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Genome-wide identification of chromatin-enriched RNA reveals that unspliced dentin matrix protein-1 mRNA regulates cell proliferation in squamous cell carcinoma

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ABSTRACT

Chromatin-enriched noncoding RNAs (ncRNAs) have emerged as key molecules in epigenetic processes by interacting with chromatin-associated proteins. Recently, protein-coding mRNA genes have been reported to be chromatin-tethered, similar with ncRNA. However, very little is known about whether chromatin-enriched mRNA is involved in the chromatin modification process. Here, we comprehensively examined chromatin-enriched RNA in squamous cell carcinoma (SQCC) cells by RNA subcellular localization analysis, which was a combination of RNA fractionation and RNA-seq. We identified 11 mRNAs as highly chromatin-enriched RNAs. Among these, we focused on the dentin matrix protein-1 (DMP-1) gene because its expression in SQCC cells has not been reported. Furthermore, we clarified that DMP-1 mRNA was retained in chromatin in its unspliced form in SQCC *in vitro* and *in vivo*. As the inhibition of the unspliced DMP-1 mRNA (unspDMP-1) expression resulted in decreased cellular proliferation in SQCC cells, we performed ChIP-qPCR to identify cell cycle-related genes whose expression was epigenetically modified by unspDMP-1, and found that the CDKN1B promoter became active in SQCC cells by inhibiting unspDMP-1 expression. This result was further validated by the increased *CDKN1B* gene expression in the cells treated with siRNA for unspDMP-1 and by restoration of the decreased cellular proliferation rate by simultaneously inhibiting *CDKN1B* expression in SQCC cells. Further, to examine whether unspDMP-1 was able to associate with the *CDKN1B* promoter region, SQCC cells stably expressing PP7-mCherry fusion protein were transiently transfected with the unspDMP-1 fused to 24 repeats of the PP7 RNA stem loop (unspDMP-1-24xPP7) and we found that unspDMP-1-24xPP7 was efficiently precipitated with the antibody against mCherry and was significantly enriched in the *CDKN1B* promoter region. Thus, unspDMP-1 is a novel chromatin-enriched RNA that epigenetically regulates cellular proliferation of SQCC.

1. Introduction

Long non-coding RNAs (lncRNAs) are defined as molecules longer than 2 kb without apparent protein-coding potential that regulate complex cellular behaviors [1,2]. Aberrant expression of chromatin-associated lncRNAs dysregulates chromatin remodeling, and is highly correlated with cancer initiation and progression [3,4]. However, the lncRNA *Tsx*, which is transcribed from the X-inactivation center and is involved X chromosome inactivation, may function as a protein coding frame [5]. The micropeptides

Abbreviations: DMP-1, dentin matrix protein 1; ncRNAs, non-coding RNAs; SIBLINGs, small integrin-binding ligand N-linked glycoproteins; SQCC, squamous cell carcinoma; unspDMP-1, unspliced DMP-1 mRNA.

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transcribed from lncRNAs were reported to possess key biological functions [6,7]. Thus, the RNA distinction between mRNAs and lncRNAs is currently obscure. Interestingly, RNA immunoprecipitation assay identified not only lncRNAs but also a large number of mRNA transcripts bound to chromatin-associated proteins [8]. Therefore, we speculate that some chromatin-associated mRNAs possess chromatin modifying ability, and are likely to be chromatin-associated lncRNAs.

Dentin matrix protein-1 (DMP-1) belongs to the family of small integrin-binding ligand N-linked glycoproteins (SIBLINGs). Many SIBLINGs members have been reported to exhibit dominant effects for matrix mineralization through their phosphorylation and to evoke several cell responses [9,10]. DMP-1 is known to have dominant effects on systemic diseases such as rickets [11,12]. Specific expression patterns of SIBLINGs proteins have been observed in both benign and metastatic cancer tissues [13–15]. Squamous cell carcinoma (SQCC) is the most common cancer type in the head and neck region [16,17], and among the SIBLINGs, secreted phosphoprotein-1 (SPP-1) expression was elevated in both tumor and tumor-free margin samples [18].

Here, we provide the first evidence of unspliced DMP-1 mRNA (unspDMP-1) being retained in chromatin in SQCC cells, and we therefore examined the ncRNA-like functions of unspDMP-1 by analyzing its effects on epigenetic alteration of promoter activities for cell-cycle related genes.

2. Materials and methods

2.1. Cell culture

The human head and neck SQCC cell lines Ca9-22, HO-1-u-1, and HSC-2 were provided from the RIKEN Cell Bank (Tsukuba, Japan). Cell culture conditions were described in the Appendix.

2.2. RNA subcellular localization analysis and bioinformatic analysis

Refer to the Appendix for the details.

2.3. Unspliced form identification

Refer to the Appendix for the details.

2.4. Quantitative PCR (qPCR) analysis

Refer to the Appendix for the details.

2.5. RNA immunoprecipitation (RIP)

Refer to the Appendix for the details.

2.6. Transient transfection of siRNA

Refer to the Appendix for the details.

2.7. Determination of cell proliferation rate

Refer to the Appendix for the details.

2.8. Chromatin immunoprecipitation (ChIP) and ChIP array

Refer to the Appendix for the details.

2.9. RNA labeling with PP7-mCherry

Refer to the Appendix for the details.

2.10. Statistical analysis

Statistical analysis was performed with the Student's *t*-test (Fig. 3A-C, and E, and Fig. 4C) and one-way analysis of variance (ANOVA), followed by Dunnett's test (Fig. 3F-H). $^{*}p < .05$, $^{**}p < .01$, or $^{***}p < .001$ was considered significant.

3. Results

3.1. Comprehensive subcellular RNA fractionation identified the unspliced DMP-1 gene as a highly chromatin-enriched mRNA in SQCC cells

For genome-wide exploration of chromatin-enriched RNAs, we comprehensively compared nuclear and chromatin RNA fractions of Ca9-22 SQCC cells by RNA-seq analysis. We detected 18,643 RNA species. After removing the RNA species whose FPKM values in either the chromatin or nuclear fraction were below the detection limit, RNA species were plotted on the X (nuclear fraction) and Y (chromatin fraction) axes as log10 (Fig. 1A). Although the role of mRNA is a protein coding frame, the mRNA distribution equivalently converged to a slope of 1 rather than inclining to the nuclear fraction compared with non-coding RNAs, antisense RNAs, and pseudogenes. Successful RNA fractionation was confirmed using the expected chromatin/nucleus ratios of *cis*-acting KCNQ1OT1, *trans*-acting HOTAIR, and protein coding ACTB with 5.01, 1.78, and -2.15 (log10), respectively (data not shown).

To identify RNAs that selectively and abundantly localize to chromatin, we evaluated RNAs whose chromatin FPKM values were greater than 10 and whose expression ratios of chromatin/nuclear were higher than 50-fold (Supplemental Fig. 1). We identified 20 RNAs, including 11 kinds of mRNAs, 8 kinds of ncRNAs, and one pseudogene. Among the identified mRNAs, we focused on the cancer-related gene, DMP-1, which belongs to the family of SIBLINGs. The visualization of read counts for DMP1 and another SIBLING, SPP-1, demonstrated their opposing preference for subcellular localization by DMP-1 and SPP-1 chromatin- and nucleus-enrichment, respectively (Fig. 1B). The read counts for DMP1 exhibited intronic RNA expression unlike the typical protein-coding gene SPP-1, which was selectively transcribed from exonic regions.

Thus, we hypothesized DMP-1 was retained in chromatin without efficient splicing in SQCC cells, and performed RT-PCR using several primer pairs to identify spliced and unspliced forms of DMP-1 (Fig. 1C). As shown in Fig. 1D, RT-PCR using forward and reverse primers designed for exon 3 (Ex3) and Ex6 (upper left panel) had positive bands at approximately 5 kbp (blue arrow) with genomic DNA (lane: b) and at 200 bp (yellow arrow) for DMP-1 cDNA (lane: c). Interestingly, a positive band was observed with reverse-transcribed RNAs from Ca9-22 (lane: d) as well as from different SQCC cells, HO-1-u-1(lane: f), and HSC-2 cells (lane: h) equivalent to genomic DNA. Similar specific unspliced forms were also observed if forward primers designed for Ex2 were utilized (upper middle panel). These results indicated that only unspliced DMP-1 mRNA (unspDMP-1) was in these SQCC cells. If forward primers were designed in intron 2 (Int2) (upper right panel) and Int1 (lower left panel), the bands in Ca9-22, HO-1-u-1, and HSC-2 cells migrated into approximately 5.1 and 7.0 kbp, respectively. The sizes of these bands were equivalent to the band sizes in

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