Altered Expression of RET Proto-oncogene Product in Prostatic Intraepithelial Neoplasia and Prostate Cancer


Background: The RET proto-oncogene encodes a protein that belongs to the tyrosine kinase growth factor receptor family. Germline point mutations in RET are found in individuals with multiple endocrine neoplasia (MEN) syndromes, and gene rearrangements have been reported in papillary thyroid cancers. We recently identified transcripts of the RET proto-oncogene in human prostate cancer xenografts and prostate cancer cell lines by means of reverse transcription–polymerase chain reaction analyses. The purpose of this study was to investigate Ret protein expression in human prostate tissue.

Methods: Ret protein expression was evaluated immunohistochemically in formalin-fixed, paraffin-embedded whole-prostate sections. The prostate specimens were obtained from 30 patients with prostate cancer after radical prostatectomies. Ret protein expression was compared in tumor foci and benign prostate tissue. Medullary thyroid carcinoma tissue associated with an MEN syndrome and papillary thyroid cancer tissue served as positive controls. Results: Ret appeared to be overexpressed in high-grade (histopathologically advanced) prostate intraepithelial neoplasia (PIN) and prostate cancer when compared with its expression level in benign prostate secretory epithelium. In addition, there was an apparent increase in Ret protein expression with decreased cellular differentiation, i.e., increasing Gleason pattern. Conclusion: Expression of the RET proto-oncogene in benign prostate epitheli-um, high-grade PIN, and histopathologically advanced prostate cancer suggests that RET may play a role in the growth of both benign and neoplastic prostate epithelial cells.

RET proto-oncogene encodes a protein in the tyrosine kinase growth factor receptor family. We recently identified (1) the RET proto-oncogene transcript in CWR22 (2), a primary human prostate cancer xenograft, while analyzing the general tyrosine kinase expression by the reverse transcription–polymerase chain reaction. This finding was intriguing because RET expression is thought to be enhanced in normal cells of neuroendocrine lineage, such as those of the adrenal medulla, but has not been reported to be expressed in prostate tissue. We expanded this study to other prostate cancer xenografts and cell lines and found RET expression in all cases (1). RET is a proto-oncogene originally identified as a rearranged gene in transformed NIH3T3 cells (3). This gene has been mapped to chromosome 10q11.2 (4). The genetic alterations of RET are well documented in the dominantly inherited multiple endocrine neoplasia (MEN) syndromes 2A and 2B, familial medullary thyroid cancer, and papillary thyroid carcinoma (5,6). In papillary thyroid carcinoma, the altered gene product has been localized to the cytoplasm (7). Allelic loss at various sites on chromosome 10 has been reported in prostate cancer, but none has been associated directly with the RET locus (8). In the context of the known tumorigenic potential and the molecular evidence for receptor tyrosine kinase activity in the prostate, we hypothesized a possible role for the RET gene product, the protein designated Ret, in prostate neoplasia.

Materials and Methods

Tissues

Formalin-fixed, paraffin-embedded, wholenumt prostate sections were obtained from 30 patients with prostate cancer after radical prostatectomies performed during the period of March 1988 through August 1994 and were provided to us through the Western Division of the Cooperative Human Tissue Network of the National Cancer Institute, Bethesda, MD. All slides from each prostate were evaluated by two pathologists (D. M. Dawson and G. T. MacLennan). The slides were stained with hematoxylin–eosin and were reviewed for histopathology. A single slide was selected from each prostate specimen that contained high-grade (histologically advanced) prostatic intraepithelial neoplasia (PIN), areas that contained no neoplasia, and areas with carcinoma. Some prostate specimens were excluded because their paraffin-embedded tissues had been almost depleted after having been used in other studies, and inadequate amounts of tissue remained in the paraffin blocks. After appropriate sections were identified, the paraffin blocks were cut further to obtain more sections for our current study. All slides studied contained fields showing both benign prostate and carcinoma tissues. In three instances, the PIN lesions had been depleted and were no longer available in the new sections cut for this study; however, we retained these three prostate specimens in this study. Grading was performed by the method of Gleason (9). Gleason’s grading classifies histopathologic patterns into five (1 through 5) categories; pattern 1 is the most differentiated pattern, and pattern 5 is the least differentiated pattern. The sum of the two most predominant Gleason patterns for each case is recorded as the total Gleason score. Gleason scores of 2–10 were represented in the series studied. The two most extensive Gleason patterns were recorded for each case (total of 60 patterns in 49 tumors) for evaluation with the observed expression of Ret; as reviewed in detail earlier (10), other pathologists (11,12) have found that most prostates resected because of prostate cancer contain multiple cancers. Sections of two medullary thyroid cancers (of type MEN 2A) and two papillary thyroid cancers served as positive control tissues that demonstrated cytoplasmic staining for Ret as described previously (7,13).

Immunohistochemical Analyses

Serial, 5-μm, wholemount prostate sections were deparaffinized in xylene and rehydrated through graded alcohols. Antigen retrieval was performed by digesting with 0.1% trypsin (Flow Laboratories, Inc., McLean, VA) for 5 minutes at 37 °C (13). Sections were rinsed thoroughly with 10 mM phosphate-buffered saline (PBS) prepared according to the procedure of Mayer and Walker (14) and adjusted to pH 7.4. Endogenous biotin was blocked with 100 μg/ml streptavidin (Sigma Chemical Co., St. Louis, MO) in blocking solution (10% goat serum in PBS) for 15 minutes in a humidified chamber (15). Unoccupied streptavidin-binding sites were blocked with 100 μg/ml biotin (Sigma Chemical

*Affiliations of authors: D. M. Dawson, E. G. Lawrence, T. P. Pretlow (Department of Pathology), G. T. MacLennan, T. G. Pretlow (Departments of Pathology and Urology), S. B. Amini (Department of Epidemiology and Biostatistics), H.-J. Kung, D. Robinson (Departments of Molecular Biology and Microbiology), M. I. Resnick (Department of Urology), Case Western Reserve University Medical Center, Cleveland, OH; E. D. Kursh, Department of Urology, The Cleveland Clinic Foundation.

Correspondence to: Thomas G. Pretlow, M.D., Institute of Pathology, Case Western Reserve University, 2085 Adelbert Rd., Cleveland, OH 44106.

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REPORTS 519
Co.) in blocking solution for 15 minutes. The blocking solution also served to prevent nonspecific binding of antibodies. Rabbit polyclonal anti-Ret antibody (#SC-167; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), recognizing the C-terminus amino acids 784–801 of the RET proto-oncogene product, was diluted 1:50 in blocking solution. Endogenous peroxidase was blocked by immersion of the slides in 3% H2O2 in 30% methanol for 10 minutes following application of the goat anti-rabbit IgG. Detection of bound antibody was performed by use of streptavidin–biotinylated horseradish peroxidase (Amersham Life Science Inc., Arlington Heights, IL) diluted 1:100 with blocking solution and incubated for 30 minutes as previously described (16). The sections were then submerged in substrate (1% diaminobenzidine in 10 mM phosphate buffer [pH 7.3]) with heavy metal enhancement ([0.02% Ni(SO4)2·(NH4)6SO4·6H2O and 0.02% CoCl2·6H2O] [17] with modification) for 20 minutes at room temperature. All sections were rinsed with PBS after each stage of the procedure. Sections were then counterstained with 1% methyl green, air-dried, and mounted in Clearium (Sur-gipath, Richmond, IL). Adjacent hematoxylin–eosin-stained slides were reviewed in parallel with the immunohistochemical preparations for histopathologic correlation. Immunohistochemically prepared slides were graded according to staining intensity on a scale of 0 (no staining) to 4+ (the maximum staining present in the control medullary thyroid carcinoma) (Fig. 1).

Control experiments for antibody specificity were performed by peptide neutralization of the Ret antiserum according to the directions of the manufacturer, i.e., overnight incubation at 4°C with a 10-fold excess of Ret control peptide (Santa Cruz #SC-167P); similar control overnight incubations were carried out in which two irrelevant peptides were substituted in parallel for the Ret control peptide. The irrelevant peptides were the Met peptide (sequence VDTRPASFWETS) obtained commercially (Santa Cruz #SC-10P) and a peptide made in our facility, 13 amino acids in length (sequence VDTRPASFWETS) obtained commercially (Santa Cruz #SC-167P); similar control overnight incubations were performed by peptide neutralization of the Ret antibody. Original magnification ×208.

Results

The most notable findings were in areas of PIN and prostate cancer (Table 1). All variants of PIN (flat, tufted, micro-papillary, and cribriform patterns) were identified (19) and showed increased expression of Ret in secretory (luminal) epithelium in comparison with the secretory epithelium of benign glands that generally did not express Ret. Armitage trend analysis (18) was used to evaluate if there was any association between Gleason pattern and immunohistochemical results. All P values were two-sided.

Statistical Analyses

Statistical analyses were performed with StatXact Version 2.2 (Cytel Software Corp., Cambridge, MA). Fisher’s exact test was used to compare the proportions of lesions that expressed Ret. Armitage trend analysis (18) was used to evaluate if there was any association between Gleason pattern and immunohistochemical results. All P values were two-sided.

The histopathologic findings on the corresponding adjacent hematoxylin–eosin-stained slides. Areas of PIN partially involving a gland could be delineated by immunohistochemical staining. Rare, isolated, benign-appearing glands with mild proliferative activity stained focally and discretely for Ret, comparable to the intensity in PIN (1+).

Forty-nine tumors were identified in the histologic sections used for this study from the prostate tissue specimens of 30 patients; the criteria of Greene et al. (11) were used to define multiple separate tumors in the same prostate. The relative frequencies of Gleason’s five patterns were similar in our study and in Gleason’s review (9) of 2911 patients studied by him and his collaborators; e.g., less than 4% of the 2911 patients’ tumors contained any Gleason pattern 1 tumor, and more than 75% of the patients’ tumors contained Gleason pattern 3 tumor as either the predominant pattern or the second most extensive histologic pattern. With the exception of tumors that contained only one Gleason pattern, the two most extensive Gleason histopathologic patterns in each histologic section were evaluated for their immunohistochemical expression of Ret. Ret appeared overexpressed in most moderately differentiated to poorly differentiated tumors but not in well-differentiated lesions (Table 1). More than 75% of areas with Gleason patterns 3, 4, or 5 showed increased Ret (2+ to 3+ in intensity); in contrast, the epithelium in foci of prostate cancer of Gleason
patterns 1 and 2 and several areas of pattern 3 expressed minimal (1+) or no Ret (Table 1). Examples of tumors with adjacent low-grade (Gleason pattern 1, 2, or 3) and high-grade (cribriform pattern 3, 4, or 5) tumor were striking and highlighted the marked differences that we observed with increasing Gleason grade (Fig. 3, C and D). For those slides that contained the smallest amount of a particular Gleason pattern, all of the available tumor on each slide was evaluated; the smallest amount of tumor evaluated consisted of two microscopic fields (×100). On average, five representative microscopic fields were evaluated. For Gleason patterns 1 and 2, equivocal or very weak staining was observed to involve less than 10% of the tumor; the remaining tumor showed no immunohistochemically detectable expression of Ret. For Gleason patterns 4 and 5, more than 75% of each Gleason pattern showed staining. Tumors with Gleason pattern 3 were more heterogeneous. As Table 1 shows, 18 of 29 tumors exhibited staining (1+ to 3+) in greater than 75% of each tumor; 11 of 29 tumors exhibited areas with no staining in 25% or more of the tumor. The trend in increased expression of Ret as a function of decreasing differentiation (increasing Gleason pattern) was striking (two-sided P for trend <.002). The difference in the expression of Ret by prostate cancer and benign secretory epithelium was significant (P = .001).

The expression of Ret by benign tissues included basal cells of normal and hyperplastic prostatic glands (Fig. 3, E and F), transitional epithelium of the prostatic urethra, seminal vesicle or ejaculatory duct epithelium, and ganglion cells of the neurovascular bundle. The basal layer was accentuated in areas of inflammation, post-inflammatory repair, and atrophy that reflect the enriched basal cell population seen in these conditions. With the exception of the rare foci described above, benign secretory cells did not express immunohistochemically detectable Ret. Variable, weak-to-moderate staining of the stromal and vascular smooth muscle was present.

### Discussion

Our study corroborates observations with other potential biomarkers such as...
comparisons of benign, dysplastic, and malignant prostatic epithelium have shown phenotypic similarities of PIN and prostate cancer (20–23) and lends support for PIN as a possible precursor lesion. The staining patterns that we have observed raise interesting questions that have been discussed by many pathologists. One of these questions pertains to the relationship between prostate cancer and PIN. Several views have been expressed (19,24) about the possibility that some variants of PIN may be histopathologically similar to carcinomas growing inside prostatic ducts. The expression of RET that we observed casts little light on this question; however, all high-grade PIN lesions that we observed, including five that were remote from the boundaries of tumor, expressed Ret with similar intensity. One might wonder if the relatively intense expression of Ret by high-grade PIN and weak expression of Ret by well-differentiated tumor would imply that PIN is not a precursor lesion for low-grade tumors. We do not believe that we have sufficient data to answer this question. We should note that, in addition to PIN, other putative preneoplastic lesions of the prostate have potential importance (22,25–27). The histopathologic evidence that Ret is expressed at high levels by basal cells in benign prostate, by the epithelial cells in high-grade PIN, and by most prostatic carcinomas suggests that the possible role of Ret in the growth of prostatic epithelial cells needs further investigation.

As noted above, the present immunohistochemical study was begun because of our observation of the expression of the RET transcript in a primary human prostate cancer xenograft. In the consideration of the potential biologic importance of RET expression in human prostatic tissues, the reported functions of RET in other cells may be helpful. Overexpression and structural alteration of RET have been shown to correlate with neoplastic transformation of the follicular cells in papillary thyroid cancer and in human fibroblasts (3,6,7,28). Tumors of neural crest origin express high levels of RET transcripts and/or gene product (13,29). Activation of RET protein with ligand binding leads to receptor dimerization and signal transduction; this outcome results in proliferation via the mitogen-activated protein kinase pathway (30,31), growth (32), and development (32,33). The recent discovery linking glial cell line-derived neurotrophic factor (33) and neurturin (34) to Ret signal transduction may lead to a further explanation for the mechanism of proliferation. Receptor dimerization and constitutive activation have been demonstrated in papillary thyroid carcinoma (7). In light of the above findings, it is conceivable that the overexpression of Ret can lead to proliferation and overgrowth of prostatic epithelial cells. These observations, in conjunction with the role of RET in inherited MEN syndromes, warrant further investigation for possible mutations in RET in prostate cancer. Although there are no known genetic alterations of RET in prostatic tissues, we cannot exclude the possibility of mutations or gene rearrangements with the data that are currently available.

References

UV-Radiation-Specific p53 Mutation Frequency in Normal Skin as a Predictor of Risk of Basal Cell Carcinoma

Allal Ouhtit, Hisayoshi Nakazawa, Bruce K. Armstrong, Anne Kricker, Ernest Tan, Hiroshi Yamasaki, Dallas R. English*

Background: A strong association has been found between skin cancer and exposure to UV radiation. The p53 tumor suppressor gene (also known as TP53), which is frequently mutated in human cancers, is believed to be an early target in UV radiation-associated skin carcinogenesis. We have previously developed a sensitive, polymerase chain reaction-based method capable of detecting and quantifying a UV radiation-specific mutation in the p53 gene (codons 247 and 248: AAC → AAT) in normal skin. We have used this method to examine whether UV radiation-specific mutation frequency is associated with risk of basal cell carcinoma (BCC) and with sun exposure. Methods: This case–control study in Australia involved 53 case subjects with BCC and 75 control subjects. DNA was isolated from normal skin (mirror-image anatomic site to the cancer site for case subjects and a randomly selected site for control subjects) and assayed for p53 mutation. Relationships between p53 mutation frequency and risk of BCC, sun sensitivity, or sun exposure were estimated by use of odds ratios (ORs) and 95% confidence intervals (95% CIs). Results: Case subjects were more likely to have a p53 mutation than control subjects (OR = 3.1; 95% CI = 1.3–7.1). In addition, the odds of BCC increased monotonically with increasing frequency of p53 mutation. No statistically significant associations could be demonstrated between p53 mutation frequency and age, sex, sensitivity to the sun, pigmented characteristics, total lifetime sun exposure, or sun exposure to the biopsy site. Conclusions: Our results indicate that tandem CC → TT mutations involving codons 247 and 248 of the p53 gene are associated with an increased risk of BCC but cannot be used as an accurate measure of total UV-radiation exposure. [J Natl Cancer Inst 1998;90:523–31]

Carcinogenesis is a complex multistage process associated with the accumulation of critical genetic alterations. Some of these alterations can be directly linked to exposure to specific carcinogens; these carcinogen-specific gene changes are called “molecular signatures” of carcinogens. We have proposed that such molecular signatures may serve as dosimeters of cumulative exposure to carcinogens and as predictors of cancer risk (1). Skin cancer provides an ideal experimental model to pursue such an idea because it is largely associated with one agent (UV radiation) (2), and UV radiation-specific gene changes have been detected (3).

The tumor suppressor gene p53 (also known as ATP53) is mutated frequently in human cancers. These mutations can cause the loss of p53’s tumor suppressor functions, which include regulation of the cell cycle, apoptosis, and DNA repair (4–6). Mutation of p53 is considered to occur early in the process of UV radiation-associated skin carcinogenesis (7–12). We have previously developed a sensitive, allele-specific polymerase chain reaction (AS-PCR) assay (13,14) to detect UV-specific mutations at two distinct hotspots of the p53 gene (3,15). We chose to detect CC to TT tandem base mutations, especially at the boundary of p53 codons 247 and 248 (i.e., AAC_CGG → AAT_TGG) because of the

Notes

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*Affiliations of authors: A. Ouhtit, H. Nakazawa, H. Yamasaki, Unit of Multistage Carcinogenesis, International Agency for Research on Cancer, Lyon, France; B. K. Armstrong, New South Wales Cancer Council, Woolloomoooloo, Australia; A. Kricker, National Breast Cancer Centre, Woolloomoooloo, Australia; E. Tan, Department of Dermatology, Royal Perth Hospital, Australia; D. R. English, University of Western Australia, Perth.

Correspondence to: Hiroshi Yamasaki, Ph.D., Unit of Multistage Carcinogenesis, International Agency for Research on Cancer, 150 cours-Albert-Thomas, Lyon F-69372, France. E-mail: Yamasaki@iarc.fr

See ‘Notes’ following “References.”