Editorial

The hunt for the perfect biomarker in nasopharyngeal carcinoma—the *RRAS* "race" beyond Epstein-Barr virus?

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Introduction

Nasopharyngeal carcinoma (NPC) is a distinct malignancy from other epithelial head and neck cancers, given its specific geographical and demographic distribution. It is endemic in parts of East and Southeastern Asia, and North and East Africa, and is more common in men than women (3:1 ratio) (1). The endemic variant of NPC is invariably associated with the Epstein-Barr virus (EBV). However, the interplay between EBV infection, genetic susceptibility and environmental factors leading to the tumorigenesis of NPC remains elusive (2). Several studies have now provided us with an overview of the genomic landscape of this tumour, confirming prior studies that showed the presence of genomic instability, epigenetic dysregulation and an enrichment of immune cells that are characteristic of this viral-associated tumour (3-5). As a consequence of these new insights, there has been substantial interest to investigate for new biomarkers that may help to screen for early disease or better stratify patients for risk of distant metastases. For these clinical purposes, cell free EBV DNA (cfEBV DNA) that is measurable in the plasma remains the most robust biomarker to date, but limitations including false positive results, and the stronger correlation with distant than local recurrence highlights the need for other biomarkers that may complement this molecular assay (6). It is in this context that Xiao and colleagues investigated for new molecular targets in NPC, identifying a plausible relationship between *RRAS* and NPC that could suggest its potential role as a biomarker for tumorigenesis and aggression.

Mutational landscape of NPC

The earliest model of NPC tumorigenesis was based on the concept of monoclonal expansion of an EBV-infected epithelial cell (7). EBV infection of the epithelial cell was thought to be an early event, and the onset of EBV-associated NPC was recently shown to be dependent on viral strain and host genetic factors (8). Upon cell entry, virus maintenance is promoted by activation of the cyclin D1 pathway and p16 inactivation, and it is in these persistently EBV-infected cells where latent gene proteins like LMP1 and LMP2 drive clonal expansion through various signaling pathways (9,10). Another cellular consequence of EBV infection in the nasopharyngeal epithelium is the inactivation of *RASSF1A* and *CDKN2A* that often results in genomic instability characterised by high frequencies of copy number deletions at the 3p and 9p chromosome arms (3,4).

The next molecular hallmark of NPC is the presence of global hypermethylation of the epigenome, which highlights another mechanism of inactivation of tumour suppressor genes like *RASSF1*, *CDKN2A* and *THY1*. Other molecular pathways including the MAPK, Hedgehog, Wnt and TGF-β signalling pathways were also reported to be dysregulated by DNA hypermethylation in NPC. This raises the potential to develop epigenetic biomarkers in NPC (5). As reported by Jiang and colleagues, they developed a signature comprising of six hypermethylated genes, namely *CCNA1*, *WIF1*, *RASSF1*, *UCHL1*, *TP73*, and *SFRP1*, and showed that patients with a lower

methylation status (based on the average median Z-score in the quantitative methylation analysis as the cut-off) corresponded to a superior disease-free survival and overall survival following chemoradiotherapy (11). These hypermethylated genes could also be utilised in combination with cfEBV DNA tests for other clinical scenarios including screening and early detection, and surveillance of disease recurrence post-treatment (5). Nonetheless, while promising, it can be technically challenging to implement these epigenomic biomarkers in the clinic, given the need for substantial tumour material.

Finally, it has been recently shown that the NF-κB signaling pathway is a key regulator of tumour aggression, and driver of intratumoral clonal heterogeneity in NPC (10). It was observed that somatic mutations in the negative regulators of this pathway, namely *CYLD*, *TRAF3* and *NFKBIA*, were observed in patients who were more likely to recur; along with the discovery of other downstream aberrations in major histocompatibility complex (MHC) class I genes, *ERBB-PI3K* and *MAPK* signaling axes, and chromatin remodeling, which were all prognostic for unfavourable disease (2-5). These findings point to the role of immune dysregulation as another key molecular trait in NPC that is involved in phenotypic diversity of this tumour.

Role of *RRAS* gene in NPC

Although aberrant *RAS* GTPase signalling primarily by methylation of *Ras* GTPase-activating-like protein (*RASAL*) has been suggested to drive tumour aggression in NPC, the role of *RRAS*, a *Ras*-related GTPase located on chromosome 19q13.3, is unknown in NPC (12). To complicate matters, *RRAS* has been reported to fulfil simultaneous functional roles as an oncogenic driver, like in cervical cancer (13), and a tumour suppressor in breast cancers (14). Here, Xiao and colleagues elucidated the role of *RRAS* as a tumour suppressor in NPC, taking into account previous findings by Jin and colleagues that suggested that epigenetic silencing of *RASAL* resulted in increased oncogenic signalling of the *RAS* GTPase axis. They first showed that *RRAS* mRNA expression was lower in NPC cell lines compared to normal controls; in support, siRNA knockdown experiments of *RRAS* promoted tumour growth, colony formation and invasive capacity (15).

Nonetheless, little is known about the relative importance of *RRAS* against the more frequent and characteristic mutations in NPC. Of note, gene amplifications and mutations of the *PIK3CA* gene have also been detected in NPC, and *PIK3CA* is a known oncogenic driver that is associated with poor prognosis in several tumour types (16). Interestingly, this gene was found to interact with *RRAS* in the protein-protein interaction (PPI) network analysis by Xiao *et al.*, although the mechanistic basis underpinning this interaction was not elucidated (15).

Previous *in vivo* studies had suggested that *RRAS* plays a role in downregulating angiogenesis, which could mean that the low expression of this gene is necessary to provide for a favourable microenvironment for tumour growth (17,18). If so, this could explain the observations by Xiao and colleagues that low *RRAS* expression was an adverse prognostic factor. Additionally, *RRAS* may contribute to more aggressive variants through its alleged interaction with *PIK3CA* or involvement in *PI3K/MAPK* signaling pathways. Of note, Sawada and colleagues investigated the effects of irradiation on *RRAS* knockout mice with tumour implants, demonstrating that the knockout effect of *RRAS* resulted in poorer responses to radiotherapy as compared to controls (18).

RRAS gene—biomarker and "druggable" target?

With the recent evolution in molecular targeted therapies, there have been increasing efforts to accurately risk stratify NPC patients at risk of disease relapse for treatment intensification. In this regard, there were recent proposals to incorporate cfEBV DNA with conventional TNM stage classification, which yielded better models that could more accurately distinguish high-risk and low-risk individuals, even for the same TNM stage (19). It is therefore prudent to query if *RRAS* can complement cfEBV DNA to further improve these clinicomolecular models? The findings demonstrated by Xiao *et al.* are only preliminary, but they do suggest that low expression of RRAS was significantly associated with advanced clinical stages and inferior survival rates. Future validation in external cohorts will confirm its clinical utility.

Despite decades of research and development efforts, the direct targeting of *RAS* has been largely ineffective. As such, efforts have been redirected to target upstream regulators and downstream effectors of *RAS*, such as kinase inhibitors (*EGFR/ERBB2* inhibitors) and the inhibition of the *RAF-MEK-ERK* cascade (*BRAF-V600E* inhibitors) (20,21). However, these primarily target oncogenic mutations of *HRAS*, *NRAS*, and *KRAS*; and therapies targeting the loss-of-function mutations in *RRAS* have been limited, owing to the general challenges faced in restoring the function of an inactivated tumour suppressor gene. Conventional wisdom

points to the notion that tumour suppressor genes are "hard" to design drug therapies for. As such, existing therapeutic strategies to target aberrant pathways linked to this family of genes have revolved around targeting molecules that regulate these tumour suppressor genes; and downstream pathways that have been consequently activated by the loss-of-function of these genes (22). A prime example would be the inhibition of PI3K-AKT-mTOR signaling axis that result from the inactivation of *PTEN*. Another area of interest would be the targeting of epigenetic mechanisms like the global promoter hypermethylation seen in NPC, for example using inhibitors of DNA methyl transferases that reverse hypermethylation of DNA in patients with myelodysplastic syndrome (23). The interaction of *RRAS* with *PIK3CA* identified by Xiao and colleagues in their PPI analysis is also intriguing as recent evidence suggests that *PIK3CA* mutations may be targeted using p110α-selective inhibitors (24). That said, the mechanisms underlying the interaction between *RRAS* and *PIK3CA*, as well as its relation to the *PI3K/AKT* signaling pathways, require further elucidation for these potential targets to be even considered a theoretical possibility.

Since the inactivation of *RRAS* has been postulated to have downstream upregulation of tumour angiogenesis and hypoxia levels, another possibility would be to utilise *RRAS* as a predictive biomarker for anti-vascular endothelial growth factor (VEGF) antibody therapies. Of note, a phase II single-arm trial previously showed favourable overall survival rates of 90.9% at 2 years with the addition of bevacizumab to standard chemoradiotherapy for loco-regionally advanced NPC (RTOG 0615) (25). Hence, it may be beneficial to re-examine the role of VEGF blockade in advanced NPCs or even in radioresistant subclones. Furthermore, since tumour hypoxia and angiogenic pathways have long been implicated in the development of radioresistance, added scrutiny into this gene may even yield valuable insights into this poorly understood phenomenon.

The era of high throughput next-generation sequencing and complex bioinformatics approaches have facilitated an acute wave of efforts to widen the search for better prognostic and predictive biomarkers. The findings elicited by Xiao *et al.* have provided insights into the functional aspects of *RRAS* and its role in the NPC genomic framework. This is a new finding using a contemporary PPI method, and will facilitate future work of developing a more comprehensive understanding of the molecular processes that are associated with NPC. Hopefully, this will help to yield a biomarker that is in fact predictive of treatment efficacy and vulnerability to targeted therapeutics, rather than merely informing on tumour aggression and recurrence. The question of going beyond cfEBV DNA in search of a companion biomarker for NPC should be a matter of when and not if.

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