Inhibition of Human Osteosarcoma Cell Migration and Invasion by a Gene Silencer, Pyrrole–Imidazole Polyamide, Targeted at the Human MMP9 NF-κB Binding Site

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Osteosarcoma is one of the most prevalent bone tumors, occurring mostly in adolescence. However, no noticeable progress has been achieved in developing new therapeutic agents for this disease. Matrix metalloproteinase 9 (MMP9), a type IV collagenase, is a known anticancer target and is overexpressed in osteosarcomas. MMPs can degrade components of the extracellular matrix and are known to be involved in tumor invasion and metastasis. In the present study, we designed and synthesized a pyrrole–imidazole polyamide (HN.49), a gene-silencing agent that specifically targets the nuclear factor-kappa B (NF-κB) binding site of the human MMP9 promoter. We then examined the effect of HN.49 on the enzyme activity of MMP9 and the migration activity of osteosarcoma cells in vitro. It was clearly shown that HN.49 polyamide reduced the expression level of MMP9 mRNA and the enzymatic activity of MMP-9 in SaOS-2 cells. Moreover, HN.49 polyamide inhibited migration and invasion by SaOS-2 cells in vitro wound-closure and matrigel-invasion assays. These results indicate that HN.49 may be a potential therapeutic agent for inhibiting the invasion and metastasis of osteosarcoma.

Key words pyrrole–imidazole polyamide; matrix metalloproteinase 9; osteosarcoma

Osteosarcoma is one of the most common primary malignant tumors of bone and occurs mainly in adolescents and young adults. There have been significant advances in its treatment in the recent years with a limb-salvage surgery and various chemotherapies. The elapse-free survival rates have been improved from less than 20% to 60%. However, these chemotherapies are not fully effective, and as a result, approximately 20% of all osteosarcoma patients still die owing to tumor metastasis. Therefore, the research should focus on developing new therapies for osteosarcoma.

Matrix metalloproteinase 9 (MMP9), 92-kDa type IV collagenase (gelatinase B, MMP9), contains fibronectin-like domains for collagen-binding and is capable of degrading types I, IV, V, VII and XI collagens and laminin. There is increasing evidence that MMP9 expression is elevated in malignant cancers compared with benign or non-invasive tumors and that for the role of MMP9 in tumor invasion in vitro and in vivo. Metastatic lesions of osteosarcoma in children have been shown to be strongly positive for MMP9 in immunohistochemical analysis. Hence, a compound that pre-transcriptionally deregulates MMP9 expression needs to be evaluated as a potentially useful MMP9 silencer for cancer therapy. Studies of the DNA minor groove binder of naturally occurring antitumor/antiviral antibiotics, including duocarmycin A and distamycin A, indicated the development of designable DNA recognition molecules in a sequence-dependent manner, including pyrrole–imidazole polyamide (PI polyamide). Since PI polyamides are low molecular organic compounds resistant to nucleolytic enzymes, PI polyamide could be transported across cell membrane and subsequently into cell nuclei without any delivery system and may not be influenced by any catabolic enzymes or metabolic enzymes, such as nucleases and p450 enzymes, even in animals. Hence, PI polyamides show great promise as new medicines that might inhibit transcriptional control of target gene. Indeed, we have developed PI polyamides as gene silencers, including the design and synthesis of PI polyamides that target MMP9 effectively. We demonstrated significant improvement of colon tumor metastasis to liver by targeting to the human MMP9 AP1 binding site.

In the present study we designed and synthesized a PI polyamide to bind to an eight-base pair sequence partially overlapping the functional nuclear factor-kappa B (NF-κB) site (HN.49) and examined the ability of HN.49 polyamide compound to block MMP9 expression and exhibit biologically relevant effects in human SaOS-2 osteosarcomas cell line.

MATERIALS AND METHODS

Synthesis of Polyamide Targeting Human MMP9 PI polyamide HN.49 targeting human MMP9 (Fig. 1A) was designed to span the boundary of the NF-κB binding site (−605 to −599) of the human MMP9 promoter (Fig. 1B). The mismatch PI polyamide, which could not recognize NF-κB binding site on MMP9 promoter was also designed and synthe-

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sized. Polyamide was synthesized according to the previously described methods, and concentration of the polyamide dissolved in distilled water was measured according to the methods described elsewhere. The purity and the molecular size of both PI polyamides used in this study were measured by HPLC and mass spectrometry (Supplementary Fig. 1).

DNA-Binding Assay Gel shift assay was performed to examine the binding affinity of HN.49 to target DNA double strand, as described previously. In brief, fluorescein isothiocyanate (FITC)-labeled DNA oligo nucleotide corresponding to −613 to −592 (5’-ggctggggaattccactggggc-3’) and its anti-sense oligo nucleotide were annealed to make double-strand oligo. One micro mole of oligo DNA was incubated with 1 µM of match or mismatch polyamides for 1 h at 37°C. The reaction mixtures were separated by electrophoresis on 4–20% gradient polyacrylamide gel in Tris–borate–ethylenediaminetetra-acetic acid (TBE) buffer, and then visualized by LAS4000 (FUJIFILM, Tokyo, Japan).

Cell-Type and Culture Conditions Human cervical carcinoma cells Hela used in this study were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Nacalai Tesque, Kyoto, Japan) containing 100 µg/mL streptomycin, 100 units/mL penicillin and 10% fetal bovine serum (FBS), and human osteosarcomas cells SaOS-2 were grown in McCoy’s 5A (Invitrogen Life Technologies, Corp., Carlsbad, CA, U.S.A.) containing 100 µg/mL streptomycin, 100 units/mL penicillin and 10% FBS. All of the cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C.

Chromatin Immunoprecipitation Assay (ChIP) Assay ChIP analysis was conducted as previously described previously with some modifications. When HeLa cells in 100-mm dishes were grown to confluence, cells were treated with or without 1 µM of match or mismatch polyamides. After incubation for 4 h, protein–DNA complexes were fixed by 1% formaldehyde in phosphate buffered saline (PBS). The fixed cells were washed and lysed in sodium dodecyl sulfate (SDS)-lysis buffer and sonicated on ice. The samples were centrifuged, and the soluble chromatin was precleared by incubation with
sheared salmon sperm DNA–protein agarose A slurry (Upstate) for 30 min at 4°C with rotation. After centrifugation at 15000 rpm for 1 min, one portion of the precleared supernatant was used as DNA input control, and the remains were sub-divided into aliquots and then incubated with a nonimmune rabbit immunoglobulin G (IgG; Upstate), IgG Ab to NF-B p65 (were from Santa Cruz), respectively, overnight at 4°C. The immunoprecipitated complexes of Ab–protein–DNA were collected by using the above protein A beads, and washed successively with low-salt buffer, high-salt buffer, LiCl buffer, and Tris–ethylenediaminetetraacetic acid (EDTA) (pH 8.0), and then eluted with elution buffer according to the manufacturer’s instructions (Upstate, Temecula, CA, U.S.A.). The cross-linking of protein–DNA complexes was reversed by incubation with 5 mM NaCl at 65°C for 4 h, and DNA was purified by proteinase K (Sigma) digestion, phenol–chloroform extraction, and ethanol precipitation. After washing, the DNA pellet was resuspended in H$_2$O and subjected to polymerase chain reaction (PCR) amplification with the forward (5'–TGT CCC TTT ACT GCC CTG A-3') and reverse (5'–ACT CCA GGC TCT GTC CTC TCT TT-3'), which were specifically designed to detect the promoter region (−657 to −484) of MMP-9. PCR products were separated by electrophoresis on agarose gels.

Wound-Healing Migration Assay Wound-healing migration assay was performed as described before. Briefly, SaOS-2 cells (1×10$^4$) were seeded in individual wells of an eight-well BD Falcon™ CultureSlides (BD Biosciences, Bedford, MA, U.S.A.). Once cells reached a confluent state, cell layers were wounded with a plastic micropipette tip having its theoretical target sequence on MMP9 promoter, ChIP assay was conducted using antibody to NF-κB p65. As shown in Fig. 2B, an infinitely lower yield of PCR product of MMP9 promoter region was detected by PCR in the precipitant of the cells treated with HN.49 than in that of the cells treated with mismatch polyamide or non-treated cells. No difference was observed in the precipitant made by using nonimmune rabbit IgG. These results strongly indicated that HN.49 could bind to its theoretical target sequence on MMP9 promoter and inhibit the binding of NF-κB to its recognition sequence.

Zymography Analysis Substrate gel zymography of the activity of MMP9 crude proteins from culture supernatants of SaOS-2 cells was performed. Briefly, 500 μL aliquot containing 3×10$^5$ SaOS-2 cells added to each of the triplicate wells. After incubation for 48 h, polyamides were added in a culture medium without FBS as described previously. The supernatant was collected after a 48-hour incubation, mixed with Novex™ Tris–Glycine SDS Sample Buffer (2X) and resolved and separated in a 10% Zymogram (Gelatin) gel. Gels were then washed in renaturing buffer and developing buffer, according to the manufacturer’s instructions (Invitrogen Life Technologies Corp.). Finally, gels were digitized using the method described previously. The density of the bands was determined using LuminaVision software Ver. 3.2 (Mitani Corp., Tokyo, Japan).

Statistical Analysis All values were expressed as the mean±S.E., and statistical significance was analyzed using the Steel–Dwass test. A p value of <0.05 was considered significant.

RESULTS Binding of HN.49 to the Promoter Region of MMP9 To verify whether HN.49 could bind to the NF-κB consensus sequence on the promoter region of MMP9 gene, gel shift assay was performed. Oligo DNA treated with HN. 49 showed clear mobility shift compared to the negative control or the DNA treated with or mismatch polyamide (Fig. 2A). To corroborate whether HN.49 could interfere the binding of NF-κB to its recognition sequence on the MMP9 promoter, ChIP assay was conducted using antibody to NF-κB p65. As shown in Fig. 2B, an infinitely lower yield of PCR product of MMP9 promoter region was detected by PCR in the precipitant of the cells treated with HN.49 than in that of the cells treated with mismatch polyamide or non-treated cells. No difference was observed in the precipitant made by using nonimmune rabbit IgG. These results strongly indicated that HN.49 could bind to its theoretical target sequence on MMP9 promoter and inhibit the binding of NF-κB to its recognition sequence.

Decreased Production of MMP9 by HN.49 Treatment We analyzed the expression level and enzyme activity of MMP9 in SaOS-2 cells treated with HN.49 or dimethyl sulfoxide (DMSO) by real-time RT-PCR and zymography analysis. Real-time RT-PCR analysis demonstrated that expression level of MMP9 mRNA in the cells treated with 3 μM and 10 μM of HN.49 polyamide were significantly lower than those treated with DMSO (Fig. 3A). Gelatinolytic activity of the cells treated with HN.49 were also decreased (Fig. 3B). Those result clearly showed that HN.49 polyamide could reduce the expression level of MMP9 transcript, resulting in the reduction of the enzyme activity of MMP9.

The Mobility and Invasion of SaOS-2 Was Decreased by HN.49 Treatment To evaluate the role of HN.49 in cell-migration ability and tumor-cell invasion, we treated SaOS-2 cells with various concentrations of the polyamide. In the wound-healing assay, cells treated with DMSO showed quick closing of the wound area within 48 h (Fig. 4A). In contrast, treatment with HN.49 inhibited the migration of those cells.

The matrigel invasion assay was performed by incubating SaOS-2 cells with various concentrations of polyamide for 48 h. We also analyzed the invasive ability of those cells treat-
ed with DMSO as a control. Cells treated with DMSO demonstrated extensive invasion through Matrigel-coated Transwell inserts. By contrast, HN.49-treated wells showed markedly less number of invasive cells (Fig. 4B).

**DISCUSSION**

While the 5-year survival rate for patients with osteosarcoma has improved with therapy combining 4 drugs, cisplatin, adriamycin, ifosfamide, and high-dose methotrexate, there has not been remarkable progress in chemotherapy in the past 20 years.\(^{22}\) Patients with no or a decreased response to chemotherapy have poor prognosis in the early stages mainly due to pulmonary metastasis. Under these circumstances, high expectations are placed on the development of new methods of chemotherapy for patients who do not show a favorable response, and therefore, up to the present, there have been some reports regarding potential drugs.\(^{23,24}\) Meyers \textit{et al.} conducted a prospective clinical study to examine the effectiveness of chemotherapy using pamidronate as a conventional bisphosphonates (BPs), in addition to cisplatin, doxorubicin, and methotrexate, involving 40 patients with osteosarcoma, and reported that this protocol resulted in the high durability of tumor-type prosthesis, without impairing the effect of the other 3 drugs.\(^{23}\) Furthermore, Xin \textit{et al.} treated osteosarcoma cell lines with risedronate as a third generation BPs \textit{in vitro},\(^{24}\) and suggested that tumor invasion may have been inhibited as a result of MMP2 and MMP9 activity inhibition, without fully clarifying the mechanisms in which risedronate inhibits MMP activity.\(^{24}\) Although BPs’ safety has been partly established, their anti-tumor effects still remain unclear. In line with this, the development of new drugs for primary malignant osteosarcoma should be required to improve the prognosis of the patients. MMP-9 is considered to be particularly good targets for anticancer drugs because it catalyzes type IV collagen, which is major components of the basement membrane including the blood vessel basement membrane. The expression of MMP-9 correlated with an aggressive, advanced invasive or metastatic tumor phenotype.\(^{25,26}\) Restricted MMP-9 activity was associated with fewer metastatic pulmonary lesions, whereas increased activity was associated with a high metastatic potential.\(^{27,28}\) Foukas \textit{et al.} reported that the survival rate of patients with stage IIB osteosarcoma significantly decreased when MMP9 expression was promoted; in such cases, the primary cause of death was pulmonary metastasis.\(^{29}\) This finding suggests the possibility of establishing anti-invasion and anti-metastatic effects on osteosarcoma by specifically inhibiting MMP9 expression. In addition, in clinical environments, these effects may improve survival and limb-salvage rates, as well

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**Fig. 3. Decreased Amounts of MMP9 Transcript and Enzymatic Activity in SaOS-2 Cells Treated by HN.49**

(A) Real-time RT-PCR analysis to determine MMP9 expression level. Cells were treated with or without various concentration of HN.49 for 48h, and the expression levels of MMP9 were determined. The numbers indicate the concentration of HN.49. Data are denoted as the mean±S.E. (n=3 for each group). p<0.05 (*). (B) Concentration-dependent decreased MMP9 activity by gelatin-based zymography of culture medium of tumor cells treated with polyamide. After treatment with various doses of polyamide for 48h, the culture media were estimated. Equal amounts of conditioned media were electrophoresed on 10% Zymogram (Gelatin) gel. The numbers on the panel indicate the concentration of HN.49, and the numbers under the panel indicate the relative density of the bands determined by LuminaVision software.
as the postoperative limb function, by preventing metastasis and reducing the range of invasion. With this in mind, we designed a conventional “non-alkylating” PI polyamide compound targeting the NF-κB binding site of human MMP9. In the present study, we found that the MMP9-inhibitor HN.49 polyamide could bind to NF-κB recognition site in the promoter sequence of MMP9, and also could inhibit the binding of NF-κB to this sequence. In addition, it was shown that HN.49 significantly reduced the expression level of MMP9 mRNA and enzyme activity of MMP9 in human osteosarcoma cell line SaOS-2. Furthermore, HN.49 significantly inhibited migration and invasion activity of SaOS-2 cells in vitro. We confirmed that HN.49 never affected the viability and growth rate of SaOS-2 cells (data not shown). Those results indicate that HN.49 has a potential to inhibit invasion and metastasis of tumor cells over expressing MMP9. Previously, our group reported that MMP9 inhibitor HN.50, which was designed to recognize to AP-1 binding site on MMP9 promoter, inhibited the expression level of MMP9, migration and invasion activity of breast and cervical cancer cell lines in vitro. Furthermore, HN.50 suppressed liver metastasis of human colon cancer cells injected to spleen of the nude mice, when the polyamide was applied intravenously. In preliminary experiments, HN.50 did not suppress the SaOS-2 cell migration effectively (data not shown). Those results also encourage us to develop another PI polyamide targeting for MMP9 as anti-cancer agent for tumors including osteosarcoma. At last we have found HN.49 targeting NF-κB binding site of MMP9 may be an attractive potential to be an anti-metastatic activity to osteosarcoma, although further investigation including in vivo analysis using the human osteosarcoma xenoplastic model are needed to confirm this hypothesis. We still don’t have enough data about the target specificity and side effects of HN.49. Since the recognition sequence of HN.49 is 7 base pair, there could be many other sites to which HN.49 could bind in whole genome. We have been trying to develop PI polyamides with longer recognition sequences. We believe these efforts will let us able to develop new therapeutic agents with higher selectivity and lower side-effect.

Fig. 4. HN.49 Treatment Reduces the Migration Activity and Invasiveness of SaOS-2 Cells in Vitro
(A) Confluent cell cultures were wounded with plastic micropipette tips (top). Cells were photographed 48h after wounding by phase contrast microscopy (bottom). The numbers indicate the concentration of HN.49. (B) The indicated cells were seeded on a Matrigel-coated membrane with or without polyamide. After 48h, the cells on the upper aspect of the filter were removed. The cells that had passed through the membranes were stained with a Diff-Quik kit and counted. The invasion was quantified as described in Materials and Methods. The numbers indicate the concentration of HN.49. Values are mean ±S.E. of three experiments. *p<0.05 (*).
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