Identification and characterization of the hypoxia response regulatory elements in the accessible genome

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Abstract:

Hypoxia is commonly observed in the solid tumor and contributes to the drug resistance in cancer therapy. Deciphering the epigenetic feature under the hypoxia condition in the solid tumor is critical for us to understand the tumorigenesis and design the precision therapy. Using the time series of ATAC-seq data under the hypoxia treatment from the epithelia cells, we identified the hypoxia response regulatory elements (HRREs) in the accessible genome. We found that these different HRREs have unique genomic features and are enriched with different transcriptional factors (TFs). Our study provides insights into the chromatin structure response to the hypoxia treatment and identifies useful genomic features for a better understanding of the hypoxia biology in the solid tumor.

Key Words: Hypoxia, Chromatin, regulatory elements.

32 **Introduction:**

33 Hypoxia is commonly observed in the solid tumor¹⁻⁴. The cancer cells in the solid tumor are
 34 found to alter their behaviors and metabolism under the hypoxia microenvironment, and
 35 further resist the cancer therapy^{5,6}. In the molecular level, hypoxia induces the production of
 36 the hypoxia-inducible factors (HIFs) and other transcriptional factors (TF), modulates the
 37 chromatin structure, and further changes the gene expression in the cancer cells⁷⁻¹⁰. Thus, a
 38 detailed characterization of the chromatin structure changes under the hypoxia could help us
 39 to understand the tumorigenesis in the hypoxia microenvironment of the solid tumor and to
 40 design a precision therapy.

41 ATAC-seq technology is a powerful tool to decipher the chromatin structure with high
 42 sensitivity¹¹. Previously, we demonstrated that the low level of oxygen could modulate the
 43 chromatin accessibility in the mouse breast tumor cell line 4T1 with ATAC-seq technology,
 44 and we observed that the openness of a large number of regulatory elements are dynamically
 45 changed during the time series of hypoxia treatment. We concluded that the hypoxia
 46 microenvironment alone could reshape the transcriptional factor (TF) motif openness in the
 47 genome wide¹². However, to better understand the gene regulation under the hypoxia
 48 environment, a detailed characterization of the dynamic changes of the hypoxia response
 49 gene regulatory element is needed. Here, we performed a systematic annotation of the
 50 hypoxia response gene regulatory elements in the 4T1 cells, and identified three different
 51 types of hypoxia response gene regulatory elements (HRREs). We also found that there are
 52 different genomic features and TFs bindings on different types of HRREs.

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55 **Results:**

56 **Identification of the hypoxia response gene regulatory elements**

57 To characterize the hypoxia response gene regulatory elements, we included all the ATAC-
58 seq data from the 4T1 cells under 1% O₂ for various time points (0, 6, 12, 24, 36, 48, and 72
59 h)¹². We used the 0h as the normoxia control for the time series comparison. When we
60 compared the ATAC-seq peaks from each time point of hypoxia treatment with normoxia
61 control, we identified 6,750 significant differential peaks ($|\log FC| > 1$, FDR < 0.01) (**Figure 1a**,
62 See **methods**). The openness of these significant differential peaks is modulated with the
63 hypoxia condition; thus, these 6,750 peaks are termed hypoxia response gene regulatory
64 elements (HRREs). Among these HRREs, a large proportion of regions, 5,015/6,750 (74.3%),
65 start to open under the hypoxia condition, and only a small part of regions, 1,735/6,750
66 (25.7%), start to close with the hypoxia treatment (**Figure 1a**).

67
68 Next, we investigated the dynamics of these HRREs across the time points (see **Methods**).
69 Interestingly, we found the openness of 4,722 HRREs follows some trend to change during
70 the hypoxia treatment. With the unbiased cluster methods¹³, we identified three different
71 types of changes among these 4,772 HRREs, and named them clusters 1-3 (**Figure b-e**). The
72 HRREs in cluster 1 (n = 2,229) came to open gradually under the hypoxia treatment
73 compared with the control and reached the highest accessibility at 72h. We observed that the
74 HRREs in cluster 2 (n = 1,358) closed gradually during the hypoxia treatment. We also found
75 that the openness of HRREs in cluster 3 (n = 1,135) fluctuated during the time series of
76 hypoxia, but had the highest accessibility after the 72h hypoxia treatment (**Figure 1f, 1g**).

78 **Characterization of the hypoxia response gene regulatory elements**

79 Different types of HRREs under the hypoxia condition indicated different parts of genome
80 responses differently to the hypoxia microenvironment. To understand the detailed
81 mechanism of how different HRREs react differently to the hypoxia, we explored the
82 genomic features of different HRREs in a systematic manner. When we performed the
83 genomic annotation with all the HRRE peaks, and three different HRREs separately (**Figure**
84 **2a**), we found that the genomic annotation of HRREs in clusters 1 and 3 were similar to all
85 the merged accessible peaks (**Figure 2a**), but the genomic annotation of HRREs in cluster 2
86 was quite different from the others (**Figure 2a**). In cluster 2, around 25% of the HRREs are
87 from the promoter-TSS regions. This observation is further confirmed from the distance
88 annotation strategy on the different groups of HRREs (**Figure 2b**), where it is clearly shown
89 that more than 25% of the HRREs in cluster 2 are located within 1k distance from the
90 transcriptional starting site. At the same time, the proportion of intergenic regions or distance
91 between 10K-100k in cluster 2 is significantly less than the other clusters. Since the HRREs
92 in the clusters start to close under the hypoxia treatment, our result indicates that a big
93 proportion of promoter regions in cluster 2 react to the hypoxia treatment. It was reported that
94 the histone modification, H3K27ac, is associated with a higher activation of transcription and
95 is found at both the proximal and distal regions of the transcription start site¹⁴. Thus, we
96 assumed that the HRREs in cluster 2 are enriched with the H3K27ac histone modification.
97 When we used the H3K27ac ChIP-Seq data from the normoxia condition to calculate the
98 H3K27ac distribution in the HRREs from each cluster (See **Methods**)¹⁵, we found indeed

that there is a significant enrichment of H3K27ac in cluster 2 compared with clusters 1 and 3 (**Figure 2c**). The significant enrichment of H3K27ac in cluster 2 indicates that a big proportion of transcriptional active sites in the normoxia become silent in the hypoxia condition.

It was shown that the accessibility of genomic regulatory elements is regulated by the transcription factors binding on the genome^{16,17}. To understand what types of transcriptional factors regulate the different clusters of HRREs, we performed a motif enrichment for each cluster of HRREs and identified the unique transcriptional factors (TF) for each cluster (**Figure 2d**). (See **Methods**). We found that there are 152 TFs enriched in cluster 1(**Table 1**), 76 TFs enriched in cluster 2(**Table 2**), and 91 TFs enriched in cluster 3(**Table 3**) (See **Methods and Supplementary materials**). Interestingly, there are 42 shared TFs enriched in all the clusters (significant, $q < 0.05$) (**Table 4**), and the common TFs in three different clusters demonstrated that there are common mechanism for three HRREs under the hypoxia treatment. After excluding the common TFs, we obtained a list of unique TFs identified for each cluster (See **Table 1-3**), and these specific TFs regulate the dynamic changes of chromatin accessibility in each cluster under the hypoxia treatment. Even Clusters 1 and 3 have the tendency of opening under the 72h hypoxia treatment, but the unique list for their TFs is quite different. There are 52 unique TFs for cluster 1, and 6 TFs for cluster 3. Surprisingly, the hypoxia induced protein factors, HIF1a and HIF2a, are only enriched in cluster 1, not the others (**Figure 2d**). The enrichment of hypoxia factors in cluster 1 probably explains why it is continuously open during the entire treatment time. Even cluster 3 has a tendency to stay open after 72 h; however, there is some fluctuation in openness during the time series treatment, which is probably because of the lack of hypoxia factor regulation on the HRREs in cluster 3. At the same time, it also indicates that the HRREs in cluster 3 are regulated differently from cluster 1 (**Figure 2d**). In cluster 2, 15 unique TFs were identified. The Motif bHLHE41, Nkx6.1, and CRE could be seen when regulating the chromatin structure¹⁸⁻²⁰, and it indicates that the decrease in the chromatin accessibility in cluster 2 is regulated by these 15 TFs under the hypoxia environment.

In summary, the unique genomic feature and TFs enrichment in the different clusters of HRREs potentially reflect the different mechanisms in the reactions to the hypoxia microenvironment.

Discussion:

It was clearly shown that the hypoxia condition affects the gene expression²¹. Numerous studies have demonstrated that the hypoxia microenvironment in the solid tumor contributes to cancer development and progression^{6,14,22,23}. The chromatin structure plays an important role in controlling gene expression²⁴. Thus, being able to decipher the chromatin regulation under the hypoxia is vital to understand the solid tumorigenesis and to design a potential therapy strategy. Here, we performed the fine characterization of how the genomic regulatory elements respond to the hypoxia across the time series hypoxia treatment in the mouse epithelial cell line. We also identified three types of hypoxia response regulatory elements (HRREs) in the genome, and found that these different types of HRREs with different

genomic features are enriched with different TFs. Our study provides insights into the chromatin structure response to the hypoxia treatment and identifies useful genomic features for a better understanding of the hypoxia biology in the solid tumor.

Acknowledgements

This work is supported by the Swedish Research Council (VR-2016-06794, VR-2017-02074 to X.C), Beijer Foundation, Jeassons Foundation, Petrus och Augusta Hedlunds Stiftelse, Göran Gustafsson's prize for younger researchers, Vleugel Foundation, and Uppsala University.

Author contributions

X.L. and X.C. designed the study, and wrote the manuscript together. X.C. supervised the study.

Competing Financial Interests statement:

The authors declare no competing financial interests.

Materials and Methods:

Data availability:

All the data used in this study were downloaded from the Gene Expression Omnibus under accession number GSE112091.

Data processing:

ATAC-seq raw data is aligned to mm9, using Bowtie2²⁵ with the ‘-very-sensitive’ parameter. The properly paired reads with mapping quality larger than 30 were kept, and the duplication reads were removed with Picard. Peak calling was performed with MACS2 using the parameters -q 0.01 -nomodel -shift 0, and the peaks overlapping with the genome black list in the ENCODE were discarded.

Identification of the differential peaks and clusters:

Deseq2²⁶ was used to identify the differential peaks by comparing the ATAC-seq peaks from each time point of hypoxia treatment (6h, 12h, 24h, 36h, 48h, and 72h) with ATAC-seq peaks from the normal controls. The differential peaks list is identified with the following cut-off with: p-value < 0.01, FDR < 0.01, and the absolute value of log2 fold change > 1. The unbiased clusters from the differential peaks were tested with Mfuzz¹³. The Pearson correlation of each cluster was used to verify the independence of each cluster. The heatmaps of each cluster were generated using Deeptools²⁷. Gene-element associations were then filtered to a distance of 100kb from element to TSS with HOMER²⁸. KEGG and GO analyses for each cluster were conducted with GREAT²⁹.

Genomic annotation and TF enrichment:

The genomic annotation of each cluster was performed based on the genomic segmentation of 3’UTR, 5’UTR, exon, intergenic, intron, non-coding, promoter-TSS, and transcription terminal sites. The distance annotation of each cluster was chosen by using the transcription start site of genes as the reference point, and the genome being separated into 5 parts: <1kbp, 1~10kbp, 10~100kbp, 100~1000kbp, and >1000kbp. The H3K27ac ChIP-seq data of 4T1 cells is downloaded from Gene Expression Omnibus¹⁵. The differential peaks from each cluster were annotated with H3K27ac histone marker¹⁵. Transcription factors for each cluster were determined using HOMER with a cut-off of q-value < 0.05. The unique TFs for each cluster are identified.

References:

- 1 Li, X. F. & O'Donoghue, J. A. Hypoxia in microscopic tumors. *Cancer Lett* **264**, 172-180, doi:10.1016/j.canlet.2008.02.037 (2008).
- 2 Walsh, J. C. *et al.* The clinical importance of assessing tumor hypoxia: relationship of tumor hypoxia to prognosis and therapeutic opportunities. *Antioxid Redox Signal* **21**, 1516-1554, doi:10.1089/ars.2013.5378 (2014).
- 3 Harris, A. L. Hypoxia--a key regulatory factor in tumour growth. *Nat Rev Cancer* **2**, 38-47, doi:10.1038/nrc704 (2002).
- 4 Gilkes, D. M., Semenza, G. L. & Wirtz, D. Hypoxia and the extracellular matrix: drivers of tumour metastasis. *Nat Rev Cancer* **14**, 430-439, doi:10.1038/nrc3726 (2014).
- 5 Brizel, D. M., Sibley, G. S., Prosnitz, L. R., Scher, R. L. & Dewhirst, M. W. Tumor hypoxia adversely affects the prognosis of carcinoma of the head and neck. *Int J Radiat Oncol Biol Phys* **38**, 285-289, doi:10.1016/s0360-3016(97)00101-6 (1997).
- 6 Muz, B., de la Puente, P., Azab, F. & Azab, A. K. The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. *Hypoxia (Auckl)* **3**, 83-92, doi:10.2147/HP.S93413 (2015).
- 7 Koukourakis, M. I. *et al.* Hypoxia-inducible factor (HIF1A and HIF2A), angiogenesis, and chemoradiotherapy outcome of squamous cell head-and-neck cancer. *Int J Radiat Oncol Biol Phys* **53**, 1192-1202, doi:10.1016/s0360-3016(02)02848-1 (2002).
- 8 Kumar, H. & Choi, D. K. Hypoxia Inducible Factor Pathway and Physiological Adaptation: A Cell Survival Pathway? *Mediators Inflamm* **2015**, 584758, doi:10.1155/2015/584758 (2015).
- 9 Choudhry, H. & Harris, A. L. Advances in Hypoxia-Inducible Factor Biology. *Cell Metab* **27**, 281-298, doi:10.1016/j.cmet.2017.10.005 (2018).
- 10 Ortiz-Barahona, A., Villar, D., Pescador, N., Amigo, J. & del Peso, L. Genome-wide identification of hypoxia-inducible factor binding sites and target genes by a probabilistic model integrating transcription-profiling data and in silico binding site prediction. *Nucleic Acids Res* **38**, 2332-2345, doi:10.1093/nar/gkp1205 (2010).
- 11 Buenrostro, J. D., Giresi, P. G., Zaba, L. C., Chang, H. Y. & Greenleaf, W. J. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. *Nat Methods* **10**, 1213-1218, doi:10.1038/nmeth.2688 (2013).
- 12 Chen, X. *et al.* Joint single-cell DNA accessibility and protein epitope profiling reveals environmental regulation of epigenomic heterogeneity. *Nat Commun* **9**, 4590, doi:10.1038/s41467-018-07115-y (2018).
- 13 Futschik, M. E. & Carlisle, B. Noise-robust soft clustering of gene expression time-course data. *J Bioinform Comput Biol* **3**, 965-988, doi:10.1142/s0219720005001375 (2005).
- 14 Carnero, A. & Lleona, M. The hypoxic microenvironment: A determinant of cancer stem cell evolution. *Bioessays* **38 Suppl 1**, S65-74, doi:10.1002/bies.201670911 (2016).
- 15 Sonzogni, O. *et al.* Reporters to mark and eliminate basal or luminal epithelial cells in culture and in vivo. *PLoS Biol* **16**, e2004049, doi:10.1371/journal.pbio.2004049 (2018).
- 16 Haberle, V. *et al.* Transcriptional cofactors display specificity for distinct types of core promoters. *Nature* **570**, 122-126, doi:10.1038/s41586-019-1210-7 (2019).
- 17 Tsankov, A. M. *et al.* Transcription factor binding dynamics during human ES cell differentiation. *Nature* **518**, 344-349, doi:10.1038/nature14233 (2015).

242 18 Montagner, M. *et al.* SHARP1 suppresses breast cancer metastasis by promoting
243 degradation of hypoxia-inducible factors. *Nature* **487**, 380-384,
244 doi:10.1038/nature11207 (2012).

245 19 Li, H. J. *et al.* NKX6.1 functions as a metastatic suppressor through epigenetic
246 regulation of the epithelial-mesenchymal transition. *Oncogene* **35**, 2266-2278,
247 doi:10.1038/onc.2015.289 (2016).

248 20 Wen, A. Y., Sakamoto, K. M. & Miller, L. S. The Role of the Transcription Factor
249 CREB in Immune Function. *J Immunol* **185**, 6413-6419,
250 doi:10.4049/jimmunol.1001829 (2010).

251 21 Chi, J. T. *et al.* Gene expression programs in response to hypoxia: Cell type
252 specificity and prognostic significance in human cancers. *Plos Med* **3**, 395-409,
253 doi:ARTN e47
254 10.1371/journal.pmed.0030047 (2006).

255 22 Jing, X. *et al.* Role of hypoxia in cancer therapy by regulating the tumor
256 microenvironment. *Mol Cancer* **18**, 157, doi:10.1186/s12943-019-1089-9 (2019).

257 23 Petrova, V., Annicchiarico-Petruzzelli, M., Melino, G. & Amelio, I. The hypoxic
258 tumour microenvironment. *Oncogenesis* **7**, doi:ARTN 10
259 10.1038/s41389-017-0011-9 (2018).

260 24 Perino, M. & Veenstra, G. J. Chromatin Control of Developmental Dynamics and
261 Plasticity. *Dev Cell* **38**, 610-620, doi:10.1016/j.devcel.2016.08.004 (2016).

262 25 Langmead, B. & Salzberg, S. L. Fast gapped-read alignment with Bowtie 2. *Nat*
263 *Methods* **9**, 357-359, doi:10.1038/nmeth.1923 (2012).

264 26 Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and
265 dispersion for RNA-seq data with DESeq2. *Genome Biol* **15**, 550,
266 doi:10.1186/s13059-014-0550-8 (2014).

267 27 Ramirez, F., Dundar, F., Diehl, S., Gruning, B. A. & Manke, T. deepTools: a flexible
268 platform for exploring deep-sequencing data. *Nucleic Acids Res* **42**, W187-191,
269 doi:10.1093/nar/gku365 (2014).

270 28 Heinz, S. *et al.* Simple combinations of lineage-determining transcription factors
271 prime cis-regulatory elements required for macrophage and B cell identities. *Mol Cell*
272 **38**, 576-589, doi:10.1016/j.molcel.2010.05.004 (2010).

273 29 McLean, C. Y. *et al.* GREAT improves functional interpretation of cis-regulatory
274 regions. *Nat Biotechnol* **28**, 495-501, doi:10.1038/nbt.1630 (2010).

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279 **Figures:**

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281 **Figure 1. Identification of hypoxia response gene regulatory elements**

- 282 a) Total accessible chromatin sites from different time points of hypoxia treatment were
283 compared to the control (under normoxia condition). The more accessible chromatin
284 sites are colored in orange, and the more close chromatin sites are colored in green.
285 b) The cluster of the hypoxia response gene regulatory elements (HRREs) following the
286 time series of hypoxia treatment. C1 = cluster 1, C2 = cluster 2, and C3 = cluster 3.
287 c) The representative genome browser track from the different cluster of HRREs.
288 cluster1 (Hif1a), cluster 2 (Est1), and cluster 3 (Slc12a4). The grey box indicates the
289 location of HRREs.
290 d) The centroid distance curve to determine the number of clusters for HRREs.
291 e) The pearson correlation among different HRREs clusters.
292 f) The average openness (calculated by RPKM) at each time point for each HRRE
293 cluster.
294 g) Left: ATAC-seq signals for cluster 1 (C1), cluster 2 (C2), and cluster 3 (C3) at
295 different time points. Right: the chromatin openness curve at different time points.

Chen_Figure2

Figure 2. Characterization of hypoxia response gene regulatory elements

- a) The genomic annotation of different hypoxia response gene regulatory elements (HRREs) clusters. C1 = cluster 1, C2 = cluster 2, C3 = cluster 3, and All = all the accessible peaks from pool samples of all time points.
- b) The annotation of the distance to transcription start sites (TSSs) from each HRRE clusters: cluster 1 (C1), cluster 2 (C2), and cluster 3 (C3).
- c) The enrichment of Histone marker H3k27ac for each HRRE cluster: 1 (C1), cluster 2 (C2), and cluster 3 (C3).
- d) The heatmap of enriched transcriptional factor motifs on each cluster of HRREs. C1 = cluster 1, C2 = cluster 2, and C3 = cluster 3.