Discovery and Clinical Application of a Novel Prostate Cancer Marker

α-Methylacyl CoA Racemase (P504S)

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Abstract

The recent discovery of the overexpression of P504S/α-methylacyl coenzyme A racemase (AMACR) in prostate cancer is a successful example of translating an advanced molecular finding into clinical practice. AMACR (P504S) has been proven to be one of the few biomarkers that can help distinguish cancer from benign cells, with high sensitivity and specificity for prostate carcinoma. It is the first gene identified by the analysis of complementary DNA microarray profiles from prostate tissue to be used as a tissue tumor marker in clinical practice and to improve the diagnosis of prostate cancer. This review focuses on the study of AMACR (P504S) expression in prostate cancer, premalignant lesions, benign prostate tissues, and other normal and malignant tissues and a discussion of its clinical usefulness. We emphasize the interpretation of the AMACR immunohistochemical results in routine surgical pathology practice and also discuss the potential future applications of this marker and the possible role of AMACR in the pathogenesis of cancer development.

Prostate carcinoma is the most common form of extracutaneous cancer in men and the second leading cause of death, accounting for more than 37,000 deaths per year in the United States.[1] The wide use of serum prostate-specific antigen (PSA) screening has resulted in an increased detection of patients with prostate cancer.[2] Tissue examination of a prostate needle biopsy or transurethral resection specimen of prostate is mandatory for the diagnosis of prostate cancer and permits patients to receive appropriate therapy. However, tissue diagnosis can be difficult and inaccurate if the cancer is very limited, because the establishment of a pathologic diagnosis requires the presence of a combination of multiple histologic features of tumor cells such as pattern of growth, nuclear atypia, absence of basal cells, and the presence of characteristic extracellular material in malignant glands.[3-5] No single morphologic feature of prostatic adenocarcinoma can be used reliably by itself. In addition, many benign conditions can mimic the morphologic features of prostate cancer, despite their benign biologic behavior.

Overdiagnosis (false positivity) may cause unnecessary treatment of men without prostate cancer and lead to incontinence or impotency. Underdiagnosis (false negativity) may delay effective treatment to patients with prostate cancer and may lead to recognition of disease at a more advanced stage. Unfortunately, there is small but significant error rate in the pathologic diagnosis of prostate cancer in general practice because discrimination between benign and malignant glands can be difficult in needle core biopsy specimens. The accuracy of pathologic diagnosis of prostate cancer may be improved by the application of a more objective and reliable tumor-specific marker.

PSA is the most commonly used biomarker for the diagnosis and the prediction of prognosis in prostate cancer.[6,7] However, PSA is not a cancer-specific marker, as it is present in

benign and malignant prostatic epithelial cells.[8] Serum PSA levels frequently are elevated in benign conditions such as benign prostatic hyperplasia (BPH) and prostatitis.[9,10] Consequently, patients with an elevated serum PSA level must undergo a biopsy to confirm or exclude the presence of prostate cancer. Other biomarkers, including prostate acid phosphatase (PAP),[11,12] prostate-specific membrane antigen,[13,14] prostate inhibin peptide,[15] PCA-1,[16] PR92,[17] prostate-associated glycoprotein complex,[18] PD41,[19] 12-lipoxygenase,[20] p53,[21] p27,[22] hepsin,[23,24] PIM-1 kinase,[23] and EZH2[24,25] are expressed in prostate carcinoma. However, up to now, none of these markers have been used by pathologists to distinguish benign from malignant glands because they lack sensitivity or specificity for prostate carcinoma in formalin-fixed tissue samples.

Benign prostate glands contain secretory epithelial cells that express PSA and PAP and basal cells that lie beneath the secretory cells. Basal cells are oriented parallel to the basement membrane and might be inconspicuous in benign glands. Because basal cells are absent in prostate adenocarcinoma, high-molecular-weight cytokeratin (34βE12)[26-29] and p63[30] immunostains specific for basal cells have been used as ancillary tools for the diagnosis of prostate cancer. The identification of the basal cells of prostate glands indicates the presence of benign glands.[26,27,29-31] However, a limitation of using this negative marker for the diagnosis of carcinoma is that basal cells can have a patchy or discontinuous distribution in some benign lesions (ie, adenosis). Consequently, negative staining for basal cell staining in a few glands suggestive of cancer is not proof of their malignancy.[29]

Discovery of P504S/α-Methylacyl Coenzyme A Racemase as a Prostate Cancer Marker

Recent advances in molecular biology have had a great impact on the clinical practice of medicine. In particular, newly developed techniques such as RNA subtraction hybridization and complementary DNA (cDNA) microarrays permit the identification and comparison of genes expressed differentially in malignant and benign cells. In 2000, Xu et al[32] using cDNA library subtraction in conjunction with high-throughput microarray screening identified 3 proteins, including P503S, P504S, and P510S, from benign and malignant prostate tissue. Xu et al[32] reported that P504S was a 382-amino-acid protein, which had been identified as human α-methylacyl coenzyme A racemase (AMACR). AMACR has a role in the β-oxidation of branched-chain fatty acids and fatty acid derivatives.[33] P504S messenger RNA (mRNA) was overexpressed in about 30% (microarray screening) to 60% (quantitative polymerase chain reaction analysis) of prostate tumors and is low to undetectable in normal tissues.[32]

In 2001, Jiang et al[34] reported P504S (AMACR) as a new molecular marker for prostate carcinoma. By using a rabbit monoclonal antibody (P504S, clone 13H4), a total of 207 clinical cases were studied, including 137 cases of prostate carcinoma and 70 cases of benign prostate from prostatectomies (n = 77), prostate needle biopsies (n = 112), and transurethral prostate resections (n = 18), to verify P504S/AMACR expression in tissue sections. Formalin-fixed, paraffin-embedded tissue blocks were used. The slides underwent antigen retrieval technique with a 0.1-mol/L concentration of citrate buffer, pH 6.0, in an 800-W microwave oven. The staining was done in an automated immunostainer with an avidin-biotin complex staining procedure used as the detecting system.[34]

Results showed that this new marker displayed 2 features making it an attractive marker for prostate carcinoma: (1) P504S/AMACR is a marker with high sensitivity for prostate carcinoma. All 137 cases of prostate carcinomas showed strongly positive expression of P504S/AMACR **Table 1** regardless of Gleason grade (2 to 5).[34] Positive P504S staining was defined as continuous, dark cytoplasmic staining or apical granular staining in epithelial cells **Image 1A**, which can be observed easily at

Table 1
Summary of Immunohistochemical Staining for Discovery of P504S/AMACR as a Prostate Cancer Marker

Reference	Antibodies	Specimens	Sensitivity (%)	Specificity (%)
Jiang et al[34]	Monoclonal*	207 clinical cases	100† (n = 137)	88‡ (n = 194)
Rubin et al[35]	Polyclonal§	342 TMAs and 94 needle biopsy specimens	97‖ (n = 94)	100‖ (n = 94)
Luo et al[36]	Polyclonal§	168 CaP cases with standard slides and TMAs	96¶ (n = 142)	97¶ (n = 144)
Beach et al[37]	Monoclonal*	405 clinical specimens	82† (n = 186)#	79‡ (n = 377)

AMACR, α-methylacyl coenzyme A racemase; CaP, carcinoma of the prostate; TMAs, tissue microarrays.
* A rabbit monoclonal antibody to AMACR (P504S, clone 13H4 originally from Corixa, Seattle, WA) available from Zeta, Sierra Madre, CA.
† Positive staining in malignant glands = continuous dark cytoplasmic staining or apical granular staining in epithelial cells.
‡ Positive staining in benign glands = focal or weak or noncircumferential staining.
§ A polyclonal anti-AMACR antibody is not commercially available.
‖ Positive staining in both benign and malignant glands = moderate or strong staining intensity in 94 cases of needle biopsy specimens.
¶ Using a cutoff of ≥100 for scoring of immunohistochemical staining as positive for both benign and malignant glands.
# 186 prostate biopsy specimens with prostate carcinoma.

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low-power magnification. A diffuse staining pattern (>75% of tumor positive) was seen in 92% of cases regardless of Gleason score. P504S/AMACR also was strongly positive in high-grade prostatic intraepithelial neoplasia (PIN). Furthermore, if high-grade PIN partially involved a prostatic gland, the expression of P504S/AMACR was present only in the PIN, but not in the normal epithelial cells of the same gland.[34] The study showed that the expression of high-molecular-weight cytokeratin and P504S/AMACR was mutually exclusive.[34] (2) P504S/AMACR is a marker with high specificity for prostate carcinoma. In contrast with carcinomas, 88% of benign prostate tissue samples, including benign cases and benign prostate tissue adjacent to carcinomas, were completely negative for P504S/AMACR.[34] The other 12% of cases (Table 1) showed only focal and weak positivity (a single cell or groups of epithelial cells with a discontinuous and weakly granular staining pattern) **Image 1B** for P504S/AMACR in the large normal or BPH glands. Moreover, the small benign glands, which can mimic cancer, including atrophy, basal cell hyperplasia, inflammatory glands, and urothelial epithelium/metaplasia and most cases of adenosis, did not show any expression of P504S/AMACR. Therefore, when used in conjunction with histologic criteria, the P504S/AMACR staining pattern should be a useful adjunct in the distinction of benign from malignant glands.[34]

In 2002, Rubin et al[35] and Luo et al,[36] using cDNA microarrays and a polyclonal antibody to AMACR, confirmed the increased expression of AMACR in prostate cancer. Rubin et al[35] reported that significant overexpression of AMACR in prostate cancer was found in 3 of 4 independent DNA microarray analyses (128 specimens) and tissue microarray specimens, including 17 metastatic prostate cancers. Rubin et al[35] also studied 94 prostate needle biopsy specimens and demonstrated 97% sensitivity and 100% specificity of AMACR in the detection of prostate cancer (Table 1).[35]

Luo et al[36] found that more than 95% of prostate cancers stained positively for AMACR, whereas less than 4% of histologically normal prostate epithelium was positive (Table 1). They also demonstrated 81% and 93% AMACR positivity in 32 metastatic prostate cancers from non–hormone-refractory disease and 14 hormone-refractory metastatic prostate cancers, respectively.[36] Luo et al[36] showed that AMACR and p63 could be used in combination in the same tissue slide, as these markers are located in the cytoplasm and the nucleus, respectively. They concluded that AMACR is a new positive marker that complements the traditional basal cell stains to enhance prostate cancer diagnosis.[36]

Later, Beach et al[37] studied 405 clinical specimens, including 376 prostate needle biopsy specimens with the P504S monoclonal antibody, and reported that 153 (82%) of 186 biopsy specimens with prostate carcinoma were positive for AMACR, while 21% of the foci of benign prostate epithelium showed focal, faint, and noncircumferential luminal staining (Table 1). Circumferential luminal to subluminal and diffuse cytoplasmic staining was the most specific staining pattern of AMACR for prostate carcinoma and almost never was associated with benign prostate tissue.[37] No positive staining was found in the specific small

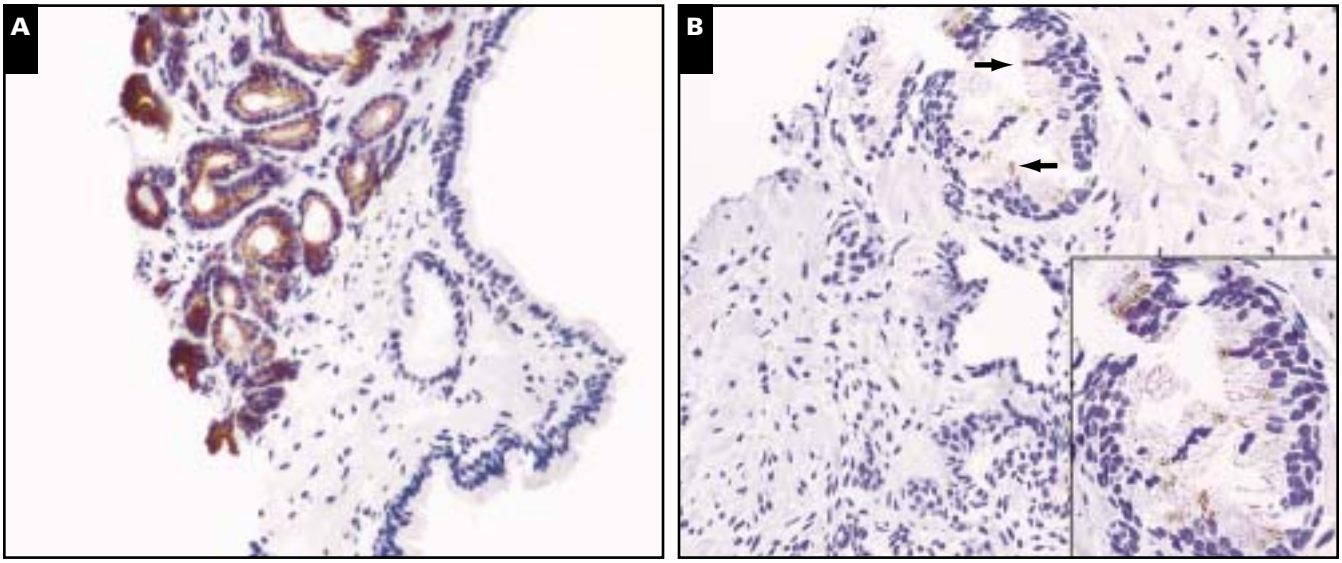

Image 1 Prostate needle biopsy specimen showing characteristic immunohistochemical staining patterns of α-methylacyl coenzyme A racemase (AMACR) between malignant and benign glands: *strong positive* AMACR with an intense, continuous, granular cytoplasmic staining pattern in carcinoma glands (**A**) compared with adjacent benign glands with negative staining or *weak positive* AMACR in individual cells only (**B**; arrows and insert) (**A** and **B**, original magnification ×200).

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gland proliferation of postatrophic hyperplasia, transitional metaplasia, and basal cell hyperplasia.[37] Leav et al[38] reported AMACR (P504S) expression in prostate cancer of the transition zone. They found that all 25 cases with Gleason grade 1 carcinoma were positive for AMACR, although the staining was less intense in grade 1 than in higher grade carcinomas.[38]

In summary, a number of studies from several institutions have demonstrated that P504S/AMACR is an important positive tissue marker for prostate carcinoma regardless of tumor grade, with a sensitivity ranging from 82% to 100% and a specificity ranging from 79% to 100%, even with different criteria for positive stains in benign and malignant glands (Table 1). It has the potential to be a useful marker for prostate carcinoma in clinical pathology practice.

A Useful Marker for the Diagnosis of Small Foci of Prostatic Adenocarcinoma in Needle Biopsy Specimens

The emphasis on the early detection of prostate cancer by mass screening of men has led to an increasing number of small foci of cancer encountered in prostate needle biopsy specimens.[2,3,39,40] Establishing a definitive diagnosis of malignancy in prostate needle biopsy specimens with minute foci of adenocarcinoma is a major diagnostic challenge for surgical pathologists.

The majority of diagnostic problems in prostate needle biopsy specimens are related to small infiltrating malignant glands that usually are graded as Gleason score 6 (3 + 3). Several factors contribute to the difficulty in diagnosis of limited prostate cancer in needle biopsy specimens. First, the malignant cells can be limited to a few glands that might be overlooked easily. Second, there is no single histologic feature specific and sufficient for the diagnosis of prostate cancer. The diagnosis is based on the combination of architectural and cytologic change.[3-5] Third, many benign prostatic conditions such as small, crowded glands; atrophy; inflammatory atypia; and basal cell hyperplasia might mimic prostate cancer histologically.[41] Fourth, the consequences associated with incorrect diagnosis can be serious, such as unnecessary prostatectomy or radiation associated with adverse complications owing to a false-positive diagnosis or delay of effective treatment owing to a false-negative diagnosis. Finally, because of sampling variations, a small focus of prostate cancer in the biopsy specimen might not necessarily represent a tumor of insignificant volume,[40,42,43] or the tumor might not be sampled during rebiopsy. Therefore, it is important to make a definitive diagnosis using limited material if possible.

Because negative staining for high-molecular-weight cytokeratin in a few atypical glands might be insufficient for a definitive diagnosis of malignancy,[29] a positive diagnostic marker specific for prostatic adenocarcinoma might enhance our ability to diagnose limited prostate cancer. Whether small foci of carcinoma can be detected reliably by AMACR is of crucial importance in its clinical application.

We studied 73 cases with a small focus (≤1 mm in diameter) of prostatic carcinoma and 69 benign prostate samples.[44] AMACR immunoreactivity was found in 69 (95%) of 73 cases of carcinoma but not in any benign prostate tissue samples (0/69) or benign glands adjacent to malignant glands **Image 2**. The 34βE12 immunostaining confirmed the absence of basal cells in the focus of carcinoma in all 73 cases. In our study, most of the cases (>95%) with a minute focus of small infiltrating glands of cancer had a Gleason score of 6 (3 + 3). We concluded that using AMACR as a positive marker along with basal cell–specific 34βE12 as a negative marker could help confirm the diagnosis of limited prostate cancer and reduce the chance of misdiagnosis in a prostate needle biopsy specimen.[44]

Recently, Magi-Galluzzi et al[45] studied large numbers (209 cases) of prostate needle biopsy specimens with small foci (<5% of a core) of prostate carcinoma, including 34 cases from their institution and 175 cases from outside consultations. Of small foci of prostate carcinoma, 88% were positive for AMACR. They found that the sensitivity varied among the different groups: 100% for the in-house cases and 80% to 87% for cases from outside institutions, which they suggested possibly related to differences in fixation and

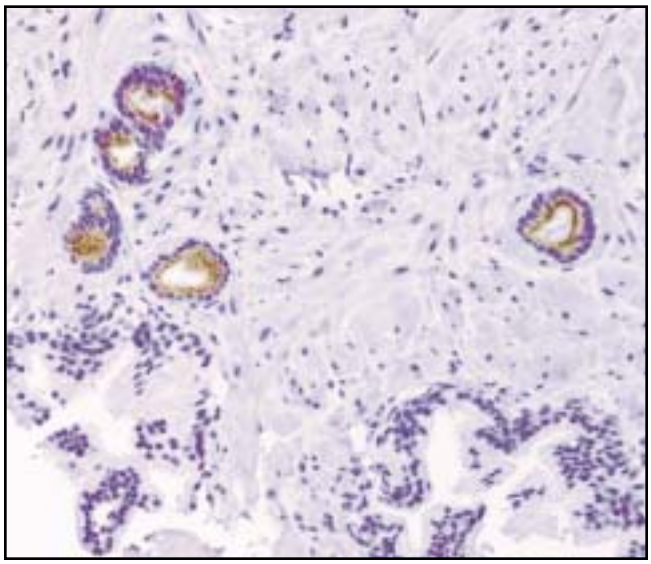

Image 2 Immunohistochemical staining of α-methylacyl coenzyme A racemase (AMACR) showing that a small focus of prostate carcinoma glands with minimal nuclear atypia was positive for AMACR (original magnification, ×200).

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processing in different pathology laboratories. Although it is extremely important to recognize negative staining of AMACR in some small cancers, they concluded that positive staining for AMACR could increase the level of confidence in establishing a definitive malignant diagnosis from the needle biopsy specimen.[45]

Both studies[44,45] have demonstrated that AMACR/P504S could be used successfully as part of the routine surgical pathology workup of difficult prostate biopsy specimens with "suspicious" small glands.

We suggest that using AMACR/P504S as a positive marker along with basal cell–specific 34βE12 and/or p63 as negative markers could help confirm the diagnosis when small atypical glands are identified by routine H&E staining. **Figure 1** illustrates 4 immunohistochemical staining patterns: (1) If small, focal atypical glands stain with basal cell markers but not with AMACR/P504S, the diagnosis is benign. (2) When atypical glands are positive for 34βE12/p63 and AMACR/P504S, malignancy can be ruled out. The differential diagnoses include high-grade PIN,[34-37] adenosis,[46] and even some benign glands[34-37] based on the findings on H&E staining. (3) If small atypical glands, excluding high-grade PIN and nephrogenic adenoma, are negative for basal cell markers but positive for AMACR/P504S, a malignant diagnosis is established. (4) In the scenario that small atypical glands are negative for 34βE12/p63 and AMACR/P504S, the diagnosis might be malignant or benign. In our experience, the likelihood of negative staining of both 34βE12/p63 and AMACR/P504S in small focal carcinoma in needle biopsy specimens is rare (<6%).[44] However, because Magi-Galluzzi et al[45] reported a variable sensitivity (80%-100%) for the diagnosis of minimal prostatic cancer, it is important to recognize that some small focal cancers might be negative for AMACR/P504S.[44,45]

In summary, positive basal cell stains (scenarios 1 and 2) can help rule out malignant or PIN lesions (Figure 1), whereas positive staining for AMACR in small atypical glands with absence of basal cells (scenario 3) can establish a definitive malignant diagnosis when high-grade PIN and nephrogenic adenoma have been excluded (Figure 1). Staining pattern 4 is an uncommon finding. We will address this issue again in the "Cautions in Interpretation of AMACR/P504S Immunohistochemical Results for Clinical Diagnosis of Prostate Cancer" section.

Boosts Diagnostic Resolution of Atypical Foci

Atypical small acinar proliferation, focus of atypical glands, and *focal glandular atypia* are the terms used by

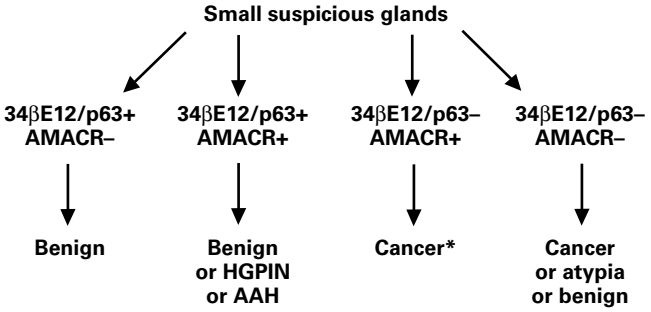

Figure 1 Schematic outline for the diagnosis of small, focal prostate carcinoma on needle biopsy by basal cell and AMACR/P504S immunohistochemical analysis. * Caution should be exercised in interpreting rare, small glands adjacent to HGPIN and nephrogenic adenoma with AMACR staining and absence of basal cells. Rare, small glands adjacent to HGPIN might represent out-pouching of the PIN glands, as PIN may exhibit a discontinuous staining pattern for basal cells. 34βE12, high-molecular-weight cytokeratin; AAH, atypical adenomatous hyperplasia; AMACR, α-methylacyl coenzyme A racemase; HGPIN, high-grade prostatic intraepithelial neoplasia.

pathologists in the 1.5% to 9.0% of prostate biopsy specimens[39,47-52] with diagnostic uncertainty. Focus of atypical glands includes a spectrum of cases with histologic atypia, but most cases are classified as atypia because of the small size of the abnormal focus. "Atypia suspicious of but not diagnostic for malignancy" is a valid diagnosis when we have "uncertainty," and pathologists often recommend repeated biopsy. Although atypia is not a pathologic entity, it conveys a 42% to 45% predictive value for carcinoma on repeated biopsy.[53,54]

In a recent study, Jiang et al[55] examined 41 foci of "atypical cases" with a combination of P504S/AMACR and 34βE12 stains on prostate needle biopsy specimens. The data showed that more than half of the diagnostically uncertain atypical foci were classified definitively when this combination of antibodies was used. Of the foci, 375 were diagnosed as cancer by at least 2 pathologists after analysis of a combination of P504S/AMACR and 34βE12 stains.[55] Among the definitive malignant diagnostic foci, 53% were resolved by 34βE12 staining alone. In the remaining 47% of cases, the addition of P504S permitted a definitive diagnosis.[55] These data suggest that positive staining for AMACR can increase the level of confidence in establishing a definitive malignant diagnosis in atypical cases with negative basal cell staining **Image 3**. The use of AMACR and a basal cell marker in combination might not only make uncertain diagnoses less frequent but also obviate the need for a number of repeated biopsies.

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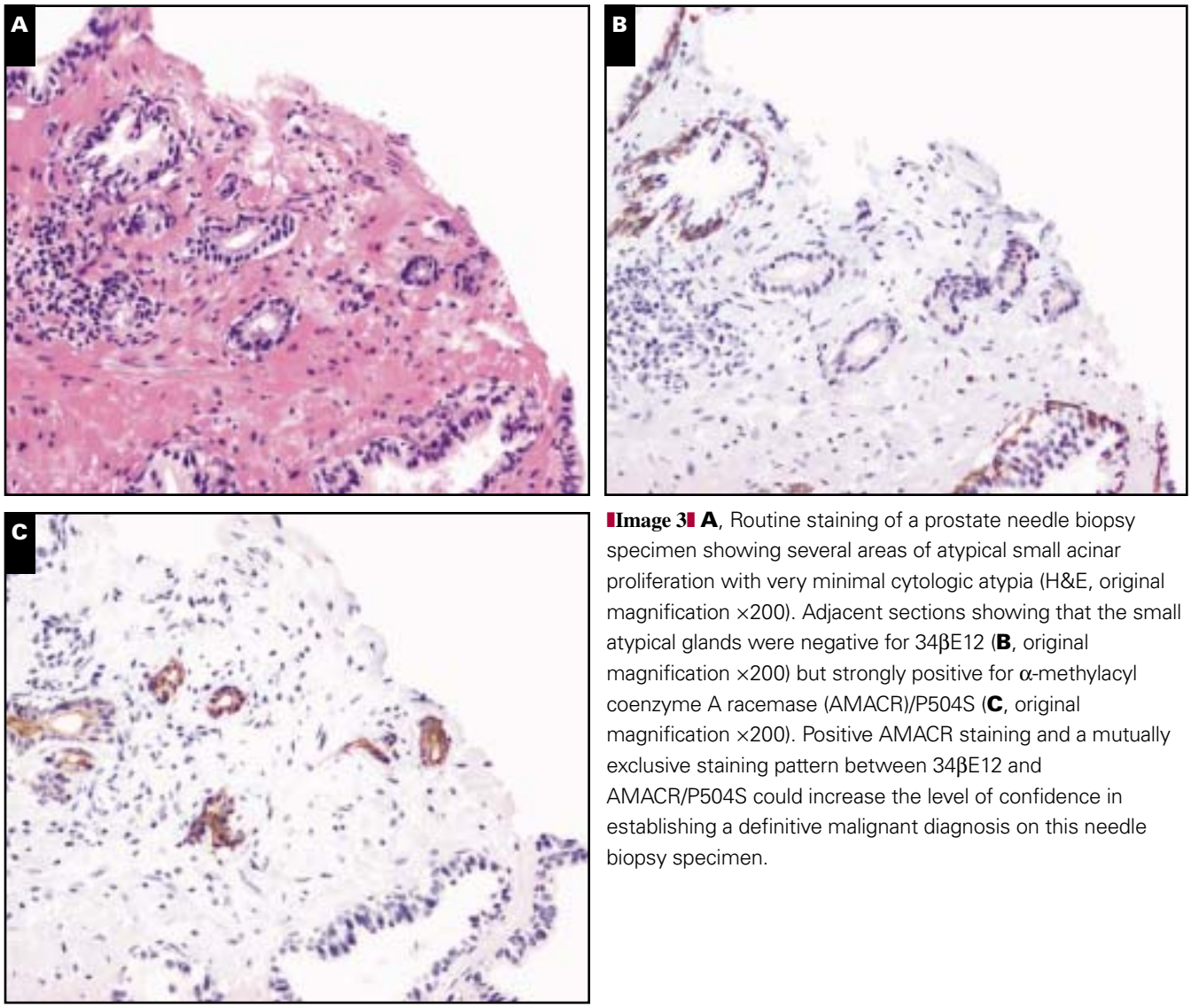

Image 3 **A**, Routine staining of a prostate needle biopsy specimen showing several areas of atypical small acinar proliferation with very minimal cytologic atypia (H&E, original magnification ×200). Adjacent sections showing that the small atypical glands were negative for 34βE12 (**B**, original magnification ×200) but strongly positive for α-methylacyl coenzyme A racemase (AMACR)/P504S (**C**, original magnification ×200). Positive AMACR staining and a mutually exclusive staining pattern between 34βE12 and AMACR/P504S could increase the level of confidence in establishing a definitive malignant diagnosis on this needle biopsy specimen.

AMACR in Variants of Prostatic Adenocarcinoma

Foamy gland and pseudohyperplastic carcinomas are 2 uncommon variants of prostatic adenocarcinoma. Foamy gland carcinoma, characterized by abundant xanthomatous-appearing cytoplasm, often demonstrates small and condensed nuclei without nucleolar prominence. Pseudohyperplastic carcinoma is composed of malignant large branching glands mimicking benign prostatic hyperplasia, although nuclear atypia typically is present. These 2 variants of prostatic adenocarcinoma might be difficult to recognize in a needle biopsy specimen. Studies of AMACR expression in these variants of prostatic adenocarcinoma are diagnostically important.

Beach et al[37] found that 5 (83%) of 6 prostatectomy specimens with pseudohyperplastic patterns of prostate carcinoma were positive for P504S/AMACR. After studying needle biopsy specimens, Zhou et al[56] reported that 68% of foamy gland carcinomas were positive for AMACR while 77% of pseudohyperplastic prostate carcinomas were positive for AMACR by immunohistochemical analysis with the P504S monoclonal antibody for AMACR. With a polyclonal AMACR antibody, the positivity rates were 62% for foamy gland carcinoma and 70% for pseudohyperplastic carcinoma, which is not significantly different from the results obtained with the monoclonal antibody. The mean percentages of stained glands in positive cases were 74.4% (range, 25%-100%) with P504S and 78.9% (range, 20%-100%) with polyclonal AMACR in foamy gland cancer and 91% (range, 10%-100%) with P504S and 86.7% (range, 10%-100%) with polyclonal AMACR in pseudohyperplastic cancer.

We also conducted a quantitative analysis of 23 cases of foamy gland carcinoma of the prostate using predominantly radical prostatectomy specimens (unpublished data). Our study

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demonstrated that 72% of malignant foamy glands were positive for AMACR. Quantitative immunohistochemical analysis using the ChromaVision Automatic Cellular Imaging System (ACIS; ChromaVision Medical System, San Juan Capistrano, CA) showed that AMACR/P504S staining intensity in foamy gland carcinoma was 57.4 (this value is an average staining intensity of the selected areas), which was significantly higher than the 15.8 found in benign prostatic glands but lower than the 110.2 found in ordinary prostatic adenocarcinoma.

These studies confirmed that AMACR was positive in a subset of but not all foamy gland and pseudohyperplastic prostate carcinoma cases. The AMACR staining was less intense in foamy gland carcinoma than in the ordinary carcinoma. In clinical practice, Zhou et al[56] suggested that when a diagnosis of foamy gland or pseudohyperplastic variant of cancer is favored on routinely stained sections and stains for basal cells are negative, positive staining for AMACR can provide an additional level of confidence to establish a definitive malignant diagnosis.

Atrophic prostate carcinoma is another rare variant of prostate adenocarcinoma. It is difficult to distinguish from benign atrophy in a needle biopsy specimen. P504S/AMACR was demonstrated in rare cases of atrophic patterns of prostate cancer.[32,35] Farinola and Epstein[57] studied the expression of AMACR in 15 needle biopsy specimens with small foci of atrophic prostate cancer. AMACR was expressed in 10 of 15 cases (67%) of atrophic prostate cancers while 5 cases were negative for AMACR. In a comparable study of small ordinary prostate cancers diagnosed in needle biopsy specimens, they found that approximately 90% were positive for AMACR. Therefore, they concluded that atrophic prostate cancers were not positive as frequently as ordinary prostate cancers.

AMACR in Prostate Adenocarcinoma After Therapy

A number of effective therapeutic modalities currently are used for patients with prostate cancer. These treatments, particularly radiation and hormonal therapy, often induce significant histologic changes not only in prostate cancer cells but also in adjacent benign prostatic glands. It is well documented that these histologic changes might present a major challenge for pathologists in the diagnosis of recurrent or persistent prostate cancer.

Radiation therapy, including external beam and internal seed implants, might provide curative therapy for some patients with prostate cancer. However, on routine H&E-stained sections, benign epithelial cells in these irradiated glands demonstrate nuclear enlargement, prominent nuclear irregularity, and hyperchromasia, mimicking prostatic adenocarcinoma.[58-60] Because the confirmation of the presence of cancer in the irradiated prostate is critical for initiating additional local therapy, it would be helpful to have a positive marker to facilitate the challenging distinction between postradiation atypia and adenocarcinoma.

Beach et al[37] found that 4 of 5 specimens with radiation-treated carcinoma were positive for P504S/AMACR. Yang et al[61] studied 80 prostate glands, including 40 radiated specimens (28 adenocarcinomas and 12 benign) and 40 nonradiated prostate specimens (20 adenocarcinomas and 20 benign). All 48 cases of carcinoma (28/28 radiated and 20/20 nonradiated specimens) showed strongly positive AMACR immunostaining, while AMACR was negative in all radiated and nonradiated benign prostate specimens and in the radiated benign glands adjacent to carcinoma. The results demonstrate that AMACR immunostaining facilitates the distinction between postradiation prostatic adenocarcinoma and radiation-induced atypia in benign prostatic epithelium.

Amin et al[62] studied P504S/AMACR expression in 26 postradiation therapy (PRT) prostate cancer patients and showed P504S/AMACR expression in 94% of PRT prostate carcinomas. They also found that down-regulation of P504S/AMACR appeared in PRT cancers with the extent of treatment effects.[62]

Another major type of therapy for patients with prostate cancer is androgen-deprivation hormonal therapy. Treatments include castration (orchiectomy), medical administration of luteinizing hormone releasing hormone analogs, and androgen receptor (AR) blockers. This type of therapy causes similar atrophic changes in prostate cancer cells and benign prostatic glands as well as in stromal inflammatory infiltrates. Rubin et al[35] reported a significant decrease in AMACR expression in the metastatic hormone-refractory prostate cancers compared with hormone-naive-localized prostate cancers. Luo et al[36] reported that 93% (13/14 cases) of hormone-refractory metastatic cancers were positive and 71.4% were strongly positive for AMACR, whereas Beach et al[37] found that 8 of 8 specimens of hormonally treated cancers were positive for P504S. Kuefer et al[63] found that AMACR expression in the hormone-sensitive cell line, LNCaP, after exposure to antiandrogen treatment was unchanged, whereas PSA, known to be androgen-regulated, demonstrated decreased expression. Recently Zha et al[64] reported that expression of AMACR was independent of AR-mediated signaling. AMACR could not affect the stabilization of AR itself or modulate the expression of the AR-targeted gene in vivo, and AR could not regulate the expression of AMACR. These data suggested that AMACR expression is hormone-independent[63,64] and that AMACR might be used as a marker to monitor cancer after hormonal therapy.

High-Grade PIN and AMACR

The importance of studying AMACR in potential precancerous lesions of the prostate is 2-fold: first, to

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possibly permit us to understand prostatic carcinogenesis and develop chemopreventive measures, and second, for distinguishing these precancerous lesions from prostate cancer, which is important for the practicing pathologist.

High-grade PIN, which consists of architecturally benign prostatic acini or ducts lined by cytologically atypical cells, is considered a precursor[65-67] of many moderately to poorly differentiated peripheral zone prostatic adenocarcinomas. Finding high-grade PIN in a prostate needle biopsy specimen is clinically significant because the risk of carcinoma on rebiopsy ranges from 27% to 79%.[68-75] Several reports have shown the expression of AMACR in high-grade PIN in addition to prostate cancer.[34-37] These findings suggest a possible role of AMACR in early prostatic carcinogenesis.

However, the positive rate of AMACR reported in high-grade PIN was variable, ranging from 13% to 72%. One of the reasons is that different specimens were used for analysis, particularly biopsy material and tissue microarray cores, which sample only a small portion of the prostate. Recently, we conducted an extensive analysis of the expression of AMACR in approximately 4,000 high-grade PIN glands from 138 radical prostatectomy specimens (unpublished data). Of the high-grade-PIN cases, 94% were positive for AMACR. However, only 41.10% (1,617/3,934) of the prostatic glands involved by high-grade PIN showed AMACR immunoreactivity. This finding indicated that variable AMACR reactivity was caused by sampling different areas of high-grade PIN with or without AMACR reactivity.

The detection of AMACR in PIN established another biochemical link between high-grade PIN and prostate cancer development, which further supports the notion that high-grade PIN is a precursor lesion for prostate cancer. Whether AMACR can serve as a molecular marker to monitor the early development of prostate cancer and detect other potential precursor lesions remains to be seen.

The presence of AMACR immunoreactivity in high-grade PIN also suggests that it is necessary to exclude PIN before a diagnosis of cancer can be made if AMACR immunostaining is used. Because of the difference in the presence of basal cells in PIN and the absence of basal cells in prostate cancer, using a combination of AMACR and basal cell markers (34βE12 or p63) is recommended for differential diagnosis.

Atypical Adenomatous Hyperplasia and AMACR

Atypical adenomatous hyperplasia (AAH) is characterized by a well-circumscribed lobule of closely packed, crowded, small glands without significant cytologic atypia.[66,76,77] The prevalence of AAH has been reported to be 1.6% to 19.6% in transurethral prostate resection specimens and 23% in radical prostatectomy specimens.[77-79] This wide range could be due to variable diagnostic criteria used by pathologists. Most cases of AAH are found in the prostatic transition zone where low-grade prostatic adenocarcinoma arises.

AAH can be difficult to distinguish from low-grade prostatic adenocarcinoma because of their architectural similarities.[80] However, AAH typically lacks significant cytologic atypia despite exhibiting abnormal architectural features similar to those of low-grade prostatic adenocarcinoma. Consequently, AAH can be confused with prostate cancer or a lesion suggestive of prostate cancer. However, the distinction between AAH and carcinoma is imperative because the prognosis and treatment are very different. The presence of patchy basal cells, which can be demonstrated by immunostaining for high-molecular-weight cytokeratins (34βE12), is a characteristic of AAH. In contrast, prostatic adenocarcinoma usually lacks basal cells. However, basal cell staining alone might be insufficient in some cases to reach a definite diagnosis, because patchy basal cell staining can be indistinguishable from negative staining, particularly if the material is limited. Therefore, a marker positive for prostate cancer will be valuable in making a definitive diagnosis.

Yang et al[46] studied 40 cases of AAH by immunohistochemical analysis using the P504S monoclonal antibody and a basal cell–specific marker specific for 34βE12. AMACR was undetectable in the majority of cases of AAH (33/40 [83%]), focally expressed in 4 (10%), and diffusely positive in only 3 cases (8%). Interestingly, 2 of 7 AMACR-positive AAH cases were found adjacent to adenocarcinomas, which were strongly positive for AMACR. All BPH cases were negative for AMACR (0/20 [0%]), and all prostatic carcinoma cases used in the study (20/20 [100%]) showed diffuse AMACR staining pattern. Gupta et al[81] recently found that 31% of cases of AAH expressed P504S/AMACR. These findings suggest that AAH is a heterogeneous entity and that AMACR immunostaining can be helpful in distinguishing the majority of AAH cases from carcinoma. The combination of AMACR/P504S and 34βE12 will help to distinguish AAH from prostatic adenocarcinoma, particularly in prostate needle biopsy specimens.

Benign Conditions of the Prostate and AMACR

As discussed previously, in cDNA expression microarray analysis, AMACR was found to be frequently overexpressed in prostatic adenocarcinomas compared with benign prostate tissue samples.[32,35,36] Western blot analysis demonstrated 36-fold overexpression of AMACR/P504S in

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prostate carcinoma compared with benign prostatic tissue.[34] However, a small amount of AMACR occasionally can be detected in benign prostatic epithelium, particularly by immunohistochemical analysis. The focal positive rates of the AMACR-positive staining with a monoclonal antibody (P504S) in benign secretory cells have been reported to be 12% to 21% in benign glands.[34,37] Other studies with a polyclonal antibody have reported similar findings.[35,36]

Typical benign prostate glands do not express AMACR at all or express very low levels. In contrast with the strong, coarse, granular staining in prostatic cancer cells (Image 1A), AMACR staining in benign prostatic secretory epithelium is almost always *focal, weak, and noncircumferential* with fine granules in the cytoplasm (Image 1B) and can be observed only with high magnification.[34,37,44] A diffuse staining pattern was not found in benign prostate glands. Moreover, the small, benign glands, which can mimic cancer, including atrophy, basal cell hyperplasia, inflammatory glands, and urothelial epithelium/metaplasia and most cases of adenosis, did not show any expression of AMACR by immunohistochemical analysis with a monoclonal antibody (P504S).[34] Rarely, scant individual positive AMACR cells could be found in florid basal cell hyperplasia (2 of 15 cases).[82] The shape and distribution of the AMACR-positive cells seemed to correspond to the chromogranin-positive neuroendocrine cells in the adjacent sections of the benign hyperplastic glands.[82] On needle biopsy material, we also occasionally observed the presence of scattered individual benign cells positive for AMACR, which might represent neuroendocrine cells but not basal cells in benign prostate glands. However, the number, shape, and distribution pattern of these benign AMACR cells are obviously different from those of cancer cells. Therefore, when used in conjunction with histologic criteria, the AMACR/P504S staining pattern should be a useful adjunct for distinction of benign from malignant glands.

We used the ChromaVision ACIS to evaluate the intensity and percentage of positivity in 10 cases of prostatic adenocarcinoma compared with the intensity and positivity in adjacent benign prostatic tissue in the same section. Six different areas of cancer or benign tissue were analyzed for each case. With ACIS, the percentage of positivity is the area detected by the brown threshold divided by the sum of the area detected by the brown and blue background (nuclear staining) thresholds. The intensity is calculated by masking out all areas not selected by the brown threshold and calculating the integrated optical density of brown within the remaining area. As measured by the ACIS, the average percentage of AMACR-positive staining was 45.7% in prostatic carcinoma and 0.02% in benign prostatic tissue ($P < .01$). The average intensity of AMACR-positive cells was 105.9 in prostatic carcinoma and 16.1 in benign prostatic tissues ($P < .02$).

When using a monoclonal antibody (P504S), Beach et al[37] failed to detect the expression of AMACR in postatrophic hyperplasia (PAH), whereas Rubin et al,[35] using a polyclonal antibody, found overexpression of AMACR in PAH. The differences might be due to the use of different antibodies that might exhibit different specificity for benign prostate glands.[83] Kunju et al[83] compared a monoclonal antibody (P504S) and a polyclonal antibody with AMACR in benign, atypical, and malignant prostate tissue samples. They found 68% of benign glands with weak expression of AMACR by the polyclonal antibody compared with only 7% of benign glands stained with P504S, although the polyclonal antibody displayed higher sensitivity for prostate cancer (100%) than the monoclonal antibody (94%).[83] The specificity of the antibodies might not be the only reason to explain the differences because by using the monoclonal antibody (P504S), we found that some PAH specimens were weakly positive for AMACR. Therefore, further study is needed to compare the expression of AMACR in PAH by using monoclonal and polyclonal antibodies that might recognize variants of AMACR.

Generally, benign hyperplastic glands (BPH) are negative for AMACR.[34,37] However, Leav et al[38] found that 8 BPH samples juxtaposed to carcinoma expressed AMACR. In contrast, AMACR was not found in any other BPH nodules. This suggests that carcinomas in the transition zone might arise from an AMACR-positive transition lesion within a subset of BPH nodules.[38]

Another benign condition, which may be present in the prostatic urethra, is nephrogenic adenoma. We found that some nephrogenic adenomas showed focal or diffuse strong AMACR immunoreactivity (unpublished data). Recent molecular genetic studies of nephrogenic adenomas have shown that they are derived from shedding renal tubules,[84] which express AMACR.[36,85] The expression of AMACR in nephrogenic adenoma supports this hypothesis. Because nephrogenic adenomas may be found in the prostatic urethra, caution should be exercised, as AMACR is not indicative of cancer in this lesion.

AMACR in Various Malignant Neoplasms and Normal Tissues

Because AMACR is highly expressed in prostate cancer, it is very important to determine its expression in normal tissues and other malignant tumors. Luo et al[36] reported that AMACR was found in hepatocytes, kidney tubules, salivary glands, and absorptive cells in the small and large intestines. By using a monoclonal antibody (P504S), Jiang et al[85] studied 222 different normal tissues. AMACR protein was detected in hepatocytes, renal tubular epithelial cells,

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bronchial epithelial cells, mucosal epithelial cells of the gallbladder, and the brush border of colonic mucosa,[85] confirming the findings of Luo et al.[36]

Zhou et al[86] tested 96 tumors with a polyclonal antibody, in tissue microarray sections, and they reported that AMACR was detected in colorectal, ovarian, breast, bladder, lung, and renal cell carcinomas, lymphoma, and melanoma. The greatest overexpression was seen in colorectal carcinoma, with positive staining in 92% of cases followed by 60% of ovarian and more than 30% of breast carcinomas. Of the colonic adenomas, 75% also expressed AMACR.[86] Therefore, they suggested that AMACR is potentially an important tumor marker for several cancers and their precursor lesions, especially those linked to high-fat diets.[86]

By using a monoclonal antibody (P504S), Jiang et al[85] studied the expression of AMACR in 539 malignant tumors and found that AMACR was expressed in a high percentage of adenocarcinomas arising from organs that constitutively express AMACR, including 17 (81%) of 21 hepatocellular carcinomas and 18 (75%) of 24 renal cell carcinomas. A number of carcinomas arising from tissues normally not expressing AMACR also were positive for the antigen, including 9 (31%) of 29 urothelial carcinomas and 4 (27%) of 15 gastric adenocarcinomas.[85] In addition, 250 cases of adenocarcinomas from lung, breast, pancreas, bile duct, adrenal gland, salivary gland, ovary, thyroid, and endometrium were negative or rarely positive for AMACR.[85]

A number of differences were found between the study by Zhou et al[86] and the study by Jiang et al.[85] They reported high expression of AMACR in breast and ovarian carcinomas and melanoma, whereas Jiang et al[85] found rare AMACR positivity in breast (9/61 [15%]) and ovarian (2/27 [7%]) carcinomas and no expression of AMACR in melanoma. The differences between the results of the studies might be due to the use of different antibodies. As previously mentioned, the polyclonal antibody for AMACR might exhibit less specificity for prostate carcinoma than the P504S monoclonal antibody.[83]

Jiang et al[87] also studied the expression of AMACR in 176 colorectal carcinomas and reported significant up-regulation of AMACR mRNA in colon carcinomas compared with normal tissue. There was very low or no expression of AMACR protein in normal colonic tissue, but AMACR was highly expressed in 76% and 75% of well-differentiated and moderately differentiated colon carcinomas, respectively,[87] which supports previous findings obtained with the polyclonal antibody for AMACR.[86] The poorly differentiated carcinomas of the colon showed a much lower frequency of positivity.[63,87]

Although AMACR is not expressed in the majority of carcinomas, the expression of AMACR in several nonprostatic carcinomas indicates that AMACR has limited value in determining the primary site of a metastatic carcinoma.

Function of AMACR and Its Role in the Pathogenesis of Cancer Development

AMACR is expressed at appreciable levels and is transported to the peroxisomal and mitochondrial compartments in a variety of tissues, including liver, kidney, skeletal muscle, gallbladder, and brain.[88] It is an essential enzyme for the degradation of branched-chain fatty acids by β-oxidation and catalyzes the conversion of several (2*R*)-methyl branched-chain fatty acyl-CoAs to their (*S*)-stereoisomers,[33] and Mubiru et al[89] and Shen-Ong et al[90] recently found 5 versions (IA, IB, IIA, IIAs, and IIB) of AMACR transcripts from human prostate cancer. AMACR IA, the most abundant form, encodes a 382-amino-acid protein. AMACR IIA contains an alternative fifth exon and encodes a 288-amino-acid protein. AMACR IIAs uses an alternative splice acceptor site in the alt5 exon. The B forms of AMACR seem to be alternative spliced versions of the IA and IIA forms in which the common 160-base-pair exon 3 is absent. Only the predominant AMACR IA contains the previously identified peroxisomal targeting signal (PTS1) peptide, while the other 4 variants are basic proteins that lack the peroxisomal targeting signal peptide. These observations have implications for the cellular localization and function of these AMACR variants.[89,90]

As a result, AMACR is a required component of the oxidative metabolism and biosynthetic pathways of branched-chain fatty acids and bile acids, respectively. High levels of branched-chain fatty acids have been found in some dietary sources such as beef, milk, and dairy products. Overexpression of AMACR in prostate and colon carcinomas and their precursor lesions (high-grade PIN and adenoma)[63,87] is of particular interest because epidemiologic and animal studies have shown an association between dietary factors and an increased rate of prostate and colon cancer.[91,92] Premalignant lesions, high-grade PIN, and colon adenomas express AMACR, whereas hyperplastic polyps of the colon, which are not associated with colon cancer, show low or no expression of AMACR.[87] Taken together, these findings suggest a potential role for AMACR in the early stage of cancer transformation and in the subsequent progression of carcinoma.

Recently, Mobley et al[93] reported that 2 branched fatty acids, including pristanic acid and phytanic acid, which are a major component of dairy and beef products,[94] markedly increased AMACR protein expression in LNCaP (an androgen-responsive prostate cancer cell line) but not the NPrEC cells (a normal prostate basal epithelial cell line). The findings provide a link between the consumption of dietary fatty acids and the enhanced expression of AMACR in prostate cancer cells.[93] However, AMACR expression is not hormone-dependent because neither

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antiandrogen nor androgen has any apparent effect on AMACR expression.[63,93]

Although exogenous fatty acids increase AMACR levels in prostate cancer cells, the molecular mechanisms by which AMACR influences the development of prostate cancer are not clear. Zheng et al[95] reported sequence variants of AMACR in germline DNA samples from hereditary prostate carcinoma families and suggested that these polymorphisms in AMACR might be associated with prostate cancer risk. Recently Zha et al[64] reported that increases of AMACR at the protein and mRNA levels in clinical specimens of prostate carcinoma were accompanied by increased enzymatic activity as well. They also demonstrated that small interference RNA against AMACR, but not the control inverted small interference RNA, reduced AMACR expression and significantly decreased proliferation of the androgen-responsive prostate cancer cell line (LAPC-4).[64] The mechanism of this growth inhibition seemed to be completely independent of androgen action.[64] It is still unknown whether overexpression of AMACR results in tumor transformation and why AMACR is so selectively up-regulated in prostate cancer. The role of AMACR in the development of prostate and other cancers should be studied further.

Cautions in Interpretation of AMACR/P504S Immunohistochemical Results for Clinical Diagnosis of Prostate Cancer

Although the findings suggest that AMACR/P504S is an excellent marker of prostate adenocarcinoma, caution should be exercised in interpreting the immunohistochemical staining pattern.

It is critical to recognize that the ratio between the AMACR levels in prostatic adenocarcinoma and benign secretory epithelium is very high, so that a comparison between malignant (Image 1A) and benign (Image 1B) glands in the evaluation of immunohistochemical staining patterns is essential. For example, if the background is minimal, weak staining with a circumferential luminal to subluminal staining pattern is specific for prostate carcinoma. If the background is high, strong diffuse cytoplasmic staining is required to consider the staining pattern consistent with cancer.

The sensitivity of AMACR/P504S immunostaining varies from 80% to 100% for conventional adenocarcinoma of the prostate.[34-37,45] Although it is uncommon, focal nonreactivity of AMACR/P504S has been reported in prostate cancer.[34,44,45] Magi-Galluzzi et al[45] demonstrated that the sensitivity of AMACR staining for small focal cancer might vary in specimens derived from different pathology laboratories. Variants of prostate cancers, including foamy gland cancer, pseudohyperplastic adenocarcinoma, and atrophic adenocarcinoma that are particularly difficult to diagnose, are less frequently (62%-77%) positive for AMACR.[56] It is important to recognize that negative AMACR/P504S staining in small suspicious glands does not necessarily indicate a benign diagnosis (Figure 1). In suspicious cases with negative AMACR staining, the diagnosis of prostate cancer should be based on architectural and cytologic changes in routine histologic sections in combination with the absence of basal cells.[43]

Background AMACR staining in smooth muscle and weak granular staining in benign glands have been reported[34-37,45] and could lead to false-positive results. However, this pattern of staining can be distinguished readily from the dark and circumferential positive staining pattern of malignant glands, which is rarely found in benign prostate glands, by using a monoclonal antibody (P504S). Since benign glands usually are lined by basal cells, the combination of AMACR/P504S and 34βE12 or p63 can easily recognize benign glands if both markers are positive in the same gland. Because P504S (cytoplasmic staining) is a rabbit monoclonal antibody and p63 (a basal cell marker, nuclear staining) is a mouse monoclonal antibody, these antibodies can be used in combination with a single chromogen and 1-step immunohistochemical staining.[36] We also have found that 2 chromogens with AMACR/P504S, 34βE12, and p63 immunococktails **Image 4A** are sensitive enough to detect small focal carcinoma in a prostate biopsy specimen.

Two possible premalignant lesions, high-grade PIN[34-37,45] and AAH,[44,96] might exhibit some or low reactivity for AMACR. Both PIN and AAH retain basal cells and positive immunostaining for 34βE12 or p63 can help in distinguishing PIN **Image 4B** and AAH from prostate cancer. However, rare, small glands adjacent to high-grade PIN with AMACR staining and absence of basal cells might represent out-pouching of the PIN glands, as PIN might exhibit a discontinuous staining pattern for basal cells. Careful examination of multiple levels of the slides, the number of atypical glands, the distance to high-grade PIN, and the staining patterns of AMACR (the dark and circumferential positive staining) and 34βE12/p63 (total absence of basal cell staining) in small atypical glands is crucial to determine whether the small atypical glands adjacent to high-grade PIN represent tangential cutting of PIN or true invasive carcinoma.

In summary, we strongly recommend the use of AMACR/P504S as a positive marker along with basal cell–specific 34βE12 or/and p63 as the negative markers in the study of problematic cases suggestive of focal cancer based on routine histologic examination.

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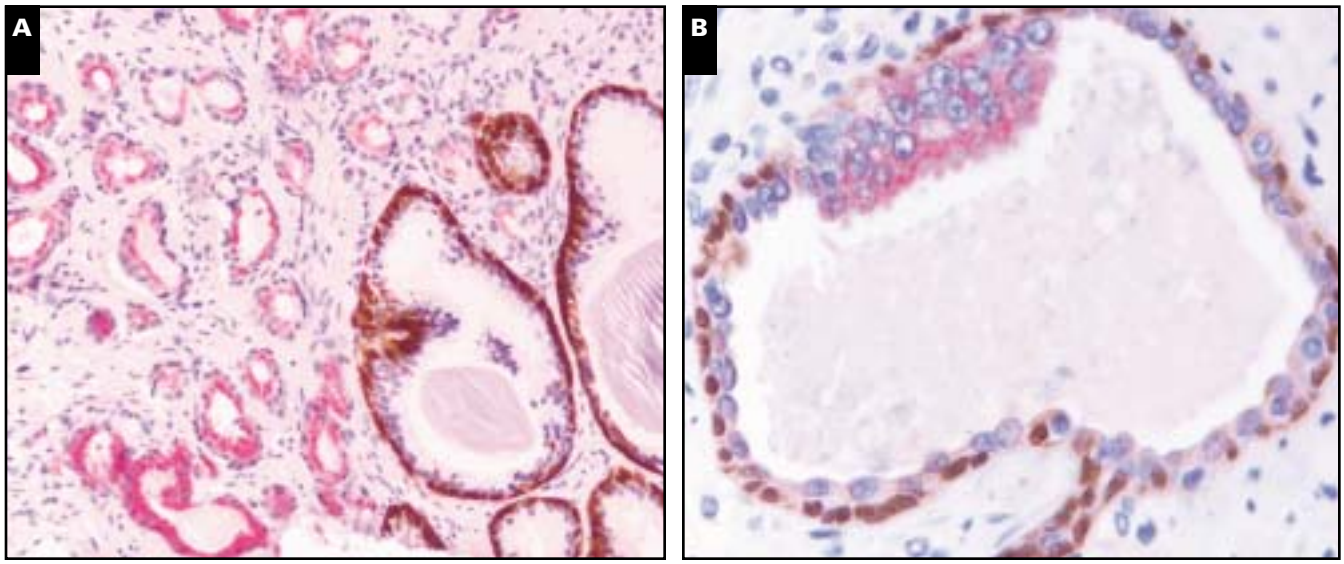

Image 4 **A**, Immunohistochemical stains with double colors (red, α-methylacyl coenzyme A racemase [AMACR]; brown, p63 and 34βE12) and triple antibodies (P504S, p63, and 34βE12) showing a mutually exclusive staining pattern between basal cell stains and AMACR. Benign but not malignant glands were positive for p63 (brown nuclear staining) and 34βE12 (brown cytoplasmic staining), while malignant but not benign glands were positive for AMACR (red cytoplasmic staining; original magnification ×200). **B**, High-grade prostatic intraepithelial neoplasia (PIN) with basal cell staining partially involving the prostate gland showing expression of AMACR (red cytoplasmic staining) only in the high-grade PIN but not in the normal epithelial cells (original magnification ×400).

Implications for Research and Clinical Application

There might be broad application for AMACR in the diagnosis of prostate cancer. In distinction to PSA, AMACR is highly selective for prostate cancer. An antibody-based or quantitative polymerase chain reaction–based serum or seminal fluid test for AMACR, which is unlikely to be affected by benign prostatic diseases, could improve on the current PSA test. In addition, if an AMACR antibody is conjugated with a fluorescent or radioactive indicator, the uptake and binding of AMACR antibody by prostate cancer cells might delineate the entire tumor and provide preoperative information about tumor volume for primary or metastatic prostate cancer.

Conclusion

AMACR (*P504S*) is the first gene identified from prostate cancer by cDNA microarrays to be suitable for clinical practice and to potentially improve the diagnosis of prostate cancer. AMACR displays several attractive features for a prostate cancer marker: (1) With significant overexpression at the mRNA and protein levels, AMACR is one of the few gene products consistently detected in prostate cancer cells by conventional methods such as immunohistochemical analysis. (2) AMACR is a marker with a high specificity for prostate adenocarcinoma. In contrast with carcinoma, most benign cases and the benign prostate tissue adjacent to carcinoma were negative for AMACR. Some benign prostatic conditions mimicking cancer, including atrophy, basal cell hyperplasia, and inflammatory glands, do not express AMACR. (3) AMACR is present in different grades and types of prostate cancer.

Because a rabbit monoclonal antibody to P504S/AMACR (Zeta, Sierra Madre, CA) has been commercially available and has been used as a standard immunohistochemical stain in clinical practice, it is important to recognize that its sensitivity might vary from laboratory to laboratory. Also focal, weak, and noncircumferential staining patterns in benign-appearing glands should not be interpreted as indicative of a malignant diagnosis. A combination of AMACR/P504S and basal cell stains is recommended to resolve clinically difficult cases.

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