Whole-genome profiling of nasopharyngeal carcinoma reveals viral-host co-operation in inflammatory NF-κB activation and immune escape

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Interplay between EBV infection and acquired genetic alterations during nasopharyngeal carcinoma (NPC) development remains vague. Here we report a comprehensive genomic analysis of 70 NPCs, combining whole-genome sequencing (WGS) of microdissected tumor cells with EBV oncogene expression to reveal multiple aspects of cellular-viral co-operation in tumorigenesis. Genomic aberrations along with EBV-encoded LMP1 expression underpin constitutive NF-κB activation in 90% of NPCs. A similar spectrum of somatic aberrations and viral gene expression undermine innate immunity in 79% of cases and adaptive immunity in 47% of cases; mechanisms by which NPC may evade immune surveillance despite its pro-inflammatory phenotype. Additionally, genomic changes impairing TGFBR2 promote oncogenesis and stabilize EBV infection in tumor cells. Fine-mapping of CDKN2A/CDKN2B deletion breakpoints reveals homozygous MTAP deletions in 32-34% of NPCs that confer marked sensitivity to MAT2A inhibition. Our work concludes that NPC is a homogeneously NF-κB-driven and immune-protected, yet potentially druggable, cancer.
Asopharyngeal carcinoma (NPC) is an etiologically complex tumor with a unique combination of epidemiologic, histologic, and viral features. NPC affects ~129,000 new patients each year (GLOBOCAN 2018), with the highest incidence occurring in South-East Asia, North Africa, and in the Inuit population in the northern regions of North America. NPC is characterized by poorly differentiated malignant epithelial cells residing in a complex microenvironment with heavy lymphocyte infiltration, giving rise to the appearance of a lymphoepithelioma with an apparent inflammatory phenotype. Malignant cells are uniformly positive for the Epstein-Barr virus (EBV), a herpesvirus widespread in the human population. EBV persistent latent infection of B lymphocytes is causally linked to several B-cell malignancies including Burkitt lymphoma, Hodgkin disease, AIDS-related lymphoma, diffuse large B-cell lymphoma, along with a somewhat unexpected association with epithelial tumors such as NPC and a small subset of gastric carcinomas.

Recent functional and genomic studies have implicated NF-κB pathway activation (e.g., TNFAIP3, MYD88) and immune evasion (e.g., PDL1/CD274, PDL2/PDCD1LG2) in the pathogenesis of EBV-associated lymphomas. However, there is little understanding of how somatic genetic aberrations and viral infection co-operate in the malignant process. In a paradox particular to NPC, it is unclear how viral and somatic factors may interact to establish the tumor’s inflammatory phenotype while rendering the malignant cells apparently obscured from detection by host immunity. Furthermore, the consistent presence of EBV in NPC is puzzling given that the epithelial cells from which NPC derives do not normally sustain a latent virus infection. This raises a long-standing question as to whether NPC development involves cellular genetic changes that favor EBV persistence.

As an immediate background to the present study, whole-exome, genome, and targeted DNA-sequencing studies of primary and recurrent NPCs have revealed various somatic alterations that drive constitutive nuclear factor kappa B (NF-κB) signaling activation in up to 40% of cases. Our previous work, in this context, isolating the malignant NPC cells from their abundant lymphocytic infiltrate by microdissection, used exome sequencing to identify two driver genes in the NF-κB pathway, Traf3 and Cyl7. Both genes are frequently inactivated by focal homozygous deletions or rearrangements, highlighting the importance of large chromosomal aberrations or structural variants (SVs) in NPC development. The complex nature of genomic changes in NPC underscored the need for whole-genome characterization in conjunction with the evaluation of EBV gene expression, to provide further insights into the interplay of somatic aberrations and viral effects in disease pathogenesis.

In this work, we employed whole-genome sequencing (WGS) to comprehensively delineate the genome landscape of 63 microdissected tumors, 5 patient-derived xenografts, and 2 cell lines of NPC. This included analysis of etiology-associated coding and non-coding mutational signatures, detection of recurrent SVs, and fine mapping of chromosome- and gene-level copy changes across the genome. This approach illuminated multiple routes to constitutive NF-κB activation, as well as immune evasion mechanisms, both involving somatic changes and viral factors. We also uncovered a prevalent mutational signature associated with homologous recombination (HR) repair defects in NPC, revealing a prominent involvement of double-stranded DNA repair defects in NPC pathogenesis. Resulting structural alterations implicate two targets with critical impacts: recurrent TGFBR2 inactivation that promotes EBV persistence in tumor cells and MTAP deletion (adjacent to the Cdkn2a/Cdkn2b loci) with marked sensitivity to MAT2A inhibition potentially applicable to a broad subset of NPC patients. Our findings characterize NPC as universally driven by pro-inflammatory NF-κB signaling counterbalanced by multiple mechanisms of immune protection.

### Results

**Mutational signatures and significantly mutated genes.** To establish a comprehensive catalog of genomic aberrations in NPC, we conducted WGS of 63 EBV-positive, non-keratinizing microdissected tumors from Southern Chinese patients (58 primary, 4 recurrent, 1 metastatic; Supplementary data 1), as well as 7 recently established cell lines and PDX models. We achieved genome-wide average sequence coverage of 83× for tumors and 51× for paired normal controls. WGS enabled identification of both coding (n = 54,588) and non-coding (n = 885,442) simple somatic mutations, which include single nucleotide variants (SNVs), small insertions, and deletions (indels) (Supplementary Data 2, Supplementary figure 1). An orthogonal validation rate of 96% (82/85 events with >10% allelic-fraction, Supplementary data 3) was achieved by target-capture deep sequencing.

To provide clues regarding NPC etiology, we examined the single base changes observed which confirmed that the predominant type of genome-wide substitution is C-to-T transitions at NpCpG sites. We also observed recurrent evidence of Signature 3 (present in 64.3% (45/70) cases), which was not reported by previous exome-sequencing studies. Mutual Signature 3 is associated with defects in DNA double-strand break repair by HR (Fig. 1A). In addition to HR defects (Signature 3), we found contributions from deamination of 5-methyl-cytosine (Signature 1, 98.6% cases), defective DNA mismatch repair (Signatures 6, 15, 20, and 26, 98.6% cases) and APOBEC/AID (Signatures 2 and 13, 44.3%). Together, these mutational signatures provide insight into NPC development that may underlie the acquisition of specific driver mutations. In a recent meta-analysis study of the published exome-sequencing data sets in 402 NPC patients, all four mutational signature classes we identified were confirmed.

To nominate coding and non-coding mutations that drive NPC, we employed ActiveDriverWGS. This identified 11 significantly mutated coding genes and one regulatory region that largely converge on NF-κB signaling (Fig. 1B). Consistent with previous studies, TP53 was the most significantly mutated gene (n = 10; q = 1.1 × 10⁻⁶), followed by Traf3, Nfkb1a, Aebp1, and Nlrc5; all of which have been reported to regulate NF-κB signaling. In addition, significant somatic aberrations detected in Hla-A and Nlrc5 suggest impairment of antigen presentation while Pten mutations may activate the PI3K pathway. We also found four coding genes, which were significantly mutated, namely Plin4, Muc21, Slc35g5, and Ervwv-1. Further studies would be required to define the function of these mutations in NPC.

Among all the SNVs and indels detected, 94% were located in non-coding regions. These include 1480 mutations in putative promoters (The Eukaryotic Promoter Database, 5997 in predicted Enhancers (ENCODE), and 4699 in TF-binding sites (ENCODE). The sole significantly mutated non-coding regulatory region in our cohort, ENSR00000289706, is a CTCF binding site ~16 kb downstream from Cys1 and ~22 kb upstream of Rrm2 (Fig. 1B). Notably, a single recurrent mutation (chr2:10097565 C>T) was identified in four tumors from three patients (Supplementary figure 2). Despite reports from several genome sequencing studies conducted thus far, this represents the first recurrent mutation identified in the non-coding region of NPC genome. Furthermore, this mutation results in a gain of a putative Nfkb1/p50 binding sequence upstream of Rrm2 which has been reported to be associated with NF-κB-signaling activation. Of note, mutations in this region were also reported in the International Cancer Genome Consortium (ICGC).
pan-cancer data set, including three occurrences of this specific point mutation\(^9\). Future studies would be required to confirm the predicted functional effect of this and other, less-frequent non-coding mutations.

**Recurrent copy number and structural alterations.** We identified somatic copy number alterations (SCNAs) using Varscan2 and Sequenza, followed by significance analysis using GISTIC (v2.0.23) (Fig. 1C, supplementary data 4). We detected polyploidy in 15.9% of patient tumors (10/63) and the median percent genome altered (PGA) was 30% (range 9–92%) (Fig. 1C). GISTIC analysis revealed frequent arm-level chromosomal losses and amplification, including frequent gains in chr.1q, 3q, 7q, 8q, 12p, and 12q, and frequent losses in chr. 3p, 9p, 11q, 14q, and 16q (Supplementary figure 3). This analysis defined homozygous deletion of 9p21.3 (CDKN2A/CDKN2B) as the most frequently altered chromosome region, impacting 37.1% of cases (Fig. 2)\(^1,2\).

**Fig. 1 Whole-genome landscape of NPC.** A Somatic gene alterations and mutational signatures of 70 NPC tumors. For each tumor, the number and types of SNVs and SVs, mutation signatures, whole-genome doubling, and percent genome altered (PGA) were shown in the top panels, as well as somatic changes detected in NF-κB, DNA repair, and TGF-β pathways, expression of EBV-encoded LMP1, BNLF2a and EBERs, clinical staging and tumor type. B Significantly mutated genes and regulatory elements (q < 0.1) identified in NPC. The altered genes in NF-κB pathways are indicated. C Copy number alterations in NPC. GISTIC copy number variations analysis showing recurrent amplification and deletion of multiple chromosomal regions.
Through the fine mapping of deletion breakpoints within 9p21.3, we noted frequent homozygous deletions of a potential druggable target, MTAP (34%), and distally, loss of type I interferon genes (e.g., IFNA1, IFNA2, IFNA8, IFNE; 16%) that may be associated with loss of antiviral response in early NPC2,3 (Fig. 2). In addition, significantly focal amplified regions harboring potential drivers were found on chr. 11q13 (CCND1), 9p24.3 (JAK2, CD274, PDCD1LG2) and 12p13.3 (TIGAR/CD9/LTBR Fig. 1C). Together, these recurrent SCNAs are consistent with the previous genome-wide studies and accurately defined NPC-associated genes including CD274 and the type I interferon genes2,8,20. Importantly, using microdissected tumor samples and WGS, we detected the highest frequencies of homozygous deletions of the critical tumor suppressor genes CDKN2A, TGFBR2, TRAF3, and potential synthetic lethal target MTAP amongst NPC genomic reports thus far8–14.

Chromosomal rearrangements were detected using a combination of three SV calling algorithms (Manta (v1.2.2), DELLY (v0.7.7), NovoBreak (v1.1.3)) followed by filtration and annotation using MAVIS (v1.8.4) (Supplementary data 5). Using this

**Fig. 2 Recurrent structural and chromosomal alterations in NPC.** Circos plot showing recurrent SVs and common CNVs in NPC. Frequencies of copy number losses and gains are shown in the outer and inner circles respectively. Selected cancer genes involved in the recurrent inter- and intrachromosomal SVs (i.e., genes with ≥3 times altered by SVs) are indicated. Homozygous deletions and structural alteration breakpoints identified in CDKN2A/B loci, MTAP, and cluster of type I IFN genes at 9p13.3, TGFBR2 at 3p24.1, and TRAF3 at 14q32.3 are shown. The DNA sequences spanning the breakpoints of deletion of TGFBR2 identified in NPC43 and Xeno-47 were confirmed by Sanger DNA sequencing.
approach, we identified a total of 3486 high-confidence SVs, including translocations, inversions, inverted translocations, insertions, deletions, and duplications (Fig. 1A). On average, NPCs harbored 50 SVs per tumor (ranging from 5 to 208), consistent with previously reported rates in head and neck squamous cell carcinoma, lung and colorectal adenocarcinomas (Fig. 1A, suppl. Figure S1). Mapping of recurrent SVs with common chromosomal aberrations revealed substantial overlap with recurrent CNAs including multiple regions on chromosome 3p, 9p, 11q, 14q, and 16q, harboring known tumor suppressors and cancer genes in NPC (Fig. 2). While the heterozygous loss of chr 3p is a known feature of NPC (64/70, 91.4% in our cohort), we also discovered recurrent homozygous deletions and SVs of TGFB2 at 3p24.1 in 11.4% of our tumors (Fig. 2). In addition, recurrent breakpoints of SVs targeting cancer genes commonly occurred in frequently deleted regions, e.g., CDKN2A on 9p21.3, MAML2 and ATM on 11q12-22, RAD51B, and TRAF3 on 14q 24-23, CYLD and NLRCS on 16q12.1-13 (Fig. 2). Taken together, we conclude that combinations of homozygous deletions and structural alterations are common mechanisms for biallelic inactivation of tumor suppressors in NPC, highlighting the need for comprehensive genome-wide profiling for this cancer type, in particular. Consistent with previous reports, recurrent oncogenic TACC3-FGFR3 fusions were also detected in our NPC cohort (2/70, 2.9%)\(^2\).

**EBV and somatic changes co-operate to sustain NF-kB activation.** By integrating all SNV, SV, and CNA data, we have established a comprehensive catalog of genomic changes implicating multiple oncogenic pathways in NPC. The most prevalently altered pathway encodes NF-kB signaling (44/70; 62.9%) (Figs. 1A and 3A). In addition to our previously reported somatic changes of NF-kB-negative regulators [TRAF3 (21.4%), CYLD (11.4%), NFKBIA (10%), NLRCS (8.6%), and TNAIP3 (5.7%)], we have identified additional alterations impacting this pathway including amplifications of LTBIR (20%) and recurrent homozygous deletions and rearrangements of BIRC2 (5.7%) and BIRC3 (8.6%). In particular, LTBIR amplification that went unreported in our previous exome study is a demonstrated driver for NF-kB activation in NPC and multiple myeloma\(^11,20-22\).

The EBV oncoprotein, LMP1, has been demonstrated to drive NF-kB pathway activation in NPC, via tumor necrosis factor receptor/TRAF3 interactions\(^3\). In this cohort, high LMP1 expression was found in 32.8% (23/70) of tumors (Fig. 3A, B). Previously, we reported the discovery of mutual exclusivity between somatic and LMP1-driven NF-kB-activating events in NPC which we have confirmed herein (Fig. 3B; two-sided Fisher’s exact test \(p = 3.5 \times 10^{-6}\)). With additional genome variations, our current WGS study further strengthened this finding, as co-selected NF-kB-activating mechanisms in 90% (63/70) of this tumor cohort with a high degree of mutual exclusivity (two-sided Fisher’s exact test \(p = 7.4 \times 10^{-7}\)). Our findings solidly define NPC as a ubiquitously NF-kB-driven malignancy. In addition, LMP1 is also known to activate PI3K/AKT and MAPK signaling pathways\(^3\). Although rare somatic alterations of PI3K pathways were observed in NPC cases with LMP1 overexpression, similar viral-somatic mutual exclusivity was observed between altered PI3K signaling pathway and LMP1 expression (Supplementary figure 4A, B).

**Impaired immune machinery by EBV and somatic changes.** With inflammatory NF-kB signaling, persistent expression of various viral RNAs (EBERs) and immune antigens (e.g., EBNA1, LMP1, LMP2) in nearly all NPC, immune evasion is believed to be critical for NPC pathogenesis. The abundantly expressed EBER transcripts are known to activate IRF3 signaling, which induces type I interferons to elicit innate immunity against EBV-infected NPC cells\(^23-25\). Indeed, using WGS we uncovered genomic losses of multiple types I interferon genes, including INF1A, IFNA2, IFNA8, and IFNE by a homozygous deletion in 15.7% of NPC (Fig. 2). Importantly, the inactivation of TRAF3 or CYLD by somatic alterations also attenuates IRF3 signaling. Furthermore, LTBIR amplification and LMP1 overexpression inactivate TRAF3 activity, thereby inhibiting the innate immune responses for EBV infection through impairment of IRF3 signaling and type I interferon production\(^2,23\). In summary, to avoid the EBV-induced innate immune response by attenuating IRF3 signaling, either acquisition of somatic alterations or overexpression of LMP1 were demonstrated in a total of 55/70 (78.6%) clinical specimens (Fig. 4A).

In terms of adaptive immunity, our data revealed SVs and aberrations of immune checkpoint machinery (PD-L1/CD274; Supplementary figure 5), and antigen presentation molecules including MHC class I (HLA-A, HLA-B), class II genes (HLA-DQA1, HLA-DQB2, HLA-DRB1, HLA-DRB5), and their major transcriptional activators (NLRCS and CIITA) in 2270 (31.4%) of NPCs (Fig. 4A). Such alterations could impair antigen presentation by tumor cells allowing them to escape from immune surveillance. In fact, loss-of-function (LOF) alterations in NLRCS and CIITA were consistent with the loss of MHC class I and class II expression in NPC tumor cells (Fig. 4B and Supplementary figure 6). Notably, inactivating mutations in MHC class I genes (NLRCS, HLA-A, HLA-B) were significantly associated with the presence of APOBEC-related mutational signature (two-sided Fisher’s exact test \(p = 0.0022\), but not the DNA mismatch repair defect mutational signature. It is likely that the NPCs with high APOBEC3 activity resulting in the presence of the APOBEC mutational signature also acquire inactivating alterations in MHC class I molecules to enable escape from CD8+ cytotoxic T-cell response. Indeed, a significant relationship between mutational burden and alterations in adaptive immunity-related genes including CD274, NLRCS, HLA-A, HLA-B, CIITA, HLA-DRB1, HLA-DRB5, HLA-DQB2 (two-sided Wilcoxon signed-rank test \(p = 0.00028\)). These findings suggested that viral factors and somatic events both contribute to defective DNA mismatch repair and APOBEC3 mutational signatures resulting in high TMB, which subsequently favors the selection and clonal expansion of EBV-infected NPC cells bearing somatic alterations in adaptive immunity-related genes.

In addition to somatic events, we also explored potential viral-associated mechanisms for evading host immune responses. Expression of BNLF2a, an EBV lytic gene inhibiting the cellular transporter associated with antigen processing (TAP) protein has been shown to be expressed in gastric cancers latently infected with EBV\(^26,27\). From RNA-sequencing data sets of EBV-associated PDXs, we also observed BNLF2a transcription in these NPC models (Fig. 4C, D and Supplementary figure 7). BNLF2a expression was confirmed in the majority of latently infected EBV-positive tumor cells in two NPC PDXs using RNA in situ hybridization (RISH) of a BNLF2a-specific probe. Because of the overlap of BNLF2a and exon 3 of LMP1 genes, the cases with positive RISH signals for LMP1-specific probe deemed “undeﬁned”. Nevertheless, high levels of BNLF2a transcript alone were detected in at least 13 NPC tumors available for RISH (Fig. 4D).

Notably, a total of 47.1% (33/70) of NPC tumors had either BNLF2a expression or somatic aberrations in antigen presentation and immune checkpoint genes. A trend for mutual exclusivity between high tumor expression of BNLF2a and somatic alterations for immune evasion was observed (two-sided Fisher’s exact test \(p = 0.24\)) (Fig. 4D). These complementary viral
and somatic events impairing antigen presentation and immune checkpoints may contribute to the clonal selection for the immune escape of EBV-infected cells during NPC development. This observation of impaired innate and adaptive immunity supports the notion that NPC tumors are protected by both viral and somatic mechanisms of immune evasion in patients, representing the second viral-somatic co-selected mechanisms (after NF-κB activation) for NPC tumorigenesis.

TGFBR2 loss is a driver event in NPC. Dysregulation of TGF-β/SMAD signaling has been shown to initiate cancer formation and disease progression in various human cancers. Here our data shows a 24.3% (17/70 tumors) rate of TGF-β/SMAD pathway gene alteration in NPC, targeting TGFBR2, TGFBR3, ACVR2A, and SMAD4 (Fig. 5A). It has been shown that TGFBR2 loss protects EBV-infected NPC cells from autocrine or paracrine TGF-β-mediated cytostasis and differentiation through Smad signaling. In fact, we found TGFBR2 aberrations in NPC patient tumors, cell lines (NPC43, NPC53) and PDXs (Xeno-32, Xeno-38, Xeno-47) (Fig. 5A). Strikingly, using RISH we observed downregulation of TGFBR2 in the majority of clinical tumor samples when compared with adjacent histological normal epithelial cells and infiltrating lymphocytes (Fig. 5B). Similar findings were observed in NPC models (Supplementary figure 8A). TGFBR2 is a major tumor suppressor residing on chr. 3p, the most frequently deleted chromosomal region in NPC and its precancerous lesions. To date, however, the role of TGFBR2 loss in the pathogenesis of NPC remains poorly defined. Here, we demonstrated that ectopic TGFBR2 expression inhibited cell proliferation and restored phosphorylation of Smad2/3 in EBV-positive NPC43 cells in response to TGF-β stimulation (Fig. 5C). Similiar findings were observed in NPC models (Supplementary figure 8A).
resistance to TGF-β-induced growth inhibition (Fig. 5E). To examine the effect of disrupting TGF-β/SMAD signaling on the outcome and stability of EBV infection in these immortalized NPE cells, the parental NP460 and TGFBR2 knockout NP460KO cells were infected with a green fluorescent protein (GFP)-tagged recombinant EBV (Akata strain), and the EBV-positive cells were isolated using FACS and grown for 7 and 14 days as we described previously31. This experiment demonstrated that the efficiency of persistent EBV infection was significantly increased in NPE cells with TGFBR2 inactivation (Fig. 5F and Supplementary figure 8B). Our findings establish TGFBR2 loss as a direct driver for NPC tumorigenesis, playing a pivotal role in attenuated TGF-β signaling and establishment of EBV latency.

Genomic aberrations in DNA repair and other oncogenic pathways. While somatic alterations of TP53 and other DNA double-strand break repair genes (e.g., ATM, BARD1, BRCA2,
CCND2, CDK4, and CDKN2A/CCND1/CDK4 deletion and structural alterations of alterations affecting 74.3% (52/70) of NPC cases at the whole-cycle regulation. Through mechanisms other than genomic instability, such as cell defects did not experience a different outcome, indicating the in disease-free and overall survival (Supplementary figure 11). This is consistent with previously reported roles of the characteristic of chromothripsis, leading to homozygous loss of KMT2D member. Specifically, NPC-24T harbored an ATM-NCOR1 fusion leading to truncation of ATM at exon 36, whereas NPC-38T displayed a focal region on chr13q with numerous breakpoints, characteristic of chromothripsis, leading to homozygous loss of BRCA2 (Supplementary figure 10). The most commonly altered DNA repair gene TP53 was associated with a significant reduction in disease-free and overall survival (Supplementary figure 9B). However, patients with mutations in other HR-related gene defects did not experience a different outcome, indicating the TP53-altered status is likely associated with disease aggressiveness through mechanisms other than genomic instability, such as cell cycle regulation.

We also observed alterations to the cell cycle machinery alterations affecting 74.3% (52/70) of NPC cases at the whole-genome level. Aside from the aberrations of TP53, homoygous deletion and structural alterations of CDKN2A ranked at the top (42.9%; 30/70 cases), followed by amplification of CCND1, CCND2, CDK4, and CDK6 (31.4%; 22/70) (Fig. 6, Supplementary figure 11). This is consistent with previously reported roles of the CDKN2A/CCND1/CDK4 axis in NPC cell growth and persistent EBV latent infection in NPE cells.1,2,3

Frequent genomic events were also found within the NOTCH signaling pathway (14/70; 20%) along with members of those affecting the chromatin modification machinery (29/70; 41.4%) (Fig. 6, Supplementary figure 10). In the NOTCH pathway, relatively common alterations, in NOTCHI (5/70; 7.1%), and MAML2 (5/70; 7.1%) were noted (Supplementary figure 12). Identification of LOF events, such as inactivating gene rearrangements of NOTCH1 and MAML2 support their tumor-suppressive roles in NPC, similar to that described in other head and neck cancers (Supplementary figure 13)32. Multiple recurrent aberrations impairing chromatin modification machinery included ARIDIA (5.7%), ARIDIB (10%), CHD6 (4.2%), KMT2C (5.7%), KMT2D (11.4%), KDM6A (8.6%), and EP300 (10%) (Fig. 6, Supplementary figure 12). Interestingly, LOF alterations were also commonly found in a cluster on chr 3p21.3 region encompassing genes of the SWI/SNF complex (BAP1 and PBRM1) and chromatin remodeling (SETD2 and BAP1) (11/70, 15.7%) (Supplementary figure 12).

MTAP deletion is druggable in primary and recurrent NPC. Previous exome studies have shown that there are many potentially druggable targets scattered across the NPC genome.2,8–11,13,14. Our whole-genome study also revealed hot-spot aberrations of common drug targets including PIK3CA, EGER, BRAF, MET, and ERBB2, yet these appeared infrequently (totaling 2.9%, 2/70). Druggable fusion events were identified in 7.1% (5/70) of tumor specimens: FGFR3-TACC3 (n = 2), NTRK2 fusions (n = 1), ALK (n = 1), and ROS1 (n = 1). In the case of NTRK and ROS1 fusions, the FDA-approved pan-cancer drugs, larotrectinib, and entrectinib are readily available for clinical treatment in these subsets of NPC patients. Recently in classic Hodgkin lymphoma, a 9p24.1 amplification involving CD274 (PD-L1) and PDCD1LG2 (PD-L2) was shown to predict response to nivolumab outcomes.33 As a target for immunotherapy, PD-L1 displayed structural alterations in only 5.7% (4/61) of NPC cases (Fig. 4). Amidst the positive clinical trial results of anti-PD-1 immunotherapy in NPC with 20–34% overall response rates, it would be important to determine if PD-L1 genomic aberrations can predict outcomes for PD-1/PD-L1 targeting in clinical settings.34–36. In a recent phase II clinical study of PD-1 inhibitor for recurrent and metastatic NPC, Wang et al. identified a potential association of genomic amplification in 11q13 regions and ETV6 alterations with poor response.14 Thus, comprehensive evaluation of both viral factors and somatic alterations targeting host immunity in these clinical trials may help to identify patients who may respond to immunotherapies.

Strikingly, an emerging drug target, MTAP (methylthioadenosine phosphorlyase) was identified as a commonly deleted gene by WGS, accounting for 34% (24/70) of NPC cases. MTAP is a key enzyme for polyamine metabolism and salvaging of adenine and methionine. Studies have revealed the pharmacologic vulnerability of MTAP-deficient tumors through targeting the MAT2A/PRMT5 axis37–40. MTAP resides next to CDKN2A on chr. 9p21.3 and is often co-deleted with CDKN2A, a frequently deleted tumor suppressor in human cancers. In fact, homozygous deletions of MTAP and CDKN2A are almost in complete overlap in our NPC cohort and several PDXs including xenogen-76 (Fig. 2). In an independent cohort of recurrent NPC, FISH analysis concluded that 32% (16/50) of aggressive tumors harbored MTAP homozygous deletion (Fig. 7A, supplementary data 6). The loss of MTAP expression was confirmed in these cases by IHC staining (Fig. 7B and supplementary figure 14A). In addition, the co-
deletion of MTAP and CDKN2A were also confirmed in 7–16.7% of NPC samples in previous whole-exome and WGS studies (Supplementary figure 14B)12,14.

We further examined the responsiveness of MTAP-deficient NPC to MAT2A inhibition in vitro and in vivo. MTAP-null C666-1 cells were generated by CRISPR and subjected to treatment with MAT2A siRNA, PRMT5 siRNA and chemical inhibitor treatments (Fig. 7C–F). As shown in Fig. 7C, the level of symmetric dimethylarginine (SDMA) was markedly reduced in MTAP-null C666-1 cells in response to MAT2A and PRMT5 siRNA treatments, indicating significant inhibition of PRMT5 activity. Knockdown of MAT2A and PRMT5 significantly suppressed the growth of MTAP-null C666-1, but not that of the parental MTAP-WT C666-1 cells (Fig. 7D). Importantly, MTAP-null C666-1 cells demonstrated heightened sensitivity (~8.7-fold more sensitive) to the MAT2A inhibitor, FIDAS-5,
compared with MTAP-wild-type NPC cell lines (Fig. 7E). FIDAS-5 treatment resulted in a reduction in the level of SDMA, accompanied by p53 expression in MTAP-null C666-1 cells (Fig. 7F). In vivo, FIDAS-5 elicited marked antitumor activity in an MTAP-deficient NPC PDX, Xeno-76 while no such antitumor effect was observed for MTAP-WT C666-1 tumors (Fig. 7G, H). In Xeno-76-transplanted mice treated with FIDAS-5, the level of circulating EBV DNA was significantly reduced, associated with an obvious reduction in tumor burden (Fig. 7I). Marked suppression of SDMA was accompanied by the increase of cleaved caspase 3, p53, BAX, and involucrin in the MTAP-deficient Xeno-76 tumors (Fig. 7J). At last, FIDAS-5 treatment resulted in the occurrence of tumor cells with keratinization phenotype and involucrin expression in Xeno-76, but not in MTAP-WT C666-1 tumors (Fig. 7H and Supplementary figure 14C). Here, we documented the therapeutic vulnerability of MTAP-deficient NPC by a MAT2A inhibitor. This precision strategy warrants future assessments in clinical studies, which might well impact a large subset of NPC patients.

Discussion

The present WGS study establishes a comprehensive whole-genome landscape of a large NPC cohort (n = 70), which can serve as a useful resource for the broader community. In addition to genome-wide mutations, rearrangements, and accurate copy number (CN) changes our study incorporates EBV viral gene expression, revealing multiple important insights into NPC.
Tumorigenesis further establishing the interrelated roles for both host somatic alterations and EBV gene expression impacting multiple cellular mechanisms, namely NF-κB activation, immune evasion, and persistent infection with EBV8–11,13,14.

Firstly, our results show that constitutive activation of the NF-κB in inflammatory pathways occurs in as high as 90% of NPC either through somatic alterations or expression of the virus-encoded LMP1 oncogene, implicating aberrant NF-κB activation as a ubiquitous hallmark of this EBV-associated malignancy. The dominance of NF-κB activation in NPC that we have identified is consistent with recent genome-wide CRISPR-based gene knockout screens and functional studies showing that perturbation of the NF-κB...
**Methods**

**NPC cell lines, PDxs, and patient tumor samples.** All 63 NPC tumor tissues (including two specimens from the same donor) were collected by endoscopy or surgery, embedded in optimal cutting temperature compound, and stored at −70 °C. DNA samples extracted from microdisected frozen tissue sections were subjected to WGS. For all patients, corresponding blood samples were collected as normal control. Written patient consents were obtained from all patients in this study according to institutional clinical research approval. The study protocol was approved by the Joint Chinese University of Hong Kong-New Territories East Clinical Research Ethics Committee at the Chinese University of Hong Kong. Hong Kong SAR. EBV infection status in these tumor samples was confirmed by EBER in situ hybridization. The detailed information of the tumors was provided in supplementary data 1. Three NPC cell lines (NPC43, NPC38, NPC53) and four patient-derived xenografts (xeno-23, xeno-32, xeno-47, xeno-76) recently established by us were included for WGS. Among these samples, 15 tumor specimens, 3 cell lines, and 4 xenografts were also included in our previous exome and genome sequencing studies. For functional and preclinical studies, the immortalized NPC cells (NPC69, NPC40) and authenticated EBV-positive cell lines (C666-1, C17C) and PDxs (xeno-2117, C15, C17) established by us and Prof. Pierre Busson were also used. The establishment of MTAP-deleted C666-1 and MTAP knockout NPC40 cells is described in supplementary methods. All NPC cell lines are commonly deleted in glioblastoma (40%), melanoma (25%), and pancreatic adenocarcinoma (25%), unlike NPC however, those cancers are commonly TP53-mutated. Since the majority of MTAP-deleted NPC harbors wild-type p53, the high levels of p53 induction triggered by MT2A inhibitor may underlie the potent tumor suppressor activity observed in NPC in contrast to other MTAP-deleted largely TP53-mutated cancers. At present, a phase I clinical trial of MTAP inhibitor AG-270 (NCT03435250) is ongoing for patients with advanced or refractory solid tumors and lymphomas. We believe our findings will encourage future clinical trials examining MTAP inhibitors in MTAP-deleted NPC, including recurrent and metastatic diseases, which are often deadly. This precision medicine strategy, together with potential biomarker-driven immunotherapy may ultimately improve treatment outcomes for patients with advanced NPC.

**Common pathway significantly inhibited the growth of EBV-positive NPC cells.** These studies, in conjunction with our current findings, conclude the essential role of constitutively activated NF-κB signaling in NPC, a feature distinct from the heterogeneous genomic landscapes of other head and neck cancers.

Second, we identified the mutually exclusive involvement of somatic alterations and overexpression of various viral genes (LMP1, BNLF2a) targeting innate and adaptive immunity in 91.4% of NPCCs. This viral and somatic co-selection is the second viral-somatic collaboration we identified herein. This feature likely arises to counteract the inflammatory environment owing to persistent EBV infection, a unique feature of NPC. Under normal circumstances, the abundantly expressed viral RNAs (e.g., EBERs) would trigger potent innate immune response via IRF3-activated type I interferon production while the presentation of multiple immunogenic viral antigens (e.g., EBNA1, LMP2) would facilitate the cytotoxic T-cell responses in EBV-infected NPC.

Thus, effective evasion from immune responses is believed to play a key role in the tumorigenesis of this virally associated cancer. In essence, our study defined a panel of genomic and viral events interfering with innate and adaptive immune responses in 78.6% and 47.1% of tumors, respectively, uncovering major mechanisms for NPC immune evasion. Notably, we confirmed the overexpression of the viral TAP inhibitor BNLF2a in the latent EBV-infected cells of 18.6% of NPC tumors, which could counteract host immune response. The identification of BNLF2a expression, PDL1 alterations, and other somatic alterations impairing the antigen presentation machinery may inform immunotherapy-related biomarkers or strategies in NPC.

Third, WGS revealed consistent loss of expression and frequent somatic LOF alterations of CDKN2A and TGFBR2, corroborating their key driver roles in NPC development. In addition to its known role in suppressing cell proliferation and differentiation, CDKN2A and TGFBR2 inactivation in persistent EBV infection in NPC. Disruption of TGF-β signaling in NPE cells may facilitate the maintenance of the EBV genome via suppression of cellular differentiation. Since deletion of chr. 3p and 9p which harbor the TGFBR2 and CDKN2A loci, respectively, are consistently found in precancerous lesions of NPC, pointing to a central role for CDKN2A loss and impaired TGF-β signaling in creating a susceptible NPE cell population capable of supporting EBV latency during early cancer development.

Finally, a combination of genomics and therapeutic investigation demonstrated the pharmacologic vulnerability of MTAP-deleted NPC, which accounts for 32–34% of our cohort. MTAP is also commonly deleted in glioblastoma (40%), melanoma (25%), and pancreatic adenocarcinoma (25%). Unlike NPC however, those cancers are commonly TP53-mutated. Since the majority of MTAP-deleted NPC harbors wild-type p53, the high levels of p53 induction triggered by MTAP inhibitor may underlie the potent tumor suppressor activity observed in NPC in contrast to other MTAP-deleted largely TP53-mutated cancers. At present, a phase I clinical trial of MTAP inhibitor AG-270 (NCT03435250) is ongoing for patients with advanced or refractory solid tumors and lymphomas. We believe our findings will encourage future clinical trials examining MTAP inhibitors in MTAP-deleted NPC, including recurrent and metastatic diseases, which are often deadly. This precision medicine strategy, together with potential biomarker-driven immunotherapy may ultimately improve treatment outcomes for patients with advanced NPC.

In conclusion, we have established a comprehensive genome landscape of NPC, and greatly enriched our understanding of NPC etiologies and driver events for tumorigenesis. We demonstrate further the interplays between EBV and somatic alterations, which are co-selected during NPC evolution. Most importantly, our findings provide a rational basis for future precision therapy trials potentially impacting >30% of primary, advanced and recurrent NPC, for the classes of MT2A and PRMT5 inhibitors.
Whole-genome sequencing. For tumor and normal samples, 1 μg of genomic DNA was subjected to WGS with a standard 100-bp paired-end read using the Illumina HiSeq 2000 platform (Illumina, San Diego) based on the manufacturer’s instructions. Sequencing libraries were constructed with 500 bp insert length. Raw sequence reads were processed and aligned to the hg38 human reference genome using bwa-mem (v0.7.15). Local realignment was performed around indels using GATK (v3.6-0) and duplicates were marked using Picard (v2.6.0). Mean target coverage 10× was achieved for the normal and tumor samples, respectively. BAM files are deposited to the European Genome-phenome Archive under the accession EGAS00001004705.

Variant calling. Somatic SNVs and small insertions and deletions (indels) were detected using four callers and several downstream filters (see Supplementary Methods). Significantly mutated coding genes and non-coding regulatory elements were identified using CADD (v14.1). DriverWGS (v0.0.1). CADD scores with mutation scores above background were filtered to include those with more than three non-synonymous mutations excluding notorious passengers. Non-coding calls were limited to open chromatin regions (by ATAC-seq in detailed regulatory elements; detailed in Supplementary Methods). SVs were called using three callers and refined using several downstream filters (see Supplementary Methods).

CN calling. Allele-specific CN profiles were generated using Varscan2 (v2.3.6) and the R (v3.3.0) package, Sequenza (v2.1.0). Significantly mutated non-coding variant (chr2: 10097565 C > T) were generated using a custom R script (See Supplementary Methods). Whole-genome duplication was inferred using code described by López S et al.19.

RNA in situ hybridization. Expressions of BNLF2a, LMP1, and TGFBR2 transcripts were detected by RNAscope 2.0 RISH assays using BNFL2a, LMP1, and TGFBR2-specific probes (Advanced Cell Diagnostics, USA) as previously described13,14. A RNAscope probe V-HHV4-BNLF2-C2 was used for the detection of BNFL2a transcripts while the probes V-HHV4-LMP1 and BA-V-EBV-LMP1-2E1 were used to confirm the presence of LMP1 transcription signals in the NPC specimens. The cases with LMP1 transcripts signals were excluded from BNFL2a detection analysis because of the overlap of the LMP1 gene and exon 3 of LMP1. BNFL2a and TGFBR2 expression was assessed by assigning a proportion score and an intensity score. The proportion score relates to the percentage of tumor cells with a positive signal (0–100). The intensity of the signal in the tumor cells was scored as 0 for none; 1 for weak, 2 for intermediate, and 3 for strong. The gene expression score was calculated as the product of the proportion and intensity scores, ranging from 0 to 300.

Immunohistochemical staining. MHC-I, MHC-II, PDL1, LMP1, MTAP protein expressions were determined on formalin-fixed, paraffin-embedded sections by immunohistochemical staining. After de-waxing, the sections were subjected to antigen retrieval and staining by the automated slide processing system BenchMark XT (Ventana Medical System Inc., Tucson, AZ, USA) as previously published18. The primary antibody used in this study was anti-LMP1 mouse monoclonal antibody (diluted 1:1000, clone C1-4, Dako, Agilent Technologies, USA), anti-PDL1 (22C3, Dako, Agilent Technologies, USA), anti-HLA Class I A/B/C (diluted 1:1000, clone EC8R-8, ab7038, Abcam, USA), anti-HLA Class II C2/C3 (diluted 1:2000, clone 6C6, ab55152, Abcam, USA), anti-MTA9 (diluted 1:400; 4158, Cell Signaling, USA), anti-Involucrin (diluted 1:100, clone 55S, MA5-11803, Invitrogen, USA) and p53 (diluted 1:100, clone DO-1, SC-126, Santa Cruz, USA).

Immunoblotting. Total cell lysates extracted from the xenografts and cell lines were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane for immunoblotting as described41. Primary antibodies used in this study were anti-TGFBR2 (diluted 1:1000, clone D-2, sc-17799, Santa Cruz, USA), anti-SMA2D (diluted 1:1000, clone D43B4, 5339, Cell Signaling, USA), anti-pSMA2D (diluted 1:1000, clone 128D4, 310122, Cell Signaling, USA), anti-LMP1 (diluted 1:1000, clone BNLF2a, LMP1, and TGFBR2 (diluted 1:1000, clone C1-4, Dako, Agilent Technologies, USA), anti-HLA Class I A/B/C (diluted 1:1000, clone EC8R-8, ab7038, Abcam, USA), anti-HLA Class II C2/C3 (diluted 1:2000, clone 6C6, ab55152, Abcam, USA), anti-MTA9 (diluted 1:400; 4158, Cell Signaling, USA), anti-Involucrin (diluted 1:100, clone 55S, MA5-11803, Invitrogen, USA) and p53 (diluted 1:100, clone DO-1, SC-126, Santa Cruz, USA).

In vitro drug study. In general, 5 × 103 cells in 100 μl of medium were seeded into 96-well plates in triplicate overnight and then treated with various concentrations of FIDAS-5 (Merck Millipore, USA) for 144 hrs. At the end of treatment, the medium was refreshed and 10 μl of CCK-8 reagent (Dojindo Molecular Technologies, USA) was added to each well and incubated at 37 °C for 4 h. The absorbance was then measured at 450 nm. Each sample was performed in triplicate. The relative growth rate was calculated by dividing absorbance at indicated time points by the absorbance at day 1 after cell plating.

In vivo mouse experiments. Mice harboring flank tumors of 3–4 week-old female athymic mice with a starting weight of 18–22 g and allowed to grow to ~50 mm3. Mice were kept within animal room limits of 20–23 °C and 40–60% humidity. The mice run on a 12 h light/dark cycle that from 7 am to 7 pm. Eight mice per group were used. FIDAS-5 (40 mg per kg) or vehicle (corn oil) was administered via intraperitoneal injection once daily. Mice were weighed and tumors were measured with a caliper every 3 days. When the tumor sizes exceeded 1000 mm3, mice were killed, and tumor and blood samples were collected for analysis. Tumor volume was calculated by the formula 0.5 × l × w2, where l and w are tumor length and width, respectively. All animal care and experimental procedures were approved by the University Animal Experimentation Ethics Committee (AEELC), the Chinese University of Hong Kong. The animal license was obtained from the Hong Kong Government, Department of Health.

Data availability. The raw data in the BAM files for the WGS sequencing from this study have been deposited in the European Genome-phenome Archive (EGA) under accession number: EGAS00001004705 in the hyperlink. Access to this data set in EGA can be requested from UHN Genomics Data Access Committee (Contact person: Natalie Stockle, Email: natalie.stockle@uhn.ca). Full mutation and copy number calls are included as part of the supplementary information and the remaining data are available from the authors upon request. A publicly available WGS data set from a cohort of 12 NPC samples deposited at the Sequence Read Archive (SRA, https://submit.ncbi.nlm.nih.gov/sra/) was used for validation of MTAP deletion in NPC12 (https://doi.org/10.1093/carcin/bgy108). The accession codes of these NPC and corresponding normal blood samples are: RSR6431671, RSR6377819, RSR631672, RSR631673, RSR6377821, RSR631674, RSR6377822, RSR631667, RSR631670, RSR6377824, RSR631677, RSR6377825, RSR631678, RSR6377826, RSR6377827, RSR631668, RSR6377828, RSR6377829, RSR631675, RSR6377829, RSR631676, RSR6377830. In addition, simple somatic mutations and SV counts of human cancers reported by Campbell et al. 2017 (https://doi.org/10.1101/162784) was used for comparison with those detected in NPC samples in this study. The data set is available in web-link: https://www.biorxiv.org/content/10.1101/162784v1 supplementary material. The NFkB1 binding site motif of the significantly mutated non-coding variant (chr2: 18097565 C > T) is available in: http://jaspar.genereg.net/matrix/MA01051. Gene v26 is available at: https://www.gencodegenes.org/human/release_26.html. Cosmic mutation signatures v2 (https://cancer.sanger.ac.uk/signatures/signatures_v2/) was used for determining the mutational signatures of NPC samples. Igenomes hg38 reference is available at: http://igenomes.illumina.com.s3-us-west-2.amazonaws.com/Homo_sapiens/UCSC/ hg38_Homo_sapiens_UCSC_hg38.tar.gz. Source data are provided with this paper.

Received: 6 October 2020; Accepted: 19 May 2021; Published online: 07 July 2021
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Acknowledgements

T.J.P. was supported by the Princess Margaret Cancer Foundation; Canada Research Chairs program; Canada Foundation for Innovation, Leaders Opportunity Fund, CF-130338; and the Ontario Ministry of Research and Innovation. Ontario Research Fund Small Infrastructure Program. K.W.L. was supported by Research Grant Council, Hong Kong (Areas of Excellence Scheme – AoE/M-401/20; Theme-based Research Scheme - T12-401/13-R; Collaborative Research Fund - C4001-18GF, C7027-16G, C5012-15E; Core Utilities of Cancer Genome and Pathobiology, Focused Innovations Scheme and Faculty Strategic Research (4620513) of the Faculty of Medicine, and VC Research Impact Fund - R4015-19F, R4017-18), the Health and Medical Research Fund (05162386, 13142201), Core Utilities of Cancer Genome and Pathobiology, Focused Innovations Scheme and Faculty Strategic Research (4620513) of the Faculty of Medicine, and VC’s One-off Discretionary Fund (VCF2014017, VCF2014015), the Chinese University of Hong Kong. C.M.T. was supported by Research Grant Council Hong Kong (T12-401/13-R; Collaborative Research Fund - C4001-18GF, C7027-16G, C5012-15E; Core Utilities of Cancer Genome and Pathobiology, Focused Innovations Scheme and Faculty Strategic Research (4620513) of the Faculty of Medicine, and VC’s One-off Discretionary Fund (VCF2014017, VCF2014015), the Chinese University of Hong Kong. C.M.T. was supported by the Health and Medical Research Fund (05162386, 13142201), General Research Fund (14113620, 17103151, Faculty Innovation Award (FIA20/20A/01) and NSFC/RGC joint Research Scheme (N_HKU7535/18 and 81611680336) of Research Grant Council Hong Kong, S.W.T. was supported by Research Grant Council Hong Kong (CRF-C7027-16G; GRF-17104167, GRF-17141197, GRF-17114818, GRF-17122420, NSFC/RGC-N_HKU7535/18 and 81861168033). L.F.Y. was supported by Fundamental Research Grant Scheme (FRGS/S1/2019/SK008/UM/026) from the Ministry of Higher Education Malaysia, Newton-Ungku Omar Fund MR/P013201 (H016-2017) from the Academy of Sciences Malaysia and Medical Research Program, and University of Malaya Impact-Oriented Interdisciplinary Research Grant Programme (IBRG008A-19ENV); V.W.Y.L. was supported by the Research Grant Council, Hong Kong (General Research Fund – 17114814, 17121616, 14168517; Research Impact Fund - R4015-19F, R4017-18), the Health and Medical Research Fund (HMIR#P15160091) from the Food and Health Bureau, The Government of the Hong Kong SAR, University-Industry Collaboration Program (UM/329; Innovation and Technology Fund, Hong Kong government, Hong Kong SAR and Lee’s Pharmaceutical
Author contributions
K.W.L. and T.J.P. have led the study, designed and conducted the experiments, analyzed the data, interpreted the results, written the initial draft, and created the figures with the input of the senior authors J.P.B., K.F.T., V.W.Y.L. and G.T.C. who contributed equally. The following authors were involved in the genome sequencing, cell-based and in vivo experiments, developed methodologies, analyzed clinical samples, helped writing the manuscript, and approved the final version: Y.Y.C., C.M.T., B.B.Y.M., J.K.S.W., E.P.H., M.K.F.M., C.C., S.V., Y.Y.Y.O., P.K.S., J.Y.K.C., I.C.P., L.F.Y., C.W.D. The following authors analyzed the genome sequencing data, developed methodologies and/or helped with the interpretation of data, helped writing the manuscript, and approved the final version: K.Y.Y., S.D.L., S.E.G., S.P., M.W., J.S.H.K., L.Y., S.W.T., L.S.Y., F.F.L. and A.T.C.C. supervised the research, provided material or funding support, helped writing the manuscript and approved the final version.

Competing interests
All authors declare no competing interests directly related to the current study. V.W.Y. Lui receives funding from a University-Industry Collaboration Program (UIM/329) by the Innovation and Technology Fund, Hong Kong government, and Lee’s Pharmaceutical (Hong Kong Limited) for the period of 2018–2020, and also served as a scientific consultant for Novartis Pharmaceutical (Hong Kong) Limited (Oct 2015–Oct 2016). B.B. Y. Ma received speaker’s honorarium and serves in the advisory board of Bristol-Myers Squibb (BMS), MSD and Novartis, Hong Kong. She has received a research grant from Novartis and Boehringer Ingelheim. E.P. Hut received speaker’s honoraria and serves in the advisory board of Merck Sharp & Dohme (MSD) and Merck Serono. A.T.C. Chan received research and travel grants from MSD, Pfizer, and Roche. The other authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-24348-6.

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Peer review information Nature Communications thanks Erik Flemington and the other, anonymous reviewer(s) for their contribution to the peer review of this work. Peer review reports are available.

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