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Original Article

Screening of *TRIM* and *TLR4* single nucleotide polymorphisms in patients with different period of clinical osteosarcoma in China

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Abstract: Osteosarcoma is the most common cancer-caused dead and the prognosis remains incompletely understand. Clinical manifestations of osteosarcoma are caused by gene mutations in interstitial cells range from abnormal cell proliferation to other tumor metastasis. Given the important role of the gene mutations in the clinical outcome of osteosarcoma, it is essential to analyze functional single nucleotide polymorphisms (SNP) in tumor-related genes contribute to define the clinical manifestations of human osteosarcoma. DNA samples from retrospective cohort of osteosarcoma individuals in China were used to determine the frequency of SNPs in *TRIM* and *TLR4* genes. DNA samples were obtained a total of 142 patients with different period of clinical osteosarcoma. Genotyping of *TRIM*-412 G/A and *TLR4* Asp299Gly and Thr399Ile SNPs was performed by PCR-restriction fragment length polymorphisms. The associations between Allele, genotype, frequencies and SNPs were evaluated in clinical groups. Results showed that The G allele in *TRIM*-412 G/A SNP was found more frequently in individuals with osteosarcoma, and the *TLR4* Asp299Gly and Thr399Ile SNP was more frequent in patients with osteosarcoma. No significant differences in allele frequencies for *TRIM* and *TLR4* were found in patients with different period of clinical osteosarcoma. In conclusion, these results suggest that *TLR4* gene may contribute to the etiology of osteosarcoma.

Keywords: Osteosarcoma, *TRIM, TLR4*, SNP

Introduction

Osteosarcoma is one of the most common malignant tumors and the prognosis remains incompletely understand due to the paucity of effective therapeutic targets that significantly influences quality of life and mean survival rate of the patients with osteosarcoma [1, 2]. Osteosarcoma is also a typical systemic malignant disease that mainly leads to common symptom of bone and joint pain, lump, claudication and fatigue in patients [3-5]. Many reports have been proposed strategies of targeted therapies evaluated by the pediatric preclinical testing program for osteosarcoma and further promoted hypothesis-driven drug discovery and development in the search for new therapies for this pediatric disease [6-8]. However, the poor survival has not been improved for patients with osteosarcoma.

Tumor early diagnosis is crucial for the treatments of human osteosarcoma that contributes to long-term survival for clinical patients [9]. Single nucleotide polymorphism (SNP) refers only polymorphism of a single base mutations in DNA, which has be found to relate with tumor classification and progression based on DNA copy number aberrations determined using SNP arrays [10]. In addition, SNP arrays in heterogeneous tissue indicated that special SNP can be regarded as mutations that may explain part of the "missing" heritability in cancer by highly accurate collection of both germline and somatic genetic information from unpaired single tumor samples [11, 12]. Further,

Table 1. Baseline characteristics of the study groups

Characteristic	Osteosarcoma	Health	*P* value
Age-years. Mean (range)	12.4-34.6	12.8-26.4	0.142
Male; % (n)	(49.2%) 70	(48%) 24	0.0284
Female; % (n)	(50.8%) 72	(52%) 26	0.0186
Clinical stage: % (n)			
I	(38.1%) 54	N (A)	
II	(24.6%) 35	N (A)	
III	(21.1%) 30	N (A)	
IV	(16.2%) 23	N (A)	

SNP microarray analyses have revealed copy number alterations and progressive genome reorganization during tumor development in SVT/t driven mice breast cancer [13]. However, the relationships between *TRIM-14* and *TLR4* SNP and osteosarcoma have not been well understood.

Evidences have showed that *TRIM-14* and *TLR4* play vital role in tumor progression and metastasis, which has been identified as a gene promoting angiogenesis, invasion and apoptotic resistance of bone cancer [14, 15]. The aim of this study was to investigate the frequency of SNPs in *TRIM-14* and *TLR4* genes in individuals with clinical osteosarcoma in China, and to explore its relationships with the clinical progression with osteosarcoma.

Materials and methods

Study design, subjects and sampling

A total of 142 osteosarcoma patients and 150 healthy volunteers were recruited in this analysis. The age was 12.4-34.6 and 13.2-38.6 years old in patients and healthy volunteers, respectively. Human biological samples were collected from years May 2010 to March 2016 in Zhejiang Hospital. The frequencies of SNPs in osteosarcoma-related genes were designed in this retrospective pilot case-control study. Inclusion criteria for individuals with osteosarcoma were diagnosed by tumor histopathology. Patients with osteoarthritis, orthopedic surgery and cancer history were excluded in this study. The characteristics of patients with osteosarcoma and healthy volunteers were summarized in **Table 1**. All patients were required to write informed consent with signature.

DNA genotyping

10 μg of genomic DNA were isolated from osteosarcoma or normal bone cells and used for PCR amplification. Genotyping of TRIM-14 G/A, and TLR4, Asp299Gly (A/G) and Thr399Ile (C/T) was conducted by PCR-restriction fragment length polymorphisms (RFLP) as described previously [16, 17]. All primer sequences, restriction endonucleases and fragment sizes are shown in **Table 2** and PCR products were analyzed by electrophoresis on a 3% agarose gel stained with ethidium bromide.

Quantitative real time PCR (qRT-PCR) analysis

Total RNA was obtained from different clinical stage osteosarcoma cells by using RNAeasy Mini Kit (QIAGEN, Gaithersburg, MD). TRIM-14 and TLR4 expression levels in osteosarcoma and normal bone cancer cells were determined by applying a qRT-PCR [18] (Invitrogen, CA, USA). All the forward and reverse primers were synthesized by Invitrogen. Relative mRNA expression level changes were calculated by $2^{-\Delta\Delta Ct}$. The results are expressed as the n-fold way compared to control.

Western blotting

Osteosarcoma cells were homogenized in lysate buffer containing protease-inhibitor and were centrifuged at 8000 rpm/min at 4°C for 10 min. The supernatant of mixture were used for analysis of purpose protein. All antibodies were purchased from Abcam (Shanghai) trading co. LTD (Abcam, Shanghai, China) For western blotting, rabbit anti-human primary antibodies: TRIM-14 (1:1000, ab90541, Abcam) and TLR4 (1:1000, ab22048, Abcam) and β-actin (1:1000, ab8826, Abcam) were added after blocking (5% skimmed milk) for 1 hour at 37°C and then incubated horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibodies for 24 hours at 4°C. The results were visualized by using chemi-luminescence detection system (Amersham Biosciences, Piscataway, NJ).

Statistical analysis

Data were shown as mean ± SD and analyzed by students t test. Multiple groups were ana-

Table 2. Primer sequences and restriction endonucleases used for RFLP analysis and fragment sizes for identification of SNPs

SNP	Primers (5'-3')	Restriction endonuclease	Fragment sizes (bp)
TRIM-14 A/C	F, 5'-TCATTCTATGTGCTG GAG ATG G-3	*Mae* III	CC: 128 + 96
	R, 5-TTTGGGGGAAGTGGG TAAGAGT-3'		TT: 236
			TC: 426 + 238 + 98
TLR4 G/A (Asp299Gly)	F, 5'-GATTAGCATATCTAG ACTACTACCTCCATG-3'	I *Nco* I	AA: 214
	R, 5'-GATCAACTTCTGAAA AAGCATTCCCAC-3'		GG: 230 + 32
			GA: 236 + 218 + 46
TLR4 C/T (Thr399Ile)	F, 5'-GGTTGCTGTTCTCAA AGTGATTTTGGGAGAA-3'	*Hinf* I	CC: 418
	R, 5'-ACCTGAAGACTGGAG AGTGAGTTAAATGTT-3'		TT: 392 + 40
			CT: 414 + 378 + 52

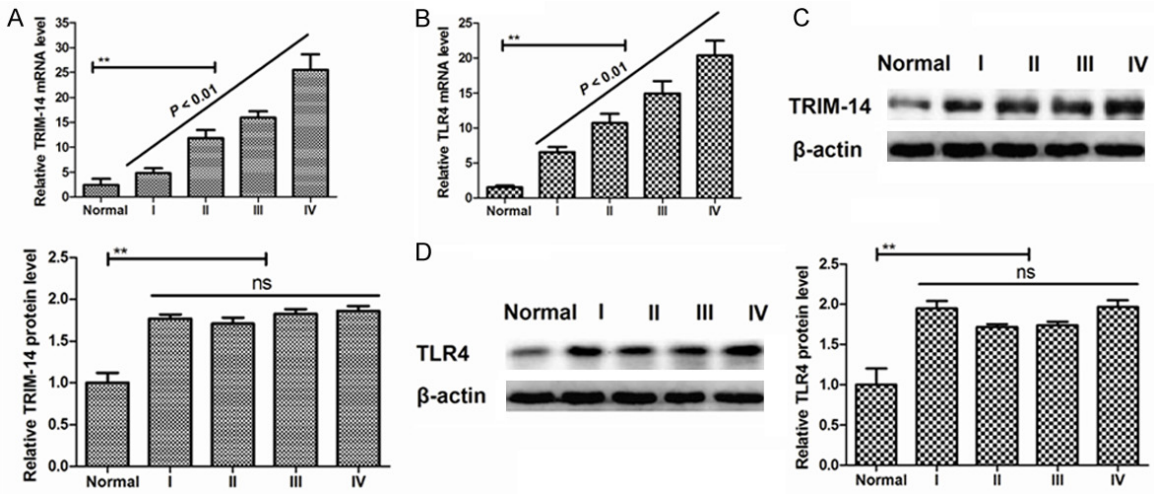

Figure 1. *TRIM-14* and *TLR4* gene and protein expression in different clinical stage osteosarcoma. A. *TRIM-14* mRNA expression levels is up-regulated in osteosarcoma tissues compared to normal bone cells. B. *TLR4* mRNA expression levels is up-regulated in osteosarcoma tissues compared to normal bone cells. C. TRIM-14 protein expression levels is up-regulated in osteosarcoma tissues compared to normal bone cells. D. TLR4 protein expression levels is up-regulated in osteosarcoma tissues compared to normal bone cells. **P<0.01.

lyzed by one-way variance (ANOVA). All data were analyzed using SPSS Statistics 19.0 and Graphpad Prism version 5.0 with the help of Microsoft Excel. Allele and genotype frequencies were calculated in each group (I-IV patients and healthy individuals) using direct counting. Hardy-Weinberg equilibrium (HWE) and the differences between allele and genotype frequencies were calculated using Fisher's exact test or Chi-square test. 95% confidence intervals (CI) and odds ratios (OR) were presented using logistic regression models to assess the magnitude of association between osteosarcoma patients and healthy volunteers groups. Hardy-Weinberg tests were used to check the observed genotype distribution in patients and in the healthy control group. Results of allele and genotype frequencies were determined by STATA SE 12.1 software. Minor allele frequencies (MAF) were calculated using the prop.test function on R 3.1.1 software. *P<0.05 was considered statistical differences.

Results

TRIM-14 and TLR4 gene and protein expression in different clinical stage osteosarcoma

TRIM-14 and *TLR4* gene and protein expression levels were investigated in different clinical stage osteosarcoma. Results showed that

Table 3. Genotype, allele frequency and MAF of *TRIM-14* gene polymorphisms in individuals with different stage of osteosarcoma

SNPs		Frequency; %					OR (95% CI)
		Normal	I	II	III	IV	
Genotype	AA	13.2	4.6	4.5	6.6	5.2	0.28 (0.12-1.10)
	GG	5.4	12.6	16.2	13.6	13.8	1.38 (0.12-1.10)
	GA	4.8	8.5	13.5	17.5	21,4	2.42 (0.12-1.10)
Allele	A	5	4	4	5	5	1.06 (0.12-1.10)
	C	6	10	12	12	14	1.32 (0.12-1.10)
MAF	0.174	0.126	0.247	0.185	0.546	0.622	

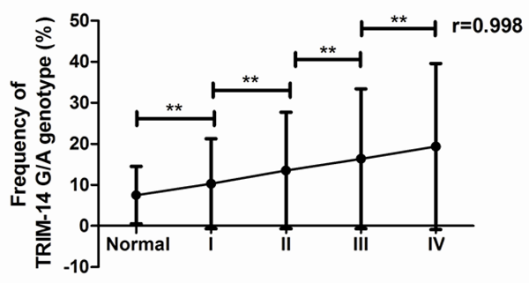

Figure 2. Correlation analysis between the genotype nucleotide GA and clinical osteosarcoma stage in *TRIM-14* gene.

TRIM-14 and *TLR4* mRNA expression levels were up-regulated in osteosarcoma tissues compared to normal bone cells (**Figure 1A**, **1B**). We also showed that *TRIM-14* and *TLR4* protein expression levels were increased in clinical osteosarcoma tissues (**Figure 1C**, **1D**). Data analysis showed that *TRIM-14* and *TLR4* protein expression levels were increased in clinical stage osteosarcoma (**P<0.01, osteosarcoma tissues vs. normal tissue). These results showed that *TRIM-14* and *TLR4* gene and protein expression levels were up-regulated in clinical osteosarcoma tissues compared to adjacent normal tissues.

TRIM-14 SNP in different clinical stage osteosarcoma

DNA samples were collected form a total of 142 patients with different clinical stage osteosarcoma and used to *TRIM-14* SNP analyses in this study. The distribution of the genotypic variants for *TRIM*-412 G/A SNPs met the HWE (Fisher's exact test). Alignment of sequence data of 142 patients in clinical I-IV stage osteosarcoma revealed the following SNPs: Genotype nucleotide AA was decreased, while genotype nucleotide GG and GA was increased in patients in clinical I-IV stage osteosarcoma compared to healthy control ($p<0.05$, OR = 0.28-2.42, 95% CI (0.12-1.10). The frequencies of the A allele in the -400 position of the promoter region of the *TRIM-14* gene was similar between each group, while the C allele was found more frequently in patients with osteosarcoma compared to healthy control (12.5% vs. 6.6%, **Table 3**). Notably, significant differences in allele, genotype frequencies and MAF values in *TRIM*-412G/A were found between the normal samples and osteosarcoma samples ($r = 0.998$. $p<0.01$, osteosarcoma tissues vs. normal tissue, Supplementary Figures 1, 2). The genotype nucleotide GA was positively correlated with clinical osteosarco-ma stage (**Figure 2**).

TLR4 SNP in different clinical stage osteosarcoma

TLR4 SNP was investigated in DNA samples in 142 patients with different clinical stage osteosarcoma. *TLR4* genotyping were obtained from 142 clinical I-IV stage osteosarcoma samples (**Table 4**). Results showed that *TLR4* Asp299Gly was frequencies AA and AG genotypes in osteosarcoma DNA samples compared to healthy controls [AA, OR = 0.76, 95% CI (0.35-2.28), AG, OR = 1.66 95% CI (0.38-3.21)]. The CC genotype is frequencies in osteosarcoma samples, while is significant difference in among clinical I-IV stage osteosarcoma samples. Findings demonstrated that Thr-399Ileu of the *TLR4* frequencies of TT and CT were not significantly between normal and osteosarcoma DNA samples, while CC was more frequency in osteosarcoma samples compared to healthy controls. We found that the frequencies of the G allele in the -1210 position of the promoter region of the *TLR4* Asp299Gly gene was similar between osteosarcoma samples and healthy controls, while the A allele was found more frequently in patients with osteosarcoma. Minor allele frequency (MAF) and co-segregation analysis with *TLR4* Asp-299Gly SNP showed significant differences between the normal samples and osteosarco-

Table 4. Genotype, allele frequency and MAF of *TLR4* gene polymorphisms in individuals with different stage of osteosarcoma

SNPs		Frequency; %					OR (95% CI)
		Normal	I	II	III	IV	
TLR4 Asp299Gly							
Genotype	AA	4.1	7.3	12.2	10.7	13.6	0.76 (0.35-2.28)
	AG	3.6	8.5	9.4	10.4	9.2	1.66 (0.38-3.21)
	CC	5.0	7.5	10.6	15.8	21.4	3.12 (0.70-6.48)
Allele	G	8.0	8.0	7.5	8.2	8.3	2.48 (0.96-6.58)
	A	42.3	53.4	56.6	52.3	56.4	4.18 (1.20-10.26)
MAF		0.082	0.012	0.024	0.035	0.020	
TLR4 Thr399Ile							
Genotype	TT	6.8	7.6	6.2	6.6	6.4	1.48 (0.47-3.66)
	CT	7.4	7.5	6.8	7.5	8.0	4.18 (1.06-12.26)
	CC	3.2	6.4	8.7	12.4	17.8	6.35 (2.08-15.30)
Allele	C	6.6	12.4	18.6	26.2	34.6	7.20 (1.90-14.46)
	T	4.6	10	12	12	14	1.32 (0.12-1.10)
MAF		0.074	0.086	0.038	0.025	0.044	

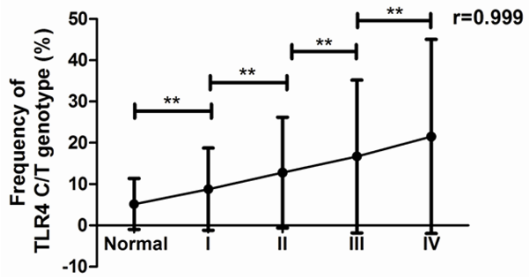

Figure 3. Correlation analysis between the genotype nucleotide CT and clinical osteosarcoma stage in *TLR* gene.

ma samples. No significant differences were found between osteosarcoma groups. Results also showed that C and T alleles in the -784 position of the promoter region of the *TLR4* Thr399Ileu were significant differences between the normal samples and osteosarcoma samples. However, there is no significant among osteosarcoma groups. We also indicate that genotype nucleotide CT was positively correlated with clinical osteosarcoma stage (**Figure 3**, P<0.01, R = 0.999). These results showed that frequencies of Thr399Ileu of the *TLR4* are differences between the normal samples and osteosarcoma samples and C/T genotype of *TLR4* gene is positively correlated with clinical stage of osteosarcoma.

Discussion

Osteosarcoma cancer is a disease that cells occur in skeleton and affiliates and present aberrant growth and migration. In the current study, we investigated the frequency of SNPs in *TRIM* and *TLR4* genes in osteosarcoma samples from clinical I-IV stage patients. Polymorphisms in promoter and coding regions of *TRIM* and *TLR4* genes have shown associations with the progression of human cancers [19, 20]. The divergence in the frequency of the genotypic variants for *TRIM*-412 G/A SNPs between normal samples and osteosarcoma samples reflected the distinct mechanisms of *TRIM-14* overexpression. We also found that frequencies of CC are higher in DNA samples form osteosarcoma and were concordant with upregulation of *TLR4* gene and protein in patients with osteosarcoma.

Recently, the functions of single nucleotide polymorphisms for the diagnosis and progression and of osteosarcoma have been wildly investigated in different genes, which related the risk or metastasis of osteosarcoma [21, 22]. Tripartite motif containing-14 (TRIM-14) is identified as an important member of TRIM family proteins, which presents the promontory effects on growth, invasiveness and resistance to cisplatin-induced tumor apoptosis [23]. Previous studies have suggested that TRIM-14 overexpression could induce an aggressive phenotype in cancer progression through regulation of the NF-κB signaling pathway [24]. Therefore, understanding the role of TRIM-14 is necessary for tumor research and treatment in human tumorigenesis and metastasis. The current study analyzed *TRIM-14* SNPs in clinical osteosarcoma samples. Results showed that TRIM-14 is overexpressed in osteosarcoma tissues since significant differences in allele, genotype frequencies and MAF values in *TRIM*-412 G/A between normal samples and osteosarcoma samples. Interestingly, the frequencies of the A allele in the -400 position of the promoter region of the *TRIM* gene was similar between each group, while the C allele

was found more frequently in patients with osteosarcoma, which may contribute to TRIM-14 overexpression in osteosarcoma tissues.

Studies suggest that toll like receptor 4 (TLR4) plays an important role in the initiation and/or maintenance of pathological pain state in bone cancer pain [25]. Genetic variants in TLR4 gene correlate with many human disease, such as myeloproliferative disorders, infectious diseases and inflammatory disease [26-28]. However, the role of TLR4 SNP in patients with osteosarcoma has not reported yet. Previous reports have indicated that TLR4 co-segregating polymorphisms Asp299Gly and Thr399Ile in TLR4 have been shown to increase the risk of Gram-negative infections, sepsis, and severe malaria [29-31]. In this study, we explored *TLR4 SNP in different clinical I-IV stage osteo*sarcoma samples. Our results showed that *TLR4* Asp299Gly was frequencies between groups for the AA and AG genotypes (72.4 and 17.8%, respectively) and AC genotype is frequencies in osteosarcoma samples, while is not significant difference in among clinical I-IV stage osteosarcoma samples. No significant differences were found between osteosarcoma groups, but genotype nucleotide CT was positively correlated with clinical osteosarcoma stage.

In conclusion, the current study found a significant association between *TRIM* and *TLR4* genes polymorphism and clinical osteosarcoma stage. The results of SNPs *TRIM* and *TLR4* genes indicate that the efficiency of used associated tumor-related genes to predict clinical stage of human osteosarcoma using the SNPs of *TRIM* and *TLR4*, which may contribute to understand the pathology of osteosarcoma and provide possible target therapies.

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Disclosure of conflict of interest

None.

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References

[1] Li YF, Yu CP, Wu ST, Dai MS and Lee HS. Malignant mesenchymal tumor with leiomyosarcoma, rhabdomyosarcoma, chondrosarcoma, and osteosarcoma differentiation: case report and literature review. Diagn Pathol 2011; 6: 35.

[2] PosthumaDeBoer J, Witlox MA, Kaspers GJ and van Royen BJ. Molecular alterations as target for therapy in metastatic osteosarcoma: a review of literature. Clin Exp Metastasis 2011; 28: 493-503.

[3] Zhao J, Zhang X, Liu J and Li J. Primary osteosarcoma of the breast with abundant chondroid matrix and fibroblasts has a good prognosis: a case report and review of the literature. Oncol Lett 2013; 6: 745-747.

[4] Navalkele P, Jones SM, Jones JK, Salazar JD, Toy PC, Iyer RV and Herrington B. Osteosarcoma tumor thrombus: a case report with a review of the literature. Tex Heart Inst J 2013; 40: 75-78.

[5] Liu JJ, Liu S, Wang JG, Zhu W, Hua YQ, Sun W and Cai ZD. Telangiectatic osteosarcoma: a review of literature. Onco Targets Ther 2013; 6: 593-602.

[6] Sampson VB, Gorlick R, Kamara D and Anders Kolb E. A review of targeted therapies evaluated by the pediatric preclinical testing program for osteosarcoma. Front Oncol 2013; 3: 132.

[7] Vijayamurugan N and Bakhshi S. Review of management issues in relapsed osteosarcoma. Expert Rev Anticancer Ther 2014; 14: 151-161.

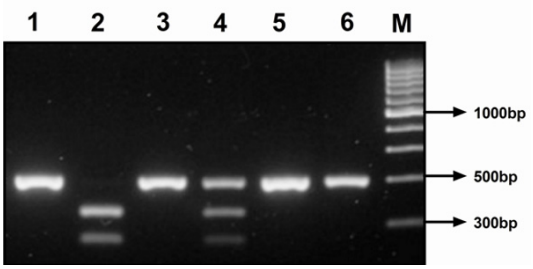
[8] He JP, Hao Y, Wang XL, Yang XJ, Shao JF, Guo FJ and Feng JX. Review of the molecular pathogenesis of osteosarcoma. Asian Pac J Cancer Prev 2014; 15: 5967-5976.

[9] Ene R, Sinescu RD, Ene P, Popescu D, Cirstoiu MM and Cirstoiu FC. Proximal tibial osteosarcoma in young patients: early diagnosis, modular reconstruction. Rom J Morphol Embryol 2015; 56: 413-417.

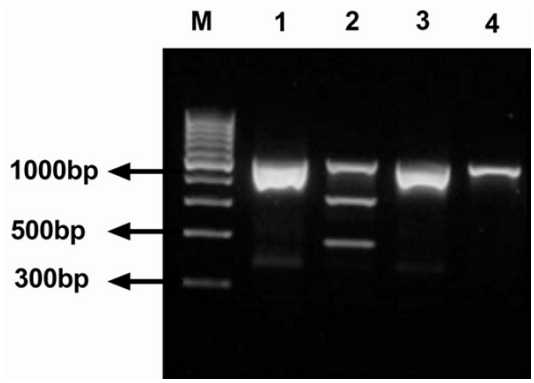
[10] Wang Y, Makedon F and Pearlman J. Tumor classification based on DNA copy number aberrations determined using SNP arrays. Oncol Rep 2006; 15 Spec no.: 1057-9.

[11] Letouze E, Sow A, Petel F, Rosati R, Figueiredo BC, Burnichon N, Gimenez-Roqueplo AP, Lalli E and de Reynies A. Identity by descent mapping of founder mutations in cancer using high-resolution tumor SNP data. PLoS One 2012; 7: e35897.

[12] Zin R, Pham K, Ashleigh M, Ravine D, Waring P and Charles A. SNP-based arrays complement classic cytogenetics in the detection of chromosomal aberrations in Wilms' tumor. Cancer Genet 2012; 205: 80-93.
[13] Standfuss C, Pospisil H and Klein A. SNP microarray analyses reveal copy number alterations and progressive genome reorganization during tumor development in SVT/t driven mice breast cancer. BMC Cancer 2012; 12: 380.
[14] Hatakeyama S. TRIM proteins and cancer. Nat Rev Cancer 2011; 11: 792-804.
[15] He W, Liu Q, Wang L, Chen W, Li N and Cao X. TLR4 signaling promotes immune escape of human lung cancer cells by inducing immunosuppressive cytokines and apoptosis resistance. Mol Immunol 2007; 44: 2850-2859.
[16] Santin I, Castellanos-Rubio A, Hualde I, Castano L, Vitoria JC and Bilbao JR. Toll-like receptor 4 (TLR4) gene polymorphisms in celiac disease. Tissue Antigens 2007; 70: 495-498.
[17] Suzuki S, Hosomichi K, Yokoyama K, Tsuda K, Hara H, Yoshida Y, Fujiwara A, Mizutani M, Shiina T, Kono T and Hanzawa K. Primary analysis of DNA polymorphisms in the TRIM region (MHC subregion) of the Japanese quail, Coturnix japonica. Anim Sci J 2013; 84: 90-96.
[18] Xiao S, Wang J and Xiao N. MicroRNAs as non-invasive biomarkers in bladder cancer detection: a diagnostic meta-analysis based on qRT-PCR data. Int J Biol Markers 2016; 31: e276-85.
[19] Song J, Kim DY, Kim CS, Kim HJ, Lee DH, Lee HM, Ko W and Lee G. The association between Toll-like receptor 4 (TLR4) polymorphisms and the risk of prostate cancer in Korean men. Cancer Genet Cytogenet 2009; 190: 88-92.
[20] Herquel B, Ouararhni K and Davidson I. The TIF1alpha-related TRIM cofactors couple chromatin modifications to transcriptional regulation, signaling and tumor suppression. Transcription 2011; 2: 231-236.
[21] Jin G, Wang M, Chen W, Shi W, Yin J and Gang W. Single nucleotide polymorphisms of nucleotide excision repair and homologous recombination repair pathways and their role in the risk of osteosarcoma. Pak J Med Sci 2015; 31: 269-273.
[22] Tie Z, Bai R, Zhai Z, Zhang G, Zhang H, Zhao Z, Zhou D and Liu W. Single nucleotide polymorphisms in VEGF gene are associated with an increased risk of osteosarcoma. Int J Clin Exp Pathol 2014; 7: 8143-8149.
[23] Nenasheva VV, Kovaleva GV, Uryvaev LV, Ionova KS, Dedova AV, Vorkunova GK, Chernyshenko SV, Khaidarova NV and Tarantul VZ. Enhanced expression of trim14 gene suppressed Sindbis virus reproduction and modulated the transcription of a large number of genes of innate immunity. Immunol Res 2015; 62: 255-262.
[24] Su X, Wang J, Chen W, Li Z, Fu X and Yang A. Overexpression of TRIM14 promotes tongue squamous cell carcinoma aggressiveness by activating the NF-kappaB signaling pathway. Oncotarget 2016; 7: 9939-9950.
[25] Pan R, Di H, Zhang J, Huang Z, Sun Y, Yu W and Wu F. Inducible lentivirus-mediated siRNA against TLR4 reduces nociception in a rat model of bone cancer pain. Mediators Inflamm 2015; 2015: 523896.
[26] Trejo-de la OA, Torres J, Perez-Rodriguez M, Camorlinga-Ponce M, Luna LF, Abdo-Francis JM, Lazcano E and Maldonado-Bernal C. TLR4 single-nucleotide polymorphisms alter mucosal cytokine and chemokine patterns in Mexican patients with Helicobacter pylori-associated gastroduodenal diseases. Clin Immunol 2008; 129: 333-340.
[27] Speletas M, Liadaki K, Kalala F, Daiou C, Katodritou E, Mandala E, Korantzis I, Ritis K, Zintzaras E and Germenis AE. TLR4 single nucleotide polymorphisms and thrombosis risk in patients with myeloproliferative disorders. Thromb Res 2008; 122: 27-32.
[28] Mera-Ramirez A, Castillo A, Orobio Y, Gomez MA and Gallego-Marin C. Screening of TNFalpha, IL-10 and TLR4 single nucleotide polymorphisms in individuals with asymptomatic and chronic cutaneous leishmaniasis in Colombia: a pilot study. BMC Infect Dis 2017; 17: 177.
[29] Mockenhaupt FP, Cramer JP, Hamann L, Stegemann MS, Eckert J, Oh NR, Otchwemah RN, Dietz E, Ehrhardt S, Schroder NW, Bienzle U and Schumann RR. Toll-like receptor (TLR) polymorphisms in African children: common TLR-4 variants predispose to severe malaria. Proc Natl Acad Sci U S A 2006; 103: 177-182.
[30] Lorenz E, Mira JP, Frees KL and Schwartz DA. Relevance of mutations in the TLR4 receptor in patients with gram-negative septic shock. Arch Intern Med 2002; 162: 1028-1032.
[31] Agnese DM, Calvano JE, Hahm SJ, Coyle SM, Corbett SA, Calvano SE and Lowry SF. Human toll-like receptor 4 mutations but not CD14 polymorphisms are associated with an increased risk of gram-negative infections. J Infect Dis 2002; 186: 1522-1525.



Supplementary Figure 1. PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) *TRIM* genotyping. Lanes 1, 3 and 5: undigested samples; lanes 2, 4: AccI digested sample; lane 6: homozygous wildtype sample; Lane M: 100 bp DNA ladder.



Supplementary Figure 2. PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) *TLR4* genotyping. Lanes 1 and 3: undigested samples; lane 2: Pme I digested sample; lane 4: homozygous wildtype sample. Lane M: 100 bp DNA ladder.