INTRODUCTION

Bladder cancer (BC) is the most common malignancy of the urinary tract. According to the American Cancer Society, approximately 79,000 new cases of BC and over 18,000 deaths were estimated to have occurred in the USA alone in 2017. Recent precision medicine showed that mutations in BC are frequently observed in FGFR3, RAS and PIK3CA genes, all of which correlate with RAS signaling networks. Among these networks, that of KRAS, in particular, is extremely complicated. Moreover, KRAS regulates more than 10 effector signaling pathways, and its expression is promoted mainly by receptor tyrosine kinases (RTK), including FGFR3. Previous studies reported on the networks of KRAS. In addition, microRNAs (miR) that directly target KRAS signaling impede KRAS-driven tumorigenesis. Moreover, miR-143 is strongly downregulated in several cancers, including BC. Moreover, miR-143 is strongly downregulated in several cancers, including BC. Furthermore, miR-143 silences KRAS and MSI2, which further downregulates KRAS expression through perturbation of the MSI2/KRAS cascade.

KEYWORDS
bladder cancer, KRAS, miR-143, Musashi-2, RNA-binding protein
Musashi-2 (MSI2). MSI1 and MSI2 share approximately 75% amino acid identity in their overall structure and belong to a family of RNA-binding proteins. MSI2 post-transcriptionally regulates mRNA processing by binding to the recognition motifs located at the 3'UTR of target mRNAs, similar to MSI1. MSI2 preferentially interacts with an ACCUUUUAAGAA motif and other poly-U sequences. UAG motifs, and UAG-containing motifs ± additional flanking nucleotides. The Musashi proteins were first linked to cancer based on studies showing elevated expression of MSI1 in gliomas, medulloblastomas, and hepatomas. MSI2 was identified as part of a translocation event with HoxA9 in chronic myeloid leukemias that preserved MSI2 RNA-binding motifs, also implicating MSI2 in cancer development. The past several years have been marked by a surge of reports elucidating the frequency and mechanisms of involvement of MSI2, in particular, in multiple forms of human cancer, including BC. Like MSI1, moreover, Dong et al. reported that MSI2 is directly regulated in a negative way by miR-143.

In the present study, we clarified the correlation between KRAS and MSI2, both of which are targets of miR-143. Notably, knockdown of MSI2 induced downregulation of KRAS, and overexpression of MSI2 upregulated KRAS without causing an increase in the level of KRAS mRNA. These results indicated that MSI2 post-transcriptionally regulated KRAS expression. Furthermore, by using a luciferase reporter assay and surface plasmon resonance (SPR), we demonstrated that MSI2 positively regulated KRAS expression through direct binding to the target sequence UAGUA in the 3'UTR region of KRAS mRNA. Taken together, our findings indicated the extremely potent anticancer activity of synthetic miR-143 (syn-miR-143), and it enabled us to clarify and better understand the role of the novel miR-143/MSI2/KRAS cascade in human BC.

2 | MATERIALS AND METHODS

2.1 | RNA immunoprecipitation

RNA immunoprecipitation (RIP) was carried out with a RIP-assay Kit (Medical & Biological Laboratories Co., Ltd., Aichi, Japan) according to the manufacturer's instructions.

2.2 | RNA-stability measurements

The RNA polymerase II transcriptional inhibitor 5,6-dichlorobenzimidazole riboside (DRB) was procured from Tokyo Chemical Industry (Tokyo, Japan). T24 cells were seeded on the day prior to transfection with the cDNA plasmid encoding MSI2 or control vector. The cells were treated with DRB at 24 hours after transfection. Cellular RNA was harvested at time 0, 2, 4, 6, and 8 hours and used for qRT-PCR analysis of KRAS mRNA. RNA half-lives were calculated from linear regression of log-transformed expression values. ANCOVA was carried out on the resulting regression lines to assess statistical significance.

2.3 | Human tumor xenograft model

Animal experimental protocols were approved by the Committee for Animal Research and Welfare of Gifu University (approval no. H30-42). BALB/cSCL-nu/nu (nude) mice were obtained from Japan SLC (Shizuoka, Japan). Human bladder cancer T24 cells were inoculated into the back of each mouse. At 7 days after the inoculation, we confirmed engraftment of the tumors. When the tumor size had reached approximately 100 mm³, treatment was started. siRNA or miRNA carried by Lipofectamine RNAi MAX (Invitrogen, Carlsbad, CA, USA) was injected into the tumor every 2 days for a total of three times. Each group contained three mice. Tumor volume was calculated by the formula: 0.5236 L1 (L2)², where L1 is the long axis and L2 is the short axis.

Other methods are shown in Data S1.

3 | RESULTS

3.1 | Impact of KRAS on proliferation of bladder cancer cell lines

To investigate the function of KRAS as an oncogene in human BC, we first assessed the association between cell growth and KRAS and that between it and HRAS in BC cell lines T24 and 253JB-V. Knockdown of HRAS by use of siRNA significantly suppressed cell proliferation, and knockdown of KRAS resulted in a more potent growth inhibition than that obtained with knockdown of HRAS (Figure 2A). In addition, KRAS effector signaling proteins, AKT and ERK1/2, were downregulated by both knockdowns (Figure 1B). Of note, this knockdown was more prominent in T24 cells, which have an HRAS mutation, not a KRAS one. These results suggested that KRAS contributed considerably to cell proliferation in BC, as did HRAS.

3.2 | Syn-miR-143 directly silences the key genes of KRAS networks and MSI2

Previously, we reported that miR-143 inhibited cell proliferation with apoptosis through silencing PI3K/AKT and MAPK signaling pathways, which are major growth-related effector signal pathways in KRAS networks in BC. As shown in Figure 2A, the expression levels of miR-143 were extremely downregulated in both T24 and 253JB-V cells. Recently, we developed a chemically modified miR-143 that has potent RNAse-resistant anticancer activity (Figure S1). This syn-miR-143 silences not only KRAS but also KRAS effector signaling molecules, AKT and ERK. To clarify how KRAS networks contribute to carcinogenesis and cell growth in BC, we introduced syn-miR-143 into T24 cells, which induced apoptosis to a greater extent than that obtained with Ambion miR-143 (Ambion, Carlsbad, CA, USA), probably as a result of extreme silencing of KRAS networks (Figure S2). As shown in Figure 2B, ectopic expression of syn-miR-143 led to significant growth inhibition in both cell lines. Western blot analysis
indicated that syn-miR-143 strongly decreased the expression of KRAS protein and its effector signaling proteins AKT and ERK1/2. Interestingly, RNA-binding protein MSI2 was also downregulated. MSI2 was recently reported as a target of miR-143 in cervical cancer\cite{30} and has the fourth-most frequent genetic alterations in BC across almost all major cancers as assessed in The Cancer Genome Atlas (TCGA) BC cohort using the cBioPortal for Cancer Genomics (cBioPortal; Figure S3), and has specific target sequences recognized by miR-143 according to in silico prediction tools in TargetScan. In addition, treatment with antagomiR-143 reversed the growth inhibition and lowered protein levels of both KRAS and MSI2 elicited by syn-miR-143 (Figure 2C). To examine whether miR-143 directly bound to the 3’UTR region of MSI2 mRNA, we cloned the 3’UTR of MSI2 mRNA containing the possible miR-143 binding site in a reporter plasmid. As a result, luciferase activity of wild-type pMIR-MSI2 was inhibited after cotransfection with miR-143 and the reporter plasmid DNA in T24 cells (Figure 2D). In contrast, decrease in luciferase activity was abrogated in the case of mutated binding sites. Together, these results indicated that miR-143 could silence MSI2 expression at the translation step and inhibit BC cell proliferation, in part, through suppression of MSI2 expression. Hence, next we focused on the oncogenic function of MSI2 and the interaction between MSI2 and KRAS.

### 3.3 Musashi-2 is upregulated in clinical tumor samples of BC

We examined the expression of MSI2 in 10 samples from BC patients by western blot analysis. Clinicopathological findings of the patients are shown in Table S1. As shown in Figure 3A, MSI2 was upregulated in six of the 10 clinical BC samples examined compared with its level in normal bladder tissues in the same patients, and expression of miR-143 was downregulated in all cases according to qRT-PCR results (Figure 3B). Therefore, all cases of MSI2 overexpression corresponded to downregulation of miR-143. In addition, qRT-PCR analysis of MSI2 mRNA showed upregulation in T24 and 253JB-V cells, and the increase was more prominent in T24 cells than in 253JB-V cells (Figure 3C). Based on these results, we focused on T24 cells in the following experiments.

### 3.4 Relationship between MSI2 and KRAS or HRAS in T24 cells

To examine the interaction between MSI2 and KRAS, we carried out knockdown and overexpression of MSI2 by using siRNA (Figure S4) and MSI2 expression vector (pF5A-MSI2), respectively. Knockdown of MSI2 by siR-MSI2 induced cell growth inhibition along with
downregulation of KRAS (Figure 4A). In contrast, overexpression of MSi2 promoted cell proliferation and upregulated KRAS (Figure 4B). Importantly, no significant change in KRAS mRNA levels was observed in the case of either silencing or overexpression of MSi2 (Figure 4A,B). On the contrary, expression levels of HRAS mRNA corresponded to those of MSi2. Taken together, these data suggested that KRAS and HRAS were downstream of MSi2 and that MSi2 may have post-transcriptionally regulated the transcripts of KRAS and HRAS. Notably, the expression of MSi2 was also affected by KRAS or HRAS, because either knockdown of KRAS or HRAS caused a decrease in the expression of MSi2 protein (Figure 4C). Also, silencing of either KRAS or HRAS caused the expression of HRAS or KRAS, respectively, to decrease. These results indicated that MSi2 and KRAS or HRAS are coordinated with each other, although it is difficult to clarify how MSi2 interacts with KRAS and HRAS, given the complicated nature of RAS signaling networks.

**FIGURE 2** Ectopic expression of microRNA (miR)-143 induces significant downregulation of KRAS and Musashi-2 (MSi2) through RNA interference. A, Relative expression levels of miR-143 in T24 and 253JB-V cells. B, Dose-dependent effect of miR-143 on cell viability and protein expression levels of the target genes. C, Above effects of miR-143 were verified in cells treated with antagomiR-143. D, Luciferase activities after cotransfection with control or miR-143 and wild-type or mutant-type pMIR vectors having the predictive miR-143 binding site in the 3’UTR of MSi2 mRNA (positions 173-179: #1 and 180-187: #2) to the mature miR-143. Colored (red and green) sequences of two sites indicate the predicted binding sites for miR-143. The nucleotide sequence of the mutated site is shown in blue. **P < .01. Means + SD indicated by error bars are shown. NS, not significant.
3.5 | Musashi-2 directly binds to mRNA of KRAS

To clarify whether or not MSI2 bound directly to mRNAs of KRAS and HRAS, using BC cells, we first carried out MSI2-immunoprecipitation (IP) followed by qRT-PCR. Western blot analysis, carried out as a quality check, showed that MSI2 was detected in input and MSI2-IP samples only, not in the control IgG-IP sample. qRT-PCR findings showed that the MSI2-immunoprecipitated RNA fraction was significantly enriched in KRAS mRNA (Figure 5A), whereas there was no enrichment of HRAS mRNA in IgG or MSI2 fractions. These data suggested that MSI2 directly bound to KRAS but not to HRAS. Therefore, the data on HRAS observed in Figure 4A,B were supposedly as a result of an indirect impact of MSI2.

In addition, to determine the direct interaction between MSI2 and KRAS mRNA, we cloned the predicted MSI2 binding site UAGUA in the 3’UTR region of KRAS mRNA in a reporter plasmid vector (Figure 5B). Results of the luciferase reporter assay indicated that activity of wild-type pMIR-KRAS was decreased by siR-MSI2 compared with that obtained with control siRNA, indicating that the activity paralleled the level of MSI2 protein expression. On the contrary, decrease in the activity of the pMIR vector was almost canceled when the mutated MSI2 binding site AUCAU was used. Thus, these data clearly showed the promoting roles of MSI2 in the translation step of luciferase mRNAs.

To examine direct interaction between MSI2 and UAGUA, we next used the SPR assay. To this end, a recombinant MSI2 protein containing the two RNA-binding domains was expressed (Figure S5) and immobilized on a sensor-chip surface. A synthetic 15-mer KRAS mRNA containing UAGUA or its scrambled sequence as a control was injected over the sensor chip. As shown in the left panel of Figure 5C, the UAGUA sequence gave the highest binding response to the immobilized MSI2 (reaching 50 resonance units at 10 μmol/L). In addition, a dissociation constant (Kd) of UAGUA for MSI2 was 2.5-fold lower than that for the control RNA (right panel of Figure 5C), suggesting that MSI2 preferentially bound to the UAGUA sequence. Collectively, these data showed that MSI2 directly interacted with KRAS mRNA by recognizing and binding to one of the specific UAGUA sequences.

3.6 | Musashi-2 post-transcriptionally enhances translation of KRAS

To investigate how MSI2 regulates the processing of KRAS mRNAs after transcription, we first examined the localization of MSI2 in cells by using immunofluorescence. MSI2 was located mainly in the cytoplasm in T24 and 253JB-V cells (Figure 6A) in agreement with the reports in a public database (The Human Protein Atlas).

Cellular localization of MSI2 and the findings in the current study suggest that MSI2 might have the ability to regulate the
stability or translation of target mRNAs. To determine whether MSI2 could regulate the stability of KRAS mRNA, we estimated the rate of mRNA decay after treatment with DRB. Time-course RNA decay curves for KRAS mRNA were prepared from qRT-PCR data after DRB treatment of cells transfected with pF5A-control or pF5A-MSI2. As a result, the half-life of KRAS mRNA was not significantly changed in either case, whereas overexpression of MSI2 was achieved in the case of pF5A-MSI2 transfection (Figure 6B). These data thus showed that MSI2 functioned to enhance the translation of KRAS mRNA rather than to stabilize the mRNA, the finding of which is well supported by the results given in Figure 4A,B. To further validate that MSI2 regulated the translation, we assessed the expression of translational initiator elf4E by western blot analysis. Notably, knockdown of MSI2 induced the downregulation of elf4E (Figure 6C). These data suggested that MSI2 played a role in enhancing translation. Furthermore, western blot analysis showed that MSI2 and KRAS were co-upregulated in six cases of 10 clinical BC samples compared with their expression in normal bladder tissues (Figure 6D). In Lee’s cohort, there was a significant positive correlation of mRNA expression levels between MSI2 and KRAS in human BC (Figure 6E). These data suggested that the patients, which had abundant MSI2 mRNA, could have increment of KRAS protein.

**FIGURE 4** Relationship between Musashi-2 (MSI2) and KRAS or HRAS in expression profiles of T24 cells. A, Effects of MSI2 knockdown using siRNA on cell growth (left panel). Protein and mRNA expression levels of KRAS and HRAS after siR-MSI2 transfection (middle and right panels). B, Effects of MSI2 knockdown and overexpression on cell growth (left panel). Protein and mRNA expression levels of KRAS and HRAS in MSI2-silenced and -overexpressed cells (middle and right panels). C, MSI2 and RAS protein profiles after transfection with siR-KRAS or siR-HRAS. *P < .05; **P < 0.01. Means + SD indicated by error bars are shown. NS, not significant.
expression through efficient translation by MSI2. Furthermore, we showed that the silencing effect of KRAS by ectopic expression with syn-miR-143 was certainly canceled by overexpression of MSI2 (Figure S6). This finding suggested that downregulated expression of MSI2 by miR-143 was significant in the growth of BC cells.

Collectively, these data indicated that MSI2 functioned to accelerate the translation of KRAS mRNA in the cytoplasm and had crucial roles as a KRAS enhancer in BC cells. This machinery was closely correlated with KRAS networks, in which there was a positive circuit for enhancement of KRAS mRNA expression by KRAS effector signaling (AKT and ERK)\(^\text{10}\) (Figures 7, S7 and S8).

3.7 | MicroRNA-143/MSI2/KRAS cascade on T24 cell-xenografted tumors in nude mice

We have clarified the novel miR-143/MSI2/KRAS cascade in vitro. To further validate the cascade between miR-143, MSI2 and KRAS, we examined the antitumor effect by using syn-miR-143 and siR-MSI2...
in vivo. As shown in Figure 8A, growth suppression of tumors was observed in the groups treated with siR-MSI2 or syn-miR-143. In addition, the tumor-suppressive effect of syn-miR-143 was greater than that of siR-MSI2. Western blot analysis of the tissue samples from grafted tumors showed that MSI2 was significantly silenced in both treated groups. Furthermore, decreased expression of KRAS

FIGURE 7  Schematic diagrams showing the roles of Musashi-2 (MSI2) in KRAS networks and the association of MSI2 with KRAS mRNA. MicroRNA (miR)-143/MSI2/KRAS cascade (left panel) and possible machinery for MSI2-mediated enhancement of the translation of KRAS mRNA (right panel)
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4 | DISCUSSION

In the current study, we clarified a novel network operating miR-143, MSI2 and KRAS (Figure 7). We were also able to show that the expression of KRAS was affected by MSI2 through binding of the latter to KRAS mRNA; the finding of which was validated by RNA-IP, SPR, and the expression profiles of the genes involved. Previously, we showed that mir-143 directly silences KRAS signaling networks, and Dong et al. reported that miR-143 also targets RNA-binding protein MSI2. However, the association between these targets of miR-143, KRAS and MSI2 had not been previously reported. Given the earlier reports that MSI2 has been suggested to interact preferentially with the UAG-containing motifs in the 3′UTR region of its target RNAs, we predicted the binding site in KRAS mRNA to be UAGUA and showed by a using luciferase reporter assay and the SPR technique (Figure 5B,C) that MSI2 protein preferentially binds to the sequence. In addition, it was earlier reported that MSI2 has functions to affect the stabilization or translation of its target mRNAs. Based on our results, MSI2 did not impact the stability of KRAS mRNA despite its direct binding to it (Figure 6B). Given that MSI2 positively regulated the translational initiator eIF4E (Figure 6C), we propose that MSI2 functioned to enhance the translation of KRAS mRNA rather than its stabilization. With regard to the role of translational regulation, MSI2 is regulated by site-specific phosphorylation, which converts MSI2 from a repressor to an activator of target mRNA translation, and MAPK and Ringo/CDK contribute to MSI2 regulatory phosphorylation, as does MSI1. MAPK also contributes to the translational machinery including eIF4E. MSI2 may be included in the cascades, and MAPK positively regulates eIF4E through phosphorylation of MSI2. In addition, given that the RAS signaling pathways, KRAS and HRAS could regulate MSI2 through MAPK indirectly. Indeed, it is reasonable that MSI2 was downregulated in cells by knockdown of KRAS and HRAS, in which ERK1/2 was also inhibited (Figures 1B and 4C).

We demonstrated an association between the specific sequence UAGUA and the ability of MSI2 to enhance the translation of its target mRNA. However, the SPR technique showed that the control sequences, despite the absence of a UAG motif, gave a weak binding response to MSI2 protein (Figure 5C). These data suggested that the specificity of MSI2 binding to transcripts may not be so high. The impact of MSI2 on its target RNAs could be due not only to binding ability, but also to other mechanisms. The two RNA recognition motif (RRM) of MSI2 are possibly involved in the mechanism. Biochemical and structural studies have suggested that RRM1 contributes the majority of the binding energy and specificity, whereas RRM2 has a more supportive role. In addition, Bennett et al. reported that these two RRM may provide a mechanism for MSI2 to distinguish its veritable targets. However, this machinery is presently barely understood and further validation is warranted.

In the present study, we clarified that MSI2 directly targeted KRAS, promoting translation of its mRNA. The RTK/RAS pathway has been reported to be involved in the regulation of cell proliferation in several cancers. Among these signaling pathways, in particular, up to 80% of non-muscle invasive BC (NMIBC) harbor activating point mutations in FGFR3, which activate the RAS/MAPK pathway. Moreover, the alteration of KRAS occurs more frequently than that of HRAS. Thus, KRAS and KRAS signaling networks are dominant pathways in BC. Previously, we have clarified the signaling networks. The “positive circuit” through the constitutive KRAS activation-stimulation of effector signaling pathways (PI3K/AKT and MAPK) occurs in colorectal cancer, resulting in enhanced proliferative activity.
nuclear KRAS transcription. As shown in Figure S7, this cascade was also seen in BC cell lines and, again, the “positive circuit” also occurred in HRA signaling networks. These data suggested that KRAS and HRAS interacted with each other, indicating that either MSI2 or miR-143 indirectly affected the expression of HRAS by regulating KRAS. Indeed, inhibition of the signals of ERK and AKT occurred, resulting in indirect regulation of HRAS expression in the case of treatment with siR-MSI2 or syn-miR-143 (Figures 2B and S8).

Recently, it was reported that miR-143 has a significant antitumor role in BC. Lin et al. reported that transfection of BC cells with miR-143 significantly inhibited cell proliferation through decreased expression of RAS protein. Wang et al. showed that overexpression of miR-143 inhibited cell proliferation in BC. Furthermore, we demonstrated previously that syn-miR-143 functions as a tumor suppressor in BC cells. Lin et al. reported that transfection of BC cells with miR-143 significantly inhibited cell proliferation in BC. In an earlier study, we also showed that miR-143 directly targets KRAS signaling networks. In the present study, we clarified the novel association between MSI2 and KRAS, both of which are targets of miR-143, validating the interaction between MSI2 and the UAGUA sequence of the KRAS transcript in vitro (Figures 4 and 5, S6). Previously, genome-wide analyses demonstrated that MSI2 binds to a multitude of target genes. Fox et al. validated targets C-MET, BRD4, and HMGA2 in pancreatic cancer, and Park et al. validated 48 genes in hematopoietic stem cells and 11 genes in leukemia stem cells. As such, MSI2 binds to a great number of targets, KRAS cannot be listed at the top in genome-wide analysis of MSI2 ribonucleoproteins. Syn-miR-143 has allowed us to propose the possibility of association between the two proteins, resulting in a better understanding of the novel miR-143/MSI2/KRAS expression system (Figure 7).

Collectively, we showed that miR-143 directly impacted MSI2 expression through its RNAi action, which also effectively inhibited KRAS networks as a novel mechanism in human BC. Moreover, this evidence was confirmed by the results of an in vivo experiment (Figure 8). Taken together, these findings indicated the complicated nature of KRAS networks and the tight control of their maintenance.

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DISCLOSURE

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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