International Journal of Clinical Oncology and Cancer Research
2017; 2(4): 93-98
http://www.sciencepublishinggroup.com/j/ijcocr
doi: 10.11648/j.ijcocr.20170204.14

The Role of Ageing, Inflammation and Maspin in the Early Stages of Prostatic Malignant Transformation

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To cite this article:

Kalin Petrov Kalchev, Alexander Ivanov Hinev, Alkwin Wanders, Alexander Georgiev Otsetov. The Role of Ageing, Inflammation and Maspin in the Early Stages of Prostatic Malignant Transformation. *International Journal of Clinical Oncology and Cancer Research*. Vol. 2, No. 4, 2017, pp. 93-98. doi: 10.11648/j.ijcocr.20170204.14

Received: June 29, 2017; **Accepted**: July 26, 2017; **Published**: August 25, 2017

Abstract: Prostate cancer (PCa) progression is an intricate step-wise process, starting from malignant transformations in the benign prostatic epithelium. The hallmark of PCa is the long period of its development from premalignant transformations in the benign prostatic epithelium towards clinically active carcinoma. Along with the age, inflammation, race and genetics, which are well-recognized risk factors for prostatic carcinoma, epigenetics play also a significant role in the initiation and progression of prostatic carcinoma. DNA methylation in the CpG gene islands is a key player in the regulation of gene expression and silencing, and significantly contributes to the disease development. Progression of cancer is associated with the loss of several tumor suppressor genes such as Maspin through mutations or epigenetic silencing. Epigenetic silencing of Maspin seems to be one of the earliest somatic change, contributing to the development of prostatic carcinoma in the human prostate.

Keywords: Prostate Carcinogenesis, Field Effect, Epigenetics, Ageing, Chronic Inflammation, Maspin

1. Introduction

Prostate cancer (PCa) is one of the major contributors to malignancy related mortality among men worldwide and represents a significant social and financial burden for each healthcare system [1, 2]. In 2017 over 161 360 new cases (19%) of prostate cancer (PCa) in the USA will be diagnosed, and estimated 26,730 (8%) men will die of this disease [1]. Despite the significant progress made into diagnostics and treatment of PCa, its etiology and biological behavior are not completely understood. Nevertheless, studies discussing PCa development have shown that the onset of prostatic adenocarcinoma is starting from a **field effect** - primary cellular insult due to the complex interplay between different biochemical, cellular, histological, genetic, and epigenetic factors which that affect cellular homeostasis and lead to the development of premalignant foci and molecular lesions, preceding histological malignant changes. Also, it was shown that the progressive accumulation of cellular alterations leads to a PCa development [3-5]. These events are characterized by the loss of tumor suppressor genes, activation of oncogenes, promotion and activation of cell proliferation and damage of the regulation of apoptosis – a hallmark of cancers.

2. Material & Methods

2.1. Sample Collection

Prostate specimens in this study were collected from 41 patients undergoing radical prostatectomy for prostatic cancer in Norrland's University Hospital, Umeå, Sweden from 2013 to 2014. Institutional review board approval was obtained for medical records review to retrospectively collect clinical and pathological data on each patient such as age (y/o), Gleason score, TNM grade, and prostate volume (PV).

2.2. Tissue Processing

We used fixed in formalin and paraffin embedded prostate cancer tissue samples. All 41 radical prostatectomy tissue samples were counterstained with hematoxylin & eosin (H&E). We assessed the degree of involvement in the tissue samples the number of normal glands, basal cell hyperplasia (BCH), atrophy, high-grade intraepithelial neoplasia (HGPIN), and prostate cancer lesions.

2.3. Immunohistochemistry (IHC)

All study slides were stained with P63/P504 to delineate the basal cell membrane and to distinguish BCH glands from some difficult HGPIN cases. Immunostaining with Maspin primary antibody (Ab-1 (#MS-1767-R7, Ready-to-use, Termo Scientific, CA, USA) was carried out. Briefly, 4-pm sections of formalin fixed, paraffin-embedded tissue were dehydrated with progressively decreased concentrations of alcohol. Next, endogenous peroxidase was blocked with 3% H202, followed by washing with PBS. The sections were first incubated with the primary antibody overnight at 4°C temperature. Secondary incubation with Dako REALTM EnVision/ HRP Rabbit/Mouseready-to-use biotinylated anti-mouse antibody was carried out for 30 minutes at room temperature. The slides were developed with 3-3'-diaminobenzidine (DAKO REALTM, Denmark) as a chromogen for 1 minute. The slides were finally dehydrated and mounted with Accu-Mount medium (Baxter, IL). The immunohistochemical staining of cells for Bcl-2 and p53 was recorded as strongly positive, weakly positive, or negative. The Ki-67-positive cells (nuclei) were counted at a 400× power. At least 1,000 cells (nuclei) were counted for each case. The Ki-67 index was generated by dividing the total number of the positive cells (nuclei) by the total number of the cells (nuclei) counted.

2.4. Evaluation of Maspin Immunostaining and Statistical Analysis

All slides were quantified using Panoramic Viewer Software (Version 1.15.2 SP2 RTM, 3DHISTECH Ltd, 2012, Budapest, HUNGARY). Maspin positive staining was identified by the presence of marked diffuse (DAB) nucleus or cytoplasm staining in the prostatic basal and luminal cells. Maspin staining was scored by its intensity on 4-tiered scale: 0-absent; 1- weak; 2-medium; 3-strong expression. The representative areas at 40× magnification were selected for each sample. At least 10 fields of each slide were evaluated and the total Maspin expression was calculated as previously described [30]. Statistical analysis was performed with SPSS 24 software.

3. Results

The mean age in our study cohort was 66,1 year old (range: 56-76 y/o). The vast majority of patients were with Gleason 7(3+4), (n=18). Interestingly, the highest mean prostate volume (PV=49.2 cc) was in the subset of patients with Gleason 6 (3+3). Maspin was found to be specifically associated with prostate epithelial cells but not with stromal tissues. Benign basal cells expressed Maspin at a low to medium level. Part of the atrophic basal cells also expressed Maspin, in a higher intensity, compared to normal glands. Although the basal cell layer in HGPIN was often disrupted, the remaining basal cells expressed Maspin in a higher intensity than normal and atrophic basal cells. As expected, in cancer lesions Maspin progressively downregulated from low-grade PCa to high-grade PCa, which correlated with the loss of basal cell layer. However, in basal cells of BCH Maspin was dramatically upregulated in all of series of prostatectomy samples (100%). Interestingly, in all BCH lesions Maspin was dramatically upregulated compared with other morphological lesions in the prostate specimens (Figure 1). We also found an association between the age, number and Maspin expression in BCH lesions *(p=0.05)*.

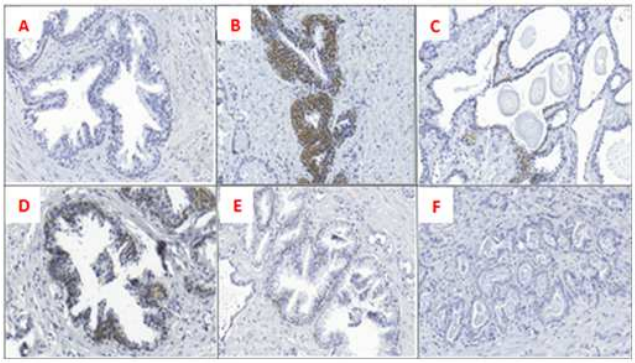

Figure 1. *Maspin expression patterns in different prostatic morphological types. A. Absent to weak Maspin staining in normal glands. B. Maspin overexpression in BCH; C. Absent to medium Maspin expression in atrophy. D. Maspin expression in HGPIN (strong to moderate). E. Maspin expression in HGPIN (low to absent). F. Absent Maspin expression in prostate cancer*

4. Discussion

Prostate cancer originates from its premalignant forerunners. Among the several proposed PCa precursors, to date high-grade prostatic intraepithelial neoplasia (HGPIN) is the only accepted putative forerunner due to its molecular, morphological and epidemiological similarities close to prostatic adenocarcinoma. Prostatic intraepithelial neoplasia (PIN) was first described in the 1960 by *McNeal* as "intraductal dysplasia" and later, in 1986, more precisely characterized by *McNeal* and *Boostwick* [6, 7]. Despite the significant progress made in the understanding of prostate cancer development, the driving mechanisms of progression from benign epithelium to pre-cancerous lesions (i.e. HGPIN) are unclear. Recent data suggest that the onset of precancerous transformations represents a mechanism, triggered by many factors, involving genetic mutations and epigenetic changes such as DNA methylation, most probably accumulated overtime. Therefore, it is important to understand whether the premalignant transformations are characterized only by some specific morphological

alterations or specific changes in gene expression might mirror the onset of pre-malignant reprogramming.

In 1999 *De Marzo et al.* proposed another plausible PCa precursor – proliferative inflammatory atrophy (PIA). PIA is frequently observed in prostatic biopsies and demonstrates genetic alterations common for both HGPIN and PCa. However, to date the role of PIA as premalignant precursor of PCa is still not proven [8]. Among the several possible precursors of prostatic adenocarcinoma like post-atrophic hyperplasia (PAH), atypical adenomatous hyperplasia and proliferative inflammatory atrophy (PIA), currently only HGPIN is an accepted PCa forerunner. HGPIN has specific morphologic features resembling the prostatic carcinoma - prominent nucleoli, nuclear enlargement, hyperchromasia and crowding. HGPIN is characterized by cellular proliferation with progressive changes in cellular phenotype and genotype, thus showing a continuum between benign prostatic epithelium and cancer. Importantly, HGPIN initiation and development is age-dependent. It was shown that the presence of the early PIN lesions can be detected as early as in the third and forth decade of male's life and its incidence is increased with age [13]. However, the process of initiation and transformation of normal prostatic epithelium towards PIN lesions is not clear and the mechanisms triggering the onset of this conversion necessitate further elucidation. According to several criteria, prostatic lesion can be considered as premalignant if: 1/ presents at an earlier stage than the neoplasm; 2/ demonstrates similarities (cellular, histological and architectural) with cancer; 3/ epidemiological relationship must be revealed [9]. In the clinical practice neither MRI, nor the computer tomography or ultrasonography can identify the presence of HGPIN in the prostate and the only method of its detection is the prostatic biopsy, associated with side effects and morbidity. Currently, markers that are widely used for the identification of HGPIN in the prostate biopsy tissue samples are AMACR and NKX3.1. It will be reasonable to ask if other specific markers might identify the early pre-malignant changes in the visibly benign prostatic epithelium. Also, it is important to understand when the early alterations in the benign epithelium occur. Studies have shown that the onset of these events begins at a particular point of male's life. The development of prostatic adenocarcinoma starts from the benign epithelium and possibly, the specific gene expression in the benign cells might mirror the onset of the initial cellular insult. The prostate is considered as vulnerable organ where the normal epithelium, under the influence of different exogenous and endogenous factors undergoes transformations, giving rise to specific genetic and cellular alterations. Based on the zonal variation in cell morphology and phenotype, studies demonstrated that the prostate carcinogenesis is characterized by predilection sites. The zonal specificity of basal cells between peripheral zone (PZ) and transition zone (TZ) were demonstrated [10]. Because PZ is a predominant site for prostatic carcinoma development (85%) versus TZ (31%), it was suggested that in contrast to TZ, PZ possess more different biological properties and certain unique epigenetic signatures [11].

Aging, Inflammation, DNA Methylation and Prostate Cancer Development

Significant number of evidence suggests that the old age, familial heredity and ethnicity are the most important risk factors for PCa. Among them, the older age and race are the greatest non-modifiable risk factors, playing an important role in the PCa development. *Ageing* is a slow, time-dependent decline of a set of multiple biological functions. Recently, the age was proposed as an independent epigenetic factor, leading to gene silencing through DNA methylation. In 2015, *Bechis and al.* reported that ageing is an important independent epigenetic factor that affects 5AR2 gene expression by DNA methylation in the benign prostatic tissues. Their study demonstrated that the attachment of methyl group to the carbon group of *5-alpha* reductase *2* leads to gene silencing. Epigenetic changes are critically important for the pathogenesis of prostatic carcinoma. In the last decade, efforts were made for the identification of specific changes and alterations in the gene machinery, leading to gene silencing and carcinogenesis. Epigenetic changes in PCa include histone modification, DNA methylation and noncoding miRNA. They also may lead to the promotion of prostate malignancy. Cell senescence is recognized as an essential factor, significantly contributing to cellular stress and alteration of the cellular genome. It is well recognized that PCa is a primarily disease in elderly and its age dependence was demonstrated in many studies [12]. This malignancy is extremely rare in men younger than 50 years of age; at the same time about 80% of diagnosed men are 65 years old and the average patient age is 70 years [13-15]. In support to these observations, *Siegel et al.* demonstrated an increasing trend in prostate cancer incidence at age intervals: up to 49 y/o, the incidence is 0.3%; (50 and 59 y/o) – 1.9%; (60-69 y/o) - 5.4%, then reaches its peak in 70 y/o or older – 9.1% [1]. However, not only prostatic carcinoma is characterized by the silencing of some critical genes. HGPIN, the precursor of PCa is also characterized by the DNA methylation of specific genes such as APC, RARB, and GSTP1 - in a lesser extent [7]. The role of epigenetics in the onset and progression of prostate cancer is widely discussed and many studies have demonstrated that the aberrant promoter hypermethylation occurs due to ageing in the normal prostatic tissues [12, 15]. The mechanism of DNA methylation involves addition of methyl group to the 5-carbon of cytosine in a CpG island, a gene promoter region, rich in CpG dinucleotides. The process is catalyzed by DNA methyltransferase (DNMT) enzyme that alters the chromatin structure, recruits methylated DNA-binding proteins and prevents transcription factor binding, leads to gene silencing and may alter the normal cellular homeostasis (Figure 2).

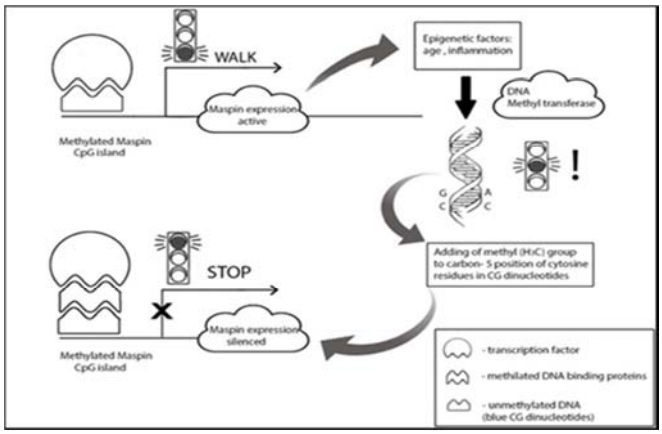

Figure 2. *Addition of methyl group to the gene promoter regions that are rich in CpG dinucleotides alters the chromatin structure, recruits methylated DNA-binding proteins, prevents transcription factor binding, and leads to gene silencing, which in term gives rise to PCa.*

Together with the oxidative stress and inflammation, this process significantly contributes to cancer development. During its growth, the prostate is constantly exposed to various damaging agents that alter the prostate cells homeostasis. Later, the rate and number of cellular alterations exceeds the ability of the cell's self-repair, which in turn triggers the onset of a cascade of different pathways, leading to cancer development, invasion and metastasis [15, 16]. Epigenetic alterations are first described in 1983 [17] and since then a growing number of studies demonstrated the role of promoter methylation of CpG islands in malignant diseases, also including prostate cancer. DNA methylation is one of the most common epigenetic mechanisms, affecting the gene expression and significantly contributes to cancer progression. DNA methylation is categorized as hypermethylation and hypomethylation. DNA methylation occurs also in benign tissues. Thus, in 1993 *Ono et al.* have reported the association between increased methylation and inactivation of collagen a1 gene with its inactivation. Likewise, *Issa et al*., [18, 19] have shown the relationship between CpG methylation of human estrogen receptor and the advanced age [18, 19]. Silencing of 5-alpha reductase 2 gene was also described as an effect of CpG methylation islands [20]. DNA hypermethylation is the best studied epigenetic event in PCa, targeting genes responsible for DNA damage repair (GSTP1), tumor suppressor gene Maspin (also known as SERPINB5), apoptosis (RASSF1), inflammatory response (2-PTGS2), cell-cycle control (CDKN2A), metastases (cadherins (CD44) and tissue metallopeptidase (TIMP). Inflammation and its relationship to ageing. Role of inflammation in DNA methylation.

The inflammation is a critical component of tumor progression. Many cancers arise from sites of infection, chronic irritation and inflammation. The inflammation was shown to contribute to the development of premalignant changes in the prostatic epithelium mainly through the oxidative stress and cytokines. In the last decade, many studies have shown that the inflammation can induce DNA methylation through the accumulation of free radicals, exceeding the cell's ability to neutralize and eliminate them. The influence of ageing on severity of inflammation or vice versa is widely disputable. On one hand, the inflammation significantly contributes to cell senesce through many mechanisms such as cytokines and free radicals. On the other hand, the older age characterized by the accumulation of free radicals, also contributes to the development of chronic inflammation. Therefore, ageing and chronic inflammation may act independently or in combination, leading to the development of aberrant changes in the normal cell genome. In support to this hypothesis, several studies have shown that chronic inflammation is age-related and the authors have reported its strong association with DNA hypermethylation [21-23]. As the inflammation increases with the advanced age, the incidence and rate of methylation also increases [21]. Possibly, these events might reflect the initial steps in PCa development. DNA hypermethylation is best studied in GSTP1 gene in prostatic carcinoma (1071 cases/total of 24 studies) and it was demonstrated that GSTP1 methylation can be detected in over 81% of PCa cases. In addition, a strong relationship with the advanced age was also demonstrated. Hyper methylation of GSTP1 gene plays significant role in the early prostatic carcinogenesis, namely in PIA (6.3%) and HGPIN (68.8%). The mechanism leading to age-dependent changes in DNA methylation is very intricate and occurs at different stages of human life [16]. This association between ageing and DNA methylation is not completely understood but recent evidence suggests the role of increased DNMT1 in prostatic tissues [21, 24]. For example, the relationship between age and inflammation was shown in a study, describing specific patterns of the expression of DNMT1 in T-lymphocytes during certain stages of human life (i.e. newborns, middle aged and elderly subjects) [25]. Furthermore, *Lopatina et al.*, *2002* reported the association between methyltransferase expressions and senescence in cultured fibroblasts [26]. *Ge R et al., 2015* reported the evidence of the impact of age-related DNA methylation and inflammatory changes (TNF-a, NF-kB, and IL-6) in the benign prostatic tissues [21]. These studies demonstrated the role of CpG hyper methylation in non-cancerous lesions, suggesting that a subset of specific genes can be methylated also in benign tissues [27, 28]. However, several genes such AMACR and NKX3.1 are specifically methylated in the *pre- and malignant prostatic tissues.* Detection of DNA hyper methylation in the prostatic premalignant precursors is an interesting and promising area of research. Therefore, it is reasonable to suggest that the methylation of these genes might mirror specific early events in the malignant conversion [29]. Of importance is also to note that tissue-specific DNA methylation will invite future studies to explore the utilization of zone-specific DNA methylation as a gene signature for the early detection and possible prevention of the patients at risk for prostate cancer.

Maspin and its role in the early malignant prostatic transformation. Maspin (also known as SERPINB5) is a gene, expressed in a tissue-specific pattern. Maspin is epigenetically regulated and its aberrant methylation is

associated with its CpG silencing, which is suggested as an early somatic change in human PCa. An interesting preliminary finding from our laboratory is the Maspin over expression in a subset of basal cell hyperplasia cells, which is in contrast with a previously reported finding of *Pierson et al* [2002], where the Maspin upregulation was detected in the preserved foci of basal cells in HGPIN. Maspin is a mammary serine protease inhibitor that is a tumor protective gene, co-expressed with GST-π in the normal prostatic glands. Maspin plays important role in the cellular response to oxidative stress and together with GST-based cellular defense machinery, actively regulates the cellular homeostasis. In addition, Maspin inhibits urokinase-type plasminogen activator on cell surface and its loss is associated with the destruction of basal cell layer membrane, followed by propagation of tumor cells and cancer progression. Loss of Maspin is associated with poor prognosis in patients with PCa. Maspin expression is down regulated in breast, prostate, gastric and melanoma cancers but upregulated in pancreatic, gallbladder and colorectal cancers. Although the Maspin expression is regulated by promoter methylation, in the normal cells that express Maspin, their promoter is not methylated. Unlike *Pierson et al*, who showed Maspin upregulation in HGPIN lesions [30], in our preliminary study, we found Maspin overexpression *basal cell hyperplasia* (BCH). This finding is intriguing because this pattern of Maspin expression might represent early event, leading to formation of the field effect. Moreover, Maspin upregulation may reflect the onset of the earliest epigenetic dysregulation in the normal epithelial cell population of the prostate. However, the lack of dysplasia in the BCH cells cannot suggest that the basal cell hyperplasia might be another plausible PCa precursor. Possibly, Maspin upregulation mirrors a primary cellular insult or defense mechanism towards specific external or internal factors, lately leading to further malignant transition. Also, one may hypothesize the possibility of BCH as a metaplasia and plausible precursor of the basal cell carcinoma of the prostate (BCC). In conclusion, it will be interesting to understand the triggering mechanisms leading to Maspin upregulation in BCH, as well as at what time point of male's life these events occur.

The understanding of mechanisms in Maspin expression, occurring in the visibly benign prostatic epithelium may provide useful information about the earliest steps of prostate cancer malignant transformation.

5. Conclusions

There is a growing body of evidence supporting the role of ageing, inflammation and DNA methylation in the early events of cancer development. The understanding of mechanisms of Maspin dysregulation at the earliest steps of prostate cancer development can significantly contribute to the early PCa detection. The patterns of Maspin expression in the prostatic premalignant lesions might be a useful new diagnostic tool for the prediction of progression of PCa and also may help for the stratification of men at risk of PCa. Further studies will help to understand the potential role of Maspin in the diagnostics and personalized treatment of patients with prostate cancer.

Acknowledgements

This work was supported by Cancerforskningsfonden i Norrland /Lions Cancerforskningsfond-Umeå, LP 17-2156, Umeå, Sweden.

We thank the Institute for Medical Biosciences, Department of Pathology at the Umeå University, Umeå, Sweden. We also express our gratitude to Mr. Petar Palovski, IT Department, Varna Medical University, Varna, Bulgaria for his technical and design support of this paper.

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