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Haplotype Effects on Matrix Metalloproteinase-1 Gene Promoter Activity in Cancer Cells

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Abstract

Increased expression of matrix metalloproteinase-1 (MMP1) is associated with poor prognosis in cancers. Several single nucleotide polymorphisms (−1607GG>G, −839G>A, −755G>T, −519A>G, −422T>A, −340C>T, and 320C>T) in the MMP1 gene promoter have recently been identified. In this study, we assessed the functional effects of these polymorphisms on MMP1 gene promoter activity in cell lines of melanoma (A2058 and A375), breast cancer (MCF7 and MDA-MB-231), lung cancer (A549 and H69), and colorectal cancer (HT-29, SW-620) by comparing the promoter strengths of 10 most common haplotypes deriving from these polymorphisms. In A2058 cells, the GG-G-G-A-T-T-T and GG-G-G-A-C-T haplotypes had 2-fold higher promoter activity than the GG-G-T-A-T-T-C, GG-G-G-A-A-T-T, GG-G-G-A-T-T-C, and GG-G-G-A-A-C-T haplotypes, which in turn, had 3-fold higher promoter activity than the G-G-T-A-A-C-T, G-A-T-G-T-T-T, G-A-T-G-A-C-T, and G-A-T-G-A-T-G haplotypes. In A375 and MDA-MB-231 cells, high expression haplotypes include not only the −1607GG-bearing haplotypes but also the G-A-T-G-A-T-T haplotype containing the −1607G allele. A similar trend was detected in A549 cells. In addition, in A549 cells, the GG-G-G-A-T-T-T haplotype had >2-fold higher promoter activity than several other −1607GG-bearing haplotypes. In MCF7 cells, the GG-G-G-A-T-T-T and G-G-T-A-A-C-T haplotypes had 1.5- to 4-fold higher promoter activity than the other haplotypes. These results suggest that the polymorphisms exert haplotype effects on the transcriptional regulation of the MMP1 gene in cancer cells, and indicate a need to examine haplotypes rather than any single polymorphism in genetic epidemiologic studies of the MMP1 gene in cancers. (Mol Cancer Res 2007;5(3):221–7)

Introduction

There is substantial evidence indicating that matrix metalloproteinases (MMP) play important roles in cancer development and progression. This group of enzymes are capable of degrading various components of the extracellular matrix, and have long been associated with cancer cell invasion and metastasis, which entail breaking down the extracellular matrix barriers (1, 2). Recent studies have revealed that MMPs can also influence cancer cell proliferation and apoptosis, as well as the immune response against cancers (3).

Members of the MMP family have different substrate specificities and expression patterns. Matrix metalloproteinase-1 (MMP1, also called interstitial collagenase) is one of the few enzymes that can degrade native fibrillar interstitial collagens which are major constituents of the extracellular matrix (4). MMP1 is expressed at very low levels in normal adult tissues, but its expression is increased in tumors (5). The prognosis of patients with tumors expressing high levels of MMP1 is poorer than those with low expression (5-7). The expression of the MMP1 gene is under intricate transcriptional regulation, which involves a number of DNA elements in the promoter of the gene (8).

It has been shown that transcriptional regulation of the MMP1 gene is influenced by a single nucleotide polymorphism located at position −1607 (relative to the transcriptional start site) in the MMP1 gene promoter (9). The polymorphism is due to an insertion or deletion of one guanidine, resulting in one allele (referred to as −1607G or 1G) containing a sequence of GAA whereas another allele (referred to as −1607GG or 2G) had a GGAA sequence instead. The GGAA sequence in the −1607GG allele forms the core of a consensus DNA element recognized by the Ets transcription factor which up-regulates MMP1 transcription (9). A number of genetic epidemiologic studies have indicated that the polymorphism is associated with susceptibility to and/or invasiveness of various types of cancer, including cutaneous malignant melanoma, breast cancer, lung cancer, and colorectal cancer (10-15).

Studies in several other genes have provided a paradigm in which the transcription of a gene can be influenced by multiple polymorphisms located in the promoter of that gene which act in concert to exert haplotype effects (16, 17). We recently

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identified a number of polymorphisms in the MMP1 gene promoter (18). In the present study, we investigated whether these polymorphisms rendered haplotype effects on MMP1 gene promoter activity in cancer cells.

Results

Linkage Disequilibrium between the MMP1 Gene Promoter Polymorphisms and Frequencies of Haplotypes

We genotyped a group of 277 unrelated British Caucasian individuals for the −1607GG>G, −839G>A, −755G>T, −519A>G, −422T>A, −340C>T, and 320C>T polymorphisms in the MMP1 gene promoter, to assess linkage disequilibrium between the polymorphisms and to identify common haplotypes for subsequent promoter activity assays.

We detected substantial, but not complete, linkage disequilibrium between the polymorphisms (Fig. 1). Three of the above seven polymorphisms, i.e., −755G>T (rs498186), −422T>A (rs475007), and −340C>T (rs514921) have been studied in the international HapMap project, and have been shown to be located in a recombination hotspot (Fig. 2).

Haplotype analyses using the THESIAS, Haploview, and PHASE programs identified 45 different haplotypes deriving from the seven polymorphisms in the Caucasian subjects studied. The majority of these haplotypes had very low frequencies. We selected the 10 most common haplotypes (each with a frequency of >2.5%; Table 1) for promoter activity assays.

Haplotype Effects of MMP1 Gene Promoter Activity in Cancer Cells

We carried out transfection and luciferase assays of the 10 most common MMP1 promoter haplotypes in two melanoma cell lines (A2058 and A375), two breast cancer cell lines (MCF7 and MDA-MB-231), two lung cancer cell lines (A549 and H69), and two colorectal cancer cell lines (HT-29 and SW-620), as MMP1 gene variation had been associated with risk and/or metastasis of these cancers (10-15). In these experiments, for each of the 10 MMP1 haplotypes, the corresponding MMP1 promoter was cloned into the pGL3-basic vector. The cancer cells were transfected with these different constructs, followed by luciferase assays as detailed in Materials and Methods.

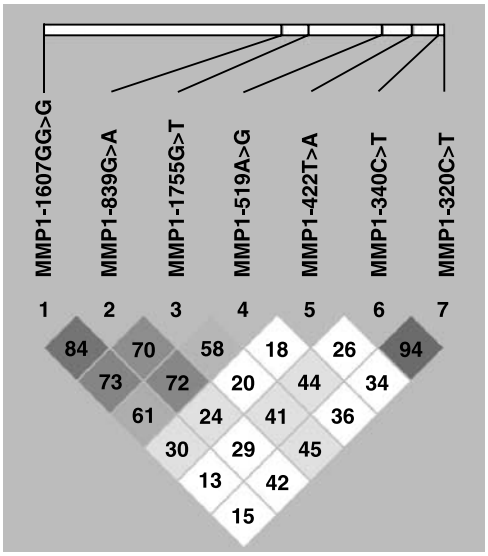

FIGURE 1. Linkage disequilibrium plot. Coefficients (*D'*) of pairwise linkage disequilibrium coefficients between the seven polymorphisms in the MMP1 gene promoter.

In A2058 cells, the haplotypes containing the −1607GG allele (GG-G-G-A-T-T-T, GG-G-G-A-T-C-T, GG-G-G-A-A-C-T, GG-G-T-A-T-T-C, GG-G-G-A-A-T-T, and GG-G-G-A-T-T-C) had 3- to 6-fold higher promoter activity than the haplotypes with the −1607G allele (G-A-T-G-A-T-T, G-A-T-G-A-C-T, G-A-T-G-T-T-T, G-G-T-A-A-C-T; Fig. 3). Among the −1607GG-containing haplotypes, the GG-G-G-A-T-T-T and GG-G-G-A-T-C-T haplotypes had ∼2-fold higher promoter activity than the GG-G-T-A-T-T-C, GG-G-G-A-A-T-T, GG-G-G-A-T-T-C, and GG-G-G-A-A-C-T haplotypes (Fig. 3).

In A375 cells, the −1607GG-bearing haplotypes had >2-fold higher promoter activity than the −1607G-bearing haplotypes, with the exception of the G-A-T-G-A-T-T haplotype (containing the −1607G allele), which had a similar promoter activity with the −1607GG-containing haplotypes (Fig. 3).

In MCF-7 cells, the G-G-T-A-A-C-T haplotype had the highest promoter activity, followed by the GG-G-G-A-T-T-T haplotype. The promoter strength of the remaining haplotypes was 1.5- to 4-fold lower than the G-G-T-A-A-C-T and GG-G-G-A-T-T-T haplotypes (Fig. 3).

The results in MDA-MB-231 cells were similar to those in A375 cells, with the haplotypes containing the −1607GG allele having at least 2-fold higher promoter activity than the haplotypes containing the −1607G allele, except the G-A-T-G-A-T-T haplotype, which had similar promoter activity to the 1607GG-containing haplotypes (Fig. 3).

In A549 cells, the GG-G-G-A-T-T-T haplotype had a significantly higher promoter activity than the other haplotypes including several −1607GG-bearing haplotypes (Fig. 3). In addition, there was a trend towards higher promoter activity possessed by the −1067GG-bearing haplotypes and the G-A-T-G-A-T-T haplotype, compared with the promoter activity of the other −1607G-bearing haplotypes (Fig. 3).

In H69, HT-29, and SW-620 cells, no significant difference was detected among the haplotypes. It was noted that in these three cell lines, the pGL3-basic vector produced similar luciferase levels to those produced by the constructs containing the MMP1 promoter (cloned into the pGL3-basic vector). This is in contrast with the A5028, A375, MCF7, MDA-MB-231, and A549 cells in which the levels of luciferase produced by the pGL3-basic vector were significantly lower than those produced by the constructs containing the MMP1 promoter.

Discussion

The main finding of our study was the presence of a haplotype effect on MMP1 gene promoter activity in cancer cells, which could not be fully explained by any one of the polymorphisms in isolation but seems to result from more than one functional polymorphism in the MMP1 gene promoter.

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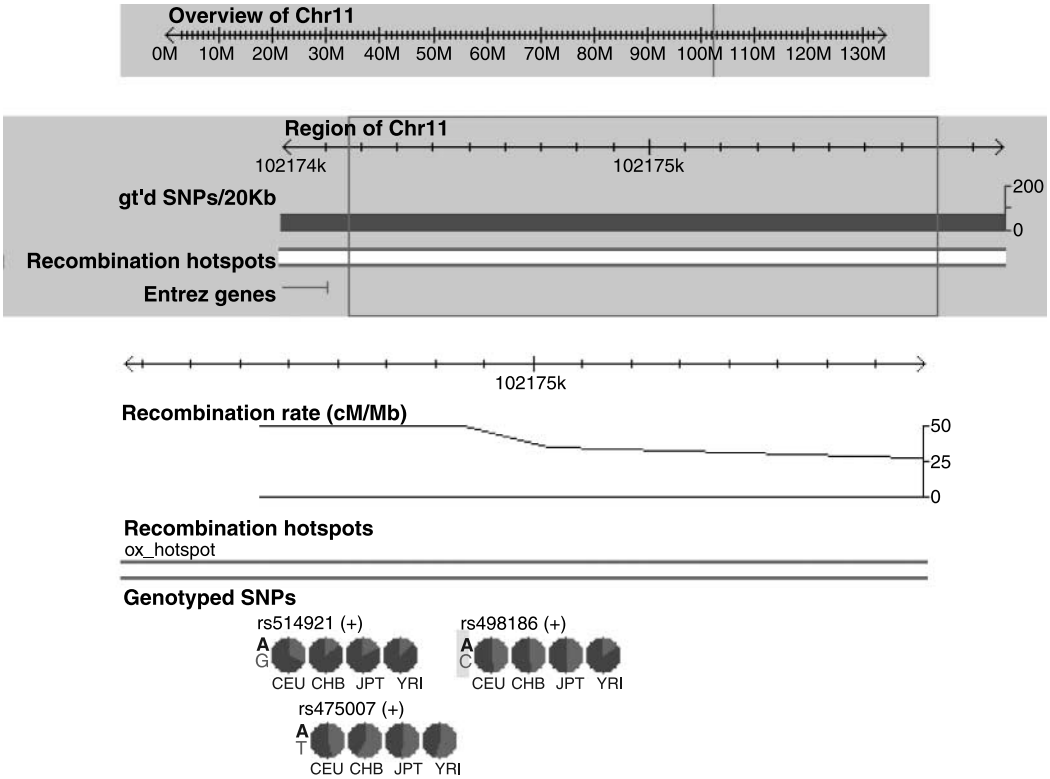

FIGURE 2. Recombination rates. Information obtained from the HapMap database showing a recombination hotspot around the MMP1 gene rs498186 (−755G>T) polymorphism (*shaded*).

In A2058 melanoma cells, all haplotypes containing the −1607GG allele had significantly higher promoter activity than the haplotypes with the −1607G allele, which is in concordance with results from previous studies on the functional effect of the −1607GG>G polymorphism, whereas the other MMP1 gene promoter polymorphisms had not been taken into account (9, 19). Furthermore, an important finding of our study is that in A2058 melanoma cells, there were significant differences among the six haplotypes that contain the −1607GG allele, such that the GG-G-G-A-T-T-T and GG-G-G-A-T-C-T haplotypes had significantly higher promoter activities than the GG-G-T-A-T-T-C, GG-G-G-A-A-T-T, GG-G-G-A-T-T-C, and GG-G-G-A-A-C-T haplotypes, indicating that MMP1 promoter activity is under the influence of not only the −1607GG>G polymorphism but also additional polymorphisms in the MMP1 gene promoter.

Table 1. Haplotype Frequency

Haplotype	Frequency		
	THESIAS	Haploview	PHASE
G-A-T-G-A-T-T	0.141	0.144	0.170
GG-G-G-A-T-T-T	0.112	0.116	0.129
G-A-T-G-T-T-T	0.105	0.103	0.092
GG-G-G-A-T-C-T	0.100	0.091	0.100
GG-G-T-A-T-T-C	0.060	0.059	0.065
GG-G-G-A-A-C-T	0.045	0.046	0.053
GG-G-G-A-A-T-T	0.039	0.036	0.042
G-G-T-A-A-C-T	0.032	0.032	0.031
GG-G-G-A-T-T-C	0.029	0.027	0.031
G-A-T-G-A-C-T	0.028	0.026	0.025

NOTE: The table shows the 10 most common haplotypes and their frequencies estimated using the THESIAS, Haploview, and PHASE programs. The haplotypes were derived from the −1602GG>G, −839G>A, −755G>T, −519A>G, −422T>A, −340C>T, and 320C>T polymorphisms, with G-A-T-G-A-T-T denoting a haplotype encompassing −1602G, −839A, −755T, −519G, −422A, −340T, and 320T; GG-G-G-A-T-T-T denoting a haplotype encompassing −1602GG, −839G, −755G, −519A, −422T, −340T, 320T, and so on.

The influence of multiple polymorphisms on MMP1 promoter activity was also detected in A375 melanoma cells, MCF7, and MDA-MB-231 breast cancer cells, and A549 lung cancer cells. In A375 and MDA-MB-231 cells, the high expression haplotypes included not only the −1607GG-bearing haplotypes but also the G-A-T-G-A-T-T haplotype, which has the −1607G allele. A similar trend was seen in A549 cells. In addition, in A549 cells, the GG-G-G-A-T-T-T haplotype had significantly higher promoter activities than most of the remaining −1607GG-bearing haplotypes. In MCF7 cells, we found that the GG-G-G-A-T-T-T and G-G-T-A-A-C-T haplotypes had significantly higher promoter activity than the other haplotypes, and among these other haplotypes, those encompassing the −1607GG allele generally had higher promoter activities than those containing the −1607G allele.

In H69, HT-29, and SW-620 cells, no significant difference was detected among the haplotypes. However, it was noted that in these three cell lines, the pGL3-basic vector produced similar luciferase levels to those produced by the constructs

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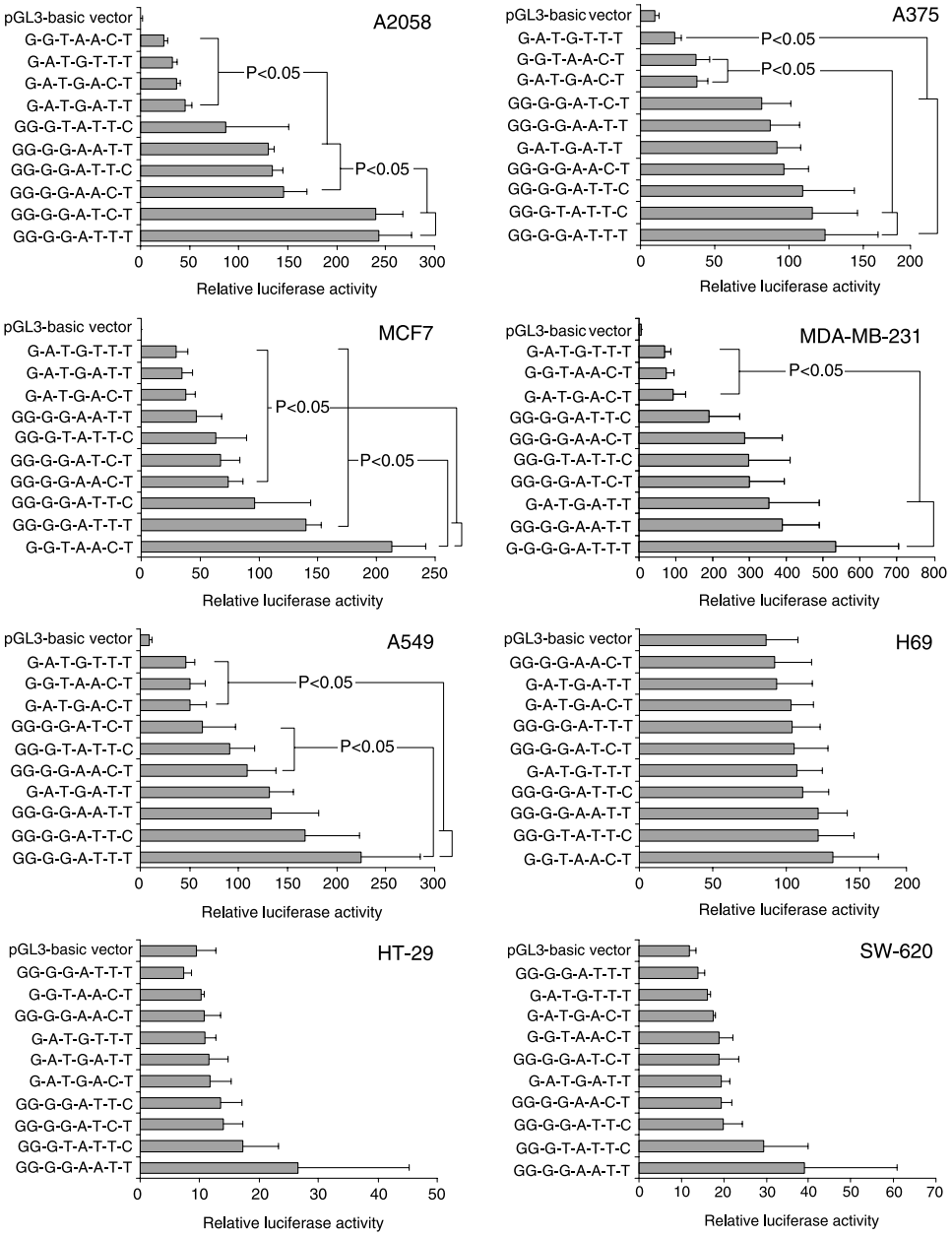

FIGURE 3. MMP1 gene promoter activity in cancer cells. Columns, relative transcriptional activity of the 10 different MMP1 promoter haplotypes in A2058 (melanoma), A375 (melanoma), MCF7 (breast cancer), MDA-MB-231 (breast cancer), A549 (lung cancer), H69 (lung cancer), HT-29 (colorectal cancer), and SW-620 (colorectal cancer) cells. Promoter activities were measured by luciferase assays as described in Materials and Methods. pGL3-basic vector columns, mean background luciferase expression levels from three independent experiments; bars, SD.

containing the MMP1 promoter. As the plasmid constructs used to transfect the cells were generated by cloning each of the MMP1 haplotypic promoter into the pGL3-basic vector, it is possible that the results in these three cell lines were confounded by the high levels of "leaky" expression of the luciferase reporter gene of the pGL3-basic vector. In contrast, in the A5028, A375, MCF7, MDA-MB-231, and A549 cells, the levels of luciferase produced by the pGL3-basic vector were significantly lower than those produced by the constructs containing the MMP1 promoter.

Overall, these results suggest that MMP1 promoter activity is dependent on the combinations of the −1607GG>G and other polymorphisms in the MMP1 gene promoter. Studies in the interleukin-6 and cholesteryl ester transfer protein genes have shown that there is functional cooperation/interaction between promoter polymorphisms in those genes (16, 17). It is possible that functional cooperation/interaction also occurs between the polymorphisms in the MMP1 gene promoter. In this study, it was noted that the GG-G-G-A-T-T-T haplotype had the highest promoter activity in most of the cell lines examined. The composition of this haplotype differs from the other haplotypes studied by having the combination of the −1607GG (or −839G or −519A, which are present in all six −1607GG-bearing haplotypes studied), −422T, −340T, and −320T alleles.

Our study showed that the haplotype effects on MMP1 promoter activity differ among some of the cancer cell lines studied, suggesting a cell line–dependent effect. The haplotype

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effects differ in some cell lines of the same types of cancer (e.g., between A5058 and A375 cells of melanoma, and between MCF7 and MDA-MB-231 cells of breast cancer), suggesting that the haplotype effects are not dependent on cancer types, but might be related to the biological variability among the different cancer cell lines. The haplotype effects in these cancer cell lines are also different from the haplotype effect in THP1 monocyte/macrophage cells examined in our previous study (18). Cell type–specific effects of polymorphisms on gene promoter activity have been reported for other genes, e.g., the MMP2, MMP8, and interleukin-6 genes (16, 20, 21). As the effects of functional polymorphisms in gene promoters probably arose from influences on binding of transcription factors, cell type–specific effects of promoter polymorphisms might reflect the differences in the repertoires of transcriptional factors and their activity in different types of cell. A database search for transcription factor binding sites showed that the sequences at the MMP1 promoter polymorphic sites have high degrees of similarity with various consensus elements recognized by transcription factors (Table 2). Although these in silico findings have not been confirmed by biological experiments, they do point to the possibility that some of the polymorphisms might have effects on transcription factor binding.

Our study provides functional data supporting the rationale for studying haplotypes, rather than a single polymorphism, in genetic epidemiology studies. To date, most genetic epidemiology studies of MMP1 gene variation in relation to cancers have focused on the −1607GG>G polymorphism (10-15). The results of our study indicate a need for genotyping additional polymorphisms in the MMP1 gene promoter and undertaking haplotype analysis, as typing the −1607GG>G polymorphism alone cannot fully segregate the various MMP1 haplotypes that differ in promoter activity. Our study showed that the degree of linkage disequilibrium between the polymorphisms in the MMP1 gene promoter was substantially lower than the degree of linkage disequilibrium between polymorphisms in some other genes, for example, the MMP3 gene (22). In the MMP3 gene, four out of the five polymorphisms located in the promoter region were in almost complete linkage disequilibrium, and accordingly, there are only three major haplotypes (22). These three haplotypes can be distinguished by genotyping just two polymorphisms, i.e., −1612 5A>6A and −709A>G (22). In contrast, in the MMP1 gene, in which linkage disequilibrium between polymorphisms was substantially weaker, to partition the different haplotypes would require the genotyping of five of the seven polymorphisms, e.g., −1607GG>G, −755G>T, −422T>A, −340C>T, and 320C>T. The low degree of linkage disequilibrium between the MMP1 gene polymorphisms likely reflects the presence of recombination hotspots at this genomic locus. Further studies will be required to determine the MMP1 haplotypes and their frequencies in other populations.

In summary, this study shows that the MMP1 promoter polymorphisms exert haplotype effects on MMP1 promoter activity in cancer cells. These results indicate a need to examine haplotypes, rather than a single polymorphism, in genetic epidemiologic studies of the MMP1 gene in cancers.

Materials and Methods

Determination of Linkage Disequilibrium between Polymorphisms and Identification of Common Haplotypes

A group of 277 unrelated British Caucasian individuals were genotyped for the following polymorphisms in the MMP1 gene promoter: −1607GG>G, −839G>A, −755G>T, −519A>G, −422T>A, −340C>T, and 320C>T. For each polymorphism, a DNA sequence containing the polymorphic site was amplified by PCR and the amplicons were digested with an appropriate restriction endonuclease which specifically cleaved one of the two alleles. Digests were subjected to gel electrophoresis, and DNA was detected by poststaining of the gel with Vistra Green and visualized using a fluorimager to determine genotypes.

The THESIAS program, which implements the Stochastic-EM (expectation-maximization) algorithm (23); the Haploview program, which uses an accelerated EM algorithm (24); and the PHASE version 2.1 program, which employs a Bayesian

Table 2. Results of *In silico* Search for Putative Transcription Factor Binding Sites Using the Genomatix SNPinspector Program

Position	Allele	Transcription factor	Strand	Core similarity	Matrix similarity
−1602	GG	Ecotropic viral integration site 1 encoded factor, amino-terminal zinc finger domain	(−)	1.000	0.958
	G	GATA-binding factor 3	(−)	1.000	0.958
		Brn-2, POU-III protein class	(−)	0.933	0.886
−839	G	PAX-5 B cell–specific activator protein	(−)	1.000	0.733
	A	PAX-1	(+)	0.750	0.626
		Thyrotrophic embryonic factor/hepatic leukemia factor	(+)	0.784	0.802
−755	G	Neuron-specific olfactory factor	(+)	1.000	0.900
	T	—			
−519	A	Pancreas transcription factor 1, heterotrimeric transcription factor	(+)	0.857	0.791
	G	Nuclear factor 1	(−)	1.000	0.987
−422	T	Myoblast determining factor	(−)	1.000	0.983
		PAX-3 binding site	(+)	0.780	0.779
		Atp1a1 regulatory element binding factor 6	(+)	1.000	0.985
	A	—			
−340	C	Hepatic nuclear factor 4	(+)	0.750	0.762
	T	Abd-B–like homeodomain protein Hoxb-9	(+)	1.000	0.940
−320	C	—			
	T	Grainyhead-like 3	(+)	1.000	0.879

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method (25, 26), were used to examine the presence and extent of linkage disequilibrium between the polymorphisms, and to determine the frequencies of haplotypes derived from the polymorphisms.

Reporter Gene Construct Production

For each of the 10 most common haplotypes derived from the above polymorphisms, a 1.9 kb DNA fragment of the MMP1 gene promoter was generated by PCR in which DNA from an individual carrying that haplotype was used as a template. Each of the 10 amplicons were first cloned into the pCR-Blunt II-TOPO vector (Invitrogen, San Diego, CA) and then subcloned into the pGL3-basic vector (Promega, Madison, WI) with the MMP1 gene promoter being placed upstream of the firefly luciferase reporter gene. All constructs were sequenced to verify that the cloned MMP1 gene promoter was in the desired orientation and free from misincorporation of nucleotide during the PCR.

Cell Culture

The A2058, A375, MCF7, MDA-MB-231, A549, H69 cell lines were obtained from the European Collection of Cell Cultures. HT-29 and SW-620 cells were kindly given by Dr. Simon Joel, Institute of Cancer, Barts and The London School of Medicine, London, United Kingdom.

A2058, A375, A549, and SW-620 cells were cultured in DMEM containing 4.5 g/L glucose and 1.5 g/L sodium bicarbonate, supplemented with 4 mmol/L of L-glutamine, 10% heat-inactivated fetal bovine serum, 100 μg/mL of streptomycin and 100 units/mL of penicillin. MCF7 cells were cultivated in Eagle's minimal essential medium with Earle's balanced salt solution supplemented with 2 mmol/L of L-glutamine, 1.0 mmol/L of sodium pyruvate, 0.1 mmol/L of nonessential amino acids, 10% heat-inactivated fetal bovine serum, 100 μg/mL of streptomycin, and 100 units/mL of penicillin. The MDA-MB-231 breast cancer cell line was cultured in Leibovitz's L15 medium supplemented with 2 mmol/L of L-glutamine, 1.0 mmol/L of sodium pyruvate, 0.1 mmol/L of nonessential amino acids, 10% heat-inactivated fetal bovine serum, 100 μg/mL of streptomycin, and 100 units/mL of penicillin. H69 and HT-29 cells were propagated in RPMI 1640 with 2 mmol/L of L-glutamine adjusted to contain 1.5 g/L of sodium bicarbonate, 4.5 g/L of glucose, 10 mmol/L of HEPES, 1.0 mmol/L of sodium pyruvate, 10% fetal bovine serum, 100 μg/mL of streptomycin, and 100 units/mL of penicillin. All cells were cultured at 37°C with 5% CO_2, except MDA-MB-231 cells, which were incubated in 100% air at 37°C.

Transient Transfection and Luciferase Reporter Assay

Each of the above cell lines was transfected with each of the reporter gene constructs described above, the promoterless pGL3-basic vector plasmid (to serve as a reference for background luciferase expression level), or the pGL3-control plasmid (containing a SV40 promoter resulting in strong expression of luciferase in mammalian cells). Transfection of A2058, A375, MCF7, A549, HT-29, and SW-620 cells was carried out with the use of FuGene 6 (Roche, Basel, Switzerland), whereas transfection of MDA-MB-231 and H69 cells was done with the use of Effectene (Qiagen, Chatsworth, CA). The pRL-TK (Promega) plasmid which contains a *renilla* luciferase gene was cotransfected into the cells to serve as a reference for transfection efficiency. The activities of firefly luciferase and *renilla* luciferase in the transfected cells were measured using a dual-luciferase assay kit (Promega) with a Glomax luminometer (Turner BioSystems, Sunnyvale, CA). MMP1 promoter activity was measured as the ratio of firefly luciferase activity of MMP1 promoter construct versus *renilla* luciferase activity, divided by the ratio of firefly luciferase activity of pGL3-control plasmid versus *renilla* luciferase activity. The mean (and SE) from at least three independent experiments are presented.

Acknowledgments

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