Integrative analysis in head and neck cancer reveals distinct role of miRNome and methylome as tumour epigenetic drivers

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Abstract

Background: Head and neck cancer is the sixth most common malignancy worldwide, with the relatively low 5-year survival rate, mainly because it is diagnosed at a late stage. Infection with HPV is well known etiology, which affects nature of these cancers and patients' survival. Besides, it is considered that the main driving force for this type of cancer could be epigenetics. In this study we aimed to find potential epigenetic biomarkers, by integrating miRNome, methylome, and transcriptome analyses.

Methods: From the fresh head and neck cancer tissue samples, we chose a group for miRNome, methylome and transcriptome profiling, in comparison to adequate control samples. Bioinformatics analyses are performed in R v4.2.2. Count normalization and group differential expression for mRNA and the previously obtained miRNA count data was performed with DESeq2 v1.36. Gene set enrichment analysis was performed and visualized using gProfiler2 v0.2.1 Identification of miRNA targets was performed by querying in miRTarBase using multiMiR v1.18.0. Annotation of CpG sites merging into islands was obtained from RnBeads.hg19 v1.28.0. package. For the integrative analysis we performed kmeans clustering using stats v4.2.2 package, using 8-12 clusters and nstart 100.

Results: We found that transcriptome analysis divides samples into cancers and controls clusters, with no relation to HPV status or cancer anatomical location. Differently expressed genes (n=2781) were predominantly associated with signaling pathways of tumour progression. We identified a cluster of genes under control of the transcription factor E2F, that are significantly underexpressed in cancer tissue, as well as T cell immunity genes and genes related to regulation of transcription. Among overexpressed genes in tumours we found those that belong to cell cycle regulation and vasculature. A small number of genes

were found significantly differently expressed in HPV positive *versus* HPV negative tumours (for example NEFH, ZFR2, TAF7L, ZNF541, and TYMS).

Conclusions: In this comprehensive study on an overlapping set of samples where the integration of miRNome, methylome and transcriptome analysis were performed for head and neck cancer, we demonstrated that the majority of genes were associated exclusively with miRNome or methylome and, to a lesser extent, under control of both epigenetic mechanisms.

Keywords: head and neck cancer, HPV, miRNome, methylome, transcriptome, integrative analysis

Background

Head and neck cancer is the sixth most common malignancy worldwide, with head and neck squamous cell carcinoma (HNSCC) accounting for more than 90% of cases. Unfortunately, HNSCC often gets diagnosed in a late phase when it is challenging to treat the tumour, which contributes to poor 5-year survival rate, currently at 66% (Johnson et al. 2020). Even though this type of cancer arises from a single cell type, i.e. the squamous cell, HNSCC is surprisingly of very heterogeneous nature (Canning et al. 2019). Primary site of tumour origin contributes to tumour heterogeneity, with most common sites being oral cavity, oropharynx, larynx, and sinonasal tract (Yan et al. 2011). Besides tumour location, the infection with high-risk human papilloma virus (HPV) types, especially in oropharyngeal area predominately affects HNSCC nature and patients' survival (Kobayashi et al. 2018). HPV positive and HPV negative HNSCC can substantially differ in terms of etiology, genetics and epigenetics (Joseph and D'Souza 2012; Johnson et al. 2020). HPV-positive HNSCC mostly affect younger population, with no or low levels of smoking and/or alcohol use (Gillison et al. 2015).

72 Moreover, unlike the HPV-negative group, preferable site of tumour origin for HPV-positive
73 HNSCC is oropharynx and it has been reported in many Western countries that this group of
74 patients is associated with better overall survival and better response to therapy (Marur et
75 al. 2010). There has been significant shift in the incidence of HPV-positive oropharyngeal
76 squamous cancer in Western countries, where HPV is associated to HNSCC in almost 70% of
77 cases (Marur and Forastiere 2016), and in fact, increase in 5-year survival of overall HNSCC
78 cases could be contributed to higher prevalence of HPV-associated HNSCC (Johnson et al.
79 2020).
80 Up to date, many studies reported better outcomes for HPV-positive HNSCC (Perri et al.
81 2020; Kofler et al. 2014; Sun et al. 2021), however the consensus on specific biomarkers,
82 which could improve diagnostic, prognostic and/or therapeutic approaches is still missing.
83 Lack of consensus biomarkers could partially be owed to differences in studied populations
84 sometimes inadequately registered tumour site of origin and possibly to the lack of
85 stratification by the HPV status. Therefore, we consider the cohort presented below to be a
86 specific case of tumours in terms of lifestyle, i.e. higher smoking and alcohol intake rates.
87 In this study, we aimed to identify epigenetic patterns in HNSCC since the main driving force
88 for the HNSCC development is considered to be epigenetics, rather than genetics. We have
89 previously analyzed the content and transcriptional levels of whole genome microRNA
90 (miRNA) profile (miRNome) in HPV-positive and HPV-negative oral and oropharyngeal
91 HNSCC samples using next generation sequencing and validated with qRT-PCR. We have also
92 analyzed the whole genome methylation profile (methylome) in the same set of samples
93 using the whole genome methylation array and validated by pyrosequencing. In this study,
94 we assessed the whole genome transcriptome analysis on the same sample set and
95 combined the mRNA, miRNA and methylation information to elucidate relevant epigenetic
96 mechanisms and their interactions. Motivated by the limited literature regarding integrative

97 studies on epigenetic changes, we set out to elucidate whether miRNAs or DNA methylation
98 have more prominent impact on HNSCC development. Moreover, we aimed to identify key
99 mechanisms and/or cellular signaling pathways of genes that have been differentially
100 expressed in HNSCC compared to healthy controls. Consequently, we provide a better
101 understanding of the epigenetic influence on the development and progression of HPV-
102 positive and HPV-negative HNSCC, shedding light on mechanisms underlying prognostic,
103 diagnostic and therapeutic strategies. In addition, integrative analysis points out key target
104 genes and signaling pathways deregulated in HNSCC in Croatian and similar populations.
105

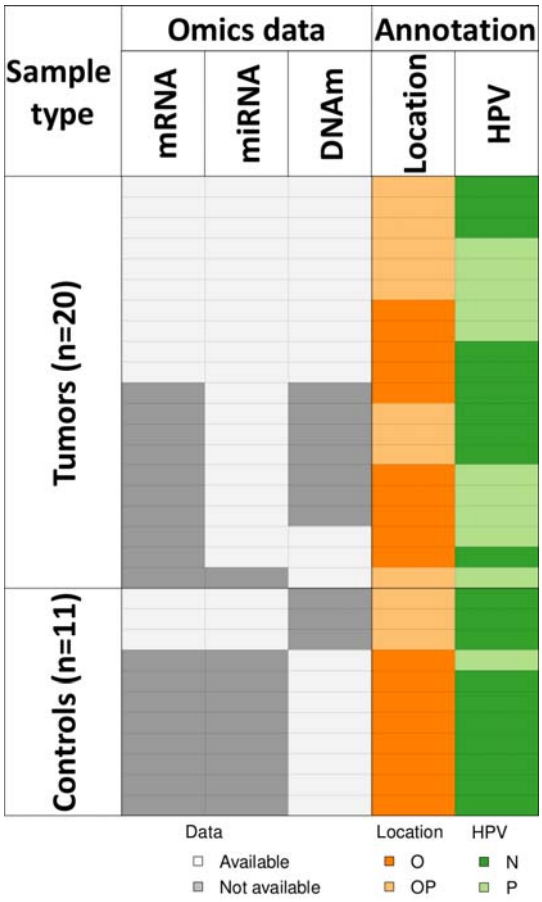
106 ## Material and Methods

107 ### Patient material

108 A total of 61 fresh oropharyngeal (OP) and oral (O) HNSCC primary tumour samples have
109 been obtained from Dubrava Clinical Hospital in the 2015-2018 time period. Twenty-two of
110 them and three non-malignant tonsil control samples were included in the miRNA profiling
111 (Božinović et al. 2019), column 2 in **Table 1**. Of 22 sequenced samples, two were excluded,
112 due to one sample being a recurrent tumour and one outlier. miRNome analysis and
113 detailed patients' socioepidemiologic and clinical data have been described in our previous
114 study . From the previously published analysis of HNSCC methylome (Milutin Gašperov et al.
115 2020), of 22 samples included in miRNA study, 12 HNSCC samples were included in the
116 whole genome methylation profiling (column 3, **Table 1**), as well as one additional sample
117 that had methylome but no transcriptome and miRNome profile. Eight healthy swabs of oral
118 mucosa were included in methylome profiling, which served as control samples .
119 For the transcriptome profiling, we selected 10 fresh HNSCC samples (6 oral and 4
120 oropharyngeal) with previous miRNome and methylome data (column 1, **Table 1**). Three

fresh tonsil tissues from the miRNome dataset were included in transcriptome profiling as control tissue. HPV status was assessed by PCR in all samples as described previously .

Table 1. Samples used in integrative analysis. Sequenced samples are shown in white. O=oral, OP=oropharyngeal, P= HPV-positive, N=HPV-negative.



Transcriptome profiling

As stated above, 13 samples were selected for transcriptome analysis in the current study to complement previously obtained omics results. Sample annotation for samples used in transcriptome analysis and subsequent integrative epigenetic study is presented in **Table 1**. Transcriptome was assessed using TrueSeq Stranded mRNA (Illumina) kit for library preparation, followed by sequencing on Nextseq500 instrument using NextSeq HIGH 150

cycles (Illumina) reagents on high output flow cell in paired end mode. Debarcoding was performed using Basespace (Illumina) platform. Quality control of sequencing data was performed with FastQC v0.11.9. Sequences were aligned to the human reference genome GRCh37 downloaded from GENCODE (released in 2013) and quantified using salmon v1.5.0, (Patro et al. 2017) reporting estimated fragment counts per transcript.

Bioinformatics preprocessing

All bioinformatics analyses listed hereafter were performed in R v4.2.2, and refer to R language packages, unless otherwise stated. The preprocessing consisted of transcriptome gene count determination, reprocessing of miRNA counts (for a subset of samples from Božinović 2019) and reprocessing of differentially methylated CpGs (for a subset of samples from Milutin Gašperov 2020), here expanded to CpG islands.

Count normalization and group differential expression for mRNA and the previously obtained miRNA count data was performed with DESeq2 v1.36. Pre-filtering of the data set was performed to reduce genes that have nearly no information about gene expression by filtering out genes and miRNAs with total counts across all samples less than 10. Count variance stabilization transformation was performed with regularized logarithm transformation (DESeq2 rlog function) and used as input to calculate principal components with plotPCA function from DESeq2 and Euclidean sample distances with dist function from the stats v4.2.2 package. Counts normalization was performed with DESeq function with log fold change shrinkage. We calculated differential expression in tumour *versus* control and HPV positive *versus* negative samples (**Table 1**). Differentially expressed genes (DEGs) and miRNA (DEmiR) were selected with BH-adjusted $p<0.05$ set as significant.

Gene set enrichment analysis (GSEA) was performed and visualized using gProfiler2 v0.2.1 on DEGs using annotated human genes as the background set for the hypergeometric test.

156 We applied the tailor-made multiple test correction method Set Counts and Sizes (SCS)
157 (Raudvere et al. 2019) and a p-value threshold of 0.05. GSEA was performed across multiple
158 databases: Gene Ontology: Biological Processes (GO:BP) (Ashburner et al. 2000), Kyoto
159 Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al. 2019), Reactome (Fabregat et
160 al. 2018), miRTarBase (Chou et al. 2018) and TRANSFAC (Matys et al. 2006). We used these
161 parameters for all subsequent GSEA.
162 Identification of miRNA targets was performed by querying significant DEGs against DEmiRs
163 using multiMiR v1.18.0 and the miRTarBase database (Huang et al. 2022), selecting only
164 experimentally validated miRNA-gene interactions.
165 Previously published DNA methylation (DNAm) data (Milutin Gašperov et al. 2020) was
166 loaded with RnBeads v2.14.0. Differential methylation (DM) was calculated comparing
167 tumour *versus* control groups on CpG sites and islands (CGIs). Annotation of CpG sites
168 merging into islands was obtained from RnBeads.hg19 v1.28.0 package. All results are
169 presented on CGIs unless otherwise stated. CGI DM was defined as the quotient of
170 methylation between groups across all CpG sites in a CGI, with significance level set to FDR-
171 adjusted p<0.05. Promoter region annotations were obtained from the RnBeads package,
172 defined as 1500 bases upstream and 500 bases downstream of the transcription start site.
173 CGI targets were assigned where CGI overlapped a promoter region.

174 **Integrative analysis**

175 Workflow for comparing all omics data and identifying specific mechanisms in HNSCC is
176 summarized in **Figure 1**.

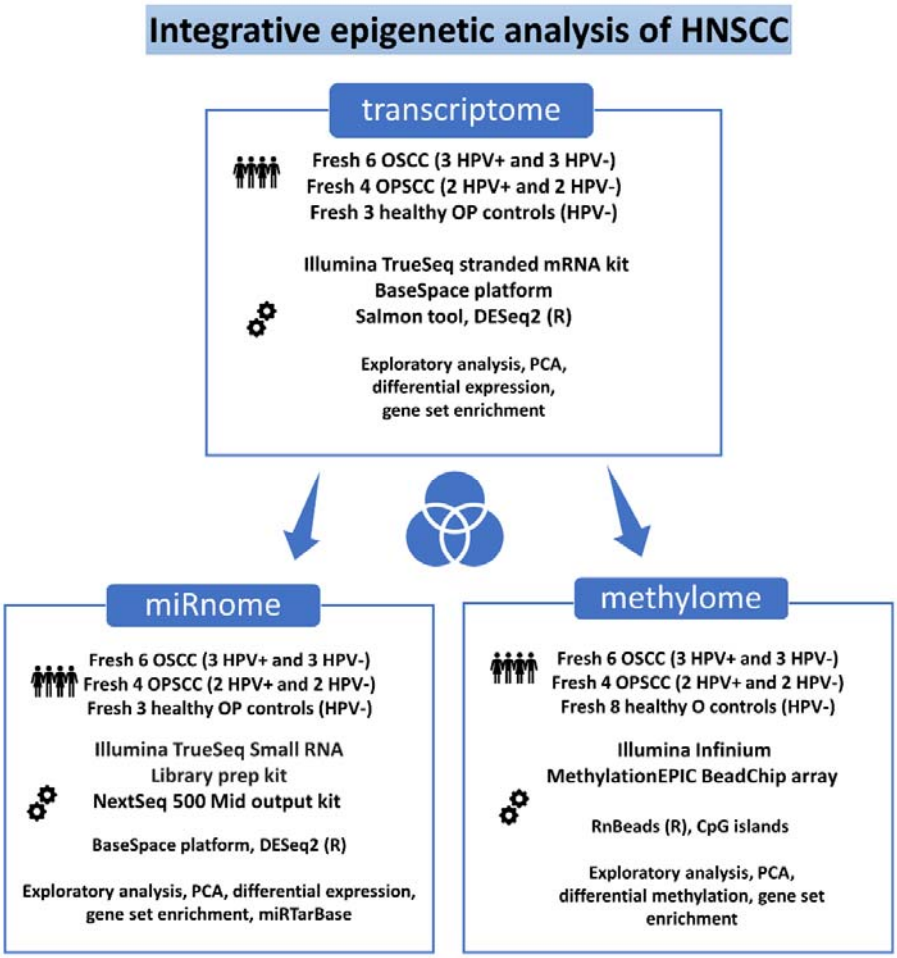


Figure 1. Schematic representation of integrative epigenetic HNSCC study.

Three -omics datasets were compared, centered on identified DEGs (**Supplemental table S1**). Multiple miRNA-gene pairs were reduced by selecting only the miRNA with highest absolute value of Spearman correlation of counts (mirRNA_Rho) for the given DEG. Multiple gene–CGI pairs were not as common, and the CGI with the lowest p-value was selected for every DEG. Resulting table contained DEGs and their associated miRNA and/or CGI. We assessed the interplay of DEmiRs, DM CGIs and DEGs in two ways: by obtaining simple overlap of DEGs targeted by DEmiRS or DM CGIs and kmeans clustering. The simple overlap was based on whether DEG's expression levels were associated with DEmiR's activity and/or CGI

188 methylation levels. Kmeans clustering was performed on log-transformed, centered and
189 scaled values of fold change for DEGs and DEmiRs, and DNAm quotient. We performed
190 kmeans clustering using stats v4.2.2 package, using 8-12 clusters and nstart 100, with the
191 best performing result for 11 clusters.
192

193 ## Results

194 ### Transcriptome profiling

195 The whole transcriptome profiling was performed on tissue samples from 13 individuals with
196 different anatomical locations and HPV infection status (**Table 1**). The top two principal
197 components of the transcriptome (**Figure 2a**), which explain 55% of the variance in the
198 samples, splits samples into two clusters of tumour and control samples, with no obvious
199 contribution of HPV status or tumour anatomical location. We observe the same pattern in
200 miRNome and methylome data (**Figure 2b** and **c**).

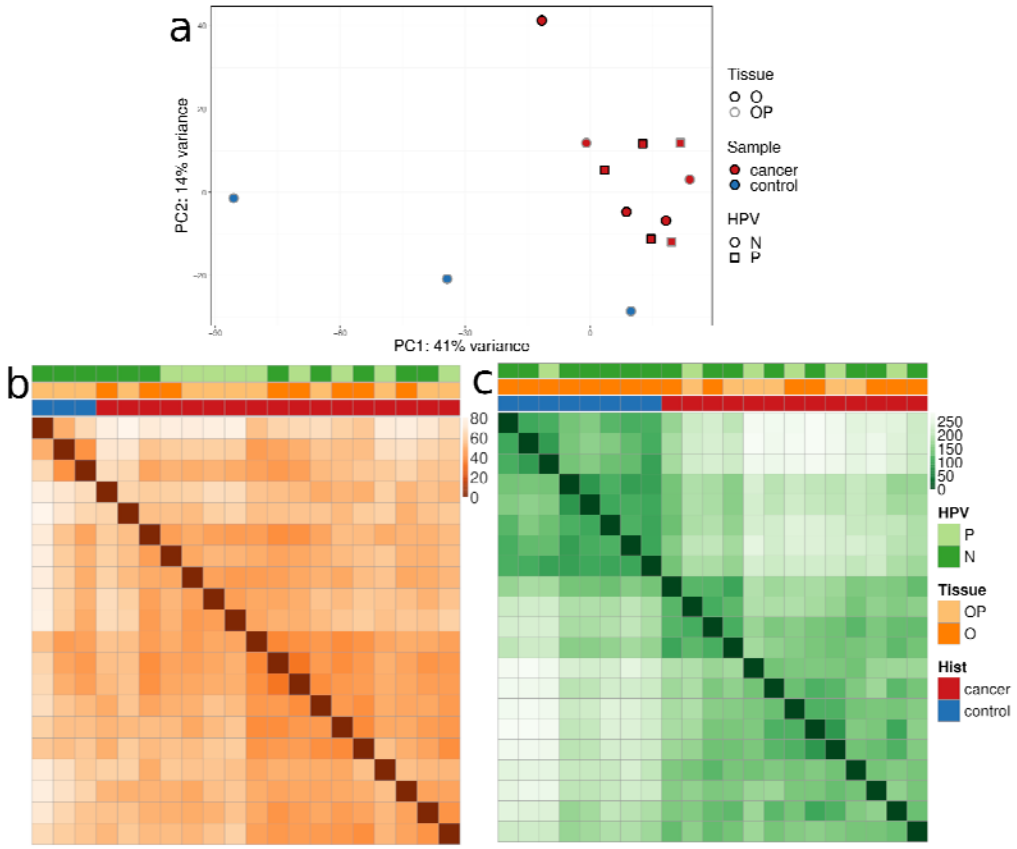
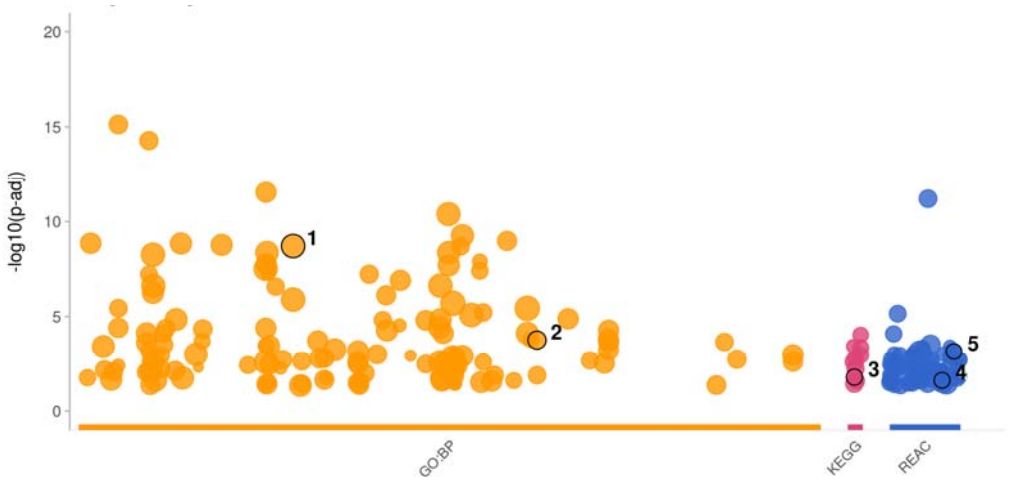


Figure 2. Exploratory analysis of three omics data sets: a) PCA plot for transcriptome data; b) sample distance heatmap for miRNome data; c) sample distance heatmap for methylome. N - HPV-negative, P - HPV-positive O - oral tumours, OP - oropharyngeal tumours.

Differential expression and GSEA

Exactly 18104 genes had no less than 10 detected reads across samples. Differential expression analysis revealed that 1248 (6.9%) of genes were significantly overexpressed and 1533 (8.5%) were significantly underexpressed in tumour (BH-adjusted $p<0.05$), when compared to transcriptomes of control samples. GSEA on DEGs showed enrichment of signaling pathways associated with tumour progression, such as cell cycle regulation and oncogenic signaling pathways such as NF-kB (**Figure 3**), for a full list of terms see **Supplemental table S2**.



id	source	term id	term name	term size		p value
1	GO:BP	GO:0032502	developmental process	4582		2.0e-09
2	GO:BP	GO:0070661	leukocyte proliferation	235		1.8e-04
3	KEGG	KEGG:04064	NF-kappa B signaling pathway	102		1.6e-02
4	REAC	REAC:R-HSA-453276	Regulation of mitotic cell cycle	88		2.4e-02
5	REAC	REAC:R-HSA-8852276	The role of GTSE1 in G2/M progression after G2 checkpoint	79		7.0e-04

Figure 3. Gene enrichment analysis of tumour *vs.* control samples. Databases shown are GO:BP (orange), KEGG (pink), and REACTOME (REAC, blue). The highlighted terms correspond to key concepts associated with cancer progression and regulation.

Differential miRNA and DNA methylation assessment

Integration of 514 significant DEmiRs based on MiRNome data (Božinović et al. 2019) with the database containing experimentally supported miRNA-target gene interactions yielded 3042 interactions among 90 different miRNAs and 1227 target genes, hereafter termed DEmiR-DEGs. In our analysis Most of these genes were regulated by a single miRNA, however some were associated with multiple miRNAs. Notably, two genes with the highest number of associated DEmiRs, *BCL2* and *MYC*, which play a role in regulating cell proliferation and apoptosis, and were associated with 18 and 16 miRNAs, respectively. In addition, some miRNAs were associated with many target genes, harboring potential therapeutic value by potentially affecting many cancer-related signaling pathways. An extreme example is a frequently reported tumour suppressor hsa-miR-26b-5p putatively

230 forming interactions with 255 unique DEGs. Within our dataset hsa-miR-26b-5p showed a
231 high negative expression correlation with known oncogenes such as *MMP10* (Rho= -0.81,
232 p<0.005), *IGSF3* (Rho= -0.79, p<0.005) and *ARNTL2* (Rho= -0.79, p<0.005) (**Supplemental**
233 **table S1**).

234 Overall methylation levels in our samples showed that more than 300,000 CpG sites were
235 hypomethylated in tumour samples compared to controls (FDR-adjusted p<0.05), while
236 72,000 were hypermethylated (**Supplemental table S3**). We also noted a larger number of
237 hypomethylated CGIs (6557) than hypermethylated (5457), although the difference is not as
238 pronounced as in CpG sites (**Supplemental table S4**). The DM CpGs predominantly fell into
239 gene bodies, but also some fell in intergenic regions corresponding to repeated elements.
240 These regions consistently showed a larger number of hypomethylated CpGs compared to
241 hypermethylated ones (**Supplemental figure S1**).

242 To identify deregulated tumour suppressors in our samples, we used the TSGene database
243 2.0 (Zhao et al. 2016) containing tumour suppressors curated from several thousand
244 publications combined with expression and mutational profiles. We pinpointed 10 genes
245 that were underexpressed and contained hypermethylated CGIs in the promoter region in
246 our samples (**Supplemental table S5**).

247 To assess the effects of transcription factors on DEGs that had differentially methylated CGIs
248 (DM-DEGS) within our dataset we performed GSEA using transcription factor database
249 TRANSFAC. We found associations with several groups of transcription factors including E2F,
250 SP/KLF and AP-2 families (**Supplemental table S6**).

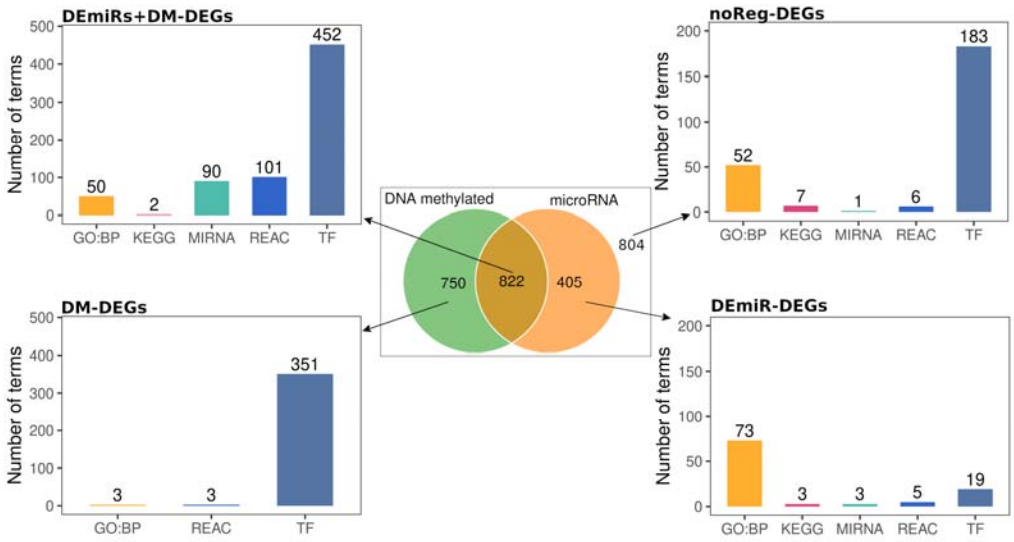
251 Of note we found the enrichment of genes regulated by the E2F family of TFs (SCS-adjusted
252 $p<10^{-43}$), which play a critical role in cell cycle control. Furthermore, GSEA on the same
253 dataset using the Reactome database showed enrichment of genes involved in cell cycle
254 regulation such as G1/S Transition and G2/M Checkpoints (SCS-adjusted $p<10^{-4}$), supporting

255 the above findings.

256 **Integrative analysis: Influence of miRNome and methylome on transcription on**

257 **transcriptional activity in HNSCC**

258 Each DEG was grouped based on the association with DEmiR or DM CGI, shown in **Figure 4**.
259 We obtained four groups: DEGs associated only with DEmiR (DEmiR-DEGs), DEGs associated
260 only with DM CGIs (DM-DEGs), DEGs associated with both DEmiR and DM (DEmiR+DM-
261 DEGs), DEGs not associated with either DEmiR or DM CGIs (noReg-DEGs). The groups had
262 different proportion of differentially represented GSEA terms, i.e. GO:BP, KEGG, miRTarBase
263 (MIRNA), Reactome and TRANSFAC (TF) (**Figure 4**, **Supplemental table S7**). The DM-DEGs
264 group were enriched by genes regulated by TFs but no significant results of GO biological
265 pathways was detected. The DEmiR-DEGs group were enriched with pathways related to
266 signaling and immunological systems. The noReg-DEGs group was enriched with immunological
267 processes and muscle system processes. DEmiR+DM-DEGs were enriched with general regulatory
268 processes: biological, metabolic and cellular processes. These terms are high level terms in GO
269 biological processes, suggesting that genes in the intersection have broad regulatory roles.



270

271 **Figure 4.** DEGs between tumour and control samples, in association with DEmiR or DM
272 promoters. Four panels show number of terms from GSEA for each group.

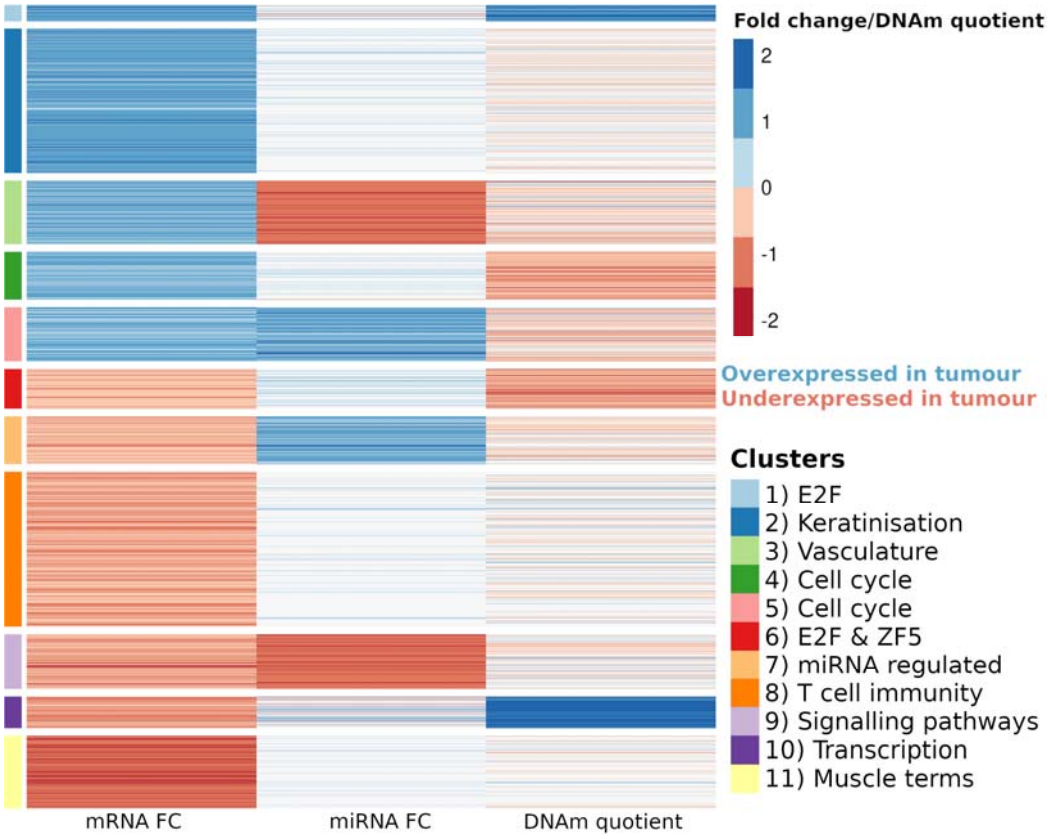
273 Clustering of DEGs with accompanying DEmiR and DM levels revealed a critical distinction in
274 the epigenetic factors driving HNSCC. The identified clusters predominantly demonstrated
275 either significant DEmiRs or DM. This suggests that in HNSCC, gene expression is
276 predominantly influenced by either miRNA regulation or methylation, but not both
277 concurrently. This key finding illuminates the mutually exclusive roles of miRNA and
278 methylation in shaping the gene expression landscape in HNSCC.

279 It is generally believed that miRNA and DNAm have an inhibitory effect on gene expression,
280 and this pattern was evident in clusters 3, 4, 7 and 10. In addition, we observed miRNA
281 expression and methylation patterns in clusters 1, 5, 6, and 9 that were indicative of
282 noncanonical regulatory activity. It is important to mention that due to multiple target genes
283 of miRNAs there was a significant overlap of underexpressed miRNAs in clusters 3 and 9
284 (73%) and overexpressed miRNAs in clusters 5 and 7 (77%).

285 GSEA analysis of each cluster linked expression and epigenetic patterns to biological function.
286 Representative terms that best described each cluster are shown in **Figure 5** and the full
287 GSEA results for each cluster are shown in **Supplemental table S7**. DM-DEGs in clusters 1, 4,
288 6 and 10 were highly enriched with genes regulated by the E2F family of TFs. Most of the
289 genes from the E2F family were not differentially expressed in our samples (**Supplemental**
290 **figure S2**), although it has been previously shown that the methylation status of CpG sites of
291 E2F binding motif influences the binding of the transcription factor (Campanero, Armstrong,
292 and Flemington 2000; Yin et al. 2017). In addition, cluster 6 contained underexpressed genes
293 with hypomethylated CGIs and was enriched with genes regulated by the ZF5 transcription
294 factor, which can function as a repressor (Numoto, Yokoro, and Koshi 1999). Clusters 4 and 5
295 were associated with similar terms related to various cell cycle processes but have different

296 patterns of miRNA expression and DNAm.

297



298

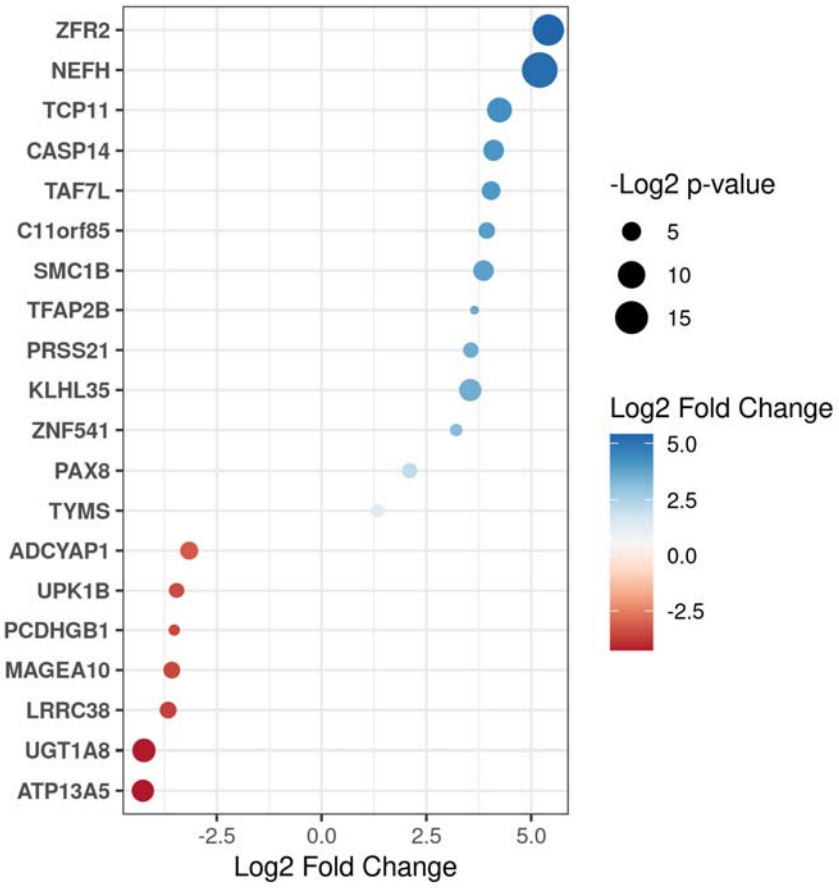
299 **Figure 5.** Kmeans clustering on integrated transcriptome, miRNome and methylome data on
300 DEGs. Fold change values for transcriptome and miRNome and DNAm quotient were scaled
301 and normalized prior to clustering. Eleven clusters are functionally described by applying
302 GSEA over several databases (GO:BP, KEGG, REACTOME, MIRNA and TRANSFAC).

303 Influence of HPV

304 HPV influence on gene expression was found to be inconclusive (**Figure 2**). We observed a
305 small number of genes and miRNAs, and no DM CGIs or sites that were significantly changed
306 while comparing HPV positive and negative tumour sample groups (FDR-adjusted $p<0.1$).
307 Number of DEGs associated with HPV status was 44, of which 25 were overexpressed
308 (**Supplemental table S9** and **S10).** Among these, we focused on the selection of DEGs found

309 to be functionally relevant and/or previously found to be differentially expressed in HPV-

310 associated cancers (**Figure 6**).



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312 **Figure 6.** Top 20 DEGs in HPV positive compared to HPV negative tumour patients.

313

314 Discussion

315 In this study, we aimed to integrate miRNome and methylome analysis with transcriptome

316 analysis on the same samples to elucidate whether miRNAs or DNAm have more prominent

317 impact on HNSCC development. The main goal was to identify key mechanisms and/or

318 cellular signaling pathways of DEGs in HNSCC compared to healthy control samples.

319 Herein, we observed that transcriptome analysis divided samples into two major clusters:

320 tumour and control samples, with no obvious contribution of HPV status or tumour
321 anatomical location. Similar results were seen in the miRNA expression profile study and
322 DNAm study in an overlapping set of samples (**Figure 2 b** and **c**) as previously published
323 (Božinović et al. 2019; Milutin Gašperov et al. 2020). This suggests that these tumours may
324 share similar cells of origin or common pathways during tumourigenesis.
325 The emergence of tumour progression pathways among DEGs (n=2781, **Figure 3**) resembles
326 transcriptomes of radioresistant HNSCCs, where hypoxia response, p53 pathway, NF-kB
327 pathway and inflammatory response were found abnormally activated. Interestingly, some
328 authors found that NF-kB and other transcription factors signatures differentiate HPV-
329 positive and HPV-negative HNSCCs.
330 Using unsupervised clustering, we found a cluster of genes under control of the transcription
331 factor E2F, that were significantly underexpressed in tumour samples in comparison to
332 controls (**Figure 5**, cluster 6). This is in line with overall hypomethylation in tumours and
333 points to exclusive control of overexpressed miRNAs. The genome-wide hypomethylation of
334 CpG sites is a known phenomenon that occurs in most cancers (Gama-Sosa et al. 1983;
335 Debernardi et al. 2021). E2F family members expression was also found to be negatively
336 regulated by miRNAs in other cancers, like lung cancers, pediatric brain tumours, and
337 chronic myeloid leukemia. In addition, genes that were significantly associated with the E2F
338 were found in clusters 1, 5 and 10 (**Supplemental table S6**).
339 In cluster 8, we found genes related to T cell immunity underexpressed in tumour samples in
340 comparison to control samples (**Figure 5**), mostly found to be targets of overexpressed
341 miRNAs, and in less extent associated with hypermethylation. This finding is in line with
342 trends detected in several previous methylation studies of head and neck cancers (Moody et
343 al. 2020; Anić et al. 2023), and in cervical cancers (Farkas et al. 2013; Milutin Gašperov et al.
344 2014).

345 Among underexpressed genes in tumours, we observed a cluster of DM-DEGs related to
346 regulation of transcription (**Figure 5**, cluster 10). Our result showed that the cluster mostly
347 contains genes with hypermethylation of CGIs in promoter regions and some genes that
348 were targeted by overexpressed miRNAs. This implies that the hypermethylation of CGIs in
349 promoter region of genes in this cluster may have disrupted binding of transcription factors
350 required for their expression, that is a well-studied phenomena (Eden and Cedar 1994;
351 Pikaart, Recillas-Targa, and Felsenfeld 1998; Bird 2002).
352 Among overexpressed genes in tumours were those that belong to cell cycle regulation
353 (**Figure 5**, clusters 4 and 5) and vasculature (**Figure 5**, cluster 3). Herein, we found cell cycle
354 genes have mostly hypomethylated promoter regions. On the other hand, our result showed
355 that genes involved in vascularization in tumours are DEmiR+DM-DEGs, likely influenced by
356 both epigenetic drivers. Moreover, other authors found a link with DNAm and cardiovascular
357 diseases directly rather than changes driven by miRNA expression (Goossens et al. 2019), or
358 that both epigenetic changes are involved in vascular diseases, claiming that, the most
359 prominent gene cluster is activated via hypomethylation (Aavik et al. 2015). Interestingly,
360 the same authors found keratin mRNAs upregulated in atherosclerosis and concluded that
361 DNA hypomethylation may play a role in keratin gene activation and ectopic keratin
362 expression (Aavik et al. 2015). Keratins are characteristic to epithelial cells and are not
363 expressed in normal arteries and induction of keratin expression in atherosclerosis has been
364 detected even earlier (Slomp et al. 1997). Our study detected similar findings in HNSCC;
365 keratin mRNAs were found upregulated, that was partially under control of DNAm and
366 partially under control of miRNome (**Figure 5**, cluster 2).
367 Herein, we found that cluster 5 (**Figure 5**) contained upregulated miRNA and their
368 corresponding upregulated target genes. Conversely, cluster 9 displayed downregulated
369 miRNA and corresponding downregulated target genes. This positive correlation of miRNAs

370 and target genes has been previously observed in multiple studies (Tan et al. 2019; Xu et al.
371 2020). While our results could imply either a direct or indirect effect of miRNA on DEmiR-
372 DEGs potentially through negating the action of repressive miRNA, protein complexes (Tan
373 et al. 2019), it is important to note that our findings represent correlation, not causation.
374 Further studies are required to establish the causal mechanisms involved. In addition, it is
375 worth considering that miRNAs often have multiple targets, and the interactions in existing
376 databases may be derived from high-throughput methods that may not reflect the full
377 complexity of in vivo biological interactions.
378 A small number of genes were found significantly differentially expressed in HPV positive
379 *versus* HPV negative tumours (**Figure 6**) in our study, a subset of which was previously found
380 differentially expressed in head and neck cancer and related to HPV status, i.e. *NEFH*, *ZRF2,*
381 *TAF7L, ZNF541* and *TYMS*. Notably, some authors have already found hypermethylation of
382 *NEFH* (protein kinase binding and microtubule binding), in pharyngeal squamous cell
383 carcinoma, which significantly correlated with HPV positivity (Nakagawa et al. 2017). *ZFR2*
384 gene codes for RNA-binding protein characterized by its domain associated with zinc fingers
385 and zinc ions. It was found overexpressed in HPV-positive HNSCC patients when compared
386 to HPV-negative patients and has been flagged as prognostic for the cervical cancer cases
387 (Tripathi et al. 2020). The expression of the transcription factor *TAF7L* (TAF7-like RNA
388 polymerase II) has been already found increased, even 220-fold, in HPV positive HNSCCs
389 (Slebos et al. 2006). *ZNF541* encodes a zinc finger protein supposed to be a component of
390 chromatin remodeling complexes. The hypomethylation and higher expression of this gene
391 was also observed in HPV-positive oropharyngeal squamous cell carcinoma (OPSCC) and
392 significantly associated with a better overall survival, independent of HPV status (Camuzi et
393 al. 2021). *TYMS* gene codes for thymidylate synthetase that catalyzes the methylation of
394 deoxyuridylate to deoxythymidylate. In HPV-positive cancers they have found it

395 overexpressed, indicating that HPV-positive oropharyngeal cancers may be more resistant to
396 5-fluorouracil chemotherapy than HPV-negative (Lohavanichbutr et al. 2009).
397 To our knowledge, we presented the most comprehensive study on an overlapping set of
398 samples where the integration of miRNome, methylome and transcriptome analyses in
399 HNSCC was conducted. We acknowledge certain limitations of our study, including the
400 relatively small sample size, which may affect the validity of our findings. In addition,
401 obtaining control samples that are genuinely representative of healthy tissue poses its own
402 set of challenges. Associating miRNome and methylome with transcriptome profiles, we
403 found many DEGs in HNSCC in comparison to control samples, that were mainly associated
404 with cell cycle regulation, transcription, immunity and tumour development. We
405 demonstrated the majority of genes belong to clusters associated exclusively with miRNome
406 or methylome and, to a lesser extent, under control of both epigenetic mechanisms.
407

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Declarations

Ethics approval and consent to participate

All patients provided informed consent to participate at the time of primary cancer treatment. The collection of samples was approved by Bioethical Bord of the Ruđer Bošković Institute (BEP-3748/2-2014) and the Ethical Board of the Clinical Hospital Dubrava (EP- KBD-10.06.2014).

Consent for publication

Not applicable - no personally identifiable information is presented in the manuscript.

Availability of data and materials

Code used is available at https://github.com/PKatarina/HNSCC_Grce

Raw datasets analyzed during the current study are available in the Figshare repository under the following DOI identfiers:

Transcriptome https://doi.org/10.6084/m9.figshare.24721746

miRNome https://doi.org/10.6084/m9.figshare.24721731

Methylome https://doi.org/10.6084/m9.figshare.24721728

All datasets and raw data will be deposited in ArrayExpress repository as well. Upon publication links will be replaced by accession numbers

Due to the file size restrictions Supplementary table S3 is deposited at Figshare repository https://doi.org/10.6084/m9.figshare.24722268

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

NMG, IS, MG and AB designed the study. KB, ED, JK, NS, IS and KM collected and processed the data. KM performed the analyses. KM, KB and NMG wrote the first draft of the manuscript. MG, IS and AB supervised and acquired funding for the study. All authors contributed to the writing of the final manuscript. All authors read and approved the final manuscript.

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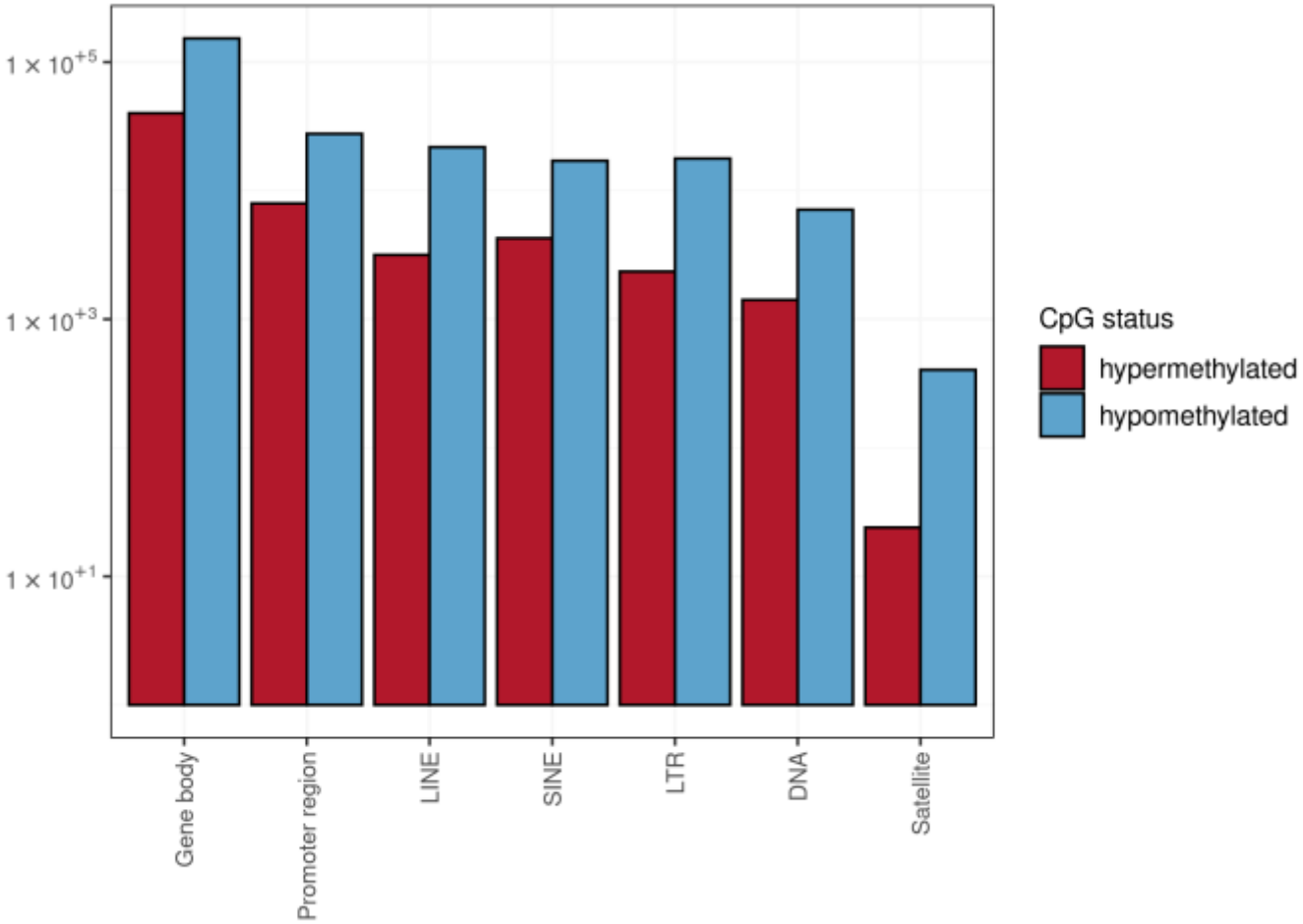
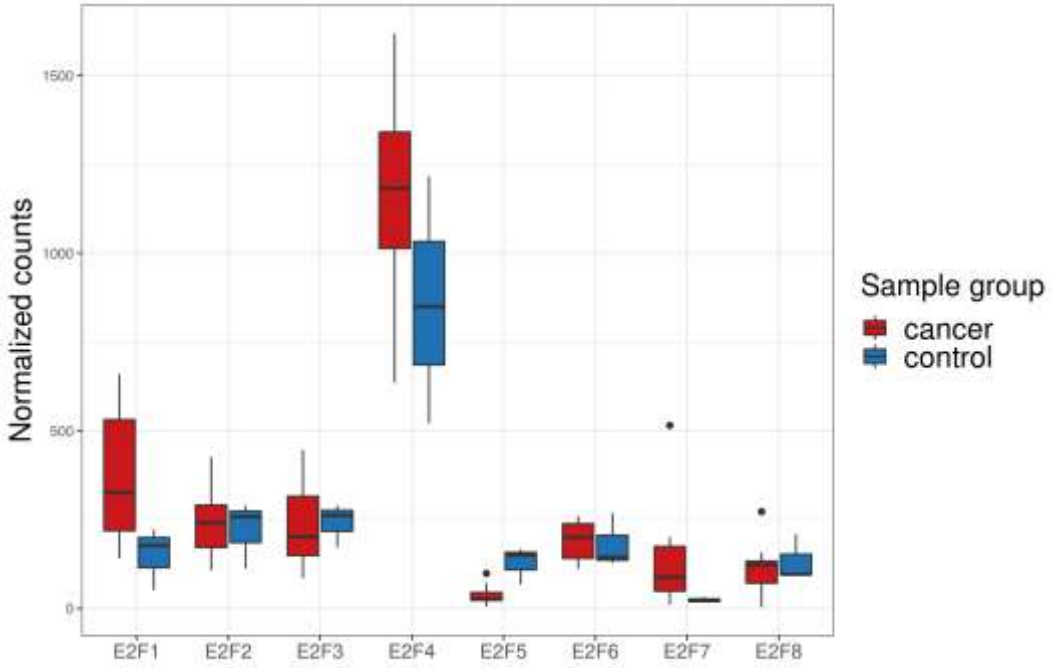
Supplemental figures

Integrative analysis in head and neck cancer reveals distinct role of miRNome and methylome as tumour epigenetic drivers

Katarina Mandić[1]*, Nina Milutin Gašperov[2]*, Ksenija Božinović[2], Emil Dediol[3], Jure Krasić[4], Nino Sinčić[4,5], Magdalena Grce[2], Anja Barešić[1]**, Ivan Sabol[2]**



Suplemental figure 1. Significant number of hypermethylated and hypomethylated CpGs in cancer vs. control groups across all genomic elements (Chi squared p < 2x10^-16). Labels: Gene body – across exons and introns; Promoter region – from 500 to 2000 distance from canonical transcription start site; LINE – Long interspersed nuclear elements; SINE – Short interspersed nuclear elements; LTR – Long terminal repeat elements which include retroposons; DNA – DNA repeat elements; Satellite – Satellite repeats. Annotation for genomic elements were used from Jin et al., 2015.



Supplemental figure 2. Expression of E2F family genes in cancer vs control samples.