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Clinical Relevance of Biomarkers in Prostate Cancer: The Role of NKX3.1, AMACR, and Ki-67 in Risk Stratification – A Comprehensive Clinicopathological Analysis

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Rezumat

Relevanța clinică a biomarkerilor în cancerul de prostată: rolul NKX3.1, AMACR și Ki-67 în stratificarea riscului - o analiză clinicopatologică cuprinzătoare

Introducere: Stratificarea precisă a riscului, esențială pentru abordarea terapeutică (în special chirurgicală) a cancerului de prostată, se bazează pe criteriile histopatologice standard. Heterogenitatea biologică a acestei neoplazii necesită identificarea de markeri complementari care să reflecte mecanismele moleculare ale progresiei tumorale. Scopul acestui studiu a fost de a evalua corelația dintre markerii imunohistochimici de metabolism (AMACR, NKX3.1) şi proliferare (Ki-67) şi agresivitatea histopatologică în ADK (adenocarcinomul prostatic).
Metode: Acest studiu clinicopatologic retrospectiv, unicentric, a inclus 385 de pacienți cu leziuni prostatice din cadrul Spitalului Clinic de Urgență Sf. Apostol Andrei Constanța (2023–2024). Din aceştia, 198 de cazuri de ADK au fost selectate pentru analiza imunohistochimică principală. Cazurile au fost clasificate conform sistemului Gleason şi a Grupelor de Grad. Expresia markerilor AMACR, NKX3.1 şi Ki-67 a fost evaluată prin imunohistochimie şi corelată cu Grupa de Grad, precum şi cu prezența inflamației cronice şi atrofiei glandulare peritumorale.
Rezultate: Expresia crescută a AMACR (93,9% din cazuri) şi indicele crescut Ki-67 (>20% în 29,3% cazuri) au fost semnificativ corelate cu Grupa de Grad mare ($p<0,001$). Pierderea expresiei NKX3.1 a crescut de la Grupa de Grad 1 la Grupa de Grad 4,

urmată de o frecvență mai mică în Grupa de Grad 5, indicând o asociere neliniară cu gradul histopatologic (p pentru tendință <0,001). Prezența concomitentă a inflamației cronice şi a atrofiei glandulare a fost asociată cu Grupa de Grad mare şi cu un indice Ki-67 semnificativ mai mare (p=0,001 şi p<0,001). Colorarea triplă (AMACR/p63/HMWCK) nu a evidențiat cazuri discordante în distingerea ADK de leziunile benigne care mimează cancerul de prostată.
Concluzii: Profilul imunohistochimic extins (AMACR, NKX3.1, Ki-67) oferă informații biologice valoroase corelate cu agresivitatea tumorii. Integrarea acestor markeri în evaluarea preoperatorie, alături de evaluarea histopatologică standard şi a micromediului peritumoral, ar putea contribui la o stratificare mai precisă a riscului. Aceste constatări sunt însă de natură corelativă, iar aplicabilitatea lor clinică necesită validare prin studii prospective suplimentare.

Cuvinte cheie: cancer de prostată; AMACR; NKX3.1; Ki-67; stratificarea riscului; imunohistochimie.

Abstract

Introduction: Accurate risk stratification, essential for the therapeutic approach (especially surgical) of prostate cancer, is based on standard histopathological criteria. The biological heterogeneity of this neoplasm requires the identification of complementary markers that reflect the molecular mechanisms of tumor progression. The aim of this study was to evaluate the correlation between immunohistochemical markers of metabolism (AMACR, NKX3.1) and proliferation (Ki-67) and histopathological aggressiveness in ADK (prostate adenocarcinoma).
Methods: This retrospective, single-center clinicopathological study included 385 patients with prostatic lesions from Sf. Apostol Andrei Emergency Clinical Hospital in Constanța (2023–2024). Of these, 198 cases of ADK were selected for the main immunohistochemical analysis. The cases were classified according to the Gleason system and Grade Groups. The expression of AMACR, NKX3.1 and Ki-67 markers was assessed by immunohistochemistry and correlated with Grade Groups, as well as with the presence of chronic inflammation and peritumoral glandular atrophy.
Results: Increased AMACR expression (93.9% of cases) and increased Ki-67 index (>20% in 29.3% cases) were significantly correlated with high Grade Groups (p<0.001). Loss of NKX3.1 expression increased from Grade Group 1 to Grade Group 4, followed by a lower frequency in Grade Group 5, indicating a non-linear association with histopathological grade (p for trend <0.001). The concomitant presence of chronic inflammation and glandular atrophy was associated with high Grade Groups and with a significantly higher Ki-67 index (p=0.001 and p<0.001). Triple staining (AMACR/p63/HMWCK) showed no discordant cases in distinguishing ADK from benign lesions that mimic prostate cancer.
Conclusions: The extended immunohistochemical profile (AMACR, NKX3.1, Ki-67) provides valuable biological information correlated with tumor aggressiveness. Integrating these markers into the preoperative evaluation, along with standard histopathological evaluation and the peritumoral microenvironment, may contribute to a more accurate risk stratification. However, these findings are correlative, and their clinical applicability requires validation through further prospective studies.

Keywords: prostate cancer, AMACR, NKX3.1, Ki-67, risk stratification, immunohistochemistry

Introduction

Prostate cancer remains a leading cause of oncological morbidity and mortality in men, and its therapeutic approach, especially surgical, is based on a precise risk stratification (1,2). This assessment is based primarily on standard histopathological criteria (Gleason score, Grade Groups), but the marked biological hetero geneity of this neoplasm requires the search for complementary markers that reflect the molecular mechanisms of tumor progression (3).

A promising area is the evaluation of the metabolic and proliferative profile. The enzyme α-methylacyl-CoA-racemase (AMACR), a well-established diagnostic marker, is overexpressed in ADK and indicates an intensified lipid metabolism, associated with the generation of oxidative stress (4-6). In contrast, loss of expression of the prostate-specific transcription factor NKX3.1, which is common in advanced tumors, marks the disappearance of an essential regulator of cellular differentiation and integrity and is associated with a poor prognosis (7,8). The Ki-67 proliferation index provides a direct measure of tumor growth dynamics (9).

However, the implementation of these markers in routine practice is hampered by their morphological similarity to benign lesions such as partial atrophy or atypical adenomatous hyperplasia (AAH), requiring adjunctive immunohistochemistry (e.g. p63) for diagnostic certainty (10,11). Although their individual roles are studied, an integrative analysis of the

combined expression of AMACR, NKX3.1 and Ki-67 and its correlation with histopathological grading in a Romanian surgical cohort is necessary to validate their clinical utility (12).

The present study builds on previously published work from our institution that reflects sustained expertise in diagnostic pathology and morphological research across different clinical contexts (13,14). Quantitative morphological approaches have also been applied in diverse medical settings, supporting objective tissue-based evaluation (15). These aspects support the feasibility of conducting complex immunohistochemical analyses within a high-volume tertiary care environment, where coordinated multidisciplinary activity is essential (16-18). Therefore, the present study aims to investigate, in a single-center series of 385 patients from Sf. Apostol Andrei Emergency County Hospital, Constanța, the correlations between key biomarkers (AMACR, NKX3.1, Ki-67) and aggressiveness parameters (Gleason score, Grade Group) in ADK. The ultimate goal is to evaluate the potential of an extended immunohistochemical profile to improve preoperative risk stratification, thus providing biological support for optimizing therapeutic decisions, including surgical management.

Materials and Methods

Study Objectives

This retrospective study aimed to evaluate the correlations between metabolic stress markers (AMACR, NKX3.1), proliferation index (Ki-67), and histopathological grade (Gleason score, Grade Group) in ADK.

Specific objectives were to:

1) assess AMACR expression and its correlation with grade;
2) analyze NKX3.1 loss and its association with high-grade tumors and Ki-67;
3) quantify Ki-67 and correlate it with grade and chronic inflammation;
4) evaluate the diagnostic utility of triple staining (AMACR/p63/HMWCK) for distinguishing ADK from benign mimics;
5) describe the clinicopathological features of the 385-case cohort.

Patient Selection

A retrospective search of pathology archives of Sf. Apostol Andrei Emergency County Hospital, Constanta, identified all consecutive prostate biopsy and TURP (Transurethral Resection of the Prostate) specimens from 2023–2024. After excluding cases with unsatisfactory material or unavailable blocks, 385 patients were included. Specimens were obtained via ultrasound-guided biopsy or TURP for suspected cancer or obstructive symptoms. Serum PSA was recorded pre-biopsy. Clinical imaging staging was not routinely available; for TURP specimens, pathological pT1 substage was noted when reported.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics (version 27.0, IBM Corp., Armonk, NY, USA). Continuous variables were expressed as mean ± standard deviation or median and interquartile range, as appropriate. Categorical variables were expressed as frequencies and percentages. Group comparisons were performed using the Chi-square or Fisher's exact test for categorical variables and the Student t-test, ANOVA, or Kruskal–Wallis test for continuous variables, according to data distribution. Trends across ordered Grade Groups were evaluated using the Cochran–Armitage test for trend. A two-tailed p-value < 0.05 was considered statistically significant. Normality of continuous variables was assessed prior to the application of parametric statistical tests. Comparisons of categorical immunohistochemical variables across Grade Groups were performed using the Chi-square test or Fisher's exact test, as appropriate.

The general characteristics of the study cohort are presented in *Table 1.*

Table 1. General characteristics of the study cohort (n = 385)

Characteristic	Value
Age (years)	
Mean (SD)	71.2 (±8.5)
Range	47 – 93
Main diagnosis, n (%)	
ADK	198 (51.4%)
Urothelial carcinoma (UC)	78 (20.3%)
Benign pathology*	109 (28.3%)
Gleason score in ADK (n=198), n (%)	
6	12 (6.1%)
7 (3+4)	64 (32.3%)
7 (4+3)	34 (17.2%)
8	24 (12.1%)
9	54 (27.3%)
10	10 (5.1%)
Grade Group in ADK (n=198), n (%)	
Grade Group 1	12 (6.1%)
Grade Group 2	64 (32.3%)
Grade Group 3	34 (17.2%)
Grade Group 4	24 (12.1%)
Grade Group 5	64 (32.3%)
Presence of chronic inflammation, n (%)	142 (36.9%)
Presence of glandular atrophy, n (%)	118 (30.6%)
Presence of AAH (atypical adenomatous hyperplasia), n (%)	80 (20.8%)

*Benign pathology: BPH (benign prostatic hyperplasia), AAH, atrophy, chronic prostatitis, benign proliferative lesions.

Conventional Histopathological Analysis

All samples were fixed in 10% buffered formalin, embedded in paraffin and routinely stained with Hematoxylin-Eosin (HE). Microscopic examination and histopathological classification were performed by two pathologists with experience in urological pathology (with interobserver agreement of >90%). The diagnosis of ADK was based on the standard ISUP/WHO morphological criteria. Each ADK case was classified according to the 2019 ISUP Gleason scoring system and assigned to one of five Grade Groups (Grade Group 1-5). Benign and premalignant entities (BPH, AAH, atrophy, chronic inflammation) were diagnosed according to accepted criteria.

Immunohistochemical Analysis

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue sections (2-3 μm thickness) at Sf. Apostol Andrei Emergency County Hospital, Constanta. For the markers AMACR (clone 13H4), Ki-67 (clone MIB-1), p63 (clone 4A4), and HMWCK (clone 34ßE12), we used the Zeta Max HRP detection system with DAB, following the manufacturer's protocol. Briefly, after deparaffinization and antigen retrieval by heating at 110°C for 40 minutes, endogenous peroxidase was blocked. The primary antibodies were applied for 30-40 minutes, followed by detection with the HRP Anti-Mouse/Anti-Rabbit system and DAB chromogen. Sections were counterstained with hematoxylin.

For the NKX3.1 marker (Master Diagnostica), staining was performed at CEDMOG, Ovidius University, Constanta, using the HIER-DAB method and the Master Polymer Plus Detection System (HRP) according to the manufacturer's instructions. This protocol included dewaxing, heat-induced epitope retrieval in pH8 EDTA buffer, incubation with the ready-to-use antibody, and detection with the corresponding polymer system.

The complete antibody panel, including clones, dilutions, and expected expression patterns, is summarized in *Table 2*.

Fig. 1 illustrates the representative immunohistochemical features encountered in the study.

Ethical Approval

This study was conducted in strict adherence to the ethical principles outlined in the Declaration of Helsinki. The study protocol received formal approval from the Institutional Ethics Board of the Constanța County Emergency Clinical Hospital (Approval No. 42767, 02.07.2024). Prior to inclusion, written informed consent was obtained from all participants, specifically authorizing the use of their biological samples and clinical data for research purposes.

Results

Clinicopathological Characteristics of the Cohort of Patients with ADK

Of the 385 cases included in the study, 198 (51.4%) were diagnosed with ADK. The mean age of these patients was 72.1 ± 7.8 years, and the mean serum PSA at diagnosis was 12.4 ± 8.9 ng/mL. The distribution of Gleason scores and Grade Groups, as well as their correlation with age and PSA, are presented in *Table 3*. We observed a progressive and significant increase in mean PSA values with increasing Grade Groups, from 6.8 ± 3.2 ng/mL in Grade Group 1 to 22.4 ± 10.3 ng/mL in Grade Group 5 ($p < 0.001$, Kruskal-Wallis test).

Expression of Metabolic and Proliferation Markers and Correlation with Tumor Aggressiveness

Immunohistochemical analysis revealed positive cytoplasmic expression for AMACR in 186 of 198 ADK

Table 2. Immunohistochemical antibody panel used in the study

Marker	Clone / Type	Dilution	Immunoexpresion	Primary Use
AMACR (P504S) Zeta corporation	Monoclonal, mouse	1:100	Cytoplasmic	Identification of prostatic ADK
NKX3.1 Masterdiagnostica	Monoclonal, rabbit	1:200	Nuclear	Confirmation of prostatic origin
Ki-67 (MIB-1) Zeta corporation	Monoclonal, mouse	1:200	Nuclear	Proliferation index assessment
p63 Zeta corporation	Monoclonal, mouse	1:200	Basal cell - nuclear	Basal cell marker
HMWCK (34βE12) Zeta corporation	Monoclonal, mouse	1:50	Granular cytoplasmic positivity - basal cell	Basal cell marker

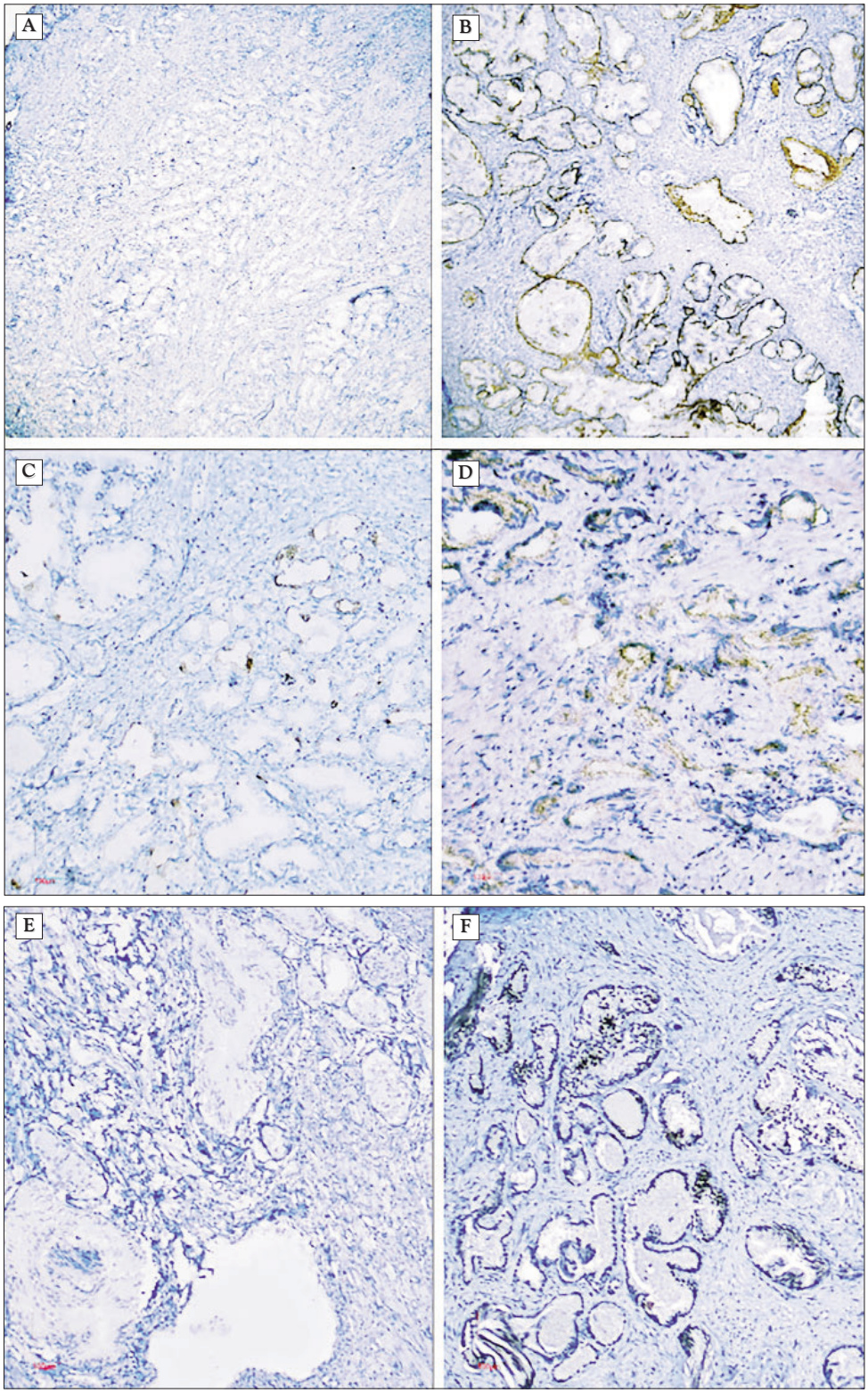

Figure 1. **(A)** HMWCK (34βE12) negative in prostatic basal cells, poorly differentiated ADK, Gleason score 4+4, grade group 3, X40; **(B)** HMWCK (34βE12) continuous positive in the basal cell layer of atrophic acini, in the background of benign prostatic hyperplasia, X100; **(C)** HMWCK (34βE12) discontinuous positive in the basal cell layer of prostatic acini, atypical adenomatous hyperplasia, X100; **(D)** AMACR intensely positive cytoplasmic in epithelial cells in malignant neoplastic proliferation, poorly differentiated ADK, Gleason score 4+5, X200; **(E)** AMACR negative in a case of cystic and partial atrophy, X100; **(F)** NKX3.1 negative (score 0) in a case of simple atrophy, in the background of benign hyperplasia of the prostate, X100;

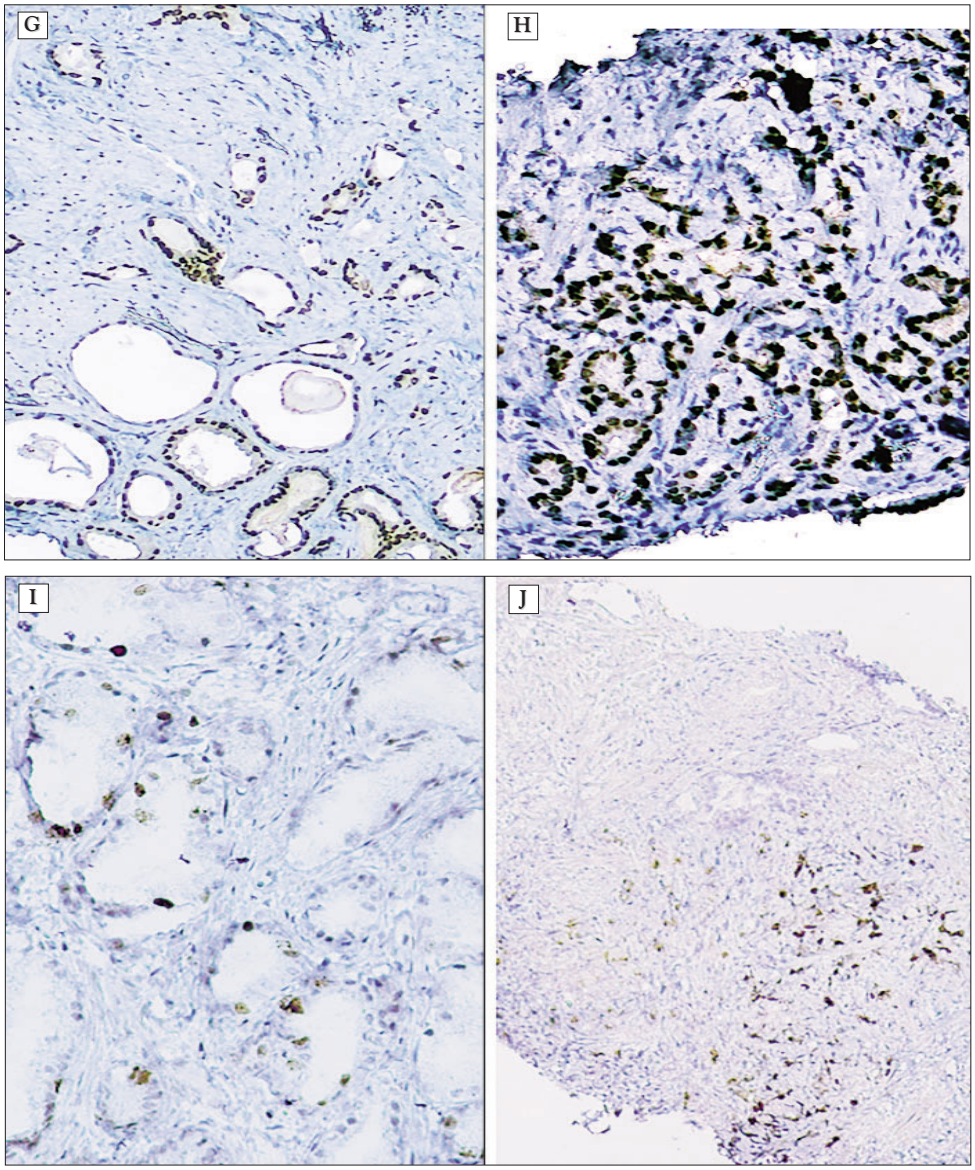

Figure 1. **(G)** NKX3.1 focal nuclear positive (score 1) in a case of moderately differentiated ADK, Gleason score 3+4, grade group 2, associated with cystic prostatic atrophy, X40; **(H)** NKX3.1 intense nuclear positive (score 2) in a case of moderately differentiated ADK, Gleason score 4+3, grade group 3, associated with cystic prostatic atrophy, X40; **(I)** Ki 67 nuclear positive in less than 10% of malignant prostatic epithelial proliferation; grade group 2 ADK, X200; **(J)** Ki 67 nuclear positive in more than 20% of malignant prostatic neoplastic cells; grade group 5 ADK, X40)

Table 3. Clinicopathological characteristics of patients with ADK, stratified by Grade Groups

Grade Group	n (%)	Mean Age ± SD (years)	Mean PSA ± SD (ng/mL)	Median PSA (IQR) (ng/mL)
1	12 (6.1%)	68.3 ± 5.1	6.8 ± 3.2	6.5 (4.1–9.0)
2	64 (32.3%)	71.5 ± 6.5	9.5 ± 4.1	8.9 (6.5–12.0)
3	34 (17.2%)	72.9 ± 7.8	13.2 ± 7.8	11.4 (8.0–16.5)
4	24 (12.1%)	73.8 ± 8.0	18.6 ± 9.5	17.0 (12.0–23.0)
5	64 (32.3%)	74.0 ± 8.2	22.4 ± 10.3	20.8 (15.0–28.0)
p-value		0.12	<0.001	<0.001

IQR = Interquartile Range; p-values calculated using ANOVA (age) and Kruskal-Wallis test (PSA).

Table 4. Correlation between Immunohistochemical Markers and Grade Groups in ADK

Grade Group	n	AMACR Positive, n (%)	NKX3.1 Loss, n (%)	Mean Ki-67 Index ± SD (%)
1	12	10 (83.3)	1 (8.3)	4.1 ± 2.3
2	64	56 (87.5)	8 (12.5)	5.9 ± 3.5
3	34	32 (94.1)	12 (35.3)	13.0 ± 5.1
4	24	24 (100)	19 (79.2)	23.5 ± 7.8
5	64	64 (100)	12 (18.8)	24.8 ± 8.2
p-value		0.002	<0.001	<0.001

cases (93.9%). AMACR expression showed a statistically significant association with Grade Group, reaching uniform positivity in high-grade tumors (p=0.002). Complete or focal loss of nuclear NKX3.1 expression was observed in 52 of the 198 ADK cases (26.3%). The frequency of NKX3.1 loss increased across Grade Groups, from 8.3% in Grade Group 1 to 79.2% in Grade Group 4. In Grade Group 5, NKX3.1 loss was observed in 18.8% of cases. This distribution indicates a non-linear relationship between NKX3.1 loss and tumor grade, with a peak frequency in Grade Group 4 (p for trend < 0.001, Cochran–Armitage test). *Table 4* summarizes the expression patterns of AMACR, NKX3.1, and Ki-67 according to Grade Groups.

Diagnostic Utility of Triple Staining (AMACR/p63/HMWCK) and the NKX3.1 Marker

Triple staining (AMACR/p63/HMWCK) was applied in 42 cases with atypical glandular architecture difficult to classify based on conventional morphology. In all 27 cases that proved to be ADK, a typical immunohistochemical pattern of AMACR+/p63- was observed. In contrast, in the 15 cases of benign "mimic" lesions (partial atrophy or atypical adenomatous hyperplasia - AAH), the pattern was the opposite, AMACR-/p63+. No cases with discordant or intermediate results were recorded in this series, supporting the diagnostic consistency of this immunohistochemical combination in challenging cases (*Table 5*). In parallel, the NKX3.1 marker was successfully used to confirm prostatic origin in 12 cases of poorly differentiated tumors (Gleason 8-10) and to exclude invasive urothelial carcinoma, which was negative for NKX3.1 in all 78 cases of urothelial carcinoma in the cohort.

Correlation Between Metabolic Microenvironment Characteristics (Inflammation, Atrophy) and Tumor Aggressiveness

When analysis was restricted to ADK cases, 86 out of 198 ADK cases (43.4%) had associated chronic active inflammation, and 74 (37.4%) had foci of glandular atrophy. The simultaneous presence of these two microenvironment characteristics was significantly associated with high Grade Group (p = 0.001) and with a significantly higher Ki-67 proliferation index (20.1% ± 8.4) compared to cases without such changes (10.8% ± 6.1, p<0.001) (*Table 6*). In addition, in a broader assessment of the peritumoral landscape, 68% (57/84) of ADK cases with Grade Group 4-5 had on biopsy at least one associated benign premalignant lesion (AAH) or atrophic focus, compared to only 19.3% (22/114) in cases with Grade Group 1-3. This separate observation reinforces the idea that a chronic microenvironment of metabolic stress and inflammation is more frequently associated with aggressive tumor phenotypes.

Discussion

This clinicopathological and immunohistochemical study, conducted on a single-center cohort of 385 patients from Romania, explores the relationship between metabolic stress, proliferative dynamics and histological aggressiveness in ADK. The main findings

Table 5. Diagnostic Utility of Triple Staining (AMACR/p63/HMWCK) in Challenging Atypical Glandular Lesions

Final Diagnosis	n	IHC Pattern	AMACR	p63/HMWCK
ADK	27	AMACR+/p63-	Positive (Strong/Diffuse cytoplasmic)	Negative (Loss of basal layer)
Benign Mimics	15	AMACR-/p63+	Negative	Positive (Intact basal layer)
Partial Atrophy	9	AMACR-/focal +/p63+	Negative/ focal positive	Positive continuous
Atypical Adenomatous Hyperplasia (AAH)	6	AMACR-/p63+	Negative	Positive continuous or focal discontinuous

Table 6. Association Between Peritumoral Microenvironment Features and Tumor Aggressiveness in ADK

Parameter	Grade Group 1-3 (n=110)	Grade Group 4-5 (n=88)	p-value
Chronic Inflammation Present, n (%)	43 (39.1%)	43 (48.9%)	0.108
Glandular Atrophy Present, n (%)	36 (32.7%)	38 (43.2%)	0.165
Both Features Present, n (%)	21 (19.1%)	35 (39.8%)	0.001
Mean Ki-67 Index ± SD (%)	8.9 ± 5.2	21.5 ± 8.9	<0.001
Ki-67 >20% (High Proliferation), n (%)	6 (5.5%)	52 (59.1%)	<0.001

show a significant correlation between: (1) increased AMACR expression and high Grade Groups (GG); (2) loss of NKX3.1 expression in Grade Group 4-5 tumors; (3) gradual increase in Ki-67 index with Grade Group; and (4) association between an inflammatory/atrophic peritumoral microenvironment and aggressive tumor phenotype. These data complement and strengthen the histopathological profile of prostate lesions in the local population, as documented in recent studies (12).

The intense and diffuse expression of AMACR, detected in 93.9% of ADK cases and significantly associated with high Grade Group, confirms its crucial role not only as a diagnostic marker, but also as an indicator of aberrant metabolism (5,19). AMACR, involved in the ß-oxidation of branched-chain fatty acids, is a major source of reactive oxygen species (ROS) in tumor cells (19). The chronic oxidative stress generated can induce mutations, genomic instability and activation of survival pathways, mechanisms directly involved in malignant progression (21,24). However, given its high prevalence across all Grade Groups, AMACR appears to function primarily as a robust diagnostic marker rather than a fine discriminator of tumor grade. Loss of NKX3.1 expression was significantly associated with higher Grade Groups, supporting its role as a marker of tumor dedifferentiation. Although a progressive increase in NKX3.1 loss was observed up to Grade Group 4, a lower frequency was recorded in Grade Group 5. The lower frequency of NKX3.1 loss observed in Grade Group 5 compared to Grade Group 4 highlights the biological hetero-geneity of high-grade ADK and suggests that NKX3.1 expression does not follow a strictly linear relationship with histological grade. These results support the interpretation of NKX3.1 as a differentiation-associated marker rather than a direct surrogate of tumor grade. These findings are consistent with previous reports describing NKX3.1 primarily as a differentiation-associated marker whose expression is variably reduced in high-grade prostate carcinomas (7,8).

The progressive increase in the Ki-67 index from Grade Group 1 to Grade Group 5, with mean values above 20% in high-risk groups, consolidates it as a robust biomarker of proliferative potential (9). Its independent prognostic value is well established (22, 23). In our study, elevated Ki-67 was closely associated not only with Grade Group, but also with loss of NKX3.1 and the presence of chronic inflammation. This indicates that accelerated proliferation may represent the phenotypic expression of multiple associated biological alterations and sustained by pro-inflammatory signals in the microenvironment. Thus, Ki-67 can be considered a phenotypic integrator of multiple molecular dysregulations that define aggressive tumors.

A key finding of the study is the strong association between the concomitant presence of active chronic inflammation and glandular atrophy with elevated Grade Groups and elevated Ki-67 (22). Chronic inflammation is a well-known etiopathogenic factor in prostate cancer, and inflammatory infiltrates constitute a constant source of ROS, cytokines, growth factors and proteolytic enzymes that can remodel connective tissue and stimulate cell proliferation (24, 25). Atrophy, often a corollary of chronic inflammation, represents a state of repeated epithelial injury and regeneration, which may favor the emergence of cell clones with oncogenic potential (11,26). The increased frequency of these changes in high-grade tumors suggests that they are not simple histological artifacts, but active components of a "permissive microenvironment" that facilitates the selection and expansion of aggressive subclones. This perspective is in agreement with recent research emphasizing the complexity and active role of the microenvironment in prostatic tumorigenesis (26).

Our results reaffirm the importance of immunohistochemistry in routine prostatic pathology. The high diagnostic reliability of triple staining (AMACR/p63) in resolving difficult diagnostic cases emphasizes that this method remains essential for the differentiation between low-grade ADK and benign mimicking lesions, such as atypical adenomatous hyperplasia (AAH) or partial atrophy (10,27). The addition of the NKX3.1 marker to the diagnostic panel is particularly valuable in two scenarios: confirmation of the prostatic origin of poorly differentiated carcinomas and their

delimitation from invasive urothelial carcinoma, a diagnostic problem with major therapeutic implications, also analyzed in other contexts of simultaneous urological cancers (20). These diagnostic challenges, as well as their impact on therapeutic strategies (including surgical), have been highlighted in recent studies on the Romanian population (27).

Conclusions

This study demonstrates significant associations between immunohistochemical markers of metabolism (AMACR), cellular differentiation (NKX3.1), proliferative activity (Ki-67), and histopathological aggressiveness in ADK. Increased AMACR expression and elevated Ki-67 index were consistently associated with higher Grade Groups, while loss of NKX3.1 expression was more frequently observed in high-grade tumors.

From a practical perspective, the combined evaluation of AMACR, p63/HMWCK, NKX3.1, and Ki-67 markers provides useful diagnostic and biological information on prostate biopsies. This expanded immunohistochemical panel could be considered an adjunct tool for risk stratification, especially in intermediate-grade tumors, and could improve diagnostic accuracy and surgical decision-making in difficult cases. However, these conclusions are generated based on histopathological correlations and do not directly assess clinical or surgical outcomes.

The retrospective design and single-center nature of the study represent its main limitations. Nevertheless, the relatively large cohort and consistent histopathological and immunohistochemical evaluation support the relevance of the observed associations.

The study has other limitations. The inclusion of both prostate biopsies and TURP specimens introduces cohort heterogeneity, as TURP specimens often come from patients with more advanced or obstructive disease. A stratified analysis by specimen type was not possible due to the limited number of cases from biopsy only, so a potential selection bias cannot be completely excluded.

A multivariate analysis was also not performed. Given the exploratory and descriptive nature of this study, we did not perform multivariate modeling to adjust for confounders such as age, PSA, or specimen type. Future studies with larger cohorts and prospective designs should include multivariate analyses to better define the independent prognostic value of these biomarkers.

Furthermore, our findings are correlative and do not directly measure clinical or surgical outcomes. Therefore, the proposed immunohistochemical panel should be considered a hypothesis-generating tool, which requires prospective validation before being implemented in current clinical decisions.

Author's Contributions

All authors contributed equally to this work and share first authorship. All authors participated in the conception and design of the study, the acquisition, analysis, or interpretation of data, and the drafting or critical revision of the manuscript. All authors have reviewed and approved the final version of the manuscript and accept responsibility for its content and integrity.

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Conflict of Interest

The authors declare no conflict of interest.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021;71(3):209-249.
2. Epstein JI, Egevad L, Amin MB, Delahunt B, Srigley JR, Humphrey PA, et al. The 2014 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma: Definition of Grading Patterns and Proposal for a New Grading System. Am J Surg Pathol. 2016;40(2):244-252.
3. Sadeghi RN, Karami-Tehrani F, Salami S. Targeting prostate cancer cell metabolism: impact of hexokinase and CPT-1 enzymes. Tumour Biol. 2015;36(4):2893-2905.
4. Valicenti RK, Thompson I Jr, Albertsen P, Davis BJ, Goldenberg SL, Wolf JS, et al. Adjuvant and salvage radiation therapy after prostatectomy: American Society for Radiation Oncology/American Urological Association guidelines. Int J Radiat Oncol Biol Phys. 2013;86(5):822-828.
5. Jiang Z, Woda BA, Rock KL, Xu Y, Savas L, Khan A, et al. P504S: a new molecular marker for the detection of prostate carcinoma. Am J Surg Pathol. 2001;25(11):1397-1404.
6. Luo J, Zha S, Gage WR, et al. Alpha-methylacyl-CoA racemase: a new molecular marker for prostate cancer. Cancer Res. 2002;62(8):2220-2226.
7. Bowen C, Bubendorf L, Voeller HJ, Slack R, Willi N, Sauter G, et al. Loss of NKX3.1 expression in human prostate cancers correlates with tumor progression. Cancer Res. 2000;60(21):6111-6115.
8. Magee JA, Abdulkadir SA, Milbrandt J. Haploinsufficiency at the Nkx3.1 locus. A paradigm for stochastic, dosage-sensitive gene regulation during tumor initiation. Cancer Cell. 2003;3(3):273-283.
9. Berney DM, Gopalan A, Kudahetti S, Fisher G, Ambroisine L, Foster CS, et al. Ki-67 and outcome in clinically localised prostate cancer: analysis of conservatively treated prostate cancer patients from the Trans-Atlantic Prostate Group study. Br J Cancer. 2009;100(6):888-893.
10. Hameed O, Humphrey PA. Immunohistochemistry in diagnostic surgical pathology of the prostate. Semin Diagn Pathol. 2005;22(1):88-104.
11. Bostwick DG, Srigley J, Grignon D, Maksem J, Humphrey P, van der Kwast TH, et al. Atypical adenomatous hyperplasia of the prostate: morphologic criteria for its distinction from well-differentiated carcinoma. Hum Pathol. 1993;24(8):819-832.
12. Roșu MC, Ionescu CA, Enciu M, Căpineanu B, Pundiche M, Dobrin N, et al. Histopathological Profile of Prostatic Lesions and the Role of Gleason Score in Surgical

Treatment Decision-Making. Chirurgia (Bucur). 2025;120(5):593-602.
13. Deacu M, Enciu M, Nicolau AA, Bălțătescu GI 1 3, Lavinia Simona Neculai-Cândea 2 4, Sorin Deacu 2 4, et al. Morphopathological features induced by SARS-CoV-2 infection - a series of 57 autopsies. Histol Histopathol. 2023;38(5):513-524.
14. Deacu S, Neculai-Cândea L, Pricop S, Aschie M, Mocanu L, Popa M. Vascular Adhesive Peptide-1 (VAP-1) Expression in Wounds – A New Vital Reaction Marker? Romanian Journal of Legal Medicine. 2021;29:347-351.
15. Popa M, Stefanescu C, Corici P. Forensic value of mandibular anthropometry in gender and age estimation. Romanian Journal of Legal Medicine - ROM J LEG MED. 2009; 17:45-50.
16. Oprinca-Muja LA, Cristian AN, Topîrcean E, Alina Cristian 1, Marius Florentin Popa 2, Roxana Cardoș 3, et al. Exploring Burnout at the Morgue During the COVID-19 Pandemic: A Three-Phase Analysis of Forensic and Pathology Personnel. Healthcare (Basel). 2025;13(5):504.
17. Halichidis S, Aschie M, Cozaru GC, Manea M, Dobrin N, Vlad SE, et al. CMV and HIV Coinfection in Women from a Region in Eastern Europe. Journal of Personalized Medicine. 2023; 13(11):1539.
18. Popa M, Stefanescu C, Corici P. Medical-legal identification methods with the aid of cheiloscopy. Romanian Journal of Legal Medicine. 2013;21:215-218.
19. Zha S, Ferdinandusse S, Denis S, Wanders RJ, Ewing CM, Luo J, et al. Alpha-methylacyl-CoA racemase as an androgen-independent growth modifier in prostate cancer. Cancer Res. 2003;63(21):7365-7376.
20. Matei E, Enciu M, Roșu MC, Voinea F, Mitroi AF, Deacu M, et al. Apoptosis-Cell Cycle-Autophagy Molecular Mechanisms Network in Heterogeneous Aggressive Phenotype Prostate Hyperplasia Primary Cell Cultures Have a Prognostic Role. Int J Mol Sci. 2024;25(17):9329.
21. Matei E, Ionescu AC, Enciu M, Popovici V, Mitroi AF, Aschie M, et al. Cell death and DNA damage via ROS mechanisms after applied antibiotics and antioxidants doses in prostate hyperplasia primary cell cultures. Medicine (Baltimore). 2024; 103(37):e39450.
22. Pollack A, DeSilvio M, Khor LY, Li R, Al-Saleem TI, Hammond ME, et al. Ki-67 staining is a strong predictor of distant metastasis and mortality for men with prostate cancer treated with radiotherapy plus androgen deprivation: Radiation Therapy Oncology Group Trial 92-02. J Clin Oncol. 2004;22(11):2133-2140.
23. De Marzo AM, Platz EA, Sutcliffe S, Jianfeng Xu, Grönberg H, Drake CG, et al. Inflammation in prostate carcinogenesis. Nat Rev Cancer. 2007;7(4):256-269.
24. Popovici V, Musuc AM, Matei E, Karampelas O, Ozon EA, Cozaru GC, et al. ROS-Induced DNA-Damage and Autophagy in Oral Squamous Cell Carcinoma by Usnea barbata Oil Extract-An In Vitro Study. Int J Mol Sci. 2022;23(23):14836.
25. Sfanos KS, Yegnasubramanian S, Nelson WG, De Marzo AM. The inflammatory microenvironment and microbiome in prostate cancer development. Nat Rev Urol. 2018;15(1):11-24.
26. Roșu MC, Zob DL, Ionescu CA, Cîmpineanu B, Pundiche M, Cozaru GC, et al. Challenges and Clinical Implications of Prostate Cancer Mimickers: A Study Conducted at a Tertiary Center. Chirurgia (Bucur). 2025;120(6):685-695.
27. Ionescu CA, Aschie M, Matei E, Cozaru GC, Deacu M, Mitroi AF, et al. Characterization of the Tumor Microenvironment and the Biological Processes with a Role in Prostatic Tumorigenesis. Biomedicines. 2022;10(7):1672.