Identification of tRNA-derived small RNAs and their potential roles in the hippocampus of nicotine exposure rats

Jiali Shao[1†], YanXia Fei[1], ChenSu[1], Wangyuan Zou[2], JinFeng Yang[1*]

[1]Department of Anesthesiology, Hunan Cancer Hospital, School of Xiangya Medicine, Central South University, Changsha 410013, Hunan, China.

[2]Department of Anesthesiology, Xiangya Hospital, Central South University, Changsha 410008, Hunan, China.

*Corresponding author. Email: 315977705@qq.com

† First author. Email: 527758565@qq.com

Keywords: tRNA-derived small RNAs (tsRNAs), small non-coding RNAs (sncRNAs), nicotine exposure

Abstract

Nicotine use is highly prevalent and brings a huge burden on individuals, society, health-care systems and economic development. The existing mechanisms underlying nicotine' actions can't illuminate all basic and clinical problems thoroughly. Transfer RNA-derived small RNAs (tsRNAs) is a novel class of small non-coding RNAs (sncRNAs), possessing potential regulatory functions in various diseases. However, the roles of tsRNAs in nicotine exposure have not been determined yet. In this study, firstly we established nicotine exposure model by subcutaneously injecting (sc.) with 0.5mg/kg of nicotine twice daily for 14 consecutive days, and conducted some behavioral observations (the pain threshold and body weight gains). Secondly, we identified the differentially expressed profiles of tsRNAs in rat hippocampus on saline or nicotine delivery conditions by using ncRNA-Seq, and then predicted the promising functions of the putative genes of the tsRNAs by bioinformatic method. The results shown that there were 26 differentially expressed tsRNAs (7 up-regulated and 19 down-regulated tsRNAs) (Fold change > 1.5; $P < 0.05$), of which the tRF-5 was the most common type. Eight tsRNAs were selected to validate the sequencing result by RT-qPCR. Then, based on the sequencing and RT-qPCR data, five candidate tsRNAs (tRF-1-T28-His-GTG-1, tRF-5c-Glu-CTC-1, tRF-5c-Glu-CTC-3, tRF-5c-Gly-GCC-2-M2, tRF-5c-Glu-TTC-4) were finally selected for further bioinformatic analysis. The GO and KEGG pathway enrichment analysis suggested that the five candidate tsRNAs might play regulatory roles through the cholinergic synapse pathways, dopaminergic synapse pathways, etc. In conclusion, our results indicated that tsRNAs were dysregulated in the rat hippocampus after nicotine exposure, and among them, tRF-5c-Glu-CTC-1 was the

most promising one, which might lay a novel foundation for further research into nicotine' actions.

Introduction

According to the 2019 National Health Interview Survey (NHIS), an estimated 50.6 million U.S. adults aged ≥18 years (20.8%) reported currently using any tobacco product. And Chinese CDC reported that in 2018, approximately 26.6% of people aged ≥15 years were current smokers in China[1, 2]. Unfortunately, cigarette smoking remains the leading cause of preventable disease and death in the United States[3]. From 1964–2012, an estimated 17.6 million deaths were related to smoking, more than 480,000 deaths yearly[4]. Cigarette smoking is linked with diseases of nearly all organs of the body, such as various cancers, respiratory diseases, cardiovascular diseases, neurological disorders, etc. In addition to public health, smoking imposed a heavy burden on social economic. Experts estimated that the annual societal costs attributable to smoking in the United States were approximately $300 billion, including $168 billion for healthcare[5, 6].

Nicotine, the primary component of tobacco, exerts multiple biological functions through which smoking increases risk for diseases. Nicotine exposure brings a series of changes in body, including stimulating the adrenal glands, activating the reward circuits, modulating neurotransmitters and receptors, and so on. The most well-known mechanisms of nicotine' actions are the role of nicotinic cholinergic and dopaminergic system. In brief, nicotine can bind and activate nicotinic acetylcholine receptors (nAChRs), which are widely distributed throughout the nervous system and body, and thus result in the release of a variety of neurotransmitters (dopamine, gamma-aminobutyric acid (GABA), serotonin, glutamate etc) in the brain, particularly dopamine[7, 8]. Besides, nicotine exposure can also produce additional neuroadaptations, including neuronal homeostatic mechanisms, neuronal

scaffolding proteins expression and epigenetic regulations[9-11]. However, there is still much to be learned and done in order to fully understand how nicotine functions.

As the non-coding RNAs (ncRNAs) have been the hot topic in many areas in recent years, many researchers pay their attention to the possibility of ncRNAs in nicotine use. Chen et al reported that ncRNAs (miRNAs, lncRNAs and circRNAs) appear to form complex interactions and impact the nicotine biosynthesis in tobacco[12], and Aliso suggested that a pattern of miRNA/mRNA regulation occurs in the habenulo-interpeduncular circuit during nicotine withdrawal[13]. As the major source of small noncoding RNAs (sncRNAs), tRNA-derived small RNAs (tsRNAs) produced by cleavage at different sites from mature or pre-tRNAs are gradually becoming the emerging research direction[14, 15]. tsRNAs were first discovered in cancer patients until 1970s and at that time, they were considered to be merely degradation products of tRNAs and have no biological functions[16]. With the rapid development of high-throughput sequencing technology, tsRNAs were revealed to function under various diseases, such as cancer, neurodegenerative diseases, metabolic disorders, and others[15, 17, 18]. As to the specific biological roles of tsRNAs, lots of researches have been done. For instance, Ivanov et al suggested that tsRNAs could inhibit translation by displacing eIF4G/A from mRNA[19]; Kim and his colleagues reported that LeuCAG3′tsRNA could modulate apoptosis by binding at least two ribosomal protein mRNAs (RPS28 and RPS15)[20]. In general, several regulatory mechanisms have been reported to date: 1) act like miRNAs to regulate gene expression, 2) regulate translation process, 3) function as epigenetic factors, 4) modulate apoptosis and immune response, etc[14, 15, 21]. Although tsRNAs have been the hot area in recent

years, the majority of studies mainly focused on the roles of tsRNAs in cancer and immune[17, 22, 23], and there have no relevant researches about the relationship between tsRNAs and nicotine use or other addictive drugs thus far. Given its various roles under physiopathological conditions, it's no doubt that exploring tsRNAs will provide a bran-new insight into nicotine exposure research.

Therefore, the aim of our study is to identify the expression profile and potential roles of tsRNAs in hippocampus after nicotine exposure. Using RNA-sequencing technologies, we identified the expression profiles of tsRNAs, and then predicted the potential roles of the candidate tsRNAs by bioinformatic methods. Our findings might reveal the potential mechanisms and novel molecular targets underlying nicotine exposure, and provide a new direction for future research in nicotine use.

Materials and methods

Animals and experiment groups

24 Adult male Sprague-Dawley rats (200±10g) were obtained from the Hunan SJA Laboratory Animal Company (Hunan, China). And then the rats were housed in groups and maintained for at least 5 days before the study in a temperature- (22±2℃) and humidity controlled (50% relative humidity) environment with 12-h light/dark cycles. The rats had food and water freely available. The experimental procedures were approved by the Animal Care and Use Committee of Hunan Cancer Hospital and conducted in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

To establish the nicotine exposure model, the rats in nicotine group (NI, n = 12) were subcutaneously injected (sc.) with 0.5mg/kg (0.5 mg/ml) of nicotine twice daily at 08: 00-09:00 am and 16:00-17:00 pm for 14 consecutive days. The rats in normal saline group (NS, n = 12) were injected with the same volume of 0.9% saline at the same time points.

To analyze the effects of nicotine on sensation, hind paw mechanical withdrawal thresholds were measured every other day using von Frey filaments test (Italy, UGO Basile) and the test was always performed between 13:00-16:00 pm[24]. The results were converted to the 50% of paw withdrawal threshold (50%PWT). The body weight was tested every day before the morning injection.

Tissue Collection and RNA isolation

On day 14, 1h after the morning injection, the rats in both groups were decapitated under deep anesthesia by 4% isoflurane inhalation for 3 minutes. The hippocampus (n=6, each group) were quickly dissected and collected on ice and fresh frozen in liquid nitrogen. Total

RNA was extracted from frozen tissues using Trizol (Invitrogen, CA, USA) according to the manufacturer's instructions. The quantity and concentration of each RNA sample were assessed using a NanoDrop ND-1000 Spectrophotometer (Thermo, USA) and the integrity was checked by agarose gel electrophoresis.

Library preparation and sequencing

The following treatments were performed before library preparation for total RNA samples to remove RNA modifications that interfered with small RNA-seq library construction: 3'-aminoacyl (charged) deacylation to 3'-OH for 3adaptor ligation, 3'-cP (2,3 -cyclic phosphate) removal to 3'-OH for 3' adaptor ligation, 5'-OH (hydroxyl group) phosphorylation to 5'-P for 5'-adaptor ligation, m1A and m3C demethylation for efficient reverse transcription. Sequencing libraries were size-selected for the RNA biotypes to be sequenced using an automated gel cutter, which were qualified and absolutely quantified using an Agilent Bio-Analyzer 2100 (Invitrogen, CA, USA). To generate the mature tRNA libraries, we removed the predicted intronic sequences (if present) and added an additional 3-terminal "CCA" to each tRNA. To generate the precursor tRNA libraries, we included 40 nucleotides of flanking genomic sequence on either side of the original tRNA sequence.

Sequencing data analysis

Image analysis and base calling were performed using Solexa pipeline v1.8 software (Off-Line Base Caller software, v1.8). Sequencing quality was examined by FastQC software and trimmed reads (pass Illumina quality filter, trimmed 3-adaptor bases by cutadapt) were aligned to mature-tRNA and pre-tRNA sequence getting from the Genomic tRNA Database using Novo Align software (v2.07.11). The remaining reads were aligned

to transcriptome sequences (mRNA/rRNA/snRNA/snoRNA/piRNA/miRNA). The abundance of tsRNAs were evaluated using their sequencing counts and normalized as counts per million of total aligned reads (CPM). The tsRNAs differentially expressed are screened based on the count value with R package edgeR. Pie plots, Venn plots, Hierarchical clustering, Scatter plots and Volcano plots are performed in R or perl environment for statistical computing and graphics of the expressed tsRNAs. The ncRNA-seq was performed by Kangcheng Bio-tech (Shanghai, China).

RT-qPCR validation

RT-qPCR was conducted to confirm the sequencing data. Eight differentially expressed tsRNAs were chosen for RT-qPCR. U6 small nuclear RNA (snRNA) was used as a reference. The RNA was reversely transcribed to cDNA using an rtStar™ tRF&tiRNA Pretreatment Kit (Arraystar, MD, USA) and an rtStar First-Strand cDNA Synthesis Kit (3' and 5' adaptor) (Arraystar, MD, USA) according to the manufacturer's instructions. Then, RT-qPCR was performed using 2XPCR master mix (Arraystar, MD, USA) with a ViiA7 Real-time PCR System (Applied Biosystems, CA, USA). The parameter settings were as follows: 95℃ denaturation (10 min), 40 amplification cycles at 95℃ (10 s), and 60℃ (60 s). After the amplification reaction was finished, the procedure was performed as follows: 95℃ (10 s), 60℃ (60 s), and 95℃ (15 s). All reactions were performed in triplicate. And the sequences of all primers are presented in supplementary table S1.

Bioinformatic Prediction

The five significantly differentially expressed tsRNAs (Fold change > 1.5 and *P*-value < 0.05) selected according to the results of the sequencing and RT-qPCR were analyzed

using bioinformatic methods. According to a previous study[25], two common algorithms were used to predict the tsRNA targets, including TargetScan and miRanda. A graph of the tsRNA/mRNA network was derived using the Cytoscape software (version 3.5.1, the Cytoscape Consortium, San Diego, CA, USA) to visualize these relationships. Then, to forecast the biological annotation and pathway of the putative targets, we used Gene Ontology (GO) to reveal the biological process, cellular component, and molecular function of the target mRNAs. Significant pathways were identified using the pathways in the Kyoto Encyclopedia of Genes and Genomes database (KEGG).

Statistical analysis

Statistical analysis was performed using Graphpad prism software (version 3.0, Chicago, IL, USA). The behaviors and PCR results are shown as the mean ± standard error of the mean (SEM). Two-tailed Student's t-test was used to compare the significant differences between the two groups. The level of significance was set at $P < 0.05$.

Database and Accession Numbers

The raw data of the tsRNA-Seq in our study were deposited at the NCBI Gene Expression Omnibus (GEO) under the accession number GSE162402.

Results

Behavioral observations: the effects of nicotine on sensation and body weight

The mechanical test revealed that on day 5, the 50%PWT of the nicotine group was significantly higher than that of the saline group (n = 12 in each group, ***$P < 0.001$), and this state continued to day 13 (Fig. 1a). The result indicated that nicotine exposure could change the pain sensation and produce an analgesic effect. Weight measure shown that on day 4, the weight increase of the nicotine group was lower, compared with the saline group(n = 12 each group, **$P < 0.01$), suggesting that nicotine exposure might impact the energy metabolism and appetite. In the days that followed, there was no significant difference about weight increase between both groups (Fig. 1b).

Expression profiles of tsRNAs in hippocampus of nicotine and saline rats

The scatter plots were created presenting the tsRNA expression variation between the nicotine group and saline group using the fold change (n=3 in each group, shown in Fig. 2a). According to the criteria of a log2 (fold change) > 1.5 and a *P*-value< 0.05, a total of 26 differentially expressed tsRNAs were finally identified, including 7 up-regulated and 19 down-regulated tsRNAs (Fig. 2b). And the hierarchical clustering heat-map for the 26 differentially expressed tsRNAs was displayed in Figure 2c. The detailed information on the top 5 up-regulated and 10 down-regulated tsRNAs ranked by fold change were presented in Table 1. In Figure 2d, the Venn diagram presented that a total of 354 were commonly expressed tsRNAs in both groups, 73 tsRNAs specifically expressed in nicotine group, and 24 tsRNAs specifically expressed in saline group.

Catalog of tRFs/tiRNAs in both groups

The pie chart demonstrates the percentage of each subtype of the tsRNAs expressed uniquely in two groups (Fig. 3a-b). Overall, except for tRF-2, the expression levels of other tsRNAs (tRF-1, tRF-3, tRF-5, tiRNA-3, tiRNA-5) in Group nicotine were increased, as compared to Group saline. In both groups, tRF-5 were the most common type. The read counts and read length for each unique read in both groups are shown in the bar chart of sequence read length distribution (Fig. 3c-d). We found that the length distribution of tsRNAs in both groups mainly concentrate on 20-24nt. The number of tsRNA subtypes against tRNA iso-decoders and the frequency of subtypes against the length of the tsRNA were shown by the stacked bar charts (Fig. 3e-h). And in these charts, we discovered that Glu-CTC, Glu-TTC, Gly-CCC, Gly-GCC, His-GTG are the most common tRNA iso-decoders of tsRNAs in both groups.

Validation for the differentially expressed tsRNAs (DEtsRNAs)

To validate the sequencing data, RT-qPCR was performed. Eight tsRNAs (3 upregulated and 5 downregulated) were chosen according to their raw expression level, the fold change value and the *p*-value (shown in Table 2). As a result, tRF-1-T28-His-GTG-1 was significantly up-regulated, and tRF-5c-Glu-CTC-1, tRF-5c-Glu-CTC-3, tRF-5c-Gly-GCC-2-M2, tRF-5c-Glu-TTC-4 were down-regulated in Group nicotine, compared with Group saline (n=3 in each group, $p < 0.05$) (Fig. 4a-b). The comparison of the results of sequencing and PCR were displayed in Table 3, and the overall results were in line with

265 the sequencing data.

266

267 **Bioinformatic Prediction**

268 The five significantly DEtsRNAs, namely, tRF-1-T28-His-GTG-1, tRF-5c-Glu-CTC-1,
269 tRF-5c-Glu-CTC-3, tRF-5c-Gly-GCC-2-M2, tRF-5c-Glu-TTC-4 were selected for further
270 bioinformatic analysis. First, we predicted the potential target genes of these five tsRNAs
271 based on TargetScan and Miranda algorithms, and the putative genes with context less
272 than -0.4 were displayed (Fig. 5). We found that there existed interactions among these
273 five candidate tsRNAs, and particularly tRF-5c-Glu-CTC-1 and tRF-5c-Glu-CTC-3 shared
274 plentiful common genes. Secondly, we conducted the GO and pathway enrichment
275 analysis of all the target genes of these five candidate tsRNAs. In terms of molecular
276 function, the general results indicated that the most significant enrichment and the most
277 meaningful terms were protein binding (GO:0005515) (see Fig. 6a, c, e, g, j). As to the
278 cellular component of these candidate tsRNAs, the results shown that the up-regulated
279 tRF-1-T28-His-GTG-1 was mainly found in spindle (GO:0005819) and intracellular
280 (GO:0005622) (Fig. 6a), whereas, the four down-regulated tsRNAs (tRF-5c-Glu-CTC-1,
281 tRF-5c-Glu-CTC-3, tRF-5c-Gly-GCC-2-M2, tRF-5c-Glu-TTC-4) were usually found in
282 intracellular (GO:0005622) and intracellular part (GO:0044424) (Fig. 6c, e, g, j). Then the
283 major biological processes of these candidate tsRNAs were involved in localization
284 (GO:0051179), establishment localization (GO:0051234), transport (GO:0006810) and cell
285 surface receptor signaling pathway (GO:0007166) (Fig. 6a, c, e, g, j).

286 According to the KEGG enrichment analysis, the most significantly enriched pathways

of tRF-1-T28-His-GTG-1 were protein processing in endoplasmic reticulum, cell adhesion molecules, and cocaine addiction (Fig. 6b). For the four down-regulated tsRNAs (tRF-5c-Glu-CTC-1, tRF-5c-Glu-CTC-3, tRF-5c-Gly-GCC-2-M2, tRF-5c-Glu-TTC-4), MAPK signaling pathway, Axon guiding, dopaminergic synapse, cholinergic synapse, glutamatergic synapse, neurotrophin signaling pathway are mainly identified in general (Fig. 6d, f, h, k). Two important pathways (the cholinergic synapse and dopaminergic synapse pathways) of tRF-5c-Glu-CTC-1 involved in the mechanism of nicotine' actions were displayed in Figure 7, and the other major pathways were shown in supplementary Figure S1.

Discussion

In this study, we reported the expression profiles of tsRNAs in the rat hippocampus after nicotine exposure for the first time. The sequencing data revealed that there were 26 differentially expressed tsRNAs, involving 7 up-regulated and 19 down-regulated tsRNAs, in the rat hippocampus after nicotine exposure. Subsequently, 8 DEtsRNAs were chosen to validate by RT-qPCR and among them, 5 candidate DEtsRNAs for further bioinformatic predictions. The GO and KEGG pathway analysis demonstrated that the potential targeted genes and the putative pathways of the 5 DEtsRNAs mainly focused on several critical signaling channels, which played important roles in nicotine' actions. Taken together, our findings suggest that these dysregulated tsRNAs may play regulatory roles on how nicotine functions.

There is a consensus that nicotine exposure can induce changes in pain perception and body weight in animal models and human studies[26-28]. Recent research indicated that nAChRs agonists (such as nicotine, epibatidine, choline) might make up a novel class of analgesics for pain management[29-31]. In our previous research, we found that short-term use of nicotine could produce analgesic effects, but long-term use or withdrawal led to hypersensitivity, and further investigation indicated that nicotine could alter pain sensitivity by affecting the expression of the pain related factor[24]. In this study, when the rats were continuously injected with nicotine, the paw withdrawal threshold increased and the body weight gains decreased, which was in accordance with our previous study.

Nicotine exerts its functions largely through the widespread nAChRs. Substantial researches suggested that nicotine exposure could upregulate nAChRs by multiple

processes, including changes in receptor assembly, trafficking, and degradation[32-34]. In recent years, genetic factors is becoming the research focus about nAChRs, and variants in the CHRNA5-CHRNA3-CHRNB4 gene cluster are the most studied associated with nicotine' actions[35, 36]. Besides, Cameli et al pointed out that genetic variations in CHRNA7 and CHRFAM7A were related to nicotine addiction[10].In our study, we found that tRF-5c-Glu-CTC-1, tRF-5c-Glu-CTC-3, tRF-5c-Glu-TTC-4 might be involved in the cholinergic synapse pathway, and in turn influence the excitability of neurons and glial cells. Further analysis indicated that the putative genes of these above tsRNAs included CHRM1, CHRM3, CHRNA3, CHRNA4 and CHRNA7, which encoded variant subunits of nAChRs. So we presumed that tRF-5c-Glu-CTC-1, tRF-5c-Glu-CTC-3, tRF-5c-Glu-TTC-4 might function in nicotine' actions by modifying the genes expression of nAChRs' subunits, thus influencing the structure and activity of nAChRs.

In addition to nAChRs, there exited some other possible mechanisms involved in nicotine' actions. On the one hand, nicotine exposure can produce widespread neuroadaptations in nervous system, including dopaminergic (DA) synapses, GABAergic circuitry, glutamatergic synapses, and so on. On the other hand, these neuroadaptations were in turn to participate in the nicotine' actions[37-39]. Recent studies shown that genetic variations in DA, GABA could regulate nicotine' actions. For instance, Bühler and his colleagues summarized that apart from nAChRs genes, variations in ANKK1, DRD2 and GABA were likewise associated with nicotine-related phenotypes[35]. Liu et al constructed the networks of candidate genes associated with nicotine addiction, and concluded that cholinergic receptors (CHRNA1, CHRNA4, CHRNA7), dopamine receptors (DRD1, DRD2,

353 DRD3) and GABA receptors (GABRA1, GABRA2, GABRA4) were involved in diverse
354 biological functions[40]. Other researches about genetic modulations in DA (COMT, GCH1,
355 and DRD2) and GABA indicated these changes could affect the modulation of DA in pain
356 pathway[41, 42]. In our study, we predicted the target genes of these five candidate tsRNAs,
357 and found that tRF-5c-Glu-CTC-1 might regulate the expressions of COMT, DRD2,
358 GABRA4, whereas tRF-5c-Glu-CTC-3 might regulate DA related genes COMT and DRD2.
359 The KEGG pathway analysis indicated that tRF-5c-Glu-CTC-1 probably participated in the
360 dopaminergic synapse, GABAergic synapses and glutamatergic synapses pathways, while
361 tRF-5c-Glu-CTC-3 might merely modulate the dopaminergic synapse and glutamatergic
362 synapses pathways, not GABAergic synapses.
363 Apart from the above neurotransmitters, previous researches suggested that nicotine
364 shared the similar functional molecules, signal paths and acting sites with some other
365 addictive drug, such as cocaine, morphine[11, 43, 44]. We found in our study that the
366 candidate tsRNAs could modulate the pathways of cocaine, morphine and amphetamine
367 addiction. To be specific, tRF-5c-Glu-CTC-1 took part in all the three paths of cocaine,
368 morphine and amphetamine addiction, tRF-1-T28-His-GTG-1 and tRF-5c-Glu-CTC-3 took
369 part in cocaine addiction, and tRF-5c-Gly-GCC-2-M2 in morphine addiction. Hence, we
370 speculated that those candidate tsRNAs might likely contribute to nicotine addiction in a
371 similar way. More researches are needed.
372 According to the KEGG pathway analysis, we also discovered that except the above
373 pathways, the putative genes of the five candidate tsRNAs were also enriched in some
374 other critical paths, such as MAPK signaling pathway, mTOR signaling pathway,

neurotrophin signaling pathway, which have been suggested to involve nicotine' actions in body[45-47]. And further analysis indicated that the majority of the putative pathways of tRF-5c-Glu-CTC-1 were associated with the previously known mechanisms of nicotine' actions, so we regard tRF-5c-Glu-CTC-1 as the most promising candidate for further study in nicotine' actions.

Generally, tsRNAs with lengths of 18–40 nucleotides, include two main types based on the length and cleavage sites on tRNA or pre-tRNA: tRNA-derived fragments (tRFs) and tRNA-derived, stress-induced RNAs (tiRNAs). tiRNAs are usually the products of angiogenin cleavage of mature tRNAs at the anticodon site during stress, which contain two subtypes tiRNA-5 and tiRNA-3. While tRFs derive from cleavage on any sites of mature or pre-tRNAs, which contain four subtypes tRF-5, tRF-3, tRF-1, tRF-2[14, 15, 48]. Growing evidence indicated that the functions of tsRNAs depended on their subtype and specific subcellular localization. Some researchers stated that cytosolic and mitochondrial tiRNAs could repress protein translations and be associated with apoptosis initiation[49, 50]. Kumar et al remarked that in HeLa cell line, tRF-5s are mostly nuclear while tRF-3s and -1s are cytoplasmic, and further study in human HEK 293 cells suggested that tRF-5s and tRF-3s are associated with Argonautes 1, 3 and 4, and then target mRNAs in a manner similar to miRNAs[51]. Zhang and his colleagues reported that in monocytes/dendritic Cells, td-piR$^{(Glu)}$ (tRF-5) could interact with PIWI protein and play a role in regulation of chromatin remodeling in somatic cells, just like piRNAs[52]. According to the known literature of tsRNAs as well as our data, the most promising candidate tRF-5c-Glu-CTC-1 belonged to tRF-5, so we postulated that it's likely that tRF-5c-Glu-CTC-1 might function by interacting

with Ago proteins or PIWI protein, and have post-transcriptional regulations similarly to miRNAs or epigenetic modulations like piRNAs. In nicotine exposure model, nicotine exposure could upregulate the expression of nAChRs and downregulate tRF-5c-Glu-CTC-1 whose downstream genes CHRM1, CHRM3, CHRNA4 and CHRNA7 could encode the subunits of nAChRs shown in our data. Given the negative regulatory relationship between tRF-5c-Glu-CTC-1 and its putative genes, we conjecture that nicotine exposure may reduce the biosynthesis of tRF-5c-Glu-CTC-1, then alleviate its suppression on the genes expression of nAChRs subunits, and thereby upregulate nAChRs, which lay the foundation for nicotine' actions. Further research is needed in future.

There are some limitations about our study. First, we only detect the profiles of tsRNAs on nicotine exposure condition, not nicotine withdrawal, and lack the dynamic detection of tsRNAs at different time point. Secondly, our research is preliminary and further functional research is needed to identify the specific locations and functions of the candidate tsRNAs in vitro and in vivo. Finally, detection of tsRNAs in human trial is hopefully to conduct in future.

To sum up, our study provided the differentially expressed profiles of tsRNAs in rat hippocampus after nicotine exposure for the first time. Further bioinformation analysis revealed that the tsRNAs might be a new class of regulatory molecules in nicotine related researches, which might function by regulating some important signaling pathways, such as the cholinergic synapse, dopaminergic synapse. Among them, tRF-5c-Glu-CTC-1 is the most promising one, and probably act in a manner of miRNAs or piRNAs. In a word, our study paves a new road for research into nicotine' actions, especially in addiction and

419 nicotine-mediated analgesia, and provides novel modulatory targets for this area.

420

421

422

423

424

425

426

427

428

429

430

431

432

433

434

435

436

437

438

439

440

Supplementary information

Additional file 1: Table S1. Sequences of primers used for RT-qPCR assay

Additional file 2: Figure S1. Mapping of several important putative pathways of the 5 candidate tsRNAs

Abbreviations:

tsRNAs: tRNA-derived small RNAs; sncRNAs: small noncoding RNAs; DEtsRNAs: differentially expressed tsRNAs; tRFs: tRNA-derived fragments; tiRNAs: tRNA-derived, stress-induced RNAs; PWT: paw withdrawal threshold; CPM: counts per million of total aligned reads; BP: Biological process; CC: Cellular component; MF: Molecular function; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; RT-qPCR: Quantitative real-time PCR.

Acknowledgements

Not applicable.

Authors' contributions

J-LS performed the experiments, collected and analyzed the data, and drafted the manuscript. Y-XF performed the experiments. CS and W-YZ revised the manuscript. J-FY designed the study, supervised the project and experiments and revised the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by grants from Hunan Province:"100 Talents Plan" on Overseas High-Level Talents Introduction (20174300015).

Availability of data and materials

Please contact the author for data requests

Ethics approval and consent to participate

All experiments were approved by the Animal Care and Use Committee of Hunan Cancer Hospital, and performed following the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Monica E. Cornelius, Teresa W. Wang, Ahmed Jamal, Caitlin G. Loretan, Linda J. Neff. Tobacco Product Use Among Adults — United States, 2019. Morbidity and Mortality Weekly Report, 2020. 69(46):1736-42.
2. Chinese CDC. 2018 China Tobacco Control Report. http://www.chinacdc.cn/ jkzt /sthd_3844/slhd_4156/201905/t20190530_202932.html. Accessed 30 May 2019.
3. US Department of Health and Human Services. The health consequences of smoking-50years of progress: a report of the Surgeon General. Atlanta, GA:2014. https://www.ncbi.nlm.nih.gov/books/NBK179276/pdf/Bookshelf_NBK179276.pdf
4. Theodore R.H, Rafael M, Kenneth E. W, Clare M, Jihyoun J, Suresh H.M, and David T.L. Tobacco control and the reduction in smoking-related premature deaths in the United States, 1964-2012. JAMA, 2014. 311(2):164-71.
5. National Institute on Drug Abuse. Tobacco, Nicotine, and E-Cigarettes Research Report. 2020:1-59.
6. Xin X, Ellen E.B, Sara M.K, Sean A.S and Terry F.P. Annual healthcare spending attributable to cigarette smoking: an update. Am J Prev Med, 2015. 48(3):326-33.
7. Ruthie E.W, Shannon L.W, Mariella D.B, John A.D. Nicotinic acetylcholine receptors and nicotine addiction: A brief introduction. Neuropharmacology, 2020:1-30.
8. Roger L. Papke. Merging old and new perspectives on nicotinic acetylcholine receptors. Biochemical Pharmacology, 2014. 89(1):1-11.
9. Tamaki Hayase. Epigenetic mechanisms associated with addiction-related behavioural effects of nicotine and/or cocaine: implication of the endocannabinoid

507 system. Behav Pharmacol, 2017. 28(7):493-511.
508 10. Cameli C, Elena B, Maria D.P, Giuliano G, Stefano C, et al. Genetic variation in
509 CHRNA7 and CHRFAM7A is associated with nicotine dependence and response to
510 varenicline treatment. Eur J Hum Genet, 2018. 26(12):1824-31.
511 11. Ju W, Wenji Y, Ming D.L. Genes and pathways co-associated with the exposure to
512 multiple drugs of abuse, including alcohol, amphetamine/methamphetamine,
513 cocaine, marijuana, morphine, and/or nicotine: a review of proteomics analyses. Mol
514 Neurobiol, 2011. 44(3):269-86.
515 12. Xi C, Shuo S, Fangjie L, Enhui S, Lu L, et al. A transcriptomic profile of topping
516 responsive non-coding RNAs in tobacco roots (Nicotiana tabacum). BMC Genomics,
517 2019. 20(856):1-14.
518 13. Alison P.C, Junko T, Rubing Z.S, Ciearra B.S, Susanna M, et al. Integrated miRNA-
519 /mRNA-Seq of the Habenulo-Interpeduncular Circuit During Acute Nicotine
520 Withdrawal. Sci Rep, 2020. 10(813):1-14.
521 14. Hak K.K, Ji-Hyun Y and Mark A.K. Transfer RNA-Derived Small RNAs: Another
522 Layer of Gene Regulation and Novel Targets for Disease Therapeutics. Mol Ther,
523 2020. 28(11):1-18.
524 15. Siqi L, Zhengping X and Jinghao S. tRNA-Derived Small RNA: A Novel Regulatory
525 Small Non-Coding RNA. Genes 2018. 9(246):1-15.
526 16. Ernest B, B.S Baliga, Charles W.G, C.W Kuo, Sidney B, et al. High Turnover Rate
527 of Transfer RNA in Tumor Tissue. CANCER RESEARCH, 1977. 37:3362-66.
528 17. Yangyang C, Yue H, Xiaowei W, Mingjie Z, Yiqin X, et al. Hypoxia-induced tRNA-

529 derived fragments, novel regulatory factor for doxorubicin resistance in triple-
530 negative breast cancer. J Cell Physiol, 2018:1-12.
531 18. Kanako S, Sherif R, Kuniyasu N and Teiji T. Stress Induced tRNA Halves (tiRNAs)
532 as Biomarkers for Stroke and Stroke Therapy; Pre-clinical Study.
533 Neuroscience,2020.434: 44-54.
534 19. Pavel I, Mohamed M.E, Judit V, Steven P.G and Paul A. Angiogenin-induced tRNA
535 fragments inhibit translation initiation. Mol Cell, 2011. 43(4):613-23.
536 20. Hak K.K, Gabriele F, Shengchun W, Wei W, Yue Z, et al. A transfer-RNA-derived
537 small RNA regulates ribosome biogenesis. Nature, 2017. 552(7683):57-62.
538 21. Shawn M.L, Marta M.F and Pavel I. The role of RNA modifications in the regulation
539 of tRNA cleavage. FEBS Lett, 2018. 592(17):2828-44.
540 22. Domenico R, Judith L and Sandra B. Emerging roles of novel small non-coding
541 regulatory RNAs in immunity and cancer. RNA Biol, 2020. 17(8):1196-1213.
542 23. Paraskevi K, Panagiotis G.A, Sotirios G.P, Vasiliki P, Andreas S, Christos K.K. A
543 novel, mitochondrial, internal tRNA-derived RNA fragment possesses clinical utility
544 as a molecular prognostic biomarker in chronic lymphocytic leukemia. Clin
545 Biochem,2020. 85:20-26.
546 24. Yanping Z, Jinfeng Y, Alec S, Robert W, Jiao W, et al. The mechanism of chronic
547 nicotine exposure and nicotine withdrawal on pain perception in an animal model.
548 Neurosci Lett, 2020. 715:1-22.
549 25. Licong S, Xiaxia H, Wenjun Z and Yi Z. Expression profiles of tRNA-derived
550 fragments and their potential roles in ovarian endometriosis. Epigenomics, 2020.

123(3):1-15.

26. Joseph W.D, Bryan W.H, Emily L.Z, Jesse D.K, and Stephen A.M. Acute analgesic effects of nicotine and tobacco in humans: a meta-analysis. Pain, 2016. 157(7):1373-81.
27. Iboro C.U, Claire A.D, Brooke A.M, Chandrika A, Keith G, et al. Nicotinic modulation of descending pain control circuitry. Pain, 2017. 158(10):1938-50.
28. Petros Y.D, Maria P, Constantinos A, Christina T, Georgia K, et al. Effects of cigarette smoke exposure and its cessation on body weight, food intake and circulating leptin, and ghrelin levels in the rat. Nicotine Tob Res, 2013. 15(1):206-12.
29. Matthews AM, Fu R, Dana T, Chou R. Intranasal or transdermal nicotine for the treatment of postoperative pain. Cochrane Database Syst Rev, 2016(1):1-125.
30. Weiwei L, Jingyi C, Benjamin H.W, Lanting H, Jing F and Yun W. Antinociceptive effects of novel epibatidine analogs through activation of alpha4beta2 nicotinic receptors. Sci China Life Sci, 2018. 61(6):688-695.
31. Deniz B, Mine S.G, Pamela F, Roger L.P and M. Imad D. New Insights on Neuronal Nicotinic Acetylcholine Receptors as Targets for Pain and Inflammation: A Focus on alpha7 nAChRs. Curr Neuropharmacol, 2018. 16(4):415-425.
32. Brandon H, Rahun S, Weston A.N, Crystal N.D, Diana F.G. Nicotine exploits a COPI-mediated process for chaperone-mediated up-regulation of its receptors. J Gen Physiol, 2014. 143(1):51-66.
33. Brandon J. Henderson, Henry A. Lester. Inside-out neuropharmacology of nicotinic drugs. Neuropharmacology, 2015. 96:178-193.

573 34. Morgane B, Sylvie G, Monica Mameli E, Isabelle C.T, Nicolas M, et al. Long-term
574 effects of chronic nicotine exposure on brain nicotinic receptors. Proceedings of the
575 National Academy of Sciences, 2007. 104(19):8155-60.
576 35. Kora M.B, Elena G, Fernando R.F, Victor E.A, Javier C.C and Jose Antonio L.M.
577 Common single nucleotide variants underlying drug addiction: more than a decade
578 of research. Addict Biol, 2015. 20(5):845-71.
579 36. Bader C, Kees J.K, Scott M, Philip A.S, Alexandra P, et al. Low smoking-exposure,
580 the adolescent brain, and the modulating role of CHRNA5 polymorphisms. Biol
581 Psychiatry Cogn Neurosci Neuroimaging, 2019. 4(7):672-9.
582 37. Jieun K, Ju H.Y, In Soo R, Sumin S, Sunghyun K and Eun S.C. Interactions of
583 Glutamatergic Neurotransmission and Brain-Derived Neurotrophic Factor in the
584 Regulation of Behaviors after Nicotine Administration. Int J Mol Sci, 2019. 20(12):1-
585 12.
586 38. Beverley M.H, Brian A.W, Rahul S, Bruce N.C, Cheng X, et al. Transcriptional
587 regulation by nicotine in dopaminergic neurons. Biochem Pharmacol, 2013.
588 86(8):1074-83.
589 39. Yijin Y, Nicole A.B, Veronica J.K and Ryan M.D. Differential Nicotinic Modulation of
590 Glutamatergic and GABAergic VTA Microcircuits. eneuro, 2019. 6(6):1-12.
591 40. Meng L, Rui F, Xinhua L, Feng C, Ju W. Pathways and networks-based analysis of
592 candidate genes associated with nicotine addiction. PLoS One, 2015. 10(5):1-17.
593 41. Roel R.I. van Reij, Elbert A.J. Joosten and Nynke J. van den Hoogen. Dopaminergic
594 neurotransmission and genetic variation in chronification of post-surgical pain. Br J

595 Anaesth, 2019. 123(6):853-864.
596 42. Caijuan L, Yanying L, Yi T, Shiqin X, Xiaofeng S, et al. The Etiological Contribution
597 of GABAergic Plasticity to the Pathogenesis of Neuropathic Pain. Molecular Pain,
598 2019:1-28.
599 43. Dragana J, Valentina B, Anna R.C, Ezio C. Nicotine, cocaine, amphetamine,
600 morphine, and ethanol increase norepinephrine output in the bed nucleus of stria
601 terminalis of freely moving rats. Addict Biol, 2019. 26(1):1-9.
602 44. Christian P.M and Judith H. The role of serotonin in drug use and addiction. Behav
603 Brain Res, 2015. 277:146-92.
604 45. Jenni H, Teemu P, Ida S, Richa G, Jadwiga B, et al. Genome-wide association study
605 in Finnish twins highlights the connection between nicotine addiction and
606 neurotrophin signaling pathway. Addict Biol, 2019. 24(3):549-561.
607 46. Yonghong D, Wenhui S, Guannan X, Ailan Y, Qinghe W and Zongwang Z. CX3CR1
608 Mediates Nicotine Withdrawal-Induced Hyperalgesia via Microglial P38 MAPK
609 Signaling. Neurochem Res, 2015. 40(11):2252-61.
610 47. Xi X, Xueliang S, Baohui Z, Hui Z, Tao Z. Nicotine alleviates chronic stress-induced
611 anxiety and depressive-like behavior and hippocampal neuropathology via
612 regulating autophagy signaling. Neurochem Int, 2018. 114:58-70.
613 48. Yijing S, Xiuchong Y, Linwen Z, Tianwen L, Zhilong Y and Junming G. Transfer RNA-
614 derived fragments and tRNA halves: biogenesis, biological functions and their roles
615 in diseases. J Mol Med (Berl), 2018. 96(11):1167-76.
616 49. Sherif R, Teiji T and Kuniyasu N. The cell and stress-specific canonical and

noncanonical tRNA cleavage. J Cell Physiol, 2020:1-15.

50. Sherif R,, Kuniyasu N and Teiji T. tRNA cleavage: a new insight. Neural Regen Res, 2020. 15(1):47-52.

51. Pankaj K, Jordan A, Suresh B.M and Anindya D. Meta-analysis of tRNA derived RNA fragments reveals that they are evolutionarily conserved and associate with AGO proteins to recognize specific RNA targets. BMC Biology, 2014. 12(78):1-14.

52. Xue Z, Xin H, Chao L, Jun L, Qifei H, et al. IL-4 Inhibits the Biogenesis of an Epigenetically Suppressive PIWI-Interacting RNA To Upregulate CD1a Molecules on Monocytes/ Dendritic Cells. J Immunol, 2016. 196(4):1591-603.

Figure legends

Figure 1. Behavioral observations. a) 50% paw withdrawal threshold (PWT) of both groups. The mechanical pain sensation of each rat was measured and recorded by 50%PWT, and the data were presented as mean ±SEM (n = 12 in each group), $^{***}P \leq 0.001$. b) Body weight changes of both groups. The body weight of each rat was recorded every day, and the difference values within every 4 days were calculated and analyzed using Student's t-test. The data were presented as mean ±SEM (n = 12 in each group), $^{**}P \leq 0.01$.

Figure 2. Expression profiles of tsRNAs sequencing data in both groups (n=3). a) The scatter plots of differentially expressed tsRNAs. tsRNAs above the top line (red dots, up-regulation) or below the bottom line (green dots, down-regulation) indicate more than 1.5-fold change between the two compared groups. Gray dots indicate non-differentially expressed tsRNAs. b) The volcano plots of differentially expressed tsRNAs. The vertical dotted lines manifest 1.5-fold changes (log2 scaled) up and down, respectively, and the horizontal dotted line shows a p-value of 0.05 (-log10 scaled). The red and green points denote the significantly up- and down-regulated tsRNAs. c) Hierarchical clustering heat-map for the 26 differentially expressed tsRNAs. The up- and down-regulated tsRNAs are colored in red and blue, respectively. d) Venn diagram based on the number of commonly expressed and specifically expressed tsRNAs. The number of tsRNAs commonly expressed in both groups were shown in red color, the specifically expressed tsRNAs in nicotine group in blue color, and the specifically expressed tsRNAs in saline group in orange color.

Figure 3. Differences and characterizations of tsRNAs expression profiles between both groups. a) and b) The Pie charts of tsRNAs subtypes in saline and nicotine group. The colors represent the tsRNAs subtypes. The values in brackets represent the numbers of tsRNAs subtypes. c) and d) The Bar charts show the total read counts against the read lengths for the complete adapter-trimmed read set. e) and f) The Stacked Bar Charts of the number of tsRNAs subtypes against tRNA iso-decoders in saline and nicotine group, respectively. The X axes represents tRNA iso-decoders and the Y axes show the number of all subtype tsRNAs against tRNA iso-decoders. g) and h) The Stacked Bar Charts of the Frequency of Subtype against Length of the tsRNAs in saline and nicotine group, respectively. The X axes represents length of tsRNAs and the Y axes show the frequency of the subtype against length of tsRNAs. The color represents the subtype tsRNAs.

Figure 4. Validation of 8 dysregulated tsRNAs using RT-qPCR in both groups. Compared with the saline group, tRF-1-T28-His-GTG-1 was significantly up-regulated in the nicotine group (a). tRF-5c-Glu-CTC-1, tRF-5c-Glu-CTC-3, tRF-5c-Glu-TTC-4, tRF-5c-Gly-GCC-2-M2 were all significantly down-regulated in the nicotine group(b). The data were normalized using the mean ±SEM (n = 3 per group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Figure 5. The tsRNA/mRNA network analysis. The network included the five candidate tsRNAs and their predicted target mRNAs (Nodes in green color is the up-regulated tsRNA, nodes in yellow color are the down-regulated tsRNAs, nodes in red color are mRNAs).

683

684 **Figure 6.** The GO annotations and pathways of target mRNAs regulated by the five
685 candidate tsRNAs. a) and b) represented the tRF-1-T28-His-GTG-1, c) and d) the tRF-5c-
686 Glu-CTC-1, e) and f) the tRF-5c-Glu-CTC-3, g) and h) the tRF-5c-Gly-GCC-2-M2, j) and k)
687 the tRF-5c-Glu-TTC-4. The bar plot shows the top 10 enrichment score values of the
688 significantly enriched GO annotations for molecular function (in blue), cellular
689 component(in green), biological processes (in light red) and the significantly enriched
690 pathway(in dark red), respectively.

691

692 **Figure 7.** Mapping of the involving signaling pathways of tRF-5c-Glu-CTC-1. a) the
693 cholinergic synapse and b) the dopaminergic synapse. Orange marked nodes are
694 associated with tRF-5c-Glu-CTC-1.