Prediction of m6A Reader substrate sites using deep convolutional and recurrent neural network

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ABSTRACT

N6-methyladenosine (m6A) is the most prevalent post-transcriptional modification in mRNA since it could regulate some significant biological functions with the binding of some m6A reader proteins. Multiple readers exist in the human genome, however, the binding specificity was not clarified due to the limited wet experiments on this topic. Therefore, we devised a deep learning approach which incorporated CNN and RNN frameworks together to predict the epitranscriptome-wide targets of six m6A reader proteins (YTHDF1-3, YTHDC1-2, EIF3A). We also utilized layer-wise relevance calculation to obtain contribution of each input feature. Our model achieved state-of-the-art performance with the average AUROC of 0.942 in EIF3A full transcript, compared with 0.929 in CNN-only framework and 0.817 in Support Vector Machine (SVM) method under same condition. Besides, we identified the optimal sequence length (1001bp) in the m6A reader substrate prediction. The results provide new insight into epitranscriptome target prediction and functional characterization of m6A readers.

CONCEPTS

• Applied computing • Life and medical sciences • Bioinformatics

KEYWORDS

Deep learning, convolutional neural network, recurrent neural network, m6A, Readers.

1 Introduction

In addition to protein and DNA, RNA can also be modified. Among numerous RNA modification, mRNA methylation that occurs in the N6-position of adenosine is called N6-methyladenosine (m6A) and is the most prevalent internal modification on eukaryotic mRNA [1]. On average, within 1000 nucleotides, one or two m6A residues can be found [2, 3]. m6A is usually found in stop codon, 3' untranslated region (UTR), and long internal exon [4, 5]. m6A has aroused the interest of many scientists due to its important biological roles in altering gene regulation, gene stability maintenance and cell renewal and differentiation [6]. Meanwhile, some researchers established an online database DRUM to help query the methylation sites of disease-related m6A [7]. m6A also involves in RNA metabolisms such as mRNA translation, degradation, splicing, nuclear export and folding [8, 9].

There are three types of m6A regulators: writer, erasers and readers. m6A is catalyzed by m6A methyltransferases, namely writer, removed by m6A demethylases, which also named as eraser, and then recognized by reader protein [1]. RNA reader proteins can bind RNA, and each reader has a specific binding site to perform the corresponding functions [1]. In previous research, a prediction framework WHISTLE was developed, aiming to predict whole-transcriptome m6A site [10]. Please note, we only focus on YTH family and EIF3 reader in this project.

m6A is recognized by the YT521-B homology (YTH) domain-containing proteins. YTH domains exist in 174 different proteins [11]. Early studies have shown that they play an important role in RNA metabolism [8, 9]. A search of the human genome revealed five proteins containing the YTH domain, namely YTHDF1-3 and YTHDC1-2, which are stored in the mammalian genome [6]. According to their main sequences and the structure of their domains, the proteins in the YTH domain are classified into three groups: YTHDC1 (DC1 family), YTHDC2 (DC2 family), and YTHDF1-3 (DF family) [6], which means YTH domain family has five YTH domain-containing proteins in humans, that is, YTHDC1, YTHDC2, YTHDF1, YTHDF2, and YTHDF3. From the sequence alignment, YTHDF1, YTHDF2, and YTHDF3 are similar to each other, whereas YTHDC1 and YTHDC2 are considered as different subtypes [6].

Although these five readers all came from YTH family, they play different roles in the cell to modulate gene regulation, DNA repair, and cell fate determination [12]. YTHDC1 is the only known reader in the nucleus as a nuclear protein, it involves in exon alternative selection during gene splicing by binding to SRSF3 (serine/arginine-rich splicing factor 3) [13]. YTHDC2 is an RNA helicase that recognizes the YTH domain of m6A, by forming complexes with MEIOC (meiosis-specific coiled-coil domain-containing protein) enabling it to regulate RNA levels during meiosis [14]. YTHDF1–3 are cytoplasmic m6A readers, by binding to m6A, YTHDF1 improves the translation efficiency of mRNA independent of m7G cap [15]. YTHDF2 is the first protein which is related to m6A being studied [16]. YTHDF2 uses the CCR4- NOT deadenylase complex to destroy and further decay the target mRNA in mRNA clearance [17].

To understand the role of YTH family readers, their structure is also important. The crystal structure of YTH Domain, alone and together, GG (m6A) CU RNA, helps to explain the reason of m6A recognition and sequence selectivity [18]. The YTH domain shares a conservative α/β fold structure, which is composed of 4 or 5αhelices and six β strands. After that, these six β strands form a barrel-shaped structure, which is a β barrel, with α helices packed against the β strands to stabilize the hydrophobic core [18]. In the YTHDC1 m6A complex, the RNA molecule locates in the positively charged groove of the protein, and m6A is hidden in the three hydrophobic residues W377, W428, and L439 [18]. To be specific, the methylation between m6A's methyl groups and the ring of amino acid residues form the basis for m6A's specific recognition [18].

For the RNA binding protein that lacks YTH domain-containing m6A readers, eILF3 (eukaryotic translation initiation factor 3) was considered as the most complex eukaryotic translation initiation factor [19]. It can specialize in translation initiation, in IRES-mediated translation initiation, eIF3 directly identifies the structure of the IRES (Internal ribosomal entry) [20]. eIF3 plays an important role in protein translation such as dissociating the post-termination 80S ribosome, participating in the 43S pre-initiation complex formation and stimulating mRNA binding with 43S pre-initiation complex [21].

To date, there is a prediction model or comprehensive online platform that can be used to annotate the internal modification function of mRNA, such as m7GHub [22]. In this project, a new computational model was designed to predict the preferential binding sites in the m6A marked transcripts for 5 YTH family proteins (YTHDC1, YTHDC2, YTHDF1, YTHDF2, and YTHDF3) and eIF3 m6A reader, considering that wet-lab experiments are expensive and time-consuming. The combination of CNN (Convolutional Neural Networks) and RNN (Bidirectional LSTM Network) were used to predict binding sites. One hot encoding was used as an encoding method. After the model was built, ROC and PR curve were drawn to evaluate and interpret the generalization ability of the deep learning model. The performance was compared with SVM (support vector machine) machine learning algorithm and CNN framework only. Additionally, the product of inputs and its corresponding gradient was calculated to estimate the contribution of each input feature.

2 Material and Methods

2.1 Collection of m6A sites and the target sites of m6A readers

In this experiment, the base-resolution and high-resolution m6A sites (DRACH motif) were downloaded from m6A -Atlas [23]. We mainly focused on six types of m6A readers recognized by Par-CLIP or iCLP, including YTHDC1, YTHDC2, YTHDF1, YTHDF2, YTHDF3, EIF3A (Table 1). For YTHDC1 and YTHDC2, the total sites are 16,664, 1,234 respectively, the number of genes is 4722, 275, the cell line used both from HeLa to HEK293T. Similarly, for three YTHDF proteins (YTHDF1-3), the total sites are 25,597, 28,970 and 7,253 respectively, the number of genes is 6,714, 6,677 and 3,495, the cell lines used covered from HeLa, Huh7, HEK293T. The remaining EIF3A was used in HEK293T cell line, the total sites and the gene number are 756 and 470 respectively. For the benefits of modeling, we consider m6A reader substrates sites as positive samples and those non- m6A reader substrates sites as negative samples. To minimize the bias in selecting the polyA RNAs, we prepared the full transcript data and mature RNA data. In detail, mature RNA data exclude the sites on the intron region while the full transcript data covered either the exon or intron region.

Table 1 Target sites of m6A readers identified by Par-CLIP or iCLIP

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Dataset | Reader | Source | Site | Total | Gene | Cell line |
| D1 | YTHDC1 | GSE74397 | 482 [24] | 16,664 | 4,722 | HeLa |
| D2 | GSE58352 | 2,633 [18] |
| D3 | GSE71096 | 2,430 [25] |
| D4 | GSE78030 | 12,309 [26] | HEK293T |
| D5 | YTHDC2 | GSE98085 | 1,183 [27] | 1,234 | 275 | HeLa |
| D6 | GSE78030 | 131 [26] | HEK293T |
| D7 | YTHDF1 | GSE63591 | 4,541 [15] | 25,597 | 6,714 | HeLa |
| D8 | GSE83438 | 2,527 [28] | Huh7 |
| D9 | GSE78030 | 20,694 [26] | HEK293T |
| D10 | YTHDF2 | GSE49339 | 22,688 [17] | 28,970 | 6,677 | HeLa |
| D11 | GSE83438 | 5,147 [28] | Huh7 |
| D12 | GSE78030 | 6,280 [26] | HEK293T |
| D13 | YTHDF3 | GSE86214 | 2,608 [29] | 7,253 | 3,495 | HeLa |
| D14 | GSE83438 | 177 [28] | Huh7 |
| D15 | GSE78030 | 5,082 [26] | HEK293T |
| D16 | EIF3A | GSE65004 | 45 [30] | 756 | 470 | HEK293T |
| D17 | GSE73405 | 731 [31] |

2.2 Deep learning model construction

To build the deep learning model, we used Keras v2.3.0 and R v4.0.2 to conduct the learning part and process the raw data for prediction. For the data preparation part, we used R to extract n nucleotides (bp) of flanking sequence centered on the target adenosine, ranging from 251 to 2001bp to explore a suitable length. The processed sequence data were then inputted to Python3 for encoding, in this case, we chose One-hot encoding method for better model interpretability, for instance, A (1,0,0,0), C (0,1,0,0), G (0,0,1,0), T (0,0,0,1). The overall framework can be seen in **Figure 1**. Each sequence was then transformed to an n4 matrix and fed into two combinations of 1D convolution (Conv1D) layer and max-pooling layer. For the first combination, we set 90 kernels with size equaled 5 and applied L2 regulation to prevent overfitting. The rectified linear unit (ReLU) was used as the activation function to provide our necessary non-linearity. The following max-pooling layer was set in size equaled 4 with strides 2 to reduce the dimension of output from the previous layer. The dropout rate was incorporated to 0.25 to further reduce the possibility of overfitting. A second 1D convolution (Conv1D) layer with 100 filters and size equaled 3 to extract the feature of the previous data. Similarly, the ReLU function and L2 regulation were applied. However, the max-pooling size was 10 with 1 stride, under which circumstance could the model achieve higher performance.

The recurrent neural networks long short-term memory (LSTM) layer was used to aggregate the outputs of CNNs for predicting the RBP binding [32], in this case, the m6A readers’ substrates sites. LSTM processed sequentially of the sequence element, hoping to capture the inter-dependencies between motifs. Moreover, the fully connected layer with 1000 neurons would receive the output from LSTM layer, and the non-linear activation function n, sigmoid, would calculate the prediction probability in each training class. The overall tuning process was used the loss function, binary cross-entropy to conduct the weight-tuning, optimizing the learning process, additionally, we found that Adam is the most suitable for this task. Finally, the output would be the probability of being m6A reader substrate sites.

2.3 Training strategy and performance evaluation

We separated each gene data set into three categories, training, testing, validation dataset, the ratio was 8:1:1 respectively. Moreover, to reduce the bias caused by imbalanced data samples, we ensured the same number of positive and negative samples in each category. The early stopping method was included to reduce the unnecessary computation during the learning process and the patience was designed as 10. The loss plot was drawn to document the training procedure and monitor the potential overfitting.

To validate the model performance, four commonly used performance metrics, including area under the ROC curve (AUC) [33], area under the Precision-Recall curve (PR-AUC) [34], accuracy (ACC) [35] and Mathew’s correlation coefficient (MCC) [36]. The formula of ACC and MCC are demonstrated as follows:

(1)

where TP and TN are denoted as True Positive and True Negative, FN and FT are denoted as False Negative and False Positive. To sum up, the higher the performance metrics value, the more accurate the prediction. Additionally, we compared the performance with the previous research using machine learning method, Support Vector Machine (SVM) and the CNN framework only to determine the optimal choice [37].

We also exploited DeepExplain's epsilon-LRP method (gradient-based) [38] to calculate the contribution in each feature input. With the assistance of this approach, we could rank the nucleotides’ significance in identifying the m6A readers’ substrates. Moreover, we extend the sequence upstream/downstream length from 50bp to 250bp, hoping to cover more information in determining each nucleotide contribution.

A screenshot of a cell phone

Description automatically generated

**Figure 1: Overall architecture of the deep learning model**

The sequence data are encoded by One-hot method and fed into the convolution layer and followed by the pooling layer twice to extract the significant features. The LSTM layer learns the long-term dependencies between sequence data generated by convolution layers. The flatten layer combines the previous kernels into a vector and inputs to the fully connected layer to calculate the probability of being m6A reader substrate site

3 Results and discussion

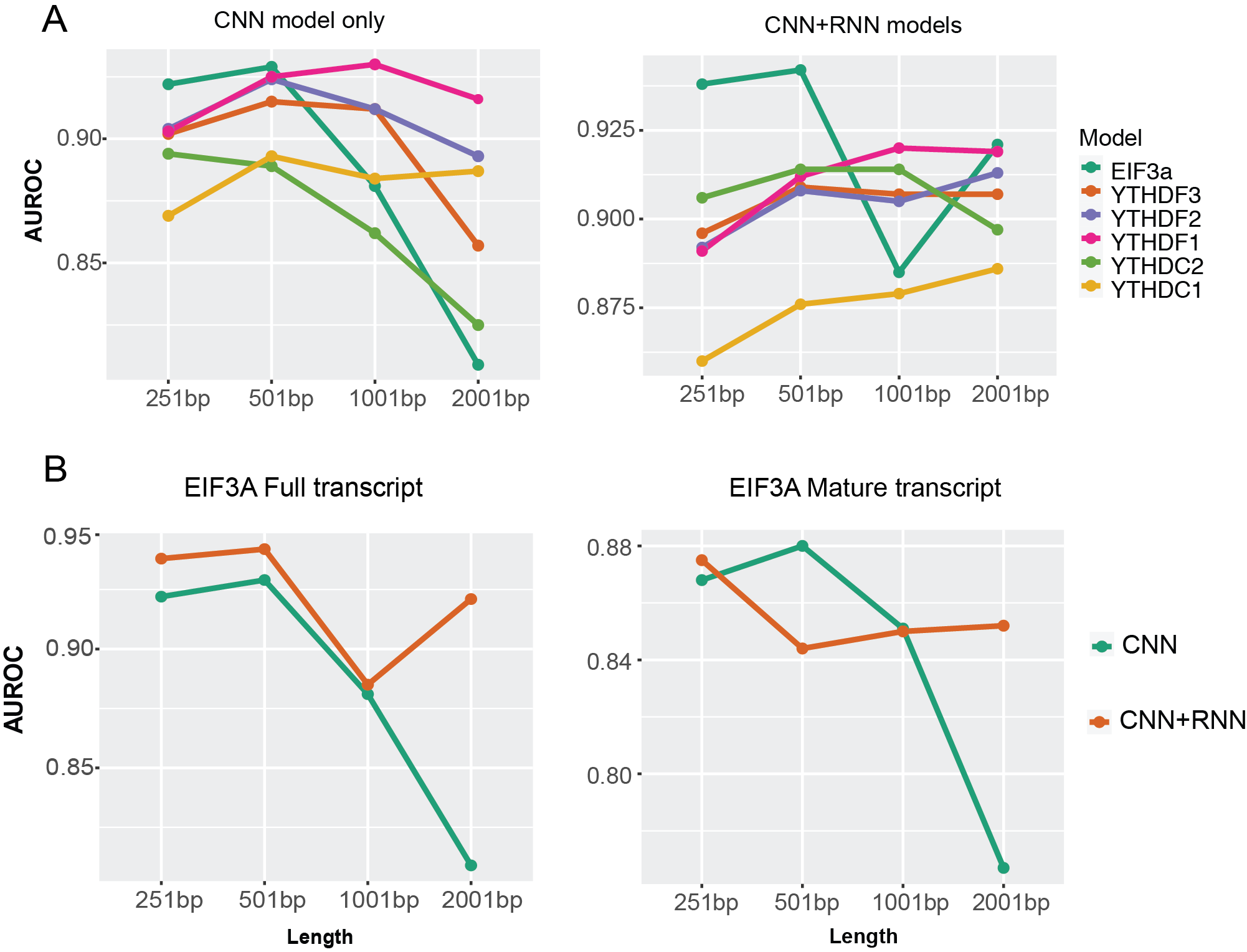
3.1 Model comparison

To compare the performance of machine learning and deep learning models in predicting m6A reader, models with six different classifiers were trained: machine learning algorithm including SVM (Support Vector Machine), RF (Random Forest), XGBoost and deep learning algorithm CNN (Convolutional Neural Network), and the combination of CNN and RNN algorithm (Recurrent Neural Networks, specifically, Bidirectional LSTM Network), were built. Six mammalian m6A reader binding site datasets were used to train models, namely, EIF3a, YTHDF1-3, YTHDC1 and YTHDC2. For tranditional machine learning model, a commonly used transcript with size of 41bp was used and encoded by One-hot method. For the two deep learning models, full transcripts and mature transcripts with the length of 1001bp were chosen and encoded by One-hot as well. The performance metrics for six models were summarized in **Table 2**. The model with CNN classifier achieved better prediction performance (AUROC = 0.929, PRAUC = 0.939) compared with SVM model, with AUROC of 0.817 and PRAUC of 0.815. The combination of CNN and RNN classifiers achieved highest performance among the other models (AUROC = 0.942, PRAUC = 0.95). The AUROC for SVM model is respectively 12.06% and 13.27% lower than the CNN model and CNN + RNN model, which suggested that that deep learning algorithms are more powerful in characterizing m6A sites.

**Table 2:** Performance metrics of EIF3A full transcripts were chosen to demonstrate the AUROC, PRAUC, MCC and Accuracy for CNN, SVM and CNN+RNN models.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | AUROC | PRAUC | MCC | | ACC |
| LR | 0.789 | 0.785 | | 0.429 | 0.714 |
| XGBoost | 0.806 | 0.817 | | 0.517 | 0.758 |
| RF | 0.818 | 0.825 | | 0.504 | 0.751 |
| SVM | 0.811 | 0.816 | | 0.503 | 0.751 |
| CNN | 0.929 | 0.939 | | 0.736 | 0.868 |
| CNN+RNN | 0.942 | 0.95 | | 0.742 | 0.868 |

Classifiers might achieve varied performance on different datasets. To assess the fitness between the 6 reader binding site datasets and the two deep learning classifiers, models were built on full transcripts and their performance were analyzed. Similarly, different size of full transcripts were encoded with One-hot method. As shown in **Figure 2(A)**, models using CNN classifier achieved theoretically good performance with overall AUROC larger than 0.8. It seems that the CNN classifier fit the YTHDF1 binding datasets better than other reader binding sites, with overall AUROC exceeding 0.9 and highest AUROC of 0.93. In addition, CNN model achieves good performance with YTHDF2 binding datasets as well, with highest AUROC of 0.929. It is noticeable that the performance of CNN models with EIF3a varied dramatically along with the size of transcript, from 0.96 to 0.81, which suggests that the performance of CNN classifier is depend on the size of transcripts. Similar trends can be seen in the YTHDC2 datasets, the trained model with different input size achieves different AUROC score, with optimal input transcript size of 251bp (AUROC = 0.89).



**Figure 2:** (A) Compared the performance of CNN model and CNN+RNN model in the prediction of six m6A reader substrates under different length in full transcripts. (B) Compared the AUROC value in either full transcript or mature transcript when predicting the EIF3A reader substrates.

Regarding the fitness of CNN+RNN classifier with the six reader datasets, models shows similar performance for YTHDF3, YTHDF2, YTHDF1, YTHDC2 datasets (with AUROC around 0.9). Moreover, the model trained with these four datasets as well as the YTHDC1 datasets (with AUROC around 0.875) seems transcript-size independent since lines are relatively stable. Interestingly, the performance of models trained with EIF3a datasets varied greatly from length to length (AUROC varied from 0.88 to 0.94). The structure variation between YTH family protein and EIF3a might contribute to the difference on model performance.

To assess the feasibility of the two classifiers, namely CNN and RNN, performance of models was interpreted and compared. **Figure 2(B)** compares the performance of models with different size of EIF3a transcripts. As indicated, the combination of CNN and RNN classifier achieves overall better performance than the CNN classifier for both full transcript and mature transcript. Since the trend of line graph for CNN+RNN model is more stable than the line for CNN model, we can infer that the combination of CNN and RNN makes the model less dependent on the length of transcript used.

A screenshot of a cell phone

Description automatically generated

**Figure 3** Feature importance scores in EIF3A full transcript prediction. We both extracted upstream/downstream 50bp and upstream/downstream 250 bp of the sequence to rank the contribution of each nucleotide in determining the binding site. In each position, the higher score it gains, the higher contribution towards the binding sites.

3.2 Layer-wise relevance calculation

Each input feature was calculated to obtain its contribution to the results by DeepExplain’s epsilon-LRP method. The feature importance plots were based on the EIF3a binding site datasets **(Figure 3).** The higher score that the position gets, the larger probability that the center nucleotide is an EIF3a reader binding site if this nucleotide present at that position. As shown in the graph, positions located around the predicted m6A sites got significantly higher scores than other positions, which means those positions are more important in determining whether the center nucleotide is m6A reader substrate site or not. Additionally, the prediction of modification site would benefit from taking sequence more than 50bp upstream or downstream the predicted site since they include positions with high importance score

Specifically, a site would be less likely to be m6A modification site if the adenosine represents in 100bp downstream since the majority of position within this sequence got importance scores smaller than 0. In comparison, the presence of cytosine in 50 upstream/downstream the predicted site tends to boost the chance of the center nucleotide being modified. No specific patterns were found for guanine and thymine as the importance plot present a shape like the sine function.

The results showed that if those positions 34bp, 59bp, 11bp, 58bp, 27bp, 49bp, 72bp upstream, 21bp, 27bp, 24bp, 25bp, 116bp downstream the modification site is cytosine, the site would more likely to be the EIF3a reader binding site. In addition, the probability of the modification site being EIF3a substrate site would decrease if guanosine was found on positions 21bp, 71bp, 33bp, 32bp, 31bp, 22bp upstream the center site or uridine was found on positions 54bp upstream or 53bp downstream the center site. The screened top 20 nucleotides that will decrease the change of the site being EIF3a modification site include: adenosines on positions 39bp, 27bp, 47bp, 61bp, 10bp, 12bp, 23bp, 170bp, 157bp, 51bp, 14bp, 226bp, 52bp upstream the center nucleotide, cytosine on positions 93bp upstream and 185bp downstream the center nucleotide, guanosine on positions 92, 97bp downstream the center site as well as uridines on positions 56bp, 63bp upstream the modification site.

Conclusion

In this experiment, we developed a deep learning method combining both CNN and RNN frameworks to predict the m6A reader substrate sites from the sequence derived feature. Six m6A readers (YTHDC1-2, YTHDF1-3, EIF3A) were investigated and divided into mature and full transcripts parts to reduce the potential bias existing in polyA selection step during RNA library construction. We compared the performance with the traditional machine learning algorithm SVM and CNN framework only and identify the optimal sequence input length, which is 1001bp. Our model achieved outstanding performance, with average AUROC over 0.85, outperforming the other two frameworks. Feature importance scores were calculated and interpreted to demonstrate the impacts in determining the m6A reader substrates.

However, this study was restricted to the human genome only and could not extend to other important organisms, mainly owing to the scarce base-resolution epitranscriptome profiling data. In the future, with the existence of other species data, for instance, yeast, it would be promising to apply the model to predict the epitranscriptome target and functional characterization on other organisms. Additionally, more encoding methods could be incorporated to extract the important feature and combined features, such as genomic features, could improve the overall performance.

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