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Shortened microsatellite d(CA)21 sequence down-regulates promoter activity of matrix metalloproteinase 9 gene

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Abstract One characteristic elements in the promoter of the matrix metalloproteinase 9 (MMP-9) gene is the d(CA) repeat. To investigate whether this element regulates the transcription of the MMP-9 gene and its enzymatic activities, we sequenced the promoter region isolated from esophageal carcinoma cell lines. TE9 cells with low MMP-9 enzymatic activity had the number of d(CA) repeats shortened from 21 to 14 or 18. TE8, TE10 and TE11 cells with high MMP-9 activities had 21 or 23 d(CA) repeats. Luciferase assays using MMP-9 promoter containing 18, 14 or 0 d(CA) repeats showed transcriptional activities which were 50, 50 or 5%, respectively, of the level achieved with promoter containing 21 d(CA) repeats. Sequence analysis of the promoter of 223 Japanese subjects revealed that most had two alleles with 20, 21 or 22 d(CA) repeats, whereas six had one or two alleles with 14, 18 or 19 d(CA) repeats. We postulate that length alteration of the d(CA) repeat causes phenotypic differences among carcinoma cells and that microsatellite instability may contribute to the polymorphism of d(CA) repeat length.

Key words: Microsatellite; d(CA) repeat; Matrix metalloproteinase 9; Gene transcription; Esophageal cancer

1. Introduction

Supercoiling of DNA plays an important regulatory role in replication, recombination and transcription. Environmental changes induce many non-B-DNA conformations which influence these processes [1–4]. One dramatic structural transition in DNA is that from a right-handed B-conformation to a left-handed Z-DNA structure [5], which is thought to be involved in gene regulation [6–12]. The most common structural transitions with the potential to form Z-structures involve sequences d(TG)n/d(CA)n, which are commonly found in many genes [7,8,13–17]. Recently, the chromosomal instability associated with d(CA)n repeats has received considerable interest and this phenomenon is frequently seen in neoplastic cells ([18–20], for review [21]). However, such mutations in d(CA) repeat length in the microsatellite DNA do not participate in chemically induced carcinogenesis [22].

We previously reported [23,24] that phenotypic differences exist among human esophageal squamous cell carcinoma cell lines (TE8, TE9, TE10, and TE11) based on our determinations of enzymatic activity of MMP-9 (also called gelatinase B and 92-kDa gelatinase), which is thought to play an important role in metastasis of carcinoma cells [25,26]. Of these cell lines examined, only the TE9 cells exhibited negligible MMP-9 enzymatic activity, even when stimulated by epidermal growth factor (EGF). The TE9 cell line was originally isolated from a poorly differentiated esophageal carcinoma, and does not require MMP-9 for its malignant properties. However, the other three cell lines, which had higher MMP-9 enzymatic activity, were isolated from well-differentiated carcinomas, and required MMP-9 for malignancy. Since the MMP-9 promoter includes d(CA) repeats which have the potential for microsatellite instability [27], we sought to determine the sequence of the promoter region of the MMP-9 gene that includes an unstable d(CA)21 repeat. This type of analysis would reveal whether there is any correlation between differences in MMP-9 enzymatic activities and regulation of MMP-9 gene expression by variations in d(CA) repeat length in the promoter.

In this study, we report that there are distinct differences in d(CA) repeat length in the promoter region of the MMP-9 gene isolated from TE8, TE9, TE10 and TE11 cell lines. Moreover, the number of d(CA) repeats correlates with the transcriptional activity of the promoter and the intrinsic MMP-9 enzyme activity of a particular cell line. We also discovered a d(CA) repeat polymorphism within the MMP-9 promoter in the Japanese population.

2. Materials and methods

2.1. Construction of reporter genes for luciferase assay

Two oligonucleotide primers selected from the sequences of the MMP-9 promoter region (5′-AATCCAGGACTTCGTGA and 3′-GAGGAATTTCGGGGGTGTTGT) and the genomic DNA as the template were used for the polymerase chain reaction (PCR) to obtain a 1868-bp DNA fragment of the MMP-9 promoter region (−1879 to −12 from the transcription start site) [27,28]. The DNA fragment was subcloned into pGEM-T Easy vector (Promega, Madison, WI, USA) and was sequenced (ALF Express, Pharmacia Biotech, Piscataway, NJ, USA). Mutated forms of the 1826-bp DNA fragment that contained 21, 18, 14 or 0 d(CA) repeats were amplified by PCR [29] and were cloned upstream of the luciferase gene in the pGL_3 basic vector (Promega, Madison, WI, USA) (Fig. 1).

2.2. Luciferase assay

TE9 cells (10^5 cells) suspended in PBS were transfected with 10 μg of each plasmid using electroporation (0.3 kV and 950 μF; Gene Pulser II, Bio-Rad, Hercules, CA, USA). The transfected cells were

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Abbreviations: MMP-9, matrix metalloproteinase 9; EGF, epidermal growth factor; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; EMSA, electrophoretic mobility shift assay

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cultured overnight in 12-well plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), and then exposed to EGF (PeproTech, London, UK) (50 ng/ml) for 30 h. Next the cells were lysed using a commercial luciferase assay buffer (Promega, Madison, WI, USA). Aliquots of 20 μl each of the supernatant was reacted with 470 mM luciferin solution (Toyo Inki Ind. Co., Tokyo, Japan). The intensity of the luminescence was measured with a luminometer (Bio-Orbit Oy, 1253 Luminometer, Turku, Finland).

2.3. Determination of number of d(CA) repeats

To determine the length of the d(CA) repeat in the MMP-9 promoter region, genomic DNA was isolated from the esophageal carcinoma cell lines and from peripheral blood cells, and subjected to PCR amplification. Two oligonucleotide primers (0.2 mM) 5′-TTGCCTGACTTGGCAGTGGAGACTGC-3′ (−210 to −192 nt) and 5′-TGTTGTGGGGGCTTTAAGGAG-3′ (−33 to −13 nt), based on the human MMP-9 gene sequences, were used for PCR in a reaction mixture (25 μl) containing 1×PCR buffer, 200 ng of template DNA, dNTPs (2.5 mM) and exTaq polymerase (Takara, Tokyo, Japan). After initial denaturing at 94°C for 5 min, PCR was performed for 35 cycles of denaturing at 94°C for 1 min, annealing at 60°C for 1 min and polymerase extension at 72°C for 1 min. The final polymerase extension was for 10 min at 72°C. The PCR products were cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) and were sequenced using fluorescent primers and an automated DNA sequencer (ALF Express, Pharmacia Biotech, Piscataway, NJ, USA). In addition, PCR products were also generated using a fluorescence-labeled forward primer (5′-ACTTGGCAGTGGAGACTGC-3′ corresponding to positions −208 to −185 nt) and the same 3′ end primer (−33 to −13 nt) as mentioned above, and were either sequenced (fluorescent PCR, [30]) or compared with DNA size markers to determine the length of the d(CA) repeats. For the latter method, an aliquot of each PCR product was denatured by heating and then quickly chilled on ice and fractionated on a 14% polyacrylamide gel containing 7 M urea. The DNA bands were visualized by silver staining and the length of the d(CA) repeat was estimated by comparison with DNA size markers [31].

2.4. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from cultured TE9 cells as previously described [32] and analyzed by EMSA as described by Tanimoto et al. [33]. Briefly, a 54-bp oligonucleotide, 5′-TGCCACACACACACACACACACACACACACACACACACACACACCCTGACCC-3′ (a synthetic d(CA)21 repeat (10 pmol) corresponding to nucleotides −93 to −85) was annealed to the primer, 5′-GGGTCAGGGT-3′ (100 pmol) and filled in by *Escherichia coli* Klenow DNA polymerase in the presence of dNTPs and [α-^{32}P]dCTP to obtain the labeled double-stranded DNA fragment. In the same way, probes for d(CA)14, d(CA)18, d(CA)20 and d(CA)22 repeats were made. The product size and the labeling efficiencies were verified by non-denaturing polyacrylamide gel electrophoresis (PAGE), and quantitated by scintillation counting. For EMSA, DNA probes (3.2–4.0×10^5 cpm/μl) and nuclear extracts were mixed in 20 μl of reaction buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, and 0.25 mg/ml poly(dI-dC) poly(dI-dC) and incubated for 30 min at room temperature. The DNA-protein complexes were separated on a 4% polyacrylamide gel (29:1) and detected by autoradiography.

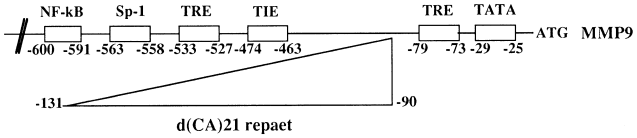

Fig. 1. Sequence of promoter region in the MMP-9 gene. Schematic presentation of *cis*-acting elements and d(CA)21 repeat sequence in the promoter region of the MMP-9 gene.

3. Results and discussion

3.1. Sequence of the MMP-9 gene promoter in carcinoma cells

In the MMP-9 producing TE10 cells, the promoter sequence was exactly the same as reported previously and the number of d(CA) repeats was 21 in both alleles (Figs. 1 and 3a) [28]. Sequence analysis of the MMP-9 promoter isolated from TE8 and TE11 cells showed the presence of d(CA)21 and d(CA)23 repeats. In contrast, sequence analysis of the promoter obtained from the MMP-9-low-producing TE9 cells revealed that the d(CA) repeats were shortened to 14 or 18 (corresponding to nucleotides −90 to −131) in one allele, whereas the other allele contained the normal d(CA)21 repeat length (Fig. 3a).

Since the number of d(CA) repeats determined by fluorescent PCR and silver staining was exactly the same as that determined by sequencing (Fig. 3a,b), most of the experiments described below were performed by the silver staining of fluorescent PCR product to determine the number of d(CA) repeats.

3.2. MMP-9 promoter activity by luciferase assay

The 1868-bp DNA fragment comprising the MMP-9 promoter/luciferase reporter constructs containing the d(CA) repeat was transfected to TE9 cells and the luciferase activities were determined. The results showed that the promoter activities were dependent on the length of the d(CA) repeat: the d(CA)18, d(CA)14 and d(CA)0repeats had 50%, 50% and 5% of the activity, respectively, obtained with the d(CA)21 repeats (Fig. 2a). This result indicated that reduction in MMP-9 gene expression in TE9 cells correlates with shortening in the d(CA) repeat length. Even the basal activities (in the absence of EGF) seen in TE9 cells transfected with d(CA)18, 14 and 0 repeats were much less than that of cells transfected with the construct containing d(CA)21 repeat. However, treatment by EGF still increased the transcriptional activity of the MMP-9 promoter 2.5-fold, compared with the basal activity in the absence of EGF treatment for each d(CA) repeat construct (Fig. 2b). Taken together, these findings indicate that the d(CA)21 repeats are required for basal activity of the

Table 1
Numbers of d(CA) repeats in 223 Japanese subjects

Number of CA repeats	14	15	16	17	18	19	20	21	22	23	Total
18	1				2						
19										1	
20							3				
21	1				1	1	18	91		4	
22							4	68	26	1	
23										1	
Subtotal	2				3	1	25	159	26	7	223
%	0.9				1.4	0.5	11.3	71.9	11.8	3.2	100

MMP-9 promoter. These results were reproducible in other tumor cell lines, HeLa and HT1080 cells (Fig. 2c,d), as well as TE8 and TE11 cells (data not shown). The TE10 cell line was not efficiently transfected by electroporation and therefore was not tested in this study.

Our previous study using TE9 cells [24] revealed that the MMP-9 enzymatic activity is negligible compared to the values in other cell lines, TE8, TE10 and TE11. However, transcriptional activity of the MMP-9 promoter containing the d(CA)14 repeats in TE9 cell line was only 50% less compared to the cell line containing the d(CA)21 repeats. Even if one allele with the d(CA)14 repeat is active, the TE-9 cells should have much higher MMP-9 expression and enzymatic activity. This suggests that the MMP-9 enzymatic activity in TE9 cells is regulated not only by transcription, but also at other steps, such as post-translational event, translation, or processing of pro-enzyme to active enzyme.

Tae et al. [15] reported that the d(CA)28 repeats located in the promoter region of the acetyl-CoA carboxylase gene suppressed approximately 70% of the basal promoter activity. This regulation of the promoter was dependent on the CCAAT box element and deletion of the d(CA)28 repeat stimulated transcription. In our study, the d(CA)21 repeat had the opposite effect; complete deletion of the d(CA)21 repeat strongly suppressed the promoter activity (to less than 10%) of the MMP-9 promoter. Interestingly, however, the MMP-9 promoter lacks the CCAAT box motif. Other reports have demonstrated that d(TG)n d(CA)n-containing Z-DNA located upstream of the rat prolactin gene, which lacks the CCAAT box motif, inhibits promoter activity, whereas the d(CA) repeat in the promoter region of the cytosolic phospholipase A2 gene does not affect promoter activity [16,34]. Therefore, the function of the d(CA) repeat seems variable among genes. Sato and Seiki reported that the d(CA)21 repeat does not activate transcription of the MMP-9 gene in HT1080 cells [28]; however, the vector constructs we used in the luciferase assay differed from theirs; we used 1826–1868 bp long DNA fragments of the 5′ flanking region that contained variations only in the d(CA)21 repeats, whereas they used short deletion mutants that lacked sequences that were upstream of the d(CA)21 repeat, as in other studies as well [15–17]. One possible explanation for the differences in the promoter activities is that the long 5′ flanking sequence deleted by Sato and Seiki contains other unknown *cis*-acting elements, which may play a specific DNA conformational role involving d(CA) repeats.

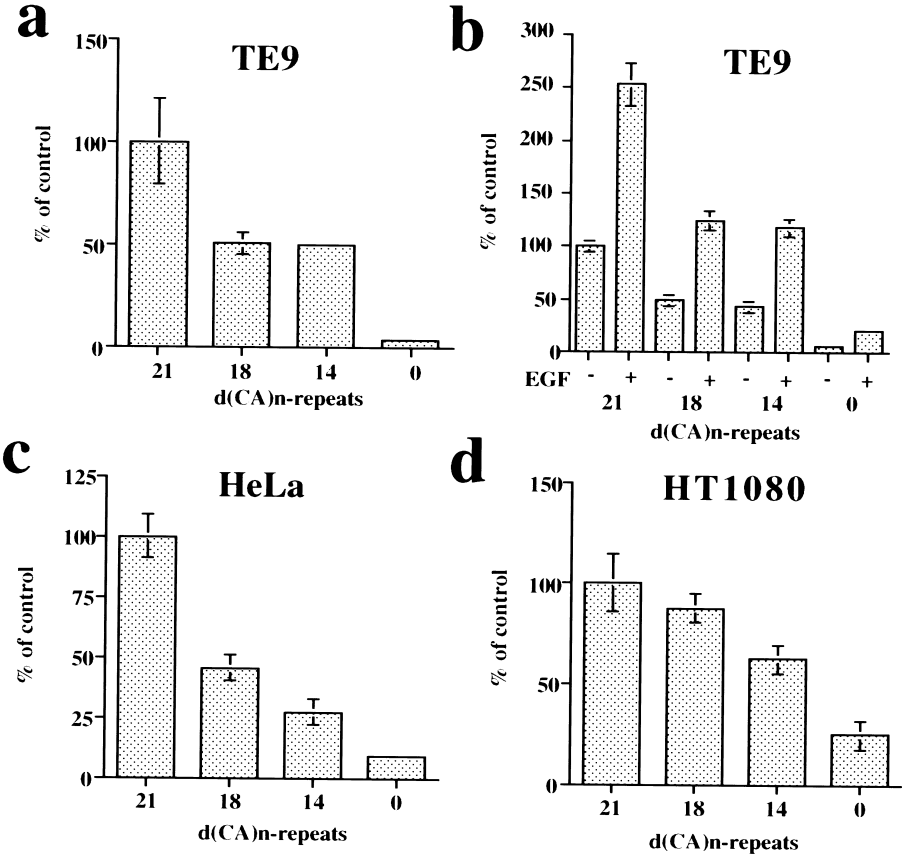

Fig. 2. Promoter activity of the MMP-9 gene correlates with the length of the d(CA) repeat. a: TE9 cells were transfected with pGL_3-basic vector containing the d(CA)21, d(CA)18, d(CA)13, or d(CA)0 repeat. When 1868-bp DNA including the d(CA)21 repeat was transfected into TE9 cells, maximal luciferase activity was observed, whereas when promoter/reporter plasmid of 1826–1862-bp DNA fragments containing d(CA)18, d(CA)13 and d(CA)0 repeat was used, the luciferase activities were significantly decreased compared with that of the d(CA)21 repeat. b: Effect of EGF (50 ng/ml) on luciferase activity in TE9 cells transfected with the mutant reporter plasmids. Although EGF still enhanced the promoter activity from d(CA)14, 18 and d(CA)0 promoter constructs, the activities were much less than that observed with the d(CA)21 repeat. c and d: HeLa and HT1080 cells transfected with the mutated luciferase vectors. The luciferase activities were calculated as percentage of the control (the value obtained with the d(CA)21 repeat in the absence of EGF = 100%).

3.3. Heterogeneity of the MMP-9 d(CA) repeat number in the Japanese population

Next, we asked whether the heterogeneity of the d(CA) repeat number observed among the carcinoma cell lines is common in genes isolated from individuals from the Japanese population. The number of d(CA) repeats was determined by fractionating PCR-amplified DNA fragments on denaturing polyacrylamide gel, followed by silver staining [31]. Some of the samples were also analyzed by sequencing and fluorescent PCR [30] to confirm the results of electrophoretic analysis. Most of the 223 Japanese people tested had two MMP-9 alleles that contained 20 or more d(CA) repeats. However, 2.8% of the people tested had one or two alleles containing less than 20 d(CA) repeat length (Table 1). Few people were homozygous in shortened repeat phenotype, and varied considerably among normal individuals. Therefore, the variation in d(CA) repeat length is not unique to carcinoma cells. The results are summarized in Table 1.

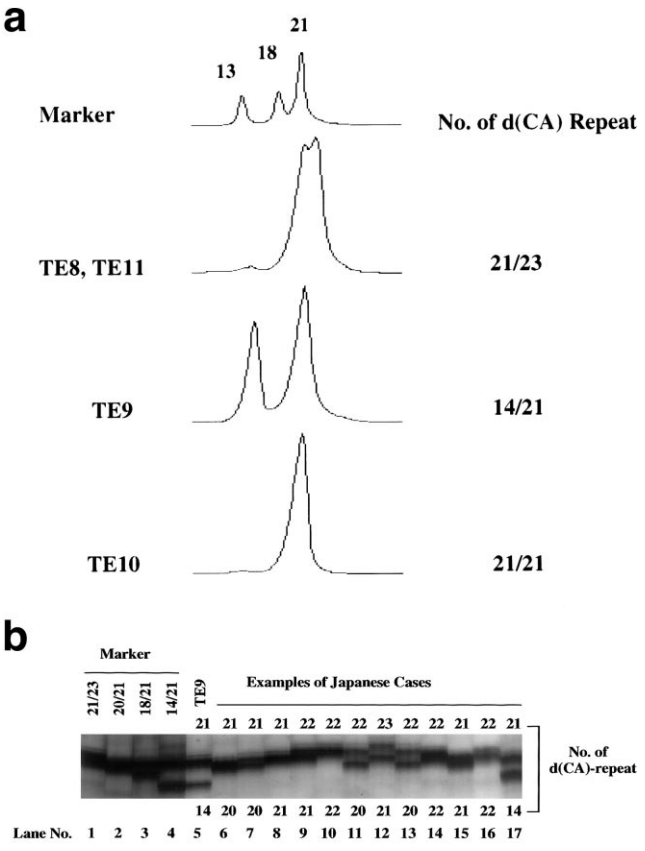

Fig. 3. Analysis of the number of d(CA) repeats. a: Analysis of d(CA) repeats analyzed by fluorescent PCR from carcinoma cell lines. The number of d(CA) repeats was determined from positions of peaks compared with those of DNA size markers. The numbers above the peaks in the marker lane represent the numbers of d(CA) repeats; 13: d(CA)13; 18: d(CA)18; 21: d(CA)21. b: Analysis of d(CA) repeats in the promoter region of genomic DNA isolated from peripheral blood of Japanese subjects. PCR products including d(CA) repeat sequences of approximately 198 bp were separated on denaturing polyacrylamide gel (14%) and were visualized by silver staining. Lanes 1–4: size marker; lane 5: PCR product from TE9 cell representing a combination of d(CA)21 and d(CA)14; lanes 6–17: PCR products from Japanese cases showing variations in d(CA) repeats.

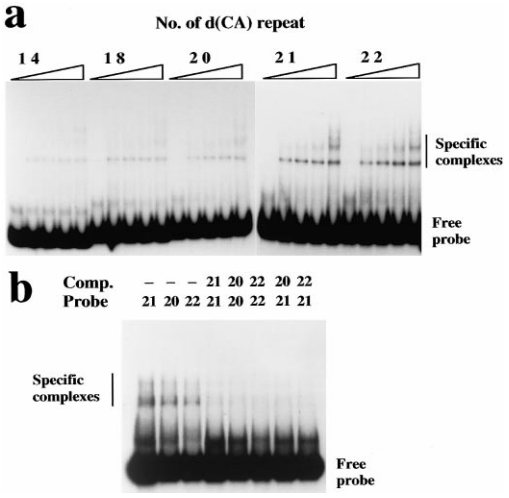

Fig. 4. EMSA from TE9 cell nuclear extract using synthetic d(CA) repeat probes. The nuclear extracts were incubated with each labeled synthetic d(CA)n probe (10^4 cpm/lane). The numbers represent probes of each d(CA) repeat length (14: d(CA)14; 18: d(CA)18; 20: d(CA)20; 21: d(CA)21; 22: d(CA)22). a: Each probe was incubated with increased amounts of the nuclear extracts (0, 2.5, 5, 10 and 20 μg/lane from left to right, shown as triangles). b: In competition studies, 5 μg/lane nuclear extract was used. Homologous and heterologous cold competitors (Comp.) were mixed in the reaction in 100-fold excess.

3.4. Presence of d(CA) repeat-binding protein(s) in nuclear extracts from TE9 cells

The TE9 cell nuclear extracts contained d(CA) repeat-binding proteins that formed specific complexes with all of the d(CA) repeat probes tested (Fig. 4a). This binding was completely abolished by a 100-fold excess of either the homologous cold competitors or heterologous competitors including different numbers of d(CA) repeats (Fig. 4b). The d(CA)21 probe showed a much higher binding affinity than the d(CA)14, d(CA)18, and d(CA)20 probes. These results are consistent with those of the luciferase assays, indicating that the number of d(CA) repeats correlates with the binding affinity of the nuclear protein(s) with the promoter and regulation of transcriptional activity.

We found that the length of the d(CA) repeat was closely related to the transcriptional activity of the MMP-9 promoter and that heterogeneity of the d(CA) repeat exists in the Japanese population. MMP-9 plays an important role in tumor growth, invasion and metastasis [25,26,35–37] and is essential for functions of many cell types, for example macrophages [38], lymphocytes [39] and trophoblasts [40]. In addition, development, embryogenesis, tissue remodeling and many pathologic conditions relate to the expression of MMP-9. We postulate that mutations of microsatellite DNA in the promoter region of MMP-9 may exhibit pleiotropic effects among different cell types and that microsatellite instability and/or variations exist among the general population.

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