Osteosarcomas (OSAs) are the most common primary malignant tumor of bone with the exception of hematopoietic malignancies, although only approximately 900 new cases occur annually in the United States. OSA has a bimodal age distribution, usually occurring in patients between 10 and 25 years of age (75%) and in patients > 40 years of age.  

Human leukocyte antigen (HLA)-G, a non-classical major histocompatibility complex (MHC) class I molecule, plays an important role in the regulation of the immune response. HLA-G has a restricted distribution in normal tissue cells, and is primarily expressed on trophoblastic cells, thymic epithelium, pancreas, and intestines. Clinical evidence in support of the role of HLA-G in immunosuppression is primarily derived from studies that have focused on correlating the level of HLA-G expression and clinical outcome in pregnancy and organ transplantation, which represent two major conditions for a host response to non-self tissues. HLA-G is an important immunotolerant molecule with the capability of inhibiting immune cell functions, such as natural killer (NK) cell, T lymphocye, and dendritic cell activities. The role of HLA-G in suppressing local immunity suggests that cancer cells utilize HLA-G overexpression during tumor development to help evade host immunosurveillance, a strategy similar to the one, which occurs in pregnancy and organ transplantation.

The HLA-G gene generates multiple protein isoforms by alternative splicing of a single mRNA, giving rise to four membrane-bound isoforms (HLA-G1mb to -G4mb), and three soluble isoforms (HLA-G5s to -G7s), which are generated by the presence of a stop codon in intron 4. The 14-bp sequence polymorphism of the HLA-G gene was
first reported by Harrison et al. in 1993. The 14-bp sequence polymorphism of the **HLA-G** gene is located in the 3’ untranslated region (UTR) of exon 8 of the **HLA-G** gene. Studies involving the 14-bp polymorphism have been performed in different ethnic populations. The frequencies of the different **HLA-G** alleles vary between different ethnic populations, ranging from 43.5 to 61.25% in the case of allele with a 14-bp deletion. All of the **MHC-G** genes, which have been studied in primates (chimpanzees, gorillas, and orangutans), include the 14-bp sequence. Thus, the polymorphism is a type of deletion rather than an insertion.

Several recently published studies have regarded the relationship between the **HLA** 14-bp polymorphism and disease. The majority of the studies have involved complications of pregnancy, some of which have suggested that the 14-bp polymorphism might be associated with pre-eclampsia and recurrent spontaneous abortion. Few studies have also described the relationship between tumors, such as bladder and cervical cancer, and the 14-bp polymorphism. Indeed, the present study is the first report regarding the relationship between **OSA** and the 14-bp polymorphism. Thus, the polymorphism is a type of deletion rather than an insertion.

The aim of this study was to determine association between the 14-bp insertion/deletion polymorphism and **OSA**.

### MATERIALS AND METHODS

#### Samples and genomic DNA extraction

Eighty-eight cases of conventional OSA samples were employed for the present study, and DNA extraction was successful in 75 cases (45 males and 30 females, mean age ± standard deviation [SD], 26.1 ± 17.5 years) (Table 1). One hundred eighty-three healthy control patients (130 males and 53 females, mean age ± SD, 38.7 ± 18.2 years) who were registered at our hospital for regular health check-ups were recruited (Table 1). Fifty-eight South Korean and 17 Argentinean patients who were diagnosed with **OSA** between 1985 and 2004 at the Kyung Hee Medical Center in Korea, and the Hospital of the University of Buenos Aires in Argentina, respectively, were also enrolled for the study. Both, the South Korean and Argentinean patients comprised one each of ethnic group (European descent). Research protocols for the use of human tissues were approved by and conducted in accordance with the policies of the Institutional Review Board at the Kyung Hee University Hospital.

#### DNA extraction and genotyping assays

Genomic DNA was extracted from formalin-fixed, paraffin-embedded tumor tissues obtained from patients with conventional **OSA** and the peripheral blood of healthy controls by using a genomic DNA isolation reagent kit (Qiagen, Hilden, Germany). Genotyping for the 14-bp insertion/deletion polymorphism in the 3’ UTR of the **HLA-G** gene was performed by a polymerase chain reaction (PCR) by using the fluorescence-labeled sense (5’-GTGATGGGCTGTTAATAGGTACCC-3’) and antisense primers (5’-GGAAGGAATGCAGTTCAGCAT- GA-3’). This primer pair generates a **HLA-G** 14-bp*1 product when the 14-bp sequence is present and a **HLA-G** 14-bp*0 product when the 14-bp sequence is deleted. PCR was carried out as described by Tripathi et al. by employing the following cycling profile: initial denaturation at 94°C for 5 minutes; and 39 cycles at 94°C for 30 seconds, 58°C for 40 seconds, and 72°C for 1 minute; with a final elongation step of 72°C for 10 minutes. The PCR products were analyzed by a gene scan (3730xl DNA Analyzer, Applied Biosystems, Foster City, CA, USA) (Fig. 1).

#### Statistical analysis

The genetic data between **OSA** and control patients were analyzed. For analysis of genetic data, SNP Stats (http://bioinfo.iconcologia.net/index.php) and SPSS ver. 18.0 (SPSS Inc., Chicago, IL, USA) were used. A logistic regression model was used for odds ratio, 95% confidence intervals, and p-values. For the statistical tests, the level of significance was set at 0.05.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Control n (%)</th>
<th>Korean OSA n (%)</th>
<th>Argentinian OSA n (%)</th>
<th>Korean OSA vs Control</th>
<th>Argentinian OSA vs Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>OR (95% CI)</td>
<td>p-value</td>
<td>OR (95% CI)</td>
<td>p-value</td>
</tr>
<tr>
<td><strong>HLA-G</strong> 14-bp*0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>270 (73.8)</td>
<td>80 (69.0)</td>
<td>26 (76.0)</td>
<td>1</td>
<td>127 (0.80-1.99)</td>
<td>0.312</td>
</tr>
<tr>
<td>96 (26.2)</td>
<td>38 (31.0)</td>
<td>8 (24.0)</td>
<td>1</td>
<td>127 (0.59-2.09)</td>
<td>0.731</td>
</tr>
</tbody>
</table>

At the **HLA-G** 14-bp insertion/deletion locus, 0 stands for the 14 bp deletion and 1 for the 14 bp insertion. p-values are calculated from logistic regression analysis. **HLA-G**, human leukocyte antigen-G; **OSA**, osteosarcoma; **n**, number of subjects; **OR**, odds ratio; **CI**, confidence interval.
**RESULTS**

The *HLA-G* genotype frequencies of the Korean control group were present in the Hardy-Weinberg (HW) equilibrium. There was a significantly different distribution for the genotype of the 14-bp insertion/deletion polymorphism between the Korean OSA and Korean control groups. Specifically, there was an increased frequency of heterozygote 210 bp/224 bp genotypes in the Korean OSA group when compared to the Korean control group (62.1% vs 40.4%, *p* = 0.002) (Table 2). However, the allele frequency of the 14-bp insertion/deletion polymorphism in the Korean OSA group was not significantly different from the

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**Table 2.** Genotype frequencies of the *HLA-G* 14-bp insertion/deletion polymorphism in osteosarcoma patients and controls

<table>
<thead>
<tr>
<th>Model</th>
<th>Control n (%)</th>
<th>Korean OSA n (%)</th>
<th>Argentinean OSA n (%)</th>
<th>Korean OSA vs Control</th>
<th>Argentinean OSA vs Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td></td>
<td></td>
<td>OR (95% CI)</td>
<td>p-value</td>
</tr>
<tr>
<td>Co-dominant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>HLA-G</em> 14-bp*0/<em>HLA-G 14-bp</em>0</td>
<td>98 (53.6)</td>
<td>22 (37.9)</td>
<td>9 (53.0)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>HLA-G</em> 14-bp*0/<em>HLA-G 14-bp</em>1</td>
<td>74 (40.4)</td>
<td>36 (62.1)</td>
<td>8 (47.0)</td>
<td>2.17 (1.18-3.99)</td>
<td>0.002</td>
</tr>
<tr>
<td><em>HLA-G</em> 14-bp*1/<em>HLA-G 14-bp</em>1</td>
<td>11 (6.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>NA</td>
<td>0.210</td>
</tr>
<tr>
<td>Dominant</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>HLA-G</em> 14-bp*0/<em>HLA-G 14-bp</em>0</td>
<td>98 (53.6)</td>
<td>22 (37.9)</td>
<td>9 (53.0)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>HLA-G</em> 14-bp*0/<em>HLA-G 14-bp</em>1</td>
<td>85 (46.5)</td>
<td>36 (62.1)</td>
<td>8 (47.0)</td>
<td>1.89 (1.03-3.45)</td>
<td>0.037</td>
</tr>
<tr>
<td><em>HLA-G</em> 14-bp*1/<em>HLA-G 14-bp</em>1</td>
<td>11 (6.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Recessive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>HLA-G</em> 14-bp*0/<em>HLA-G 14-bp</em>0</td>
<td>172 (94)</td>
<td>58 (100.0)</td>
<td>17 (100.0)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>HLA-G</em> 14-bp*0/<em>HLA-G 14-bp</em>1</td>
<td>11 (6.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

At the *HLA-G* 14-bp insertion/deletion locus, 0 stands for the 14 bp deletion and 1 for the 14 bp insertion.

*p*-values are calculated from the Fisher exact test.

*HLA-G*, human leukocyte antigen-G; OSA, osteosarcoma; n, number of subjects; OR, odds ratio; CI, confidence interval; NA, not applicable.
Korean control group (p = 0.312) (Table 1).

In the Argentinean OSA group, there were no differences in allele and genotype frequencies of the HLA-G bp insertion/deletion polymorphism when compared to the Korean control group (Tables 1, 2). However, the Argentinean control group was not involved in our study, thus these results were of limited value. The Argentinean OSA group had an increased frequency of the heterozygote genotype than the expected HW frequencies (47.0% vs 36.5%), even though the difference was not statistically significant (p = 0.05). The Argentinean OSA group had an increased frequency of the heterozygote genotype than the expected HW frequencies (47.0% vs 36.5%), even though the difference was not statistically significant (p = 0.05). The ob served result was likely a reflection of the low number of cases, which was not statistically significant (p = 0.52), which was likely a reflection of the low number of cases.

Based on the clinical analysis, the heterozygote genotype of the 14-bp insertion/deletion polymorphism was found to be in significant correlation with the absence of soft tissue extension (82.9% vs 66.4%, p = 0.019) (Table 3). Seven cases of Korean patients were not included in this analysis because of inadequate clinicopathologic data. The other clinicopathologic variables did not reveal any significant association with the genotype of the 14-bp insertion/deletion polymorphism.

### DISCUSSION

The purpose of this study was to determine the relationship between the 14-bp insertion/deletion polymorphism and OSA. Recent studies have demonstrated associations between OSA and polymorphisms, such as the glutathione S-transferase (GST) polymorphism, MDM2 SNP 309 polymorphism, tumor protein p53 (TP53) Arg72Pro polymorphism, and limbic system-associated membrane protein (LSAMP) deletion. Salinas-Souza et al. reported that GST polymorphisms might be associated with treatment response and OSA progression. Toffoli et al. showed that MDM2 SNP 309 was associated with an increased risk of high-grade OSA in females, and TP53 Arg72Pro possessed prognostic value for overall survival. Yen et al. identified association of chromosomal aberrations involving LSAMP with disease progression in patients with OSA and revealed LSAMP as a novel tumor suppressor gene.

HLA-G expression has been detected in a wide range of human cancers, including lung carcinoma, renal cell carcinoma, mesothelioma, breast carcinoma, glioma, ovarian cancer, and colorectal carcinoma. These studies encompass important biological and clinical implications for HLA-G expression in human tumor tissues. However, till date the association between OSA and HLA-G has not been studied.

In the present study, we demonstrated that the OSA group had an excess number of the heterozygotes in Korean population (62.1% vs 40.4%, p = 0.002). The Argentinean OSA group had an increased frequency of the heterozygote genotype than the expected HW frequencies (47.0% vs 36.5%), even though the difference was not statistically significant (p = 0.52). The observed result was likely a reflection of the low number of cases, and further study is needed with respect to large number of cases and other ethnic populations.
Heterozygosis for the HLA-G 14-bp insertion/deletion polymorphism has been reported to be associated with recurrent fetal loss and systemic lupus erythematosus (SLE). The former studies have indicated a significant increase in the number of heterozygotes for the 14-bp polymorphism (HLA-G 14-bp*0/HLA-G 14-bp*1) in women with recurrent spontaneous abortions versus women without recurrent spontaneous abortions. In patients with SLE, the heterozygote group exhibited lower SLE disease activity indexes than the homozygous deletion and insertion groups.

In OSA patients, although the effect of HLA-G heterozygote is not clear, we suggest two possible explanations for the same. First, heterozygote excess may represent a hitch-hiking effect. Genetic hitch-hiking refers to the process by which the frequency of a gene changes due to selection operating upon linked genes. An excess of heterozygosis within or near the HLA-G gene may influence HLA-G 14 bp polymorphism. Tan et al. observed that the promoter of HLA-G was extraordinarily polymorphic and provided strong evidence of balancing selection at the HLA-G promoter region, which is characterized by two different lineages of human haplotypes and may have different promoter activity. These differences could result in different spatiotemporal patterns of expression that meet different immunologic tissue needs. Therefore, the HLA-G heterozygote had different levels of expression of HLA-G mRNA, according to various conditions and disease states.

The other explanation is that the 14-bp deletion may directly influence HLA-G mRNA stability and concentration. It has been postulated that there may be a direct association between the 14-bp sequence and an altered pattern in the HLA-G mRNA isoform. Therefore, the mRNA forms, which lack 92 bases, were shown to be more stable than the complete transcripts. These findings indicate that the HLA-G gene possessing the +14 bp haplotype may produce more stable HLA-G mRNAs, and therefore may evade the attack of immune cells, including T-lymphocytes, NK cells, and dendritic cells.

Furthermore, the heterozygote genotype of the 14-bp insertion/deletion polymorphism was significantly correlated with absence of soft tissue extension. Soft tissue extension is known to be a poor prognostic factor in OSA. However, the other clinicopathologic variables did not demonstrate any significant association with the genotype of the 14-bp insertion/deletion polymorphism. Further studies on the relationship between the heterozygote genotype and OSA are warranted.

In conclusion, the Korean OSA group had an increased frequency of heterozygote genotypes than the healthy population. We also postulate that HLA-G heterozygote patients may be more susceptible to OSA.

REFERENCES
14. Castelli EC, Mendes-Junior CT, Viana de Camargo JL, Donadi EA.


