# Absence of a Correlation between the Presence of a Single Nucleotide Polymorphism in the Matrix Metalloproteinase 1 Promoter and Outcome in Patients of Chondrosarcoma

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## ABSTRACT

***Purpose:*** **Increased levels of matrix metalloproteinase 1 (MMP-1) expression have been associated with poor outcome in chondrosarcoma. The existence of a single nucleotide polymorphism creating an Ets-binding site in the MMP-1 promoter may be one mechanism for elevated MMP-1 transcription. The aim of our study was to identify the prevalence of this single nucleotide polymorphism (SNP) in chondrosarcoma patients, to determine its correlation with disease outcome, and to discern whether it could serve as a prognostic marker in patients with chondrosarcoma.**

***Experimental Design:*** **Sixty-seven chondrosarcoma specimens were selected sequentially from an established tumor bank with a median duration of 47 months follow-up (range, 24 to 179 months). DNA was extracted, amplified with PCR, and sequenced to determine presence (GG) or absence of the Ets-binding site created by the SNP.**

***Results:*** **Eighteen (27%) samples were homozygous for the absence of the Ets site, 34 (51%) were heterozygous for the SNP, and 15 (22%) were homozygous for the SNP. The 5-year overall survival rate for patients was 78, 80, and 84%, respectively ($P = 0.5527$). The disease-free survival rate was 16, 63, and 76%, respectively ($P = 0.0801$). The 5-year disease-free survival rate for patients with the homozygous G/G genotype was 16%, compared with 71% for patients who were either homozygous or heterozygous for the GG allele ($P = 0.0444$).**

***Conclusions:*** **Despite a statistical correlation between MMP-1 gene expression and outcome in chondrosarcoma, this study demonstrates an absence of a correlation between the presence of the SNP and prognosis in patients with chondrosarcoma.**

## INTRODUCTION

Chondrosarcoma is a malignant primary bone tumor that does not respond to current chemotherapy or radiation treatment, and wide surgical resection remains the primary mode of treatment. Histologic grading, which is subjective, is the current standard to predict prognosis of human chondrosarcoma. An objective form of prognostication would be of clinical value and could serve as the basis of a novel therapeutic intervention.

Matrix metalloproteinases (MMPs) play an important role in the modeling and remodeling of the extracellular matrix in both the normal and diseased physiologic states. It has been demonstrated that MMPs can contribute to the processes of tumor invasion and metastasis by influencing the capacity of tumor cells to transverse tissue boundaries (1). It has been previously shown that patients with high levels of interstitial collagenase (MMP-1) expression have poorer outcome, especially in esophageal, colorectal, and melanoma tumors (2–4). Several studies indicate that MMP activity or expression could be a useful parameter for determining prognosis in patients with chondrosarcoma (5, 6). Increased levels of MMP-1 expression were found in patients with recurrence, as compared with chondrosarcoma patients that remained disease-free (7, 8). Thus, increased MMP-1 expression may denote more aggressive tumor activity and dissemination that is facilitated by extracellular matrix degradation and a complex sequence of biological signaling that ensues (9).

Many molecules that induce MMP-1, such as nuclear factor-κB, tumor necrosis factor α, and transforming growth factor β, require the activator protein 1 (AP-1) site to exert their influence and implicate c-fos and c-jun in MMP transcriptional regulation (10–12). The level of MMP-1 expression, and hence, its potential to mediate connective tissue degradation and tumor progression, can be influenced by a genetic variation in the MMP-1 promoter (13–15). One proposed mechanism that could augment MMP-1 expression is the presence of an Ets transcription factor-binding site that results from the addition of a single guanine base at −1607 bp in the MMP-1 promoter sequence adjacent to an AP-1 site (15). At this loci, either a single guanine or a double quanine is found with a sequence of 5′-AAAGAT-3′ or 5′-AAAGGAT-3′. Sequencing of the promoter in human leukocytes from genomic libraries revealed that 30% have a homozygous double guanine genotype at this locus. The addition of a second guanine base 5′-AAAGGAT-3′ results in the creation of the triplet 5′-GGA-3′, which constitutes a consensus sequence for an Ets-binding site (PEA-3) upstream from the

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AP-1–binding site (16). This single nucleotide polymorphism (SNP), located within a PEA3 site, has been termed an oncogene-responsive unit and has been correlated with prognosis in several human malignant tumors, including esophageal, colorectal, and melanoma (2–4, 17). The resultant interaction of these two sites and the synergistic effect of Ets- and AP-1–binding factors lead to increased levels of expression on the MMP-1 gene in ovarian carcinoma (18, 19), colorectal carcinoma (20–22), lung carcinoma (23), endometrial carcinoma (24), as well as melanoma (25). If the presence of the Ets-binding site correlates with the level of MMP-1 expression in chondrosarcoma, this could provide the basis for objective prognostication and possibly a novel therapeutic target.

The aim of our study was to identify the prevalence of this SNP in the tumor tissue of chondrosarcoma patients to determine the correlation with disease outcome.

## MATERIALS AND METHODS

Sixty-seven tumor specimens, obtained during resections from January 1980 through July 2001, were selected sequentially from an established chondrosarcoma tumor bank at the Mayo Clinic Foundation. The selection criteria included available follow-up records on the patients, details of the operative and adjuvant therapy, and appropriate processing of the resected specimen. Approval from the Institutional Review Board was obtained. The patient group consisted of 22 females and 45 males and ranged in age from 16 to 87 years (mean, 54 years). The three-tiered histologic grading system, described by Lichtenstein and Jaffe (26), was used. Cytologically, increased cellularity and cytological atypia are the most important features, and these characteristics are used to determine the grade of the chondrosarcoma. The patients were followed for a minimum of 2 years or until death. The median duration of

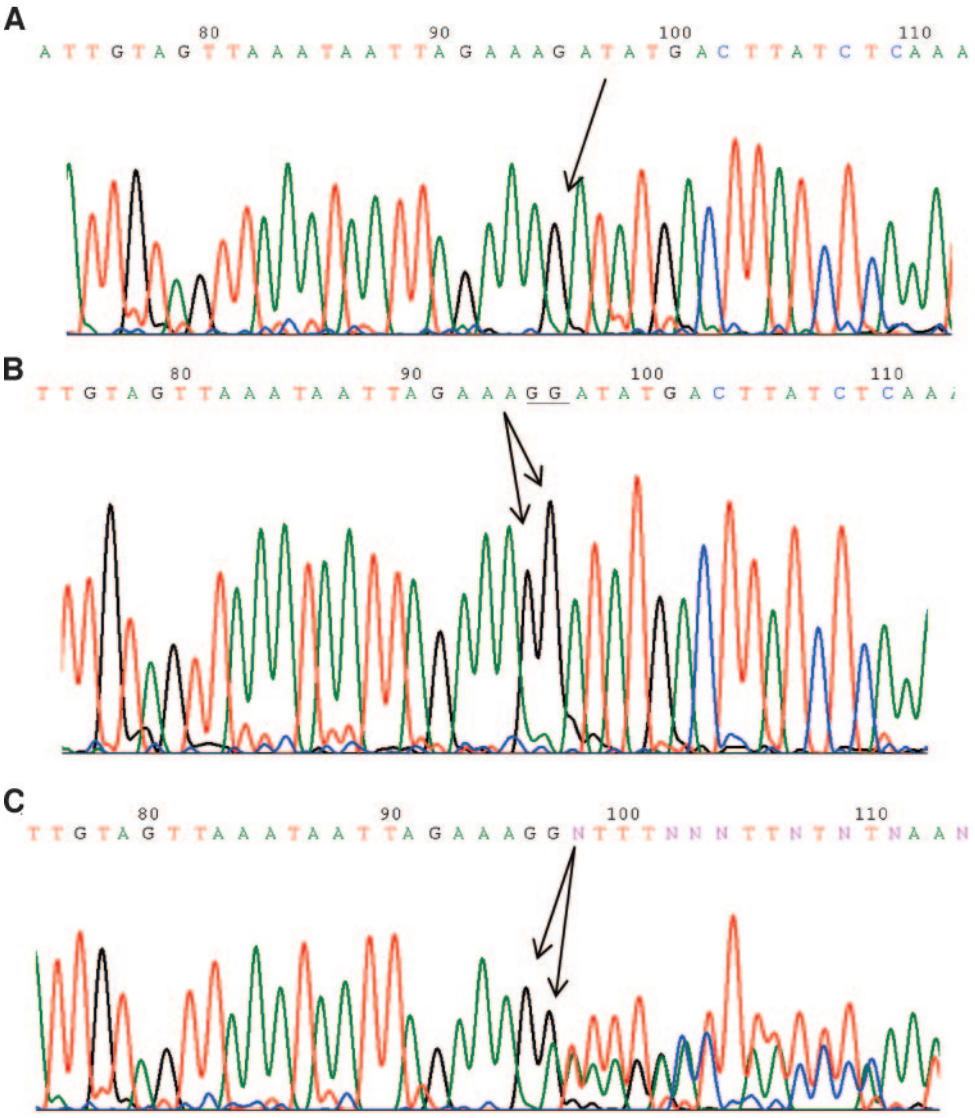


*Fig. 1* *A*, homozygous G/G genotype with single G peak and clean sequence. *B*, homozygous GG/GG genotype with double G peak and clean sequence. *C*, heterozygous G/GG genotype with double G peak and uninterpretable sequence.

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*Table 1* The table shows the frequency of patient characteristics with different MMP-1 promoter SNP genotypes

| | | G/G | | G/GG | | GG/GG | | |
|---|---|---|---|---|---|---|---|---|
| Variables | Category | *N* | % | *N* | % | *N* | % | Mantel-Haenszel $\chi^2$, *P* |
| Gender | Female | 6 | 33 | 11 | 32 | 5 | 33 | 0.9956 |
| | Male | 12 | 67 | 23 | 68 | 10 | 67 | |
| Age (y) | <50 | 12 | 67 | 19 | 56 | 6 | 40 | 0.1305 |
| | ≥50 | 6 | 33 | 15 | 44 | 9 | 60 | |
| Tumor grade | 1 | 7 | 39 | 18 | 53 | 10 | 67 | 0.0813 |
| | 2 | 7 | 39 | 12 | 35 | 4 | 27 | |
| | 3 | 4 | 22 | 4 | 12 | 1 | 7 | |

follow-up of the living patients was 47 months (range, 24 to 179 months).

The DNA was extracted from tumor tissue using a commercial kit (Pure Gene, Gentra, Minneapolis, MN). PCR primers and sequencing primers were designed with the aid of a commercial program (Gene Fisher, California Polytechnic State University, San Luis Obispo, CA). Primers for PCR amplification were as follows: sense, 5′-GAGTCACTTCAGTGGCAA-3′; and antisense, 5′-TGTCTTGGGTACTGGTGA-3′. PCR conditions were optimized to produce only the single band of interest with the FastTAQ PCR kit (Roche, Indianapolis, IN). The reaction conditions were as follows: 250 ng of DNA, 200 μmol/L deoxynucleotide triphosphate, 3.75 mmol/L $MgCl_2$, 0.4 μmol/L of each primer, and 1.25 units of TaqDNA polymerase. PCR reactions were denatured for 5 minutes at 94°C, amplified for 25 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 45 seconds at 72°C, followed by a 7-minute extension at 72°C.

The amplification product was run on a 2% agarose gel with ethidium bromide to confirm the presence of a single 470-bp PCR product. The PCR products were purified with a QIAquick PCR Purification kit (Qiagen, Valencia, CA), sent to the Molecular Biology Core Facility (Mayo Clinic Foundation, Rochester, MN) for preparation of the sequencing reactions with a Big Dye Terminator v3.0 Cycle Sequencing kit with Amplitaq DNA polymerase, FS (Applied Biosystems, Foster City, CA), and evaluated with an Applied Biosystems 3730 DNA analyzer. DNA sequencing was performed to identify the presence (GG) or absence (G) of the SNP at the bp of interest, as well as to determine whether the tumor sample genotype was heterozygous (G/GG) or homozygous (GG/GG) for the SNP. Sequences that were homozygous for either the single guanine (G/G; Fig. 1*A*) or the double guanine (GG/GG; Fig. 1*B*) have a clear sequence downstream from the locus of interest. The G/GG heterozygous sequence (Fig. 1*C*) was not interpretable downstream of the SNP locus as a result of the overlapping sequence created by the insertion of the guanine base in one allele. The PCR amplicon was sequenced in both directions with forward (5′-CTGATGCCTCTGAGAAGAGGAT-3′) and reverse (5′-CACTTCAGCACCTTATGGTGT-3′) sequencing primers to confirm the identity of the SNP at −1607 bp in the MMP-1 promoter. Having concurrent sequence in both directions virtually eliminates the possibility of compression artifacts because compression does not typically occur at the same site on both strands. Template concentration was titered, and only sequences with optimal peak amplitudes were used for genotype determination.

**Sample Size and Data Analysis.** This is a retrospective study in which 67 tumor specimens were collected during the study timeframe. This population has an 81% power to detect a 35% difference in 5-year survival using the log-rank statistics with α level < 0.05. Statistical correlation of SNP genotypes with gender, age, and tumor grade was determined using Mantel-Haenszel $\chi^2$ analysis for categorical variables. The estimated rates of overall survival and disease-free survival (neither local recurrence nor metastasis) were calculated according to the Kaplan Meier method (27). Log-rank statistics were used to compare survival differences between groups. The Cox proportional hazard model was adopted to perform the multivariate analysis to ascertain the independent prognostic parameters. *P* values < 0.05 were considered statistically significant.

## RESULTS

At the time of last clinical follow-up, 36 patients (54%) were alive without disease, 15 (22%) were alive with disease, 4 (6%) were dead of other causes without disease, and 12 (18%) had died of the disease. Histologic evaluation by experienced musculoskeletal pathologists indicated 35 patients had a grade 1 lesion, 23 patients a grade 2 lesion, and 9 patients a grade 3 lesion. Amplification of the MMP-1 promoter and sequencing indicated that 18 (27%) samples were G/G homozygous without

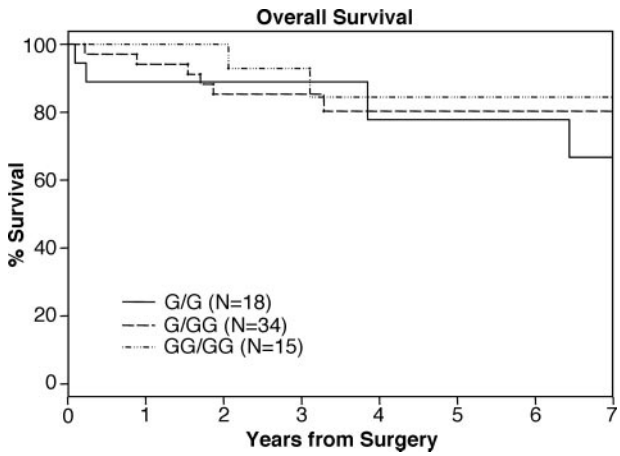


*Fig. 2* Kaplan-Meier curve for the overall survival rate stratified by the MMP-1 promoter SNP genotype. Overall two-sided *P* = 0.5527. Comparison of the G/G *versus* G/GG genotypes produced *P* = 0.8564. Comparison of G/G with GG/GG genotypes produced *P* = 0.2887.

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the SNP, 34 (51%) were G/GG heterozygous for the SNP, and 15 (22%) were GG/GG homozygous for the SNP.

The patient characteristics for each genotype of the MMP-1 promoter are presented (Table 1). The genotype had no correlation identified with gender, age, or tumor grade. The 5-year overall survival rate for G/G homozygous patients was 78%, compared with 80 and 84% in G/GG heterozygous and GG/GG homozygous patients, respectively ($P = 0.5527$; Fig. 2). The local recurrence rate for patients with the G/G genotype was 50%, compared with 26.47 and 26.67% in patients with the G/GG and GG/GG genotypes, respectively ($P = 0.1338$; Table 2). The metastatic rate for patients with the G/G genotype was 33.33%, compared with 23.53 and 0% in patients with the G/GG and GG/GG genotypes, respectively ($P = 0.0221$). The disease-free survival rate of patients with the G/G genotype was 6%, compared with 63 and 76% in patients with the G/GG and GG/GG genotypes, respectively ($P = 0.0801$; Fig. 3). There is not a significant difference in overall survival or disease-free survival between the three genotypes.

Statistical evaluation of overall and disease-free survival rates was performed after grouping patients who possessed the GG allele into one group (G/GG + GG/GG) and comparing it to the patients who were homozygous for single G allele. The 5-year overall survival rate for patients with the G/G allele was 78%, compared with 81% in patients with the GG allele ($P = 0.5465$). The 5-year disease-free survival rate for patients with the homozygous G/G genotype was 16%, compared with 71% in patients with the GG allele ($P = 0.0444$; Fig. 4). The prognosis for disease-free survival in patients with the GG allele was better than those homozygous for the single G.

*Table 2* Comparison of local recurrence and metastasis rates between different MMP-1 promoter SNP genotypes

| | G/G ($N = 18$) | G/GG ($N = 34$) | GG/GG ($N = 15$) | $P$ |
|---|---|---|---|---|
| Local recurrence rate | 9/18 (50%) | 9/34 (26.47%) | 4/15 (26.67%) | 0.1338 |
| Metastatic rate | 6/18 (33.33%) | 8/34 (23.53%) | 0/15 (0%) | 0.0221 |

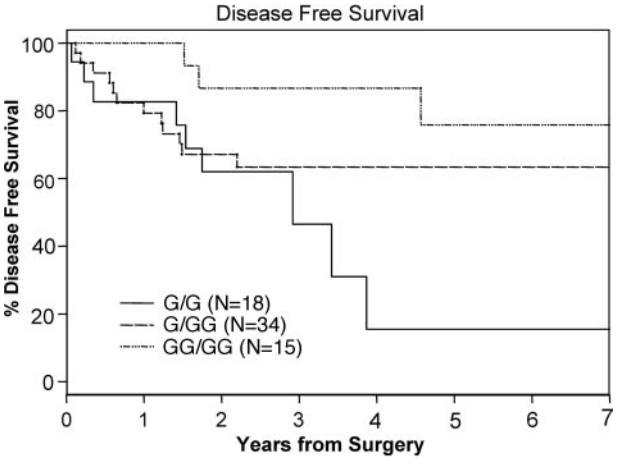


*Fig. 3* Kaplan-Meier curve illustrating the disease-free survival rate of different MMP-1 promoter SNP genotypes. Overall two-sided $P = 0.0801$. Comparison of the G/G *versus* G/GG genotypes produced $P = 0.2200$. Comparison of the G/G *versus* GG/GG genotypes produced $P = 0.0090$.

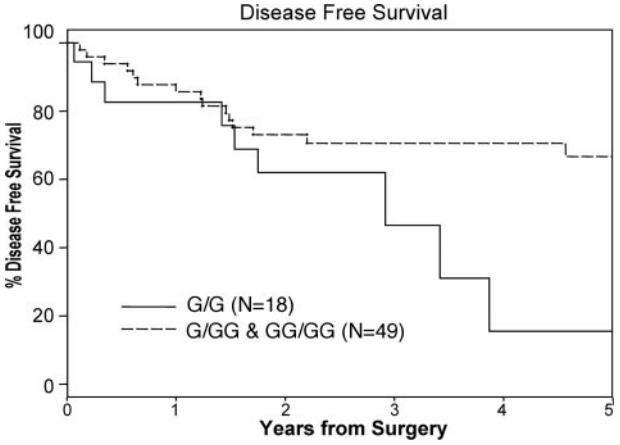


*Fig. 4* Kaplan-Meier curve demonstrating a significant difference in the disease-free survival rates when comparing the G/G genotype group to a combined group containing both G/GG and GG/GG genotypes. Overall two-sided $P = 0.044$.

A Cox proportional hazard model was constructed to determine statistically significant independent prognostic variables (Table 3). Gender, age, and the presence of the SNP did not reach statistical significance as independent prognostic factors. The tumor grade was the most dominant prognostic variable of disease-free or overall survival.

## DISCUSSION

The SNP located at −1607 bp in the MMP-1 promoter creates an Ets-binding site and has been reported to contribute to significantly higher transcription in normal fibroblasts and melanoma cells (15). The SNP has been proposed as a mechanism for elevating MMP-1 gene expression and for facilitating tumor progression by mediating enhanced degradation of the interstitial matrix. The mechanism mediating the increase in MMP-1 expression by the SNP has not been elucidated. In chondrosarcoma, esophageal cancer, colorectal carcinoma, and melanoma, patients with increased levels of MMP-1 expression have poorer outcome with tumor invasion and metastasis (2–4, 7, 8, 17).

It has been reported that melanoma, ovarian, and endometrial carcinoma tissue from patients carrying the GG allele contain higher levels of MMP-1 transcripts compared with those from patients not carrying this allele (18, 24, 25). Rutter *et al.* (15) presented that the SNP was not a mutation because the frequency of genotyping of 100 control individuals indicated that the distribution of this SNP in the normal population is approximately: 31%, G/G homozygous; 30%, GG/GG homozygous; and 39%, G/GG heterozygous. In contrast, in eight tumor cell lines, the frequency of GG/GG genotype increased to 62.5% (15). In the present study, the genotype frequency of G/GG heterozygotes and GG/GG homozygotes was 51 and 22%, respectively. The genotype frequencies for the MMP-1 promoter SNP in chondrosarcoma tumor tissue were similar to reported

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*Table 3* Cox proportional hazard model analysis of independent prognostic variables.

| Variable | Parameter | Estimated hazard ratio with 95% hazard ratio confidence interval | *P* |
|---|---|---|---|
| Overall survival | | | |
| Male | 0.54935 | 1.732 (0.536–5.597) | 0.3586 |
| Age ≥50 years | 0.85424 | 2.35 (0.765–7.218) | 0.1357 |
| G/GG | 0.52031 | 1.683 (0.496–5.705) | 0.4036 |
| GG/GG | −0.40257 | 0.669 (0.115–3.898) | 0.6545 |
| Grade 2 | 1.72675 | 5.622 (1.106–28.571) | 0.0374 |
| Grade 3 | 3.06471 | 21.428 (3.881–118.325) | 0.0004 |
| Disease-free survival | | | |
| Male | −0.03475 | 0.966 (0.407–2.294) | 0.9373 |
| Age ≥50 years | 0.01654 | 1.017 (0.453–2.282) | 0.9680 |
| G/GG | −0.13442 | 0.874 (0.357–2.138) | 0.7683 |
| GG/GG | −0.80352 | 0.448 (0.133–1.505) | 0.1939 |
| Grade 2 | 1.64165 | 5.164 (1.786–14.931) | 0.0024 |
| Grade 3 | 2.24818 | 9.470 (2.786–32.197) | 0.0003 |

allele frequencies for control groups and melanoma patients but were different from reported allele frequencies for ovarian and endometrial carcinoma patients, as well as tumor cell lines (Table 4). This suggests that this is a somatic rather than a tumor induced genotype.

The current study did not identify a correlation between the presence of SNP in the MMP-1 promoter and prognosis in patients with chondrosarcoma. This is despite the fact that MMP-1 expression has been correlated with outcome in this disease process. To our knowledge, this is the first report of an absence of relevance of this SNP in prognosis in patients with malignancy. There was no significant difference in overall survival ($P = 0.5527$) or disease-free survival ($P = 0.0801$) in patients with the three different genotypes at −1607 bp. Comparison of outcomes, stratifying by genotype within each tumor grade, was hampered by small sample sizes. A significant difference does exist with an inverse correlation between the presence of the SNP and disease-free survival ($P = 0.0444$). Such an observation could be explained by steric inhibition at the AP-1 site by Ets transcription factors (28). Incorporating tumor grade into the Cox proportional hazard model for overall survival or disease-free survival indicated this was not independently statistically significant. These findings indicate that the SNP is not an independent prognosticator of outcome in patients with chondrosarcoma.

The mitogen-activated protein kinase signaling pathway regulates MMP-1 gene expression by activating cofactors that interact with AP-1 and polyoma-enhancing activity–3/E26 virus (PEA3/Ets) transcription factor-binding sites located within the promoter region. The inhibition of Fra-1 expression, an AP-1 transcription factor component, preferentially down-regulates transcription from the MMP-1 promoter DNA containing the GG SNP allele, compared with DNA contain the single G allele in melanoma cells (29, 30). Ets transcription factors can positively and negatively activate transcription by interaction with coregulatory-binding partners or by regulating phosphorylation (28, 29, 31). This suggests that Ets family proteins and partner proteins may differ in various cell types (32). Alternatively, other pathways regulating MMP-1 expression may act independently of the SNP at −1607 bp (33).

Although various human tumor tissues have demonstrated coexpression of Ets factor and MMPs, there was no correlation between Ets expression and metastasis in pancreatic and thyroid carcinoma (34, 35). Many unresolved issues of Ets function still remain, and additional investigation will be required (36). Transcriptional regulation is a complex process that is often influenced by tissue-specific factors. Therefore, it is possible that MMP-1 transcriptional regulation is not directly increased by the presence of this Ets-binding site in chondrosarcoma tumors because it has been demonstrated to be in epithelial tumors. This suggests that the gene expression secondary to this SNP is tissue specific and varies functionally between different disease processes (14).

*Table 4* Major series in different tumor patients reporting the frequency of each genotype of the MMP-1 promoter

| | | | Genotype (% of total number tested) | | |
|---|---|---|---|---|---|
| | | *N* = | G/G | GG/GG | G/GG |
| Rutter *et al.* (15) | Controls | 100 | 31 | 30 | 39 |
| | Tumor cell lines | 8 | 12.5 | 62.5 | 25 |
| Kanamori *et al.* (18) | Controls | 150 | 20 | 43 | 37 |
| | Ovarian cancer | 163 | 11 | 37 | 52 |
| Nishioka *et al.* (24) | | | | | |
| | Endometrial carcinoma | 100 | 9 | 50 | 41 |
| Ye *et al.* (25) | Controls | 142 | 29 | 23 | 48 |
| | Cutaneous malignant melanoma | 139 | 24 | 29 | 47 |
| Present study | Chondrosarcoma | 67 | 27 | 22 | 51 |

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The histologic determination of tumor grade and subsequent clinical course of chondrosarcoma is subjective in nature, and more objective methods have been unsuccessfully sought to assess prognosis (37). Although the current study reveals that SNP is not an independent prognostic parameter to predict the outcome of patients with chondrosarcoma, the mechanism regulating MMP-1 gene expression remains an attractive therapeutic target. Additional elucidation of the roles and mechanisms of MMP-1 expression in chondrosarcoma may lead to development of novel therapeutic strategies.

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# Absence of a Correlation between the Presence of a Single Nucleotide Polymorphism in the Matrix Metalloproteinase 1 Promoter and Outcome in Patients of Chondrosarcoma

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