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Exome and genome sequencing of nasopharynx cancer identifies NF-κB pathway activating mutations


Nasopharyngeal carcinoma (NPC) is an aggressive head and neck cancer characterized by Epstein-Barr virus (EBV) infection and dense lymphocyte infiltration. The scarcity of NPC genomic data hinders the understanding of NPC biology, disease progression and rational therapy design. Here we performed whole-exome sequencing (WES) on 111 micro-dissected EBV-positive NPCs, with 15 cases subjected to further whole-genome sequencing (WGS), to determine its mutational landscape. We identified enrichment for genomic aberrations of multiple negative regulators of the NF-κB pathway, including CYLD, TRAF3, NFKBIA and NLRCS, in a total of 41% of cases. Functional analysis confirmed inactivating CYLD mutations as drivers for NPC cell growth. The EBV oncoprotein latent membrane protein 1 (LMP1) functions to constitutively activate NF-κB signalling, and we observed mutual exclusivity among tumours with somatic NF-κB pathway aberrations and LMP1-overexpression, suggesting that NF-κB activation is selected for by both somatic and viral events during NPC pathogenesis.
asopharyngeal carcinoma (NPC) is one of the most aggressive head and neck cancers and frequently metastasizes to distant lymph nodes and organs. Its distinct etiology linked with latent infection of the oncogenic Epstein-Barr virus (EBV) and characteristic heavy lymphocyte infiltration often distinguish NPC from other head and neck cancers. In fact, EBV-associated NPC is endemic in Southern China and Southeast Asia, contrasting with the epidemic Human Papillomavirus (HPV)-associated head and neck cancer in some Western countries. Due to the intrinsic invasiveness and asymptomatic nature of the disease, majority of NPC patients are diagnosed with advanced diseases (60–70% cases) with poor outcome. Thus far, there is no effective targeted therapy for advanced NPC.

NPC is a complex malignancy with etiology and pathology involving both EBV infection and a combination of multiple genetic aberrations. Unlike other head and neck cancers, the scarcity of NPC genomic data to date hinders the understanding of NPC biology, disease progression and rational therapy design for better treatments. The extensive stromal components of these often small-sized tumours present major challenges for comprehensive and precise genomic characterization.

Here we report the genomic landscape of the first and largest cohort of microdissected EBV-positive NPC of more than 100 cases. Our study suggests that the somatic mutation rate of NPC is substantially higher than that reported previously and reveals that majority of NPCs display activation of the NF-κB signalling pathway as a result of somatic inactivating mutations in negative regulators of NF-κB.

**Results**

**Whole-exome sequencing (WES) of microdissected NPC.** WES was performed on 111 unique tumour specimens derived from 105 unique subjects. The cohort included 78 primary tumours, 11 local recurrences and 22 metastatic tumours. Ninety-seven subjects were from Southeast Asia where NPC is endemic and eight from the United States (Supplementary Table 1 and Supplementary Data 1). The overall non-synonymous mutation rate of EBV-positive NPC was significantly lower than that of HPV-positive head and neck squamous cell carcinoma (HNSCC) and EBV-positive stomach adenocarcinoma (TCGA, USA). (\(P = 0.0196\) and 0.0004, respectively; unpaired \(t\)-test; Fig. 1a). Primary, recurrent and metastatic NPC tumours shared a similar mutational burden in our cohort and orthogonal validation of mutations by amplicon sequencing demonstrated a validation rate of 82% at selected sites. Interestingly, increasing mutational burden in primary NPC lesions \((N = 70)\) was negatively correlated with overall and disease-free survival \((P = 0.0214\) and 0.0118, respectively; Log-rank test; Fig. 1b). NPC mutational rate was not associated with age, sex or clinical stage.

The combination of EBV infection, diet and genetic factors are etiologically linked with NPC pathogenesis in endemic regions.

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**Figure 1 | Somatic mutation rates and mutational signatures of NPC tumours identified by WES.** (a) NPC tumours have significantly lower rate of non-synonymous mutations as compared with HPV-positive HNSCC and EBV-positive stomach adenocarcinoma (TCGA, USA). (b) The mutational burden, stratified by the top, mid and low quartile counts per patient, correlated with overall and disease-free survival in NPC patients \((N = 70\) NPC patients with primary tumours). (c) Mutational signatures of 70 primary and 27 recurrent or metastatic NPC cases reveal a primarily C to T transition signature. (d) The top COSMIC cancer mutation signatures in NPC.
To identify whether mutagenic processes are operative in NPC, we analysed the mutation spectrum of NPC. The predominant type of substitution was C > T transition at NpCpG sites in both primary and recurrent/metastatic tumours (Fig. 1c). Combined non-negative matrix factorization clustering and correlation with the 30 curated mutational signatures defined by the Catalog of Somatic Mutations in Cancer (COSMIC) database6,7 revealed three dominant signatures in NPC (Supplementary Data 2). The predominant signature underlies the deamination of 5-methyl-cytosine process (Signature 1), followed by defective DNA mismatch repair signature (Signatures 6 + 15 + 20 + 26), and by an APOBEC/AID signature, which was only observed in a subset of primary NPCs (Signatures 2 + 13) (Fig. 1d).

**Negative regulators of NF-κB pathway are commonly mutated.**
To identify somatically mutated genes in NPC, we employed the MutSigCV 2.0 algorithm8 with a Benjamini-Hochberg false discovery (FDR) threshold of 0.1 (Figs 2 and 3a). We identified a total of seven genes significantly mutated above background rates in Asian NPCs: TP53, NRAS, NF-κB pathway genes, CYLD, TRAF3 and NFKBIA, the major histocompatibility complex (MHC) class I gene HLA-A, and the transcriptional regulator MEDI2L, a gene previously reported to be mutated in mouse and human HNSCC9 (Supplementary Data 3 and 4). TP53 mutations were observed in 9.5% of cases, consistent with rates reported previously1,4. Yet, TP53 mutations were ~2.3-fold enriched in recurrent/metastatic NPC (15.2%; versus 6.4% in primary NPC), perhaps due to therapy-related selection. TP53 mutation status was not correlated with survival of patients with primary NPC. Our US cohort size was not large enough to enable statistically robust comparisons with the Asian cohort of significantly mutated genes.

CYLD, TRAF3 and NFKBIA are important negative regulators of NF-κB activity. CYLD is a tumour suppressor gene that deubiquitinates TRAF2, an activator of NF-κB signalling10,11. Germline mutations in CYLD are associated with familial cylindromatosis, characterized by multiple benign tumours in the head and neck region and hair follicles12. We found that the majority of CYLD mutations (8/11 mutations; 72.7%) were truncating, including nonsense and frameshift mutations. Truncating mutations of CYLD have not been identified in NPC previously, but were found at lower rates in HNSCC4. TRAF3, a key negative regulator of the non-canonical NF-κB pathway in NPC, was found to be mutated in 8.6% cases (9/105 cases), a rate higher than previously reported in NPC (1/33 cases; 3.0%) (refs 13,14). Most NPC-associated TRAF3 mutations were located in the RING finger and the MATH/TRAF domains (Fig. 3a), regions known to be essential for the suppression of NIK-activating NF-κB signalling13. Thus, mutations at these sites may lead to constitutive activation of NF-κB signals in NPC tumours. NFKBIA functions to trap NF-κB in the cytosol, thus inhibiting its activation, and was mutated in 6.7% of NPC. Deletion of this gene has been reported in glioblastoma, which was associated with poor survival15.

Additional genes which displayed recurrent mutations in our data set but did not reach statistical significance (q < 0.1) included the NF-κB pathway member NLRRC5, which was mutated in six cases (4.8%) (Fig. 3a). NLRRC5 is a potent inhibitor of NF-κB activation and Type I interferon signalling16. It competes with NEMO for binding to IKK-alpha and IKK-beta, thus blocking their activation and kinase activities. Notably, NLRRC5, is also a key transcriptional activator of MHC class I genes, including HLA-A and HLA-B. In fact, HLA-A was significantly mutated in our cohort (7.2% of cases), with predominantly recurrent truncating mutations. These findings implies that inactivation...
Recurrent mutations of chromatin remodeling genes and PI3K/MAPK activating genes in NPC. Primary NPCs are shown in light blue boxes, local recurrent and metastatic tumours in pink and green boxes, respectively. Clonal expansion in NPC recurrences and metastases by comparing variant frequencies between the primary and tumours were available from four patients for clonality analyses. Mismatch repair genes, including MSH6, were mutated in cancer: ABL1, BUB1B, NCOA1 (each mutated in three tumours), and CARS, HSP90AB1, NCOA1 (each mutated in two tumours) were uniquely found in recurrent/metastatic NPCs (n = 33). Among these, ABL1 K690del was identified in a NPC liver metastasis (HKNPC-091) and a lymph node metastasis (P-10-27817). This ABL1 mutation has been identified in melanoma by the TCGA (www.cbioportal.org). We identified two individual’s tumours with hypermutator phenotypes (HKNPC-003, HKNPC-033) with 2,051 and 817 somatic mutations in coding regions, respectively (Fig. 2); both tumours harboured mutations in mismatch repair genes, including MSH6 (HKNPC-003) and MLH1 (HKNPC-033). Both patients with hypermutated tumours had poor overall survival of ~12 and 19 months, respectively. Clonal expansion in NPC recurrences and metastases. Multiple tumours were available from four patients for clonality analyses by comparing variant frequencies between the primary and respective recurrent/metastatic lesions from the same individual. (Supplementary Fig. 2, Supplementary Data 5). Surprisingly, we noted that many mutations with previously annotated roles in cancer were not shared between the paired primary and recurrent lesions. This observation from our two primary/recurrent tumour pairs (cases HKNPC001 and HKNPC012) seems to concur with our recent findings in primary/recurrent HNSCC. In paired primary/recurrent HNSCC samples, we also found that TP53 was the only shared annotated mutated cancer gene as defined by the Cancer Gene Census, COSMIC. For these NPC patients, the recurrent or metastatic lesion appeared to show a single-nucleotide variant (SNV) clonality distribution different from that of the primary tumour, supportive of emerging new subclone(s) in recurrences. On the other hand, the liver metastases from patients HKNPC-008 and HKNPC-009 both showed a relatively higher percentage of shared mutations among two distinct regions of liver metastases (Supplementary Fig. 3; Supplementary Data 5). Frequent chromosomal deletions of Chr 3p, 14q and 16q. Regions of somatic copy number alteration were defined using WES and whole-genome sequencing (WGS) segmentation data and the GISTIC tool (Fig. 4; Supplementary Figs 4 and 5). Similar gene copy changes were observed in our WES (from formalin-fixed paraffin-embedded (FFPE) tissues) and WGS (from fresh frozen tissues) studies. Selected copy number variants (CNVs) were further validated and confirmed by
fluorescence in situ hybridization (FISH) analyses (Supplementary Fig. 6). Arm-level copy number analysis demonstrated consistent losses of 3p, 14q, 16q and recurrent 12p amplification. As we reported previously, 3p loss contributes to the inactivation of multiple NPC-related tumour suppressors including RASSF1A and FHIT\(^{3,19}\). Losses of 14q and 16q may serve as one of the mechanisms for inactivating multiple negative regulators of NF-kB pathway, such as NFKBIA (14q13), TRAF3 (14q32.3), CYLD (16q12.1) and NLRC5 (16q13). Notably, deletion of CYLD was identified in 20/97 cases (20.6%) with one case harbouring homozygous deletion (Supplementary Fig. 5). The CYLD homozygous deletion was further confirmed by FISH (Supplementary Fig. 7). Other recurrent focal copy number peaks included loss of the CDKN2A/CDKN2B locus (9p21.3; 57.6%), consistent with our previous findings\(^{20}\).

**CYLD is tumour-suppressive in NPC.** To further define the scope of genetic alterations in NF-kB signalling at the whole-genome level, we determined the structural variants from 15 whole-genome sequenced Asian NPC tumours (Supplementary Data 6). We also performed FISH analyses for CYLD, TRAF3 and NFKBIA in all Asian NPCs. For both CYLD and TRAF3, inactivating structural alterations, including translocation, tandem duplication and homozygous deletion, were identified in seven additional cases (Fig. 5a,b; Supplementary Figs 7 and 8). No additional cases with NFKBIA structural alteration were identified. The summative rate of somatic genetic events in CYLD (18.6%) and TRAF3 (17.5%) was as high as \(\sim 35.1\%\) (with one case having both CYLD and TRAF3 alterations), suggesting that CYLD and TRAF3 genomic aberrations may represent a major mechanism underlying NPC tumorigenesis.

We and others have previously reported cellular studies of TRAF3 in NPC models\(^{14}\). Here we further showed that wild type TRAF3, but not the patient-derived mutants inhibited the non-canonical NF-kB pathway in NPC cells (Supplementary Fig. 9). On the other hand, little is known about the functional importance of CYLD mutations in NPC or other cancers. Importantly, a CYLD frameshift mutation was also identified in the EBV-positive NPC cell line, C666-1, resulting in complete loss of CYLD protein expression in this cell model (Supplementary Fig. 10). Ectopic expression of CYLD wild-type gene suppressed C666-1 cell proliferation (Fig. 5c) and anchorage-independent growth on soft agar (Fig. 5d), while expression of patient-derived CYLD point and truncating mutations resulted in the loss of these tumour-suppressive activities (Fig. 5c,d). This loss-of-function activity of CYLD mutants was confirmed in another NPC cell line, HK1-EBV (Supplementary Fig. 11). Further, a higher NF-kB transcriptional activity (as measured by an NF-kB reporter assay) was observed in C666-1 cells expressing the patient-derived CYLD mutants versus wild-type (Fig. 5e). Furthermore, nuclear translocation of Bcl-3, a component of CYLD tumour-suppressive activities (Fig. 5c,d). This loss-of-function activity of CYLD mutants was confirmed in another NPC cell line, HK1-EBV (Supplementary Fig. 11). Further, a higher NF-kB transcriptional activity (as measured by an NF-kB-luciferase reporter assay) was observed in C666-1 cells expressing the patient-derived CYLD mutants versus wild-type (Fig. 5e). Furthermore, nuclear translocation of Bcl-3, a component of CYLD mutants (Supplementary Fig. 12).

**LMP1 and NF-kB pathway aberrations are mutually exclusive.** The viral oncoprotein, LMP1 is a potent activator of NF-kB signalling in NPC\(^{21}\). We detected high LMP1 expression in 25.7% of cases by immunohistochemistry and high LMP expression was associated with poor outcome, as previously reported\(^{22}\). We identified mutual exclusivity among LMP1 overexpression and NF-kB pathway aberrations dominated by CYLD, TRAF3, NFKBIA and NLRC5 events (Fig. 6; Supplementary Fig. 13). Specifically, genomic alterations in NF-kB pathway regulators were exclusive with high LMP1 expression \((P = 0.00014; \text{Fisher’s exact test, two-tailed})\), further supporting NF-kB deregulation via somatic genetic events as a core feature of NPC. Our findings provide a potential genetic explanation for the unique inflammatory feature of NPC, contributed by both somatic and viral-mediated addiction to NF-kB signalling in this EBV-associated malignancy\(^{2,5}\).
Figure 5 | CYLD and TRAF3 alterations in NPC. (a) Additional genetic alterations of CYLD and TRAF3 in NPC detected by fluorescent in-situ hybridization (FISH). Left: Five additional cases of CYLD rearrangements and 1 case of CYLD homozygous deletion were identified by FISH using a CYLD break-apart probe and 1 case of CYLD tandem repeat was confirmed by Sanger sequencing (Supplementary Figs 7 and 8). Right: Seven additional cases of TRAF3 gene rearrangements were also identified using TRAF3 break-apart probes. (b) Left: CYLD gene rearrangement as revealed by WGS. A circos plot showing the CYLD gene rearrangement (HKNPC-088T, confirmed by FISH). A scale bar representing 1μm is shown in all FISH pictures. Right: TRAF3 gene rearrangement as revealed by WGS. A circos plot showing the TRAF3 gene rearrangement (HKNPC-089T, confirmed by FISH). (c) Transient transfection of CYLD wild-type (WT) gene into an EBV-positive NPC cell line, C666-1 resulted in significant growth inhibition at day 5 (7 × 10^4 cells in 4% FBS, P = 0.0022, n = 6). Similar results were obtained in three independent experiments. Expression of CYLD mutants (p.S600F and frameshift mutant p.A527fs) revealed a loss-of-function of the tumour suppressive activity versus CYLD WT gene in C666-1 cells. CYLD WT and mutant protein expressions are shown underneath. (d) CYLD WT expression, but not the mutants, inhibited the anchorage-independent growth ability of C666-1 cells in soft-agar colony formation assay. C666-1 cells were infected with retroviral vectors expressing the CYLD WT gene and the CYLD mutants. Colonies were stained with 0.1% indonitotetrazolium chloride and counted, and presented as a bar graph, which showed cumulative results of five independent experiments (n = 15). (e) CYLD WT expression was able to reduce NF-κB activity in C666-1 cells in complete culture medium containing 10% FBS. Expression of CYLD mutants was not able to suppress NF-κB activity in C666-1 cells. The NF-κB activity was measured using the luciferase/renilla assay (Promega, USA) with the Cignal NF-κB Pathway Reporter gene system (Qiagen, USA). A cumulative plot of five independent experiments (total n = 19) showing changes in NF-κB-dependent luciferase activity in CYLD stable versus EGFP-Ctrl cells.

**Poor outcome for patients with MHC class I aberrations.** In addition to NF-κB pathway alterations, we also noted a high rate of MHC class I gene aberrations (NLRC5, HLA-A, HLA-B, HLA-C, B2M). Frequent rearrangements of HLA-A, HLA-B and HLA-C genes were identified by dRanger analysis23 (Supplementary Data 7). Twenty-nine NPC cases (30%) were MHC class I-altered (rearrangements and point mutations) (Fig. 7a). Importantly, survival analysis identified inferior outcome in these MHC class I-altered patients with primary NPC (Fig. 7b). It is likely that the deficiency of antigen presentation mechanism may represent an escape mechanism from host immunosurveillance, thus favouring rapid tumour progression. The clinical efficacy of immune checkpoint inhibitors in this genetic subgroup of NPC patients should be examined carefully as these drugs often rely on intact tumour antigen presentation in situ.

Given a prior report of an association among ERBB/PI3K activation and inferior outcome in NPC3, we assessed whether we could confirm this finding in our cohort. We noted no significant difference in outcome among cases with and without known activating mutations in these oncogenic pathways among our primary set of 70 tumours (Supplementary Fig. 14). We did, however, note that mutations in genes known to activate PI3K signalling were non-statistically enriched in recurrent/metastatic cases and found that there was an association between PI3K activating events and poor outcome among recurrent and metastatic NPC patients (Supplementary Fig. 14), in accordance with data presented in HNSCC24. This finding could have therapeutic implications in that targeted kinase inhibitors are often explored initially in the recurrent/metastatic setting.

**Somatically altered pathways of NPC.** To better define somatically altered pathways in NPC, we mapped all somatic variants identified in our mutational and copy number analyses to known functional annotations with selected genes, and summarized as a pathway diagram for NPC (Fig. 8). As noted above, frequent
somatic alterations in key nodes of the NF-κB pathways were the dominant feature in our NPC cohort, and we also observed recurrent somatic alterations in genes regulating cell cycle progression, chromatin remodelling and DNA repair. Notably, few variants which have previously been associated with response to a targeted therapeutic agent were identified, although recurrent mutations and amplifications of RAS family genes and PI3K/mTOR pathway members were observed.
In conclusion, our study suggests that NPCs sub-classified by LMP1 status are distinct in terms of clinical history and outcome. We envision that NF-κB negative regulators. As shown in our previous study, knockdown of NF-κB signals by short interfering RNAs can effectively inhibit NPC growth and survival. It can be envisioned that NF-κB inhibitors and the newly developed small molecule inhibitors targeting Bc3 can potentially inform therapy, highlighting the importance of a core feature of NPC. We also demonstrated how NPC genomics revealed a higher degree of somatic defects in NF-κB activation. This study also reveals that NF-κB deregulation, either via somatic genetic events or LMP1 overexpression, is a core feature of NPC. We also demonstrated how NPC genomics can potentially inform therapy, highlighting the importance of targeting activated NF-κB signalling in NPC patients with somatic defects in NF-κB negative regulators. As shown in our previous study, knockdown of NF-κB signals by short interfering RNAs can effectively inhibit NPC growth and survival. It can be envisioned that NF-κB inhibitors and the newly developed small molecule inhibitors targeting Bc3 can potentially be used as novel therapeutics for NPC patients. Furthermore, the discovery of MHC class I lesions in a subgroup of NPC may have biological implications for immune checkpoint inhibitors or other cytotoxic T-cell-based immunotherapies in NPC.

In conclusion, our study suggests that NPCs sub-classified by LMP1 status are distinct in terms of clinical history and outcome as well as genomic features. Future laboratory studies and precision medicine-based treatment approaches for NPC would need to consider the features of these distinct subclasses.

Methods
NPC tissue collection. All NPC tumours were collected by endoscopy or surgery. Patient consent was obtained according to institutional clinical research approval (IRB) at the Chinese University of Hong Kong, Hong Kong (for Asian cases), and at the University of Pittsburgh, USA (for US cases). The original clinical and survival data of patients were provided in Supplementary Data set.

Exome sequencing and variant calling. DNA was extracted from micro-dissected FFPE or frozen tissue samples using the Qiagen FFPE DNA extraction kit as per the manufacturer’s protocol. Solution based hybrid capture with the Agilent SureSelect method was followed by sequencing on the Illumina Hi-Seq instrument to a goal coverage of 80× as previously described. Raw FASTQ files were processed for downstream analysis using PicardTools (http://broadinstitute.github.io/picard/). BAM files are deposited to dbGAP-NHGRI (Study ID: 20055, Nasopharynx Cancer Whole-Exome Sequencing) for public use.

Variant calling (SNVs, indels) was performed using the Firehose pipeline running Mutect2, then filtering out OxoG artifacts and likely germline mutations that were previously seen in both dbSNP build 134 and 1,000 Genome data. To remove potential artifacts from FFPE samples, we filtered out variants in a variant frequency peak with <5% frequency and total coverage under 30×. The overall mutation rate and average coverage were calculated for the 105 tumour and normal pairs. Significance analysis was conducted using a local version of MutsigCV 2.0, which considers gene expression, replication time and chromatin state when calculating the background rate. To further improve our confidence in the most significantly mutated genes, we also called mutations using Strelka2 and filtered out genes for which <25% of mutations overlapped between the two methods. To assess for the most likely cancer-associated mutations, we chose mutations seen in at least three samples in the COSMIC database using Oncotator.

Figure 8 | Pathway diagram summarizing deregulation of signalling pathways and transcription factors in NPC. Key affected pathways, components and inferred functions, are summarized in the main text. The frequency (%) of genetic alterations for NPC tumours are shown. Pathway alterations including homozygous deletions, amplifications and somatic mutations are shown. Activated and inactivated pathways/genes, and activating or inhibitory symbols are based on predicted effects of genome alterations and/or pathway functions.
hum22777). Germline variants identified from NPC patients were shown in Supplementary Data 8. We used the Fluidigm Access Array microfluidic system, which allows for multiplexed, high-throughput, amplicon sequencing using custom-targeted primers for a total of 464 positions to validate a total of 82% of variants (Supplementary Data 9). These variants included both SNVs and indels in the cases targeted primers for a total of 464 positions to validate a total of 82% of variants (Supplementary Data 9). These variants included both SNVs and indels in the cases targeted.

**Identification of somatic SNV and SV** was conducted by MuTect27 and Oncotator was used to determine the CNV segments into GISTIC 2.0 with mostly standard method of using panel of normals to model noise and normalize calls. As we increased our model to more than four signatures, we did not identify any additional highly correlated COSMIC signatures.

**Whole-genome sequencing.** For the 15 NPC cases that were subjected for WGS, 1 μg of genomic DNA extracted from the microdissected frozen tumours and matched normal samples were subjected to the Illumina Whole-Genome Sequencing Service in Macrogen (Seoul, Korea). Standard Illumina protocols and Illumina paired-end adapters were used for library preparation from the fragmented genomic DNA. Sequencing libraries were constructed with 500 bp insert length. WGS was performed using the Illumina HiSeq 2000 platform with a standard 100-bp paired-end read previously described29. Mean target coverage of 40 × was achieved for the normal and tumour samples, respectively. The raw sequence reads were processed and aligned to the hg19 human reference genome using Isaac aligner30. BAM files are deposited European Nucleotide Archive (ENA) (Accession number PRJEB12830). WGS of matched normal and tumour samples of NPC patients. Identification of somatic SNV and SV was conducted by MuTect27 and Manta24, respectively. To evaluate the sensitivity of somatic SV results, we used only one SV result when different calls share breakpoints within 1,000 base pairs. We predicted target copy number and copy number aberrations and of heterozygosity in NPC genome using TITAN2.3. Copy number SNVs of normal sample and the tumour-adjacent copy numbers of sites were extracted by samtools/bcf tools.39. The identified somatic copy number aberrations and SVs of each NPC were visualized by CIRCOS34.

**Pathway diagram for NPC.** To calculate recurrent percentages, we considered an alteration to be activating if it landed in a known oncogene and was either a COSMIC mutation (in at least three samples) or highly amplified. Similarly, we considered alteration to be inactivating if it was nonsense or frameshift in a COSMIC mutations or homozygous deletions. Highly recurrent single copy gains or losses were considered potentially activating or inactivating, respectively. Missense or other non-synonymous mutations were taken into consideration as unknown functional effect.

**Fluorescence in situ hybridization.** FISH analysis was conducted to detect the CYLD, TRAF3 and NFKBIA in FFPE NPC tissue sections. Commercially available dual-colour (orange and green) flanking probes of CYLD, TRAF3 and NFKBIA were used (Empire Genomics, USA). FISH analysis was performed as we previously described39. In each case, at least 100 nuclei were analysed. The break-apart signals were scored if orange and green signals separated by one signal diameter or only single signal were detected. Cases with translocation of CYTD, TRAF3 and NFKBIA were defined as >20% of breakapart signals in the tumour cells.

**LMP1 immunohistochemical staining.** The expression of LMP1 was determined in FFPE NPC sections by immunohistochemical staining. After de-waxing, the sections were subjected to antigen retrieval and staining in the automated slide processing system Benchmark XT (Ventana Medical Systems Inc., Tucson, AZ) with the OptiView Amplification kit (Ventana Medical Systems Inc.). The primary antibody used in this study was anti-LMP1 mouse monoclonal antibody (CS1-4, Dako). The LMP1 expression was assessed by assigning a proportion score and an intensity score. The proportion score was assigned to the percentage of tumour cells with positive membrane and cytoplasmatic staining (0–100). The intensity score was assigned for the average intensity of positive tumour cells (0, none; 1, weak; 2, intermediate; 3, strong). The LMP1 staining score was the product of proportion and intensity scores, ranging from 0 to 300. The LMP1 expression was categorized into absence/low (score 0–100) and high (score 100–300).

**Immuno blotting.** Immuno blotting was performed as previously published37. All antibodies were used from Cell Signaling Technologies, USA. These are antibodies against CYLD (#12797) and actin (#8456).

**Generation of CYLD mutant cells.** The CYLD wild-type (WT), and various CYLD mutants generated were gene synthesis and cloned into the pMXs-puro vector (Cell Biolabs, USA). The pMXs-puro-EGBP plasmid was used as control. The retroviral packaging cell line, Plat A cells were used to generate infective retroviruses for C666-1 and HK1-EBV infection according to the manufacturer’s instructions. Seven days after infection, cells were collected for the confirmation of CYLD mutant expression by immunofluorescence. Nuclei were plated at low density (1 × 10^4 per well) in RPMI (for C666-1) or DMEM (for HK1-EBV) with 4% FBS and assessed for proliferation at 48 h. Mutant-induced cell growth, compared with the EGBP vector control or CYLD WT, was assessed by MTT assay. C666-1 and HK1-EBV cell lines were previously established and authenticated in our laboratory. No mycoplasma contamination was detected in these cell lines. An unscropped images of western blot membranes for the expression of CYLD WT and mutant protein is shown in Supplementary Fig. 15.

**Soft aga colony formation assay.** C666-1 stable cells expressing the CYLD WT and various CYLD mutants were plated at a density of 1 × 10^5 cells per well of a 6-well plate. Cells were grown in 0.35% soft agar for 21–24 days. Colonies were then stained with 0.1% indonitrotetrazolium chloride (Sigma-Aldrich, USA) for 48 h, and counted under a stereomicroscope.

** Luciferase assay.** C666-1 cells were co-transfected with CYLD WT or various CYLD mutants with Cignal NF-κB Pathway Reporter Assay (Qiagen, USA). After 48 h, luciferase activity was measured using the Dual-Luciferase Reporter Assay (Promega, USA). Luciferase counts were normalized to Renilla counts for each sample.

**Immunofluorescence staining for Bcl-2 translocation.** C666-1 cells were co-transfected with the NFκB translocation and constellation (CST) siRNA and HA-tagged CYLD WT or various CYLD mutants. At 48 h post-transfection, cells were fixed with 4% paraformaldehyde in PBS for 10 min. Cells were then permeabilized and blocked with blocking buffer (3% BSA, 0.1% Triton X-100 in PBS). After washing with PBS, cells were incubated with the corresponding primary antibodies (Anti-HA.11, 1:1,000 from BioLegend, USA; Anti-βC (C-14), 1:400 from Santa Cruz, USA), and then in fluorophore-conjugated secondary antibodies (Alexa Fluor 488 and Alexa Fluor 555 from Invitrogen, USA). Cells were finally washed with PBS and counterstained with 4,6-diamidino-2-phenylindole to label the nuclei. The fluorescence images of cells were captured with the Axio Observer microscope (Zeiss) equipped with a cooled CCD (charge-coupled device) camera.

**In vivo ubiquitination assay.** HeLa cells were transfected with various CYLD-expressing plasmids as indicated. Cells were harvested at 24 h post-transfection and total protein extract was extracted with RIPA buffer supplemented with protease inhibitors. Cell lysate was then boiled for 30 min in RIPA buffer containing 1% SDS to disrupt-protein-protein interaction. The cell lysate was then cooled down on ice and further diluted with RIPA buffer to decrease the SDS concentration to 0.1% before performing immunoprecipitation. One milligram of protein lysate was incubated with 1 μg anti-FLAG (M2) antibody from Sigma-Aldrich overnight at 4°C with constant rocking. Protein G-conjugated Dynabeads (Novex, Invitrogen) was then added to pull down the protein-containing antibody complex. The beads were washed with RIPA buffer three times and incubated in 2 × Laemmli buffer at 70 °C to dissociate the protein–antibody complex. The protein samples were then separated by SDS–polyacrylamide gel electrophoresis and western blotting was performed using the antibodies as indicated. The His-tagged K48R ubiquitin plasmid was a kind gift from Dr Ramin Massoumi (Lund University, Sweden).

**Statistical analysis.** Statistical analyses to test for correlation between genomic and clinical features such as age, sex and clinical staging were performed using standard R packages. We used the Fisher’s exact test for discrete variables, the Log-rank test for continuous variables, and the Bonferroni method of multiple testing correction.

**Data availability.** The WES and WGS data that support the findings of this study have been deposited in dbGAP-NHGRI (Study ID: 20055, Nasopharynx Cancer Whole-Exome Sequencing) and European Nucleotide Archive (ENA) (Accession number PRJEB12830), respectively. All remaining data are included in the manuscript and Supplementary Information or available from the authors upon request.

**References**


