The Korean Journal of Pathology 2011; 45: 485-490
http://dx.doi.org/10.4132/KoreanJPathol.2011.45.5.485

14-bp Insertion/Deletion Polymorphism of the *HLA-G* Gene in Osteosarcoma Patients

Ahrim Moon · Su Kang Kim[1]
Joo-Ho Chung[1] · Ki Yong Na
Liliana G. Olvi[2]
Eduardo Santini-Araujo[2,3]
Youn Wha Kim · Yong-Koo Park

Departments of Pathology and [1]Pharmacology, School of Medicine, Kyung Hee University, Seoul, Korea; [2]Laboratory of Orthopaedic Pathology, [3]Department of Pathology, School of Medicine, University of Buenos Aires, Buenos Aires, Argentina

Received: August 2, 2011
Accepted: September 19, 2011

Corresponding Author
Yong-Koo Park, M.D.
Department of Pathology, Kyung Hee University Hospital, School of Medicine, Kyung Hee University, 1 Hoegi-dong, Dongdaemun-gu, Seoul 130-702, Korea
Tel: +82-2-958-8742
Fax: +82-2-958-8730
E-mail: ykpark@khmc.or.kr

*This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korean Government (MEST; No. 20100028333).

Background: The major histocompatibility complex class I, G (human leukocyte antigen-G [*HLA-G*]) gene plays a vital role in the suppression of immune responses. Recently, a number of studies have reported an association between *HLA-G* and diseases (pregnancy complications, organ transplantation, and tumors). Some of the studies have revealed that the 14-bp insertion/deletion polymorphism might be associated with various diseases. The aim of the present study was to explore a possible influence of the 14-bp insertion/deletion polymorphism on osteosarcoma. **Methods:** Genomic DNA was extracted from 75 formalin-fixed, paraffin-embedded tumor tissues derived from patients with conventional osteosarcoma (OSA) and 183 peripheral blood samples of healthy controls. Fifty-eight cases were South Korean patients with OSA and 17 cases were Argentine patients with OSA. The *HLA-G* 14-bp insertion/deletion polymorphism at exon 8 of the *HLA-G* locus was analyzed by polymerase chain reaction. **Results:** There was a significantly different distribution profile for the 14-bp genotypes between the Korean OSA and Korean control groups. Specifically, there were more 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). **Conclusions:** The results suggest that *HLA-G* heterozygote patients may be more susceptible to OSA in the Korean population.

Key Words: *HLA-G*; 14-bp insertion/deletion polymorphism; Osteosarcoma

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.[1] OSAs have a bimodal age distribution, usually occurring in patients between 10 and 25 years of age (75%) and in patients >40 years of age.[1]

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 trophoblatic cells, thymic epithelium, pancreas, and intestines.[2,3] 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.[4] HLA-G is an important immunotolerant molecule with the capability of inhibiting immune cell functions, such as natural killer (NK) cell, T lymphocyte, and dendritic cell activities.[5-7] The role of HLA-G in suppressing local immunity suggests that cancer cells utilize HLA-G over expression 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.[8]

The 14-bp sequence polymorphism of the *HLA-G* gene was

first reported by Harrison *et al.*[9] 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.[9,10] 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.[11] All of the *MHC-G* genes, which have been studied in primates (chimpanzees, gorillas, and orangutans), include the 14-bp sequence.[12] Thus, the polymorphism is a type of deletion rather than an insertion.[12]

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.[11,13] Few studies have also described the relationship between tumors, such as bladder[14] and cervical cancer,[15] and the 14-bp polymorphism. Indeed, the present study is the first report regarding the relationship between OSAs and the 14-bp polymorphism.

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´-GTGATGGGCTGTTTAAAGTGTCACC-3´) and antisense primers (5´-GGAAGGAATGCAGTTCAGCATGA-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.*[16] 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 1. Allele frequencies of the *HLA-G* 14-bp insertion/deletion polymorphism in osteosarcoma patients and controls

Allele	Control n (%)	Korean OSA n (%)	Argentinean OSA n (%)	Korean OSA vs Control		Argentinean OSA vs Control	
				OR (95% CI)	p-value	OR (95% CI)	p-value
HLA-G 14-bp*0	270 (73.8)	80 (69.0)	26 (76.0)	1		1	
HLA-G 14-bp*1	96 (26.2)	36 (31.0)	8 (24.0)	1.27 (0.80-1.99)	0.312	1.12 (0.59-2.09)	0.731

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.

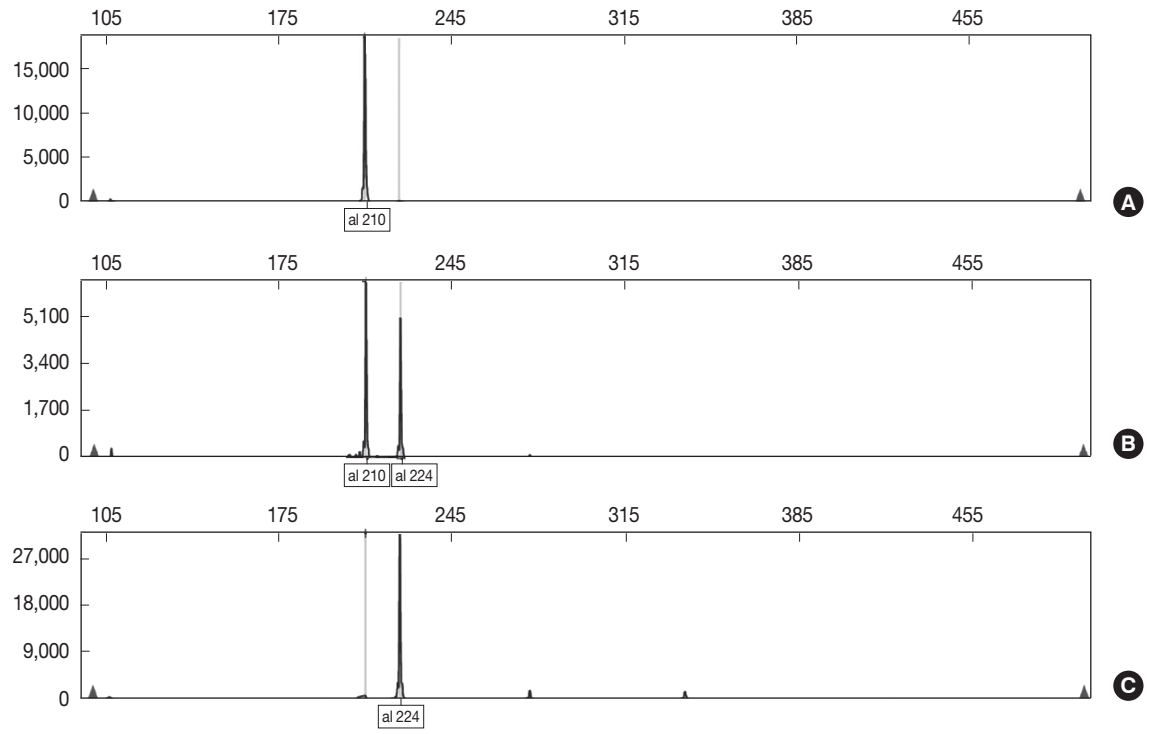

Fig. 1. The genotypes of human leukocyte antigen-G (*HLA-G*) 14-bp insertion/deletion polymorphisms as analyzed by gene scan. (A) *HLA-G* 14-bp*0/*HLA-G* 14-bp*0 genotype of *HLA-G.* (B) *HLA-G* 14-bp*0/*HLA-G* 14-bp*1 genotype of *HLA-G.* (C) *HLA-G* 14-bp*1/*HLA-G* 14-bp*1 genotype of *HLA-G*.

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

Model	Control n (%)	Korean OSA n (%)	Argentinean OSA n (%)	Korean OSA vs Control			Argentinean OSA vs Control		
				OR (95% CI)	p-value	p-value[a]	OR (95% CI)	p-value	p-value[a]
Co-dominant									
HLA-G 14-bp*0/*HLA-G* 14-bp*0	98 (53.6)	22 (37.9)	9 (53.0)	1			1		
HLA-G 14-bp*0/*HLA-G* 14-bp*1	74 (40.4)	36 (62.1)	8 (47.0)	2.17 (1.18-3.99)	0.002		1.18 (0.43-3.20)	0.350	
HLA-G 14-bp*1/*HLA-G* 14-bp*1	11 (6.0)	0 (0.0)	0 (0.0)	NA		0.210	NA		1.000
Dominant									
HLA-G 14-bp*0/*HLA-G* 14-bp*0	98 (53.6)	22 (37.9)	9 (53.0)	1			1		
HLA-G 14-bp*0/*HLA-G* 14-bp*1	85 (46.5)	36 (62.1)	8 (47.0)	1.89 (1.03-3.45)	0.037		1.02 (0.38-2.77)	0.960	
HLA-G 14-bp*1/*HLA-G* 14-bp*1									
Recessive									
HLA-G 14-bp*0/*HLA-G* 14-bp*0	172 (94)	58 (100.0)	17 (100.0)	1			1		
HLA-G 14-bp*0/*HLA-G* 14-bp*1									
HLA-G 14-bp*1/*HLA-G* 14-bp*1	11 (6.0)	0 (0.0)	0 (0.0)	NA	NA	0.071	NA	NA	0.604

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

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

Table 3. Correlation of the *HLA-G* 14-bp insertion/deletion polymorphism with clinicopathological variables in osteosarcoma patients

		Total cases			Korean cases		
		n	Heterozygote (%)	p-value	n	Heterozygote (%)	p-value
		69			52		
Sex	M	42	23 (54.8)	0.326	33	19 (57.6)	0.245
	F	27	18 (66.7)		19	14 (73.7)	
Age (yr)	≤25	44	25 (56.8)	0.559	28	17 (60.7)	0.982
	>25	25	16 (64.0)		24	16 (66.7)	
Subtype	Osteoblastic	53	32 (60.4)	0.768	36	24 (66.7)	0.527
	The others	16	9 (56.3)		16	9 (52.9)	
Tumor size (cm)	<8	40	23 (56.1)	0.703	30	18 (60.0)	0.336
	≥8	29	18 (62.1)		22	14 (63.6)	
Stage	I	13	10 (76.9)	0.215	13	10 (76.9)	0.244
	II-IV	56	31 (55.4)		39	23 (59.0)	
Soft tissue extension	Absent	50	34 (82.9)	0.019[a]	33	26 (78.8)	0.002[a]
	Present	19	7 (36.8)		19	7 (36.8)	
Distant metastasis	Absent	47	28 (59.6)	0.970	33	22 (66.7)	0.527
	Present	22	13 (59.1)		19	11 (57.9)	
Recur	Absent	61	37 (60.7)	0.564	45	29 (64.4)	0.697
	Present	8	4 (50.0)		7	4 (57.1)	

Seven cases of Korean patient are not included in this analysis because of inadequate clinicopathologic data.
[a]Significant difference, $p<0.05$ (chi-square test).
HLA-G, human leukocyte antigen-G; M, male; F, female.

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%; data not shown). However, this difference 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 36.8%, $p=0.019$) (Table 3). Seven cases of Korean patients were not included in this analysis because of inadequate clinicopathologic data. The other clinicopathologic variables did not revealed 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,[17] *MDM2* SNP 309 polymorphism,[18] tumor protein p53 (*TP53*) Arg72Pro polymorphism,[18] and limbic system-associated membrane protein (*LSAMP*) deletion.[19] Salinas-Souza *et al.*[17] reported that *GST* polymorphisms might be associated with treatment response and OSA progression. Toffoli *et al.*[18] showed that *MDM2* SNP 309 was associated with an increased risk of high-grade OSA in females, and *TP53* Arg-72Pro possessed prognostic value for overall survival. Yen *et al.*[19] 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,[20] renal cell carcinoma,[21] mesothelioma,[22] breast carcinoma,[22] glioma,[23] ovarian cancer,[24] and colorectal carcinoma.[25] 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[16,26] 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.[16,26] In patients with SLE, the heterozygote group exhibited lower SLE disease activity indexes than the homozygous deletion and insertion groups.[27]

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.[28] An excess of heterozygosis within or near the *HLA-G* gene may influence *HLA-G* 14 bp polymorphism. Tan *et al.*[29] 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 spaciotemporal 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.[10] Therefore, the mRNA forms, which lack 92 bases, were shown to be more stable than the complete transcripts.[30] 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.[4]

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 as one of the 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

1. Goorin AM, Abelson HT, Frei E 3rd. Osteosarcoma: fifteen years later. N Engl J Med 1985; 313: 1637-43.
2. Kovats S, Main EK, Librach C, Stubblebine M, Fisher SJ, DeMars R. A class I antigen, HLA-G, expressed in human trophoblasts. Science 1990; 248: 220-3.
3. Crisa L, McMaster MT, Ishii JK, Fisher SJ, Salomon DR. Identification of a thymic epithelial cell subset sharing expression of the class Ib HLA-G molecule with fetal trophoblasts. J Exp Med 1997; 186: 289-98.
4. Sheu J, Shih IeM. HLA-G and immune evasion in cancer cells. J Formos Med Assoc 2010; 109: 248-57.
5. Bahri R, Hirsch F, Josse A, *et al.* Soluble HLA-G inhibits cell cycle progression in human alloreactive T lymphocytes. J Immunol 2006; 176: 1331-9.
6. Bahri R, Naji A, Menier C, *et al.* Dendritic cells secrete the immunosuppressive HLA-G molecule upon CTLA4-Ig treatment: implication in human renal transplant acceptance. J Immunol 2009; 183: 7054-62.
7. Wang SS, Han JY, Wu XW, *et al.* A study of HLA-G1 protection of porcine endothelial cells against human NK cell cytotoxicity. Transplant Proc 2004; 36: 2473-4.
8. Fujii T, Ishitani A, Geraghty DE. A soluble form of the HLA-G antigen is encoded by a messenger ribonucleic acid containing intron 4. J Immunol 1994; 153: 5516-24.
9. Harrison GA, Humphrey KE, Jakobsen IB, Cooper DW. A 14 bp deletion polymorphism in the HLA-G gene. Hum Mol Genet 1993; 2: 2200.
10. Hviid TV, Hylenius S, Rørbye C, Nielsen LG. HLA-G allelic variants are associated with differences in the HLA-G mRNA isoform profile and HLA-G mRNA levels. Immunogenetics 2003; 55: 63-79.
11. Yan WH, Lin A, Chen XJ, *et al.* Association of the maternal 14-bp insertion polymorphism in the HLA-G gene in women with recurrent spontaneous abortions. Tissue Antigens 2006; 68: 521-3.
12. Castro MJ, Morales P, Martínez-Laso J, *et al.* Evolution of MHC-G in humans and primates based on three new 3'UT polymorphisms. Hum Immunol 2000; 61: 1157-63.
13. Hylenius S, Andersen AM, Melbye M, Hviid TV. Association between HLA-G genotype and risk of pre-eclampsia: a case-control study using family triads. Mol Hum Reprod 2004; 10: 237-46.
14. Castelli EC, Mendes-Junior CT, Viana de Camargo JL, Donadi EA.

HLA-G polymorphism and transitional cell carcinoma of the bladder in a Brazilian population. Tissue Antigens 2008; 72: 149-57.
15. Simões RT, Gonçalves MA, Castelli EC, *et al.* HLA-G polymorphisms in women with squamous intraepithelial lesions harboring human papillomavirus. Mod Pathol 2009; 22: 1075-82.
16. Tripathi P, Abbas A, Naik S, Agrawal S. Role of 14-bp deletion in the HLA-G gene in the maintenance of pregnancy. Tissue Antigens 2004; 64: 706-10.
17. Salinas-Souza C, Petrilli AS, de Toledo SR. Glutathione S-transferase polymorphisms in osteosarcoma patients. Pharmacogenet Genomics 2010; 20: 507-15.
18. Toffoli G, Biason P, Russo A, *et al.* Effect of TP53 Arg72Pro and MDM2 SNP309 polymorphisms on the risk of high-grade osteosarcoma development and survival. Clin Cancer Res 2009; 15: 3550-6.
19. Yen CC, Chen WM, Chen TH, *et al.* Identification of chromosomal aberrations associated with disease progression and a novel 3q13.31 deletion involving LSAMP gene in osteosarcoma. Int J Oncol 2009; 35: 775-88.
20. Urosevic M, Kurrer MO, Kamarashev J, *et al.* Human leukocyte antigen G up-regulation in lung cancer associates with high-grade histology, human leukocyte antigen class I loss and interleukin-10 production. Am J Pathol 2001; 159: 817-24.
21. Bukur J, Rebmann V, Grosse-Wilde H, *et al.* Functional role of human leukocyte antigen-G up-regulation in renal cell carcinoma. Cancer Res 2003; 63: 4107-11.
22. Kleinberg L, Flørenes VA, Skrede M, *et al.* Expression of HLA-G in malignant mesothelioma and clinically aggressive breast carcinoma. Virchows Arch 2006; 449: 31-9.
23. Wiendl H, Mitsdoerffer M, Hofmeister V, *et al.* A functional role of HLA-G expression in human gliomas: an alternative strategy of immune escape. J Immunol 2002; 168: 4772-80.
24. Sheu JJ, Shih IeM. Clinical and biological significance of HLA-G expression in ovarian cancer. Semin Cancer Biol 2007; 17: 436-43.
25. Hansel DE, Rahman A, Wilentz RE, *et al.* HLA-G upregulation in pre-malignant and malignant lesions of the gastrointestinal tract. Int J Gastrointest Cancer 2005; 35: 15-23.
26. Xue S, Yang J, Yao F, Xu L, Fan L. Recurrent spontaneous abortions patients have more -14 bp/+14 bp heterozygotes in the 3'UT region of the HLA-G gene in a Chinese Han population. Tissue Antigens 2007; 69 Suppl 1: 153-5.
27. Veit TD, Cordero EA, Mucenic T, *et al.* Association of the HLA-G 14 bp polymorphism with systemic lupus erythematosus. Lupus 2009; 18: 424-30.
28. Barton NH. Genetic hitchhiking. Philos Trans R Soc Lond B Biol Sci 2000; 355: 1553-62.
29. Tan Z, Shon AM, Ober C. Evidence of balancing selection at the HLA-G promoter region. Hum Mol Genet 2005; 14: 3619-28.
30. Rousseau P, Le Discorde M, Mouillot G, Marcou C, Carosella ED, Moreau P. The 14 bp deletion-insertion polymorphism in the 3' UT region of the HLA-G gene influences HLA-G mRNA stability. Hum Immunol 2003; 64: 1005-10.