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ORIGINAL RESEARCH

First Indonesian Nasopharyngeal Cancer Whole Epigenome Sequencing Identify Tumour Suppressor CpG Methylation

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Introduction: Nasopharyngeal cancer (NPC) is a multifaceted disease characterized by genetic and epigenetic modifications. While Epstein–Barr virus (EBV) infection is a known risk factor, recent studies highlight the significant role of DNA methylation in NPC pathogenesis. Aberrant methylation, particularly at CpG sites, can silence tumour suppressor genes, promoting uncontrolled cell growth. This study aims to analyse the methylation patterns in Indonesian NPC patients through whole-epigenome sequencing.

Methods: Seven clinical nasopharyngeal cancer samples were collected and confirmed histopathologically. DNA was extracted, sequenced using Oxford Nanopore technology, and aligned to the GRCh38 human reference genome. Methylation analysis was performed using modkit and statistical analysis with R software. Enriched pathways and processes were identified using ClusterProfiler in R, and gene overlap analysis was conducted.

Results: The analysis identified both globally hypermethylated and hypomethylated NPC samples. Key tumour suppressor genes, such as *PRKCB, PLCB3, ITGB3, EPHA2, PLCE1, PRKCD, CDKN2A, CDKN2B, RPS6KA2, ERBB4, LRRC4, AKT1, PPP2R5C, and STK11* were frequently hypermethylated and confirmed to have lower expression in an independent NPC transcriptome cohort, suggesting their role in NPC carcinogenesis. Enriched KEGG pathways included PI3K-Akt signalling, ECM–receptor interaction, and focal adhesion. The presence of EBV DNA was confirmed in all samples, implicating its role in influencing methylation patterns.

Discussion: This study provides comprehensive insights into the epigenetic landscape of NPC, underscoring the role of CpG methylation in tumour suppressor gene silencing. These findings pave the way for targeted therapies and highlight the need for region-specific approaches in NPC management.

Keywords: nasopharyngeal cancer, epigenome, methylation, Epstein–Barr virus, whole-genome sequencing

Introduction

Nasopharyngeal cancer development (carcinogenesis) is a complex process involving both genetic and epigenetic alterations.[1,2] While Epstein–Barr virus (EBV) infection is a major risk factor, epigenetic changes like DNA methylation are now known to play an increasingly important role. These changes involve adding methyl groups (chemical modifications) to DNA, often in regions called CpG sites. This disrupts normal gene expression and can silence tumour suppressor genes, promoting uncontrolled cell growth and cancer development. Aberrant methylation patterns are particularly pronounced in nasopharyngeal cancer, making them promising targets for diagnosis and potentially even future therapies.

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Recent studies indicate methylation could disrupt cellular pathways critical for preventing cancer, ultimately contributing to nasopharyngeal carcinoma (NPC) development.[3–5] One key pathway involves tumour suppressor genes, which normally act as brakes on cell division.[6] Methylation can silence these genes, allowing cells to divide uncontrollably. Studies have identified frequent methylation of genes like DAPK (Death-Associated Protein Kinase) in NPC patients.[7] Disabling DAPK through methylation disrupts a cell's self-destruction pathway, enabling damaged or abnormal cells to survive and potentially progress to cancer. There are many other pathways (such as PI3K, JNK, ERK, JAK, and RAS) when expressed abnormally may also contribute to cancer progression.[8,9]

Furthermore, methylation can tamper with DNA repair mechanisms. Normally, cells have processes to fix errors in their DNA. However, methylation can silence genes responsible for DNA repair, such as *CCND1, CDKN2A*, etc. leaving mutations unchecked.[10] This accumulation of mutations can lead to uncontrolled cell growth and eventually contribute to NPC formation. Studies suggest a link between EBV infection, a major NPC risk factor, and abnormal methylation patterns.[9,11] EBV may trigger or influence these methylation events, creating a favourable environment for cancer development.[9]

Due to the aberrant methylation in NPC, one hypothesis is using hypomethylating agents in hypermethylated NPC type to reactivate silenced genes through demethylation.[12] It is expected that it could restore normal cellular functions and potentially increase the efficacy of immune or chemotherapeutic strategies. Early trials of azacitidine and similar agents in other hypermethylated tumours have shown promise,[13] with evidence of re-expression of silenced genes and increased sensitivity to other treatments.[14] However, studies specifically investigating the use of hypomethylating agents in NPC remain limited with mixed result, with some preclinical data suggesting their potential to modulate key oncogenic pathways and improve therapeutic outcomes, but clinical trial has not shown tangible benefit.[15–17]

In general, nasopharyngeal cancer (NPC) is a cancer with a relatively low frequency of somatic mutation compared to other solid cancer.[18] However, NPC has a higher frequency of aberrant methylation.[18] It was found that NPC is generally globally hypermethylated, but recent findings suggested that there are also globally hypomethylated subtypes of NPC.[19] The environmental factors are known to influence DNA methylation patterns.[20] Thus, NPC methylation signatures from a certain geographical region may differ with other regions. Here, we reported the Indonesian clinical NPC whole-epigenome sequencing and an in-depth analysis focus on CpG island methylation.

Method

Biospecimens

Seven clinical nasopharyngeal cancer samples were collected from the Radiation Oncology Cipto Mangunkusumo National General Hospital biobank. All samples were fresh frozen nasopharyngeal cancer biopsy tissue. The tissue collection was done through nasoendoscopy biopsy procedure for suspected nasopharyngeal cancer patients. Macroscopically apparent tumour was biopsied with some portion of it being stored in the fresh frozen tissue form in the biobank, and another portion was processed for histopathological examination. All seven samples were confirmed histopathologically to be undifferentiated non-keratinized nasopharyngeal carcinoma. This study was conducted in accordance with the principles of the Declaration of Helsinki. Ethical committee approval (KET-149/UN2.F1/ETIK/PPM.00.02/2023) from Faculty of Medicine, Universitas Indonesia ethical board, was obtained before the specimen collection. Inform consent was obtained before the biopsy procedure, and it was clearly stated that some portion of their biopsy tissues would be stored in the biobank for further molecular and genomic examination.

DNA Extraction

Fresh frozen samples were processed by thawing at room temperature. A minimum of 25 mg of tissue was used. The specimens were then mechanically dissected until very small pieces. DNA extraction was done using the Qiagen DNA Mini kit. The extraction protocol was followed in adherence to the manufacturer's protocol for tissue DNA extraction. The extracted DNA was then measured for concentration and purity by using Qubit fluorometers and NanoDrop Microvolume Spectrophotometers, respectively. A minimum DNA of 1 µg was required for further library preparation and NGS sequencing. DNA purity was between A 260/280 of 1.8–2.0.

Library Preparation and NGS Sequencing

Library preparation protocol used was Ligation sequencing DNA V14 (SQK-LSK114) from Oxford Nanopore. Library preparation was carried out in strict adherence to manufacturer protocol. The extracted DNA was further sequenced using Promethion 2 Solo NGS device from Oxford Nanopore. One promethion flow cell was used for each sample. The DNA was sequenced in its native form, and no PCR step is involved to preserve the modified bases (methylation information). The sequencing ran for 96–120 hours or until all pores from the flow cell was used up. In between the sequencing run, the flow cells were washed and reloaded with the library when the pore occupancy dropped to below 20%. The sequencing output was in the form of POD5 raw data.[21]

Basecalling and Alignment

After sequencing, the raw data was further basecalled using MinKNOW software powered by Dorado from Oxford Nanopore.[22] Basecalling was done in super accuracy mode with dna_r10.4.1_e8.2_400bps_sup@v5.0.0 basecalling model with 5mC and 5hmC detection mode.[23] Minimum sequencing quality score was 10, any bases below a quality score of 10 were excluded. Alignment was performed toward GRCh38 human reference genome. Minimum read depth required for further methylation analysis was 5.[24] All bases below read depth 5 were excluded from methylation analysis. A separate alignment toward NC_007605 human gammaherpesvirus 4 (Epstein–Barr virus) was done. This was to confirm the presence of EBV DNA within our nasopharyngeal cancer specimen.

Methylation and Statistical Analysis

Methylation information of 5mC (5-methylcytosine) from all NPC specimens was piled up using modkit tool from Oxford Nanopore. A normal nasopharyngeal epithelium whole-epigenome methylation information was obtained in bigwig formats as a comparison from public GEO database (Biosample SAMN18923230, sample dataset GSM5270798_WGBS0024, available from https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM5270798). The data was converted to non-binary BED format using BigWigToBedGraph tool from UCSC. The normal nasopharyngeal epithelium CpG sequences were originally aligned toward the hg19 normal human reference genome. Therefore, a conversion was done from hg19 to GRCh38 normal human reference genome using Lift Genome annotation from UCSC to enable a comparable region because all our cancer samples were aligned toward GRCh38 normal human reference genome.

The individual sequencing position with methylation information from the normal nasopharynx and all NPC samples were further processed using R software. The methylation value in each position was in the percentage, and then a match of chromosome and position coordinate was made between the normal nasopharynx and each NPC sample. Global methylation was calculated by averaging the methylation value of all NPC samples and one normal sample. Then, an independent *t*-test of the mean between normal and each NPC sample was used to determine the statistical significance with p value less than 0.05 considered statistically significant. Global average methylation below the normal sample, which was statistically significant, was considered hypomethylated and vice versa. Visualization of the methylation analysis was done globally for all matched CpG regions across all NPC samples and normal sample in a heatmap by CpG region clustering. Heatmap visualization was done using Complex Heat Map package in R.[25,26]

The whole CpG region in entire human genome was extracted from UCSC table browser. This CpG region was used to calculate the average methylation value in normal and NPC samples. An independent *t*-test was performed to calculate the statistical significance of the average methylation value between normal and each NPC sample. A significant level of p-value less than 0.05 was used to indicate a real difference in methylation value. A computation extracting the methylation value of cancer sample subtracted to normal sample methylation value was done to determine which CpG regions were more hypermethylated or hypomethylated relative to the normal sample. Then, filtering was done to separate hypermethylated and hypomethylated CpG regions.

The hypermethylated CpG regions were further processed to compute for overlap toward all known genes using the R package TxDb.Hsapiens.UCSC.hg38.knownGene database. Then, the hypermethylated gene sets were extracted and processed for methylation enrichment analysis using ClusterProfiler in R.[27] Methylation enrichment analysis was done by utilising pathways and processes from Kyoto Encyclopaedia of Genes and Genomes (KEGG) database and Gene Ontology

(GO) database for biological process (BP), molecular function (MF), and cellular component (CC). Methylation enrichment hierarchical network analysis map was visualized using hierarchical plot from R package ClusterProfiler.[27] Each KEGG enrichment pathway analysis from all enriched samples was analysed for gene overlaps and visualised using upset plot.

The enriched genes, pathways and processes were extracted, and genes overlap computation was done using R to pinpoint which genes had the highest intersection among all hypermethylated pathways and processes. If a hypermethylated gene is found to be involved in many hypermethylated pathways and processes, it is more likely to be more important biologically. The hypermethylated genes were checked whether they are known tumour suppressor genes or not toward Tumour Suppressor Gene database (TSGene) from University of Texas.[28] Furthermore, those enriched hypermethylated tumour suppressor gene were validated using independent nasopharyngeal cancer transcriptome profiling (data obtained from European Bioinformatics Institute, available from https://www.ebi.ac.uk/gxa/experiments-content/E-GEOD-12452/resources/DifferentialSecondaryDataFiles.Microarray/normalized-expressions).[29,30] The hypermethylated tumour suppressor genes with significantly lower expression in NPC tissue relative to normal nasopharyngeal tissue (p value <0.05 and FDR value 0.05) were reported as likely to the driver of carcinogenesis in NPC.

Result

All 7 samples were successfully mapped against EBV genome, confirming the presence of EBV in all these NPC specimens. EBV 5mC methylation in sample 1, sample 2, and sample 4 was remarkably higher than the other samples. The statistics of the EBV methylation value is presented in Table 1. The EBV methylation value distribution across all samples is presented in the violin plot in Figure 1.

All the NPC methylation data are stored and made available in GEO database with accession number GSE272926. The global CpG methylation profile underwent unsupervised clustering using the Complex Heatmap R package to determine the methylation cluster relative to the normal nasopharyngeal epithelium. Among seven NPC samples, there were four samples that were globally hypermethylated, and the other three samples exhibit global methylation profile comparable to normal nasopharyngeal epithelium. The statistics of all the NPC samples and normal nasopharynx methylation value is presented in Table 2. The methylation value distribution across all NPC samples and normal nasopharynx is presented in the violin plot in Figure 2. The global CpG methylation heatmap of all NPC samples and normal sample is shown in Figure 3.

Analysis of Methylated KEGG Pathways and GO Processes

Methylation enrichment analysis based on the KEGG pathway identified enriched KEGG pathways for five NPC samples (S1, S2, S3, S5, S6) from the hypermethylated CpG regions. There were 53, 11, 52, 21, and 54 KEGG enriched pathways for samples S1, S2, S3, S5 and S6, respectively. The complete list of enriched pathways for all those samples is available in Supplementary Data 1. The most common CpG hypermethylated enriched KEGG pathways include calcium signalling

Table 1 EBV Methylation Value Statistics in Percentage Across All Samples

Sample	Min	Q1.25%	Median	Mean	Q3.75%	Max	SD
S1	0	94	97.44	90.14	98.81	100	21.62
S2	0	83.33	93.75	82.15	100	100	27.97
S3	0	0.35	1.57	21.37	22.12	100	35.06
S4	50	83.77	92.31	89.74	100	100	12.19
S5	0	0	0	22.20	28.57	100	35.74
S6	0	0	1.71	20.72	21.82	100	34.09
S7	0	0	0	20.89	18.18	100	35.80

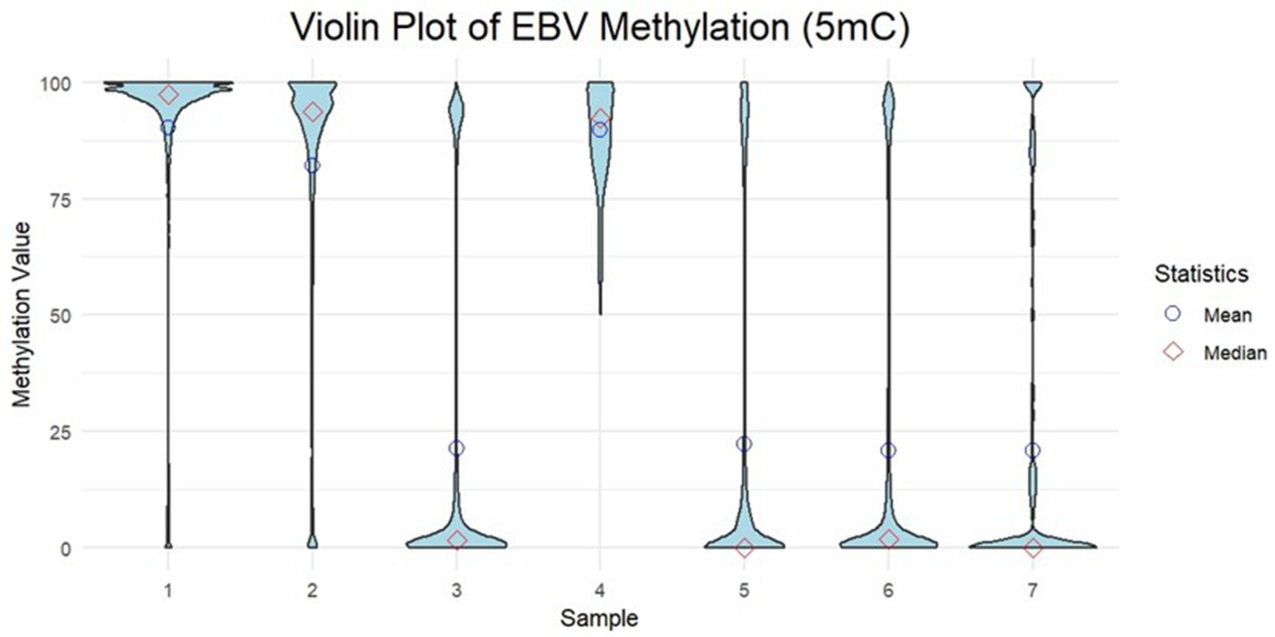

Figure 1 EBV Methylation value distribution across all samples (filtered for coverage above 5x).

pathway, ECM–receptor interaction, morphine addiction, neuroactive ligand–receptor interaction, nicotine addiction, Rap1 signalling pathway, and signalling pathways regulating pluripotency of stem cells which are enriched in all five NPC samples.

The second most commonly enriched CpG hypermethylation KEGG pathways, which are present in four out of five NPC hypermethylated enriched KEGG pathways, are axon guidance, cAMP signalling pathway, cholinergic synapse, circadian entrainment, Cushing syndrome, focal adhesion, glutamatergic synapse, GnRH secretion, hippo signalling pathway, MAPK signalling pathway, maturity-onset diabetes of the young, phospholipase D signalling pathway, retrograde endocannabinoid signalling, and transcriptional misregulation in cancer. The frequency of all KEGG pathways that are enriched among all five NPC samples is available in Supplementary Data 2. From the hierarchical clustering analysis of the enriched hypermethylated KEGG pathways, there were five clusters of enriched KEGG pathways in all hypermethylated KEGG pathway samples. The hierarchical clustering is shown in Figure 4.

Table 2 Methylation Value Statistics in Percentage Across All NPC and Normal Nasopharynx Samples

Sample	Min	Q1.25%	Median	Mean	Q3.75%	Max	SD
S1	0	0.65	0.86	0.76	1	1	0.27
S2	0	0.58	0.83	0.72	0.93	1	0.29
S3	0	0.60	0.83	0.73	1	1	0.29
S4	0	0.54	0.78	0.69	0.90	1	0.30
S5	0	0.64	0.83	0.74	0.92	1	0.26
S6	0	0.64	0.83	0.75	1	1	0.27
S7	0	0.50	0.77	0.69	0.88	1	0.29
normal	0	0.65	0.84	0.74	0.93	1	0.27

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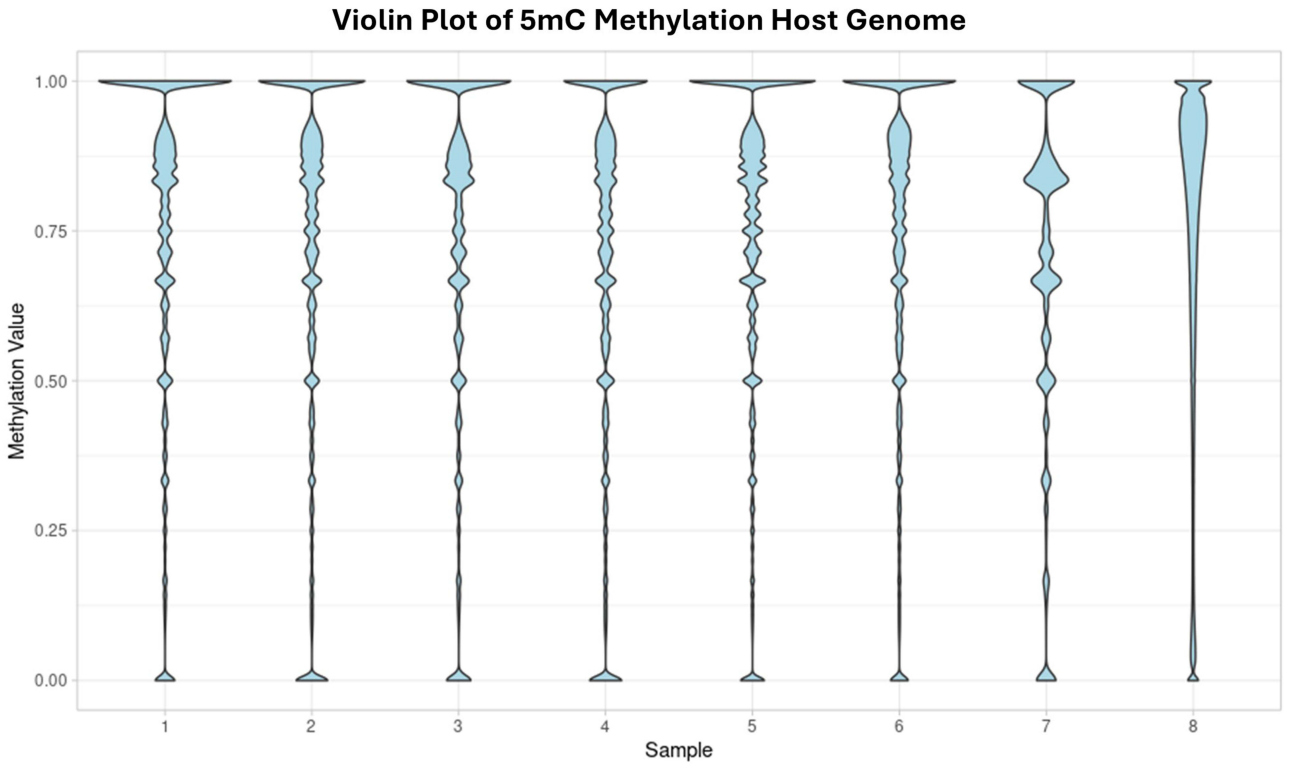

Figure 2 Methylation value distribution across all NPC samples (sample 1–7) and normal (sample 8) nasopharynx sample (filtered for coverage above 5x).

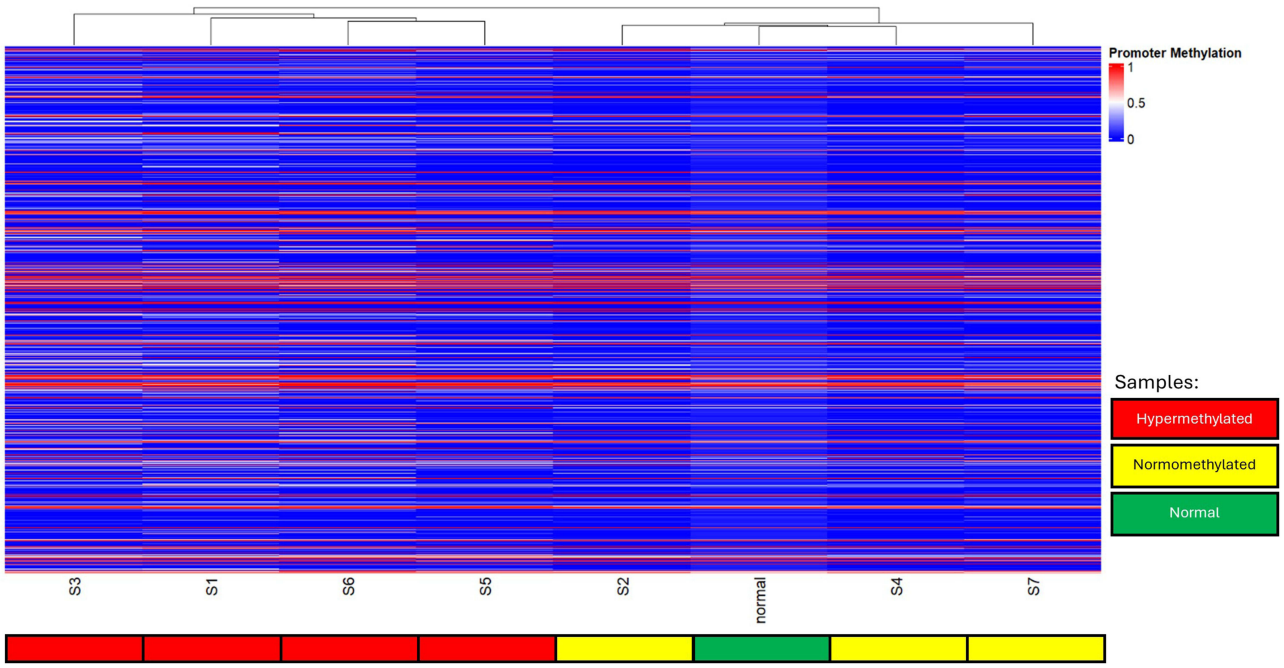

Figure 3 Heatmap of all 7 samples based on CpG regions, the global average methylation was compared toward normal nasopharynx epithelium.

From the KEGG pathway interaction analysis, it was found that PI3K-Akt signalling, focal adhesion, and ECM receptor interaction have the most overlapping hypermethylated pathways in sample S1. While the most overlapping hypermethylated KEGG pathways in sample S2 are focal adhesion and ECM receptor interaction pathways. In sample S3, the most overlapping hypermethylated KEGG pathways are proteoglycans in cancer, Wnt signalling pathway,

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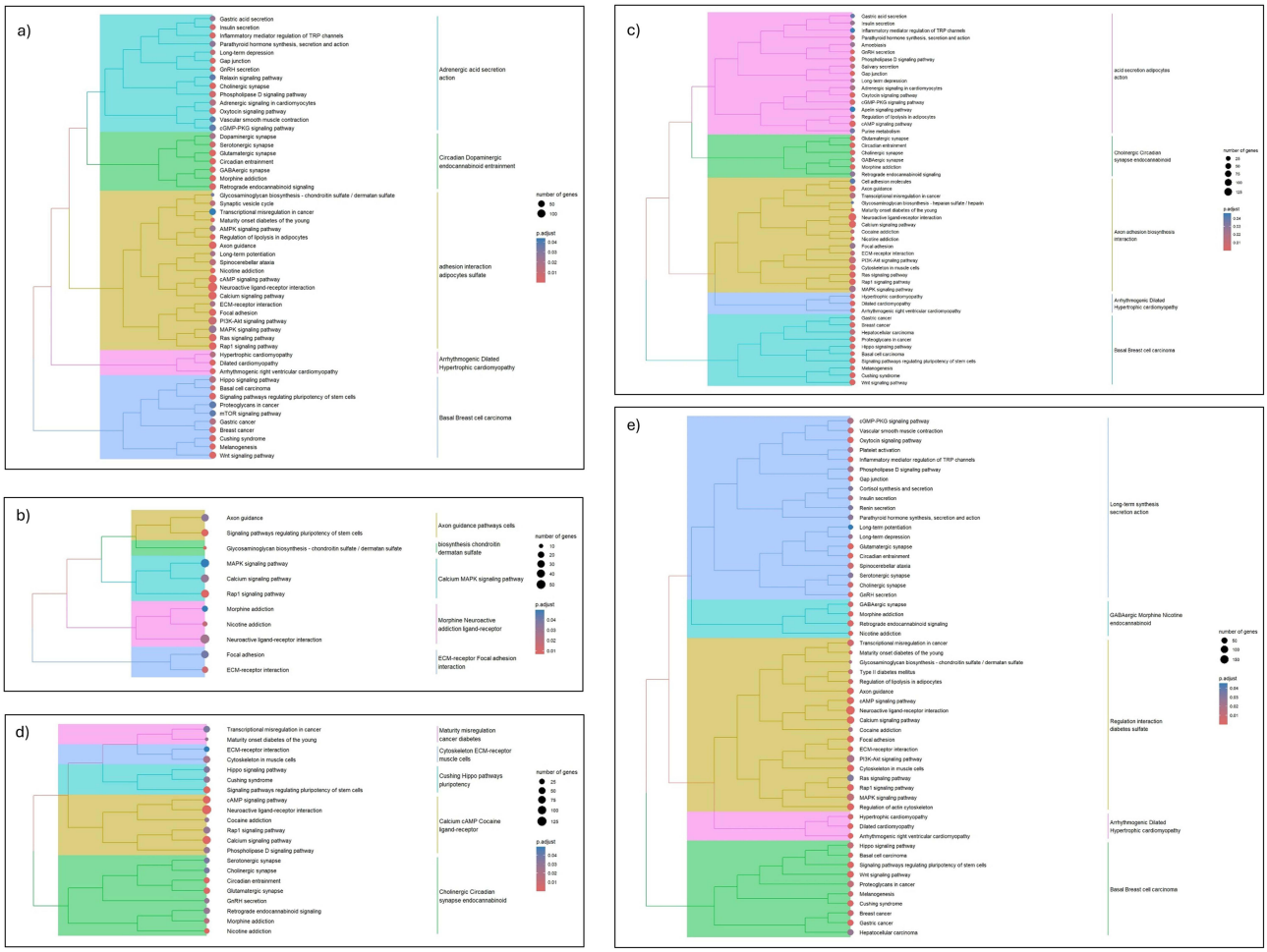

Figure 4 Hierarchical clustering of hypermethylated KEGG pathways across all samples with enriched KEGG pathways. (**a**) sample S1; (**b**) sample S2; (**c**) sample s3; (**d**) sample s5; (**e**) sample s6.

signalling pathways regulating pluripotency of stem cells, Cushing syndrome, hepatocellular carcinoma, hippo signalling pathway, breast cancer, gastric cancer, melanogenesis, and basal cell carcinoma pathway. In sample S5, cytoskeleton in muscle cells and ECM receptor interaction are the most overlapping hypermethylated pathways. In sample S6, Wnt signalling pathway, signalling pathways regulating pluripotency of stem cells, proteoglycans in cancer, gastric cancer, Cushing syndrome, hepatocellular carcinoma, hippo signalling pathway, breast cancer, melanogenesis, and basal cell carcinoma are the most overlapping hypermethylated KEGG pathways. The details of the pathway interaction analysis in the upset plot are available in Supplementary Data 3.

Methylation enrichment analysis based on GO biological process identified enriched GO BP processes for all NPC samples from the hypermethylated CpG regions. There were 901, 277, 1071, 235, 637, 962 and 2 enriched biological processes for samples S1, S2, S3, S4, S5, S6, and S7, respectively. The embryonic skeletal system morphogenesis and homophilic cell adhesion via plasma membrane adhesion molecules are two biological processes that are enriched in all 7 NPC hypermethylated CpG samples. The complete list of enriched GO biological processes for all those samples is available in Supplementary Data 4. The frequency of all GO BP processes that are enriched among all 7 samples is available in Supplementary Data 5.

Methylation enrichment analysis based on GO molecular function identified enriched GO MF processes for 6 out of 7 NPC samples from the hypermethylated CpG regions. There were 88, 29, 119, 21, 58, and 106 enriched molecular function processes for samples S1, S2, S3, S4, S5, and S6, respectively. The most common enriched GO molecular function processes are DNA-binding transcription activator activity, DNA-binding transcription activator activity RNA

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polymerase II-specific, DNA-binding transcription repressor activity, DNA-binding transcription repressor activity RNA polymerase II-specific, gated channel activity, monoatomic ion gated channel activity, and neurotransmitter receptor activity which are enriched GO MF processes in all 6 NPC samples. The complete list of enriched MF for all those samples is available in Supplementary Data 6. The frequency of all MF processes that are enriched among all 6 samples is available in Supplementary Data 7.

Methylation enrichment analysis based on GO cellular component identified enriched GO CC processes for 6 out of 7 NPC samples from the hypermethylated CpG regions. There were 87, 40, 82, 40, 77, and 88 enriched cellular component processes for samples S1, S2, S3, S4, S5, and S6, respectively. The most common enriched GO cellular component processes are adherens junction, asymmetric synapse, axon terminus, cell cortex, cell leading edge, collagen-containing extracellular matrix, dendritic spine, distal axon, excitatory synapse, GABA-ergic synapse, glutamatergic synapse, monoatomic ion channel complex, neuron projection terminus, neuron spine, neuron to neuron synapse, neuronal cell body, perikaryon, postsynaptic density, postsynaptic membrane, postsynaptic specialization, presynaptic active zone, Schaffer collateral - CA1 synapse, and synaptic membrane which are enriched GO CC processes in all 6 NPC samples. The complete list of enriched CC for all those samples is available in Supplementary Data 8. The frequency of all MF CC processes that are enriched among all 6 samples is available in Supplementary Data 9.

Analysis of Individual Methylated Genes

From all the hypermethylated CpG regions of each sample, they were matched with the known genes, and a list of genes was obtained. The gene lists underwent methylation enrichment analysis based on KEGG pathways and GO as described above. The enriched pathways and GO were extracted, and the hypermethylated genes were counted for those involved in multiple pathways and GO. The hypermethylated gene with the involvement of ten or more pathways and GO were reported. The greater the gene involvement in multiple pathways or processes, then the higher the possibility of that gene to affect the NPC carcinogenesis whenever methylated. The tumour suppressor gene was highlighted due to greater probable suppression of gene expression by methylation to cause loss of function. Validation of hypermethylated tumour suppressor genes was done using independent NPC transcriptome profiling.[29,30]

The *PRKCB* and *PLCB3* were the most common hypermethylated tumour suppressor genes among KEGG enriched pathways that intersected with 10 or more pathways and had a confirmed lower RNA expression in the transcriptome data. Other common hypermethylated tumour suppressor genes enriched in the KEGG pathways were *ITGB3, EPHA2, PLCE1, PRKCD, CDKN2A, CDKN2B, RPS6KA2, ERBB4, LRRC4, AKT1, PPP2R5C* and *STK11*. They all had lower expression in NPC compared to normal in the transcriptome data. The complete list on the number of intersected hypermethylated genes across all hypermethylated KEGG pathways, along with the tumour suppressor annotation and confirmed lower expression from independent validation toward transcriptome data for all samples are available in Supplementary Data 10.

Based on GO BP enrichment analysis, the *WT1* gene is found to be highly methylated in the NPC samples and had a confirmed lower expression in nasopharyngeal cancer sample compared to normal sample from transcriptome data. Other common hypermethylated tumour suppressor genes that intersected with 10 or more biological processes and had confirmed lower expression include *TBX5, PRKCB, ITGB3, RARB, PPARG, ERBB4, EPHA2, STK11, PPARA*, and *EPHA2*. From the GO BP analysis, there were relatively high number of hypermethylated genes that were involved in (up to 100) enriched GO BP processes.

The high number of overlapping enriched GO BP is likely due to the nature of this biological analysis where normally not all human biological processes are expressed in a cell type. Only the required processes are expressed, so this infers that methylation does likely play a role in suppressing unnecessary gene expression. The complete list on the number of intersected hypermethylated genes across all hypermethylated GO BP processes, along with the tumour suppressor annotation and confirmed lower expression from independent validation toward transcriptome data for all samples are available in Supplementary Data 11.

In the GO MF enrichment analysis, the common hypermethylated tumour suppressor genes were *PPARA, EPHA2, EPHB3, PRKCB, PPARG, ADAMTS8, EPAS1*, and *FOXO1*. They were validated to have lower nasopharyngeal cancer expression compared to normal nasopharynx from transcriptome data. They were generally involved in only 5–13

molecular functions. The complete list on the number of intersected hypermethylated genes across all hypermethylated GO MF processes, along with the tumour suppressor annotation and confirmed lower expression from independent validation toward transcriptome data for all samples are available in Supplementary Data 12.

The *ERBB4, LRRC4, ITGB3, NEURL1, PRKCB, EPHA2, HOMER2, PTPRT*, and *MAL* tumour suppressor genes were found to be hypermethylated, involved in 5–15 GO CC processes across multiple NPC samples from enriched GO CC analysis, and validated to have lower expression in NPC transcriptome data. The complete list on the number of intersected hypermethylated genes across all hypermethylated GO CC processes, along with the tumour suppressor annotation and confirmed lower expression from independent validation toward transcriptome data for all samples are available in Supplementary Data 13.

Discussion

The study on nasopharyngeal cancer (NPC) presented here marks a significant advancement in understanding the epigenetic modifications involved in this malignancy. By conducting the first whole-epigenome sequencing of NPC samples from Indonesian patients, we have identified key tumour suppressor CpG methylation patterns that may play a crucial role in the pathogenesis of NPC. Our analysis revealed varying patterns of global methylation across the NPC samples. Specifically, we observed both global hypermethylated and normomethylated states, which suggest a complex interplay of epigenetic modifications in NPC. The presence of globally hypermethylated samples aligns with previous findings that NPC exhibits a high frequency of aberrant methylation,[19] despite its relatively low frequency of somatic mutations compared to other cancers. This observation in global methylation status indicates that they could have a distinct biology and carcinogenesis process that contribute to NPC development.

The EBV genome detected from our sequencing experiment found that EBV was also methylated in NPC samples. Previous finding reported that EBV methylation pattern was consistent with the host methylation pattern.[19] There were specific EBV differentially methylated region between hypermethylated and hypomethylated EBV. Those EBV differential methylated regions have been tested by us and we found some samples where there was discordance between EBV methylation pattern and host methylation pattern. It was likely that the Southeast Asian EBV in NPC was not so similar with those reported in China and Hong Kong. Recent NPC and EBV epigenome study from Malaysian NPC sample also found this kind of methylation discordance pattern.[31] This finding worth further exploration to understand the biology of EBV methylation and host genome.

The comprehensive analysis of hypermethylated CpG regions in NPC samples by mapping hypermethylated CpG regions to known genes and performing methylation enrichment analysis, and we identified key genes involved in multiple pathways and biological processes. A critical finding of our study is the frequent hypermethylation of known tumour suppressor genes such as *PRKCB, PLCB3, ITGB3, EPHA2, PLCE1, PRKCD, CDKN2A, CDKN2B, RPS6KA2, ERBB4, LRRC4, AKT1, PPP2R5C, and STK11* from KEGG pathway analysis. These genes were also found to have lower RNA expression compared to normal nasopharynx in another independent transcriptome analysis.[30]

Among those genes identified, *PRKCB* and *PLCB3* emerged as the most prominent hypermethylated tumour suppressor genes, particularly within KEGG pathways. *PRKCB* encodes the beta isoform of protein kinase C (PKC), a family of serine/threonine kinases involved in various cellular processes, including proliferation, differentiation, apoptosis, and angiogenesis. *PLCB3* encodes a member of the phospholipase C (PLC) family, enzymes that play a vital role in intracellular signalling by generating second messengers like inositol trisphosphate (IP3) and diacylglycerol (DAG).[32] These molecules are critical for the regulation of calcium signalling, which influences various cellular functions such as cell growth, differentiation, and apoptosis.

In various cancers, PRKCB has been reported to play both oncogenic and tumour-suppressive roles, depending on the cellular context. For example, in some types of leukaemia and lymphoma, PRKCB activation is associated with oncogenic signalling, promoting cell survival and proliferation through the activation of pathways like NF-κB.[33,34] However, in solid tumours, including NPC, hypermethylation and subsequent downregulation of *PRKCB* could contribute to tumorigenesis by disrupting normal cell cycle regulation and apoptotic responses.[35,36] The loss of *PRKCB* function may lead to unchecked cell growth and resistance to apoptosis, facilitating the progression of cancer.

Furthermore, *PRKCB* has been linked to the regulation of angiogenesis, a process critical for tumour growth and metastasis.[37,38] Its downregulation could alter the tumour microenvironment, potentially promoting an aggressive cancer phenotype.[39] Given these multifaceted roles, the hypermethylation and reduced expression of *PRKCB* in NPC may represent a significant epigenetic alteration contributing to the malignancy's progression.

PLCB3 has been recognized for its role in modulating the signalling pathways that control cell proliferation and survival. Dysregulation of *PLCB3* has been observed in several cancer types,[40,41] where altered calcium signalling can contribute to malignant transformation and tumour progression.[42] In particular, *PLCB3* has been shown to interact with signalling pathways like the PI3K/AKT pathway, which is frequently activated in cancer and plays a pivotal role in promoting cell survival and growth.[42]

In the context of NPC, the hypermethylation of *PLCB3* and its reduced expression may disrupt these signalling pathways, leading to aberrant cell behaviour that supports carcinogenesis. Additionally, the role of *PLCB3* in modulating immune responses and inflammation could further influence the tumour microenvironment,[43] potentially contributing to immune evasion and sustained tumour growth.

GO Biological Processes (GO BP) enrichment analysis highlighted significant findings, with the WT1 gene showing high levels of hypermethylation and reduced expression in NPC samples. *WT1* is classified as tumour suppressor gene. However, this *WT1* tumour suppressor gene can turn out to have somehow dual role as a tumour suppressor and as an oncogene.[44] For instance, the *WT1* gene is recognized as a tumour suppressor gene, primarily associated with Wilms tumour.[44–46] It plays a critical role in regulating cell growth and maintaining normal cellular functions. Nevertheless, studies have also indicated that *WT1* may also act as an oncogene in certain contexts, adding complexity to its role in tumour biology.[45]

In the context of nasopharyngeal cancer (NPC) and epigenetic modifications, we observed significant hypermethylation of *WT1* across all NPC samples analysed. This hypermethylation corresponds with lower RNA expression levels, as confirmed by transcriptome data. If in the context of NPC, if it is considered an oncogene, then the suppression of *WT1* due to hypermethylation suggests a downregulation of its oncogenic potential, which could be beneficial in the context of cancer treatment. Our methylation analysis result can act as a guide on which genes are worthy to be further explored to determine its precise biological role.

Furthermore on the pathway analysis, there are some pathways such as WNT and TGFβ pathways where lower expression in NPC is associated with better prognosis.[47] It was found that alterations in the WNT signalling pathway were prevalent in certain TME-based subtypes of NPC,[47] consistent with our finding, further implicating this pathway in the disease's molecular landscape.[48] These findings suggest that the WNT pathway not only plays a role in epigenetic regulation but is also critical in defining the biological behaviour of NPC tumours.

Additionally, our methylation enrichment analysis identified several KEGG pathways and GO processes that were significantly enriched in the hypermethylated CpG regions. Notably, pathways such as the PI3K-Akt signalling pathway, ECM–receptor interaction, and focal adhesion were frequently enriched, highlighting their importance in NPC. The overlap of these pathways in hypermethylated samples underscores the potential mechanistic roles they play in tumour suppression and NPC progression.

The significance of the PI3K-Akt pathway in NPC is further corroborated by studies showing its involvement in cell survival, growth, and metabolism.[5] In particular, methylation changes in genes related to this pathway, such as *FANCI* and *POSTN*, have been linked to NPC progression. *FANCI*, a crucial player in DNA repair,[49] when hypermethylated, can lead to genomic instability and contribute to carcinogenesis. Similarly, *POSTN*, which is involved in the remodelling of the extracellular matrix,[50] plays a role in tumour metastasis and is also influenced by epigenetic changes. The involvement of ECM–receptor interaction and focal adhesion pathways suggests that changes in cell-matrix interactions are critical in NPC pathogenesis, potentially affecting cell migration, invasion, and metastasis.

Another significant aspect of our findings is the confirmed presence of Epstein–Barr virus (EBV) DNA in all NPC samples. This supports the established link between EBV infection and NPC.[51–53] EBV is known to contribute to NPC by influencing methylation patterns, which could explain the aberrant methylation observed in our samples. This connection suggests that EBV may trigger or exacerbate epigenetic alterations, creating a favourable environment for NPC development.

The identification of specific methylation patterns in tumour suppressor genes and key pathways offers potential biomarkers for early diagnosis and therapeutic targets for NPC.[10,54] Our findings underscore the importance of considering not only the presence of hypermethylation but also the involvement of these genes in multiple pathways and processes, as this may highlight their significance in the carcinogenic process. The distinct methylation signatures associated with NPC can be used to develop diagnostic tools that detect these epigenetic changes.[55] Additionally, targeting the methylation machinery or the affected pathways could provide new avenues for NPC treatment, potentially improving patient outcomes.

Conclusion

In conclusion, this study provides comprehensive insights into the epigenetic landscape of NPC, emphasizing the significant role of CpG methylation in tumour suppressor gene silencing and pathway dysregulation. The findings underscore the complexity of NPC epigenetics and pave the way for further research to explore the therapeutic potential of targeting these epigenetic alterations. The regional methylation signatures also suggest the need for tailored approaches in the diagnosis and treatment of NPC, considering the geographical and environmental context of the patients.

Data Access

The data has been submitted to the GEO database with accession number GSE272926.

The reviewer link to view the data: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE272926

The reviewer token to view the data: enubueiyprsbjgt

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Disclosure

The authors report no conflicts of interest in this work.

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