A Pilot Study on Prostate Cancer Risk and Pro-Inflammatory Genotypes: Pathophysiology and Therapeutic Implications

C.R. Balistreri[1], C. Caruso [1], G. Carruba[1,2], V. Miceli[1,2], I. Campisi[2], F. Listì[1], D. Lio[1], G. Colonna-Romano[1], and G. Candore[1,*]

[1]*Immunosenescence Group, Department of Pathophysiology and Biomedical Methodologies, University of Palermo, Palermo, Italy and* [2] *Experimental Oncology, Department of Oncology, ARNAS-Civico, Palermo, Italy*

Abstract: Host genetic factors are crucial risk determinants for many human cancers. In this framework, an interesting model is represented by prostate cancer (PC), which is featured by a complex pathophysiology with a strong genetic component. Multiple genes seem to influence PC risk and several single nucleotide polymorphisms (SNPs) of candidate genes modifying PC susceptibility have been identified. It is noteworthy the potential association of common SNPs in pro-inflammatory genes with PC risk, since chronic inflammation is assumed to play a key role in prostate carcinogenesis. With the aim to identify candidate genes as an experimental basis to develop new strategies for both prevention and treatment of PC, we have investigated the potential role of common SNPs of a gene cluster (TLR4, TLR2, PTGS2 and 5-Lo), involved in innate and inflammatory response, in PC cases, age-matched controls and centenarians from Sicily. Six SNPs were genotyped and their association with PC risk determined. Statistical analysis evidenced a significant association of some pro-inflammatory gene SNPs with an increased risk of PC. Furthermore, significant differences were observed comparing the three groups in the combined presence of a "high responder" pro-inflammatory profile. Overall, the present results suggest the likely association of these SNPs and PC risk, clearly motivating the need of larger studies to confirm the role of these genes in PC development and/or progression.

Keywords: Prostate cancer (PC), inflammation, genetics, TLR4, TLR2, PTGS2, 5-LO, SNP.

INTRODUCTION

Host genetic factors may play a critical role in the pathophysiology of many human cancers [1]. An interesting model is prostate cancer (PC), which represents the second principal cause of cancer-related deaths in Western males. It is likely that prostate cancers can take decades to progress to a clinically manifest disease, making their diagnosis and treatment difficult [2,3]. Over the last two decades, serum prostate-specific antigen (PSA) has been considered a valuable tool for PC surveillance. However, the age-adjusted mortality rates of PC have remained fairly constant. At the present, there is an overall consensus to consider PSA a nonspecific marker to diagnose PC. Increased serum levels of PSA have frequently been observed in several other disease states of the prostate gland than cancer [4-6]. On the other hand, different biomarkers of various stages of PC development have recently been identified as promising tools for PC diagnosis [5,7,8].

Currently, the evidence for a strong genetic background in PC development is compelling. Several case-control studies, twin studies, and segregation analyses have consistently found genetic susceptibility to PC and its clinical outcome [6,9-11]. Several candidate genes of PC risk have been identified and their interaction, either additively or epistatically, has been hypothesized. Furthermore, common single nucleotide polymorphisms (SNPs) of a series of low penetrance alleles, referred to as "genetic modifier alleles", are likely to play an important role in PC susceptibility. The list of these variants is long and the major pathways under examination include those involved in androgen action, DNA repair, carcinogen metabolism and inflammation [6]. There is also a general consensus that specific combinations of these variants, in the appropriate environmental settings, have the potential to profoundly affect the risk of PC development.

In the present study, we have assessed the potential role of some common SNPs of the Toll-Like Receptor (TLR)-4,-2, cyclooxygenase-(COX)-2 (described as PTGS2), and 5-lipoxygenase (5-Lo) genes, all encoding molecules involved in innate immunity and inflammatory response, in PC development. Genetic variants of these genes, mostly SNPs, determine pro-inflammatory genotypes known to modulate and increase risk of chronic inflammatory states and tissue damage and to create a tissue milieu favoring carcinogenesis, there by promoting development and progression of PC. In this study, we have compared selected SNPs in PC patients, in healthy controls, and in a second control group of centenarians, "exceptional individuals", who have been able to escape major common age-related diseases, including cancer [12-14]. The results of this study could help understand the pathophysiology of PC, further clarifying the association between the genetics of inflammation and PC susceptibility. In addition, they could allow the definition of a PC high risk profile, eventually leading to both early recognition of individuals at risk of disease and identification of potential molecular targets for prevention and/or treatment.

SUBJECTS AND METHODS

Patients and Controls

The study included 50 Sicilian patients with PC at the time of their admission to the Department of Oncology of Palermo ARNAS-Civico Hospital (age range 60–80 years). Controls were 125 age-matched male Sicilians in good health according to their clinical history and blood tests (complete blood cell count, erythrocyte sedimentation rate, glucose, urea nitrogen, creatinine, electrolytes, C reactive protein, liver function tests, iron, proteins). A second control group consisted of 55 male centenarians (>99 years), whose age was confirmed from records at the city hall and/or church registries. No cancer or other age-related diseases were clinically detectable in the centenarians, although some had reduced auditory and visual acuity. Because immigration and intermarriage have historically been rare in the last hundred years, the ethnicity of all participants was established by all four grandparents having been born in Sicily. The study received approval from local ethic committees and all participants gave their informed consent.

*Address correspondence to this author at the Department of Pathophysiology and Biomedical Methodologies, University of Palermo, Corso Tukory, 211, 90134, Palermo, Italy; Tel: +39 0916555931; Fax: +39 0916555933; E-mail: gcandore@unipa.it; crbalistreri@unipa.it

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Genotyping

The study material consisted of DNA samples. The DNA samples of cancer cases were obtained by prostate tissue placed into a suitable volume of RNA-*later* (RNA Stabilization Reagent, Applied Biosystems, California, U.S.A.), used to purify genomic DNA and total RNA simultaneously from each single biological sample with All-Prep DNA/RNA Mini Kit (Qiagen, Dusseldorf, Germany). Tumor samples were genotyped for SNPs of the genes selected in this study. In particular, six SNPs, located in the promoter and coding regions of TLR-4,-2, PTGS2, 5-Lo genes, were analysed (Table **1**).

Table 1. Genes (Accession Number), SNPs (Accession Number), and Substitutions Investigated in the Study

Genes	SNPs
TLR4 (NM-138554.1)	+896A/G (Asp299Gly; rs4986790) +1196C/T (Thr399Ile; rs4986791)
TLR2 (NM 003264)	+2408 G/A (Arg753Gln; rs5743708) +2029C/T (Arg677Trp; no rs available designation)
PTGS2 (NM-000963) (Cox-2 gene)	-765G/C (rs20417)
5-Lo (NM000698)	-1708 G/A (no rs available designation)

The DNA samples of the two control groups had been previously extracted from blood samples and genotyped for the six SNPs. The procedure for detecting the +896A/G and +1196C/T TLR4 and +2408 G/A and +2029C/T TLR2 SNPs (Table **1**) was based on Restriction Fragment Length Polymorphism-PCR (RFLP-PCR), restriction cleavage with Nco I, Hinf I, Mwo I and Mps I respectively (New England Biolabs, USA), and separation of DNA fragments by electrophoresis, as previously described [15,16]. The genotyping of -765G/C PTGS2 SNP was performed using RFLP-PCR and with Aci I (New England Biolabs, USA) restriction enzyme, as previously reported [17]. An allele refractory mutation system (ARMS)-PCR was utilised to determine the -1708 G/A 5-Lo SNP, as previously described [17].

Statistics

Allelic and genotypic frequencies were evaluated by gene count. The data were tested for the goodness of fit between the observed and expected genotype frequencies according to Hardy-Weinberg equilibrium, by χ^2 test. Significant differences in frequencies among the three groups were calculated by χ^2 (by 3x3, 3x2 and 2x2 tables, where appropriate). Furthermore, Odds Ratios (OR) with 95% Confidence Intervals (CI) and their significance were calculated.

RESULTS

Tables **2** and **3** show the genotype distributions, allele frequencies and the OR (95% CI) values of the TLR4 and TLR2 gene SNPs analysed in this study. Both the genotypes and the alleles of +896A/G TLR4 SNP were significantly differently distributed among the three groups (p=0.006 and p=0.001, respectively; Tables **2** and **3**).

In particular, a significant difference was observed comparing the genotype distributions and allele frequencies of this SNP in patients and centenarians (p=0.002 and p=0.0008, respectively; OR=16.8 (2.1-129.6; p=0.0008) (Tables **2** and **3**). No significant differences were instead detected in the genotype distributions and allele frequencies of this SNP between patients and age-matched controls (Tables **2** and **3**). In contrast, no significant differences were detected in the genotype distributions and allele frequencies of +1196C/T TLR4 SNP (Tables **2** and **3**).

Analyzing the data of +2408G/A TLR2 SNP among the three cohorts, a significantly different distribution was revealed of both the genotypes and the alleles (p=0.0007 and p=0.0003, respectively) (Tables **2** and **3**). In particular, a significant difference was obtained comparing patients and centenarians (p=0.004 and p=0.002 respectively; OR=8.3 (1.8-37.2) p=0.0027), while no significant differences were detected between patients and age-matched controls (Tables **2** and **3**). We did not observe any significant difference for both genotype distributions and allele frequencies of the +2029C/T TLR2 SNP (Tables **2** and **3**).

Tables **4** and **5** illustrate the genotype distributions, allele frequencies and the OR (95% CI) values of -765G/C PTGS2 and -1708G/A 5-Lo SNPs in three groups enrolled in the study. Both the genotypes and the alleles of -765G/C PTGS2 SNP were differently distributed among the three groups (p=0.01 and p=0.05, respectively) (Tables **4** and **5**). In particular, comparing the genotype distributions and the allele frequencies of this SNP of patients and centenarians, we observed a significant difference (p=0.03 and p= 0.01, respectively; OR=2.1 (1.1-3.9 p=0.01) (Tables **4** and **5**). In contrast, no significant differences were detected in the genotype distributions and allele frequencies of this SNP between patients and age-matched controls (Tables **4** and **5**). Concerning the -1708G/A5-Lo SNP, both the genotypes and the alleles were differently distributed among the three groups (p=0.0004 and p=0.0007, respectively) (see Tables **4** and **5**). In particular, there was a significant difference in the genotype distributions and the allele frequencies of this SNP between patients and centenarians (p=0.008 and respectively p =0.0005; OR=5.17(2-13.3; p=0.0005) and between patients and controls (p=0.003 and p=0.005 respectively; 2.4(1.3-4.5; p=0.005) (Tables **4** and **5**).

In summary, these pro-inflammatory alleles were overrepresented in patients compared to healthy men and underrepresented in centenarians compared to younger healthy controls.

To identify a pro-inflammatory genetic risk profile, we evaluated the frequency of "+896A+TLR4/+2408G+TLR2/+2029C+TLR2/-765G+PTGS2/-1708A+5-Lo" high pro-inflammatory" genotype among the three cohorts. By comparing it with frequency of all other combinations, we observed that this "high responder" genotype was differently distributed among the three groups (p=0.002), in particular between PC patients and centenarians (36% vs. 11%, p=0.002; OR=4.59 (1.6-12.8), p=0.004) (see Table **6**).

DISCUSSION

PC is the most common non-skin malignant cancer in Western male populations. Its incidence increases rapidly in men over 50 years of age [2]. The development of PC is based on the interaction between genetic factors and the host exposure to environmental factors, such as infectious agents, dietary carcinogens and hormonal imbalances [6,18-22]. In this complex situation, chronic inflammation seems to play a key role. The chronically altered prostatic milieu is characterized by the activation of signaling pathways that represent a possible link between inflammation and carcinogenesis. Many of these factors play a dual role in the process, promoting neoplastic progression but also facilitating cancer prevention [6,18-21].

To date, there are no adequate biomarkers to guide PC prognosis and treatment [3-5]. Several studies have focused on detecting candidate genes, in particular genes involved in DNA repair, carcinogen metabolism and inflammation [6], which are associated with genetic susceptibility to PC and the clinical outcome of this disease [10,11]. Genetic variants of these genes,

Table 2. Genotype Distributions of +896A/G (Asp299Gly) and +1196C/T (Thr399Ile) Polymorphisms of TLR4 Gene, and of +2029C/T (Arg677Trp) and +2408 G/A (Arg753Gln) Polymorphisms of TLR2 Gene in 50 PC Patients, 125 Age-Matched Controls and 55 Centenarians from Sicily

SNPs	Genotypes	PC		Age-Matched Controls		Centenarians		P1[a] (3x3 table)	P2[b] (3x2 table)	P3[c] (3x2 table)
		N	%	N	%	N	%			
+896A/G-TLR4	A/A	49	98	111	88.8	41	75	0.006[*]	NS	0.002[**]
	A/G	1	2	13	10.4	12	21			
	G/G	0	0	1	0.8	2	4			
+1196C/T-TLR4	C/C	48	96	118	94	49	89	NS	NS	NS
	C/T	2	4	7	6	5	9			
	T/T	0	0	0	0	1	2			
+2029C/T TLR2	CC	45	90	120	96	47	85	NS	NS	NS
	CT	5	10	5	4	7	12			
	TT	0	0	0	0	1	3			
+2408 G/A TLR2	GG	49	98	115	92	42	76	0.0007[§]	NS	0.004[§§]
	GA	1	2	10	8	10	18			
	AA	0	0	0	0	3	6			

All genotypes were in Hardy-Weinberg equilibrium. [*]Significant differences by $\chi 2$ test in genotype distribution of +896 A/G TLR4 SNP among the three groups were found. [**]A significant p value was also observed between patients and centenarians, analyzing by $\chi 2$ test the genotype distribution of this SNP. [§]The genotype distributions of +2408G/A TLR2 SNP were significantly distributed among three groups, and [§§]between patients and centenarians.

P1[a] = significance values calculated by $\chi 2$ test , analyzing the data of these SNPs among the three groups

P2[b] = significance values calculated by $\chi 2$ test, analyzing the data of these SNPs between patients and age-matched controls

P3[c] = significance values calculated by $\chi 2$ test, analyzing the data of these SNPs between patients and centenarians

Table 3. Allele Frequencies of +896A/G (Asp299Gly) and +1196C/T (Thr399Ile) Polymorphisms of TLR4 Gene, and of +2029C/T (Arg677Trp) and +2408 G/A (Arg753Gln) Polymorphisms of TLR2 Gene in 50 PC Patients, 125 Age-Matched Controls and 55 Centenarians from Sicily. (2x2 Comparisons Between the Different Groups with Odd Ratio (OR) and 95% Confidence Interval)

SNPs	Alleles	PC		Age-Matched Controls		Centenarians		P1[a] (3x3 table)	P2[b] (3x2 table)	P3[c] (3x2 table)	OR (95% CI)
		N	%	N	%	N	%				
+896A/G TLR4	+896A	99	99	235	94	94	85	0.001[*]	NS	0.0008 [**]	6.3 (0.8-48.5; p=0.08)a
	+896G	1	1	15	6	16	15				2.6 (1.2-5.6; p=0.01)[b]
											16.8(2.1129.6;p=0.0008)[c]
+1196C/T TLR4	+1196C	98	98	243	97	103	94	NS	NS	NS	_
	+1196T	2	2	7	3	7	6				_
											_

(Table 3) Contd….

SNPs	Alleles	PC		Age-Matched Controls		Centenarians		P1[a] (3x3 table)	P2[b] (3x2 table)	P3[c] (3x2 table)	OR (95% CI)
		N	%	N	%	N	%				
+2029C/T TLR2	+2029C	95	95	245	98	101	92	NS	NS	NS	–
	+2029T	5	5	5	2	9	8				–
											–
+2408G/ATLR2	+2408G	98	98	240	96	94	85	0.0003[§]	NS	0.002[§§]	2(0.4-9.4; p=0.5)[a]
	+2408A	2	2	10	4	16	15				4(1.7-9.3; p=0.0008)[b]
											8.3(1.8-37.2; p=0.0027)[c]

[*]A significant different frequency of this SNP among three groups and [**]in PC patients respect to centenarians was observed. [§]Besides, the allele frequencies of +2408G/A TLR2 SNP were found significantly distributed among three cohorts and [§§]between patients and centenarians.

P1[a] = significance values calculated by $\chi 2$ test, analyzing the data of these SNPs among the three groups

P2[b] = significance values calculated by $\chi 2$ test, analyzing the data of these SNPs between patients and age-matched controls

P3[c] = significance values calculated by $\chi 2$ test (3x2 table), analyzing the data of these SNPs between patients and centenarians.

In the last column we reported the OR values calculated for higher frequent alleles in PC patients vs. age-matched controls[a] , age-matched controls vs. centenarians[b]; PC patients vs. centenarians[c]

Table 4. Genotype Distributions of -765 G/C Polymorphism of PTGS2 Gene and of -1708 G/A Polymorphism of 5-LO Gene in 50 PC Patients, 125 Age-Matched Controls and 55 Centenarians from Sicily

SNPs	Genotypes	PC		Age-Matched Controls		Centenarians		P1[a] (3x3 table)	P2[b] (3x2 table)	P3[c] (3x2 table)
		N	%	N	%	N	%			
-765G/C PTGS2	GG	31	62	65	52	27	49	0.01[*]	NS	0.03[**]
	GC	15	30	46	37	13	24			
	CC	4	8	14	11	15	27			
-1708G/A5-Lo	GG	30	60	98	78	49	89	0.0004[§]	0.003[§§]	0.008[§§§]
	GA	17	34	27	22	6	11			
	AA	3	6	0	0	0	0			

All genotypes were in Hardy-Weinberg equilibrium. [*]Significant differences by $\chi 2$ test in genotype distribution of -765G/C PTGS2 SNP among the three groups were found. [**]A significant p value was also observed between patients and centenarians, analyzing by $\chi 2$ test the genotype distribution of this SNP. [§]The genotype distributions of -1708G/A5-Lo SNP were significantly distributed among three groups, and [§§§]between patients and centenarians and [§§]between patients and age-matched controls.

P1[a] = significance values calculated by $\chi 2$ test, analyzing the data of these SNPs among the three groups

P2[b] = significance values calculated by $\chi 2$ test, analyzing the data of these SNPs between patients and age-matched controls

P3[c] = significance values calculated by $\chi 2$ test, analyzing the data of these SNPs between patients and centenarians

Table 5. Allele Frequencies of -765 G/C Polymorphism of PTGS2 Gene and of -1708 G/A Polymorphism of 5-LO Gene in 50 PC Patients, 125 Age-Matched Controls and 55 Centenarians from Sicily. 2x2 Comparisons Between the Different Groups with Odd Ratio (OR) and 95% Confidence Interval

SNPs	Alleles	PC		Age-matched Controls		Centenarians		P1[a]	P2[b]	P3[c]	OR (95% CI)
		N	%	N	%	N	%	(3x3 table)	(3x2 table)	(3x2 table)	
-765G/C PTGS2	-765G	77	77	176	70	67	61	0.05*	NS	0.01**	1.4(0.8-2.4; p=0.2)[a]
											1.5(0.9-2.4; p=0.09)[b]
	-765C	23	23	74	30	43	39				2.1 (1.1-3.9; p=0.01)[c]
-1708G/A5-Lo	-1708G	77	77	223	89	104	95	0.0007§	0.005§§	0.0005§§§	2.4(1.3-4.5; p=0.005)[a]
	-1708A	23	23	27	11	6	5				2.09(0.8-5.2; p=0.1)[b]
											5.17(2-13.3;p=0.0005)[c]

*An over-expression of -765G pro-inflammatory allele among the three groups and **in PC patients respect to centenarians was observed. §Besides, the allele frequencies of -1708G/A5-Lo SNP were found significantly differently distributed among three cohorts, and §§between patients and age-matched controls and §§§between patients and centenarians.

P1[a] = significance values calculated by $\chi 2$ test, analyzing the data of these SNPs among the three groups

P2[b] = significance values calculated by $\chi 2$ test, analyzing the data of these SNPs between patients and age-matched controls

P3[c] = significance values calculated by $\chi 2$ test, analyzing the data of these SNPs between patients and centenarians

In the last column we reported the OR values calculated for higher frequent alleles in PC patients vs. age-matched controls[a] , age-matched controls vs. centenarians[b]; PC patients vs. centenarians[c]

Table 6. The Frequency of +896A+TLR4/+2408G+TLR2/+2029C+TLR2/-765G+PTGS2/-1708A+5-Lo"High Responder"(Pro-Inflammatory) Genotype Among the Three Cohorts

	+896A+TLR4/+1196C+TLR4/+2408G+TLR2/+2029C+TLR2/-765G+PTGS2/1708A+5Lo "high-responder" (pro-inflammatory) Genotype	Other Genotypes
Patients (N=50)	18	32
Age-matched Controls (N=125)	44	81
Centenarians (N=55)	6	49*

* Significant differences by $\chi 2$ test (3x3 table) in this genotype frequency among the three groups and between patients and centenarians (p= 0.002) were observed.

primarily SNPs, may modulate and be associated with increased chronic inflammation and carcinogenesis of the prostate gland. In addition, they may operate in combination to create a "risk profile" [6]. It is also generally believed that such SNPs can modify the effectiveness of therapies of various age-related diseases, including PC. Accordingly, SNPs associated with polygenic traits represent a major goal of many pharmaceutical companies to discover novel therapeutic molecular targets [23,24].

Based on the above considerations, we have analyzed the role in PC of six SNPs of the TLR4, TLR2, PTGS2 and 5-Lo genes, encoding molecules primarily implicated in innate immunity and inflammatory response. Our results indicate that pro-inflammatory SNPs of some of these genes are significantly associated with PC. Furthermore, we identified a risk profile in PC patients compared to centenarians, used in our study as an additional control group of "exceptional individuals" able to avoid major common age-related diseases, including cancer [12-14]. As we have previously demonstrated that alleles associated with age-related diseases are not

included in the genetic profile favoring longevity [25,26], a genetic background promoting pro-inflammatory responses may play opposite roles in age-related diseases, including PC, and in longevity.

On the basis of data reported herein, some inference on their translational impact can be drawn. The presence of "high-risk" alleles of the PTGS2 and the 5-LO genes may suggest the possibility of developing preventive measures using specific inhibitors of eicosanoids and/or their enzymes. For people who do not respond to (or comply with) NSAID therapy, other more sophisticated preventive approaches may be possible [6], including the use of agonists of TLR receptors in subjects who are carriers of +896A/G TLR4 and +2408 G/A TLR2 SNPs. The aim of such an approach would be to block the TLR-NF-κB signaling pathway required for induction of inflammatory responses and the release of pro-inflammatory mediators. This approach might reduce the risk of PC development [6,27]. Another possible therapeutic intervention in subjects with pro-inflammatory alleles of TLR4 and TLR2 genes might be antibody-mediated stimulation of TAM receptors involved in the inhibition of the inflammatory response. The sequential induction of this pathway, and its integration with upstream TLR and cytokine signaling networks, may limit the inflammatory response and maintain innate immune system homeostasis. A better understanding of the regulatory mechanisms of this cascade may have important implications for therapeutic intervention in human immune disorders [28,29].

Although our study presents some limitations primarily related to the relatively small number of patients and controls, it compares subjects belonging to a very homogeneous population from Sicily. Thus, our data are likely to be at least as reliable as those from studies performed on larger cohorts of patients from Northern Europe or the United States, which are ethnically matched to Caucasians in general. Conversely, as discussed by Caruso *et al.* [6], the use of centenarians as "super-controls" might enhance the possibility of identifying the genetic profile characterizing susceptibility and/or resistance to PC. It may be argued that a Bonferroni-type adjustment should be performed to correct for testing multiple polymorphisms. However, we feel that this correction is too stringent and has the potential to ignore important observations [30]. In any case, further investigations on larger homogenous populations are needed to corroborate the present evidence.

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ABBREVIATIONS

ARMS-PCR	=	Allele Refractory Mutation System-PCR
CI	=	Confidence Interval
COX-2	=	Cyclo-Oxygenase-2
OR	=	ODD Ratio
5-Lo	=	5-Lypoxygenase
PC	=	Prostate Cancer
PSA	=	Serum Prostate-specific Antigen
PTGS2	=	Prostaglandin E Synthase 2 gene of cyclooxygenase-2
RFLP-PCR	=	Restriction Fragment Length Polymorphism-PCR
SNP	=	Single Nucleotide Polymorphism
TAM	=	TAM receptor
TLR4	=	Toll-Like Receptor-4
TLR2	=	Toll-Like Receptor-2

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