Identification of a multidimensional transcriptome signature predicting tumor regrowth of clinically non-functioning pituitary adenoma

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Received November 17, 2019; Accepted May 28, 2020

DOI: 10.3892/ijo.2020.5087

Abstract. Clinically non-functioning pituitary adenoma (NFPA) represents approximately one third of all pituitary adenomas. Tumor regrowth is an important feature of NFPA; however, the effective methods with which to predict this are limited. The present study analyzed the expression of protein-coding genes and long non-coding RNA in 66 patients with NFPA. Cox regression analysis was performed to identify genes associated with regrowth or progression-free survival (PFS). Kaplan-Meier, random survival forest analysis and receiver operating characteristic curve (ROC) analyses were performed to generate a multi-protein-coding gene (PCG) and long non-coding RNA (lncRNA) signature with a maximum area under the ROC curve (AUC). In total, 1 PCG (CHST12) and 2 lncRNAs (COA6-AS1 and RP11-23N2.4) were identified that were significantly associated with tumor regrowth. The multi-transcriptome signature exhibited a high predictive accuracy for tumor regrowth, with an AUC of 0.869/0.726 in the training/testing set, and the discriminative power of this signature was better than that of age. On the whole, the present study indicates that the combined PCG and lncRNA signature may be beneficial as a marker for the prediction of the prognosis of patients with NFPA.

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Key words: clinically non-functioning pituitary adenoma, regrowth, predicting signature

Introduction

Clinically non-functioning pituitary adenoma (NFPA) is one of the most common subtypes of pituitary adenoma. Being unassociated with specific serum hormone changes, NFPA is usually detected based on symptoms, such as headaches and visual disturbance or incidental imaging examinations (1,2). Surgical resection is the primary treatment choice for NFPA; however, patients are often faced with tumor residue as the tumor may have surrounded the internal carotid artery or invaded the cavernous sinus (3,4). Approximately 12-58% of patients may experience relapse within 5 years (5). Radiotherapy is often recommended to patients with tumor residue; however, its long-term complications, such as visual defects and hypopituitarism, pose concerns (6,7). Thus, surgery remains the optimal treatment option for patients with tumor regrowth. However, in contrast to clinically functioning pituitary adenoma (FPA), for which serum hormone monitoring is used for detection, NFPA lacks an effective evaluation method, resulting in the failure of early intervention. Thus, research concerning the molecular mechanisms of tumor regrowth and the development of methods for predicting prognosis is of utmost significance.

Protein-coding genes (PCGs) are the most commonly used class of molecular marker. Numerous studies have reported that the altered expression of PCGs results in aggressive tumor behavior and may affect the prognosis of patients with NFPA (1,8,9). Long non-coding RNAs (lncRNAs) are a subgroup of non-protein-coding RNAs >200 nucleotides in length. lncRNAs play pivotal roles in the regulation of gene expression and have been used as prognostic markers in multiple tumors types (8,10). The expression of lncRNAs has been found to be related to the growth and invasive behavior of pituitary adenoma (11,12). However, there has been little research on the role of lncRNAs or its combination with PCGs as potentially novel approaches for prognostic evaluation in pituitary adenoma.

The present study aimed to identify PCGs and lncRNAs that are asscoiated with the regrowth of NFPA, and to establish a model which may be used to predict tumor regrowth based on

the PCG and lncRNA expression profiles of 66 NFPA samples through microarray analyses. The findings of the present study may provide an effective method for the prediction of the prognosis and post-operative intervention of patients with NFPA.

Materials and methods

Patients and samples. NFPA specimens from 66 patients who had undergone surgical treatment at Beijing Tiantan Hospital from October, 2007 to July, 2014 were selected, which included 34 females and 32 males with a median age of 55 years (range, 25-66 years). The median follow-up time was 78 months (range, 36-121 months). The definition of tumor regrowth was a maximum increase in tumor diameter of >2 mm on an enhanced MRI from the time of surgery to the follow-up endpoint with or without headaches, visual disturbances or other mass effect symptoms. The clinical characteristics of the patients are presented in Table I and detailed clinical information is presented in Table SI. The present study was approved by the Medical Ethics Committee of Beijing Tiantan Hospital and written informed consent for the use of the resected samples for research purposes was obtained from all patients.

Total RNA extraction and RNA microarray. Total cellular RNA was extracted from the tumor samples using the mirVana™ miRNA Isolation kit without phenol (cat. no. AM1561, Ambion; Thermo Fisher Scientific, Inc.), which were used to produce labeled fluorescent cRNA targets for the SBC human ceRNA array v1.0 (4x180 K). The prepared cRNA targets were hybridized with the slides and scanned on an Agilent Microarray Scanner (Agilent Technologies, Inc.). Following data collection with Feature Extraction software v10.7 (Agilent Technologies, Inc.), the data were normalized by quantile normalization using a package named limma from R program (bioinf.wehi.edu.au/limma).

Selection of PCGs and lncRNAs subsets related to tumor regrowth. All patients We were randomly divided into the training (n=33) and testing sets (n=33) using an algorithm called sample from R program (www.r-project.org/). In the training set, the evaluation of the association between the expression of candidate PCGs and lncRNAs, and the PFS of each patient was performed using univariate Cox regression analysis.

Selection of candidate PCGs and lncRNAs as a predictive signature. A model for selecting PCGs and lncRNAs as a predictive signature of prognosis was developed using the following formula (13,14):

$$\text{Risk score (RS)} = \sum {}^{N}i=1 \text{ (Explg x Coef)},$$

where 'N' in the formula represents the number of prognostic PCGs and lncRNAs, 'Explg' represents the expression of PCGs and lncRNAs, and 'Coef' represents the regression coefficient of PCGs and lncRNAs.

Random survival forests-variable hunting (RSFVH) analysis was used to screen 10 PCGs and lncRNAs, as previously described (13). Considering that a prediction model with a smaller number of PCGs and lncRNAs would be more practical than one with a larger number, all of the screened PCGs and lncRNAs were included. A time-dependent ROC curve was used to evaluate the specificity and sensitivity of the regrowth prediction of the RS of the 210-1=1023 signatures in the training set. The patients with NFPA in each set were separated into the high- and low-risk groups with the median RS in the training set used as the cut-off value.

Table I. Clinical characteristics of the 66 patients with NFPA.

Feature	Training set	Testing set	Entire set
Sex			
Female	15	19	34
Male	18	14	32
Age (years)			
≤52	18	15	33
>52	15	18	33
Knosp classification			
I	5	9	14
II	5	4	9
III	5	5	10
IV	18	15	33
Regrowth			
Yes	23	23	46
No	10	10	20
Invasion			
Yes	21	18	36
No	12	15	30

NFPA, non-functioning pituitary adenoma.

Statistical analysis. Kaplan-Meier survival analyses were performed to assess the regrowth distributions in the training and testing sets, and the statistical significance was assessed using the two-sided log-rank test. Chi-square tests were performed to analyze the associations among the clinical features. Multivariable Cox regression analysis was performed to evaluate the independence of the risk score from clinical features. $P<0.05$ was considered to indicate a statistically significant in the present study. All the analyses in the present study were performed in the R program (www.r-project.org) with the timeROC (cran.r-project.org/web/packages/timeROC/index.html), survivalROC (cran.r-project.org/web/packages/survivalROC/index.html) and randomForestSRC packages (cran.r-project.org/package=randomForestSRC).

Functional analysis of PCGs and lncRNAs. The co-expression association between the candidate PCGs and lncRNAs was evaluated with Pearson's correlation coefficients. The expression data used for Pearson's correlation were derived from the microarray analysis. Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment analyses of the PCGs and lncRNAs were performed to confirm the biological functions of lncRNAs. The co-expression correlations between the candidate PCGs and all other PCGs were assessed using Pearson's correlation analysis, and genes with P-values <0.05 and absolute values from the Pearson's

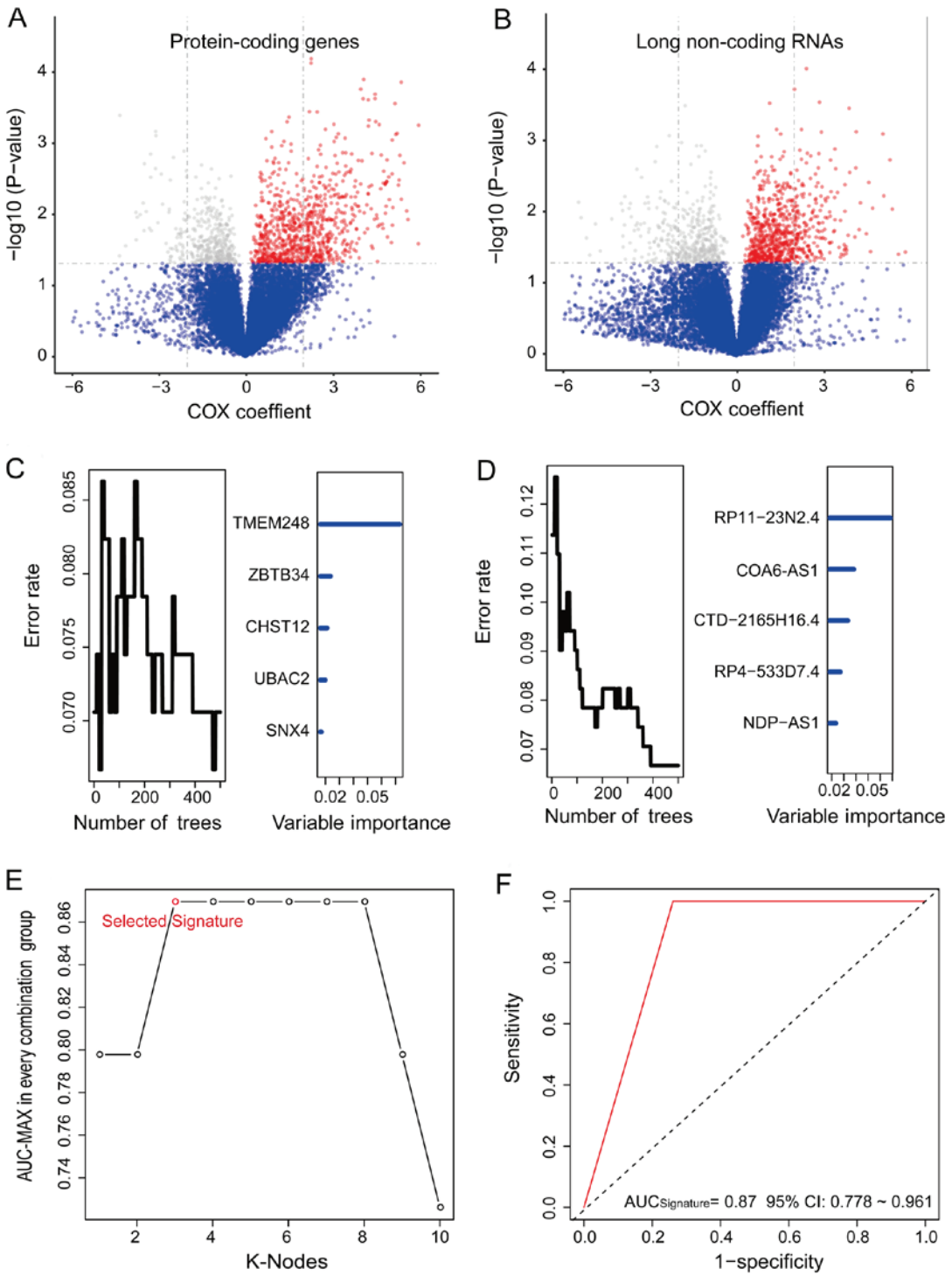

Figure 1. Identification of the candidate PCG and lncRNA markers in the training set. (A and B) Univariate Cox regression analysis of all PCGs and lncRNAs in the training set. (C and D) RSFVH analysis to identify the candidate PCG and lncRNA signatures. (E) The accuracies of all 1,023 signatures were evaluated and the 10 best accuracies are illustrated. (F) ROC analysis of the molecular signature predicting model in training set. PCG, protein-coding gene; lncRNA, long non-coding RNA; RSFVH analysis, random survival forests-variable hunting analysis.

correlation coefficient >0.6 were selected for KEGG and GO enrichment analyses. The above-mentioned analyses were performed with the clusterProfiler package (15,16).

Results

Construction of a PCG-lncRNA signature for the prediction of NFPA regrowth. Expression profiles of 66 pituitary tumor tissues were obtained using a ceRNA microarray. The expression levels of 18,829 PCGs and 19,741 lncRNAs were then determined. The 66 NFPAs were randomly divided into a training set (n=33) and a testing set (n=33). The training set was used for screening the candidate PCGs and lncRNAs associated with tumor regrowth, and the testing set was used for validating the classification power. In the selection step, all analyses were based on the training set.

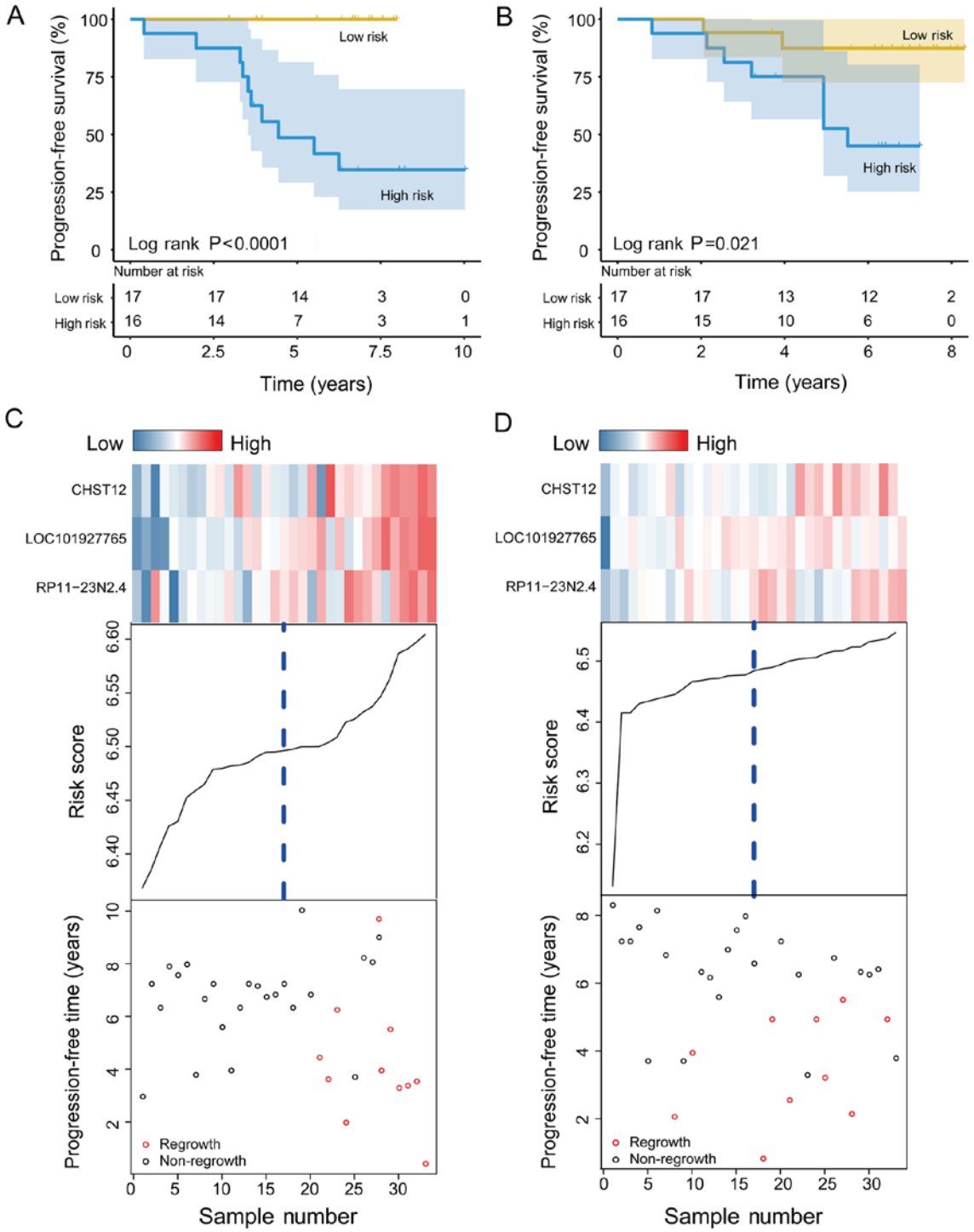

Figure 2. The molecular signature predicts the regrowth of patients with NFPA. (A and B) Kaplan-Meier analyses of patients divided into the high-risk and low-risk groups using the molecular signature in the training and testing set, respectively. The log-rank test was used to calculate P-values. (C and D) Risk score level, regrowth status and expression level of patients in the high-risk and low-risk groups. NFPA, non-functioning pituitary adenoma.

With regrowth as the dependent variable, univariate Cox regression analysis was performed. In total, 1,245 PCGs and 1,214 lncRNAs (Table SII) were identified that were significantly associated with tumor regrowth (P<0.05; Fig. 1A and B). Subsequently, 10 candidates, including 5 PCGs and 5 lncRNAs related to tumor regrowth were identified among the 2,459 members of the PCG and lncRNA set according to the permutation importance values of the random forest supervised classification (RSFC) analysis (Fig. 1C and D).

To obtain an optimized prediction signature, time-dependent ROC analysis was performed and the specificity and sensitivity of the regrowth prediction of the RS was evaluated for each patient according to the RS model of 210-1=1023 in the training set (Table SIII). The PCG and lncRNA combination with the max AUC was composed of CHST12, COA6-AS1 and RP11-23N2.4 (Fig. 1C and D; Table II). The RS of the signature was obtained as follows: RS = (4.95 x CHST12) + (3.41 x COA 6-AS1) + (1.90 x RP11-23N2.4). The AUC for the PCG and

Table II. Identification of PCGs and lncRNAs in the predicting signature and the univariable Cox association with regrowth.

Gene symbol	Coefficient[a]	P-value[a]	Expression level association with poor prognosis	Chromosome location (GRCh38/hg38)
CHST12	4.95	0.003	High	chr7: 2403588-2448484: +
COA6-AS1	3.41	0.001	High	chr1:234372807-234373593: -
RP11-23N2.4	1.90	0.007	High	chr15:52584907-52587652: +

[a]Derived from the univariable Cox regression analysis in the training set. PCG, protein-coding gene; lncRNA, long non-coding RNA.

Table III. Association of the signature with the clinical characteristics of the patients with NFPA.

	Training set			Testing set			Entire set		
Variable	Low risk	High risk	P-value[a]	Low risk	High risk	P-value[a]	Low risk	High risk	P-value[a]
Sex			> 0.99			0.62			0.63
Female	8	7		11	8		19	15	
Male	9	9		6	8		15	17	
Age (years)			0.59			0.02			0.03
≤52	8	10		4	11		12	21	
>52	9	6		13	5		22	11	
Invasion			> 0.99			0.39			0.48
Yes	11	10		11	7		22	17	
No	6	6		6	9		12	15	

[a]Data were analyzed using the Chi-squared test; P-value <0.05 was considered to indicate a statistically significant difference. NFPA, non-functioning pituitary adenoma.

lncRNA signature was 0.869 in the training set, which indicated a strong performance for regrowth prediction (Fig. 1E and F).

Predictive ability of the molecular signature for tumor regrowth. In the training set, patients were separated into the high-risk (n=16) and low-risk (n=17) group using the median RS of the signature as the cut-off. The results revealed that patients in the high-risk group had a shorter PFS than patients in the low-risk group (median PFS, 4.44 years vs. <7.97 years; P<0.001; Fig. 2A). The regrowth rate in the high-risk group was >50%, whereas that of the low-risk group was <1%. In the testing set, patients were similarly separated into the high-risk (n=16) and low-risk (n=17) groups. To confirm the predictive power of the CHST12, COA6-AS1 and RP11-23N2.4 signature, the results of Kaplan-Meier analysis for the patients in the testing set were plotted, as shown in Fig. 2B (median PFS, 5.51 years vs. <8.30 years; P=0.021). The regrowth rate of the patients in the low-risk group was approximately 12.6%, whereas that of patients in the high-risk group was 55.0%.

The regrowth status of patients with NFPA from the training and testing sets is illustrated in the dot plots (Fig. 2C and D). Moreover, the expression patterns of the 3 PCG and lncRNAs in the 2 groups are presented. Consistent with the pattern observed in the training set, patients with low-risk scores tended to have lower expression levels of the 1 PCG and 2 lncRNAs than the patients in the high-risk group.

Associations between patient clinical characteristics and the molecular signature. To better understand the associations between clinical characteristics and the CHST12, COA6-AS1 and RP11-23N2.4 signature, the associations of the signature with the clinical characteristics of the whole patient set (n=66) were investigated. Unlike age, sex, and invasion status based on Knosp classification (17,18) exhibited no association with the PCG-lncRNA signature (Table III).

To explore the independency of our signature from the clinical characteristics, multivariable Cox regression analysis was performed of the entire patient set using the PCG-lncRNA signature-based RS. It was found that the CHST12, COA6-AS1 and RP11-23N2.4 signature was indeed independent of these clinical characteristics (high-risk vs. low-risk, HR=1.47; 95% CI, 1.22-1.79; P<0.001, n=66; Table IV).

Comparison of the predictive power between the molecular signature and age. To compare the specificity and sensitivity of regrowth prediction between age and our signature, ROC analysis was performed. The predictive power of our signature was significantly better than that of age in the training/testing set (AUC=0.870/0.683 vs. AUC=0.726/0.676; Fig. 3A and B), which demonstrated that our signature is an effective for prediction.

To further understand the predictive ability of the signature regarding 3- to 5-year PFS, timeROC analysis was used within

Table IV. Univariate and multivariate Cox regression analysis of the signature of the patients with NFPA in the training, testing set and entire set.

A, Univariable analysis

	Training set (n=33)				Testing set (n=33)				Entire set (n=66)			
		95% CI				95% CI				95% CI		
Variable	HR	Lower	Upper	P-value	HR	Lower	Upper	P-value	HR	Lower	Upper	P-value
Sex												
Male vs. female	1.05	0.29	3.74	0.94	0.53	0.14	2.03	0.35	0.75	0.31	1.81	0.52
Age (years)												
>52 vs. ≤52	0.23	0.05	1.09	0.06	0.35	0.09	1.36	0.13	0.29	0.10	0.79	0.02
Knosp classification												
III IV vs. I II	1.36	0.72	2.54	0.34	1.11	0.68	1.83	0.67	1.21	0.82	1.78	0.33
Signature												
High-risk vs. low-risk	1.88	1.37	2.57	<0.001	1.24	0.92	1.66	0.04	1.47	1.23	1.76	<0.001

B, Multivariable analysis

	Training set (n=33)				Testing set (n=33)				Entire set (n=66)			
		95% CI				95% CI				95% CI		
Variable	HR	Lower	Upper	P-value	HR	Lower	Upper	P-value	HR	Lower	Upper	P-value
Sex												
Male vs. female	0.54	0.09	3.40	0.52	0.49	0.12	2.03	0.33	0.50	0.17	1.45	0.20
Age (years)												
>52 vs. ≤52	0.29	0.05	1.82	0.19	0.54	0.13	2.24	0.39	0.47	0.18	1.23	0.12
Knosp classification												
III IV vs. I II	1.39	0.66	2.92	0.38	1.14	0.66	1.96	0.64	1.20	0.82	1.75	0.35
Signature												
High-risk vs. low-risk	1.95	1.34	2.86	<0.001	1.23	0.89	1.70	0.05	1.47	1.22	1.79	<0.001

NFPA, non-functioning pituitary adenoma.

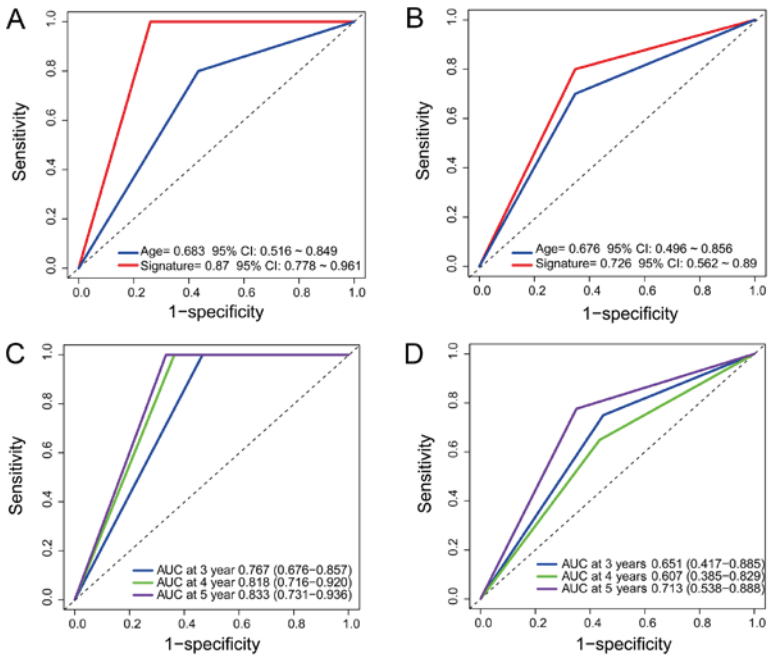

Figure 3. Comparison of the predictive power between age and the molecular signature. (A and B) The predictive power of the molecular signature compared with that of age in the training and testing set, respectively. (C and D) The predictive power of the PCG and lncRNA signature by timeROC analysis in the training and testing set, respectively.

the 2 sets. The AUC of our signature was 0.767/0.818/0.833 at 3/4/5 years in the training set and 0.651/0.607/0.713 at 3/4/5 years in the testing set, respectively (Fig. 3C and D). The results indicated that our predictive signature had a better performance for 5-year PFS than for 3- or 4-year PFS.

Functional characterization of the PCG and lncRNAs composing the prognostic signature. To explore the biological functions of the PCG and lncRNAs in our signature, the co-expression network of the one PCG and two lncRNAs with other PCGs were assessed using Pearson's correlation analysis. The expression levels of 1,076 PCGs highly correlated with those of one or more PCG and lncRNAs (Pearson's correlation coefficient >0.60, P<0.05, Table SIV). Subsequently, performed KEGG pathway and GO function enrichment analyses were performed for the co-expressed PCGs. GO function annotation analysis revealed that 1,074 PCGs were enriched in 98 GO/KEGG terms (Table SV), which indicated that the PCG and lncRNAs may be associated with important biological processes, including those involving the spliceosome and mitochondrial protein complex, etc., (Fig. 4).

Discussion

Unlike tumor regrowth in functioning pituitary adenoma, which can be monitored by monitoring the levels of specific serum hormones, tumor regrowth in NFPA is difficult to monitor. By the time patients are re-examined due to optic nerve compression symptoms, the tumor may have acquired a large volume, which presents a number of obstacles to total resection and post-operative recovery. Therefore, the present study aimed to develop novel predictive signatures that can identify early regrowth and serve as predictive markers of prognosis. The present study aimed to categorize patients into

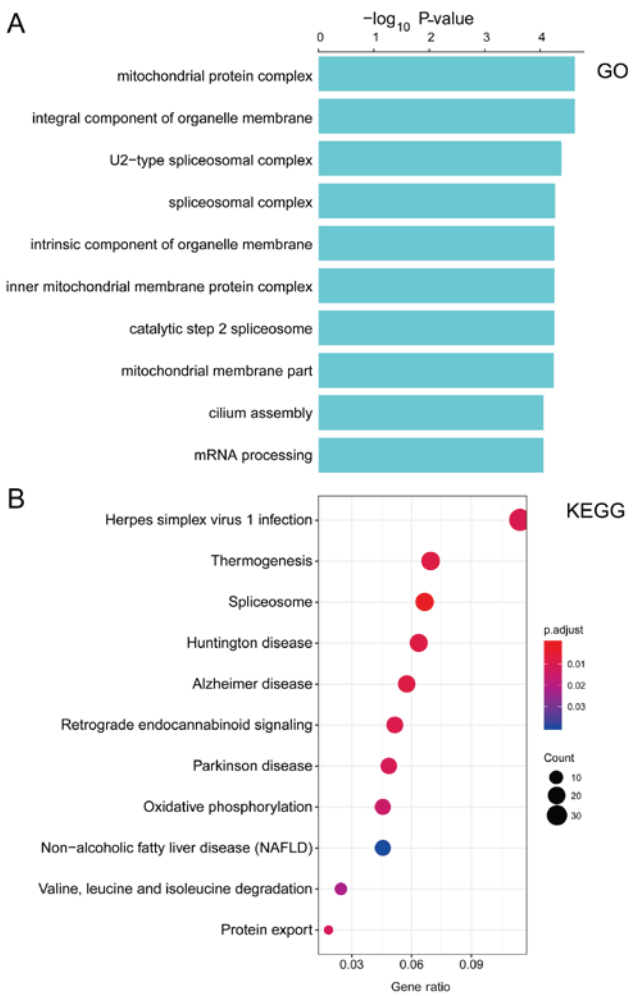

Figure 4. Functional enrichment analysis of the co-expressed PCGs with predicting PCGs and lncRNAs by clusterProfiler. (A) GO analysis. (B) KEGG analysis. PCG, protein-coding gene; lncRNA, long non-coding RNA.

a high- and low-risk group; thus the most effective and timely treatment can be applied to patients with NFPA.

A previous study focused on factors associated with tumor regrowth in NFPA with the ultimate goal of ameliorating the prognosis of patients at the post-operative stage (18). Age is recognized as an important independent factor influencing the prognosis of patients with NFPA, with a younger age being associated with a greater chance of tumor regrowth (19,20). However, the prognostic value of age is not as effective as the PCG-lncRNA signature identified in the present study. Ki-67 is another commonly used pathological prognostic evaluation index (21); however, the use of a single indicator for prognostic assessment has limitations in accurately evaluating patient prognosis. A previous study attempted to establish a statistical model that considers both combines clinical features (age and tumor volume) and molecular markers (p16, WIF1 and TGF-β) to evaluate regrowth probability among patients with NFPA post-operatively (1). In the present study, the inclusion of clinical characteristics did not improve predictive efficacy. Furthermore, unlike the above-mentioned study, a time component was added to the prognosis assessment and independently assessed the prognosis of patients at different time points.

In recent years, long non-coding RNAs have been reported in a number of types of tumor and represent a promising novel group of molecular markers for tumor biological behavior, disease diagnosis and prognosis evaluation (22-24). The expression of lncRNA H19 has been found to be decreased in pituitary adenomas, and its overexpression can markedly inhibit the growth of pituitary tumor cells; furthermore, it has been identified as a potential drug resistance marker (11). Xing *et al* (25), identified differentially expressed mRNAs and lncRNAs in clinically NFPA and normal pituitary, and constructed a mRNA-lncRNA co-expression network. However, their research failed to illustrate the regulatory mechanisms of key genes or lncRNAs or their effects on patient prognosis. Guo *et al* (26) successfully constructed a circRNA signature using the random survival forest algorithm, Kaplan-Meier analysis and ROC analysis. They randomly divided patients into a training and testing set and obtained an accuracy of 0.87 and 0.67 in each group. Compared with the single signature, the present study included PCGs and lncRNAs into the model and achieved a better accuracy in this model. In addition, the present study introduced a concept of time into our predictive signature, which could be more instructive in clinical practice. The present study found 2 lncRNAs that could be used as prognostic signatures. However, the function and regulatory mechanisms of these two lncRNA have not yet been reported. A PCG marker, CHST12, was also identified, which plays an important role in articular cartilage; however, there is limited research available on its role in tumors (27,28). The biological roles of the one PCG and two lncRNAs composing the signature in the present study are not yet fully illustrated and warrant further investigation in future studies.

There are a few limitations to the present study which need to be acknowledged. First, there is limited research available on the functions and molecular mechanisms of these PCGs and lncRNAs in tumors. The biological roles of CHST12, COA6-AS1 and RP11-23N2.4 have not yet been fully illustrated and thus require further investigation in the future. Second, there are limited sequencing data available regarding NFPA; thus, the verification of the present results with an independent validating set was not possible. In addition, further studies are required using larger cohort sizes and for the validation of the present results in the future. It should be noted that further research based on these data is still ongoing and the authors would like to share their data privately to researchers who are interested in pituitary adenoma. Finally, the application of our signature in clinical practice should be tested prospectively. Despite the above-mentioned limitations, the consistent and significant correlation of our CHST12, COA6-AS1 and RP11-23N2.4 signature with tumor regrowth indicates that this signature is a potentially potent prognostic predictive model for NFPA.

In conclusion, to the best of our knowledge, this is the first study to integrate PCGs and lncRNAs to predict tumor regrowth in patients with NFPA. The findings of the present study may provide a new aspect of prognostic evaluation and may help patients to benefit from early intervention.

Acknowledgements

The authors would like to thank Dr Yazhuo Miao and Mr. Qiuyue Fang from Capital Medical University for the selfless assistance in collecting clinical data.

Funding

The present study was funded by the National Natural Science Foundation of China (grant nos. 81771489, 81672495 and 81601205), the National High Technology Research and Development Program of China (863 Program, grant no. 2014AA020610), the Beijing Municipal Science and Technology Commission (grant no. Z171100000117002), the Beijing Talents Fund (grant no. 2015000021223ZK24) and the China National Key Research and Development Program (grant no. 2017YFC0908300).

Availability of data and materials

Further research regarding the topic of the present study is ongoing and all data in this study are available from the corresponding author on reasonable request.

Authors' contributions

SC was involved in data collection and analysis, and in the writing of the manuscript. JG and ZL were involved in data collection. CL and YZ were involved in the conception and design of the research, funding management and in the final editing of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Medical Ethics Committee of Beijing Tiantan Hospital and written informed consent for the use of the resected samples for research purposes was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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