Pre-malignant nasopharyngeal epithelial cell models

Qing-Li Kong,1,2,3 Su Guan,1,2 Bao-Hong Guo1,2 and Mu-Sheng Zeng1,2

[Abstract] Experimental models that allow investigation of nasopharyngeal carcinoma (NPC) progression could provide valuable insights of the molecular mechanism of nasopharyngeal carcinogenesis as well as potential clinical intervention. Because Epstein-Barr virus only infects humans and a few species of monkeys, representative NPC animal models have not been available so far. Attempts to provide cell models for early nasopharyngeal carcinogenesis have involved in the studies of in vitro transformation of normal finite lifespan human nasopharyngeal epithelial cells (NPEC) to immortality. The first two immortalized NPECs were established by introduction of ectopic SV40T or HPV E6/E7. In order to avoid the unrelated molecular alterations caused by the viral oncogenes, we established and characterized two immortalized NPECs by introduction of Bmi-1, an oncogene which has been demonstrated to be overexpressed in NPC cells and specimens. In addition, human telomerase reverse transcriptase (hTERT) immortalized NPECs have been established in both Tsao’s and our laboratory. Unlike the immortalized cells induced by viral oncogenes, these immortal NPECs maintain a normal p53 checkpoint, and are unlikely to have other undefined genetic lesions except presenting some molecular alterations which have been observed in NPC. Thus, the establishment of the immortalized NPECs can be used to further study the mechanism of NPC development using defined genetic elements, particularly in elucidating the role of EBV infection in NPC development.

Key words: nasopharyngeal carcinoma, immortalization, bmi-1, viral oncogene, human telomerase reverse transcriptase (hTERT)

Nasopharyngeal carcinoma (NPC) is a common cancer in Southeast Asia. In South China, especially in the Cantonese region around Guangzhou, it occurs at an incidence 100–fold higher compared with other populations in Europe and North America. Most NPCs are undifferentiated or poorly differentiated squamous carcinoma with the characteristic of a fast growth rate and highly metastatic potential, and they usually affect a relatively young population at the age of 45 years old. The etiology of NPC is multifactorial, including virological, genetic, and environmental factors. NPC is the most consistent Epstein-Barr virus (EBV) associated tumor, and EBV infection has been postulated to be an early event in the pathogenesis of NPC. Genetic alterations including deletion of chromosomes 3p and 9p, inactivation of tumour suppressor genes, RASSF1A and p16, could be detected in premalignant nasopharyngeal epithelium prior to EBV infection. Thus, the development and progression of NPC may involve accumulation of multiple genetic alterations over a long period of time. However, the precise genetic changes that are responsible for NPC progression are largely unknown.
To study the mechanisms of NPC development, various NPC derived cell lines have been established. Most of these cell lines are EBV negative, although some of them were positive at the early passages. Until now, only a few NPC cell lines, such as C666–1, stably harboring the EBV genome, were used as the EBV–positive NPC model. In addition to the established NPC cell lines, several EBV–positive NPC transplants in nude mice (e.g. C15, C17, xeno–666, xeno–2117 and xeno–1915) were used for identifying the genetic alterations and delineating aberrant signal transduction pathways in NPC. However, they are not ideal models for demonstration of the early events in NPC carcinogenesis.

Normal nasopharynx derived epithelial cells (NPEC) exhibit a limited life span, which is followed by replicative senescence. Acting as a strong tumor suppressor mechanism, replicative senescence prevents spontaneous immortalization of human cells. It has been demonstrated in both in vitro and in vivo models that bypass senescence and immortalization are the essential initial steps in tumorigenesis. Immortalization allows a cell to grow indefinitely and to go through further oncogenic steps, resulting in a fully malignant behavior. Thus, efforts to model early nasopharynx carcinogenesis have mainly focused on the studies of in vitro transformation of the normal finite lifespan human NPEC to immortality and malignancy and thereby have provided significant insights into the biology of early NPC.

Immortalization of NPEC by SV40T or HPV E6/E7

Both p53 and RB pathways play essential roles in mediating senescence of various normal cells. By direct targeting these pathways, the viral oncoproteins SV40T or HPV E6/E7 have been widely used to induce immortalization of normal human cells. Tsao et al. had established and characterized the first two immortalized NPECs by ectopic expression of SV40T or HPV E6/E7. These two cell lines have been termed as NP69SV40T and NP39E6/E7, which retain characteristics of primary NPEC cells as determined by cellular morphology, keratin profiles and response to TGF–B1 treatment. Both cell lines display high activity of telomerase, and they are supposed to be deficient in the function of p53 and RB pathways. Consistent with previous reports in HPV E6/E7 or SV40T immortalized cells, abnormal karyotypes including gains on chromosomes 5 and 20 and losses on chromosome 19 in NP39E6/E7, or gains on chromosome 8, 9, 22 and losses on 6, 11 and 16 in NP69SV40T have been observed. Interestingly, five of the observed 33 genetic alterations (gains on chromosomes 1q, 11q, 12q and 17q and losses on 3p and 9p) have been observed in a high frequency in NPC. Because these cell lines have not been fully malignantly transformed, they are widely used in various studies to serve as non–cancerous nasopharyngeal epithelial cell control.

Immortalization of NPEC by Bmi–1

Although the above mentioned cell lines have been widely used in NPC studies, it is important to note that these viral oncoproteins, capable of inactivating multiple cellular checkpoints simultaneously, are not associated with most human cancers, including NPC. In light of the goal of developing in vitro systems that model NPC progression, NPEC strains immortalized with cellular oncogenes, which also overexpressed in NPC, would provide an ideal cellular system to precisely delineate the steps involved in NPC development. Apart from human telomerase reverse transcriptase (hTERT), two cellular oncogenes, c–MyC and Bmi–1 have been described in the literature, which can immortalize certain cell types. These oncogenes are also overexpressed in a variety of cancers. We have demonstrated the overexpression of Bmi–1 in NPC samples and NPC cell lines, and the expression of Bmi–1 protein is found to correlate with the invasion of NPC primary tumor and the prognosis of NPC patients. It is interesting to note that C666, the only well known NPC cell line consistently carrying EBV, has the highest Bmi–1 expression. Consistent with a recent report that Bmi–1 is up–regulated in Hodgkin lymphoma (HL) cells by the EBV oncogene latent membrane protein–1 (LMP1) through the NF–kappaB signaling, we observed that LMP1 could induce Bmi–1 expression in NPC cells (data not shown). In NPECs, ectopic expression of Bmi–1 induces immortalization of the cells by enhancing telomerase activity and repressing p16 expression. This is the first report that Bmi–1 alone is able to immortalize p16 expressing cells. These immortal NPEC cell lines (NPEC1–Bmi1 and NPEC2–Bmi1) maintain a normal p53 checkpoint and are unlikely to have other
undefined genetic lesions, which is always the case with tumor derived cell lines and cells immortalized with viral oncogenes. Interestingly, our recent works indicate that Bmi–1 may play a major role in the initiation and progression of NPC by inducing immortalization, stem–like cancer cell self–renewal and cell invasion (data not shown). More importantly, we observed that EBV can directly infect the NPEC1 –Bmi1 and NPEC2 –Bmi1 at a higher efficiency than the control NPECs (20% vs. 2%, Cao, et al. unpublished observations) under a modified culture condition (Fig. 1). Thus, Bmi–1 immortalized NPECs can be used to further study the mechanism of NPC development using defined genetic elements, especially to demonstrate the role of EBV during NPC development.

**Immortalization of NPEC by hTERT**

Telomerase activation has been suggested to be the only step required for the immortalization of certain types of cells. 24 It has been reported that expression of hTERT and subsequent telomerase activation could extend the life span of human fibroblasts and retinal pigment epithelial cells. 24,25 In NPEC, introduction of hTERT is able to induce cell immortalization, although at a relative low frequency.7 The immortalized NPEC (termed as NP460) are mainly diploid in karyotype. Consistently, introduction of hTERT alone, we were able to induce immortalization of a NPEC. The cells keep normal karyotypes in the early passage. However, after passage 11, gain of chromosome 22 was consistently observed in the hTERT transfected cells (Guo, et al. Unpublished observation). Unlike NPECs immortalized by viral oncogenes, the hTERT immortalized cells only harbor limited genetic alterations, and most of the alterations have been previously identified in premalignant and malignant nasopharyngeal epithelial cells.7,10 These genetic alterations include downregulation of both p16 and RASSF1A expression by homozygous deletion or promoter hypermethylation, as well as upregualtion of ID1 by undefined mechanism.7,10

In order to investigate the effect of EBV on these cell lines, we used EBV to infect these cell lines and observed that EBV infected the primary NPECs and the NPECs were immortalized by SV40T or hTERT at a similar efficiency. However, EBV could infect the Bmi–1 immortalized cells at a high efficiency (Cao, et al. Unpublished observations), which suggests that Bmi–1 may deregulate the extracellular pathway required for efficient infection of EBV to NPEC. Further studies in these model systems would be greatly helpful to understand the mechanism of EBV infection of epithelial cells. In summary, immortalized NPEC models have led to substantial progress in our understanding of the early molecular alterations in NPC carcinogenesis.

**References**


5. Gallo C, Low WK, Teoh G. Association of Epstein-Barr virus with nasopharyngeal carcinoma and current status of development of cancer-derived cell lines [J]. Ann Acad Med Singapore,