NASOPHARYNGEAL CARCINOMA—REVIEW OF THE MOLECULAR MECHANISMS OF TUMORIGENESIS

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Abstract: Nasopharyngeal carcinoma (NPC) is a head and neck cancer rare throughout most of the world but common in certain geographic areas, such as southern Asia. While environmental factors and genetic susceptibility play important roles in NPC pathogenesis, the Epstein–Barr virus in particular has been implicated in the molecular abnormalities leading to NPC. There is upregulation of cellular proliferation pathways such as the Akt pathway, mitogen-activated protein kinases, and the Wnt pathway. Cell adhesion is compromised due to abnormal E-cadherin and β-catenin function. Aberrations in cell cycle are due to dysregulation of factors such as p16, cyclin D1, and cyclin E. Anti-apoptotic mechanisms are also upregulated. There are multiple abnormalities unique to NPC that are potential targets for novel treatments.

Keywords: nasopharyngeal carcinoma (NPC); Epstein–Barr virus (EBV); LMP1; tumorigenesis; review

Nasopharyngeal carcinoma (NPC) is a squamous cell carcinoma that usually develops around the ostium of the Eustachian tube in the lateral wall of the nasopharynx.1 Though rare among whites, NPC is particularly common in the southern Chinese population of Guangdong, Inuits of Alaska, and native Greenlanders.2,3 Chinese emigrants continue to have a high incidence of the disease, but the rate of NPC among ethnic Chinese born in North America is considerably lower than those born in China.4 This epidemiologic evidence implies that both environmental factors and genetic susceptibility play roles in the development of NPC. The environmental factors may include exposure to nitrosamines in salted and pickled foods.5 Certain human leukocyte antigen subtypes have been associated with NPC, as they have various genetic polymorphisms.6

The World Health Organization classifies NPC based on histology.7 Type 1, keratinizing squamous carcinoma, is characterized by well-differentiated
cells that produce keratin. Type 2, nonkeratinizing squamous carcinoma, varies in cell differentiation but does not produce keratin. Type 3 is also nonkeratinizing, but is less differentiated, with highly variable cell types (clear cell, spindle cell, anaplastic). Types 2 and 3 NPC are Epstein–Barr virus (EBV) associated and have better prognoses than type 1; EBV infection is generally absent in type 1, especially in nonendemic areas. However, more recent data state that almost all NPC tumors, regardless of histologic subtype, have comorbid EBV infections, which is strong evidence for EBV as the etiology of NPC. This close association with EBV is what makes NPC unique from other head and neck cancers.

Standard treatment for NPC is radiotherapy, but concurrent adjuvant chemotherapy improves survival rates. However, with other cancers, the prognosis of NPC depends upon tumor size, lymph node involvement, and distant metastasis (TMN staging). But NPC, in contrast to other head and neck malignancies, is highly sensitive to radiation and chemotherapy. High survival rates are reported for stage 1 and 2 diseases, but the prognosis for metastatic disease remains poor even with combined radiation and chemotherapy treatment, with disease relapse rates as high as 82%. Unfortunately, the majority of NPC is diagnosed at an advanced stage because of non-specific presenting symptoms (cervical nodal enlargement, headache, nasal and aural dysfunction), delay in seeking treatment after the onset of symptoms, and the difficulty of a thorough nasopharyngeal exam. In light of this, more targeted treatments of NPC need to be developed. To do so, the molecular changes that lead to NPC tumorigenesis must first be clarified. This review provides an overview of the major molecular mechanisms underlying the development of NPC. Figure 1 gives a summary of the pathways and their downstream effects on the tumorigenesis of NPC. Tables 1 and 2 provide a list of proteins aberrantly expressed and the percentage of NPC tumors that have these abnormalities.
EPSTEIN–BARR VIRUS

EBV is a γ-herpes virus present in over 90% of adults worldwide. Though the infection is lifelong, it usually remains harmless unless the balance between host and virus is altered. Diseases associated with EBV include those of lymphocytic origin (infectious mononucleosis, Hodgkin’s disease, and Burkitt’s lymphoma) and epithelial origin (NPC, oral hairy leukoplakia, and undifferentiated gastric carcinoma). At least 95% of NPC tumors are EBV associated. Additionally, the severity of EBV infection varies with carcinoma type, with undifferentiated carcinomas having the highest EBV titers.

EBV has tumorigenic potential due to a unique set of latent genes: latent membrane proteins (LMP1, LMP2A, and LMP2B) and EBV-determined nuclear antigens (EBNA1 and EBNA2) are the proteins predominantly expressed in NPC. LMP1 is the principal oncogene of NPC—it is required for cell immortalization and is present in 80% to 90% of NPC tumors. The LMP1 molecule includes 6 transmembrane domains and a carboxy-terminus containing 2 signaling domains called C-terminal activating regions 1 and 2 (CTAR 1 and 2). The transmembrane domains allow LMP1 to associate with the host membrane, whereas the CTAR regions directly activate a number of signaling pathways including nuclear factor κ-B (NF-κB), mitogen-activated protein (MAP) kinases, and phosphoinositol-3-kinase (PI3K). Although the basic role of LMP1 is to prevent apoptosis, it has other important functions in cancer development. LMP1-positive cells have greater mobility, leading to higher metastatic potential and faster disease progression. LMP1 is also involved in suppressing immunogenic responses against NPC; for example, LMP1 has intrinsic T-cell inhibitory properties and mediates downregulation of CD99, an important component of the anti-NPC immune response. The importance of LMP1 in tumorigenesis is illustrated by numerous studies that show the inhibition of LMP1 results in increased tumor cell sensitivity to chemotherapy.

Less is known about LMP2 and EBNA than LMP1, but with recent research their role in EBV-induced tumorigenesis is becoming more understood. Previously it was thought that LMP2 probably only mediated tumor cell survival, however, newer data show that LMP2, particularly LMP2A, has more diverse functions. LMP2A downregulates the NF-κB transcription factor and can decrease LMP1 expression. Additionally, LMP2A expression causes NPC cells to become migratory and invasive. EBNA1 is an unusual protein that binds the EBV genome to host chromosomes, and thus mediates equal partitioning of viral DNA into daughter cells during cell division and may play a role in immune evasion. EBNA2 may be involved in the transactivation of LMP1. The roles of these EBV latent genes are summarized in Figure 2.

CRITICAL PROLIFERATIVE SIGNALS

EBV infection appears to be necessary but not sufficient for tumorigenesis in NPC. Clearly, other mechanisms are required. Although there are many aberrations that contribute to tumorigenesis, the critical signals in NPC development are the Wnt pathway and transcription factors NF-κB and β-catenin.

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<th>Table 2. Proteins underexpressed in NPC.</th>
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<td>Abnormal protein</td>
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<td>WIF</td>
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<td>RASSF2A</td>
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<td>E-cadherin</td>
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Abbreviations: WIF, Wnt inhibitory factor; RASSF2A, Ras association domain family 2A; CHFR, checkpoint with forkhead-associated and RING finger domains; PTEN, phosphatase and tensin homolog.
Wnt Pathway Is Upregulated and Critical to the Intranuclear Accumulation of β-Catenin. The Wnt signaling pathway is important for normal development but is aberrantly activated in cancer. Wnt proteins bind to receptors belonging to the frizzled (Fz) family. In the canonical pathway, this activates an intracellular cascade that involves the inhibition of glycogen synthase kinase 3β (GSK-3β) and the adenomatous polyposis coli (APC) protein, ultimately resulting in the stabilization and nuclear translocation of cytoplasmic β-catenin. Nuclear β-catenin then interacts with various transcription factors to cause cellular proliferation and differentiation. Cytoplasmic β-catenin also has a role in the normal cell by binding to the intracellular domain of E-cadherin to maintain cellular adhesion.51

Abnormal Wnt signaling has been implicated in a number of cancers including head and neck carcinoma, lung cancer, colorectal cancer, melanoma, and leukemia.52 Prolonged Wnt signaling activates Akt, which together with disheveled phosphorylates GSK-3β.53 Phosphorylated GSK-3β is inactive, thus allowing β-catenin accumulation. Although the role of the Wnt pathway in NPC has not been fully explored, there is abundant evidence that aberrant Wnt signaling plays a role in NPC development. A gene expression study of NPC found the upregulation of the Fz 7 receptor and claudin 1 (a gene positively regulated by β-catenin) and the underexpression of axin 2, an inhibitory protein of the Wnt pathway.54 Most NPC tumors exhibit Wnt pathway protein dysregulation—93% have increased Wnt protein expression and 75% of tumors have decreased expression Wnt inhibitory factor (WIF), an endogenous Wnt antagonist.18,20 The expression of WIF has been shown to be silenced via promoter hypermethylation in NPC cell lines.55 These results indicate that aberrant Wnt signaling is a critical component of NPC.

Increased β-Catenin Is Critical to NPC Proliferation. β-Catenin is the key mediator of canonical signaling in the Wnt pathway. In normal cells, a protein complex of axin, APC, and GSK-3β phosphorylates β-catenin, marking it for subsequent ubiquitin-mediated degradation. Wnt pathway activation leads to deactivation of this protein complex and thus increased levels of β-catenin. Nuclear β-catenin levels are increased in 92% of NPC tumors, making nuclear β-catenin 1 of the more important components of NPC development.19,20 EBV infection increases the level of cytoplasmic β-catenin and causes its nuclear localization via increased GSK-3β.56 Mutations of the β-catenin gene are rare in NPC cells, indicating that GSK-3β is underactive because of downregulation by Wnt or Akt.52,57,58 Interestingly, while EBV infection upregulates Akt in both
Hodgkin’s lymphoma and NPC, only NPC cells showed increased levels of phosphorylated GSK-3β and intranuclear β-catenin.19 This suggests that the Wnt pathway has a more critical role in the dysregulation of β-catenin in NPC than in other EBV-associated diseases.

Intranuclear β-catenin contributes to NPC development in several ways. It activates numerous downstream proliferation signals, including c-myc and cyclin D1.52 Beta-catenin binds to the interleukin (IL)-8 promoter site and increasing IL-8 levels in NPC; IL-8 is an important angiogenic factor in NPC.59 Intranuclear β-catenin also downregulates RASSF1A expression.60 Normal RASSF1A is critical for microtubule stabilization and regulation of mitotic events; its downregulation leads to abnormal mitotic spindles and microtubule organization, ultimately resulting in aneuploidy and facilitation of transformation of cancerous NPC cells.60 Despite these developments, the full impact of aberrant β-catenin in the development of NPC remains to be completely understood. Given the importance of the Wnt pathway and β-catenin to NPC tumorigenesis, a greater understanding of these pathways is likely to contribute to the development of novel, targeted treatments for NPC.

**High Levels of NF-κB Mediate Cell Immortalization.** NF-κB normally plays 2 main roles in the cell: regulation of cell growth and modulation of inflammation. Constitutive NF-κB signaling is required for cell growth and proliferation in carcinogenesis of numerous neoplasms.61–63 Mechanisms responsible for NF-κB activation are diverse—for example, in head and neck squamous cell carcinoma (HNSCC), interleukin 1 directly induces NF-κB binding to DNA, whereas oncoproteins activate NF-κB in breast carcinoma.64,65 In NPC, LMP1 activates NF-κB through the binding of tumor necrosis factor receptor-associated factors (TRAFs).66 NF-κB dysregulation is 1 of the most important components of NPC tumorigenesis, illustrated by the fact that almost all NPC tumors exhibit NF-κB overexpression.17,18 NF-κB activation in turn leads to a myriad of different and sometimes conflicting cellular pathways. Upregulation of NF-κB results in the activation of a number of proliferative signals, including Bcl-2, cyclooxygenase 2 (COX-2), and vascular endothelial growth factor; yet it also causes accumulation of p53 and induces G2/M phase arrest in NPC cells.67,68 Furthermore, activation of NF-κB by LMP1 leads to telomerase activation and cell immortalization.69 LMP1 induces binding of the NF-κB p65 subunit to human telomerase reverse transcriptase (hTERT) in NPC cells.69 This leads to nuclear translocation of both proteins and subsequent activation of telomerase. The ultimate effect of LMP1-induced NF-κB activation appears to be LMP1-mediated immortalization.

In contrast to LMP1, LMP2A decreases NF-κB levels.47 As NF-κB is also an important regulator of the inflammatory response via expression of cytokines and chemokines,70 downregulation of NF-κB suppresses the immune response against NPC. So while LMP1 and 2A have opposite effects on NF-κB, these contradictory effects strike the balance between the antiapoptotic and anti-inflammatory levels of NF-κB that ultimately promotes tumor growth.47 However, the exact relationship of the contradictory roles of LMP1 and 2A on NF-κB remains to be fully elucidated.

**APOPTOSIS DYSREGULATION**

Aberrant apoptosis, as in all malignancies, is also required for NPC development. Inhibition of apoptosis seems to be critical to NPC tumorigenesis, second in importance only to cell proliferation. The upregulated antiapoptotic factors best identified in NPC are bcl-2, survivin, and telomerase.

**Bcl-2 Overexpression Is Important for Cell Proliferation.** Bcl-2 is an oncprotein whose overactivation interferes with apoptosis.71 The best-known mechanism of bcl-2 upregulation is in follicular lymphomas, where t(14;18) translocation of the bcl-2 gene to a site adjacent to a heavy chain immunoglobulin gene results in bcl-2 overexpression.72 Bcl-2 is upregulated in NPC in the absence of the t(14;18) translocation.73,74 Overexpression is most likely EBV associated because EBV-positive NPC cells have greater bcl-2 expression than EBV-negative NPC cells.23 However, in contrast to other upregulated pathways in NPC, bcl-2 upregulation does not appear to be LMP1 dependent. Although LMP1 transfection of lymphocytes increases bcl-2 levels, this does not occur with transfection of epithelial cells such as those in NPC.24 Furthermore, inhibition of LMP1 does not affect bcl-2 expression in NPC.45 Although the mechanism of bcl-2 upregulation is unclear at this time, its overall role in promoting cell proliferation is well documented. In fact when compared with other head and neck cancers, bcl-2 is overexpressed in a higher percentage of NPC tumors and therefore appears to be more important in NPC.
In NPC, Bcl-2 acts synergistically with LMP1 to promote a more rapid cell growth than bcl-2 alone, LMP1 alone, or LMP1 with mutant p53. Previously it was thought that this allows tumor cells to overcome the apoptotic effects of increased wild-type p53. However, a more recent study shows that bcl-2 and wild-type p53 act synergistically to increase tumor cell growth via upregulation of proliferating cell nuclear antigen, a protein required for DNA synthesis and cell proliferation. These data suggest that bcl-2 is very important to NPC tumorigenesis and that further studies to elucidate the mechanism of bcl-2 upregulation as well as understanding its role in NPC development are warranted.

Survivin Overexpression Is Critical to NPC for Avoiding Apoptosis. Survivin is a key inhibitor of apoptosis and promoter of cell proliferation, notable for its absence in normal adult tissues but presence in embryonic cells and numerous tumors. Survivin inhibits apoptosis by associating with microtubules in mitotic spindles and inhibiting cell apoptosis mechanisms, though these have not been demonstrated in NPC cells specifically. In NPC, intranuclear survivin binds cyclin-dependent kinase 4 (cdk4) and displaces inhibitory proteins p21 and p16, thus allowing cdk4 to initiate transcription of S phase proteins. LMP1 induces survivin expression and nuclear translocation, though the mechanism is unknown at this time. The net effect is increased S-phase transitions and cell proliferation. Survivin overexpression is probably the most important antiapoptotic factor in NPC development that a gene expression study found survivin overexpression in all study samples. Inhibition of survivin expression decreases NPC cell viability as well as increases NPC tumor radiosensitivity. Additionally, survivin levels have prognostic significance in NPC—patients who had tumors with low levels of survivin were less likely to have metastatic disease and had increased survival. 

High Telomerase Activity Contributes to NPC Cell Immortalization. Telomerases are enzymes responsible for the maintenance of eukaryotic chromosome telomeres and the continuous proliferation of cells, including neoplasms. Critical to telomerase activity is the hTERT. In most head and neck cancers, cell immortalization requires initial genomic instability, such as Robertsonian translocations or chromosomal losses or gains, and subsequent reactivation of telomerase via this genomic instability. Although the majority of primary NPC tumors display high telomerase activity as well, activation of telomerase is EBV dependent. LMP1 increases telomerase activity via NF-κB pathway and increased c-myc expression. NF-κB mediates transactivation of the hTERT gene and nuclear localization of the hTERT protein, whereas c-myc increases hTERT promoter activity. Telomerase is critical to the transformation of normal nasopharyngeal epithelia into malignancy. In NPC cells, 91% demonstrate hTERT expression and 85% have telomerase overactivity; conversely, cells from chronically inflamed nasopharyngeal epithelium show neither hTERT expression nor telomerase activity. Furthermore, normal nasopharyngeal epithelial cells can be transformed into immortalized cells by just activating telomerase. These findings show that telomerase is a necessary component in NPC development.

Curiously, LMP2A inhibits hTERT expression and subsequently decreases telomerase activity and cell proliferation in epithelial cells. These effects are paradoxical to the importance of EBV and telomerase to malignant transformation in NPC. Although it has been speculated that hTERT downregulation may interfere with normal B-cell function and therefore aid in the immune evasion of NPC, the role of telomerase inhibition in NPC development remains to be understood.

OTHER ABNORMAL PROLIFERATION SIGNALING PATHWAYS
A variety of proliferative signals are aberrantly activated in NPC. Although Wnt pathway is perhaps the most important of these pathways that is dysregulated in NPC, aberrations in the PI3-K, MAP kinases, and EGFR signaling also contribute to tumorigenesis.

PI3K Is Activated via LMP1 and Inactivation of Phosphatase and Tensin Homolog. The PI3 kinases are a family of kinases that are involved in a wide variety of cellular pathways—PI3K activation may have an important role in the development of normal human keratinocytes. Akt, an important downstream target of PI3K, helps regulate cell proliferation and prevent apoptosis. Overactivation of PI3K has been implicated in various squamous cell carcinomas, in which the mechanism of PI3K activation is thought to be via the overexpression of a catalytic subunit of PI3K.
While the overactivation of the PI3K pathway is critical to the development of NPC as well, its upregulation occurs by different mechanisms. LMP1 can directly activate PI3K, leading to Akt phosphorylation. As with NF-kB activation, the TRAF-binding domain of LMP1 is the active site. LMP2A can also directly activate Akt. Another potential mechanism of PI3K activation is through decreased levels of phosphatase and tensin homolog (PTEN), an inhibitor of PI3K.

PTEN is a protein tyrosine phosphatase that dephosphorylates phosphatidylinositol 3,4,5-triphosphate (PIP3), the lipid second messenger that activates Akt. It is a tumor suppressor gene with a rapidly growing importance in cancer development, especially in certain thyroid cancers that consistently demonstrated low PTEN levels. Downregulation of PTEN is found in about half of NPC tumors, making it partially responsible for the upregulation of the PI3-K/Akt pathway in NPC. Well-differentiated NPC shows a greater loss of PTEN than poorly differentiated cells. Mutation of the PTEN gene is extremely rare in HNSCC; therefore, other mechanisms are most likely responsible for the low PTEN levels observed in NPC. PTEN downregulation could be caused by epigenetic alterations to the PTEN genome (ie, promoter hypermethylation). PTEN hypermethylation has been demonstrated in laryngeal and thyroid cancer but not specifically in NPC; however, given that a number of other genes are inactivated by hypermethylation in NPC, it is likely that the PTEN gene is epigenetically altered and warrants further investigation. Alternatively, lung cancer studies have found that nicotine stimulates PTEN degradation by phosphorylating the PTEN C-terminus. Although nicotine use does not have a strong association with NPC development, it is likely that an environmental factor with similar effects on PTEN exists for NPC.

Ultimately PTEN downregulation and Akt upregulation results in decreased activity of the cell division cycle 2/cyclin B (cdc2/cyclin B) complex, inducing G2/M phase arrest in NPC cells. It also results in the upregulation of c-fos expression; c-fos is a proto-oncogene that dimerizes with Jun proteins to form the activator protein-1 (AP-1) transcription factor, which regulates expression of cell proliferation, migration, and survival. Interestingly, a study comparing malignancies with adjacent tissue found that though the adjacent tissue was histologically normal, those cells also expressed high Akt with low PTEN levels. Additionally, loss of PTEN is associated with metastatic disease—low PTEN is demonstrated in almost 80% of clinical stage III–IV tumors versus about 20% of clinical stage I–II tumors. This suggests that abnormalities in Akt and PTEN occur early in NPC tumorigenesis and contribute to disease advancement and metastasis.

MAP Kinases JNK and ERK Are Upregulated by LMP1. Located primarily in the nucleus, MAP kinases regulate gene expression by phosphorylating various transcription factors that are already bound to DNA. Their activity is regulated by a cascade involving MAPK kinase (MAPKK or MEK) and a MAPKK kinase (MAPKKK or MEKK). The MAP kinases most well studied in NPC are the c-Jun N-terminal kinase (JNK) and extracellular signal-related kinase (ERK).

Normal JNKs are activated by environmental stress and have important roles in determining cell survival or death. Prolonged JNK activation exerts a proapoptotic effect in normal cells via tumor necrosis factor (TNF)-α-induced apoptosis, and transient JNK activation can also cause cell proliferation. Usually in tumors, JNK activity is depressed and thus has antiapoptotic effects on the cells. However, in NPC, JNK is consistently upregulated, and the activating effect of LMP1 on JNK is well documented. Though the reason for this remains to be fully explained, there are several possibilities. One is that the proapoptotic effect of prolonged JNK activation is overwhelmed by the numerous proliferative signals present in NPC. Another is that other influences provide the brief decreases in JNK activation to provide the transient JNK stimuli needed for cell proliferation. Wild-type p53 has been shown to decrease JNK activity in thyroid cells; high levels of wild-type p53 are uniquely present in NPC and this may contribute to transient JNK activation. However, the most likely explanation for this pattern of JNK activation in NPC is that JNK may simply function differently in NPC and other head and neck cancers. Constitutive activation of JNK has been well demonstrated in NPC as well as oral squamous cell carcinoma (OSC) and thyroid cancers. In thyroid carcinoma, high basal JNK activation is associated with cell growth, not apoptosis. Furthermore, prolonged JNK activation in NPC has important roles in tumorigenesis, including increased p53 phosphorylation leading to its deactivation and activation of DNA methyltransferase, which is responsible for the hypermethylation and silencing
of the E-cadherin gene. These data show that constitutive JNK is unique to head and neck cancers, including NPC, and it contributes to NPC tumorigenesis. However, the significance of JNK activation as well as its unique effects in NPC development remains unknown. Studies aimed at observing the effects of inhibiting JNK signaling on NPC apoptosis or cell growth would help elucidate this question and help determine if targeting JNK is a potential therapeutic strategy.

ERK is regulated by the Ras/Mek/ERK cascade. Normal ERK activation plays a role in cell growth and differentiation. ERK phosphorylates Ets transcription factors to induce fos gene expression, which ultimately leads to transactivation of NF-κB and AP-1. This pathway regulates cellular levels of cyclin D1 and c-myc, which are important cell cycle components. Therefore, it is no surprise that upregulation of this pathway has been demonstrated in a variety of malignancies, including hepatocellular carcinoma, gastric adenocarcinoma, and renal cell carcinoma. OSCs originating in southeast Asia have higher rates of Ras overexpression and mutations and stronger association with chewing tobacco. While NPC may have similar environmental influences, Ras mutations are rare in NPC and overactivation of the ERK is primarily LMP1-dependent. LMP1 can directly activate Ras, thus overactivation of the ERK is primarily LMP1-dependent. Additionally, LMP1 induces epigenetic alterations in Ras association domain family (RASSF) proteins, which are negative effectors of Ras, and can result in Ras overactivation. Because 80% of NPC cell lines demonstrated RASSF downregulation, it is likely that the latter of the 2 mechanisms is more critical to increased ERK activity. Ultimately the cell cycle is dysregulated and cell proliferation uncontrolled. Only about half of NPC exhibits upregulated ERK, suggesting that ERK is not critical to NPC. Nonetheless, ERK contributes to NPC development as tumors exhibiting high ERK levels have poorer prognoses, with shorter overall survival rates and faster disease progression.

Epidermal Growth Factor Receptor Acts As An Intracellular Transcription Factor. The epidermal growth factor receptor (EGFR) is a 7-transmembrane receptor whose overexpression has been demonstrated in a wide variety of tumors including lung, prostate, and breast. In these cancers, EGFR overexpression or mutation causes upregulation of its signaling cascade to ultimately cause uncontrolled cell proliferation. However, EGFR overexpression has been demonstrated in only half of NPC tumors. Additionally, suppression of EGFR signaling fails to fully inhibit NPC growth, suggesting that other proliferative signals such as Wnt may play a more important role for cell growth in NPC. Interestingly, EBV exerts a unique effect on EGFR: LMP1 causes the endocytosis and nuclear accumulation of EGFR. Intranuclear EGFR acts as a transcription factor to increase cell proliferation, and cytoplasmic EGFR binds to cyclin D1 and cyclin E proteins to accelerate G1/S transition. Therefore, it appears that EGFR has a more important role as a transcription factor than as a purely proliferative signal in NPC.

TUMOR SUPPRESSORS

As with all cancers, the development of NPC involves the loss of tumor suppressors. However, the mechanisms of inhibition of tumor suppressors may be somewhat unique in NPC.

High Levels of p53 Are Found in NPC. p53 is a hallmark tumor suppressor that induces cell cycle arrest in response to DNA damage; its levels are traditionally decreased in tumors. In most head and neck cancers, low levels of p53 are due to mutations. However, p53 in NPC does not follow this classic pattern. NPC cells have increased levels of p53, with high LMP1 levels correlating with higher p53 expression. p53 mutations are relatively rare in NPC, so the vast majority of expressed p53 is wild type. The wild-type p53 fails to induce apoptosis in NPC because it is inactivated through 2 mechanisms—loss of p14 and excess ΔN-p63. p14 maintains p53 stability by inhibiting its proteolysis; in NPC, p14 levels are low via promoter hypermethylation, thus allowing for more efficient p53 degradation. p63 is actually a homologue of p53 with similar DNA binding sequences to p53. There is a mutated version of p63 in NPC, called ΔN-p63, which lacks the N-terminal transactivation domain needed to activate apoptosis. The ΔN-p63 isoform binds the normal p63 (and p53) DNA sequences, thus preventing normal p63 or p53 from binding, but fails to induce apoptosis because of loss of the N-terminal sequence.

The reason for high p53 levels in NPC is unclear. High levels of normal p53 may be advantageous to NPC development because tumor cells with normal p53 are immune to JNK-induced
EBV infection.\textsuperscript{24} In light of this, it appears that type p53 may simply be the natural response to apoptosis.\textsuperscript{135} Alternatively the increased wild-type p53 may simply be the natural response to EBV infection.\textsuperscript{24} In light of this, it appears that the loss of p53 is not critical for NPC development, so deactivation of other tumor suppressors is likely required for NPC tumorigenesis.

**p16 Activity Is Decreased.** p16 is a cyclin-dependent kinase inhibitory protein (CKI) that suppresses activity and is frequently inactivated in cancer.\textsuperscript{136} The normal function of p16 is to suppress cdk4, an enzyme that controls the G1/S checkpoint by negatively regulating cyclin D1 activity.\textsuperscript{137} Therefore, loss of p16 results in cyclin D1 overactivation and subsequent increases in G1/S phase transitions.\textsuperscript{138} Traditionally, p16 transcription is mediated by the retinoblastoma protein (pRb); inactivation of pRb, as in retinoblastoma, leads to low levels of p16.\textsuperscript{139} However, in head and neck cancers, including NPC, this is not the case, as the majority of head and neck cancers exhibit low p16 levels with high pRb levels.\textsuperscript{140,141} In HNSCC, the most common mechanism of p16 inactivation is homozygous deletion of the gene followed by hypermethylation of the gene.\textsuperscript{142} Similarly, NPC cell lines have low levels of p16 secondary to hypermethylation of the p16,\textsuperscript{99} but this epigenetic alteration may be mediated by LMP1-induced formation of a c-Jun/JunB heterodimer causing the activation of DNA methyltransferase.\textsuperscript{143} Additionally, LMP1 deactivates p16 by inducing cytoplasmic accumulation of E2F4/5 and Ets2, which are nuclear proteins required for normal p16 activity. Ets2 is a key transcription factor for p16 expression and LMP1 directly causes its translocation from the nucleus to the cytoplasm.\textsuperscript{144} E2F4/5 are proteins required for the activation of p16-induced cell cycle arrest in G1,\textsuperscript{144} and their nuclear localization is dependent on binding to nuclear pRb.\textsuperscript{145} LMP1 induces the translocation of E2F4/5 from the nucleus into the cytoplasm by causing E2F4/5 to dissociate from pRb proteins.\textsuperscript{144} LMP1 further promotes cytoplasmic accumulation of Ets2 and E2F4/5 by mediating their binding to a nuclear export protein called chromosome maintenance region 1 (CRM-1).\textsuperscript{144}

About two thirds of NPCs exhibit low p16 levels, indicating that p16 downregulation is not critical to NPC development. However, the absence of p16 is still important to NPC. Patients with NPC tumors with low p16 levels have a worse prognosis because this is associated with decreased radiosensitivity and higher rates of tumor recurrence.\textsuperscript{26,30} The reason for this may be that the greatest radiosensitivity in cells is just prior to DNA synthesis and loss of p16 increases the number of cells in S phase.\textsuperscript{146} There is some data that pretreatment of NPC patients with p16 gene therapy before radiation can improve outcomes; the presumed mechanism behind this is that by normalizing p16 levels, the cell cycle is slowed at the G1/S checkpoint and the number of cells in G1 increased.\textsuperscript{147} However, further clinical trials are needed to evaluate the effectiveness of this treatment.

**Activity of p27 Is Decreased.** p27 is a CKI that binds to S-phase kinases (ie, cdk2) to inhibit cell cycle progression.\textsuperscript{148} Phosphorylated p27 cannot regulate cdk2 activity, thus allowing the cdk2/cyclin E complex to remain activated and allows for progression of the cell cycle.\textsuperscript{149} Low p27 is well demonstrated in NPC as well as a number of tumors, including OSC, gastric cancer, and small cell lung cancer.\textsuperscript{150,151} In these latter cancers, low p27 levels are due to upregulation of ubiquitin–proteasome-mediated degradation mechanisms induced by high c-myc levels.\textsuperscript{150,152} However, in NPC cells, c-myc and p27 levels are not correlated, suggesting that other mechanisms are responsible for the low p27 activity in NPC.\textsuperscript{29} LMP1 causes this via upregulation of AKT-mediated phosphorylation of p27, thus targeting the protein for either degradation or cytoplasmic localization.\textsuperscript{45} Also, constitutively active ERK phosphorylates and deactivates p27.\textsuperscript{149} Because p27 negatively regulates cyclin E activity, reduced p27 activity results in more chromosomal instability and S-phase transitions. In addition, as elevated p27 activity is present in epithelial cells with cell–cell contact, lower levels of p27 may predispose to tumor cell metastasis.\textsuperscript{153} Low p27 is present in 68% of NPC, so it is unlikely that its downregulation is required for metastasis. However, its absence is still important to NPC progression, as reduced p27 activity has been linked to more aggressive NPC tumors as well as earlier disease recurrence after treatment.\textsuperscript{29} Therefore, p27 dysregulation plays a role in cell cycle dysregulation and chromosomal instability leading to higher-grade malignancy in NPC.

**CELL CYCLE REGULATION**

Like all cancers, development of NPC requires the derangement of the normal cell cycle. However, in NPC the abnormal expression of cell cycle proteins stems from LMP1-mediated upregulation of MAP kinases and downregulation of tumor suppressors.
Cancers. In most tumors, high cyclin E is demonstrated in a variety of head and neck tumors, which results from increased cdk4 expression.128,163 E2F binds to pRb to increase transcription of S phase proteins, including cyclin E. The net result of this is increased number of cells in S phase. While the mechanism of cyclin E upregulation is becoming clearer, the exact role cyclin E plays in NPC development remains largely unknown. The above data suggest that cyclin E plays an important role in the G1/S phase transition. Cyclin E, like cyclin D1, may have prognostic significance or may be a treatment target to enhance therapeutic success. Therefore, the mechanism of cyclin E in NPC tumorigenesis warrants further investigation. Gene studies could be done to elucidate what percentage of NPC tumors actually exhibit increased cyclin E levels. Additionally, it would be interesting to observe the effect inactivation of cyclin E has on NPC cell cycle progression.

**High Cyclin D1 Levels May Give NPC Its Unique Radiosensitivity.** Cyclin D1 is responsible for cell progression through G1 phase. Normal cyclin D1 activity is regulated by p16: cyclin D1 is active when bound to the cdk4/cdk6 complex, and displacement of cyclin D1 from this complex by p16 leads to cyclin D1 degradation and termination of G1. Overexpression of cyclin D1 enables cells with unrepaired structural or genomic damage to traverse the G1/S checkpoint, thus increasing the risk of tumor formation. Cyclin D1 is overexpressed in NPC. Underlying mechanisms include constitutively active Ras and Raf protein and, more importantly, low p16 levels. Additionally, LMP1-induced intranuclear accumulation of EGFR that can directly activate cyclin D1 transcription. The degree of cyclin D1 overexpression in NPC is comparable to that of other head and neck cancers—for example, 66% in NPC versus 64% in HNSCC. In HNSCC, high cyclin D1 levels are associated with increased local disease recurrence; conversely, high cyclin D1 levels in NPC correlate with increased responsiveness to radiotherapy and fewer local tumor recurrences in NPC. This may be due to the fact that cyclin D1 affects the number of cells in G1/S phase transition and the radiosensitivity is highest just prior to DNA synthesis. Therefore, high cyclin D1 levels may be the molecular reason behind the unique sensitivity of NPC to radiation and chemotherapy. However, the exact effects of increased cyclin D1 levels in NPC is not known, and further investigation is warranted to determine whether upregulating cyclin D1 can enhance current therapeutic success.

**Cyclin E Levels Are Increased.** The cyclin E/cdk2 complex regulates cell entry into S phase and initiation of DNA synthesis. It also negatively regulates tumor suppressor p27 by phosphorylating and causing its degradation. Dysregulation of cyclin E activity results in rapid progression through S phase and increased chromosomal instability. Increased cyclin E activity has been demonstrated in a variety of head and neck tumors, including NPC and laryngeal and oral cancers. In most tumors, high cyclin E activity occurs due to impaired cyclin E degradation. But the increased cyclin E levels in NPC are secondary to LMP1-induced nuclear translocation of EGFR, which binds to the promoter of cyclin E and increases its expression. Cyclin E activity is further enhanced by the binding of cytosolic EGFR. Additional increases in activity may be due to increased levels of activated E2F, which results from increased cdk4 expression. E2F binds to pRb to increase transcription of S phase proteins, including cyclin E. The net result of this is increased number of cells in S phase. While the mechanism of cyclin E upregulation is becoming clearer, the exact role cyclin E plays in NPC development remains largely unknown. The above data suggest that cyclin E plays an important role in the G1/S phase transition. Cyclin E, like cyclin D1, may have prognostic significance or may be a treatment target to enhance therapeutic success. Therefore, the mechanism of cyclin E in NPC tumorigenesis warrants further investigation. Gene studies could be done to elucidate what percentage of NPC tumors actually exhibit increased cyclin E levels. Additionally, it would be interesting to observe the effect inactivation of cyclin E has on NPC cell cycle progression.

**C-myc Levels May Correlate with Disease Progression.** C-myc is critical to the regulation of several important G1/S phase proteins; most notably, it sequesters inhibitory p27 from the cdk2/cyclin E complex, thus allowing for cell proliferation and progression through the G1 phase. C-myc upregulation is a common occurrence in cancers and the mechanism of its dysregulation is diverse, secondary to chromosomal translocations and point mutations in lymphoid tumors and to mutations of its regulatory proteins in other cancers such as colon, breast, and melanoma. However, in NPC, there have been reports of both increased and decreased c-myc levels. Older studies show that when compared with normal nasopharyngeal epithelia, 90% of NPC showed increased c-myc expression, and this was correlated with decreased survival. It has been shown that LMP1 increases c-myc transcription via activation of the signal transducer and activator of transcription protein 3 (STAT3) and NF-κB transcription factors. Additionally, the Ras/MAPK/ERK pathway has also been shown to upregulate c-myc transcription in human fibroblasts; although this association has not been specifically demonstrated in NPC cells, ERK overactivation is well established in NPC, making this a likely mechanism of c-myc overactivation. However, a more recent study showed that decreased c-myc levels are found in about 60% of NPC tumors and correlate with more aggressive NPC tumors with higher rates of lymph node metastasis. This study also showed that poorly differentiated (types 2 and 3) NPC is more associated with...
low c-myc levels, whereas well-differentiated (type 1) NPC has higher c-myc levels, which may explain this conflicting pattern of c-myc expression. This suggests that c-myc plays a role in disease progression, but further investigation is needed to better understand the role of c-myc in NPC development and to evaluate if targeting c-myc can slow NPC progression or metastases.

Checkpoint with Forkhead-Associated and RING Finger Domains is Downregulated via Promoter Hypermethylation. Checkpoint with forkhead-associated and ring finger domains (CHFR) is a mitotic checkpoint regulator that delays chromosome condensation in case of abnormal spindle formation.170 Gene expression of CHFR is reduced because of promoter hypermethylation in most tumors; however, the prevalence of CHFR hypermethylation is much higher in NPC—61% in NPC versus 30% in other primary head and neck cancers and 40% in colorectal cancer.31,171 This suggests that loss of CHFR expression is more common, and possibly more important, to NPC than other cancers. As a result, many NPC cells prematurely enter mitosis with unstable genomes, thus increasing the chance of chromosomal abnormalities and aneuploidy in daughter cells.31 Several chromosomal aberrations have been identified in NPC tumors—some sites correspond to proteins key to NPC development, including p16, RASSF1A, and CKIs, while a number of sites do not correspond to any known tumor suppressors or oncogenes.172,173 The prognostic and therapeutic value of CHFR remains unclear. CHFR activity can be restored in methylated cells treated with a methyltransferase inhibitor; fewer of the treated cells entered mitosis when compared with methylated cells.171 This indicates that targeting CHFR may slow the progression of tumors by reducing aneuploidy and chromosome damage. However, other studies show that detection of methylated CHFR genes is better used as a prognostic indicator because cancer cells with methylated CHFR genes actually have better prognoses and increased chemosensitivity to microtubule inhibitors such as paclitaxel.174,175 Further studies about the role of CHFR in NPC are warranted to better understand this relationship and determine if CHFR methylation can be used as a prognostic factor or treatment modality.

CELL ADHESION
Abnormal cell adhesion is critical to the invasive and metastatic potential of tumor cells. In NPC, E-cadherin and matrix metalloproteinases (MMPs) are 2 important components of cell adhesion that are dysregulated.

Decreased E-Cadherin Levels Correlate with Metastatic Disease. E-cadherin is a transmembrane glycoprotein that mediates cell communication in normal cells and as a metastasis suppressor in tumor cells.176 The downregulation of E-cadherin in NPC is due to aberrant promoter methylation of the E-cadherin gene.58 E-cadherin requires cytoplasmic β-catenin to maintain cell adhesion51; because cytoplasmic levels of β-catenin is decreased in NPC,20 cell adhesion via E-cadherin is compromised in NPC. Furthermore, E-cadherin levels are inversely proportional to disease progression: metastatic NPC tumors display lower E-cadherin mRNA and protein levels than primary NPC tumors (19% in primary tumors vs 42% in metastatic tumors).49,58 However, the proportion of NPCs that exhibit decreased E-cadherin levels is lower than for other primary HNSCC, where 88% of tumors show decreased E-cadherin expression.177 This suggests that while E-cadherin is important to NPC development, it is not as critical to metastases in NPC as in other head and neck cancers.

MMPs Are Upregulated and Correlate with Disease Progression. MMPs are type IV collagenases whose overexpression has been implicated in a number of cancers. There are 16 subtypes of MMPs that differ in structural domains that confer substrate specificity, cell-surface localization, and inhibitor binding.178 The complexity of MMP function in cancer metastasis is just now becoming clear: not only do they degrade basement membranes and extracellular matrices to allow for tumor invasion, they are also involved in activation of growth factors to promote cell growth and angiogenesis and also protect tumor cells from apoptotic signals.179–182 Interestingly, although tumor cells produce MMPs, the vast majority of MMPs come from inflammatory cells in the surrounding stroma of tumors. In HNSCC, the high level of MMP activity is secondary to overexpression of membrane type-1 MMP, a protease responsible for activating MMPs.183 In NPC, LMP1 is the driving force behind high MMP activity.

While a number of MMPs are upregulated by LMP1, current data suggest that MMP1 is the most important to NPC development. The upregulation of MMP1 in NPC far surpasses that in
other cancers: when compared with controls, MMP1 expression is increased 124-fold in NPC versus 9-fold in other head and neck cancers.\(^{184}\) LMP1 upregulates MMP1 by increasing transcription with a new Ets binding site in the MMP1 promoter and by increasing expression of MMP3, an activator of latent MMP1.\(^{185}\) A recent study found that some NPC tumors contain a MMP1 promoter polymorphism that prevents binding of the promoter repressor protein; patients with tumors that are homozygous for this polymorphism tended to have more aggressive tumors and late-stage disease at presentation.\(^{186}\) Additionally, MMP1 has the ability to activate MMP2, further enhancing overall MMP activity.\(^{187}\)

Two other MMPs consistently upregulated in NPC are MMP2 and MMP9, but considerably less is known about these 2 enzymes than MMP1. High MMP2 expression correlates with poor survival in NPC.\(^{188}\) An activating MMP2 promoter polymorphism, similar to that in MMP1, has been discovered and correlates with an increased susceptibility to developing NPC; additionally, heavy smokers with the polymorphism had the highest risk for NPC, suggesting that environmental factors such as smoking may interact with MMP2 in NPC development.\(^{189}\) MMP9 expression is associated with lymph node metastases.\(^{190}\) While polymorphisms for MMP9 have also been discovered, these do not correlate with an increased NPC risk like those for MMP1 and MMP2, suggesting that MMP9 polymorphisms are not activated.\(^{186}\) MMP9 transcription is more EBV dependent than MMP1 and 2 as their expression is upregulated by LMP1 via NF-kB and AP-1 activation.\(^{191,192}\)

These findings show that MMPs have prognostic significance in NPC and may also have therapeutic implications. Antibodies or blocking peptides directed against MMPs may prevent metastases and increase treatment efficacy. Given the diversity of effects MMPs have in other tumors, the role of MMPs in NPC pathogenesis should first be better understood and the findings applied to develop novel therapeutics for NPC.

**NEW TREATMENTS FOR NPC**

While the traditional treatment for NPC has been radiation and chemotherapy, there are several new potential treatments that specifically target the molecular aberrations of NPC.

BRD7 is a protein expressed in normal nasopharyngeal cells but is downregulated in NPC.\(^{193}\) Interestingly, its expression is unchanged in gastric and colorectal cancers, suggesting that its upregulation is unique to NPC and it is exclusively involved in NPC tumorigenesis.\(^{194}\) The uniqueness of BRD7 lies in its ability to affect multiple pathways critical to NPC development. By regulating the transcription of signaling proteins in the ERK and Rb/E2F pathways, BRD7 normalizes cyclin D1 and E activity and, therefore, the G1/S progression.\(^{195}\) BRD7 also stabilizes cell proliferation by downregulating c-jun and inhibiting the accumulation of intracellular β-catenin.\(^{195}\) Finally, BRD7 downregulates AP-1 activity, which subsequently lowers MMP activity and helps regain normal cell adhesion.\(^{195}\) A recent study confirmed these properties when ectopic administration of BRD7 to NPC cells resulted in cell growth inhibition and delayed G1/S phase progression.\(^{195}\) These data show that methods targeting the upregulation of BRD7 are promising, targeted treatment modalities for NPC. Current research has elucidated various molecular properties of BRD7, including nuclear localization sequences and promoter regulators.\(^{196,197}\) Further studies are needed to discover clinically applicable methods of increasing BRD7 in NPC, whether by simply increasing protein levels or upregulating expression. Also all studies on BRD7 thus far have used NPC cell lines; animal studies looking at the effect of BRD7 on tumor growth would be useful in determining the efficacy of this treatment modality.

Cyclooxygenase (COX)-2 overexpression has been demonstrated in many cancers, including NPC.\(^{126}\) The subsequent increase in prostacyclins are key mediators of cell proliferation, angiogenesis, and apoptosis.\(^{198-200}\) COX-2 inhibition has shown promise as a new treatment modality for other head and neck cancers: nonsteroidal anti-inflammatory drugs were protective against chemically induced tongue cancers in animals, and selective COX-2 inhibitors prevented HNSCC xenograft growth in nude mice.\(^{201,202}\) More recently, COX-2 inhibitors have been shown to decrease growth of NPC cell lines in a dose-dependent manner and reduce cyclin D1 levels.\(^{203}\) Additionally, the use of COX-2 inhibitors in NPC is promising because of their tumor suppressor effects and ability to potentiate the effects of radiotherapy, the current primary treatment for NPC.\(^{161}\)

As overactivation of the Wnt pathway is highly important to NPC development, inhibition of this
pathway is a potential treatment modality for NPC. Because nearly all NPC exhibits Wnt over-expression or WIF underexpression and this pathway is critical to the abnormal β-catenin that is also observed in all NPC, blockade of the Wnt pathway appears to be the most promising molecular treatment modality. Blocking the Wnt pathway can be achieved either by anti-Wnt antibodies or Wnt antagonists. Wnt antibodies have been studied in HNSCC cells and resulted in inhibited cell growth with induction of apoptosis. Various Wnt antagonists have been developed, the most notable of which is WIF-1. As downregulation of WIF-1 has been specifically demonstrated in NPC, attempts to normalize WIF-1 in NPC would be the most promising of Wnt-targeted treatment. The WIF-1 protein has been successfully transfected into colorectal, melanoma, and non-small cell lung cancer (NSCLC) cells and resulted in significant tumor suppression. WIF-1 gene therapy in xenograft mouse models of melanoma and NSCLC resulted in lower tumor volume and weight. The adenovirus vector used for Wnt gene therapies has been successful at blocking Wnt expression; although it is associated with significant intestinal toxicity and high mortality rates with other Wnt antagonists, these adverse effects are not seen with WIF-1 delivery. This evidence points to WIF-1 as an effective and safe potential therapy for NPC.

CONCLUSION
The mechanism of NPC tumorigenesis is complex, involving the aberrations of a large variety of pathways and the alteration in expression of numerous proteins. Normal regulation of apoptosis, cell proliferation, and cell adhesion are dysregulated. The understanding of these mechanisms has greatly increased in the past decade, generating enough data to begin the development of prognostic factors and targeted treatments for NPC. Refining prognostic factors into clinically applicable assays may aid in the detection of NPC in asymptomatic patients, in addition to staging and monitoring disease. The search for molecularly based treatments for NPC has only just begun, and so much is yet to be discovered. Improved understanding of the unique molecular mechanisms behind NPC will hopefully lead to the development of targeted treatments against NPC that slow disease progression and improve survival.

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