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Original Article

Human antigen R knockdown attenuates the invasive activity of oral cancer cells through inactivation of matrix metalloproteinase-1 gene expression

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KEYWORDS
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Abstract *Background/purpose:* The RNA-binding protein human antigen R (HuR) recognizes AU-rich elements in the 3′-untranslated regions of mRNA. The expression of cytoplasmic HuR is related to the malignancy of many carcinomas. The aim of this study is investigation of effect of HuR knockdown for invasive activity of oral carcinoma.
Materials and methods: Proliferation, invasion, real-time PCR, and reporter gene assays were performed to confirm that the knockdown of HuR downregulates the invasive activity of cancer cells. Immunohistochemical staining was performed for high invasive carcinoma, squamous cell carcinoma (SCC) and low invasive carcinoma, verrucous carcinoma (VC), to determine if the localization of cytoplasmic HuR is related to matrix metalloproteinase-1 (MMP-1) expression.

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Results: Invasive activity was significantly lower in HuR knockdown cancer cells than in control cells. A luciferase assay revealed that HuR knockdown inactivated the promoter activity of the *MMP-1* gene. The mRNA levels of the transcription factors required for MMP-1 expression, including *c-fos* and *c-jun*, were decreased in HuR knockdown cancer cells. Immunohistochemical analysis revealed the level of cytoplasmic HuR and MMP-1 in invasive carcinoma to be higher than in low invasive cancer. HuR induced MMP-1 expression in the invasive front of most SCC cases.
Conclusion: HuR knockdown attenuated the invasive activity of cancer cells by decreasing the expression of the MMP-1, at least partially. HuR localization may help determine the invasive phenotype of cancer cells and inhibit cancer cell invasion. Furthermore, in oral SCC, HuR may be related to invasive activity through the expression of MMP-1.

Introduction

Gene expression is important to regulate the decay of mRNA. Early response genes that encode mRNA include the AU-rich elements (ARE) in the 3′-untranslated regions (UTR), such as growth factors, proto-oncogenes, and cytokines.[1,2] Many proteins interact with ARE and regulate the decay of ARE-mRNA.[1–4]

Human antigen R (HuR), an RNA-binding protein, belongs to the embryonic lethal abnormal vision (ELAV)-like family.[5] HuR is related to many biological events, such as inflammation, differentiation, and carcinogenesis.[6,7] HuR can recognize ARE and export ARE-mRNA from the nucleus to the cytoplasm in a chromosome maintenance region 1 (CRM1)-dependent manner under conditions such as serum stimulation or stress.[8,9] HuR exists in the nucleus but can move between the nucleus and cytoplasm, although this mechanism of HuR translocation is not fully understood.

In normal cells, nearly all HuR exists in the nucleus; however, in many cancer cells, it is observed in the cytoplasm. Cytoplasmic HuR is increased in many cancers, including colon cancer, and contributes to malignancy.[7,10]

Cytoplasmic HuR and ARE-mRNA are increased in oral cancer cells.[11] In addition, activities characteristic of cancer cells, including a motile/invasive phenotype and anchorage-independent cell growth, are lost in HuR knockdown cells, at least partially, through effects on their cell cycle.[12] Furthermore, Woo et al. demonstrated the invasive phenotype to be attenuated in breast cancer cells by knockdown of HuR.[13]

In this study, we aimed to characterize the invasive activity of HuR knockdown cancer cells. Here, we investigated whether HuR knockdown attenuates the invasive activity of cancer cells by blocking the gene expression of matrix metalloproteinase-1 (*MMP-1*) and the association between cytoplasmic HuR localization and the invasive phenotype of oral carcinoma.

Materials and methods

Cells

HSC-2 (human oral squamous cell carcinoma), HSC-3 (human tongue squamous cell carcinoma), Ca9.22 (human gingival squamous cell carcinoma), and HT1080 cells (human fibrosarcoma cell line) were cultured at 37 °C under a 5% CO_2 atmosphere in DMEM with 10% fetal bovine serum and penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA).

HuR knockdown

Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) was used to transfect HuR siRNA[12] or control (Ctr) siRNA (Silencer Negative Control #1 siRNA; Ambion, Inc., Austin, TX, USA) according to manufacturer instructions. HuR expression was analyzed using western blotting at 48 h post-transfection.

Western blot analysis

Western blot analysis was reported previously.[14] The antibodies were β-actin (Sigma), HuR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), MMP-1 (Abcam, Cambridge, MA, USA), and horseradish peroxidase-conjugated IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

Quantitative real-time RT-PCR (qRT-PCR)

TRI reagent (Sigma) was used for the isolation of total RNA according to the manufacturer's protocol. Total RNA (1 μg) was reverse-transcribed using ReverTra Ace (Toyobo, Osaka, Japan). A Mini Opti-con system and SSO EvaGreen Supermix or SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Nenzlingerweg, Switzerland) were used for qRT-PCR.[15,16] The following primers were used for the amplification of cDNA: for *c-jun*: 5′-ggtggagttgaaagagttaagaatg-3′, 5′-tcctgaaacatcgcactatcc-3′; for GAPDH and *c-fos* were shown previously.[12] GAPDH was used for normalization. HSC-3 and Ca9.22 cells were used to evaluate the half-life ($t_{1/2}$) of total ARE-mRNA using 5 μg/mL of actinomycin D-mannitol (Sigma) at 30 or 60 min.

In vitro invasion assay

The invasion assays were reported previously,[12] and 5.0×10^4 cells (HSC-2), 1.0×10^5 cells (HSC-3 and Ca9.22), or 2.5×10^4 cells (HT1080) were used.

Reporter gene assay

HSC-2, HSC-3, Ca9.22, and HT1080 cells were transfected with HuR-siRNA or Ctr-siRNA. After 24 h, cells were transfected with reporter plasmids (pGL2-MMP-1) and the pRL-tk vector as inner control using Optifect transfection reagent (Invitrogen). The construction of plasmid pGL2-MMP-1 was previously reported; it consists of an MMP-1 promoter fragment of 563 bp (−518 to +45) subcloned into the luciferase reporter vector pGL2-Basic (Promega, Fitchburg, WI, USA).[17] Luciferase activity was estimated at 24 h using a luminometer, Lumat LB 9507 (Berthold Technologies, Wildbad, Germany) with a dual-luciferase reporter assay system (Promega).

Immunohistochemical analysis of in vivo tumors

Tissue samples were obtained from 10 cases of tongue squamous cell carcinoma (SCC) and 10 cases of verrucous carcinoma (VC) in the oral cavity (two in the tongue, three in the buccal mucosa, three in the gingiva, and one each in the lip and soft palate). These cases involved patients who consulted our hospital between January 2008 and December 2010 (Table 1). The study was approved by the Ethics Committee of Hokkaido university hospital (012−0446). Informed consent was obtained from the patients before the samples were used. The specimens were fixed using 10% buffered formalin embedded in paraffin. Four-micrometer sections were deparaffinized and subjected to antigen retrieval heat treatment in TE buffer. The slides were then immersed in 1% hydrogen peroxidase for 10 min and 1% BSA in PBS for 30 min. The immunohistochemical staining of MMP-1 (1:2000 dilutions, Abcam) and HuR (1:5000 dilutions, Santa Cruz Biotechnology, Inc.) antibodies in PBS containing 1% BSA was performed. The slides were stained with Histofine Simple Stain MAX-PO (M) R (Nichirei Bioscience, Tokyo, Japan), counterstained with hematoxylin, and visualized using the ChemMate En Vision kit/HRP (DAB) kit (DAKO, Tokyo, Japan). Other sections were stained with hematoxylin and eosin and subjected to histopathological evaluation. HuR was assessed as positive or negative and scored 1 and 2, respectively. MMP-1 was assessed as negative, low positive, moderate positive, and high positive, which were scored as 1, 2, 3, and 4 respectively.

Table 1 Clinicopathological features of squamous cell carcinoma (SCC) and verrucous carcinoma (VC).

Characteristic	SCC	VC
Age		
Median	62.5	70.5
Range	44−81	47−91
Gender		
Male	7	5
Female	3	5
Location		
Tongue	10	2
Cheek mucosa	0	3
Gingiva	0	3
Lip mucosa	0	1
Soft palate	0	1
Cytoplamic HuR		
Negative	0	7
Weak positive in the basal layer	0	3
Positive	10	0
Score average	2.0*	1.3
MMP-1		
Negative	0	2
Low positive	4	8
Moderate positive	2	1
High positive	4	0
Score average	2.7*	1.6

HuR, human antigen R; MMP-1, matrix metalloproteinase-1; *$P < 0.05$.

Statistical analysis

The control siRNA data was calculated as 100%. Statistical analysis was conducted using the unpaired Student's t-test. The results are reported as mean ± standard deviation (SD) with a $P < 0.05$ determining statistical significance.

Results

Knockdown of HuR changes the invasive phenotypes of cancer cells

To understand the effects of HuR knockdown on the invasive activity of cancer cells, the oral carcinoma cells HSC-2, tongue carcinoma cells HSC-3, gingival carcinoma cells Ca9.22, and fibrosarcoma cells HT1080 were subjected to a Matrigel invasion assay. HT1080 cells are used to examine the invasive activity of cancer cells because they display high motility and invasive activity.[18] After confirming HuR knockdown (Fig. 1A), the cells were applied to invasion chambers containing Matrigel-coated membranes. The mean invasion rates of HuR knockdown HSC-2, HSC-3, Ca9.22, and HT1080 cells decreased after 24 h compared with those of the control cells (Fig. 1B and C). These results suggest that HuR knockdown downregulates the invasive activity of cancer cells.

Knockdown of HuR alters MMP-1 promoter activity

An earlier study demonstrated that HuR knockdown leads to decreased expression of β-actin mRNA and protein.[19] We expected a decreased level of β-actin protein in our HuR knockdown cancer cells. However, as shown in Fig. 2C, the expression levels of β-actin protein in the HuR knockdown cancer cells were almost the same as in control cells. To explore why HuR knockdown attenuates the invasive activity of cancer cells, we examined the expression of the matrix metalloproteinase (MMP) gene because MMP affects the tumor microenvironment[20] and cancer progression.[21]

Since *MMP*-9 mRNA has ARE, we examined the expression of *MMP*-9 mRNA in HuR knockdown cancer cells. However, the expression was not reduced by HuR knockdown (data not shown); hence, we examined the expression MMP-1 as the next candidate of MMP. There is no ARE in MMP-1 mRNA;

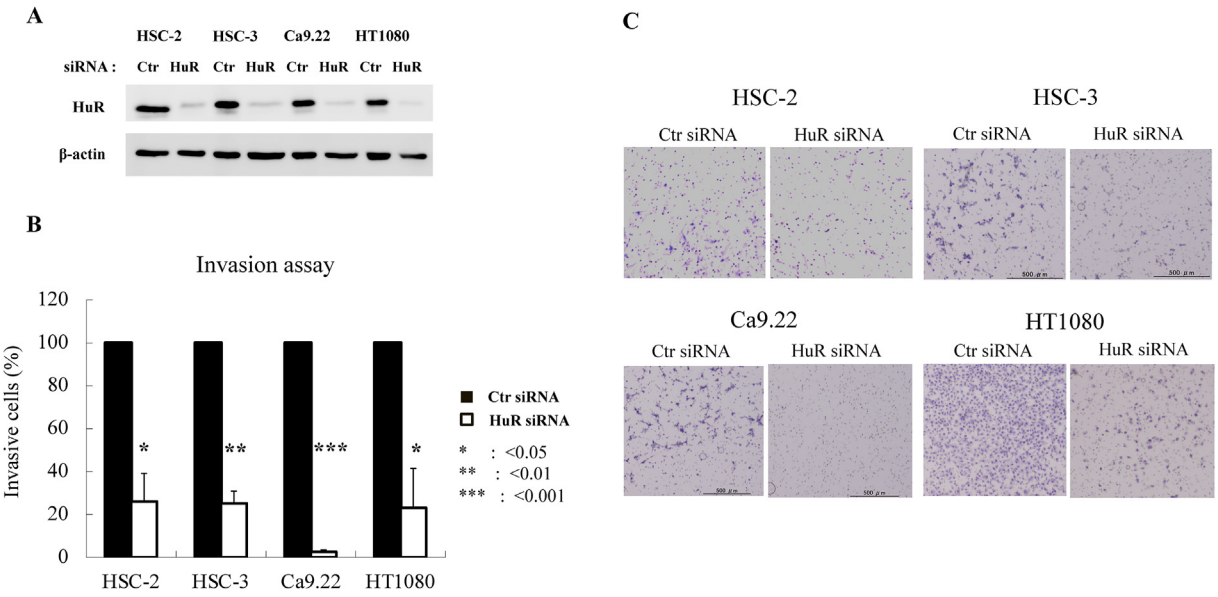

Figure 1 Knockdown of HuR decreases the invasive activity of cancer cells. (A) HuR and control (Ctr) siRNAs were used in oral cancer (HSC-2, HSC-3, and Ca9.22) and fibrosarcoma (HT1080) cells. The protein level of HuR and β-actin was detected using Western blot analysis. (B) Cancer cells transfected with HuR or Ctr siRNAs were placed in a BioCoat Matrigel Invasion Chamber. The cells on the lower side of the membrane were fixed, stained, and counted after 24 h. Mean ± SD of three independent experiments: bars, SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. (C) Phase images of HSC-2, HSC-3, Ca.922 and HT1080 cells transfected with HuR siRNA and control siRNA.

however, since the mRNA of the transcription factor activator protein 1 (AP-1) involved in the transcription of MMP-1 has ARE and AP-1 expression is implicated in cancer cell invasion,[22] we considered that MMP-1 expression is affected by HuR knockdown at the transcriptional level. We examined the promoter activity of the MMP-1 gene in each cancer cell using a luciferase reporter system. A luciferase reporter, including the MMP-1 promoter,[17] was introduced into each HuR knockdown cancer cell, and their luciferase activities were estimated (Fig. 2A). The mean activity of the MMP-1 promoter in each cancer cell was diminished by HuR knockdown compared with that of the control cancer cells (Fig. 2B). Additionally, we confirmed MMP-1 protein expression in HuR knockdown cancer cells. In HSC-2 and HSC-3 cells, the expression level of MMP-1 protein was decreased (Fig. 2C). The findings indicate that HuR knockdown reduces the expression of MMP-1 through the downregulation of the mRNA of transcription factors required for activation of the MMP-1 gene promoter.

Knockdown of HuR affects the expression of the transcription factors required for MMP-1 transcription

The transcription of MMP-1 is activated by several transcription factors, including AP-1,[23] which are associated with the invasive phenotypes of cancer cells. To explore the mechanism responsible for the downregulation of MMP-1 promoter activity in HuR knockdown cancer cells, we examined the mRNA levels of AP-1-related genes, including *c-fos* and *c-jun,* in HuR knockdown and control cells using qRT-PCR. As shown in Fig. 3A, the quantities of *c-fos and c-jun* mRNA in the HuR knockdown oral cancer cells were lower than those in the control cancer cells. We examined the stabilization of *c-fos* and *c-jun* between with and without HuR knockdown in HSC-3 and Ca9.22 using qRT-PCR with actinomycin D. The half-life of *c-fos* was 26.1 and 27.6 min in HuR knockdown HSC-3 and control HSC-3 cells, respectively (Fig. 3B). Differences were statistically significant at the 60-min time point. HuR knockdown did not alter the stabilization of *c-jun* in HSC-3 cells (Fig. 3B). The half-life of *c-jun* was 29.1 and 30.1 min in HuR knockdown Ca9.22 and control Ca9.22 cells, respectively (Fig. 3C). Differences were statistically significant at the 30-min time point. Moreover, HuR knockdown did not change the stabilization of *c-fos* in Ca9.22 cells (Fig. 3C). The results indicate that knockdown of HuR decreases the mRNA quantities of the transcription factors and stabilization of *c-fos* or *c-jun* in each cell required for the activation of MMP-1 expression.

Cytoplasmic localization of HuR is correlated with MMP-1 expression and invasive phenotypes of oral cancer cells in vivo

To determine whether cytoplasmic localization of HuR is related to the expression of MMP-1 and invasive phenotypes of carcinoma, we attempted the immunohistochemical staining of HuR in oral carcinoma cells. VC, which displays exophytic growth and low invasive potential, and tongue squamous cell carcinoma, which displays greater invasive potential, were subjected to this examination. The clinical and pathological features of these cancers are summarized in Table 1. The expression of HuR was restricted to the nucleus in seven cases of VC, and slight cytoplasmic expression was observed in three cases of VC (Tables 1 and

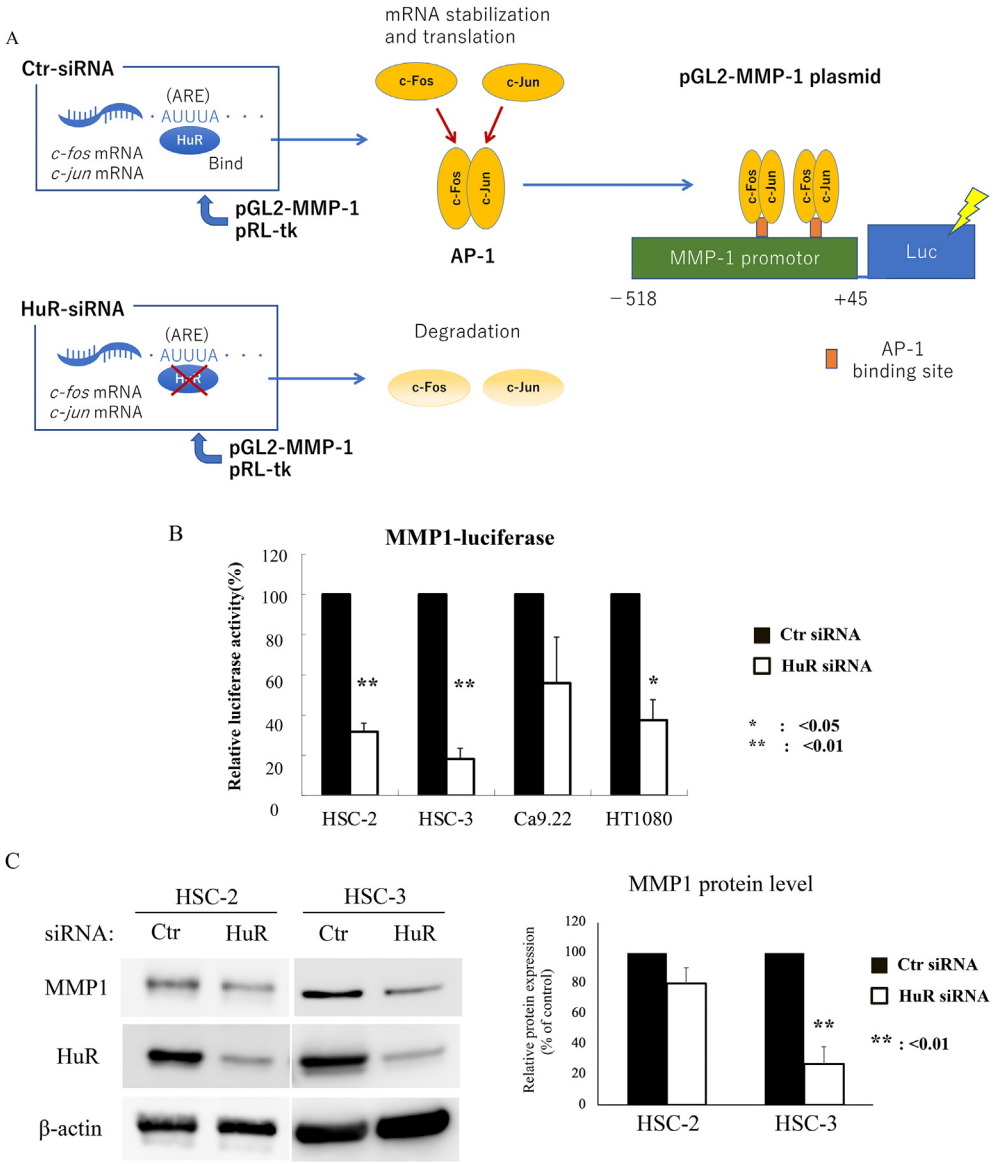

Figure 2 Knockdown of HuR decreased the expression of MMP-1 in cancer cells. (A, B) HuR knockdown and control cells were transfected with the pGL2-MMP-1 reporter plasmid and pRL-tk vector. After 24 h, the cell lysate was subjected to a luciferase assay using a luminometer. Mean ± SD of three independent experiments: bars, SD. *$P < 0.05$; **$P < 0.01$. (C) HSC-2 and HSC-3 cells were transfected with HuR and Ctr siRNAs. The protein levels of HuR, β-actin, and MMP-1 were detected using Western blot analysis. A histogram depicts the quantitative assessment of MMP-1 protein in each fraction. Mean ± SD of three independent experiments: bars, SD. *$P < 0.05$.

2, and Fig. 4E). In contrast, positive cytoplasmic and nucleic expression of HuR were identified in all cases of invasive tongue SCC (Tables 1 and 2 and Fig. 4B). Furthermore, we examined immunohistochemical staining of MMP-1. Cells expressing the cytoplasmic HuR also expressed MMP-1 in the invasive front of most SCC cases (Fig. 4C). In contrast, there is no or low MMP-1 expression in verrucous carcinoma (Fig. 4F). Mean expression scores for HuR and MMP-1 were significantly different between SCC and VC, and MMP-1 tended to be more moderately positive and highly positive in SCC than in VC, *P*-value = 0.057. Cytoplasmic HuR and MMP-1 expression was significantly different between SCC and VC. The results are in agreement with *in vitro* experiments showing HuR expression to be strongly related to the MMP-1 expression in oral SCC.

Discussion

In our previous report, we described the knockdown of HuR to downregulate the malignant phenotypes of oral cancer cells, including the degree of motility and invasion, as well as anchorage-independent cell growth.[12] In this study, we confirmed the invasive activity of HuR knockdown cancer cells. We found that MMP-1 promoter activity was

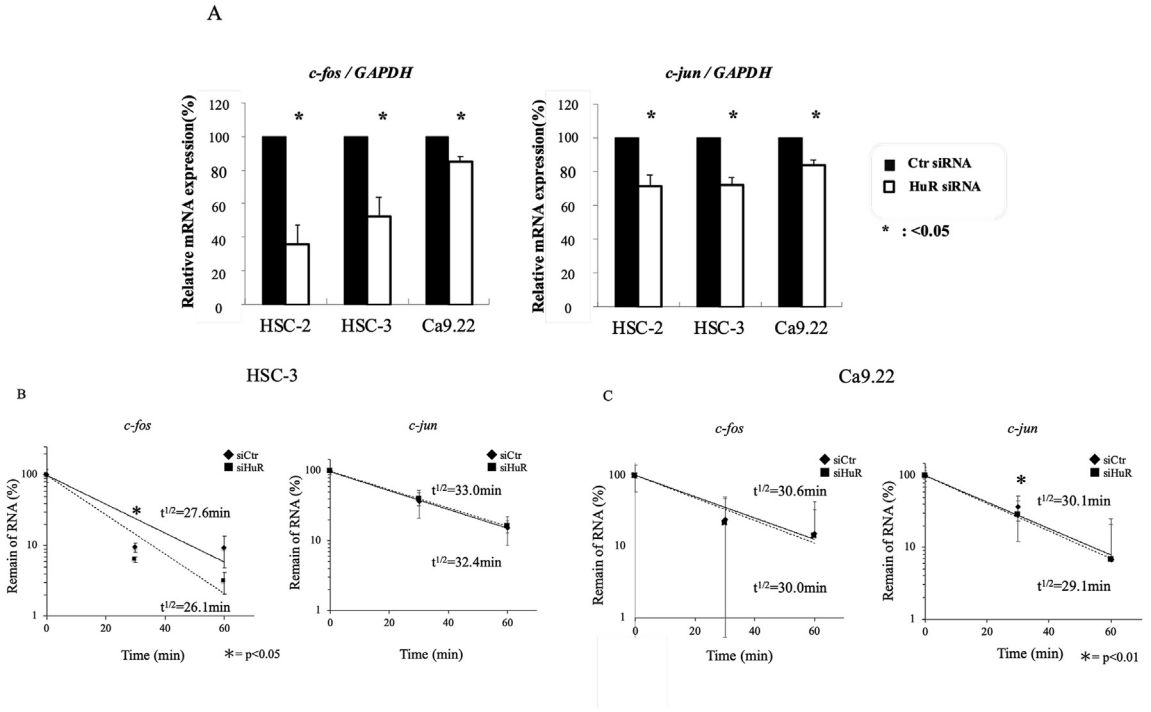

Figure 3 The amount and stabilization of mRNA encoding the transcription factor AP-1 in HuR knockdown oral cancer cells. (A) The accumulation of *c-fos* and *c-jun* mRNA in HuR knockdown oral cancer cells or control cancer cells was estimated using qRT-PCR. (B) The stabilization of *c-fos* and *c-jun* mRNA in HuR knockdown or control in HSC-3 and Ca9.22 cells was estimated using qRT-PCR. Mean ± SD of three independent experiments: bars, SD. *$P < 0.05$.

Table 2 Relationship between human antigen R (HuR) and matrix metalloproteinase-1. (MMP-1) levels in squamous cell carcinoma (SCC) and verrucous carcinoma (VC).

		SCC	VC	
Cytoplasmic HuR	negative	0	7	
	positive	10	3	$P = 0.003$
MMP-1	−/+	4	9	
	++/+++	6	1	$P = 0.057$
		SCC	VC	
cytoplasmic HuR positive		10	3	
MMP-1 ++/+++		6	1	

suppressed in HuR knockdown cancer cells via down-regulation of AP-1-related genes, including *c-fos* and *c-jun*, required for transactivation of the MMP-1 gene. Furthermore, the degree of cytoplasmic localization of HuR was significantly higher in oral SCC than in VC, which displays exophytic growth and low invasive potential. These findings suggest that HuR upregulates MMP-1 expression by activating its transcription to confer an invasive phenotype on cancer cells. Furthermore, HuR can be used as a diagnostic tool for cancer invasion, and HuR knockdown has the potential as cancer therapy.

Dormoy-Raclet et al. showed that HuR knockdown attenuated the invasive activity of HeLa cells by down-regulating β-actin mRNA expression.[19] They elucidated that β-actin mRNA was stabilized by HuR associating with a uridine-rich element within 3′-UTR. In our experiment, the level of β-actin protein was not decreased by HuR knockdown in oral cancer cells or fibrosarcoma cells (Fig. 1A). The findings suggest that knockdown of HuR decreases β-actin protein expression in a cell type-dependent manner, although additional detailed data is required to confirm this.

MMP-1 was expressed in many different malignant tumors higher than in corresponding normal tissues.[24] The transcription of MMP-1 is activated by AP-1,[23] including *c-fos and c-jun*. In hepatocellular carcinoma (HCC) patients, MMP-1 was upregulated and related to poor prognosis.[24] Furthermore, CircDLC1, circular RNA, inhibit the MMP-1 expression in the HCC to binding HuR.[25]

HuR stabilizes *MMP*-9 mRNA by binding to the ARE in the 3′-UTR of its mRNA.[26] Hence, we hypothesized that HuR knockdown affects MMP-9 expression in oral cancer cells.

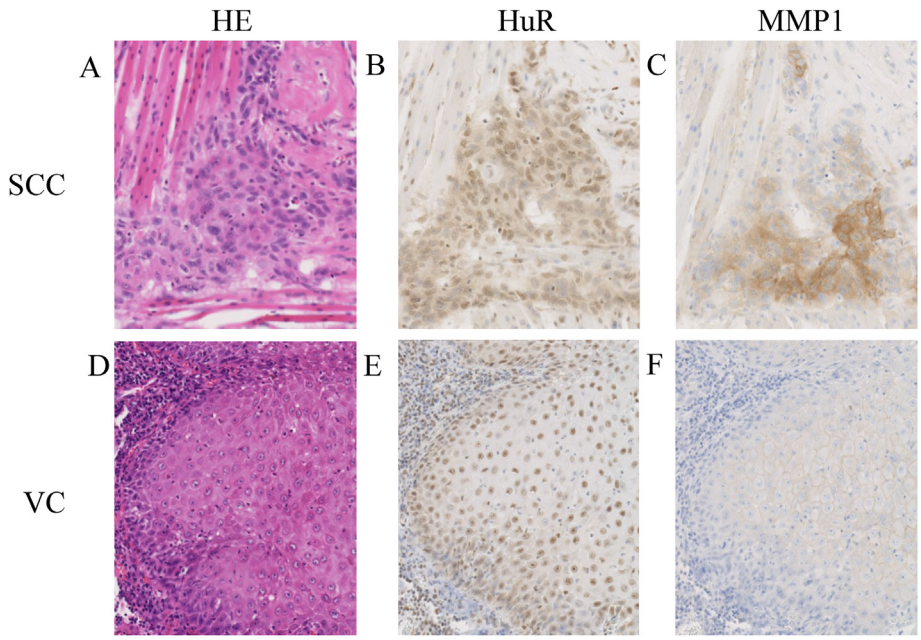

Figure 4 Immunohistochemical analysis of HuR and MMP-1 expression in verrucous carcinoma and squamous cell carcinoma. HuR and MMP-1 proteins in low invasive verrucous carcinoma (VC) and high invasive squamous cell carcinoma (SCC) were stained. (A) HE, (B) HuR, and (C) MMP-1 expression in the SCC tissue. (D) HE, (E) HuR, and (F) MMP-1 expression in the VC tissue.

To examine this possibility, we carried out a gelatin zymography assay to identify the activity levels of gelatinases, such as MMP-9 and MMP-2. However, we did not detect a difference in MMP-9 or MMP-2 activity between the control cells and HuR knockdown oral cancer cells (data not shown). The findings indicate that HuR knockdown does not affect MMP-9 and MMP-2 activity in oral cancer cells, suggesting that the effect of HuR on the stability of gelatinases depends on the cell type.

We also examined whether the cytoplasmic localization of HuR was related to invasive activity in tumor specimens. VC, a variant of SCC, displays slow, exophytic growth and a good prognosis compared to invasive SCC. We immunohistochemically stained HuR in VC and SCC to examine whether the expression of cytoplasmic HuR is upregulated in oral cancer cells. Positive cytoplasmic expression was observed in all cases of tongue SCC, whereas HuR expression was mostly restricted to the nucleus in VC. Moreover, in many cells where HuR was localized in the cytoplasm, MMP-1 was expressed. Thus, we conclude that the expression of cytoplasmic HuR and MMP-1 is closely related to the invasive activity of in vivo tumors in oral cancer cells.

In conclusion, the invasive activity of HuR knockdown cancer cells was markedly decreased compared with control cancer cells. Furthermore, HuR knockdown inactivated MMP-1 transcription through the downregulation of AP-1-related genes, including *c-fos* and *c-jun*, required for its expression. The expression of cytoplasmic HuR in SCC was higher than that in VC, which displays low invasive activity. The results suggest that HuR plays an important role in the invasive activity of cancer cells through the expression of MMP-1, and HuR knockdown could be used to inhibit cancer cell invasion.

Declaration of competing interest

The authors have no conflicts of interest to declare.

Acknowledgments

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