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Identifying Differentially Expressed Apoptosis Related Genes in Prostate Cancer

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Abstract

Apoptosis plays a pivotal role in the pathogenesis of cancer. It is important to understand the molecular mechanisms underlying prostate cancer, and how prostate cancer cells evade apoptotic mechanisms. We performed apoptosis related microarray experiments to identify differentially expressed apoptosis related genes in three prostate cancer specimens. Student's t-test and Fisher's exact test were used to compare gene expression between prostate cancer and normal specimens. We identified seven apoptosis related genes that were differentially expressed in prostate cancer. BIK, BAX, BRAF and BNIP3L were up-regulated while CD40, CARD8 and PYCARD were down-regulated. We also found evidence that the apoptosis pathway plays a role in prostate cancer.

Keywords: Apoptosis; Prostate Cancer; PCR Array

Introduction

Apoptosis is a homeostatic cellular process that has been linked to a number of physiological and pathological conditions. It plays a fundamental role in the pathogenesis of several diseases, such as cancer [1]. There are mainly three pathways by which caspases can be activated: intrinsic (or mitochondrial), extrinsic (or death receptor) and intrinsic endoplasmic reticulum pathway [2]. The intrinsic pathway is initiated when internal stimuli such as irreparable genetic damage, hypoxia, extremely high concentrations of cytosolic Ca^{2+}, and severe oxidative stress are present [3]. The extrinsic pathway is initiated when death receptors recruit adapter proteins such as the TNF Receptor-Associated Death Domain (TRADD) and the Fas-Associated Death Domain (FADD) [4]. Either stimulus activates a series of caspases and contributes to typical apoptotic morphological changes. Apoptosis is linked to the elimination of potentially malignant cells, hyperplasia and tumor progression [5]. Therefore, apoptosis and carcinogenesis include: 1) disrupted balance of pro-apoptotic and anti-apoptotic proteins, 2) reduced caspases function and 3) impaired death receptor signaling [6].

Carcinogenesis is complicated and apoptosis plays a role in cancer initiation and progression. Apoptosis is also believed to play an important role in prostate cancer. Therefore, it is important to understand the molecular mechanisms underlying prostate cancer and how prostate cancer cells evade apoptotic mechanisms that give rise to their uncontrolled growth and behavior. This is especially important when prostate cancer progresses to an apoptosis-resistant androgen-independent stage (CRPC, Castration-Resistant Prostate Cancer) [7]. Aim of our study was to perform an apoptosis related microarray study to access to association between apoptosis related genes and prostate cancer.

Materials and Methods

Study Population

We studied three prostate cancer specimens collected from patients who underwent radical prostatectomy at Suzhou Municipal Hospital. All cases were histologically confirmed as prostate adenocarcinoma. The pathological stage of prostate cancer was classified into TNM stage and tumor grade was evaluated by Gleason Score. The ethics committee at Suzhou Municipal Hospital approved this study and all patients provided written informed consent.

RNA Extraction and cDNA Preparation

Tissue specimens were homogenized with TRIzol® reagent (Invitrogen, Carlsband, CA) using a power homogenizer and incu-

bated at room temperature, followed by chloroform addition and centrifugation. Total RNA was precipitated from the supernatant with isopropanol, washed with 75% ethanol and re-suspended in 50 µl of DEPC-treated water. RNA concentration and purity were calculated after measurement on a UV spectrophotometer with 260 nm absorbance and a 260/280 nm absorbance ratio respectively.

We synthesized cDNA by Reverse Transcription (RT) with Thermoscript™ RT kits (Invitrogen), using random hexamers as amplification primers. In detail, 2.5 µg of total RNA, 50 ng of random hexamers and 1 mM dNTPs were heated at 65˚C for 5 min in order to remove RNA secondary structures and placed on ice until the addition of cDNA synthesis mix, which contains 1X cDNA synthesis buffer (50 mM Tris-acetate pH 8.4, 75 mM potassium acetate, 8 mM magnesium acetate), 5 mM dithiothreitol (DTT), 40 U RNaseOut™ and 15 U Thermoscript™ reverse transcriptase. The final mix (volume 20 µl) was incubated for 10 min at 25˚C for primer extension, and cDNA synthesis was conducted at 55˚C for 50 min. The reaction was terminated by heating it at 85˚C for 5 min. In order to remove the RNA template, cDNA was incubated at 37˚C for 20 min with 2 U of E. coliRNaseH and stored at -20˚C until used.

Quantitative Real-Time PCR Array

Quantitative real-time PCR (qRT-PCR) was done using an apoptosis PCR Array containing 96 apoptosis-related genes (Super Array Biosciences) (Table 1).

	Case 1	Case 2	Case 3
Age (year)	64	67	70
PSA (ng/ml)	8.2	6.5	10.1
cStage	T2a	T2a	T2b
pStage (TNM)	T2N0M0	T2N0M0	T3N0M0
Gleason score	4+3	3+4	4+4

Note: cStage: clinical stage; pStage: pathlogical stage

Table 1: Characteristics of prostate cancer patients.

Briefly, RNA samples were isolated using Trizol reagent. All samples had 260/280 ratios above 2.0 and 260/230 ratios above 1.7. An equal amount of RNA (2µ/g) was used for reverse transcription using RT2First Strand Kits from Super Array Biosciences. PCR reactions were done using the RT2 profiler PCR array PAHS-3012 E (Human Apoptosis PCR Array 96 HT) on the ABI Fast7900 using RT2Real-time SYBR Green PCR master mix PA-012. The total volume of the PCR reaction was 10µL. The thermocycler parameters were 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative changes in gene expression were calculated using the ΔΔCt (threshold cycle) method. Five housekeeping genes were included on the array (B2M, HPRT1, RPL13A, GAPDH, and ACTB) to normalize the RNA amounts.

Statistical analysis

Student's t-test and Fisher's exact test were used to examine gene expression status between prostate cancer and normal tissue. All statistical analyses were 2-sided and performed with SPSS 11.5 (SPSS, Chicago, IL). Statistical significance was set at the 95% level (p-value <0.05).

Results

All the three specimens had localized prostate cancer with pathologic stages that ranged from T2N0M0 to T3N0M0. Study population characteristics are summarized in (Table 1). The standard curves in the amplification were constructed based on housekeeping genes, which were used to normalize the detected apopto-

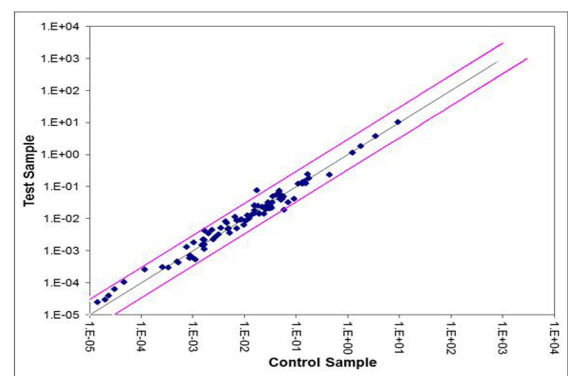

Figure 1: Fold-change in apoptosis related genes expression scatter plot in prostate cancer.

We observed that 47 apoptosis related genes were over-expressed (fold changes>1) and 42 genes were under-expressed (fold changes<0). After performing the Student's t- test to replicate values for each gene in case and control groups, we had seven genes BIK(P=0.0103), BAX(P=0.0424), BRAF(P=0.0448), BNIP3L(P=0.0394), CD40(P=0.0116), CARD8(P=0.0478) and PYCARD(P=0.0227) with significant P values (Figure2) (Table2).

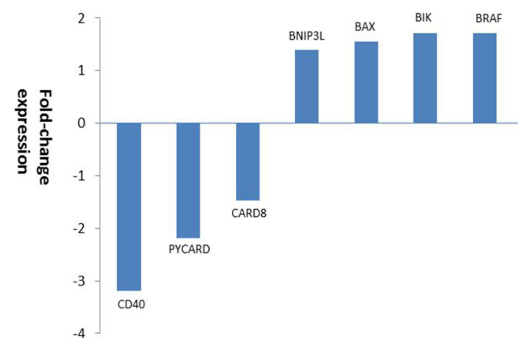

Figure 2: Magnificent expression of seven apoptosis related genes.

	Case(Ct)	Con(Ct)	Case(2^-ΔCt)	Con(2^-ΔCt)	*P* value	Fold regulation
BAX	3.78	4.42	7.30E-02	4.70E-02	0.0424	1.55
BIK	6.48	7.25	1.10E-03	6.60E-03	0.0103	1.7
BNIP3L	4.34	4.82	4.90E-05	3.50E-02	0.0394	1.39
BRAF	9.59	10.36	1.30E-03	7.60E-04	0.0448	1.71
CARD8	10.75	10.18	5.80E-04	8.60E-04	0.0472	-1.48
CD40	5.76	4.08	1.90E-02	5.90E-02	0.0116	-3.19
PYCARD	4.58	3.45	4.20E-02	9.10E-02	0.0277	-2.19

Note: Fold regulation value>1 means up-regulation, value<0 means down-regulation

Table 2: Expression analysis of apoptosis related genes in prostate cancer.

Therefore, we deduced that these seven genes were significantly different between prostate cancer and normal prostate tissue. In addition, this suggests that the genes have different functions in different regions of the same specimens.

Discussion

We conducted this study in order to determine mRNA transcription levels of apoptosis related genes using a quantitative real-time RT-PCR method. An apoptosis PCR Array contains premiere global apoptosis-related genes and we were able to detect all targeted genes in one procedure. PCR reactions were performed using the RT2 profiler PCR array PAHS-3012 E which has been used in many studies [8-10]. We successfully detected seven differentially expressed genes using an apoptosis PCR Array. The BIK gene shares a critical BH3 domain with other death-promoting proteins, such as BID, BAK, BAD and BAX [11], and is associated with apoptosis repressors Bcl-X (L) or Bcl-2 suppresses death-promoting activity [12-15] reported that the expression level of BIK was up-regulated in prostate cancer compared with normal tissue ranging from 1.28 to 2.42-fold. As a likely target for anti-apoptosis, over-expression of BIK changed the apoptosis pathway for prostate tissue. The BAX gene shares the same BH3 domain with the BIK gene and it has a similar function when it is associated with BAX-Bcl-X (L) as BIK does [15-17] reported similar results of BAX over-expression in prostate cancer tissues. The BNIP3L gene is a member of the BCL2/adenovirus E1B 19 kd-interacting protein (BNIP) family. This protein counteracts the apoptotic inducer BNIP3 and may play a role in tumor suppression [18]. BNIP3 and BNIP3L/NIX at the mitochondrial outer membrane regulates the opening of a pore in the mitochondrial double membrane in order to mediate the translocation of lysosomal proteins from the cytoplasm to the mitochondrial matrix [19]. A study by [20] observed that the expression level of BNIP3L was 2.276-fold up-regulated with a P value of 0.041.

BIK, BAX and BNIP3L all belong to the Bcl-2 family and fluctuations in their expression levels disrupted the balance of Bcl-2 family proteins. Furthermore, they inhibited the mitochondrial apoptosis pathway. This phenomenon is one cause of prostatic carcinogenesis. The BRAF protein plays a role in regulating the MAP kinase/ERKs signaling pathway, which affects cell division, differentiation and secretion. In our study, we observed that BRAF had an over-expression level of 1.71-fold [21] reported that the BRAF mutation is common in many human malignant tumors including melanoma, colorectal carcinoma and pancreatic cancer. Several studies reported BRAF over-expression, ranging from 1.0 fold to 3.67 fold [13-17,21].

The CD40 gene was previously named the TNF Receptor Super Family Member 5(TNFRSF5). This receptor has been found to be essential in mediating a broad variety of immune and inflammatory responses [22]. In the apoptosis pathway, CD40-mediated cell survival proceeds through NF-Kappa B (NFKB) dependent up-regulation of Bcl-2 family members [23]. In our data, CD40 was 3.19-fold under-expressed in prostate cancer. This result is similar to that reported by [15-17,20]. PYCARD (also named TMS1 and ASC) mediates the apoptosis pathway and is involved in activation of the mitochondrial apoptotic pathway and promotes caspase-8-dependent proteolytic maturation of BID independently of FADD in certain cell types [24]. It also mediates mitochondrial translocation of BAX and activates BAX-dependent apoptosis coupled to activation of caspase-9, -2 and -3 [25,26] reported that a direct role for aberrant methylation of the TMS1 gene in the progression of breast and gastric cancer involving down-regulation of the pro-apoptotic TMS1 gene. We observed that PYCARD was under-expressed in our samples. This finding confirms what other studies have reported [15-20]. Furthermore, growing tumors of transplanted human cancer cells in nude mice were eradicated by the activation of endogenous ASC in the tumor cells, irrespective of the form of cell death. Thus, ASC mediates distinct forms of cell death in different cell types, and is a promising target for cancer therapy [27]. CARD8 shares the same C-terminal Caspase-Recruitment Domain (CARD) with PYCARD. Both PYCARD and CARD8 mediate apoptosis involved in NFKB and pro-Caspase-1 activation [28] reported CARD8 under-expression at 1.04-fold in prostate cancer which was similar with our result. We have a limitation on this study that the sample is few. When this study began we collected few radical prostatectomy sample and when the PCR-array conducted some samples were not preserved well and ruled

out of the test. We still thought although the samples are not big enough the outcome may be useful for identifying apoptosis related genes in prostate cancer.

As a premiere global profiling platform, our experimental method is convenient. On the other hand, a main disadvantage was that we did not detect all of the genes using the combined platform. This study identified seven apoptosis related genes differentially expressed between normal prostate and prostate cancer tissue and revealed that the apoptosis pathway plays a role in prostate cancer. Although we only studied three prostate cancer specimens, all of our outcomes could be confirmed by other various studies. We will focus future work on the seven genes and seven protein expression in different prostate cell lines. Moreover, we will try to study the regulation, initiation and progression of the seven genes in prostate cancer.

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