TLR4 promoter rs1927914 variant contribute to the susceptibility of esophageal squamous cell carcinoma in Chinese population

Jiaying Li [1, 2], **Hongjiao Wu** [2], **Hui Gao** [1], **Ruihuan Kou** [3], **Yuning Xie** [1, 2], **Zhi Zhang** [3], **Xuemei Zhang** [Corresp. 2]

[1] School of Public Health, North China University of Science and Technology, Tangshan, China

[2] College of Life Science, North China University of Science and Technology, Tangshan, China

[3] Affliated Tangshan Gongren Hospital, North China University of Science and Technology, Tangshan, China

Corresponding Author: Xuemei Zhang
Email address: jyxuemei@gmail.com

Background. Toll-like receptor 4 (TLR4), as a key regulator of both innate and acquired immunity has been linked with the development of various cancers. Although the association between TLR4 rs1927914 gene polymorphism and cancer have been reported, studies on genetic susceptibility to esophageal cancer have not been reported. Thus, this study aims to analyze the relationship between genetic variations TLR4 rs1927914 and the risk of esophageal cancer.**Methods.** This study adopts case-control research strategy to investigate the association between genetic variation of TLR4 and esophageal squamous cell carcinoma (ESCC) risk and includes 480 ESCC patients and 480 health controls. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype TLR4 rs1927914 polymorphism. Taqman probe method was used to determine the genotypes of TLR4 rs11536891 and rs7873784 variants. The relationship between TLR4 genetic variation and ESCC risk was analyzed by Logistic regression model by calculating the odds ratio (*OR*) and 95% confidence interval (*95%CI*).**Results.** Compared with TLR4 rs1927914AA genotype carriers, GG carriers had a lower ESCC risk (*OR* = 0.59, 95%*CI* = 0.38-0.93, *P* = 0.023). Stratification analysis by age showed that TLR4 rs1927914GG could affect the risk of ESCC in elderly people (*OR* = 0.59, 95%*CI* = 0.36-0.97). Smoking stratification analysis indicated that rs1927914 GG carriers were related to ESCC susceptibility among non-smokers (*OR* = 0.36, 95%*CI* = 0.18-0.73). We found that TLR4 rs7873784 (G>C) and rs11536891 (T>C) conforms to complete genetic linkage, and their genotype distributions of these two SNPs among ESCC patients and normal controls were not statistically significant (*P*>0.05). Dual luciferase reporter assay suggested that TLR4 rs1927914 G allele reporter gene activity was 1.76-fold higher than A allele in KYSE30 ESCC cells.**Conclusion.** TLR4 rs1927914 variant contribute to the ESCC risk by effecting the promoter activity.

TLR4 promoter rs1927914 variant contribute to the susceptibility of esophageal squamous cell carcinoma in Chinese population

Jiaying Li[1,2], Hongjiao Wu[2], Hui Gao[1], Ruihuan Kou[3], Yuning Xie[1,2], Zhi Zhang[3], Xuemei Zhang[1,2]

[1]School of Public Health, North China University of Science and Technology, Tangshan, Hebei, China

[2]College of Life Science, North China University of Science and Technology, Tangshan, Hebei, China

[3]Affliated Tangshan Gongren Hospital, North China University of Science and Technology, Tangshan, Hebei, China

Corresponding Author:

Xuemei Zhang[1,2]

No. 21 Bohai Avenue, Caofeidian District, Tangshan/Hebei, 063009, China

Email address: jyxuemei@gmail.com

21

Abstract

Background. Toll-like receptor 4 (TLR4), as a key regulator of both innate and acquired immunity has been linked with the development of various cancers. Although the association between TLR4 rs1927914 gene polymorphism and cancer have been reported, studies on genetic susceptibility to esophageal cancer have not been reported. Thus, this study aims to analyze the relationship between genetic variations TLR4 rs1927914 and the risk of esophageal cancer.

Methods. This study adopts case-control research strategy to investigate the association between genetic variation of TLR4 and esophageal squamous cell carcinoma (ESCC) risk and includes 480 ESCC patients and 480 health controls. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype TLR4 rs1927914 polymorphism. Taqman probe method was used to determine the genotypes of TLR4 rs11536891 and rs7873784 variants. The relationship between TLR4 genetic variation and ESCC risk was analyzed by Logistic regression model by calculating the odds ratio (*OR*) and 95% confidence interval (*95%CI*).

Results. Compared with TLR4 rs1927914AA genotype carriers, GG carriers had a lower ESCC risk (*OR* = 0.59, 95%*CI* = 0.38-0.93, *P* = 0.023). Stratification analysis by age showed that TLR4 rs1927914GG could affect the risk of ESCC in elderly people (*OR* = 0.59, 95%*CI* = 0.36-0.97). Smoking stratification analysis indicated that rs1927914 GG carriers were related to ESCC susceptibility among non-smokers (*OR* = 0.36, 95%*CI* = 0.18-0.73). We found that TLR4 rs7873784 (G>C) and rs11536891 (T>C) conforms to complete genetic linkage, and their genotype distributions of these two SNPs among ESCC patients and normal controls were not statistically significant (*P*>0.05). Dual luciferase reporter assay suggested that TLR4 rs1927914 G allele reporter gene activity was 1.76-fold higher than A allele in KYSE30 ESCC cells.

Conclusion. TLR4 rs1927914 variant contribute to the ESCC risk by effecting the promoter activity.

Keyword: TLR4, Esophageal squamous cell carcinoma, Single nucleotide polymorphism, Innate immune

Introduction

Esophageal cancer, as the sixth leading cause of cancer death, is one of the most common malignant tumors worldwide(*Bray et al., 2018*). Esophageal cancer contains two common histological types: esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC). There are clear differences between EAC and ESCC that affect their distribution and incidence in the world(*Domper Arnal, Ferrández Arenas, &Lanas Arbeloa, 2015; Yang, Chen, &Tu, 2016*). In China, most of the cases of esophageal cancer are squamous cell cancer(*Lin et al., 2013*). ESCC is caused by environmental and genetic factors. Epidemiological studies have reported that tobacco smoking, alcohol drinking, ingesting hot substances and so on played a role in the development of ESCC(*Yu et al., 2018*). However, not all individuals who have been exposed to these hazards eventually get ESCC. In recent years, genetic polymorphisms have been reported to impact the development of esophageal cancer(*Hiyama et al., 2007; Yue et al., 2017*).

The interaction between the immune system and malignant cells is an impact on tumorigenicity(*TermeTanchot, 2017*). On one hand, the immune system kills or clears malignant transformed cells through a variety of mechanisms, on the other hand, malignant cells use a variety of mechanisms to escape immune system-mediated rejection for their own development. As the most studied pattern recognition receptor, Toll-like receptors (TLRs) can enhance the innate immune response and stimulate antigen-derived cells such as dendritic cells, thus activating the immune response of tumor-specific T cells and affecting the development of tumors. TLR4, as the first identified mammal TLR, is widely expressed in the mammalian cell

surface. TLR4, as the main receptor for pathogen invasion, can not only recognize extracellular antigens, but also respond to intracellular injury related factors, thus promoting the secretion of inflammatory factors and interferon. Therefore, in the tumor microenvironment, TLR4 plays a key part in tumor infiltration of immune cells or in cancer cells. Studies have shown that TLR4 is overexpressed in a variety of malignant tumors and associated with poor prognosis in cancer patients(*J. Li et al., 2017; Pandey, Chauhan, &Jain, 2018; Wang et al., 2017; Zhao et al., 2019*).

Single nucleotide polymorphisms (SNP) are one of the most common genetic variants in the genome. Over the past decade, large-scale SNP analyses, known as genome-wide association studies (GWAS), have provided a new way to identify genetic loci that may be associated with the disease, such as cancer susceptibility, survival prognosis or drug response.

In view of the important role of TLR4 in tumors, we screened out three functional SNPs located on TLR4 gene using bioinformatics methods and then performed a case-control study in Chinese population to determine whether they were correlated with the occurrence of ESCC.

Materials and methods

Study subjects

In this study, 480 ESCC patients and 480 cancer-free controls were included. Cases were recruited from Apr 2008 to Dec 2012 in Affiliated Tangshan Gongren Hospital and Tangshan Renmin Hospital of North China University of Science and Technology (Tangshan, China). Inclusion criteria: all patients were diagnosed as primary ESCC by histopathology; all specimen were genetically unrelated Han Chinese; none of the patients had received radiotherapy or chemotherapy. 480 healthy individuals were randomly recruited from the same region and matched with cases on age and sex. All participants signed the written informed consent. Institutional Review Board of North China University of Science and Technology had approved the research (12-002).

97

98 **TLR4 SNPs selection**

99 In this study, we predicted the possible functional SNPs in the regulatory region of TLR4. All
100 included SNPs located in the promoter region or the 3' untranslated region with MAF≥0.05. For
101 the SNPs in the promoter region of TLR4, transcription factor binding capability was predicted
102 by TRANSFAC program. For the SNPs located in the 3′ untranslated region, microRNA binding
103 ability was predicted using Web Server. Finally, TLR4 rs1927914 in the promoter region and
104 TLR4 rs11536891 and rs7873784 in the 3' untranslated region were selected for further
105 genotyping.

106

107 **TLR4 genotyping**

108 Each subject donated 2mL of peripheral blood. DNA was extracted using the blood DNA kit
109 provided by TIANGEN Biotech (Beijing). TLR4 rs1927914 genotyping was performed by the
110 Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The target
111 DNA fragment was amplified by PCR using the forward primer 5'-
112 TGACATGGAAAATGGAGAGATAGAGG-3' and reverse primer 5'-
113 GGACTATGATGGAGATTGAAAATGTGG-3'. PCR was performed using a 6μl reaction
114 system containing 0.05μM each primer, 10ng DNA, and 2 x Es Taq MasterMix (CWBIO,
115 Beijing, China). PCR procedure was 3 minutes at 95℃, followed by 32 cycles (30s at 95℃, 30s
116 at 56.5℃ and 34s at 72℃) and 5 minutes at 72°C for final extension. TLR4 PCR products were
117 cut by Nsi *I* and verified with 3% agarose gel. TLR4 rs11536891 and rs7873784 variants were
118 genotyped using Taqman probe method. Taqman SNP genotyping assays C_31784036_10 and
119 C_29292008_10 were design by Thermo Fisher Scientific (Waltham, USA).

120

121 **Vector construction and site-directed mutation**

122 To analyze the effect of TLR4 promoter region genetic variation on transcriptional activity, we
123 used primers 5'-GGGGTACCCCGGATTGGAAGTGCTTGGAG-3' (with Kpn *I recognition*
124 *site*) (NEB, Ipswich, USA) and 5'-CTAGCTAGCTAGAAGAAGAAAACGCCTGC-3' (with
125 Nhe *I recognition site*) (NEB, Ipswich, USA) to amplify 1832bp fragment between the promoter
126 -1762 bp to 70bp of TLR4. The PCR product was cloned into pGL3-basic reporter vector
127 (Promega, Madison, USA). Based on the sequence results, we constructed pGL3-rs1927914A
128 plasmid. The template vectors (pGL3-rs1927914 A) were then used to obtain pGL3-rs1927914G
129 vector by site-specific mutagenesis reaction using site-specific mutation kit (TIANGEN, Beijing,
130 China). All constructs were verified by direct sequencing.

131

132 **Cell culture, Transfection and luciferase assay**

133 Esophageal carcinoma cells (KYSE30) were purchased from American Type Culture Collection
134 (ATCC). Cells were cultured in DMEM medium containing 10% FBS (Gibco, Vienna, Austria)
135 and 1% penicillin and streptomycin. Cells were seeded at a density of 3 x 10^5 cells/well in 24-
136 well plate to 70-80% confluence. Cells were co-transfected with different pGL3-Basic vectors
137 and pRL-SV40 using Lipofectamine^{TM} 2000 (Invitrogen, Carlsbad, USA). Luciferase activity
138 was detected by Dual Luciferase Reporter Assay. A 13μL of cell lysate was mixed with 25μL of
139 Luciferase Assay Reagent II, and Firefly luciferase activity was measured by GloMax 20/20
140 Luminometer. Then, 25μL of 1×Stop & Glo solution was added to determine Renilla luciferase
141 activity. The ratio of Firefly and Renilla luciferase activity was presented to the level of relative
142 luciferase activity. Independent experiments were performed three times.

143

144 **Statistical analysis**

In this study, all the research data were statistically analyzed using SPSS 23.0 (SPSS, Chicago, USA). The differences of basic characteristics in cases and controls were tested by χ^2 test. The Hardy-Weinberger equilibrium (HWE) of TLR4 polymorphisms in controls were tested by χ^2 test. The correlation between the genetic variants in TLR4 and the risk of esophageal cancer were evaluated by *OR* and *95%CI*. The activity of luciferase reporter gene was compared by two independent sample t-test. $P<0.05$ indicated statistically significant.

Results

Study subjects' general demographic characteristics

The general information of all subjects was showed in Table 1. There were no significant differences in age and gender between the cases and controls ($P>0.05$). The proportion of smokers in the case group was 64.4% and in control group was 30.6% ($P<0.001$), indicating a statistical difference. However, there were no statistically significant differences in cumulative smoking among ESCC patients and healthy controls ($P = 0.149$).

The influence of TLR4 variants on ESCC risk

Table 2 showed the association of TLR4 rs1927914, rs11536891 and rs7873784 genotypes with the susceptibility to esophageal cancer. Genotypes distribution of 3 SNPs among controls group were consistent with the Hardy-Weinberg equilibrium (HWE), indicating that the selected population was well representative. The genotypes frequencies of TLR4 rs1927914 AA, GA and GG were 40.6% (195), 49.4% (237) and 10% (48) in cases and 35.2% (169), 49.6% (238) and 15.2% (73) in controls. Multivariate logistic regression analysis displayed that rs1927914 GG genotype contributed to a decrease ESCC risk (*OR* = 0.59, *95%CI* = 0.38-0.93, $P = 0.023$) when compared with AA genotype. After genotyping TLR4 rs7873784 (G>C) and rs11536891 (T>C)

169 in 100 samples, we found that TLR4 rs7873784 and rs11536891 conforms to complete genetic
170 linkage. Based on this, in further study, we only genotyped TLR4 rs11536891. There was no
171 significant difference in the distribution of TLR4 rs11536891 genotypes in the case group and
172 the control group (P>0.05).

173

174 **Stratification analysis**

175 The stratification analysis by gender, age and smoking status was used to further explore the
176 interaction effect of genetic variation of TLR4 rs1927914 on ESCC risk (Table 3). When
177 stratified by gender, there was no significant correlation between genotypes of TLR4 rs1927914
178 and the esophageal cancer risk among males and females (*OR* = 0.67, *95%CI* = 0.41-1.09; *OR* =
179 0.31, *95%CI* = 0.09-1.11). In the age stratification, median age (50-year) in controls was set as
180 cut-off value for all subjects. Our data showed that older subjects (age>50) with GG genotype
181 had a lower esophageal cancer risk than those with the AA genotype (*OR* = 0.59, *95%CI* = 0.36-
182 0.97), but not among younger subjects(*OR* = 0.53, *95%CI* = 0.18-1.55). In a stratified analysis
183 based on smoking status, we found that the GG genotype was a protective factor among non-
184 smoker (*OR* = 0.36, *95%CI* = 0.18-0.73), but not among smoker (*OR* = 0.93, *95%CI* = 0.49-
185 1.76).

186

187 **Luciferase reporter gene activity detection**

188 For further verification, we assessed the effect of TLR4 rs1927914 genetic variation on
189 transcriptional activity. We transiently transfected the recombinant plasmid with rs1927914A
190 (pGL3-Basic-A), G allele (pGL3-Basic-G) or pGL3-Basic into KYSE30 cells together with an
191 internal control plasmid to detect the expression of luciferase activity, respectively. Reporter
192 gene assay result suggested that luciferase activity driven by TLR4 rs1927914 G allele was 1.76-
193 fold higher than TLR4 rs1927914 A allele (P = 0.0043) (Figure 1).

194

195 **Discussion**

196 Because the symptoms of esophageal cancer are not obvious in the early stage, most of the
197 patients are diagnosed in the middle and late stages and often accompanied by malnutrition. In
198 addition, due to the difficulty in surgical resection of partial lesions and poor effect of drug
199 treatment, the overall 5-year survival rate is around 19%(*Moral Moral et al., 2018*). Multiple
200 large clinical studies have shown that concurrent chemoradiotherapy (CCRT) can significantly
201 improve the local control rate and the overall survival rate of esophageal cancer(*Kang et al.,*
202 *2018; Takeda et al., 2018*). Therefore, CCRT is still the standard therapy for patients with locally
203 advanced esophageal cancer who cannot receive or refuse surgical treatment. However, CCRT is
204 not tolerated in patients with advanced age, severe cardiopulmonary complications or
205 malnutrition. In the past decade, targeted therapy has brought cancer treatment into the era of
206 precision therapy with its low toxic side effects and high therapeutic efficiency. The discovery of
207 EGFR, ALK and other driving genes in lung cancer provides an example for targeted therapy of
208 malignant tumors. Therefore, it is still necessary to look for potential molecular targets to guide
209 the clinical treatment of esophageal cancer.

210 TLRs are important components of inflammatory response by effecting on innate immune
211 response So far, it has been found that TLR family has 10 members. Studies have shown that
212 TLRs members play important roles in biological processes such as inflammatory response and
213 immune response, apoptosis and angiogenesis and are closely related to the development of
214 various cancers(*Belmont et al., 2014; Dajon, Iribarren, &Cremer, 2017; Garcia et al., 2016;*
215 *Paone et al., 2010; Vijay, 2018*). TLR4 gene locates in chromosome 9q32-33. TLR4 mRNA can
216 be polyadenylation at 3'UTR to produce 5432nt and 12853nt transcripts that both encode the
217 same 839aa protein. Kutikhin et al. found that the high expression of TLR4 in cancer tissues can
218 promote the metastasis and invasion of tumor cells, and it is not suppressed by the immune
219 system(*Davoodi, Hashemi, &Seow, 2013; Kutikhin et al., 2014*). Studies have shown that TLR4

is overexpressed in ESCC tissues and associated with poor prognosis in cancer patients(*X. Li et al., 2018; Sato et al., 2020*). Therefore, reasonable inhibition of TLR4 gene expression is conducive to disease recovery.

So far, several studies have found that TLR4 polymorphisms influence cancer susceptibility, such as gastric cancer, myeloma and hepatocellular carcinoma(*Bagratuni et al., 2016; He et al., 2018; C. Huang et al., 2017*). In Chinese population, Huang et al. found that there is a significantly decreased risk of gastric cancer in individuals carrying of the allele C for the rs10116253 and allele T for the rs1927911 in TLR4(*L. Huang et al., 2014*). Similar results were found in hepatocellular carcinoma(*Minmin et al., 2011*). Song et al. found that both TLR4 rs1927911 and rs11536858 polymorphism increased the susceptibility of prostate cancer in Korean Men(*Song et al., 2009*). Our results showed that TLR4 rs1927914 variations affect the risk of ESCC. Shi et al. reported that TLR4 rs1927914 genetic variations are correlated with the hepatocellular carcinoma susceptibility(*Shi et al., 2017*). This result was in accordance with the study of Minmin, which carriers with the heterozygous genotypes for the rs1927914 were associated with HCC risk(*Minmin et al., 2011*). However, researchers didn't find the correlation between TLR4 rs1927914 and lung and gastric cancer risk(*H. Huang et al., 2010; Wu et al., 2020*). Therefore, it is suggested that TLR4 rs1927914 may be associated with occurrence of ESCC. To verify the population data, we performed the luciferase reporter assay and provided the evidence that rs1927914 G allele was associated with elevated transcriptional activity. TLR4 may promote esophageal cancer cell proliferation and inhibit apoptosis through different signal transduction pathways. For example, inhibiting NF-κB transcription factor expression inhibits esophageal cancer progression(*Kohtz et al., 2019*). At present, there are few studies on the correlation between TLR4 rs11536891 polymorphism and tumor and mainly focus on prostate cancer or colorectal cancer. Song et al. found that TLR4 rs11536891 was not associated with prostate cancer risk(*Song et al., 2009*), while Tsilidis et al. reported that TLR4 rs11536891 was related to colorectal cancer risk(*Tsilidis et al., 2009*). In our previous study, TLR4 rs11536891 gene polymorphism was not correlated with lung cancer susceptibility(Wu et al., 2020). Our

study also showed that there was no correlation between TLR4 rs11536891 gene variation and ESCC risk.

In addition to genetic factors, the development of tumors is also related to environmental factors. Epidemiological studies suggest that smoking may induce esophageal cancer risk(*DongThrift, 2017*). Thus, we performed stratification analysis for smoking status and found TLR4 rs1927914 GG genotype carriers may decrease the risk of non-smokers, indicating that tobacco smoking may be one of the risk factors for ESCC. This is consistent with the study of Chen et al(*Chen et al., 2010*). Meanwhile, we found that GG genotype is a protective factor for older subjects. These results suggest that the risk of ESCC is mainly caused by the combination of environmental and genetic factors.

Conclusion

In summary, our report showed that TLR4 rs1927914 A>G polymorphism in the promoter can affect the transcriptional activity of TLR4 further influence esophageal cancer susceptibility.

Acknowledgments

The authors thank all patients and control subjects for their participation.

Funding

This work was supported by Key Project of Natural Science Foundation of Hebei province of China (H2017209233 to X. Zhang), Leader talent cultivation plan of innovation team in Hebei province (LJRC001 to X. Zhang) and National Natural Science Foundation of China (81101483 to X. Zhang).

Competing Interest

All authors declare that they have no competing interests.

Author contributions

JL: acquisition, analysis, and interpretation of data; drafting the manuscript. HW, HG, RK, XY: DNA extraction, data collection and analysis. ZZ collection of blood samples; acquisition and interpretation of data. XZ: design of the work, analysis and interpretation of data, revision of the article, final approval of the version to be published. All authors read and approved the final manuscript.

Human Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

This research was approved by Institutional Review Board of North China University of Science and Technology had approved the (12-002).

Data Availability

The following information was supplied regarding data availability:

The raw data are available as a Supplementary File.

References

Bagratuni T, Terpos E, Eleutherakis-Papaiakovou E, Kalapanida D, Gavriatopoulou M, Migkou M, Liacos CI, Tasidou A, Matsouka C, Mparmparousi D, Dimopoulos MA, Kastritis E. 2016. TLR4/TIRAP polymorphisms are associated with progression and survival of patients with symptomatic myeloma. *Br J Haematol 172*(1) 44-47 DOI 10.1111/bjh.13786.

Belmont L, Rabbe N, Antoine M, Cathelin D, Guignabert C, Kurie J, Cadranel J, Wislez M. 2014. Expression of TLR9 in tumor-infiltrating mononuclear cells enhances angiogenesis and is associated with a worse survival in lung cancer. *Int J Cancer 134*(4) 765-777 DOI 10.1002/ijc.28413.

Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. 2018. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin 68*(6) 394-424 DOI 10.3322/caac.21492.

Chen J, Zhang N, Wakai T, Wei L, He Y, Kumagai N, Kitsu K, Wang S, Akazawa K. 2010. Effect of the interaction between the amount and duration of alcohol consumption and tobacco smoking on the risk of esophageal cancer: A case-control study. *Exp Ther Med 1*(6) 991-997 DOI 10.3892/etm.2010.152.

Dajon M, Iribarren K, Cremer I. 2017. Toll-like receptor stimulation in cancer: A pro- and anti-tumor double-edged sword. *Immunobiology 222*(1) 89-100 DOI 10.1016/j.imbio.2016.06.009.

Davoodi H, Hashemi SR,Seow HF. 2013. 5-Fluorouracil Induce the Expression of TLR4 on HCT116 Colorectal Cancer Cell Line Expressing Different Variants of TLR4. *Iran J Pharm Res 12*(2) 453-460.

Domper Arnal MJ, Ferrández Arenas Á, Lanas Arbeloa Á. 2015. Esophageal cancer: Risk

factors, screening and endoscopic treatment in Western and Eastern countries. *World J Gastroenterol 21*(26) 7933-7943 DOI 10.3748/wjg.v21.i26.7933.

Dong J, Thrift AP. 2017. Alcohol, smoking and risk of oesophago-gastric cancer. *Best Pract Res Clin Gastroenterol 31*(5) 509-517 DOI 10.1016/j.bpg.2017.09.002.

Garcia PV, Seiva FR, Carniato AP, de Mello Júnior W, Duran N, Macedo AM, de Oliveira AG, Romih R, Nunes Ida S, Nunes Oda S, Fávaro WJ. 2016. Increased toll-like receptors and p53 levels regulate apoptosis and angiogenesis in non-muscle invasive bladder cancer: mechanism of action of P-MAPA biological response modifier. *BMC Cancer 16* 422 DOI 10.1186/s12885-016-2474-z.

He B, Xu T, Pan B, Pan Y, Wang X, Dong J, Sun H, Xu X, Liu X, Wang S. 2018. Polymorphisms of TGFBR1, TLR4 are associated with prognosis of gastric cancer in a Chinese population. *Cancer Cell Int 18* 191 DOI 10.1186/s12935-018-0682-0.

Hiyama T, Yoshihara M, Tanaka S, Chayama K. 2007. Genetic polymorphisms and esophageal cancer risk. *Int J Cancer 121*(8) 1643-1658 DOI 10.1002/ijc.23044.

Huang C, Zhang H, Bai R, Wang L,Lv J. 2017. A896G and C1196T Polymorphisms Within the TLR4 Gene Abate Toll-Like Receptor 4-Mediated Signaling in HepG2 Cells. *DNA Cell Biol 36*(11) 1029-1038 DOI 10.1089/dna.2017.3892.

Huang H, Wu J, Jin G, Zhang H, Ding Y, Hua Z, Zhou Y, Xue Y, Lu Y, Hu Z, Xu Y, Shen H. 2010. A 5'-flanking region polymorphism in toll-like receptor 4 is associated with gastric cancer in a Chinese population. *J Biomed Res 24*(2) 100-106 DOI 10.1016/s1674-8301(10)60017-6.

Huang L, Yuan K, Liu J, Ren X, Dong X, Tian W, Jia Y. 2014. Polymorphisms of the TLR4 gene and risk of gastric cancer. *Gene 537*(1) 46-50 DOI 10.1016/j.gene.2013.12.030.

Kang J, Chang JY, Sun X, Men Y, Zeng H, Hui Z. 2018. Role of Postoperative Concurrent

Chemoradiotherapy for Esophageal Carcinoma: A meta-analysis of 2165 Patients. *J Cancer 9*(3) 584-593 DOI 10.7150/jca.20940.

Kohtz PD, Halpern AL, Eldeiry MA, Hazel K, Kalatardi S, Ao L, Meng X, Reece TB, Fullerton DA, Weyant MJ. 2019. Toll-Like Receptor-4 Is a Mediator of Proliferation in Esophageal Adenocarcinoma. *Ann Thorac Surg 107*(1) 233-241 DOI 10.1016/j.athoracsur.2018.08.014.

Kutikhin AG, Yuzhalin AE, Volkov AN, Zhivotovskiy AS, Brusina EB. 2014. Correlation between genetic polymorphisms within IL-1B and TLR4 genes and cancer risk in a Russian population: a case-control study. *Tumour Biol 35*(5) 4821-4830 DOI 10.1007/s13277-014-1633-6.

Li J, Yin J, Shen W, Gao R, Liu Y, Chen Y, Li X, Liu C, Xiang R, Luo N. 2017. TLR4 Promotes Breast Cancer Metastasis via Akt/GSK3β/β-Catenin Pathway upon LPS Stimulation. *Anat Rec (Hoboken) 300*(7) 1219-1229 DOI 10.1002/ar.23590.

Li X, Li H, Dong X, Wang X, Zhu J, Cheng Y, Fan P. 2018. Expression of NF-κB and TLR-4 is associated with the occurrence, progression and prognosis of esophageal squamous cell carcinoma. *Int J Clin Exp Pathol 11*(12) 5850-5859.

Lin Y, Totsuka Y, He Y, Kikuchi S, Qiao Y, Ueda J, Wei W, Inoue M, Tanaka H. 2013. Epidemiology of esophageal cancer in Japan and China. *J Epidemiol 23*(4) 233-242 DOI 10.2188/jea.je20120162.

Minmin S, Xiaoqian X, Hao C, Baiyong S, Xiaxing D, Junjie X, Xi Z, Jianquan Z, Songyao J. 2011. Single nucleotide polymorphisms of Toll-like receptor 4 decrease the risk of development of hepatocellular carcinoma. *PLoS One 6*(4) e19466 DOI 10.1371/journal.pone.0019466.

Moral Moral GI, Viana Miguel M, Vidal Doce Ó, Martínez Castro R, Parra López R, Palomo

Luquero A, Cardo Díez MJ, Sánchez Pedrique I, Santos González J, Zanfaño Palacios J. 2018. Postoperative complications and survival rate of esophageal cancer: Two-period analysis. *Cir Esp 96*(8) 473-481 DOI 10.1016/j.ciresp.2018.05.002.

Pandey N, Chauhan A, Jain N. 2018. TLR4 Polymorphisms and Expression in Solid Cancers. *Mol Diagn Ther 22*(6) 683-702 DOI 10.1007/s40291-018-0361-9.

Paone A, Galli R, Gabellini C, Lukashev D, Starace D, Gorlach A, De Cesaris P, Ziparo E, Del Bufalo D, Sitkovsky MV, Filippini A, Riccioli A. 2010. Toll-like receptor 3 regulates angiogenesis and apoptosis in prostate cancer cell lines through hypoxia-inducible factor 1 alpha. *Neoplasia 12*(7) 539-549 DOI 10.1593/neo.92106.

Sato Y, Motoyama S, Wakita A, Kawakita Y, Liu J, Nagaki Y, Nanjo H, Ito S, Terata K, Imai K, Minamiya Y. 2020. High TLR4 expression predicts a poor prognosis after esophagectomy for advanced thoracic esophageal squamous cell carcinoma. *Esophagus* DOI 10.1007/s10388-020-00732-x.

Shi G, Wang C, Zhang P, Ji L, Xu S, Tan X, Li H. 2017. Donor Polymorphisms of Toll-like Receptor 4 rs1927914 Associated with the Risk of Hepatocellular Carcinoma Recurrence Following Liver Transplantation. *Arch Med Res 48*(6) 553-560 DOI 10.1016/j.arcmed.2017.11.011.

Song J, Kim DY, Kim CS, Kim HJ, Lee DH, Lee HM, Ko W, Lee G. 2009. The association between Toll-like receptor 4 (TLR4) polymorphisms and the risk of prostate cancer in Korean men. *Cancer Genet Cytogenet 190*(2) 88-92 DOI 10.1016/j.cancergencyto.2008.12.011.

Takeda K, Umezawa R, Takahashi N, Matsushita H, Kozumi M, Ishikawa Y, Yamamoto T, Takeda K, Jingu K. 2018. Impact of change in serum albumin level during and after chemoradiotherapy in patients with locally advanced esophageal cancer. *Esophagus 15*(3) 190-197 DOI 10.1007/s10388-018-0612-1.

Terme M, Tanchot C. 2017. [Immune system and tumors]. *Ann Pathol 37*(1) 11-17 DOI 10.1016/j.annpat.2016.12.004.

Tsilidis KK, Helzlsouer KJ, Smith MW, Grinberg V, Hoffman-Bolton J, Clipp SL, Visvanathan K, Platz EA. 2009. Association of common polymorphisms in IL10, and in other genes related to inflammatory response and obesity with colorectal cancer. *Cancer Causes Control 20*(9) 1739-1751 DOI 10.1007/s10552-009-9427-7.

Vijay K. 2018. Toll-like receptors in immunity and inflammatory diseases: Past, present, and future. *Int Immunopharmacol 59* 391-412 DOI 10.1016/j.intimp.2018.03.002.

Wang K, Wang J, Wei F, Zhao N, Yang F, Ren X. 2017. Expression of TLR4 in Non-Small Cell Lung Cancer Is Associated with PD-L1 and Poor Prognosis in Patients Receiving Pulmonectomy. *Front Immunol 8* 456 DOI 10.3389/fimmu.2017.00456.

Wu H, Gao H, Li A, Xie Y, Jia Z, Yang Z, Zhang H, Zhang Z, Zhang X. 2020. Impact of Genetic Variation in TLR4 3'UTR on NSCLC Genetic Susceptibility. *J Oncol 2020* 7593143 DOI 10.1155/2020/7593143.

Yang CS, Chen X, Tu S. 2016. Etiology and Prevention of Esophageal Cancer. *Gastrointest Tumors 3*(1) 3-16 DOI 10.1159/000443155.

Yu C, Tang H, Guo Y, Bian Z, Yang L, Chen Y, Tang A, Zhou X, Yang X, Chen J, Chen Z, Lv J, Li L. 2018. Hot Tea Consumption and Its Interactions With Alcohol and Tobacco Use on the Risk for Esophageal Cancer: A Population-Based Cohort Study. *Ann Intern Med 168*(7) 489-497 DOI 10.7326/m17-2000.

Yue C, Li M, Da C, Meng H, Lv S, Zhao X. 2017. Association between genetic variants and esophageal cancer risk. *Oncotarget 8*(29) 47167-47174 DOI 10.18632/oncotarget.17006.

Zhao S, Sun M, Meng H, Ji H, Liu Y, Zhang M, Li H, Li P, Zhang Y, Zhang Q. 2019. TLR4 expression correlated with PD-L1 expression indicates a poor prognosis in patients with

412 peripheral T-cell lymphomas. *Cancer Manag Res 11* 4743-4756 DOI
413 10.2147/cmar.S203156.

414

415 Figure 1: Transient reporter gene expression assays. Luciferase expression of two constructers
416 (pGL3-rs1927914G and pGL3-rs1927914A) in KYSE30 cells co-transfected with pRL-SV40 to
417 standardize transfection efficiency. Fold increase was measured by defining the activity of the
418 empty pGL-3 Basic vector as 1. *P < 0.05.

Table 1(on next page)

Distributions of select characteristics in cases and control subjects

Table 1 Distributions of select characteristics in cases and control subjects

Variables	case (n = 480)			Controls (n = 480)		*P* value[a]
	No	(%)		No	(%)	
Sex						0.930
Male	403	84.0		402	83.7	
Female	77	16.0		78	16.3	
Age						0.162
≤50	83	17.3		100	20.8	
>50	397	82.7		380	79.2	
Smoking status						<0.001
Non-smoker	171	35.6		333	69.4	
Smoker	309	64.4		147	30.6	
Pack year of smoking[b]						
≤25	123	39.8		69	46.9	0.149
>25	186	60.2		78	53.1	

[a]Two-sidde χ^2 test

Table 2(on next page)

Gene polymorphism of TLR4 and their association with ESCC

Table 2 Gene polymorphism of TLR4 and their association with ESCC

TLR4 genotypes	Cases (n = 480)		Controls (n = 480)		OR (95%CI)	*P* value[a]
	No	(%)	No	(%)		
Rs1927914						
AA	195	40.6	169	35.2		
GA	237	49.4	238	49.6	0.91(0.68-1.22)	0.528
GG	48	10.0	73	15.2	0.59(0.38-0.93)	0.023
Rs11536891						
TT	410	85.4	410	85.4		
CT	64	13.3	68	14.2	0.96(0.65-1.43)	0.847
CC	6	1.3	2	0.4	4.59(0.87-24.25)	0.073

[a]Data were analyzed by unconditional logistic regression and adjusted for sex, age and smoking status

Table 3(on next page)

Stratified analysis between TLR4 rs1927914 genotypes and ESCC risk

Table 3 Stratified analysis between TLR4 rs1927914 genotypes and ESCC risk.

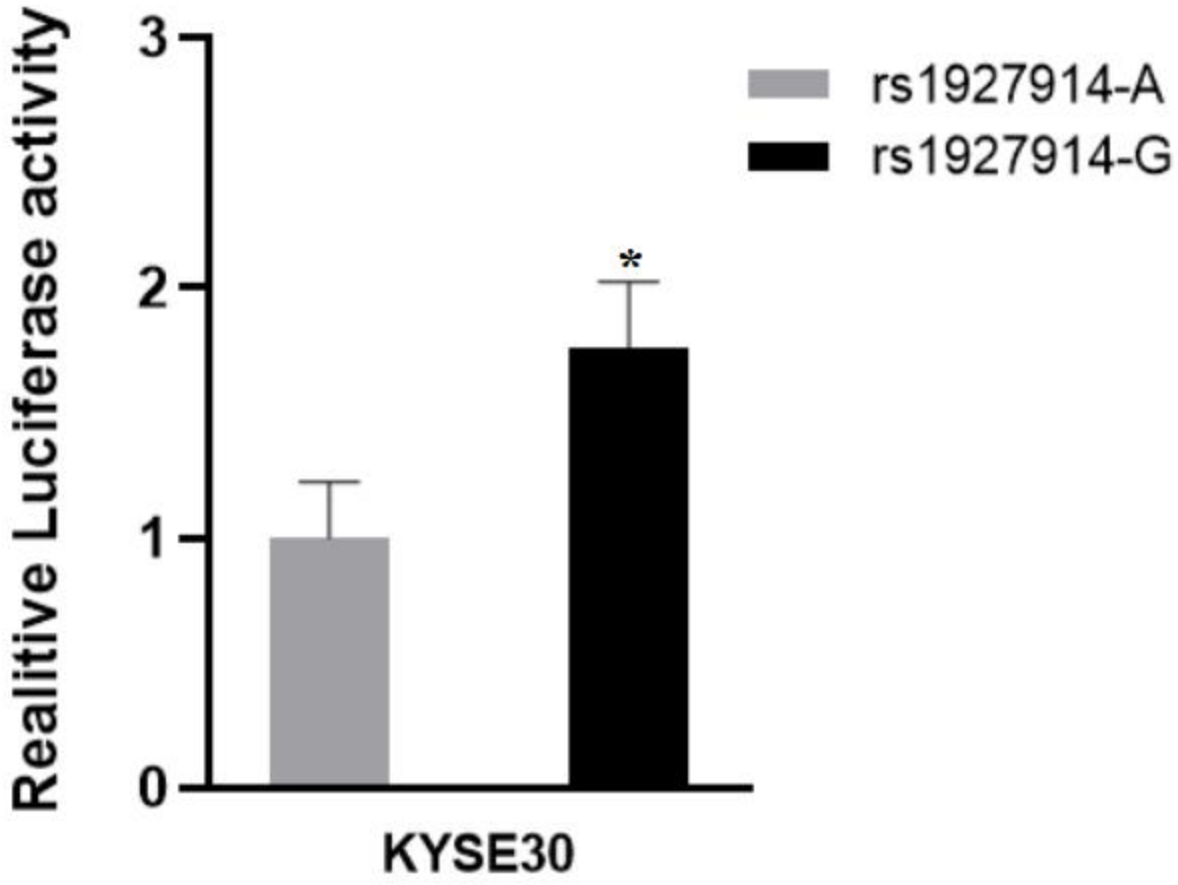
Variables	Genotypes (Cases/Controls)			GG/AA model	GA/AA model
	AA	GA	GG	OR (95%CI)[a]	OR (95%CI)[a]
Sex					
Male	195/142	237/197	48/63	0.67(0.41-1.09)	0.95(0.69-1.32)
Female	35/27	38/41	4/10	0.31(0.09-1.11)	0.73(0.37-1.44)
Age					
≤50	38/33	34/52	11/15	0.53(0.18-1.55)	0.55(0.26-1.17)
＞50	157/136	203/186	37/58	0.59(0.36-0.97)*	1.00(0.73-1.38)
Smoking status					
Non-smoker	73/109	86/170	12/54	0.36(0.18-0.73)*	0.76(0.51-1.13)
Smoker	122/60	151/68	36/19	0.93(0.49-1.76)	1.12(0.73-1.71)
Pack year of smoking					
≤25	49/28	61/35	13/6	1.26(0.43-3.68)	0.98(0.53-1.84)
>25	73/32	90/33	23/13	0.78(0.35-1.74)	1.26(0.70-2.26)

[a]Data were analyzed by unconditional logistic regression and adjusted for sex, age and smoking status

*$P < 0.05$

Table 4(on next page)

Transient reporter gene expression assays


Realitive Luciferase activity
3
2
1
0
*
rs1927914-A
rs1927914-G
KYSE30