MMP-3 gene polymorphisms and Osteosarcoma

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Received 24 January 2014; revised 08 June 2015

Osteosarcoma (OSA) is the most common adolescence cancer among all primary bone tumors next only to multiple myeloma. It has a substantially worse prognosis and ability to metastasize to lung. MMPs (matrix metalloproteinases) are among the major proteases that take part in regulation of ECM (extracellular matrix). MMPs play an active role in the formation of the osteoid tissue, rich in collagens and other ECM proteoglycans. They also take part in pro-osteoclast, osteoclast, osteoblast, and osteoid formation. Many members of the MMP gene family have been linked to human cancers. It has been shown that MMPs particularly play a role in the tumor’s acquisition of an invasive and metastatic character. In our study, the E45K and T102T polymorphisms of MMP-3 were studied using the PCR-RFLP method in 135 Turkish subjects (54 subjects with osteosarcoma and 81 healthy controls). We found that frequencies of E45K G allele (p:0,010, χ²:6,710, OR:1,429, 95%Cl:1,019-1,858) and AG genotype (p:0,001, χ²:14,753, OR:2,32, 95%Cl:1,491-3,626) were elevated in patients compared to controls. Besides, there was a significant difference in E45K AA genotype between study groups (p:0,004, χ²:8,182, OR:2,929, 95%Cl:1,38-6,19). There were no significant differences between any genotypes or allele in the control and patient groups for MMP-3 T102T polymorphism. Our findings indicate that the G allele and AG genotype of MMP-3 E45K polymorphism is associated with increased risk of osteosarcoma in adolescent population of Turkey.

Keywords: Adolescents, Cancer, E45K, Matrix Metalloproteinases, OSA, T102T

Osteosarcoma (OSA) is the most prevalent primary bone malignancy in humans. It is the third most prevalent cancer among adolescents and accounts for more than 56% of all bone tumors. It is estimated that it has an annual incidence of 4 per 1 million people with a bimodal age distribution, with two peaks, at 15-19 years and 70 years¹. OSA is a highly metastatic disease with poor overall survival. Lungs are the primary targets of the metastatic disease such that 80% of all metastases involve these organs, particularly the peripheral portions²,³. In metastatic OSAs, resistance to chemotherapy is common as revealed by the 5-year survival rate, <20%⁴,⁵. In non-metastatic OSAs, both chemotherapy and surgical therapy can be applied. In these cases the long-term survival rate does not usually go beyond 70%⁶.

Osteosarcoma possesses a complex karyotype while recurrent translocations are absent. The disease has been linked to a few certain genes having a potential role in the development and progression of the disease⁷,⁸. A series of trials conducted by Yang et al.⁹-¹¹ concluded that the alterations of gene expression of 1162 genes and 142 deletions play a role in the pathogenesis of osteosarcoma.

The extracellular matrix (ECM) hosts some structural molecules (proteins, proteoglycans, polysaccharides) as well as some enzymes, both being secreted by certain cells forming a 3-dimensional macromolecule network specific to different tissues, in a way to create cellular microenvironments or niches¹²,¹³. In case the regulation of ECM remodeling is lost, tissue integrity is jeopardized, leading to development of pathological processes including connective tissue disorders, cancer, and metastasis (tumor microenvironment)¹⁴,¹⁵. Matrix metalloproteinases (MMPs), an enzyme family that provides the ECM remodeling, are responsible for degradation of ECM elements. Biologically, MMPs have been linked to ECM degradation and turnover¹⁶,¹⁷. Although certain MMPs are expressed in bone and cartilage tissue during the normal bone development, MMPs -2, -9, -13, -14, and -16 have an important role in skeletal development, as shown by knockout mice models and human genetic diseases¹⁸,¹⁹.

Wu et al.²⁰ determined the differences in expression of the candidate genes and the protein levels in...
normal tissue and osteosarcoma. They analyzed the data with three separate statistical methods and demonstrated an interaction between the collagen formation (COL1A2 and COL5A2) and MMP-3 in osteosarcoma cells. By binding to integrins, connective tissue growth factor (CTGF) regulates the invasiveness of some human cancer cells. Studies have shown that in overexpressed CTGF levels MMP-3 and MMP-2 levels are upregulated and they boost the migratory ability of osteosarcoma cells. Both in vitro and case/control studies have provided strong evidence that MMP-2 and MMP-9 genes play a role in osteosarcoma development.

Duivenvoorden et al. examined cancer cells with bone metastasis in cancerous cell lines and showed that TGF-beta 1 altered the activities of MMPs and TIMPs. They reported that an altered MMP activity impaired the fine balance in osteoclast and osteoblast development, leading to a bone malignancy. De bart et al. studied osteosarcoma cell lines and reported that MMPs may have a role in development of osteosarcoma.

Although earlier in vitro and in vivo studies have demonstrated MMPs' effects in osteosarcoma development, no studies to date have been conducted on MMP-3 polymorphism in osteosarcoma. MMP-3 E45K (rs679620) polymorphism forms 82 residual cleavage points on the pro-MMP-3 protein sequences, activating the MMP-3 enzyme. MMP-3 T102T (rs41380244), on the other hand, has a functional importance. Here, we conducted a study in a Turkish patient population to show the relationship of the E45K and T102T polymorphisms of MMP-3 with osteosarcoma.

Materials and Methods

Patients

The study group consisted of 54 patients (24 females and 30 males) with a diagnosis of osteosarcoma (mean age: 23.60±8.61). The diagnoses were established with histological examination in all cases. The control group consisted of 81 healthy individuals (mean age: 27.03±8.97; 50 females and 31 males) with a negative family history of neoplasia. After obtaining written informed consent from the participants and approval from the Istanbul University’s Ethics Committee, blood specimens were collected in tubes containing ethylenediaminetetraacetic acid (EDTA). DNA was extracted from peripheral blood lymphocytes using the salting-out procedure.

Genotyping

Polymorphisms were genotyped using polymerase chain reaction (PCR)/restriction fragment length polymorphism (RFLP) methods. PCR was used to amplify the region of MMP-3 E45K polymorphism and the MMP-3 T102T polymorphism. PCR Primers were designed by using Primer 3 Input (version 0.4.0). Reactions were performed with 10 pmol of each primer: F:5’-AAATTTGCCCATTATTTCAGCAAG-3’, R:5’-CCCCCTCTGAACCATTACCTG-3’ for E45K polymorphism and F:5’-GTTCCITGGATGGAGGTGA-3’, R:5’-CTGTTAGGAAAAATTGAAGCA-3’ for T102T polymorphism. Template DNA (0.5-1.0 μg) was used in a PCR under stringent conditions to avoid the possibility of false positives for genotyping. Reactions were performed with 10 pmol of each primer in final volume of 50 μL containing 25 mM MgCl₂, 10 mM Tris-HCl (pH 8.4), 2mM of each dNTP and 5 unit Taq Polymerase. Amplification was carried out in a DNA Thermal for 35 cycles with denaturation steps at 94°C for 30 s, annealing at 59°C for 30 s and extension at 72°C for 30 s for E45K polymorphism and for 35 cycles with denaturation steps at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 30 s for T102T polymorphism. Restriction enzymes were determined by using NEBcutter V2.0 programme. For E45K genotypes, presence of the polymorphisms was determined by enzymatic digestion of the initial PCR product with TaqI at 60°C for 3 h. Three genotypes could be determined after electrophoresis: genotype uncut (399 bp), cut (236, 163 bp) and heterozygoyt (399, 236, 163 bp). For T102T genotypes, presence of the polymorphisms was determined by enzymatic digestion of the initial PCR product with AclI at 37°C for 3 h. Three genotypes could be determined after electrophoresis: genotype uncut (237 bp), cut (139, 98 bp) and heterozygoyt (237, 139, 98 bp). Some of the samples selected randomly were also subjecting to sequencing for validation. The results were reproducible with no discrepancy of genotyping.

Statistical analysis

Allele and genotype frequencies were determined by direct counting. Categorical variables such as genotypes and alleles were compared using Chi-Square (χ²) test. Differences in continuous variables between carriers and control subjects were tested using Student’s t test. Statistical analyses were performed with SPSS 7.5 software (SPSS Inc, Chicago, USA).
Results and Discussion

We did not observe any significant difference between the study groups according to ages (P >0.05). Table-1 shows the E45K and T102T genotypes in study groups. We observed that frequencies of E45K G allele (p:0.010, χ²:6.710, OR:1.429, 95% CI:1.019-1.858) and AG genotype (p:0.001, χ²:14.753, OR:2.32, 95% CI:1.491-3.626) were elevated in patients compared to controls. Besides, a significant difference was observed in E45K AA genotype between the study groups (p:0.004, χ²:8.182, OR:2.929, 95% Cl:1.019-1.858). The AG genotype forms a significantly more common in osteosarcoma compared to the control group (p:0.001, χ²:14.753, OR:2.32, 95%CI:1.491-3.626). Similarly, the G allele frequency of MMP-3 T102T polymorphism was significantly more common in osteosarcoma patients compared to the control (p:0.001, χ²:14.753, OR:2.32, 95%CI:1.491-3.626). There was no significant difference between any genotypes or allele in the control and patient groups for MMP-3 T102T polymorphism.

In this study, we explored the role of MMP-3 E45K and MMP-3 T102T polymorphisms in the pathogenesis of osteosarcoma in 54 patients with osteosarcoma and 81 healthy controls. Our statistical analyses showed that the AG genotype in MMP-3 E45K polymorphism was significantly more common in osteosarcoma patients compared to the control group (p:0.001, χ²:14.753, OR:2.32, 95%CI:1.491-3.626). Similarly, the G allele frequency of MMP-3 E45K was significantly higher in osteosarcoma compared to the control group (p:0.010, χ²:6.710, OR:1.429, 95% CI:1.019-1.858). No significant relationship was found between the genotypes of MMP-3 T102 T polymorphism and the patient/control group.

Table 1—Distribution of MMP-3 E45K and T102T genotypes and alleles

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>MMP-3 Patients (n=54)</th>
<th>Control (n=81)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E45K</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>14 (25.9%)</td>
<td>41(50.6%)**</td>
</tr>
<tr>
<td>AA</td>
<td>9 (16.7%)</td>
<td>20(24.7%)</td>
</tr>
<tr>
<td>AG</td>
<td>31(57.4%)***</td>
<td>20(24.7%)</td>
</tr>
<tr>
<td>Alleles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>59(54.6%)</td>
<td>102(62.9%)</td>
</tr>
<tr>
<td>G</td>
<td>49(45.4%)*</td>
<td>60 (37.1%)</td>
</tr>
<tr>
<td>MMP-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotypes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>45 (83.3%)</td>
<td>65 (80.2%)</td>
</tr>
<tr>
<td>CC</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>CG</td>
<td>9 (16.7%)</td>
<td>16 (19.8%)</td>
</tr>
<tr>
<td>Alleles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>99(91.6%)</td>
<td>146(90.12%)</td>
</tr>
<tr>
<td>G</td>
<td>9(8.4%)</td>
<td>16(9.88%)</td>
</tr>
</tbody>
</table>

**n: number of subjects**

Significant p values= *p<0.010, χ²:6.710, OR:1.429, 95%CI:1.019-1.858**; **p<0.004, χ²:8.182, OR:2.929, 95%CI:1.019-1.858**; ***p<0.001, χ²:14.753, OR:2.32, 95%CI:1.491-3.626

Osteosarcoma (OSA) ranks first among the primary malignant bone tumors in young individuals and second after multiple myeloma among all primary bone tumors. The incidence of osteosarcoma is increased in the presence of various genetic factors and conditions, such as retinoblastoma, Li-Fraumeni syndrome, Rothmund-Thomson syndrome (poikiloderma congenitale), prior radiation therapy, alterations in the p53, RD, and MDM2 oncogenes, and heterozygosity for chromosomes 3q, 13q, and 18q. Response to chemotherapy is poor among osteosarcomas that are >30% chondroblastic or osteoblastic and well differentiated. Furthermore, it has been reported that a chondroblastic subtype of the tumor have a shorter metastasis-free survival. Matrix metalloproteinase (MMP) family proteins have a wide array of functions in cellular biology, including osteoblastic differentiation. Studies examining the role MMP-10 that belongs to the same subfamily as MMP-3 in the differentiation of osteoblasts in mice myoblastic cell lines have provided important insight into heterotopic bone development. Collagen VI is a component of extracellular matrix found in many tumor types, which has an important role in tumor development. Thanks to the native alpha3 chain of collagen VI, it has the ability to bind to MMP-11 that belongs to the same group as MMP-3 and certain studies in 6 Saos (Sarcoma osteogenic) cell lines have reported that it also has a role in malignant progression as MMP-11 does.

Barthelmi et al. showed that MMP-13 and MMP-2 play an active role in proteolytic cascade in human osteoblast cultures. However, they could not find any conclusive evidence for MMP-3.

Our literature search yielded only a few functional, experimental, and animal studies examining the role of MMP-3 in the pathogenesis of osteosarcoma. To note, no studies have yet studied the genetic variants of MMP-3 in osteosarcoma development. As the first of its kind, our study demonstrated that the AG genotype of the MMP-3 E45K variant was significantly more common in osteosarcoma than the control group (p:0.001, χ²:14.753, OR:2.32, 95% CI:1.491-3.626). The AG genotype forms a cleavage point at the 82nd residue on the pro-MMP-3 protein sequence, and thus activates MMP-3 enzyme and causes protein maturation. Rearrangement of ECM (Extracellular Matrix) by increased MMP-3 protein may provide osteoblasts with a malignant character and an enhanced ability to migrate.
Conclusion
A comparison of the AG genotype with gene expressions at the tissue level may be an important target for future research. Studies on the genetic variation, gene expression levels, and immunohistochemical properties of MMP-10 and MMP-11, i.e. the members of the same MMP subfamily, are of utmost significance for clarifying the pathogenesis of this disease. Cutting the pro-MMP-3 plays a critical role in the activation and maturation of MMP-3. Our study has clearly demonstrated that the MP3 E45K variant is associated with the development of osteosarcoma.

Acknowledgement
The present work was supported by the Research Fund of Istanbul University. Project No. 19358. “Done Genetics and Bioinformatics Limited Co.” did sequencing analysis.

Competing Interests
The authors declare that they have no competing interest.

References
25. Duivenvoorden WC, Hirte HW & Singh G, Transforming growth factor beta acts as an inducer of matrix


