

PD-L1, an Important Immune Checkpoint Regulator, Is Suppressed by miR-34a in Head and Neck Squamous Cell Carcinoma

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Abstract

Background: Key molecules regulating the immune checkpoint have shed light on the efforts to control several cancers. Recently, immune checkpoint inhibitors for cancer therapy such as antibodies against programmed cell death 1 (PD-1), programmed cell death 1 ligand 1 (PD-L1), and cytotoxic T-lymphocyte associated protein 4 (CTLA4) have been developed. In head and neck squamous cell carcinomas (HNSCCs), such immune checkpoint inhibitors have come into clinical use and are expected to improve patients' prognoses. Recently, *miR-34a* has been shown to be a downstream micro RNA of TP53 that regulates PD-L1 in several types of cancer. To reveal the correlations between *miR-34a* and PD-L1 in HNSCCs in terms of clinical significance, we analyzed 19 HNSCC cell lines.

Methods: We measured the expression levels of *miR-34a* and PD-L1 in 10 HNSCC cell lines as well as in 9 of their derived acquired cisplatin (CDDP) resistant cell lines by qRT-PCR and Western blotting. Results were further analyzed by their TP53 statuses. We also investigated the changes in PD-L1 expression levels after *miR-34a* precursor- or inhibitor-mediated forced expression or suppression in HNSCC cell lines.

Results: We observed inverse correlations between both mRNA and protein expression levels of *miR-34a* and PD-L1. No significant differences in *miR-34a* levels were observed with regard to *TP53* status. Forced expression of *miR-34a* decreased PD-L1 expression, and suppression of *miR-34a* increased PD-L1 expression.

Conclusion: Our present results suggest that *miR-34a* negatively regulates PD-L1 expression, possibly in a TP53 independent manner in HNSCC.

Background

Head and neck squamous cell carcinoma (HNSCC) is the sixth most commonly diagnosed cancer and the eighth most common cause of cancer death worldwide [1]. Although molecular target therapy has been utilized lately, surgical resection and/or a combination of cisplatin-based chemo-radiotherapy or sole radiotherapy are the major therapeutic strategies for patients with HNSCC. However, poor survival rates are still reported in advanced stage patients. Recently, functions of immune checkpoint proteins such as programmed cell death 1 (PD-1), programmed cell death 1 ligand 1 (PD-L1) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) in the cancer cells have been elucidated, and inhibitory antibodies against those immune checkpoint molecules have been developed [2, 3]. In HNSCC, anti-PD-1 antibody nivolumab and pembrolizumab have been shown to be clinically effective [4, 5].

Micro RNAs (miRNAs) are a class of small noncoding RNA molecules, typically 18–25 nucleotides long, known to be among the factors regulating gene functions. miRNAs are found to play important roles in proliferation, differentiation, and migration [6, 7], and some of them work critically for suppression of carcinogenesis. *miR-34a* is known to be one of the downstream molecules of TP53 and functions as a tumor suppressor [8–11]. It could be a prognostic marker in sinonasal squamous cell carcinoma treated

with cisplatin (CDDP)-based chemoradiotherapy [12]. A number of genes targeted by *miR-34a* have been identified [13–15], and *PD-L1* was included in one such target gene in non-small cell lung cancer and acute myeloid leukemia [16, 17]. In this study, we investigated the relationship between *miR-34a* and PD-L1 in HNSCC.

Methods

Cell lines

We utilized a total of ten HNSCC cell lines (RPMI2650, HSC2, HSC3, HSC4, IMC3, IMC4, Ho1-u-1, SAS, Ca9-22, HSQ89) as well as nine their derived and previously established acquired CDDP resistant cell lines (RPMI2650CR, HSC2CR, HSC3CR, HSC4CR, IMC4CR, Ho1-u-1CR, SASCR, Ca9-22CR, HSQ89CR) [18, 19]. Culture conditions of these 19 cell lines were described previously [18, 19].

Rna Preparation

Preparation of miRNAs and total RNAs were done using mirVana miRNA Isolation kit (Applied Biosystems, Foster City, CA) and the RNeasy Mini Kit (QIAGEN, Tokyo, Japan) according to the manufacturers' instructions, and the quality of the extracted RNAs was monitored by Bioanalyzer (Agilent Technologies, Santa Clara, CA).

Quantitative Reverse Transcription-polymerase Chain Reaction (qRT-PCR)

qRT-PCR experiments were performed according to methods described previously [12]. In brief, quantification of *miR-34a* was assessed by TaqMan microRNA assay kit and an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Results were normalized by Δ Ct methods, and the RNU44 level was monitored as the internal control. Using ROX Mix (Thermo Fisher Scientific, Tokyo, Japan), 2 μ g of total RNA from each sample was used for cDNA synthesis and qRT-PCR analyses for detection of *PD-L1* expression levels. Signals were detected by an ABI PRISM 7000 Sequence Detection System, and expression levels were normalized by $\Delta\Delta$ Ct methods using beta-2-microglobulin (*B2M*) as the internal control [20]. The *PD-L1* primer pairs and the probe used for qRT-PCR are as follows: forward primer, 5'-CCC AGT TCT GCG CAG CTT C-3'; reverse primer, 5'-AGC AAA TAT CCT CAT CTT TCT G-3'; and probe, 5'-CGC GCT TCT GTC CGC CTG CAG-3'. All of these experiments were performed in triplicate.

Western Blotting

Western blotting analyses were performed as described previously [21]. In brief, total proteins from the collected cell were extracted, both before treatment and 16 hours after treatment, with 1 μ g/ml of doxorubicin, then electrophoresed on a SuperSep Ace 5–20% polyacrylamide gradient gel (Wako, Osaka, Japan), transferred to a PVDF membrane (ATTO, Tokyo, Japan), and blocked by skim milk. Then the membrane was incubated overnight at 4°C with one of the following primary antibodies: rabbit anti-human PD-L1 monoclonal antibody [28 – 8] (Abcam, Tokyo, Japan), mouse anti-human p53 monoclonal

antibody DO-7 (DAKO, Glostrup, Denmark), or mouse anti-ActB monoclonal antibody (Sigma, St. Louis, MO). Horseradish peroxidase (HRP) conjugated secondary antibodies (Amersham Biosciences, Little Chalfont, UK) were used against corresponding primary antibodies (rabbit or mouse). Signals were visualized using Enhanced ChemiLuminescence (ECL) Detection Reagent (Amersham) and digitally processed using ImageQuant LAS 4000 (GE Healthcare, Tokyo, Japan). The band intensities were digitalized by the ImageQuant software (GE Healthcare), and relative intensities were calculated by normalization using β -actin (ACTB) as the internal control.

Transfection of miR-34a precursor or inhibitor to the cell lines

Cell lines were seeded into six-well plates at a density of 2×10^5 cells per well and grown overnight. Then either the *miR-34a* precursor (Ambion Pre-miR miRNA Precursor) or the *miR-34a* inhibitor (Ambion Anti-miR miRNA Inhibitor) was transfected at the final concentration of 12.5 nM using 7.5 μ L RNAiMAX transfection reagent. After 72 hours, cell pellets were collected for further investigation.

Statistical analysis

Statistical analyses were done using Microsoft excel for Mac (ver.16.32 (19120802)) and R for Mac (R 3.5.2 GUI 1.70 El Capitan build (7612)). The Spearman's rank correlation, Kruskal-Wallis test and Welch's t test analyses were carried out. Significant difference was considered as $P < 0.05$.

Results

Expressional analyses of miR-34a in the cell lines

Initially, expression levels of *miR-34a* in the cell lines were investigated (Fig. 1). Relative expression levels of *miR-34a* (*miR-34a/RNU44* ratio) significantly varied among cell lines (0.0097-2.07, median: 0.042, $P < 0.001$ by Kruskal-Wallis test), and ratios of 0.1 or more were observed in seven cell lines (RPMI2650, HSQ89, HSQ89CR, IMC4, IMC4CR, IMC3 and HSC2CR).

Expression Analyses Of Pd-l1 Mrna And Protein

We then investigated the mRNA and protein expression levels of PD-L1 (Figs. 2a and 2b). Relative mRNA expression levels of *PD-L1* (*PD-L1/B2M* ratio) significantly varied among cell lines (0.000112-0.0494 median: 0.00198, $P < 0.001$ by Kruskal-Wallis test), and ratios of 0.01 or more were observed in six cell lines (HSC2, HSC2CR, HSC3, HSC4CR, SAS and SASCR).

Analyses of the relationships between miR-34a and PD-L1 expressions

The relationships between *miR-34a* and PD-L1 mRNA and protein were analyzed (Figs. 2c and 2d). No discrepancies between mRNA and protein expression levels were evident in PD-L1. HSC2, HSC3, HSC3CR, SAS, and SASCR showed high PD-L1 mRNA expression relative to miR-34a. In contrast, RPMI2650, HSQ89, IMC4 and IMC4CR showed higher expressions of *miR-34a* relative to PD-L1. It is notable that (1)

we observed significant inverse associations between expression levels of *miR-34a* and PD-L1 (mRNA: $r = -0.4684$ $P = 0.04476$, protein: $r = -0.7439$ $P = 0.0003931$), and that (2) we did not observe any coherence of *miR-34a* expression levels between parental and resistant cells.

Forced expression of miR-34a

Next, we induced forced expression using *miR-34a* precursor into the cell lines with lower expressions of *miR-34a*. We selected three cell lines, HSC2, HSC3 and HSC3CR, and analyzed their expressional changes of PD-L1 after introduction of *miR-34a* precursor. We observed significantly decreased *PD-L1* mRNA expression levels in response to the increase of *miR-34a* in HSC3 and HSC3CR (Figs. 3a and 3b). These results are in good accordance with those in Fig. 2.

Inhibition of miR-34a

In an opposite manner, we also performed suppression experiments using *miR-34a* inhibitor. We selected four cell lines with the higher expression of *miR-34a*, RPMI2650, HSQ89, IMC4 and IMC4CR; results are shown in Figs. 3c and 3d. We observed significantly increased *PD-L1* mRNA expression levels in response to the decrease in *miR-34a* levels in RPMI2650 and IMC4CR. Again, these results are in good accordance with those in Fig. 2.

Elucidation of miR-34a in association with TP53

Because the regulation of *miR-34a* in association with TP53 has been reported [22], the statuses of *TP53* in 10 HNSCC cell lines and a representative CDDP resistant RPMI2650CR were sequenced for exons 5–8 to detect mutations. Results are shown in Fig. 4a. Then we analyzed protein expression levels of TP53 by Western blotting with and without doxorubicin treatments; results are shown in Fig. 4a. Three of five cell lines without *TP53* mutation (RPMI2650, IMC4, and IMC3) increased their protein expression levels after doxorubicin treatment, but no significant differences of *miR-34a* levels were detected irrespective of *TP53* status (Fig. 4b).

Discussion

In this study, we demonstrated an inverse correlation between expression levels of *miR-34a* and PD-L1 in HNSCC cells. Forced expression of *miR-34a* decreased PD-L1 expression, and introduction of an inhibitor of *miR-34a* increased PD-L1. These results suggest that *miR-34a* is one of the important molecules in regulation of PD-L1 in HNSCCs, possibly in an TP53 independent manner.

PD-L1 is reported as a ligand that specifically binds to PD-1 [23]. Its important role is to suppress the immune system by binding to PD-1. A number of cancer cells express PD-L1, which results in escape from immune cells, and such cancer cells can survive. Some reports have demonstrated a significant association between PD-L1 expression and poor prognosis in various malignant tumors [24–28]. Chen et al. [29] showed that PD-L1 expression was regulated by oncogenic pro-survival signaling pathways, such as MAPK or PI3K/Akt pathways. Several investigators have also reported correlations between *miR-34a*

and PD-L1 expressions. Wang Xi et al. [16] suggested that *miR-34a* can regulate PD-L1 expression by targeting *PD-L1* mRNA in acute myeloid leukemia, and Cortez et al. [17] found that TP53 regulates PD-L1 via *miR-34a*, which directly binds to the 3' untranslated region of *PD-L1* in non-small cell lung cancer.

miR-34a is known as a tumor suppressing miRNA and its role as the tumor suppressor in HNSCC has been reported [30, 31]. In sinonasal squamous cell carcinomas, *miR-34a* expression can be an independent prognostic biomarker in patients undergoing *cis*-diamminedichloroplatinum treatment [12]. One of the key findings in the present study is that the expression levels of PD-L1 and *miR-34a* inversely associated in HNSCC; this result suggests that the PD-L1 opposes *miR-34a*. This is in good agreement with previous reports that *miR-34a* may downregulate PD-L1 expression in squamous cell carcinoma [16, 17]; this should lead to a better prognosis.

Various clinical trials of the immune checkpoint inhibitors have been performed recently, and the expression levels of the immune checkpoint proteins such as PD-L1 or tumor mutation burden are sometimes discussed as indications of such immune checkpoint inhibitors in HNSCCs [32]. We analyzed *miR-34a*, which has been reported as one of the downstream genes of TP53 [22], but the expression levels of *miR-34a* were found to be independent of TP53 statuses in the present study; expression levels of *miR-34a* varied among cells with wild-type TP53, and no significant associations were observed. It is reported that methylation-induced silencing of *miR-34a* is associated with chemoresistance in prostate cancer [33] and that *miR-34a* promoter methylation and *TP53* polymorphisms may be associated with colorectal cancer pathogenesis [34]. However, the numbers of available cells were limited and further investigations are needed.

In this study, we demonstrated that *miR-34a* is one of the important molecules in regulation of immune checkpoint proteins, but further genetic and epigenetic studies, including upstream and downstream molecules of the *miR-34a* family, will contribute to better clinical management of patients with HNSCC.

Conclusion

In HNSCC cells, *miR-34a* negatively controls the PD-L1 function, possibly independently from TP53 status. These results may contribute to open a pathway for novel and efficient ways to treat patients with HNSCC.

List Of Abbreviations

ACTB: β -actin; CDDP: cisplatin; CTLA4: cytotoxic T-lymphocyte associated protein 4; ECL: enhanced chemiluminescence; HNSCC: head and neck squamous cell carcinoma; HRP: horseradish peroxidase; miRNA: micro RNA; PD-1: programmed cell death 1; PD-L1: programmed cell death 1 ligand 1; qRT-PCR: quantitative reverse transcription-polymerase chain reaction

Declarations

Ethics approval and consent to participate

This study was approved by Ethics Committee in Tohoku University School of Medicine under the accession numbers of 2018-1-084 and 2018-1-085, and all patients provided written informed consent for this study.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors have no competing interests to declare.

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Authors' contributions

TO, YS, YK, and AH made the research plan. KH, TO, TI, YK, AO, AN, and YK contributed to collecting patients' samples. KH and TI contributed to collecting data, and TO, YS, and AH supervised experiments. KH, TO, YS, YK, and AH contributed data analysis. KH wrote the manuscript draft, and the draft was brushed-up by KH, TO, YS, and AH. All authors read and approved the final manuscript.

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Figures

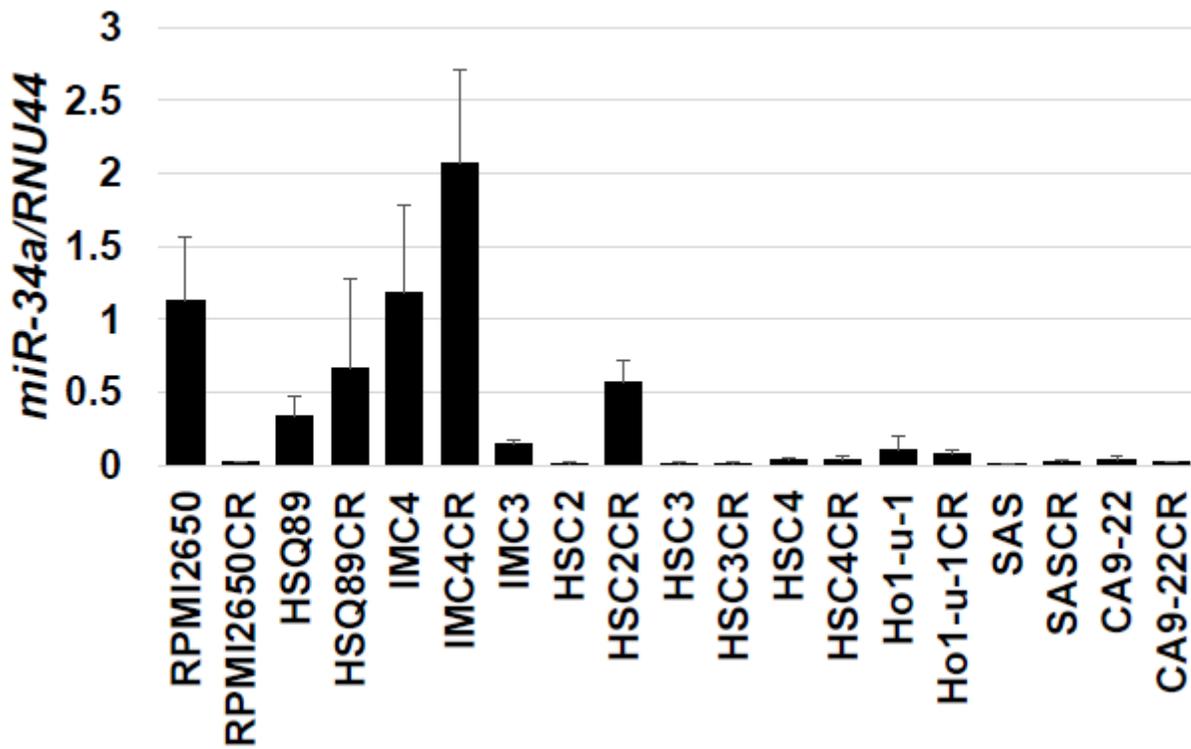


Figure 1

Expression levels of miR-34a in HNSCC cell lines analyzed in this study are shown. Expression of RNU44 is monitored as the control, and relative expression levels (miR-34a/RNU44 ratio) are displayed; significantly varied expression levels among cell lines were observed (0.0097-2.07, median: 0.042, $P < 0.001$) by Kruskal-Wallis test

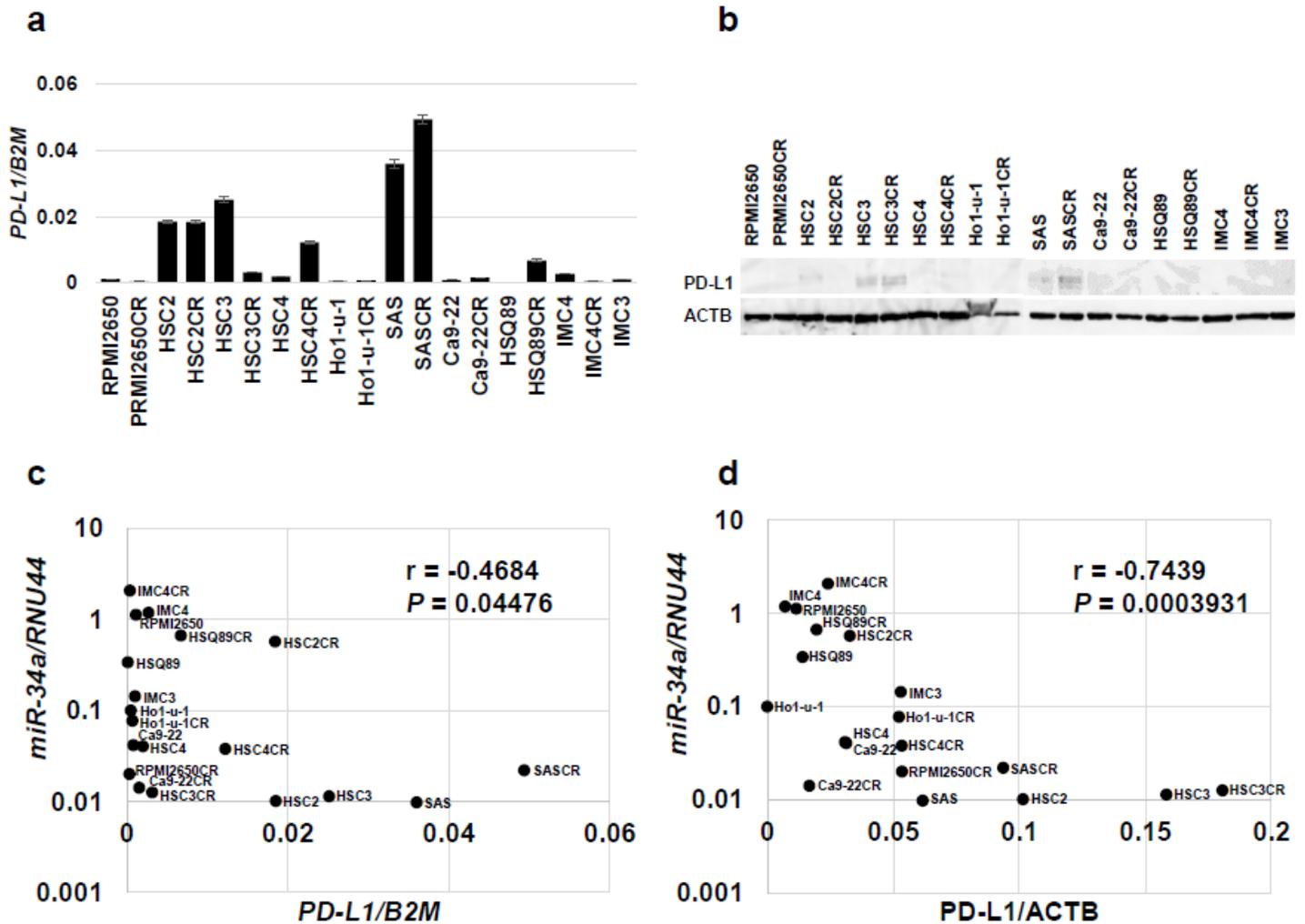


Figure 2

Expression levels of PD-L1 in HNSCC cell lines analyzed in this study are shown. Expression of beta-2-microglobulin (B2M) is used as the control, and relative expression levels (miR-34a/RNU44 ratio) are displayed; significantly varied expression levels among cell lines were observed (0.000112-0.0494 median: 0.00198, $P < 0.001$) by Kruskal-Wallis test. (b) Protein expression levels of PD-L1 by Western blotting are shown. Expression of beta-actin (ACTB) is used as the control. Full-length blots/gels are presented in Supplementary Figure 2b. (c) Relationship between miR-34a and PD-L1 mRNA in each cell line is plotted as a point diagram. (d) Relationship between miR-34a and PD-L1 protein in each cell line is plotted as a point diagram.

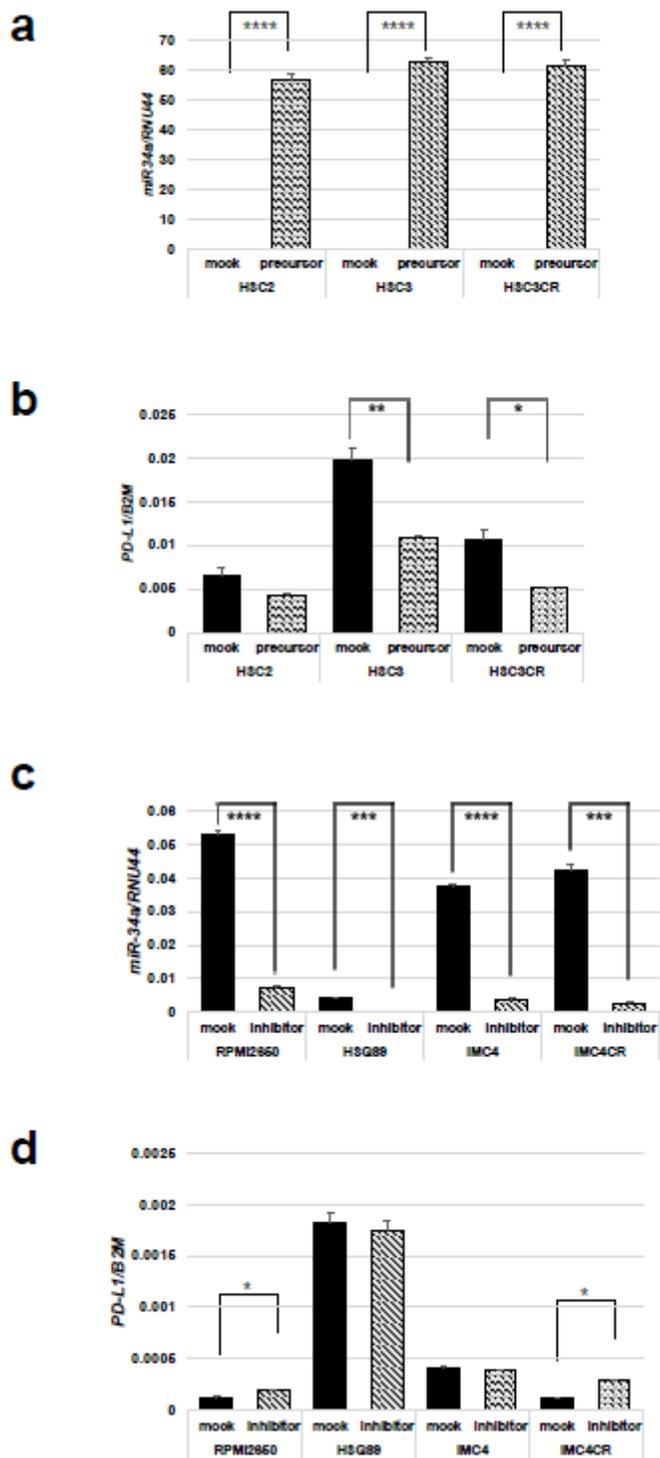


Figure 3

Results of introduction of miR-34a precursor (a, b) or inhibitor (c, d) into HNSCC cell lines. (a) Expression levels of miR-34a after precursor transfection. (b) Expression levels of PD-L1 mRNA after precursor transfection. (c) Expression levels of miR-34a after inhibitor transfection. (d) Expression levels of PD-L1 mRNA after inhibitor transfection. *, **, ***, and **** denote $P < 0.05$, $P < 0.01$, $P < 0.005$, and $P < 0.001$, respectively.

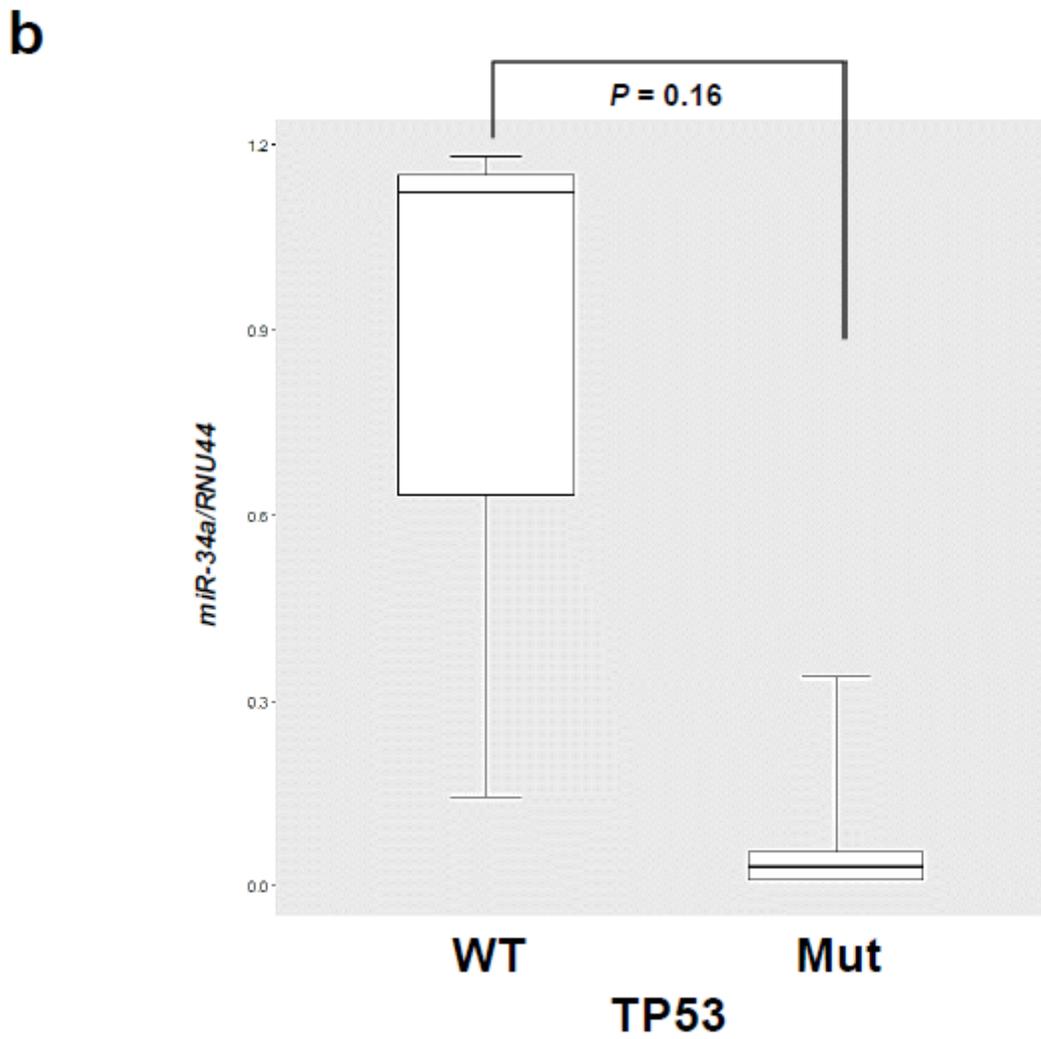
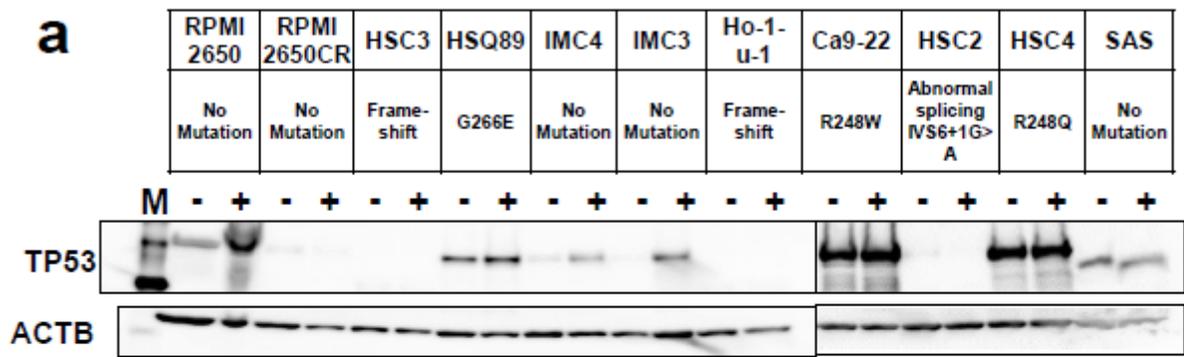


Figure 4

(a) Expression of TP53 with and without treatment of doxorubicin in HNSCC cell lines. TP53 mutation status in exons 5-8 for each cell line is also indicated. Expression of beta actin (ACTB) is used as the control. M, protein size marker; -, without doxorubicin treatment; +, with doxorubicin treatment. Full-length blots/gels are presented in Supplementary Figure 4a. (b) Box and whisker plot of miR-34a expression

after doxorubicin treatment in association with TP53 mutation status. Maximum number, 25% percentile, median, 75% percentile and minimum numbers are shown in box and whisker plots.

Supplementary Files

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