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ORIGINAL RESEARCH

Cold Tumour Phenotype Explained Through Whole Genome Sequencing in Clinical Nasopharyngeal Cancer: A Preliminary Study

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Introduction: Nasopharyngeal cancer (NPC) is a complex cancer due to its unique genomic features and association with the Epstein–Barr virus (EBV). Despite therapeutic advancements, NPC prognosis remains poor, necessitating a deeper understanding of its genomics. Here, we present a comprehensive whole genome sequencing (WGS) view of NPC genomics and its correlation with the phenotype.

Methods: This study involved WGS of a clinical NPC biopsy specimen. Sequencing was carried out using a long read sequencer from Oxford Nanopore. Analysis of the variants involved correlation with the phenotype of NPC.

Results: A loss of genes within chromosome 6 from copy number variation (CNV) was found. The lost genes included HLA-A, HLA-B, and HLA-C, which work in the antigen presentation process. This loss of the major histocompatibility complex (MHC) apparatus resulted in the tumour's ability to evade immune recognition. The tumour exhibited an immunologically "cold" phenotype, with mild tumour-infiltrating lymphocytes, supporting the possible etiology of loss of antigen presentation capability. Furthermore, the driver mutation PIK3CA gene was identified along with various other gene variants affecting numerous signaling pathways.

Discussion: Comprehensive WGS was able to detect various mutations and genomic losses, which could explain tumour progression and immune evasion ability. Furthermore, the study identified the loss of other genes related to cancer and immune pathways, emphasizing the complexity of NPC genomics. In conclusion, this study underscores the significance of MHC class I gene loss and its probable correlation with the cold tumour phenotype observed in NPC.

Keywords: nasopharyngeal cancer, genomic, MHC class I, antigen processing and presentation, copy number variation, deletion

Introduction

Nasopharyngeal cancer (NPC), characterized by its distinct genomic and biological features, remains a formidable challenge in the realm of oncology, owing to its intricate association with the Epstein–Barr virus (EBV) and sophisticated immune evasion mechanisms.[1] Evading immune surveillance is characteristic of cancer and phenotypically can be seen as a "cold" tumour, which has minimal tumour infiltrating lymphocytes. This NPC's unique genetic and virological characteristics has long perplexed the scientific community and posed significant clinical challenges. Despite advancements in therapeutic modalities, the prognosis for NPC remains suboptimal, underscoring the pressing need for a comprehensive understanding of its genomic underpinnings.

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Whole genome sequencing (WGS) has emerged as an indispensable tool, offering unprecedented insights into the genomic landscape of NPC.[2] It has potential implications for assessing how the tumourgenomics translate into its aggressive phenotype. WGS, a cutting-edge technology, allows for in-depth exploration of the entire genomic repertoire of NPC tumours, enabling the identification of molecular aberrations that may contribute to the development of various cancer hallmarks, including immune evasion.[3,4]

Here, we report on the captivating crossroads of nasopharyngeal cancer genomics, the association with immune evasion, and the NPC phenotype, through comprehensive WGS. We assessed the NPC's intricate genomics, seeking a relationship between and an association with aberrant NPC genomics, and correlated them with the relevant tumour phenotype. We aim to cast new light on molecular and genetic alterations in clinical NPC specimens that potentially drive cancer progression and render the cancer unseen by our immune system.

Materials and Methods

A freshly frozen nasopharyngeal cancer biopsy specimen was obtained. Informed consent had already been obtained from the patient for further genetic analysis. Ethical approval for collection, storage, and further molecular or genetic testing was received from the Faculty of Medicine, Universitas Indonesia's ethics committee (approval number: KET-149/UN2.F1/ETIK/PPM.00.02/2023). The specimen was collected from a patient whose nasopharyngeal cancer was clinically evident but not yet pathologically confirmed. A biopsy from the nasopharynx mass was taken as part of a routine diagnostic procedure. A small piece of biopsied tissue, around 50 mg, was concurrently collected and stored in a liquid nitrogen tank in −80°C for further molecular and genetic testing.

Pathological confirmation was obtained through examination by a senior pathologist. Information on the tumour immune microenvironment, assessed through histopathological slides, was also collected. The immune microenvironment on the histopathological slide was identified as mild, moderate or heavy tumour lymphocyte infiltration by assessing the overall region of the slides.[5] Relevant tumour phenotype and WGS finding were correlated.

DNA Extraction

Upon pathological confirmation of nasopharyngeal cancer, 30 mg of freshly frozen specimen was retrieved. It was thawed and mechanically dissected on 100 µL of phosphate-buffered saline (PBS) with a sterile surgical scalpel. Further, the sample underwent mechanical shearing with another 250 µL of PBS using an ultrasonic sonicator for 10–15 seconds, repeated until no solid granular mass was visible. The sample DNA was then extracted using the QIAamp DNA Mini kit (QIAGEN) following the manufacturer's instruction. Extracted DNA underwent quality control for assessment of its purity and quantity. DNA purity was assessed using a NanoDrop™ Spectrophotometer; DNA quantification was assessed using a Qubit™ Fluorometer (both Thermo Fisher).

DNA Library Preparation and DNA Sequencing

Extracted DNA was further processed through a library preparation step, following the protocol of the Oxford Nanopore Ligation sequencing DNA V14 (SQK-LSK114) instructions. No PCR step was involved. Sequencing of the whole genome was done using a PromethION 2 Sequencing Unit Solo from Oxford Nanopore with PromethION flow cell R10.4.1 and kit 14 chemistry. The library DNA concentration loaded was around 100–200 fmol, as suggested by the manufacturer. Sequencing was carried out for 71 hours until all pores were run out to achieve maximal data. Reloading of library DNA samples to sequencing flow cells was done twice during the whole sequencing process (when the pore occupancy dropped to around 25%), as recommended by the manufacturer's manual.

Bioinformatics

The raw sequencing data was in POD5 format.[6] The raw data was further basecalled using EPI2ME wf-basecalling (available from https://github.com/epi2me-labs/wf-basecalling) and the output was FASTQ data. The FASTQ data was assessed for quality using NanoPlot (available from https://github.com/wdecoster/NanoPlot).[7] Furthermore, alignment was carried out using EPI2ME wf-alignment (available from https://github.com/epi2me-labs/wf-alignment). An alignment reference file was downloaded from UCSC reference human genome GRCh38 and NCBI reference

genome for EBV. Alignment to the EBV reference genome was just to ensure that EBV DNA was present (commonly present concurrently with nasopharyngeal cancer).

The alignment BAM file, which was mapped to the human reference genome, then underwent copy number variation (CNV) calling using EPI2ME wf-human-variation (available from https://github.com/epi2me-labs/wf-human-variation). Single nucleotide variation (SNV) calling was done from a BAM file using an EPI2ME wf-human variation SNV calling pipeline. Annotation was carried out with SnpEff. The SNV variants were classified based on their genes' function associated with specific pathways according to those available in the Kyoto Encyclopaedia of Genes and Genomes (KEGG database). A graphical summary according to SNV types of mutation by relevant cancer-related KEGG pathways was presented. Possible driver mutation based on SNV data was screened for manually by analysing the biological function of those genes.

Visualization of aligned data was carried out using Integrative Genomics Viewer (IGV) Desktop Application.[8–10] Genes' location, names and other details within the specific human chromosomal region were obtained from ENSEMBLE database. Reported CNV copy number gains or losses were summarized. The loss or gain genes were checked to see if they contained any genes related to the human cancer pathway or immune system-related pathway based on the KEGG database. The biological explanation for loss or gain genes was analysed to find an association between cancer phenotype and genomic findings.

Results

The WGS yielded a total 53,141,702 reads and 64,805,867,700 bases; its N50 read length was 1798 bases and its median read quality was 17. The average sequencing depth was 18.7x. The reads were successfully mapped against both EBV and the human GrCh38 reference genome. It showed EBV DNA was present as it is commonly infected and remains latent in nasopharyngeal cancer.[11,12]

From the CNV calling result, it was found that there was a total loss on chromosome 6 base position 28,500,000 until 33,500,000, and a total loss on chromosome X base position 1 until 2,000,000. There was also a single copy loss on chromosome 11 base position 1 until 3,500,000, and on chromosome 15 base position 23,500,000 until 31,500,000. An overview visualization of the CNV result is shown in Figure 1.

There were 425 genes with a total loss within chromosome 6 base position 28,500,000 until 33,500,000. From those 425 genes, 22 genes are protein-coding genes and have a function related to antigen processing and presentation based on the KEGG database immune system pathway. There were 4 protein coding gene losses related to complement and

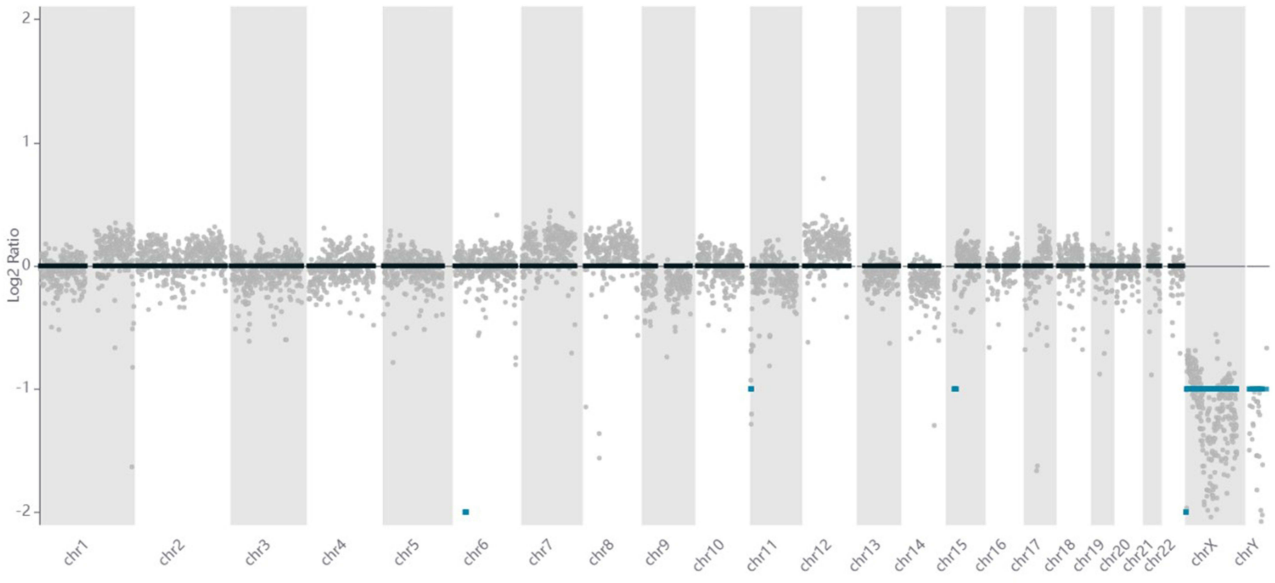

Figure 1 Overview of CNV result from WGS NPC data.

coagulation cascades. One gene loss related to Fc gamma R-mediated phagocytosis. One gene loss related to NOD-like receptor. One gene loss related to NOTCH signalling. One gene loss related to both complement and coagulation cascades, and apoptosis signalling. One gene loss was related to both ERK signalling and chemokine signalling. These genes and their related roles are summarised in Supplementary Table 1.

There were 30 genes with total losses within chromosome X base position 1 until 2,000,000. Out of 61 genes, three were protein-coding and play a role in B cell receptor signalling, T cell receptor signalling, or JAK-STAT signalling. These genes and their related roles are summarised in Supplementary Table 2. Genes with a single copy loss are summarised in Supplementary Table 3 and Supplementary Table 4. There was no significant copy number gain from this WGS sample.

The dominant gene loss within this WGS NPC sample was related to antigen processing and presentation. All MHC class I molecules, HLA-A, HLA-B, and HLA-C, were completely lost. The MHC class I molecules are necessary for normal cells to present antigens, including tumour antigens, to CD8 T cells and other immune cells. Thus, it can be inferred that tumours escape recognition because they have lost that important and necessary repertoire to tell immune cells that there is something wrong within the cells.

Gene loss was confirmed using an integrative genomics viewer (IGV; as shown in Supplementary Figure 1a). An example of loss of the HLA-A gene is shown in Supplementary Figure 1b. Identifying the loss of other genes, such as TNF, NOTCH4, IL3RA, etc., is less straightforward. Those genes are more context dependent, though they might be related to a cancer-related pathway or an immune-related pathway. The effects of loss of those genes remain uncertain.

From the SNV calling result, multiple variants were detected. These variants are summarized in Figure 2. The majority were G to A and missense variants. Among the detected variants were some that are also involved in various signalling pathways. Those pathways are known to be important and, when deranged, to be associated with cancer (Figure 3).

Among all variants, one notable driver mutation was identified. It was a mutation in the PIK3CA gene. It was a missense mutation: Human Genome Variation Society (HGVS) NM_006218.4:c.1634A>C. The change happened in position 1634 of the PIK3CA gene from A to C nucleotide, which initially coded for amino acid glutamic acid, but changed to alanine in amino acid sequence 545 (rs ID: rs121913274 SNP). There were 43 reads within that position, with 22 reads showing C instead of A, confirming the presence of the variant (see Supplementary Figure 2). There is a clinically approved targetable drug, alpelisib, for that specific mutation.[13] It works by selectively inhibiting class I PI3K p110α, which is the catalytic subunit of PI3K.[14]

Clinically, this patient had an aggressive nasopharyngeal cancer, initially diagnosed at stage IV. The tumour was localised within the nasopharynx, neck nodes, and very small early lung metastasis. Neoadjuvant chemotherapy consisting of cisplatin and 5-fluorouracil (5-FU) was administered for 3 cycles, then followed by chemoradiation to the nasopharynx and neck nodes. The local nasopharynx and regional node masses shrank, but 3 months later the lung had multiple lung metastases. The patient underwent further chemotherapy but the cancer continued to progress. The histopathological specimen from the initial biopsy revealed mild tumour infiltrating lymphocytes, which sort of excluded lymphocytes from the bulk of cancer cells. This was considered an immunologically cold tumour, which generally has a poorer prognosis. The representative histopathological finding is shown in Figure 4.

Discussion

Chromosomal instability in cancer that leads to chromosomal aberration is actually well documented.[15] Various reasons can lead to chromosomal aberration. One very plausible mechanism is a defect in mitotic DNA repair capability.[16] In normal conditions, an effective DNA sensing mechanism keeps track and activates a DNA repair response if an error occurs.[17] This is a protective mechanism. A DNA repair mechanism fixes all errors inherently occurring during the cell life cycle or cell mitosis until an exogenous assault occus that may also cause DNA error.[18]

Not all DNA repair mechanisms are error-free, however. For instance, in a double-strand DNA break (DSB), there are generally 2 main DNA repair mechanisms: non-homologous end joining (NHEJ) and homologous recombination (HR).[19] NHEJ is considered error prone, while HR is an error-free repair mechanism.[19] NHEJ is active in all cell cycles. HR is a DNA repair mechanism that typically operates during the S and G2 phases of the cell cycle, when a sister chromatid is

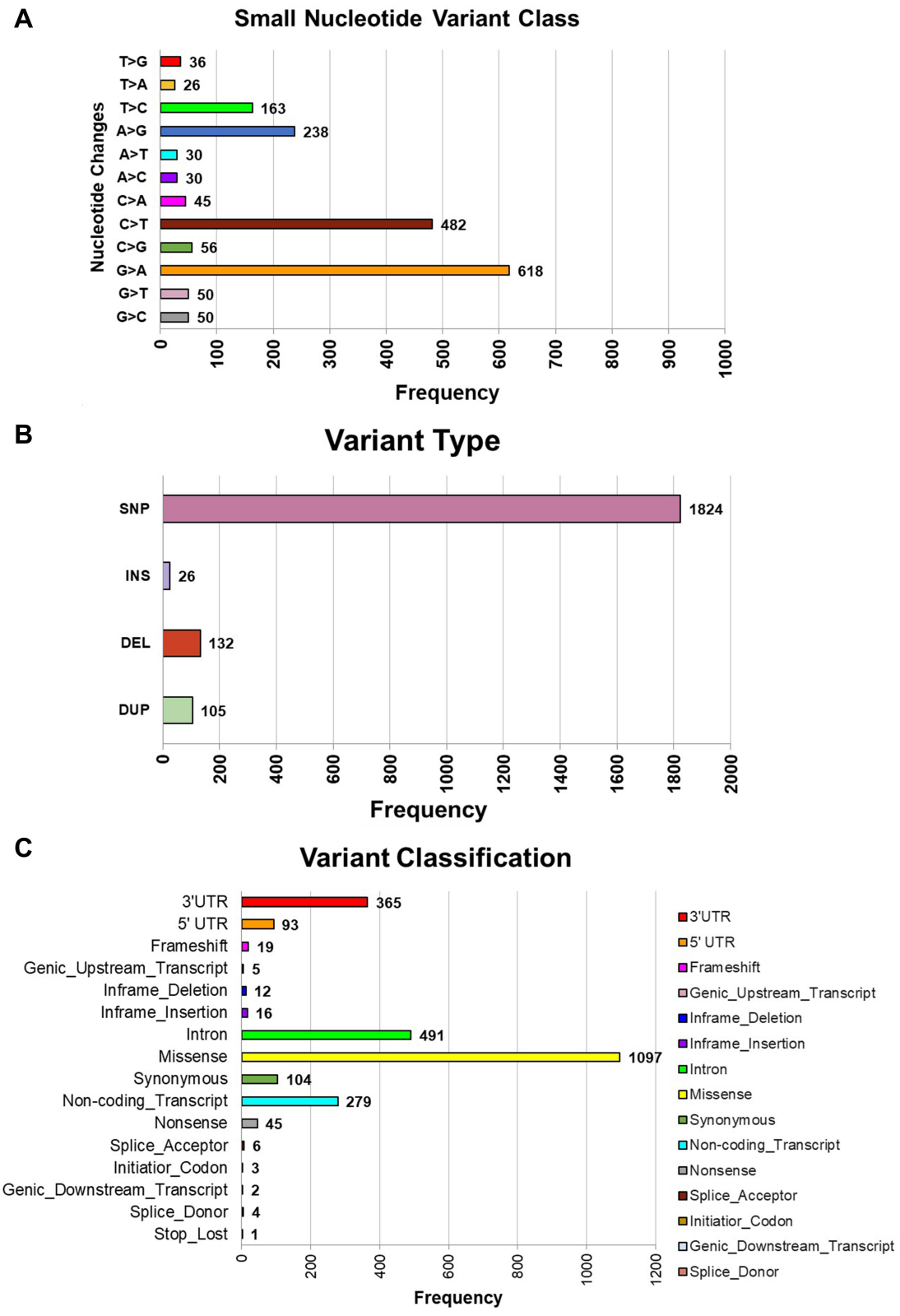

Figure 2 Summary of small variant from a comprehensive WGS of a clinical NPC. (**A**) Summary of the frequency of single base changes; (**B**) summary of variant type; (**C**) summary of variant classification.

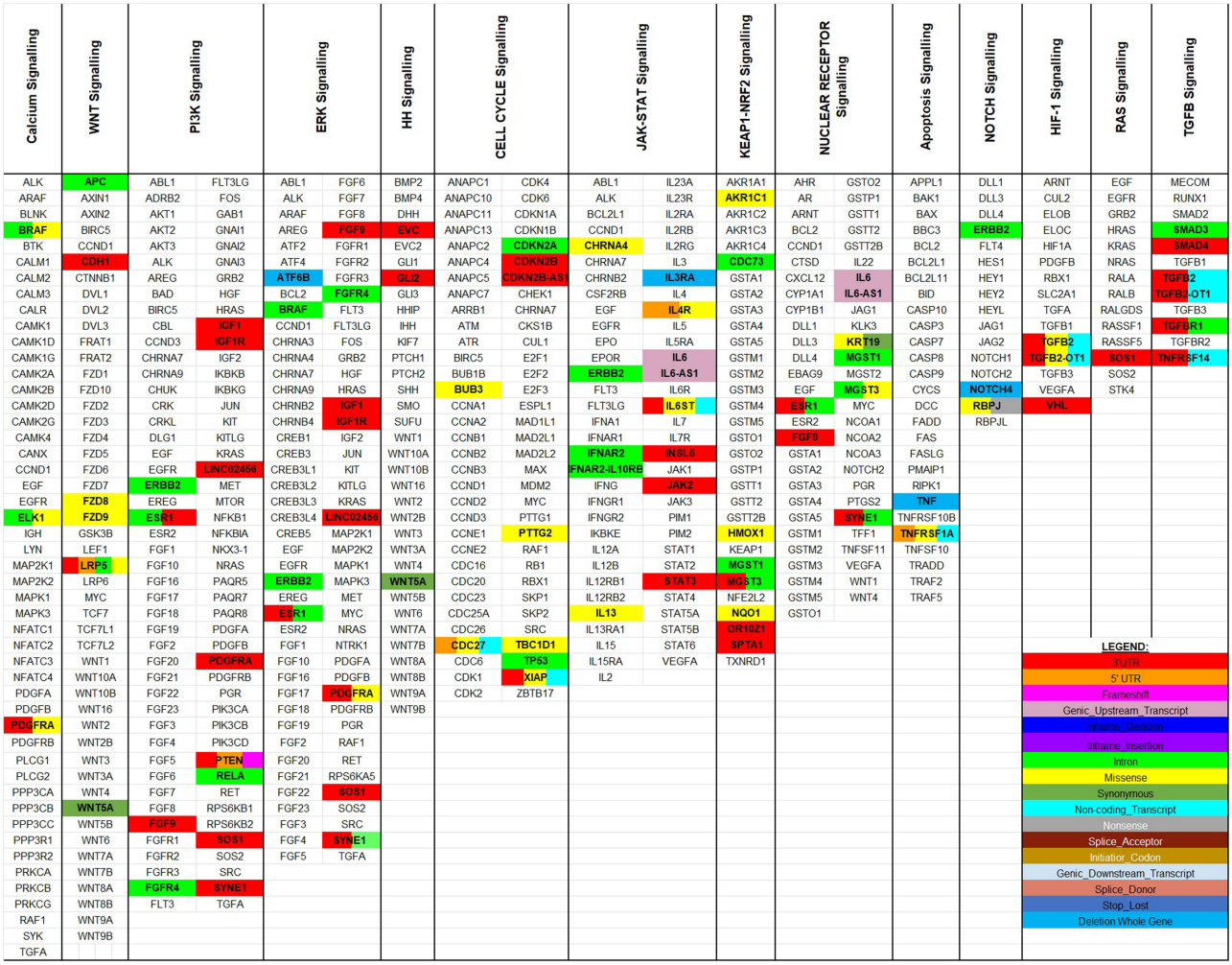

Figure 3 Important cellular pathways with the listed genes associated with those pathways. Colour coding indicates variant detected in that gene. More than 1 colour coding indicates more than 1 variant classification detected.

available as a template for repair.[20] This ensures accurate repair of double-strand DNA breaks by using the intact sister chromatid as a template.[20] Impairment of HR by any means, such as due to EBV in NPC cases, can result in reliance on an error-prone DNA repair mechanism, thus accumulating DNA mutations.[21]

Activation of homologous recombination (HR) during mitosis is generally suppressed and considered unfavourable. When HR is activated during mitosis, several problems can occur. During mitosis, the chromosomes condense and are not in a state in which HR can readily occur. Attempting HR during mitosis can result in a loss of genetic material, as there is no homologous chromatid available for repair.[16] Furthermore, activating HR during mitosis can disrupt the normal process of chromosome segregation, potentially leading to aneuploidy (abnormal chromosome number) and mitotic errors.[22] Aneuploidy can be a hallmark of cancer and is associated with genomic instability.[3]

In certain conditions or due to specific factors, HR can be mistakenly activated during mitosis. During the G2 phase, cells have mechanisms to check for DNA damage and ensure proper repair before proceeding to mitosis.[23] If DNA damage is detected but not adequately repaired, cells may enter mitosis with unresolved DNA lesions.[23] This can potentially trigger HR during mitosis. Furthermore, mutations or deficiencies in other DNA repair pathways, such as non-homologous end joining (NHEJ),[24] can lead to HR being used as a backup mechanism during mitosis when other pathways fail. This can also result in the inappropriate activation of HR.

Conditions that cause replication stress, such as stalled replication forks or excessive DNA damage, can lead to HR activation during mitosis as a means of repairing the DNA damage.[25] Some viruses can also interfere with cells' DNA

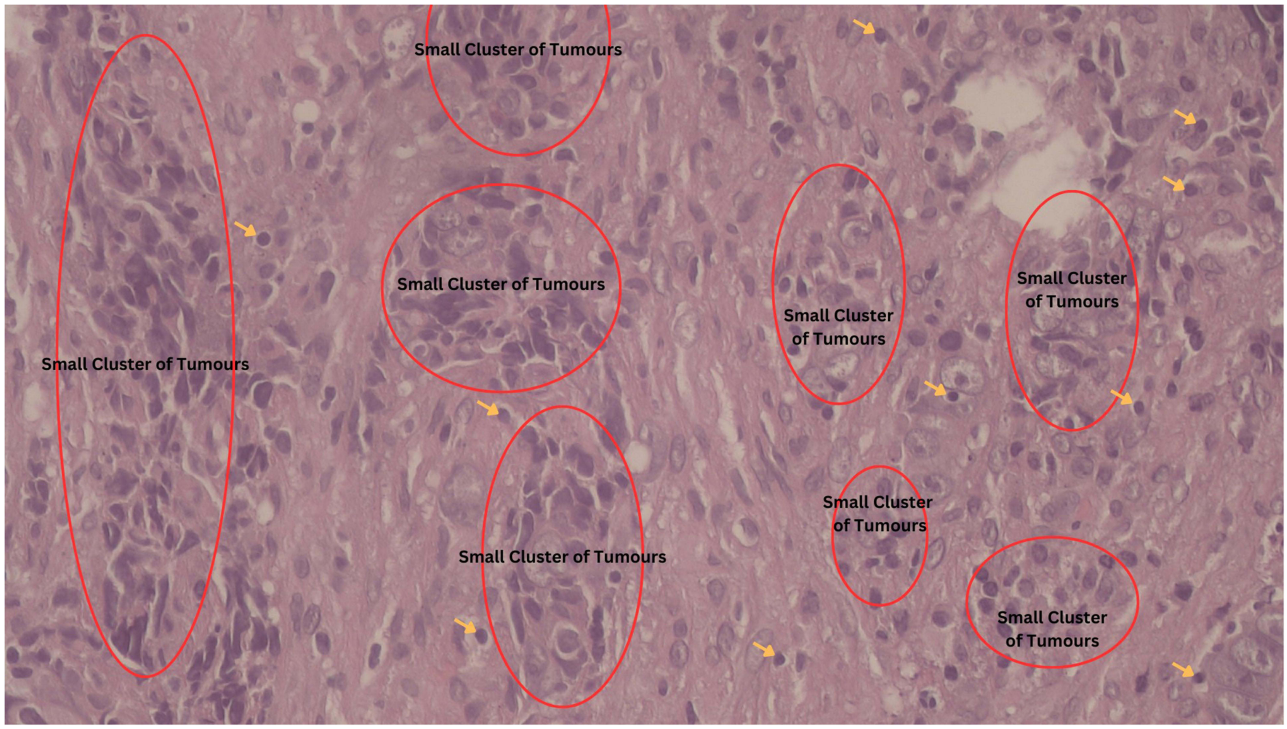

Figure 4 Histopathological examination with region of dominant cancer cells (marked in red oval circle) and lymphocytes marked by yellow arrows. There was only mild true tumour infiltrating lymphocyte. Examination was done in 100x magnification.

repair mechanisms, potentially leading to the inappropriate activation of HR.[26] Some carcinogens, such as benzene, can disrupt the regulation of DNA repair pathways and lead to genomic instability.[27] This instability can manifest as the activation of HR during mitosis.[27]

The activation of HR during mitosis is usually considered a last-resort repair mechanism and is associated with the potential for adverse consequences, including genomic instability and the generation of aneuploid cells. Cells have evolved mechanisms to tightly control the timing of HR to ensure accurate repair, primarily during the S and G2 phases, when a sister chromatid is available as a template. Inappropriate HR activation during mitosis can result in genetic abnormalities, for example loss of certain genomic regions, and can be associated with cancer development and progression.

In our WGS study of a clinical NPC sample specimen, it was revealed that there was a portion of the genome with total loss. Genomic aberration in our sample can be caused by multiple factors, including accumulated pre-existing mutations caused by many factors, including aberrant mitotic repair ability. The chronic process of genomic aberration resulted in constant selection of clones of cells that harbour important pro-survival cancer genes and deletion of anti-survival cancer genes.[28]

Our study showed that significant total losses were observed on chromosome 6, where multiple genes related to antigen processing and presentation are located. This loss encompassed essential genes like HLA-A, HLA-B, and HLA-C, which play a pivotal role in enabling normal cells to present antigens, including tumor antigens, to CD8 T cells and other immune cells.[29] The absence of these MHC-I molecules suggests that tumor cells within the NPC specimen may have acquired the ability to evade immune recognition by lacking the necessary molecular machinery to communicate with immune cells.[29]

We also confirmed that the tumour phenotype from this histopathological specimen was an immunologically cold tumour, as evidenced by the excluded tumour-infiltrating lymphocytes. There were only mild tumour-infiltrating lymphocytes, which is in line with the WGS finding on the loss of genes necessary to conduct proper antigen processing and presentation. This resulted in the absence of immune recognition of tumour cells.[30]

Various mechanisms can account for how the loss occurred, but the fact that there was a loss on those antigen processing and presentation genes can have important clinical implications. For instance, adoptive T cell transfer or CAR T cells might not work in this scenario. T cell therapy requires the presence of tumour antigen on the tumour cell

surface.[31] This was abolished in our specimen, rendering this kind of therapy ineffective. Another example, radiotherapy with a hypofractionated dosing regimen generally of around 6–20 Gy per fraction, has been known to induce upregulation of MHC class I molecules to improve the tumour neoantigen presentation to CD8 T cells.[1,30,32]

However, in the case of total loss of MHC class I related genes, we also suspected that no such upregulation can be observed. Therefore, the treatment strategy of using hypofractionated radiotherapy to act as a tumour in-situ vaccine and combined with checkpoint inhibitors might not work in this scenario. Furthermore, our study identified the loss of other genes through CNVs, including TNF, NOTCH4, and IL3RA, etc. While these genes are associated with cancer and immune-related pathways, their precise functions and impacts on NPC remain context-dependent and warrant further investigation.[33,34]

In our SNV calling, we found small variants in multiple genes that were involved in important cellular signalling. Not all those variants might have important clinical consequences. In general, mutation in genes directly regulating signalling pathways might drive the development, aggressiveness, and survival of these genes until ability to metastasise of malignant cancer.[35,36] The one that was very likely to have an important clinical relevance in our comprehensive sequencing was the mutation in the PIK3CA gene. The mutation found in PIK3CA was a missense mutation in amino acid position 545, which has been reported to be oncogenic in multiple cancer sites.[37–39] The PIK3CA inhibitors have also been shown to produce meaningful clinical tumour regression in multiple solid tumours with PIK3CA mutation.[14,40] However, clinical trial results have shown that the PIK3CA inhibitor only confers the benefit of additional cancer progression-free survival by a median of 5.3 months compared to placebo in PIK3CA mutated breast cancer.[41] Though great, this is not extraordinary; eventually, the cancer still grows.

It is very likely that cancer cells are progressively multiclonal.[42] Continuous mutation and natural clonal selection result in the most aggressive and fittest cancer stem cells surviving and proliferating. Those different clones might have completely different genomic landscapes within an individual, which drive progression and complicate treatment strategy.[43] Discovering the genomic profile of an initial site is the first step in devising an effective treatment strategy. Eventually, if the cancer continues to progress, another profiling is needed to alter the treatment strategy based on the current and main pattern of the cancer molecular landscape.

Conclusion

In conclusion, our study provides valuable insights into the genomic landscape of NPC with its driver mutation and its potential links to immune evasion, with the loss of MHC-I genes standing out as a key mechanism by which NPC tumours may escape immune recognition. The findings underscore the necessity for further research on such cancer subsets. Various mechanisms might lead to the observed phenotype of this immunologically cold tumour, such as, in our case, total loss of the MHC class I repertoire.[44,45] We need to devise a new strategy for dealing with such a tumour subset to improve therapeutic outcomes in the future.

Ethics Statement

This study has received ethical clearance from the ethics committee of the Faculty of Medicine, Universitas Indonesia (KET-149/UN2.F1/ETIK/PPM.00.02/2023). All informed consent was obtained prior to inclusion of the subject in this study.

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Disclosure

The authors report no conflicts of interest in this work.

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