

Comparative Genomic Analysis Suggests FOXP3 Facilitates Tumor Evasion against Immunosurveillance by Transcriptionally Up-regulating Proteinase Inhibitor 9

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Abstract—The serpinB9 protein, known as proteinase inhibitor 9 (PI-9) in human, can potently inhibit the proteinase, granzyme B, which is the major granzyme immune system cells use to kill intracellular pathogen infected or neoplastic cells. Overexpression of PI-9 in tumor cells is considered as a mechanism for tumor evasion against immunosurveillance carried out by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells. We analyzed the upstream, downstream and intronic regions of serpinB9 from nine vertebrate genomes. Three well-conserved non-simple-repeat regions were identified, which locate at ~8kb upstream (in human), between exons 3 and 4, between exons 4 and 5, respectively. Using the Transfac database, we found a few transcription factor binding motifs matching the conserved regions, including motifs for FOXP3, GATA-4, HNF-1, HNF-3 β , Pax, Evi-1 and Nkx2-5. We used a Poisson distribution to assess the significance of the number of occurrences of these motifs in the conserved regions. FOXP3 turned out to be significant, with p-value = 0.0015. Overexpression of FOXP3 was previously shown to be associated with poor prognosis of ovarian cancer. Our finding suggests a mechanistic link between the overexpression of FOXP3 and cancer – FOXP3 transcriptionally up-regulates PI-9, which facilitates tumor evasion by by-passing immunosurveillance.

Keywords—Comparative Genomics, SerpinB9, PI-9, FOXP3, Tumor, Immunosurveillance

I. INTRODUCTION

Serine proteinase inhibitors (Serpins) are a superfamily of proteins that fold into a conserved structure and employ a suicide substrate-like mechanism to inhibit serine or cysteine proteinases. Serpins are the largest family of proteinase inhibitors and are fundamental to the control of proteolysis in

multicellular eukaryotes [1, 2]. SerpinB9 belongs to the ovalbumin subgroup of the serpin superfamily, known as PI-9 in humans and SPI-6 in mice; both PI-9 and SPI-6 are potent inhibitors of granzyme B, which is the major granzyme that immune system cells, including cytotoxic T lymphocytes and natural killer cells (CTL & NK cells), use to kill infected or neoplastic target cells [3, 4]. PI-9 is expressed in various cells and tissues. In immune system cells, PI-9 is considered to protect those cells against misdirected granzyme B and keep the integrity of granules in cytolytic lymphocytes. In placenta, ovary and testis, PI-9 helps to maintain these organs as immune privileged sites [5-8]. Because CTL and NK cells play important roles in tumor immunosurveillance [9, 10], the capability of PI-9 to inhibit CTL and NK cells-mediated apoptosis enables it to increase tumor incidence in certain circumstances[5]. Consistently, PI-9 and SPI-6 are expressed in a plethora of primary tumor cells and tumor cell lines[5]. Overexpression of PI-9 is associated with higher-grade malignancy and unfavorable clinical outcome in melanoma and lymphoma patients [11, 12]. Studies on transcriptional regulation of PI-9/SPI-6 will shed lights on tumor initiation and progression.

Transcriptional regulation is achieved, for the most part, by the interaction between transcription factors (trans-) and their specific DNA binding sites (cis-). Based on the assumption of negative selection, comparative genomics procedures predict cis-regulatory regions by identifying conserved non-coding DNA sequences across species [13, 14]. The assumption on negative selection is supported by observations that functionally important regions tend to have a lower mutation rate than non-functional regions during evolution, and transcription factor binding sites are enriched in conserved non-coding genomic sequences [15-17].

Although PI-9's proximal promoter has been characterized, which includes an estrogen responsive unit (ERU), two NF- κ B

sites and an API site [18, 19], it seems that these sites are not enough to explain all expression patterns of PI-9 in various tissues. There might be distal promoters or enhancers that participate in the regulation of PI-9 expression. To test this hypothesis, we aligned homologous serpinB9 genes and identified transcription factor (TF) binding sites conserved in rodents and primates through a comparative genomic approach.

II. METHODS

SerpinB9 was used as a keyword to Search the Emsembl database (<http://www.ensembl.org/>). Human serpinB9 gene ID was found and subsequently used to start homologous gene search using BioMart [20]. The DNA sequences of homologous serpinB9 genes in nine species, including human, chimpanzee, macaque, mouse, rat, chicken, frog, fugu fish and zebrafish, were retrieved from BioMart. Each DNA sequence was taken from 50kb upstream of the transcriptional starting site (TSS) to 50kb downstream of 3' end of the gene. It has been shown that many genomes contain stretches of highly repetitive DNA sequences, which includes simple repeats, tandem repeats, segmental repeats and interspersed repeats. It is considered that about 50% of human genome is repetitive in nature. Thus an online program Repeatmasker (www.repeatmasker.org) was used to screen interspersed repeats and low complexity DNA sequences. The processed sequences were fed into the MultiPipMaker program (<http://pipmaker.bx.psu.edu/pipmaker/>) with default parameters to identify conserved regions. The conserved regions were extracted and subsequently scanned for TF binding sites using the Match™ program [21]. We used all vertebrate Position Specific Weight Matrices (PSWM) in TRANSFAC professional release 7, with a threshold minimizing the sum of false positive and false negative reports [22]. Independent to cross-species conservation and Transfac search, we devised a test to assess the significance of the identified binding sites as follows. Two thousand promoter regions, with the same lengths as the conserved regions identified from MultiPipMaker, were chosen randomly from human genome and mouse genome as "background sequences". The same PSWMs were used to scan these background sequences with the same threshold. The counts of hits of each PSWM in the scan on background sequences were used to derive the parameter for a Poisson distribution. The actual number of hits of the same PSWM in the PI-9 conserved regions was evaluated by this Poisson distribution to derive a p-value. The p-value represents random chance of observing the same or larger number of hits by this PSWM in a region of the same length of the serpinB9 conserved regions in multiple species.

III. RESULTS

Conserved non-coding sequences among homologous serpinB9 genes

DNA sequences of serpinB9 genes from different species were identified as described in methods. Low complexity and

interspersed DNA sequences of human serpinB9 (PI-9) were masked by RepeatMasker. The masked sequences are fed into the MultiPipMaker program with default settings <http://pipmaker.bx.psu.edu/cgi-bin/multipipmaker>. Fig. 1 is part of the MultiPipMaker result of the homologous serpinB9 genes. The gene sequence is marked by an arrow, which is the direction of transcription.

The DNA sequences are considered conserved if the conservation score (pip) computed by MultiPipMaker is bigger than 50%. The exons are well conserved throughout the nine species except for exon 1, which only appears in primates, and exon 7, with only a small fraction in the 5' end being conserved (Fig. 1). Since most of exon 7 is 3' UTR, it indicates that protein coding area is conserved. Three conserved non-coding sequence segments were identified. They appear at 8-9kb upstream of TSS (region a), between exons 3 and 4 (region b) and between exons 4 and 5 (region c), respectively (Fig. 1). Since the mouse serpinB9 (SPI-6) gene has been shown to have similar functions and tissue specific expression patterns in comparison to human PI-9 gene [5, 23], the conserved area between rodents and human might be very useful to identify some unknown cis-regulatory sites.

Two other conserved DNA segments were found besides those shown in Fig. 1. One is located at 26kb downstream of TSS, which is within two other genes, MGC3972 and serpinB1. The other is located at 50kb upstream of TSS, which is on the last exon of the serpinB6 gene. Chances for these regions to be the regulatory regions for serpinB9 genes are small, thus we only focused on the regions a, b, and c (Fig 1).

Transcription factor binding sites

We extracted the conserved non-coding sequences from human, mouse and rat, and applied the MATCH program to identify TF binding sites within them. To our surprise, within the conserved area around 8 kb upstream of TSS (region a), only a few TF binding sites were found in human and rodents (data not shown). Whereas for PI-9 (human) and SPI-6 genes (mouse), within the two conserved areas in between exons 3 and 4 (region b), and between exons 4 and 5 (region c), many TF binding sites were found (Table 1, Table 2). The conserved TF binding sites among human, mouse and rat include: Evi-1, Cdc-5, FOXP3, GATA-4, Pit-1, HNF-1, HNF-3 β , Pax, Nkx2-5 and MEF-2. Given that these TF binding sites are located within two relatively small areas and there is good conservation between rodents and primates, it is very likely that some of these sites may regulate the differential expression of PI-9/SPI-6 genes in different cell types and tissues. Among these TF binding sites, FOXP3 appeared 4 times in the human conserved regions and 3 times in the mouse conserved regions, which makes FOXP3 the most frequently appeared TF binding sites in our scan. To assess the probability that FOXP3 binding sites we found were false positives, we randomly selected 2000 genes from human and mouse respectively and retrieved the 5' upstream flank sequence to TSSs with 2.8kb for human and 2kb for mouse. The lengths of these sequences match the lengths of the actual conserved sequences in human and mouse. The repeat regions

of those sequences were masked by RepeatMasker and then scanned for FOXP3 binding sites. The occurrence rates of FOXP3 in human and in mouse are 1.074/2.8kb and 0.904/2.0kb, respectively and these rates were considered as background parameters. With these parameters (for Poisson distribution), we assessed the probability of observing the same or larger number of FOXP3 sites in background sequences of the same lengths as PI-9/SPI-6's conserved regions. This gave a p-value of 0.0015, which strongly indicated the appearance of the FOXP3 sites on the PI-9 conserved regions was not random.

IV. DISCUSSION

Quite a few conserved exon regions in serpinB9 genes were identified by MultiPipMaker. Exons 2 to 6 and the beginning of exon 7 are well conserved from human to zebrafish. The most important structure of serpinB9 is the reactive central loop (RCL), which is located at the end of the C-terminus, presumably encoded by the beginning of exon 7, thus the conservation of the beginning of exon 7 suggests function of this protein as a serpin is conserved throughout all vertebrates.

Two intronic, conserved, binding site rich regions were identified. Many of putative TFs predicted to interact with these two conserved intronic regions play important roles in

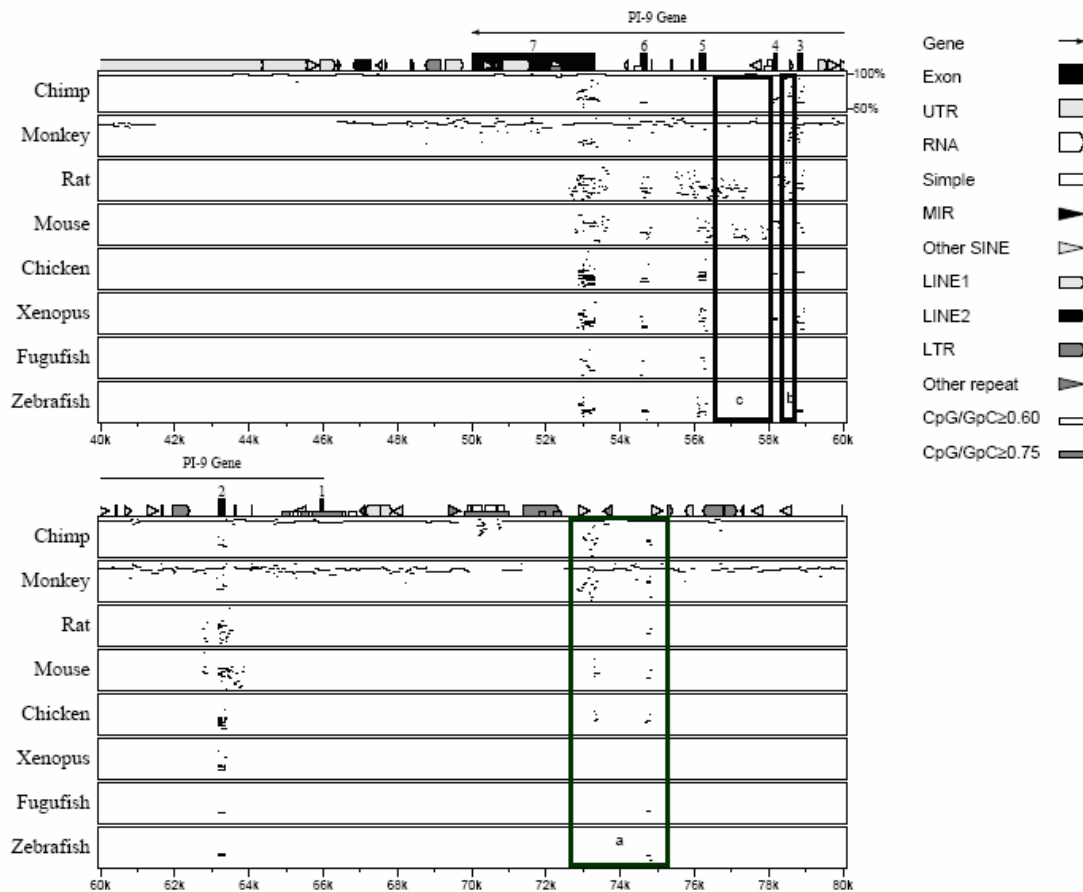


Figure 1. Part of the MultiPipMaker output for the nine serpinB9 genes.

Table 1. Transcription factor binding sites found in PI-9/SPI-6 from the region between exons 3&4 (region b)

Human (600bp)			Mouse (450bp)		
Matrix Match	Sequence	Factor	Matrix Match	Sequence	Factor
0.801	tATGTTatcttttt	Evi-1	0.860	attactTCCAG	Pax
0.812	tATCTTtttttaata	Evi-1	0.953	agtttTATTactta	HNF-3beta
0.886	agatatgttAAAATa	Evi-1	0.973	gttttaTTACtta	XFD-1
0.824	atatGTTAAaat	Cdc5	0.780	tATTTActtatgtct	Evi-1
0.795	atgtattgaacCCTGAataac	Pax-6	0.834	tttATTAAtac	Cdc5
0.954	ttgaaccctgaatAACCAgaaatgaa	AIRE	0.804	ttgcaaaGTCACc	RFX1 (EF-C)
0.869	tggctcaCATCT	GATA-4			
1.000	tgTAATCccaa	PITX2			
1.000	CACTTga	Nkx2-5			
0.970	aaaaAAATAgtgca	HNF-3beta			
0.791	ttttaaattggTTAACa	HNF-1			
0.824	tggTTAACataa	Cdc5			
0.884	caactTTTATgtt	Cdx-2			

Table 2. Transcription factor binding sites found in PI-9/SPI-6 from the region between exons 4&5 (region c)

Human (2.2kb)			Mouse (1.6kb)		
Matrix Match	Sequence	Factor	Matrix Match	Sequence	Factor
0.917	ataaAAACAgca	MEF-2	0.989	aacacATAAAcatgtg	Freac-7
0.918	taTTCATgattactgaca	Pit-1	0.813	aTTCtTgtcctttct	Evi-1
0.831	gtttgatCCAACccttc	FOXP3	0.860	tcatTTTAAaac	Cdc5
0.974	tgGGGAGttccag	NF-kappaB	1.000	cCTTTGaa	TCF-4
0.856	caaaCAAAGatca	HNF-4	0.797	gcagtGTTCTgacatgt	FOXP3
1.000	teAAGTG	Nkx2-5	0.896	ttacttaTATTT	GATA-4
0.781	aagcaggaatgCGGGAaacia	Pax-6	0.849	agatagacAGGTTc	Evi-1
1.000	gCGGGAaa	E2F-1:DP-1	0.928	tcactgtTATTT	GATA-4
0.790	aGTTAAcacaatcaaatg	HNF-1	0.925	caTTCATattctctetaa	Pit-1
0.980	gttaaCCAATca	CCAAT box	0.847	gaattGTTCTgcatc	FOXP3
1.000	ccaCCAATc	ACAAT	0.802	ttcatcaattaTTGCT	HNF-1
0.871	atatTTTAAAtgc	Cdc5	0.889	tttcaGATCT	GATA-4
0.956	ttTGTTTgtttt	FOX	0.940	ctacactCAATC	PBX
1.000	teAAGTG	Nkx2-5	0.918	tcaaTCAATt	CDP CR1
0.982	cggggGGGGGtcc	MAZR	0.809	ataaaGTTTTtccatg	FOXP3
1.000	teAAGTG	Nkx2-5	0.962	aattCCAGActtattg	Hand1:E47
0.860	gagAGCCAacgtggctggccctct	Pax-9	0.930	tgcttaTATTT	GATA-4
0.871	AGACAAAagtaa	GATA-4			
0.787	atctgaaACACCcgtga	FOXP3			
0.882	cattcTGACCtcggctcttttt	COUPTF			
0.792	gtgtGTTTgtccat	FOXP3			
0.835	tgtCCTATgtccatgt	PPAR			
0.949	cgacTGTGTtttttt	Poly A			
0.830	gtgtTTTTTtcaaac	FOXP3			
0.811	gaaaagGATTGaaaacgaaagggc	COMP1			

the immune system and may contribute to the cell and tissue specific expression of PI-9. Although the proximal promoter area of PI-9 has been characterized, the TF binding sites in the proximal promoter are not enough to explain all expression patterns of PI-9 in different tissues. For example, PI-9 is highly expressed in eyes (cornea). Since cornea is estrogen receptor negative, the estrogen responsive unit may not work.

The eye is an immune privileged site, therefore NF- κ B and AP-1 may not be active. Thus, there may exist other TF binding sites interacting with eye-specific TFs. Among the identified TFs that may interact with the conserved intronic regions (Tables 1 and 2), PITX2 and PAX-6 are involved in the morphogenesis of eyes and extra-ocular muscle [24-26]. GATA-4 is abundantly expressed in ovary and has been

shown to be related with its function [27], which indicates GATA-4 might be responsible for PI-9 expression in the ovary. The ERU in the proximal promoter may also contribute to the regulation of expression in ovary, because the ovary is an estrogen responsive organ. FOXP3, NF- κ B, MAZR, NKx2-5, AIRE are involved in the functions of the immune system [28-31]. The clustering of their binding sites in the small conserved intronic regions may explain the differential expression of PI-9 in different immune system cells. Other TF binding sites in the conserved intronic regions include Evi-1, Cdc-5, Pit-1, HNF-3 β and MEF-2. Among them, Evi-1, a zinc finger proto-oncogene, has been shown to be overexpressed in leukemic cells [32, 33], which suggests the possibility of induction of PI-9/SPI-6 in those cells by Evi-1 and consequently increases the resistance of those cells to be killed by immunosurveillance cells. Pit-1 has been found expressed in both human and mouse placenta, where PI-9/SPI-6 is abundantly expressed [34]. HNF-3 β was found to be related with mouse embryo development [35]. Cdc-5, as a regulator of mitotic entry, may suggest a link between PI-9/SPI-6 expression and cell cycle progression.

FOXP3, with most abundant binding sites in the conserved intronic regions, is critical for the function of CD25⁺CD4⁺ regulatory T cells [28, 29], which play important roles in immune regulation through suppression of other immune system cells. FOXP3 overexpression is associated with poor prognosis of ovarian cancer [28, 36]. SPI-6 transgenic and knockout studies have shown that SPI-6 can prolong the life of CD8⁺ memory T cells and protect against self-granzyme B induced apoptosis of CD8⁺ CTLs [37, 38]. Over-expression of PI-9 in CTLs enhances their potency presumably through protecting themselves against mis-directed granzyme B [8]. Thus, up-regulation of PI-9 by FOXP3 may account for protection of CD25⁺CD4⁺ regulatory T cells against mis-directed granzyme B mediated apoptosis, prolonging its life and enhancing its ability to suppress their target cells including CTLs and other CD4⁺ T lymphocytes. Overexpression of FOXP3 may induce PI-9 expression in other non-immune cells and contribute to tumor initiation and progression. FOXP3 has also been shown to be important in autoimmune diseases [28]. Because it has been shown that granzyme B cleavage of some substrates is related with some autoimmune diseases, FOXP3 induction of PI-9 may indicate a link between autoimmune diseases and PI-9.

In summary, the clustered TF binding sites in the two conserved intronic regions might be enhancers of PI-9/SPI-6 genes. These results await further biological validations by meanings of gel shift assay, DNA footprinting assay and transcription analysis in different cell types and tissues. The abundance of FOXP3 binding sites in the conserved intronic regions suggests a missing link between the regulation of PI-9 expression and tumor.

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