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ARTICLE

Exploring DNA methylation data for diagnostic classification of Diffuse large B-cell lymphoma in Dogs

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Abstract

Diffuse large B-cell lymphoma (DLBCL) is a common B-lymphocyte tumor in dogs, making up 60-70% of cases. We assessed the utility of DNA methylation data for the diagnostic classification of DLBCL in dogs. We also assessed the utility of the classification features identified in cDLBCL for diagnostic classification of DLBCL in humans. The GSE94913 cDLBCL DNA methylation dataset from the Gene Expression Omnibus (GEO) was used for analysis. Differential methylation analysis was performed between the 37 cDLBCL and seven control lymph node samples in the dataset. 1701 differentially methylated probes were identified between the cDLBCL and control lymph nodes groups. Applying recursive feature elimination on the 1701 significant probes, 20 probes were selected for machine learning classification tasks. The methylation values of these 20 probes were used to build an SVM model and create the training and testing set. 100% of the test samples were accurately classified by the SVM model. The diagnostic classification utility of the identified differentially methylated CpGs/CDS was also assessed in humans using the GSE28094 human DLBCL dataset. 95% of 98 DLBCL and leukocyte samples obtained from this dataset was correctly classified using clustering techniques on 11 CpG sites of 5 genes (*ERBB4*, *IGF2*, *PGF*, *PITX2*, *TJP1*). The utility of DNA methylation data for the diagnostic classification of DLBCL in dogs is demonstrated. Further exploration of this data type for potential biomarker discovery in cDLBCL is necessary.

Keywords: Biomarkers; Classification; DNA methylation; Dogs; Machine learning.

1. Introduction

Lymphoma is the most common hematological cancer in dogs (Mizuno et al., 2020). Its cause is unknown but genetic susceptibility and environmental factors are proposed to have contributory roles (Zandvliet, 2016). Diffuse large B-cell lymphocytes (DLBCL) is the most common tumor of B-lymphocytes in dogs, making up about 60-70% of all cases (Ferraresso et al., 2017). The advances in treatment options have been modest, with high relapse rates and a low 2-year survival rate of 20% (Aresu, 2019; Martini et al., 2019). Novel therapeutic approaches is therefore needed. Canine DLBCL (cDLBCL) shares similar molecular and clinical features with human DLBCL (hDLBCL) and is therefore a comparative model (Richards et al., 2013; Aresu et al., 2019). Knowledge obtained from studying cDLBCL could be beneficial in understanding hDLBCL disease. Clinically cDLBCL presents as a moderate to severe peripheral lymph node enlargement (Aresu et al., 2015). Diagnosis is made using a combination of flow cytometry, cytology and histopathology (Riondato and Comazzi, 2021). Prognosis and selection of optimal treatment methods after diagnosis is still a challenge (Klimiuk et al., 2021).

Molecular investigation at different omics levels have shown cDLBCL to be a heterogeneous tumor (Richards et al., 2013; Giannuzzi et al., 2019; Sirivisoot et al., 2022). Tumor and non-tumor samples were separated into different clusters, by the application of clustering methods on gene expression data (Mudaliar et al., 2013). Gene Expression profiling and immunohistochemistry was used to distinguish the subtypes of hDLBCL and cDLBCL (Richards et al., 2013). Epigenetic and genetic alterations are necessary for tumor development (Takeshima and Ushijima, 2019). Alterations in the epigenome during tumorigenesis include global changes in histone modification marks (Fraga et al., 2005; Hosseini and Minucci, 2017), deregulation of noncoding RNA networks (Liz and Esteller, 2016), global DNA methylation loss and CpG promoter islands hypermethylation of tumor suppressor genes (Baylin and Jones, 2016). Few studies have been done to characterize the epigenetic landscape of cDLBCL and these have been focused on DNA methylation. These included DNA methylation studies that focused on single gene (Sato et al., 2014; Fujiwara-Igarashi et al., 2014; Tomiyasu et al., 2014; Ferraresso et al., 2014), and genome-wide (Ferraresso et al., 2017; Aresu et al., 2019; Hsu et al., 2021). The full understanding of the molecular mechanisms underlying cDLBCL is still incomplete. This study aims to explore the utility of DNA methylation data for diagnostic classification of DLBCL in dogs. The identified classification features would also be assessed for diagnostic classification of DLBCL in humans.

2. Experimental

Dataset

The GSE94913 cDLBCL DNA methylation dataset was retrieved from the GEO database (Ferraresso et al., 2017). This dataset is composed of 37 cDLBCL samples and 7 control lymph node samples. This dataset is a microarray of 37479 CpG and coding sequences (CDS) probes, with standard microarray data normalization and processing applied.

Differential methylation analysis

Differential methylation analysis was performed between the 37 cDLBCL and 7 control lymph node samples with the limma package in R (version 4.1.1) (Ritchie et al., 2015). Significant probes were selected as those that met the threshold of p-adjusted value < 0.01 and logFC > 1.

Machine learning (ML)

Clustering of all 44 samples based on all significant probes was done using the factoextra R package (version 1.0.7) (Kassambara and Mundt, 2020). A combination of k-means and hierarchical clustering algorithm was applied to the significant probes (Kassambara and Mundt, 2020). Thereafter, the resulting data object was visualized as a dendogram (Kassambara and Mundt, 2020).

To find possible features diagnostic of cDLBCL, machine learning classification tasks were performed. Recursive feature elimination (RFE) was performed on the significant differentially methylated probes to obtain the top 20 optimal features for classification. ML model creation and classification was done using LibSVM (Chang and Lin, 2011). The feature values were scaled with a LibSVM built-in python script. Using the train_test_split function in Scikit-learn (Pedregosa et al., 2011), the data was splitted into training (70%) and testing (30%) sets. The SVM algorithm was used for the training and evaluation of the model. To determine the best parameters of the SVM model to be subsequently applied to the test samples, 5-fold cross-validation was performed. The model was applied to predict the class of the samples in the testing set. The model was evaluated using precision, recall and accuracy. Also, interaction analysis of the twenty genes identified above was performed with STRING database (version 11.5) (Szklarczyk et al., 2021). Using TRRUST (version 2) (Han et al., 2018), we performed a transcriptional regulatory analysis to find the transcription factors regulating the identified genes.

To assess whether the methylated probes identified in this study could be useful for diagnostic classification of hDLBCL, RFE was done to select the top 200 important features for classification. This was to accommodate for the highly reduced gene number in the human dataset to be used for this assessment. The GSE28094 dataset was retrieved from the GEO database (Fernandez et al., 2012). This dataset is a human DNA methylation dataset comprising 424 normal tissue samples, 1054 tumor samples and 150 non-cancerous disorders samples. The beta values of 1505 CpG sites corresponding to 808 genes were profiled in the dataset. We extracted the profiles for 49 DLBCL and 49 healthy normal leukocytes from the dataset. The available genes out of the 200 top genes identified from RFE were thereafter used as classification features. Clustering of these 98 human samples was then performed using factoextra package (Kassambara and Mundt, 2020). Interaction analysis and transcriptional regulatory analysis of the genes were performed using STRING database (version 11.5) (Szklarczyk et al., 2021) and TRRUST (version 2) (Han et al., 2018) respectively. Python and R scripts used in this study's analyses are available in https://github.com/ZEB-LASU/cDLBCL

3. Results

The differential methylation analysis between the cDLBCL and control lymph node groups identified 1701 differentially methylated probes. 100% of the 44 samples were accurately clustered using these 1701 significantly methylated probes (fig. 1). The top 20 probes for classifying samples selected by RFE are shown in Table 1. Annotation

on the Ensembl genome browser (CanFam 3.1) identified these to include eleven protein-coding genes, three non coding RNAs, and six unidentified genes (Table 1). These were used in ML training and test set building. The training set was built on 30 samples randomly selected by the train_test_split Scikit-learn function, comprising of 25 cDLBCL samples and 5 control lymph node samples. The testing set was made up of 14 samples selected by train_test_split Scikit-learn function, comprising of 12 cDLBCL samples and 2 control lymph node samples. The 5-fold cross-validation accuracy obtained was 100%. Evaluation of the SVM model on the testing set resulted in an accuracy of 100% of correctly predicted samples (Table 2). For the interaction and transcriptional regulatory analysis, no interaction and transcriptional regulatory relationships were returned.

To assess the diagnostic classification utility of the methylated genes in hDLBCL, 11 CpG sites of six genes (ERBB4, IGF2, PGF, PITX2, TJP1, PDGFA) was used as features for clustering the 98 samples obtained from the GSE28094 dataset. 95% of the samples were accurately clustered into their respective groups (fig. 2). The 5 misclassified samples were however in a separate cluster group even though placed in the same branch with the normal leukocyte tissues (fig. 2). A supervised machine learning model could not be constructed due to the difference in platforms and the small number of genes profiled in the available hDLBCL dataset. The interaction analysis between the six genes identified interactions between four of the six genes (PDGFA, PGF, ERBB4, IGF2) (fig. 3). The transcriptional regulatory analysis revealed WT1 regulating the genes IGF2 and PDGFA.

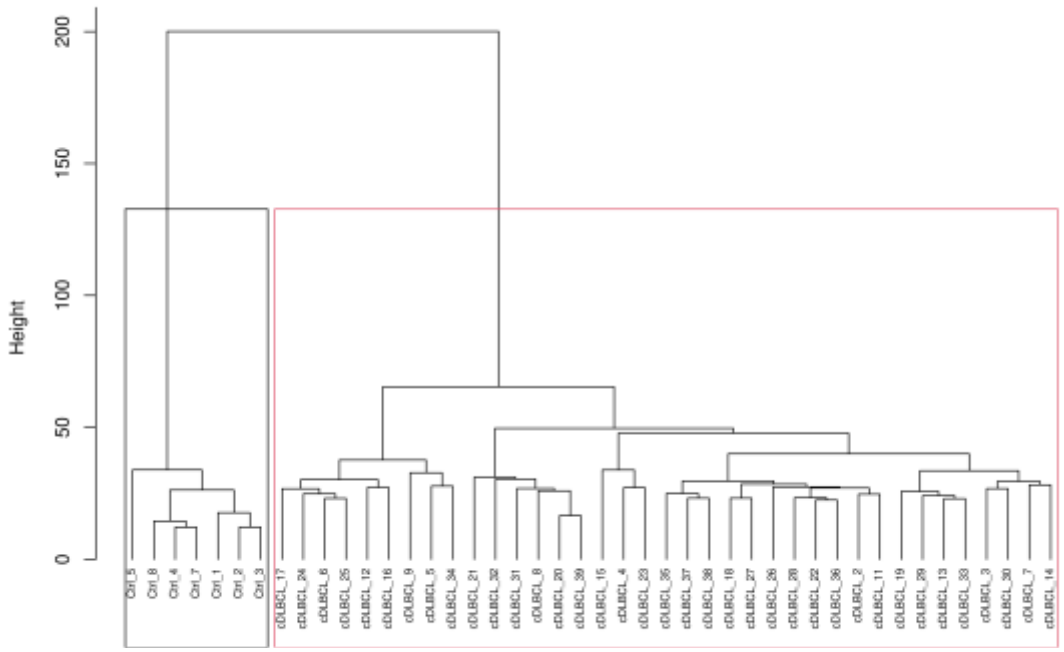

Fig. 1. Clustering of the cDLBCL and control lymph node samples using all 1701 significant methylation probes. The x-axis contains the samples and which are labelled for clear identification. Identified were two principal clusters with the 7 control lymph node samples all grouped in the left cluster (black rectangle box) while the 37 cDLBCL samples were all grouped in the right cluster (red rectangle box).

4. Discussion

The differential methylation analysis revealed expectedly that there were differences in methylation between cDLBCL and the control samples. Indeed, aberrant DNA methylation is characteristic of cancer (Dong et al., 2014), and cDLBCL (Ferraresso et al., 2017; Fujiwara-Igarashi et al., 2014; Hsu et al., 2021; Sato et al., 2018). The accuracy of the clustering despite using all 1701 significant probes substantiated the differential methylation analysis result and indicated their possible utility for classification. The high accuracy obtained by the ML model (Table 2) constructed using 20 significant probes (features) demonstrate the validity of these CpGs for diagnostic identification of DLBCL in dogs. As a limitation, the testing set was small, requiring validation in larger sample cohorts. We were able to explore the use of DNA methylation data for diagnostic classification of DLBCL in Dogs.

Applying machine learning classification techniques to cDLBCL DNA methylation data can give good results and thus can be beneficial Dysregulated expression of some of these identified top genes have been associated with several cancers including RAB7A in gastric cancer (Liu et al., 2020), RAB7A in breast cancer (Xie et al., 2019), LEF1 in melanoma (Kim et al., 2020), IGF2 in hepatocellular carcinoma (Ma et al., 2019) and adrenocortical carcinoma (Pereira et al., 2019), ST8SIA2 in lung cancer (Hao et al., 2019), DDIT4 in acute myeloid leukemia (Cheng et al., 2020) and in ovarian carcinoma (Chang et al., 2018), and PPM1H in colon adenocarcinoma (Sugiura et al., 2008) and in pancreatic cancer (Zhu et al., 2016). These genes could be potential drivers of cDLBCL in Dogs. Aberrant methylation of these genes could affect their expression. They can also serve as possible therapeutic targets upon further studies and functional studies of these identified genes should elucidate our understanding

Table 1. Twenty probes selected by recursive feature elimination and used as features for machine learning classification

Probe ID	adj-p-value	Gene name
GT_chr6:31498360-31499687_296737	2.40686e-05	TNP2*
GT_CpG_102:chr20:2792244-2793806_10609	5.00947e-19	RAB7A*
GT_CpG_133:chr8:34699507-34700781_125659	4.57457e-07	JKAMP*
GT_CpG_199:chr32:28632693-28635175_287611	1.84638e-06	LEF1*
GT_CpG_208:chr10:6070361-6072996_308205	2.76684e-22	PPM1H*
GT_CpG_217:chr3:47653366-47655868_330479	4.13005e-17	ST8SIA2*
GT_CpG_31:chr4:22936675-22937328_508162	6.84959e-12	DDIT4*
GT_CpG_35:chr25:15429047-15429606_825639	7.52139e-17	SGCG*
GT_CpG_42:chr18:46302185-46302823_622168	5.85738e-12	IGF2*
GT_CpG_57:chr22:8708474-8709185_748846	3.30485e-08	DGKH*
GT_CpG_62:chr6:17872282-17873207_782319	1.75655e-12	ENSCAFT00000077585**
GT_CpG_72:chr10:55372722-55373751_851800	1.48866e-14	ENSCAFT00000065559**
GT_CpG_82:chr19:23852100-23852984_905754	0.00054	ENSCAFT00000089251**
GT_CpG_90:chr1:104706255-104707295_948211	3.10691e-19	ENSCAFT00000044531*
GT_CpG_103:chr9:5445423-5446725_17310	8.35098e-18	
GT_CpG_33:chr23:33251449-33252124_530445	2.76684e-22	
GT_CpG_43:chr15:45025176-45025844_631179	6.43581e-19	
GT_CpG_69:chr11:62398353-62399319_830114	1.94461e-17	
GT_CpG_77:chr27:31835085-31836199_881657	1.55622e-14	
GT_CpG_99:chr6:38018350-38019848_992235	1.37342e-07	

Shown are the probe identifiers, their adjusted p-values, and corresponding gene names. *protein coding genes. **long non-coding RNA gene. Blanks are genes without annotation names

Table 2: Machine learning classification results of the testing set using SVM model

Class	**Precision**	**Recall**
cDLBCL	1.0	1.0
Control lymph node	1.0	1.0
Accuracy	100% (14/14)	

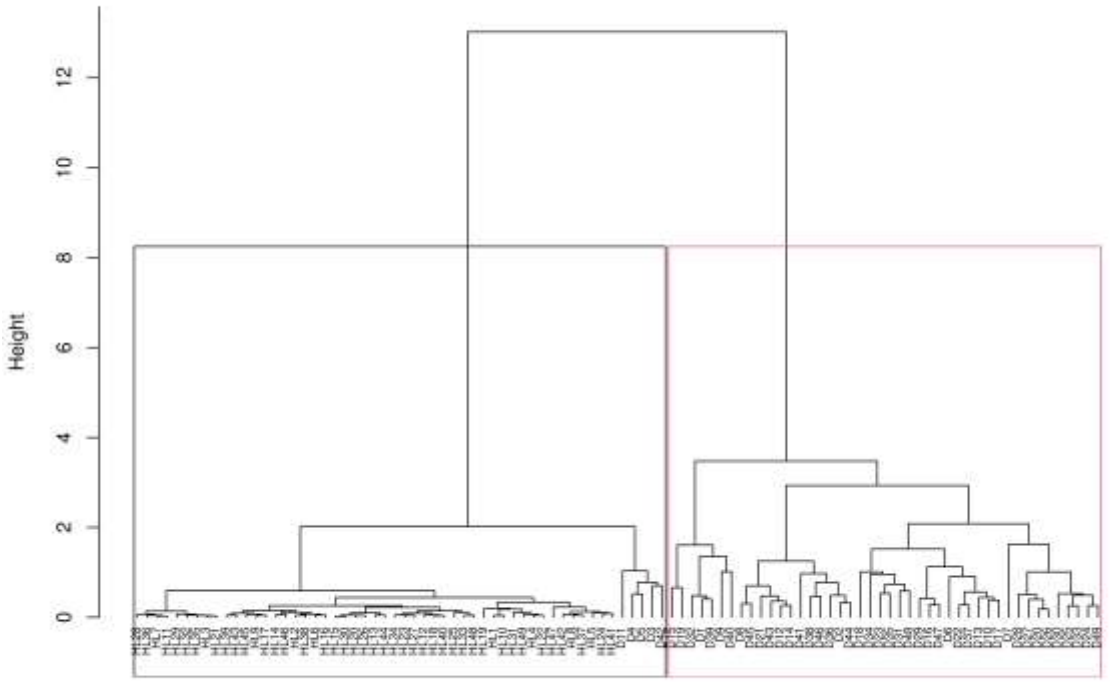

Fig. 2. Clustering of the 98 DLBCL and leukocyte samples of the GSE28094 human dataset. The x-axis contains the samples, with the DLBCL samples labelled with D and leukocyte samples labelled with HL. Identified were two main clusters with the DLBCL samples predominantly clustered in the right cluster (red rectangle box) while the leukocyte samples were predominantly clustered in the left cluster (black rectangle box). 5 DLBCL samples were misclassified.

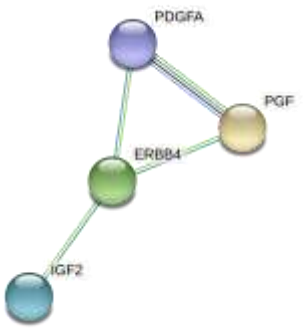

Fig. 3. Gene interaction analysis of the six (ERBB4, IGF2, PGF, PITX2, TJP1, PDGFA) genes used for clustering the hDLBCL samples.

understanding of DLBCL disease mechanisms in dogs. The unavailability of external datasets to further validate this CpG signature indicates the need for more investigative studies to characterize the epigenetic landscape of DLBCL in dogs. The unsupervised model based on 11 CpGs of six genes (ERBB4, IGF2, PGF, PITX2, TJP1, PDGFA) was able to discriminate hDLBCL samples and leukocyte samples with high accuracy (fig 2). This reinforces the molecular and clinical similarity between cDLBCL and hDLBCL (Richards et al., 2013; Aresu et al., 2019). These six genes could thus be potentially used for diagnostic identification of DLBCL in humans upon further validation. WT1 was reported to have transcriptionally regulate IGF2 and PDGFA, and has been associated with several cancers (Qi et al., 2015; Zhang et al., 2020). Diagnostic classification of tumors using DNA methylation data has been demonstrated in multiple human cancers (Capper et al., 2018; Liu et al., 2020; Giwa et al., 2021). It could therefore be possible to develop diagnostic biomarkers that are clinically useful in canine and human DLBCL.

5. Conclusion

This exploratory study demonstrates the utility of DNA methylation data for diagnostic classification of DLBCL in dogs. DNA methylation data-based diagnostics for tumor classification is becoming mainstream. Therefore, characterizing the methylation landscape of DLBCL in dogs should have clinical benefit. It will also further understanding of the disease mechanisms and aid therapeutic development as survival rate of DLBCL in dogs is still low. Further exploration of this data type for potential biomarker discovery in cDLBCL is necessary.

Declaration of Conflict of Interests

No conflict of interests to declare

Authors' Contributions

Conception: [AG]
Design: [AG]
Execution: [OA, AG]
Interpretation: [OA, AG]
Writing the paper: [OA, AG]

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