# Expression of p14$^{ARF}$, p15$^{INK4b}$, p16$^{INK4a}$, and DCR2 increases during prostate cancer progression

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**Prostate carcinoma is a hormonally driven age-related neoplasm. Cellular senescence is an age-related process where cells remain metabolically active but in a growth-arrested state at the G1 phase. p14$^{ARF}$, p15$^{INK4b}$, and p16$^{INK4a}$, which are known to regulate G1 cell cycle arrest, and the tumor necrosis factor receptor superfamily member decoy receptor 2 (DCR2), have been recently identified as senescence markers. The purpose of this study was to characterize and compare the expression of p14$^{ARF}$, p15$^{INK4b}$, p16$^{INK4a}$, and DCR2 in tissue microarrays containing cases of normal prostate, nodular hyperplasia, prostate intraepithelial neoplasia (PIN), and malignant prostate cancer tissue. We performed immunohistochemical staining for p14$^{ARF}$, p15$^{INK4b}$, p16$^{INK4a}$, and DCR2 in tissue microarray blocks containing 41 cores of normal prostate, 65 cores of nodular hyperplasia, 21 cores of PIN, 69 cores of low-grade prostate carcinoma, and 42 cores of high-grade prostate carcinoma, derived from 80 cases of prostatectomy with adenocarcinomas. We detected positive staining of p16$^{INK4a}$ in 19% of the PIN, 25% of the low-grade carcinoma, and 43% of the high-grade carcinoma specimens but none in the normal prostate and nodular hyperplasia specimens. Expression of p14$^{ARF}$ revealed very high levels of expression in normal tissues (83%), nodular hyperplasia (88%), PIN (89%), and cancer cells (100%). P15$^{INK4b}$ and DCR2 were found positive in 81 and 33% normal, 46 and 10% nodular hyperplasia, 74 and 36% PIN tissues, 87 and 89% low-grade carcinomas, and 100 and 93% high-grade carcinomas. There is an increased protein expression of senescence-associated molecular markers, indicating that cellular senescence might play a role in prostate carcinoma. Because p16$^{INK4a}$-positive cells were detected only in premalignant lesions and carcinomas but not in normal or benign tissues, p16$^{INK4a}$ may aid in the diagnosis of PIN and prostate cancer in difficult cases.**
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Accumulations of somatic mutations in older individuals contribute to the high frequency of prostate cancer occurrence. Increasing evidence shows that age-related changes affect the molecular crosstalk between stromal and epithelial cells generating a more permissive environment for tumor development.[1] Cellular senescence is an age-related process and these cells accumulate in tissues with age. Senescent cells, originally defined in fibroblasts,[2] remained metabolically active but in a growth-arrested state at the G1 phase.[3] Senescence is an extremely stable form of cell cycle arrest that cells use to protect themselves from incidental oncogenic activation and may play a role as an antitumor mechanism because of its antiproliferative capacity. However, if certain tumor suppressor genes mutate, senescent cells may be immortalized and begin to over proliferate, which can lead to the development of cancer.

It is well established that *pRb* and *p53* pathways play an important role in maintaining senescence,[4] which are activated to promote senescence by products of the *INK4a/ARF* locus. The *INK4a/ARF* locus encodes three important tumor suppressors, *p14$^{ARF}$*, *p15$^{INK4b}$*, and *p16$^{INK4a}$*. The *p16$^{INK4a}$* gene, a cell cycle-related gene, encodes a p16 protein that binds competitively to cyclin-dependent kinase 4 protein[5] and then, during the cell cycle G1 phase,

inhibits the interaction of cyclin-dependent kinase 4 protein and cyclin D1 to stimulate passage through the cell cycle.[6] The p16 protein is often highly expressed in senescent cells in culture and inactivated in a variety of human cancers.[7] *p14^ARF* shares a common exon 2 with the *p16^INK4a* transcript but has a different exon 1b and is translated in an alternative reading frame.[8–10] Loss of p14 in knockout mice also results in tumor formation.[11] *p15^INK4b* is located centromeric to the *p16/p14* gene locus and encodes proteins that are known as major inhibitors of cell proliferation. It is a negative growth regulator and a downstream effector of transforming growth factor $\beta$-induced cell cycle arrest.[12]

Decoy receptor 2 (DCR2) is one of the tumor necrosis factor-related apoptosis-inducing ligand receptors; it suppresses tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis[13] and is expressed in many normal tissues, but its expression is often silenced or downregulated by promoter hypermethylation in multiple cancer types, including cervical cancer, breast cancer, neuroblastoma, malignant mesothelioma, and prostate cancer.[14]

p14^ARF, p15^INK4b, p16^INK4a, and DCR2 have been recently identified as senescence markers.[15] The first three markers are important in G1 arrest and are often mutated or deleted in various malignancies and DCR2 can selectively induce apoptosis in cancer cells. Staining with antibodies against p14^ARF, p15^INK4b, p16^INK4a, and DCR2 revealed abundant positive cells in premalignant lung tumors, whereas malignant tumors were essentially negative.[15]

The purpose of this study was to characterize and compare the expression of p14^ARF, p15^INK4b, p16^INK4a, and DCR2 using immunoperoxidase technique in tissue microarrays containing cases of normal prostate, nodular hyperplasia, prostate intraepithelial neoplasia (PIN), and malignant prostate cancer tissue, and determine their expression in prostate cancer progression.

## Materials and methods

### Case Selection

The construction of the tissue microarray has been described previously[16] which contains 41 cores of normal prostate tissue, 65 cores of nodular hyperplasia, 21 cores of PIN, 69 cores of low-grade prostate carcinoma (Gleason grade 2 and 3), and 42 cores of high-grade prostate carcinoma (Gleason grade 4 and 5) from 80 patients who had undergone prostatectomy at the University of Rochester Medical Center for the treatment of prostatic adenocarcinoma. All identifiers were removed to protect patient's confidentiality, the use of human tissue in the study was approved by the Institutional Review Board.

### Immunohistochemical Staining

Tissue microarray slides were subjected to immunohistochemical staining. In brief, after initial deparaffinization, sections were steamed in 10 mM citrate buffer (pH 6.0) to unmask the epitopes for 20 min and then treated with protein-blocking solution (Biocare Medical, Walnut Creek, CA, USA) for 10 min. The slides were incubated against p16^INK4a (Biocare Medical; 1:100 for 30 min at ambient temperature), p14^ARF (GeneTex prediluted; 1 h at ambient temperature, San Antonio, TX, USA), p15^INK4b (Santa Cruz; 1:300 for 1 h at ambient temperature, Santa Cruz, CA, USA), and DCR2 (Stressgen; 1:250 overnight at 4°C, San Diego, CA, USA). Slides were incubated with universal secondary antibody (Biocare Medical) and horseradish peroxidase (Biocare Medical) for 15 min each. Tissues were stained for 3 min with freshly prepared 3,3′-diaminobenzidine tetrahydrochloride and then counterstained with hematoxylin, dehydrated, and mounted.

### Tissue Microarray Analysis

We visually read the array under the microscope, scoring each core individually. Cases in which no epithelial cells were found or no core was available were excluded from the study.

On the basis of expression patterns in prostate epithelial cells, p14^ARF, p15^INK4b, p16^INK4a, and DCR2 protein expression was considered positive if any positive staining was seen in the epithelial cells. Immunostained tissue microarrays slides were semiquantitatively scored by two independent pathologists (ZZ and DR) blinded to the clinicopathologic information. Quantitation was performed using a four-score grading system. Negative was defined as the total absence of staining and given a score of '0'. Presence of staining was scored as weakly positive and given a score of '1', intermediate positive score '2', and strongly positive score '3'. For the statistical analysis, cases were grouped as negative (score '0') or positive (score '1', '2', or '3').

### Statistical Analysis

The basic descriptive statistical analysis and tables were created using the Statistica software package, version 6.0 (Statsoft, Inc., Tulsa, OK, USA). Chi-square ($\chi^2$) test and Fisher's exact test were utilized for the analysis on categorical data. Results were considered statistically significant at the $P < 0.05$ level.

## Results

As shown in Figure 1, all instances of expression appeared in the cytoplasm of prostatic epithelial cells. p16^INK4a analysis showed positive staining in
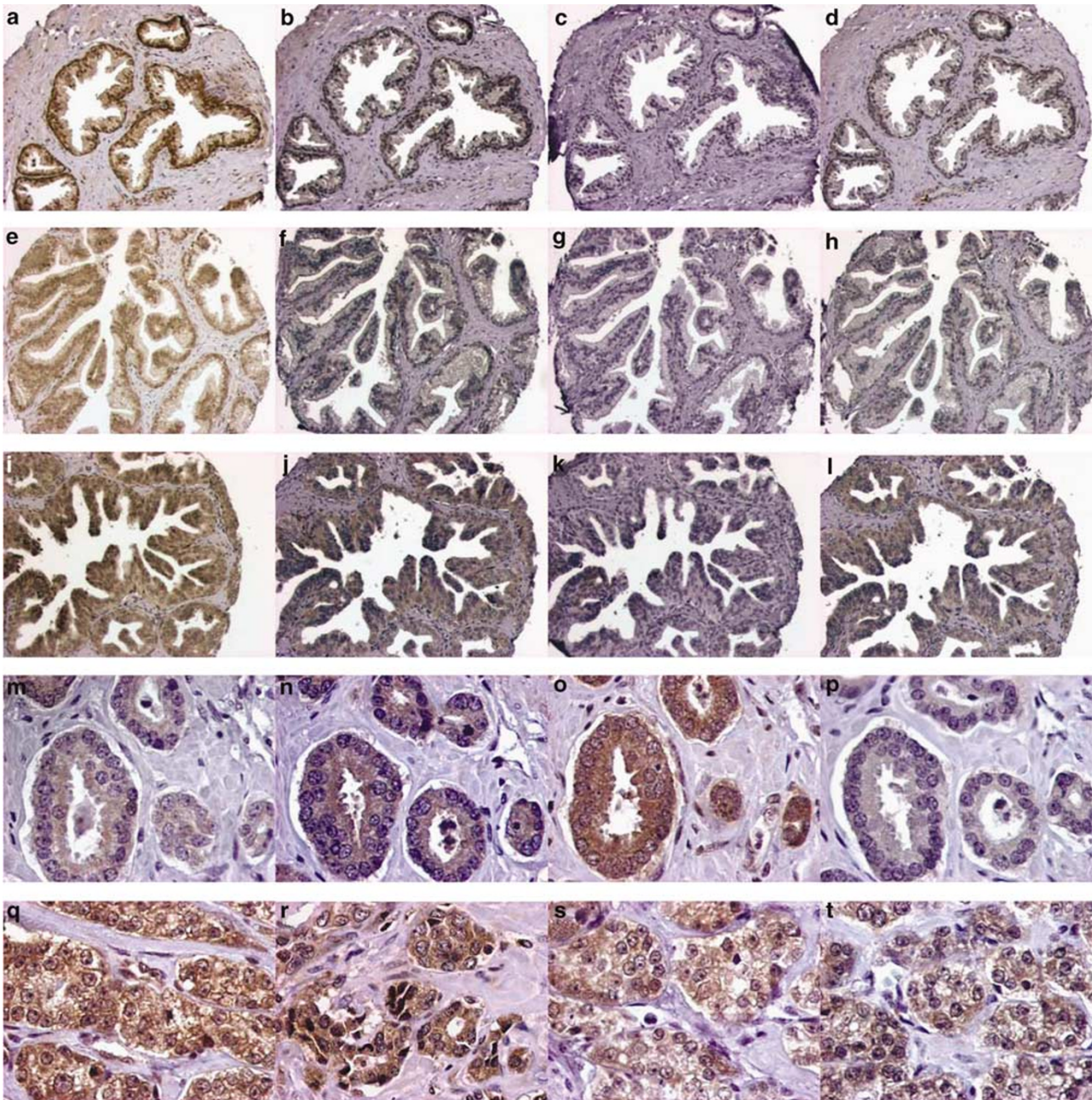
**Figure 1** Expression of different markers in prostate tissues, as shown by positive or negative immunostaining of the cytoplasm. Normal tissue ($\times 10$): (**a**) p14, positive; (**b**) p15, negative; (**c**) p16, negative; and (**d**) DCR2, negative. Nodular hyperplasia ($\times 10$): (**e**) p14, positive; (**f**) p15, negative; (**g**) p16, negative; and (**h**) DCR2, negative. PIN ($\times 10$): (**i**) p14, positive; (**j**) p15, positive; (**k**) p16, positive; and (**l**) DCR2, positive. Low-grade prostate carcinoma ($\times 40$): (**m**) p14, positive; (**n**) p15, positive; (**o**) p16, positive; and (**p**) DCR2, negative. High-grade prostate carcinoma ($\times 40$): (**q**) p14, positive; (**r**) p15, positive; (**s**) p16, positive; and (**t**) DCR2, positive.

19% of the PIN, 25% of the low-grade carcinoma, and 43% of the high-grade carcinoma specimens but none in the normal prostate tissue and nodular hyperplasia specimens. The evaluation of expression of p14$^{ARF}$ revealed very high levels of expression in both the normal tissues (83%), nodular hyperplasia (88%), PIN (89%), and cancer cells (100%). P15$^{INK4b}$ and DCR2 were found positive in 81 and 33% of normal tissues, 46 and 10% in nodular hyperplasia tissues, 74 and 36% in PIN tissues, 87 and 89% in low-grade carcinomas, 100 and 93% in high-grade carcinomas (Table 1). A statistically significant difference was found among p14$^{ARF}$, p15$^{INK4b}$, p16$^{INK4a}$, and DCR2 expression and pathologic lesions (Table 1). In all cases, the frequency of positive cores was significantly higher ($P < 0.05$) in cancer tissues than in noncancer tissues, and there was a trend towards increased

**Table 1** p14[ARF], p15[INK4b], p16[INK4a], and DCR2 protein expression in different tissues

| Lesion type | p14[ARF] | | | p15[INK4b] | | | p16[INK4a] | | | DCR2 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Positive | Negative | P-value | Positive | Negative | P-value | Positive | Negative | P-value | Positive | Negative | P-value |
| Normal | 40 (83%) | 8 (17%) | 0.59 (a) | 38 (81%) | 9 (19%) | <0.001 (a) | 0 (0%) | 41 (100%) | 1.00 (a) | 16 (33%) | 32 (67%) | <0.001 (a) |
| Nodular hyperplasia | 61 (88%) | 8 (12%) | 1.00 (b) | 32 (46%) | 37 (54%) | 0.02 (b) | 0 (0%) | 65 (100%) | <0.001 (b) | 7 (10%) | 64 (90%) | <0.001 (b) |
| Prostate intraepithelial neoplasia | 24 (89%) | 3 (11%) | 0.02 (c) | 20 (74%) | 7 (26%) | 0.22 (c) | 4 (19%) | 17 (81%) | 0.77 (c) | 10 (36%) | 18 (64%) | <0.001 (c) |
| Low-grade carcinoma | 69 (100%) | 0 (0%) | 1.00 (d) | 60 (87%) | 9 (13%) | 0.03 (d) | 17 (25%) | 52 (75%) | 0.06 (d) | 62 (89%) | 8 (11%) | 0.53 (d) |
| High-grade carcinoma | 43 (100%) | 0 (0%) | 0.01 (e) | 43 (100%) | 0 (0%) | <0.001 (e) | 18 (43%) | 24 (57%) | <0.001 (e) | 40 (93%) | 3 (7%) | <0.001 (e) |

Reference: *P*-values reflect differences in proportions between: (a) normal *vs* nodular hyperplasia; (b) nodular hyperplasia *vs* prostate intraepithelial neoplasia *vs* low-grade carcinoma; (d) low-grade carcinoma *vs* high-grade carcinoma; (e) high-grade carcinoma *vs* normal tissue. Percentages represents to the positive or negative case number ratio.

staining in high-grade prostate carcinomas (Gleason grade 4 and 5) compared with low-grade carcinomas (Gleason grade 2 and 3).

The results of immunostaining for p14[ARF], p15[INK4b], p16[INK4a], and DCR2 in association with patients' pathologic lesions are summarized in Table 1.

## Discussion

In this paper, we examined the expression of molecules implicated senescence pathway and found that p14[ARF], p15[INK4b], p16[INK4a], and DCR2 were expressed more frequently in prostate carcinomas than in benign prostatic tissues, implying that p14[ARF], p15[INK4b], p16[INK4a], and DCR2 are activated in prostate carcinoma. Meng *et al*[17] found DCR2 overexpression in human colon cancer cells, and Liu *et al*[13] found that some chemotherapy agents caused DCR2 overexpression in a human lung cancer cell line. Furthermore, Schwarze *et al*[18] found that primary prostate tumors retain the ability to senesce in culture, and the *p16/pRb* pathway is altered in 85% of primary prostate cancers.[19] These data suggest that cellular senescence may play a role in prostate cancer development.

In human cells, inactivation of p16[INK4] also facilitates the immortalization process[20] and p16[INK4] activity increased in senescent cells[21] but not in highly proliferative cells. Meanwhile, several investigators have found that overexpression of p14[ARF] and p16[INK4] also plays a critical role in the growth arrest of normal epithelial cells associated with senescence.[18–20,22] Furthermore, senescent cells have been identified in human tissues, including nodular hyperplasia, a hyperproliferative disorder of the prostate.[3] Several other studies have suggested that expression of p14[ARF], p15[INK4b], p16[INK4a], and DCR2 is widely downregulated in several solid tumors including hepatic, pancreatic, esophageal, breast, and urinary bladder carcinomas and gliomas,[6,13,23,24] suggesting that senescence pathways are inactivated in large number of these tumors. Our data showed that p16[INK4] and other markers have an increased expression during prostate tumor progression, suggesting that senescence pathways are still intact in large number of prostate cancers. Tumor and tissue heterogeneity may account for the contrast in expression between our results and those previously described by other studies.[6,13,23,24] This indicates that different types of tumor may have different mechanisms of inactivation of senescence program. The elucidation of these results may be matter of further investigation.

Most studies have used normal diploid fibroblasts as a model to study senescence.[18] However, most cancers originate from epithelial cells,[25] including prostate cancers. Recently, other cell types have been shown to demonstrate senescent activity in culture, such as lung epithelial,[15] hepatic,[6] and

pancreatic cells.[26] To our knowledge, our study is the first to determine the expression of senescence markers p14[ARF], p15[INK4b], p16[INK4a], and DCR2 in cells of human prostate tissue samples. Our data, involving the staining of normal tissue, nodular hyperplasia, PIN, and prostate carcinoma specimens for senescence markers, showed that these biomarkers were differentially expressed during prostatic cancer progression.

In summary, our study demonstrated that there is an increased protein expression of senescence-associated molecular markers indicating that cellular senescence might play a role in prostate cancer progression. In addition, p16[INK4a] staining might be a diagnostic aid particularly in prostate carcinomas and intraepithelial neoplasias for difficult cases.

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