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Original article

The expression of Toll-Like Receptors (TLRs) in testicular cancer: A case control study

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Abstract

Background: It has been suggested that malfunction of immune system may causes testicular cancer. Recently, our understanding of innate immune system has been expanded, by discovery of "Toll-Like Receptors" (TLRs). Some studies have shown that polymorphisms of TLR2 and 4 may affect on the risk of cancer. Also, the role of TLRs 3 and 9 have been shown in apoptosis and metastasis of cancer cells in animal models.

Objective: Little information is available about the influence of innate immunity on testicular malignancy. Therefore, expression of TLRs 2, 3, 4 and 9 as main components of innate immunity has been investigated in this study.

Materials and Methods: In this case control study, TLRs gene expression was examined by RT-PCR in normal testis and testicular cancer tissues. Real time quantitative PCR (Q-PCR) analysis was used to compare the relative expression of TLRs between the samples.

Results: mRNAs of TLR 2, 3, 4 and 9 were expressed in all normal and cancer samples. Q-PCR reveals that cancer samples had stronger expression of these genes compared with normal ones.

Conclusion: It seems that the different TLRs expression in testicular cancer cells may contribute to extensive signaling pathways involved in carcinogenesis.

Key words: *Toll- like Receptors (TLRs), Testicular Cancer, Innate Immunity, NF-kappa B.*

Introduction

Cancer is a major public health problem that can torment everyone in all parts of the world (1). Testicular cancer develops in the testis, a part of the male reproductive tract which produces sperm, other male reproductive cells such as Sertoli and Leydig cells and androgens (2).Testicular cancer may be due to irregularities of germ cell development during embryogenesis (3). Imperfections during male germ cell development can lead to the formation of testicular germ cell tumors (TGCTs), which are classified as teratomas, nonseminomas and seminomas (4). On the other hand, the molecular basis of most cancers remains unclear (5). With this possibility that cancers can be eliminated by specific immune responses, it can be suggested that most of cancers may be the result of an incomplete immune response in clearing abnormal cells (6).

The innate immunity, as the first line of host defense, can recognize specific molecular patterns of microbial components via a limited number of receptors called pattern recognition receptors (PRRs) (7). These receptors can recognize pathogen-associated molecular patterns (PAMPs) and activate signal-transduction pathways that induce the expression of a variety of immune-response genes. The recently identified receptors of the Toll family appear to have a major role in the induction of inflammatory responses (8). The genes that are expressed in response to TLR

signaling encode proteins which are important in several innate immune responses (6). Up to now, ten TLR members with different roles have been identified in human (9). According to their localization, TLRs are divided into two main subgroups: Cell surface and cytosolic groups (10).

On the other hand, there are some evidences that specific chronic inflammatory diseases involving TLRs signaling can lead to cancer development (11, 12). Also, high expression of some TLRs has been reported in many tumor cells, or tumor cell lines (13). In addition, numerous recent studies indicated that nuclear factor -kappa B (NF-kB) is found in a number of human malignancies (14, 15). NF-kB is one of principal transcription factors activated by TLRs signaling pathways (6). So it seems that NF-kB signaling pathway may be a link between TLRs, chronic inflammation and tumor development (16).

To date, the alteration in TLRs gene expression was not studied in human testicular cancers so we decided to study and compare gene expression of some TLRs in the normal and testicular cancer tissues. Four members of the TLRs family were selected based on their function, ligands and their location in cell (TLR2, 3, 4 and 9). TLR2 and 4 are members of cell surface group.TLR2 and its associated receptors (TLR1 and TLR6), are mainly involved in the detection of molecules derived from Gram-positive bacteria, fungi (zymosan) and synthetic lipoproteins (17-19). TLR4 recognizes lipopolysaccharide (LPS) from Gram negative bacteria (20).

In contrast, TLR3 and 9 are cytosolic TLRs. TLR3 recognizes RNA from double-stranded viruses, while TLR9 recognize sun methylated CpG DNA found richly in prokaryotic genomes and DNA viruses (21, 22). Actually, one of the most important aspects of our selection was diversity in ligand recognition (Gram negative and positive bacteria and viruses) and cell localization (cell surface and cytosolic) of these TLRs. Therefore, expression of TLRs 2, 3, 4 and 9 as main components of innate immunity has been investigated in this study.

Materials and methods

Samples collection

In this case control study, the normal testis samples (Control group) were obtained from ten men who underwent testis surgery for benign reasons such as testicular sperm extraction (TESE). Cancer samples were obtained from ten men who underwent orchiectomy because of seminoma (Case group). The samples were collected at Royan Institute, Laleh Hospital and Shariati Hospital of Tehran, Iran. Exclusion criteria were history of infection or congenital disorders of male reproductive tract. All procedures were approved by the Royan Ethics Committee. Written informed consent was obtained from all participants prior to the collection of tissue samples.

Tissue samples were immediately collected after surgeries. For genomic studies, all samples were immediately coated by RNAlater (Ambion, Huntington, UK) and then transported to the laboratory. Samples after cutting at 5mm were transferred to 2ml cryovial tubes (Greiner Bio-One, Frickenhausen, Germany) and then immersed in liquid nitrogen containers for 30 seconds. Finally, tissue samples were stored at -70°C until use in laboratory.

RNA isolation, cDNA synthesis and reverse transcription PCR (RT-PCR)

After thawing the frozen samples, tissues were removed from RNAlater and then total RNA was extracted using TRI-Reagent (Sigma, Pool, UK) according to the manufacturer's instructions that we used in our pervious study (23). Total RNA was treated with DNase I (Fermentas, Sanktleon-rot, Germany) to remove genomic DNA contamination from samples. First-strand cDNA synthesis was performed using oligodT primers and the Superscript II reverse transcriptase system (Fermentas, Germany). Negative RT controls were prepared without inclusion of the enzyme (non-reverse transcriptase controls, RT controls).

The RT-PCR was performed by combining cDNA, Platinum Blue PCR Super Mix (Invitrogen, Paisley, UK) and the forward and reverse primers for TLR2, 3, 4 and 9 (Metabion, Martinsried, Germany). The forward and reverse primer sequences used are depicted in table I. The amplification was continued for 40 cycles under the following conditions: 5 minutes at 95°C for initial denaturation, followed by 39 cycles of 45 seconds at 95°C, 45 seconds at 58-60°C (different temperature for different TLRs, Table I) and 45 seconds at 72°C. Non-

template water controls were also included to ensure lack of reagent DNA contamination (negative control). Furthermore, endometrial samples were used as positive control (23).

Beta-actin (β-actin) was used as Housekeeping gene. RT-PCR was performed to detect gene expression of TLR2, 3, 4 and 9 in normal and cancer testis samples. After PCR, all samples were transferred on 1.7% agarose gel (Sigma, UK) then electrophoresis was performed with 1x TAE buffer (Invitrogen, UK) at 95 V for 40-50 min. Results were illustrated by using an ultraviolet trans illumination and digital images were captured by Gel documentary machine (Carestream, Berlin, Germany). The PCR products were sequenced to confirm the identity of the amplified product.

Quantitative real time PCR (QPCR)

Quantitative real-time PCR (Q-PCR) was performed in triplicates with the constructed cDNAs and the same primers that were used in PCR reactions (Table I). SYBR Green Jump Start Taq Ready mix (Sigma) master mix [containing 10 µl SYBR Green, 7 µl of water, 1 µl of each primers (20 pmol/µl) and 1 µl of cDNA] was added to each well of PCR plate and amplification was performed under the following conditions: 50 cycles of 95°C for 30s, 58-60°C for 30s and 72°C for 30s (24).

Results were analyzed using Applied Biosystems SDS 7000 (Applied Biosystem, Foster, USA). The Quantitative PCR data were analyzed using the comparative CT method (25). Briefly, the difference in cycle times, ΔCT was determined as the difference between the tested gene and the reference housekeeping gene, Human ß-actin. We then obtained ΔΔCT by finding the difference between groups. The fold change was calculated as

$$FC = 2^{-\Delta\Delta CT}$$

All experiments included negative controls (no cDNA).

Statistical analysis

The results were expressed as mean±SEM. Statistical analysis was performed by using student's group t-test. P<0.05 was considered as significant.

Results

TLR2, 3, 4 and 9 expressions in the normal and cancer testis samples

Our findings indicate that TLR2,3,4 and 9 genes are expressed in all normal and testicular cancer specimens. All amplified products were of the predicted size and control experiments with non-reverse transcriptase RNA of each sample confirmed that there was no contamination of genomic DNA in the samples. Compare to housekeeping gene expression, it seems that the TLRs gene expression was more in cancer samples than normal tissues especially for TLR2 and 4 (Figure 1).

Quantitative expression of TLR2, 3, 4 and 9 between normal and cancer testis samples

Q-PCR analysis was used to investigate the relative expression of these TLR genes to compare between normal and cancer testis tissues. Although cancer samples showed significantly stronger expression of all examined TLRs compared to normal ones, this difference was more significant for TLR2 and 4 (Figure 2).

Table I. Sequence of TLRs primers used in the current investigation. Data obtained from other reports are referenced.

Gene	Forward primer	Reverse primer	Annealing temperature (°C)	Product Size (bp)	References
TLR2	5'-TCGGAGTTCTCCCAGTTCTCT-3'	5'-TCC AGTGCTTCAACCCACAA-3'	59	175	Aflatoonian et al. (2007)
TLR3	5'-GTATTGCCTGGTTTGTTAATT GG-3'	5'-AAGAGTTCAAAGGGG GCACT -3'	60	150	
TLR4	5'-CGTGGAGAC TTG GCCCTA AA -3'	5'-TTCACACCTGGATAAATCCAGC-3'	59	301	Aflatoonian et al. (2007)
TLR9	5'-TTCCCT GTAGCTGCTGCTC-3'	5'-ACAGCCAGTTGCAGTTCACC-3'	58	207	
B-actin	5'-CAAGATCATTGCTCCTCCTG-3′	5'-ATCCACATCTGCTGGAAGG-3'	60	90	Aboussahoud et al. (2010)

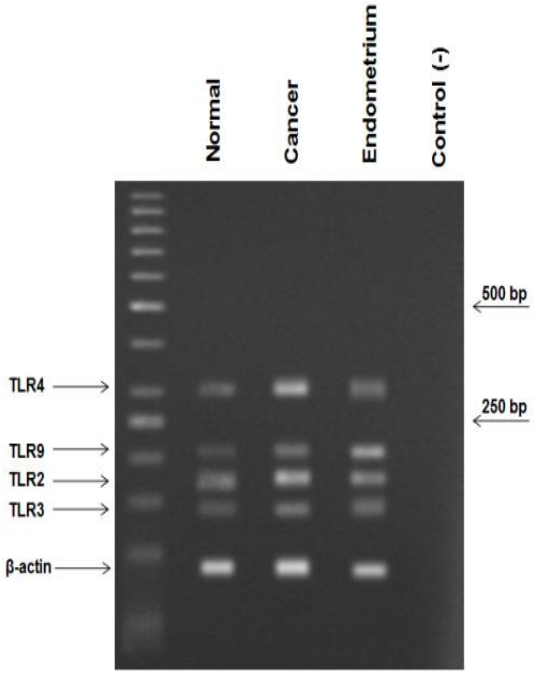

Figure 1. Results of RT-PCR for TLRs 2, 3, 4 and 9 mRNA expression in human normal and cancerous testis tissues. β- actin was used as Housekeeping gene. Also, endometrial samples and non-template water were used as positive and negative controls.

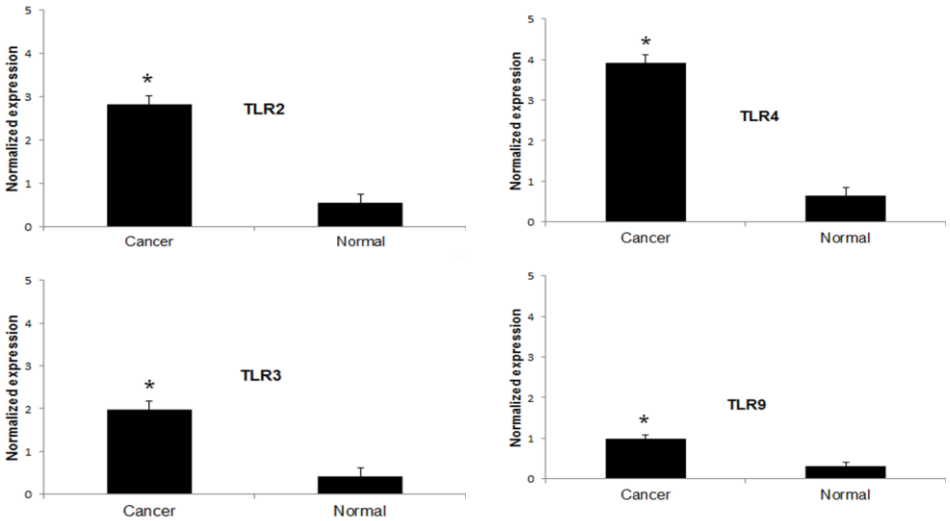

Figure 2: Mean ± SEM of normalized expression values for TLR 2, 3, 4 and 9 genes in human normal and cancerous testis tissues. *The level of statistical significance was set at $p<0.05$ and data was analyzed using student's group t-test (n=10).

Discussion

The present study revealed that the genes of TLRs 2, 3, 4 and 9 were expressed in normal and cancerous testis tissues. However, their expressions were higher in testicular cancer tissues. As testis is an organ that produces sperm, it is very important to protect spermatozoa from microbes during its formation, maturation, transit and storage (26). Our finding due to the expression of some TLRs in normal testis tissue may provide supporting evidence that TLRs as members of innate immunity play important role in the testis protection against microbial pathogens (27).

In addition, higher expression of TLR2, 3, 4 and 9 in seminoma in comparison to normal testis tissues in present study is in consistence with other studies which revealed elevated expression of some TLRs in other malignancies such as; Colon cancer, Breast

cancer, Gastric cancer, Ovarian cancer and Prostate cancer (28-32). There is one potential explanation for higher expression of these TLRs in cancerous testis tissues. Since TLRs signaling pathways lead to the activation of NF-kB and some other studies have directly demonstrated that NF-kB is often found in a number of human malignancies, it can be suggested that NF-kB may play an essential role in TLRs-induced tumorigenesis when TLRs are increased (14, 33).

Conclusion

Although this study revealed significant changes in some TLRs expression between normal and cancerous testis tissues, we could not draw any definite conclusion because of small sample size. One potential explanation was suggested for the alteration in TLRs gene expression in testicular cancer: this increase may be one of causative factors in tumorogenesis because TLRs activate NF-kB. Further studies are recommended to evaluate the difference in protein expression level, to confirm this suggestion by evaluating the expression of NF-kB, and also to investigate other TLRs gene expression in cancerous testis tissue.

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Conflict of interest

The authors declare no conflict of interest in this article.

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