kong). Again, none of these NPC cell lines contained the EBV genome despite extensive testing; further suggesting that either EBV infection was unrelated to the development of NPC; or that these cell lines were not representative of NPC tissue or both. CG-1 and CNE were both EBV-positive but after long-term culture they lost their EBV positivity.<sup>79</sup>

### *EBV Negative NPC-derived Cell Lines*

In contrast to the initially EBV+ tumour tissue, several NPC-derived cell lines were generated from EBV-tumour tissue. These included those derived from well-differentiated SCCs – CNE, NPC/HK1, NPC-TW 02 (NPC-TW 076), and NPC-TW 08.<sup>70,71,77,80-82</sup> Second generation NPC-derived cell lines were occasionally produced from the parental cell line. For example, the CNE epithelioid NPC cell line subsequently produced the CNF fusiform cell line. Importantly, neither CNE nor CNF cell lines demonstrated the presence of EBV virus particles,<sup>82</sup> again challenging a causal relationship between EBV and NPC.

### Attenuation of EBV Expression in Long-term NPC-derived Cell Lines

Expression of EBV DNA has been observed to become attenuated after longer-term culture in many cell lines, including NPC-TW 01, NPC-TW 03, NPC-TW 05, NPC-TW 06, and NPC-TW 07, etc. suggesting that EBV does

not seem to be required for the survival and proliferation (Table 1).<sup>60,71,81,82</sup> In other words, EBV might facilitate the initiation of the pathological process that eventually leads to NPC, but is not required for subsequent growth of tumour cells *in vitro*. Currently, this issue cannot be resolved since there is a lack of EBV+ NPC cell lines that are truly

Table 1. Long-term Solid Tissue Cultures Derived from NPC Tumours

Cell line	NPC WHO classification	Derived cell type	Culture method	EBV status	References
C-15	NKC	epithelial/syncytial	subcutaneous xenografting /Swiss nude mice	IP	(64)
C-17*	NKC	epithelial/syncytial	subcutaneous xenografting /Swiss nude mice	IP	(64)
C-18†	NKC	epithelial/syncytial	subcutaneous xenografting /Swiss nude mice	IP	(64)
CAO	—	—	xenografting in nude mice	IP, LP	(68)
X666	UC	epithelial/syncytial	subcutaneous xenografting a in BALB/c (nu/nu) athymic nude mice	IP, LP	(90)
C666	UC	epithelial/syncytial	RPMI 1640, 1% fetal bovine serum (FBS)	IP, LU	(67,90)
C666-1; HK666	UC	epithelial/syncytial	RPMI 1640, 1% FBS	IP, LP	(63,67)
CG1	NKC	epithelia	DMEM/F12, 5% FBS	IP	(62)
CNE-2	UC	epithelial	RPMI 1640, 10% FBS	IP	(72)
CNE-2Z	UC	epithelial	RPMI 1640, 10% FBS	IN	(76)
CNE-3	UC	epithelial	RPMI 1640, 10% FBS	IN	(65)
HNE-1	UC	epithelial	DMEM/F12	IP, LN	(60,61,74)
HONE-1	UC	epithelial	RPMI 1640	IP, LN	(60,61,74)
NPC/HK1530; Xeno-3	UC	epithelial	subcutaneous xenografting in athymic mice	IP, LN	(69)
NPC/HK1915; Xeno-2	UC	epithelial	subcutaneous xenografting in athymic mice	IP, LN	(69)
NPC/HK2117; Xeno-1	UC	epithelial	subcutaneous xenografting in athymic mice	IP, LN	(69)
NPC-TW 03	UC	epithelial	DMEM, 5% FBS	IP, LN	(70,71)
NPC-TW 06	UC	epithelial	DMEM, 5% FBS	IP, LN	(70,71)
NPC-TW 07	UC	epithelial	DMEM, 5% FBS	IP, LN	(70,71)
SUNE	UC	epithelial	RPMI 1640, 10% FBS	IP	(66)
SUNE1	UC	epithelial	DMEM, 5% FBS	UNK	(14)
NPC-TW 04	UC	epithelial	DMEM, 5% FBS	IN, LN	(70,71)
NPC-TW 09	UC	epithelial	DMEM, 5% FBS	IN, LN	(70,71)
CNE	SCC	epithelial	RPMI1640, 15% FBS	IN, LN	(80,82)
NPC/HK1	SCC	epithelial	RPMI1640, 15% FBS	IN, LN	(81)
NPC-TW 02; 076	SCC	epithelial	DMEM, 7% FBS	IN, LN	(71,77)
NPC-TW 08	UC	epithelial	DMEM, 5% FBS	IN, LN	(70,71)
NPC-TW 01; 039	SCC	epithelial	DMEM, 7% FBS	IP, LN	(71,77)
NPC-TW 05	UC	epithelial	DMEM, 5% FBS	IP, LN	(70,71)
NP69SV40T	SCC	epithelial	Immortalised by SV40T	IP, LP	(46)
NP39E6/E7	SCC	epithelial	Immortalised by HPV16E6/e7	IP, LP	(46)
NPC-BM1‡	UC	epithelial	RPMI1640, 10% FBS	UNK	(91)
NPC-F/L	UC	epithelial	EF-10, 10% FBS, co cultivated with EBV-transformed cell line.	IP, LP	(85)

IN: initially EBV-negative; IP: initially EBV-positive; LN: long-term culture EBV-negative; LP: long-term culture EBV-positive; LU: long-term culture EBV-status unknown; NKC: non-keratinising carcinoma; SCC: squamous cell carcinoma; UC: undifferentiated carcinoma; UNK: unknown.

\* Derived from metastatic tissue in the skin

† Derived from metastatic tissue in the regional lymph node

‡ Derived from metastatic tissue in the bone marrow

representative of NPC itself.

#### *Development of NPC-derived Cell Lines Using Xenografts*

Several cell lines have been derived from transplantation of a human source to an animal one. NPC-derived cell lines; C15-C18 was subcutaneously xenografted into Swiss nude mice. X666 was derived from a subcutaneous xenograft of BALB/c (*nu/nu*) athymic nude mice. NPC/HK1530 (Xeno3), NPC/HK1915 (Xeno2) and NPC/HK2117 (Xeno3) were all derived from athymic murine xenografts. The CAO NPC cell line was an EBV-positive NPC-derived from a 54-year-old male patient from Shanghai China and was propagated in a BALB/c nude mouse.<sup>83,84</sup> Again, since these cell lines are not derived from primary cultures, the relationship to NPC itself is controversial.

#### *Development of NPC-derived Cell Lines Using Other Methods*

Currently there are hybrid tumour cells known as NPC-F/L that have been developed.<sup>85</sup> These were made using a co-culture system in which EBV transformed LCL of adenoid origin was cultured in the presence of NPC tissue. NPC-F/L contains some EBV genome such as the EBNA and EA but do not have VCA. In another method, the SCC derived EBV-positive cell epithelia squamous cell carcinoma NP39E6/E7 was generated by immortalisation with HPV16E6/E7 and NPC cell line NP69SV40T, was immortalised by SV40LT Ag.<sup>46</sup> These 2 methods, however, may not be truly representative of the biological properties of NPC, as they are not from pure primary culture.

#### **Difficulties in Culturing NPC Cells *In vitro***

##### *Limited Tissue Quantity from PNS Biopsies*

There are many inherent limitations to culturing NPC cells *in vitro*. Typically, PNS biopsies provide only small tissue fragments. In order to increase the amount of available tissue, subcutaneous tumours may be first transferred to athymic mice for initial *in vivo* expansion before seeding into tissue culture flasks for *in vitro* culture.<sup>63</sup> Besides the potential introduction of murine cells, this method is likely to result in changes to the primary human biopsy tissue sample.

##### *Contamination by Other Cell Types*

It is common for biopsy tissues to be heavily infiltrated with lymphoid cells and cells of connective tissue origin. Hence, it is virtually inevitable that cell lines established from tumour explants acquire a lymphoblastoid phenotype during the initial stages of long-term cell culture.<sup>67</sup> Several investigators have used serum free medium (SFM) to inhibit the fibroblast/mesenchymal cell growth and promote tumour cell proliferation, e.g. the NPC-TW039 and NPC-TW076 cell lines.<sup>77</sup> Established NPC-derived cell lines

were later converted from SFM to complete medium containing foetal bovine serum (FBS). This may also have a dual benefit of helping to decrease the serum dependent loss of EBV status.<sup>77</sup> Other investigators have used pipetting during cell culture to remove fibroblasts; i.e. the CG-1 NPC-derived cell line.<sup>62</sup> There have been many limitations in culturing these cell lines. The following are some problems encountered when trying to establish an EBV-positive NPC cell lines. Firstly, there are not many true EBV-positive NPC cell lines available that can be used as a standard for the development of other NPC cell lines with the only exception of C-666.<sup>63</sup> However, its status as a true NPC cell line remains questionable. Secondly, the cultures lose their EBV-positivity after long periods of *in vitro* culture. Transplantation into nude mice of NPC material has not been very efficacious and most of the primary transplanted tumours are no longer available for further investigation.<sup>64</sup> Moreover, it is best if a tumour is obtained directly from primary tissue rather than from another source such as from xenograft or transformed EBV+ cell lines. This provides a more accurate reflection of the cytogenetic details of the primary tumour and thus is a better tool for study of NPC tumourigenicity.

#### **Conclusion**

Here we have presented the importance of using authentic EBV+ human NPC cell lines for research in NPC. Specifically, continuous cell lines are important tools for the study of the genetic mechanisms and environmental factors responsible for NPC, both in terms of tumourigenicity as well as its metastatic potential. Although, there were many NPC cell lines that have been established using numerous culture methods, most of them are EBV-negative after a long-term culture. Carcinomas with similar features to UNPC have been described at other sites including the thymus, tonsils, lungs, stomach, skin or cervix, and are often referred to as undifferentiated carcinoma of nasopharyngeal type (UCNT).<sup>86</sup> Some of these carcinomas showed less strong EBV-positivity, such as those derived from thymic epithelia tumours from Chinese but not western patients.<sup>87</sup>

The precise mechanism by which EBV transforms cells is becoming clearer as more research is conducted. The viral gene expression in EBV-associated tumours is limited to a few latent genes of which the most prevalent is LMP1. It is reported to act as a constitutively activated receptor that causes aberrant cellular signalling to at least 4 major pathways. The role for virus in the pathogenesis of NPC is still largely circumstantial and controversial.<sup>88</sup> For example, the squamous cell NPCs and non-keratinising NPCs vary in their degree of EBV association. Squamous cell NPCs may in fact be EBV-negative and other factors such as smoking or HPV infection may be more relevant in the

pathogenesis of these cell types. On the other hand, non-keratinising NPCs are almost always EBV-positive, suggesting that the virus is a rate-limiting step for these tumours. It will be important to define the exact timing of EBV infection of nasopharyngeal epithelial cells and to identify the factors that precede and probably pave the way for EBV infection. Clearly, further studies are required to support these findings. The complexity of these virus and host relationships suggests the existence of potential targets for gene therapy for EBV-related cancers.

The geographically constrained distribution of EBV associated NPC in Southeast Asian populations suggests that both viral and host genetics may influence disease risk. The analysis of LMP1 with reference to the geographical distribution demonstrate that LMP1 sequences show a distinct geographic structure, indicating that the southeast Asian isolates have evolved as a lineage distinct from those of Papua New Guinea, African, and Australian isolates.<sup>89</sup> Due to the fact that NPC occurs more frequently in Asia than compared to the Western regions, there is increased motivation in establishing NPC cell lines from each region.

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