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Expression of leukemia/lymphoma related factor (LRF/Pokemon) in human benign prostate hyperplasia and prostate cancer

Himanshu Aggarwal [a,*], Anshu Aggarwal [a], William J. Hunter III [b], Paulos Yohannes [c], Ansar U. Khan [c], Devendra K. Agrawal [a]

[a] Department of Biomedical Sciences, Creighton University School of Medicine, Omaha, NE 68178, USA
[b] Department of Pathology, Creighton University Medical Center, Omaha, NE 68131, USA
[c] Urology Health Center, Fremont, NE, USA

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ABSTRACT

Leukemia/lymphoma related factor (LRF), also known as Pokemon, is a protein that belongs to the POK family of transcriptional repressors. It has an oncogenic role in many different solid tumors. In this study, the expression of LRF was evaluated in benign prostate hyperplastic (BPH) and prostate cancer (PC) tissues. The functional expression of LRF was studied using multiple cellular and molecular methods including RT-PCR, western blotting, immunohistochemistry, and immunofluorescence. Paraffin-embedded human tissues of BPH and PC were used to examine LRF expression. Histological staining of the BPH and PC tissue sections revealed nuclear expression of LRF with minimal expression in the surrounding stroma. The semi-quantitative RT-PCR and western immunoblot analyses demonstrated significantly higher mRNA transcripts and protein expression in PC than BPH. High expression of LRF suggests that it may have a potential role in the pathogenesis of both BPH and prostate cancer. Further studies will help elucidate the mechanisms and signaling pathways that LRF may follow in the pathogenesis of prostate carcinoma.

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Introduction

Benign prostate hyperplasia (BPH) and prostate cancer (PC) are growing health problems that continue to present a momentous multi-disciplinary challenge to urologists, radiologists, and oncologists. BPH affects three out of four men between the ages of 60 and 69 years in the United States (Parsons, 2008). Prostate cancer is the most common non-dermatologic cancer and the second highest cause of cancer-related deaths in American men.

The molecular events associated with the initiation of prostate cancer are not precisely known. The secretory epithelial cells of the prostate gland are exposed to many external stimuli, including oxidative stress, which is mediated by numerous hormones, growth factors and neuropeptides that control their proliferation, differentiation and survival (Chen et al., 1999; Kim, et al., 1999; Pirtskhalaishvili and Nelson, 2000; Konety and Nelson, 2001; Tanji et al., 2001; Mimeault and Batra, 2006). The complex changes in the prostate micro-ecosystem involved in the initiation and progression of prostate cancer are mediated by several known growth factor-signaling cascades, including the EGFR, hedgehog, and Wnt/beta-catenin pathways (Karhadkar et al., 2004). These pathways act in a cooperative mechanism by inducing heterogeneous tumorigenic cascades that regulate cell differentiation, proliferation, migration, and survival (Karhadkar et al., 2004). A pool of prostate specific stem cells, which are implicated in cell renewal during the prostate regeneration process, has been proposed to represent the minority of epithelial cells that could provide the prostate cancer progenitor cells following the sustained activation of different growth factor signaling cascades (van Leenders et al., 2003; Schalken and van Leenders, 2003; Hudson, 2004; Palapattu et al., 2005; Hussain et al., 2003; Kumar et al., 1998).

LRF is a transcription factor that is involved in the oncogenesis of various human malignancies (Zelent, 1994). LRF is also known as Pokemon (POK erythroid myeloid ontogenic factor), FBI-1 (Factor that Binds to IST, the HIV-1 inducer of short transcripts), and OCZF (Osteoclast-derived Zinc Finger) (Agrawal et al., 2006; Stogios et al., 2007). It is a 72kDA protein encoded by the ZBTB7A gene, which resides on the 19p13.3 locus and belongs to the POK family of transcriptional repressors. POK (POZ and Kruppel) proteins are ubiquitous transcriptional repressors and many of them function in human cell differentiation and development. Various members of the POK protein family, such as BCL-6 (B-cell lymphoma-6), PLZF (promyelocytic leukemia zinc finger) and LRF have been shown to be important in the pathogenesis of various human cancers (Maeda et al., 2005a,b; Kelly and Daniel, 2006; Davies et al., 1999). High expression of LRF has been quantified in human tumors including T- and B-cell lymphomas, nerve glioma, non-small cell lung, and breast carcinomas (Maeda et al., 2005a,b; Rovin and Winn, 2005; Aggarwal et al., 2010; Apostolopoulou, et al., 2007; Zhao et

* Corresponding author. Creighton University School of Medicine, CRISS II Room 510, 2500 California Plaza, Omaha, NE 68178, USA. Fax: +1 402 280 1421.
E-mail address: dkagr@creighton.edu (H. Aggarwal).

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al., 2008; Qu et al., 2010). A recent study using prostate cancer cell lines indicated that LRF interacts with the androgen receptor in a ligand-dependent manner (Cui et al., 2010a,b).

An *in-vitro* study indicated that mouse embryonic fibroblasts, in which the ZBTB7A gene was knocked out, resisted oncogene-mediated transformation by various oncogenes. This resistance was restored when ZBTB7A expression was restored (Maeda et al., 2005a,b). The role of ZBTB7A as a proto-oncogene was also examined *in vivo* in transgenic mice that overexpressed ZBTB7A in immature T- and B-lymphoid cells. These mice developed thymic lymphomas and tumor infiltration of the bone marrow and their survival was significantly lower than of the wild-type mice (Maeda et al., 2005b).

Despite some *in vitro* evidence and animal data, studies of ZBTB7A in human malignancies and especially in solid tumors, including prostate cancer, are lacking. Also, since the overexpression of LRF has been associated with increased proliferation in cancerous tissues (Maeda et al., 2005a; Kelly and Daniel, 2006; Aggarwal et al., 2010; Zhao et al., 2008; Cui et al., 2010a), it may function in the pathogenesis of BPH. The aim of this study was to examine and quantify the expression of LRF in human prostate cancer and BPH tissues.

Materials and methods

Human prostate tissues samples

Eighteen prostate tissues (16 BPH and 6 PC) were obtained after surgery from Urology Health Center, Fremont, NE, USA. The fresh tissue specimens were collected anonymously with no link to the subjects. The tissues were transported in the University of Wisconsin solution to maintain the histological and functional integrity of the tissue specimen (Abebe et al., 1993). The fresh tissue was cut into three sections for RNA and protein analysis. The third section was fixed in paraformaldehyde and embedded in paraffin.

Nine additional paraffin-embedded tissues of prostate cancer were provided by the Department of Pathology at Creighton University Medical Center. Sections (4–5 μm) were cut using a microtome and stained with hematoxylin and eosin. All slides were reviewed by two pathologists, in a blinded manner, to confirm the diagnoses. The study was considered "exempted" by the National Institute of Health guidelines and approved by the Institutional Review Board of Creighton University.

RNA isolation and semi-quantitative RT-PCR

RT-PCR was carried out as previously described (Aggarwal et al., 2010). Briefly, cell pellets were resuspended in Trizol Reagent (Sigma, St. Louis, MO, USA) and RNA was isolated according to manufacturer's protocol. RNA (1 μg) was reverse-transcribed into cDNA and PCR was performed according to the recommended protocol using the PCR Master Mix (Promega) and primers were at a final concentration of 1 μM. PCR products were electrophoresed on a 1.5% agarose gel and photographed under UV light. Densitometric analysis of the bands was measured using the UVP Bioimaging System (Upland, CA, USA).

Western immunoblotting

Western immunoblotting was performed as described previously (Aggarwal et al., 2010). Briefly, cell pellets were suspended in a solution containing Mammalian Protein Extraction Reagent (MPER, Promega) and protease cocktail inhibitor (P1860, Sigma). Total protein was extracted according to the manufacturer's protocol and quantified using the Bio-Rad Protein Assay (Cat No: 500-0006). Protein lysates (25 μg) were resolved using 0.1% SDS/10–15% polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was placed in 5% non-fat dry milk in PBS containing Tween-20 for 1 h at room temperature to block any non-specific protein binding sites. Membranes were then incubated for 1 h in anti-GAPDH (1:2000, NB-1300, Novus Biologicals). Subsequently, the membranes were incubated for 1 h with the anti-LRF rabbit polyclonal antibody (1:200; Pacific Immunology). Membranes were washed with PBS and incubated with the horse-radish peroxidase conjugated secondary antibody solutions, anti-rabbit (1:1000, NB730H, Novus Biologicals) for LRF and anti-mouse (1:2000, NB720H, Novus Biologicals) for GAPDH. Antibody binding was detected using enhanced chemiluminescence solution (ECL, Pierce, Rockford, IL) and the density of the bands was quantified by the UVP Bioimaging System.

Immunohistochemistry

Immunohistochemistry was carried out as previously described (Aggarwal et al., 2010). Briefly, formalin fixed tissues were cut at 4–5 μm, mounted on slides and deparaffinized. For immunostaining, antigen retrieval was carried out using Target Retrieval Solution (S1699, DAKO, Carpenteria, CA) and sections were steamed for 30 min. Endogenous peroxidase was blocked by 3% hydrogen peroxide for 20 min and then subjected to the primary LRF antibody solution (1:1600; Pacific Immunology) for one hour at room temperature. Negative controls were run in parallel using pre-immune serum from rabbit (PAC-767; Pacific Immunology) or by omitting the primary antibody. Sections were washed using wash buffer (S3006, DAKO) for 10 min and exposed to biotin-labeled goat anti-rabbit IgG for 30 min. The immune conjugates were then treated with an avidin–biotin complex according to the manufacturer's instructions (PK-6101, Vectastain ABC elite kit, Vector Laboratories, CA, USA). Staining was completed using diaminobenzidine chromogen (K4010, DAKO) followed by counterstaining with hematoxylin (S3309, DAKO). Immunostained sections were photographed by an Olympus DP71 camera at 200× or 400× magnifications.

Immunofluorescence

Antigen retrieval and deparaffinization of tissue sections (5 mm) were done as previously described in the immunohistochemistry section. Sections were cooled to room temperature and prepared for immuno-labeling by blocking and permeabilizing in a solution consisting of PBS containing 0.25% Triton-X 100, 1% BSA, and 5% normal goat serum (Jackson Laboratories, West Grove, PA, USA). Sections were immunolabeled with primary rabbit polyclonal antibody LRF at an optimized concentration (1:200) for 1 h. An affinity purified goat anti-rabbit Cy2 antibody (1:200, Jackson Laboratories) was applied to sections for 1 h in the dark. Slides were rinsed with PBS containing 0.1% BSA to remove excess antibody. Fluorescence was preserved by sealing specimens with solution of equal parts of PBS and glycerol containing 1% n-propyl gallate and 1.5 mg/mL 4,6-diamindino-2-phenylindole, dilactate (DAPI). Images were obtained within 1 h of mounting.

Statistical analysis

Values of all measurements were expressed as the mean ± SE. The Graph Pad Prism 4.0 biochemical statistical package (GraphPad Software, Inc., San Diego, CA, USA) software was used to plot graphs and for statistical analysis. Nuclear staining for IHC and IF was observed by two independent observers, in a blinded manner. There was <5% inter-observer variation. Values of $p<0.05$ were considered statistically significant.

Results

Expression of LRF mRNA transcripts and protein was observed in all 22 prostate tissue samples. The representative data from the

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