Alpha-methylacyl-CoA Racemase (AMACR) Protein is Upregulated in Early Proliferative Lesions of the Breast Irrespective of Apocrine Differentiation

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PII: S0046-8177(22)00211-8

DOI: https://doi.org/10.1016/j.humpath.2022.08.002

Reference: YHUPA 5356

To appear in: *Human Pathology*

Received Date: 25 July 2022

Revised Date: 12 August 2022

Accepted Date: 16 August 2022

Please cite this article as: Gatalica Z, Stafford P, Vranic S, Alpha-methylacyl-CoA Racemase (AMACR) Protein is Upregulated in Early Proliferative Lesions of the Breast Irrespective of Apocrine Differentiation, *Human Pathology*, https://doi.org/10.1016/j.humpath.2022.08.002.

Title:

Alpha-methylacyl-CoA Racemase (AMACR) Protein is Upregulated in Early Proliferative Lesions of the Breast Irrespective of Apocrine Differentiation

Running title:
AMACR expression in breast lesions

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Summary

Alpha-methylacyl-CoA racemase (AMACR/P504S) is a mitochondrial and peroxisomal enzyme involved in the branched-chain fatty acid and bile acid metabolism. AMACR is a useful diagnostic biomarker for prostate carcinomas and several other malignancies. Its expression in apocrine breast lesions had been previously reported, but its role in breast cancer progression has not been fully investigated. One hundred fifty breast samples (80 with invasive carcinomas) were studied. The expression of AMACR protein was analyzed using the immunohistochemical method (IHC). Lesions were considered positive if AMACR was detected in ≥10% of the cells at any intensity comprising a histologically defined normal epithelial structure or a pathologic lesion. In addition, AMACR mRNA relative expression was calculated from the whole-transcript RNA-Seq performed on >20,000 diverse tumor samples using a 20,000+ hybrid-capture NGS assay with the transcript capture panel based on the Agilent SureSelect Human All ExonV7. Expression of AMACR protein was restricted to epithelia. It was uncommon in the normal breast (7/81 samples, 9%). Increasing AMACR expression was observed with proliferative epithelial lesions (18% of usual ductal hyperplasias/adenosis, 70% of atypical lesions and 72% of DCIS/LCIS). Invasive ductal carcinomas NST and invasive lobular carcinomas expressed AMACR in 64% and 46%, respectively. The highest AMACR expression was observed in luminal B and HER2-positive breast carcinomas (86-100%). Triple-negative breast carcinomas exhibited AMACR in 50% of the cases. Apocrine lesions showed strong, nearly uniform overexpression of AMACR (100% of metaplasias, hyperplasias and in situ carcinomas and 88% of invasive apocrine carcinomas were positive). RNA-Seq analysis also confirmed AMACR expression in breast carcinomas, although its median value was substantially lower with a lower standard deviation

than in prostate carcinomas. Over-expression of AMACR characterizes various proliferative, preinvasive and invasive breast lesions and is not specific to the apocrine morphology. It points to altered lipid metabolism (branched fatty acids) as one of the general characteristics of breast carcinogenesis, like several other malignancies. Its early detection may represent a potential target for cancer progression intervention.

Keywords: Breast – breast carcinoma – proliferative lesions – apocrine lesions – AMACR – Immunohistochemistry-lipidomics

1. Introduction

Branched-chain fatty acids play an essential role in the human diet from the earliest development, being present in breast milk (1). They are metabolized in peroxisomes due to the methyl groups on the carbon chains (2, 3). Lipids are degraded by α- and β-oxidation processes via several metabolic pathways. α-methyl acyl-CoA racemase (AMACR or racemase) plays an essential role in all these pathways (2, 3). AMACR regulates β-oxidation of branched-chain lipids in peroxisomes and mitochondria and promotes chiral reversal of 2-methyl acids (2-4). In healthy organs, high AMACR mRNA expression was described in the liver, kidneys, and salivary glands, while AMACR protein expression was observed in hepatocytes, renal tubules, bronchial epithelial cells and mucosal cells of the gall bladder (5). Mutations of the *AMACR* gene cause sensory-motor neuronal and liver disorders inherited in an autosomal recessive pattern (2-4). AMACR protein expression has been described in various cancers, most notably prostate cancer (6). In addition, AMACR positivity has also been reported in papillary renal cell, colorectal, and hepatocellular carcinomas (5, 7-10). Inconsistent data on AMACR expression has been reported in breast cancer (5, 8, 10, 11). Witkiewicz et al. demonstrated that AMACR expression correlated with the tumor grade in breast cancer, with the highest expression in high-grade carcinomas (11), while Nakamura et al. recently demonstrated a strong and consistent AMACR expression (~97%) in apocrine tumors of the breast (both in situ and invasive), comparable to the expression of gross cystic disease fluid protein-15 (GCDFP-15) (12). In contrast, non-apocrine breast carcinomas in their study exhibited much lower (22%) AMACR mRNA and protein expressions (12).

In the present study, we expanded investigations into AMACR expression in the breast to include normal structures, various proliferative breast lesions and various histologic/molecular types of invasive carcinomas, to better characterize its role in breast diseases.

2. Materials and Methods

2.1. Sample selection

One-hundred and fifty breast specimens (normal, benign and malignant) diagnosed at the Department of Pathology, University of Oklahoma College of Medicine, were selected for immunohistochemical testing of AMACR protein expression, including 70 non-invasive samples and 80 samples with carcinomas. Among the breast carcinomas, 72 cases were primary, and eight were metastatic (lymph nodes, liver and bone).

In addition, a retrospective analysis of AMACR mRNA expression across a diverse set of solid tumor types was assessed using whole-transcriptome RNA-Seq on >20,000 samples (Caris Life Sciences, Phoenix, AZ).

2.2. Immunohistochemistry

AMACR protein expression was analyzed by the AMACR (P504S) Rabbit polyclonal IgG antibody (Biocare Medical, cat# AVA 200G, G25) using automated procedures (Benchmark, Ventana, AZ). Lesions were considered positive if AMACR was detected in ≥10% of the cells at any intensity comprising histologically defined structures. Prostate carcinoma sections served as a positive control for AMACR expression.

In addition, the status of estrogen receptor (ER), progesterone receptor (PR), Her-2/neu, and Ki-67 markers was recorded from the routine diagnostic workup when available. The thresholds for positivity for these four biomarkers were according to their respective guidelines (13-15). The invasive carcinomas were graded according to the Nottingham combined histologic grade (modified Scarff-Bloom-Richardson grade) (16). In diagnostically challenging cases (e.g., florid/usual/ vs. atypical hyperplasia, complex sclerosing lesions, lobular neoplasia, apocrine

lesions), additional immunohistochemical stains were performed (e.g., p63, CK5/6, Calponin, E-cadherin, Androgen receptor).

Invasive ductal carcinomas have been classified into four molecular subtypes [Luminal A, luminal B, HER2-positive and triple-negative] using immunohistochemical surrogate definitions (ER, PR, Her2 and Ki-67) of intrinsic subtypes of breast cancer as proposed by the St. Gallen Consensus 2013 and ESMO 2019 guideline (15, 17).

2.3. RNA sequencing

Whole-transcript RNA-Seq was performed using a 20,000+ transcript NGS hybrid-capture assay using the Agilent V7 capture probe set. RNA-Seq is referred to as the Caris WTS assay (Whole Transcriptome Sequencing) and has used a consistent, CAP/CLIA validated assay from the first to the most current assay, with no major assay or pipeline version changes. All RNA data were processed from FASTA, checked for sufficient read depth, correct positive and negative control results per S4 flowcell, aligned to hg19 using the current STAR aligner, and analyzed for TPM (Transcripts per Million Molecules) using the current Salmon expression pipeline (18). This method normalizes and scales to the set of analyzed genes; here, we use approximately 20,000 common gene transcripts. The median value across these transcripts is 1.0, with the one percentile approaching 0.001 and the 99th percentile approaching 200. Different patient samples will have slightly different minimum and maximum values. However, samples with a minimum of 50M reads and passed all quality filters had similar data distributions at the 25th, 50th and 75th percentiles. RNA-Seq data was analyzed for fusions, splice variants, variants (INDELs and SNV's), TPM using Salmon and FPKM (Fragments Per Kilobase of transcript per Million mapped reads) using Cufflinks (19) and analyzed for variants,

copy numbers and splice variants. RNA Expression data was queried across cancer types and presented by gene as distributions of expression within the cancer groups, as defined by Caris.

2.4. Statistical analysis

Pearson's chi-squared test determined statistically significant differences between the expected and the observed frequencies in categorical variables. For 2x2 contingency tables, Fisher's exact test was applied. All statistical analyses were performed using IBM Statistical Package for the Social Sciences (IBM SPSS, version 27). A statistical significance was achieved at $p<0.05$.

3. Results

The types and frequency of breast lesions included in the study are provided in Tables 1-4. The mean age of the patients from the entire cohort was 59.15 years (median: 59.5 years, range, 27-79 years).

As a start point to assess AMACR expression in breast cancer, we utilized RNASeq analysis using a large cohort of different cancers (>20,000 cases from Caris Life Sciences). With the large number of total cases reflecting the prevalence in the general population of late-stage cancers, the distribution of expression in breast carcinoma and prostatic adenocarcinoma reflects a robust average, with the median value of AMACR expression in breast carcinoma being an order of magnitude less than prostate. Of note, the higher expression in the prostate does not automatically equate with a higher standard deviation, although in this case prostate does display a higher variance and higher expression value than the breast (20).

Immunohistochemical evaluation of the AMACR protein expression demonstrated a granular cytoplasmic expression pattern by IHC, regardless of the type of breast lesion (Figures 2-4). Occasional nuclear reactivity of AMACR protein was also observed but was considered non-specific.

The patients' age did not impact AMACR expression and distribution (p=1.0).

In the invasive cohort, AMACR expression was significantly higher in invasive carcinomas than in TDLU [50/80 (62.5%) vs. 3/30 (10%), p<0.001] or benign proliferative lesions [e.g., usual ductal hyperplasia, 1/16 (17%), p<0.001]. Although AMACR expression was a consistent feature of the apocrine carcinomas (Figures 2), it was also seen in other morphologic variants (e.g.,

ductal NST and special types such as lobular, mucinous, and micropapillary carcinomas) (Table 1, Figure 3). However, significant differences in AMACR expression were observed between different molecular subtypes of breast cancer, with the highest expression in HER2-positive and triple-negative breast cancers (Table 2, p=0.005, Chi-Square test) (Figure 3). Consequently, the expression of AMACR correlated well with the tumor grade (Table 3). Two invasive cases (one apocrine and one micropapillary carcinoma) had corresponding lymph node metastases, with the apocrine case being discordant (primary tumor negative and lymph node metastasis positive). Although the expression of AMACR in metastatic cases (n=8, 75%) appeared to be higher than in the primary carcinomas (n=72, 62%), the difference did not reach statistical significance (p=0.70).

In the invasive carcinoma cohort (n=80), we observed an increased frequency of AMACR expression from matched benign proliferative lesions (1/5) to flat epithelial atypia (FEA) (3/4) and atypical/in-situ lesions (ADH/DCIS and LCIS, 12/20, 60%) (Figure 4). Notably, three in-situ cases were discordant with their invasive counterparts: Two in-situ were positive without AMACR positivity in the invasive component, while one invasive case was positive without the AMACR expression in the associated in-situ component. Similar trends of AMACR expression were observed in the cohort of cases containing only benign and non-invasive lesions (n=70) (the results are summarized in Table 4).

A consistent, strong, uniform cytoplasmic AMACR expression characterized apocrine epithelium proliferative lesions; apocrine metaplasia (10/10), hyperplasia (6/6), apocrine DCIS (4/4), and invasive/metastatic apocrine carcinomas (7/8) were positive for AMACR (Figures 2 and 4). These observations of expression in benign/non-invasive apocrine cells (apocrine

193 metaplasia, n=13; apocrine hyperplasia, n=21, apocrine DCIS, n=1) were also observed in the

194 cohort of benign and non-invasive lesions of the breast (Table 4) when present in the samples.

195

4. Discussion

AMACR/racemase is a mitochondrial and peroxisomal enzyme that is involved in the β-oxidation of branched-chain fatty acids mediating the interconversion of (R)- and (S)-2-methyl-branched-chain fatty acyl-coenzyme As (21). AMACR expression has been previously reported in various cancers, including breast cancer. We also confirm AMACR mRNA expression across the cancers, including prostate, breast, colon, small cell lung, and neuroendocrine tumors (Figure 1).

The diagnostic utility of AMACR has been limited to prostate cancer, showing an excellent sensitivity and specificity in identifying pre-malignant (High-grade prostatic intraepithelial neoplasia) and malignant prostate epithelium compared with the negative benign prostate glands. AMACR has also shown an excellent diagnostic utility for papillary renal cell carcinoma (9).

In contrast to normal breast tissues (TDLU), which was rarely positive, we found a common AMACR expression across various proliferative lesions of the breast, including in-situ and invasive breast carcinoma of various histologies. Our RNASeq analysis, based on a large cohort of different cancers (>20,000 cases from Caris Life Sciences), further confirmed increased AMACR expression in invasive breast cancer, although its median value and standard deviation were significantly lower than in prostate carcinoma (Figure 1). At the protein level, we also confirm recent observations of a consistent and strong AMACR expression in apocrine lesions of the breast, both benign (metaplasia and hyperplasia), pre-malignant (apocrine DCIS) and malignant (invasive apocrine carcinoma). Previously, Nakamura et al. (12) found AMACR

expression in 38/39 (97.4%) of apocrine carcinomas and in 27/28 (96.4%) apocrine DCIS, consistent with the expression of the apocrine-specific biomarker GCDFP-15 (12). That study also found significantly higher mRNA AMACR levels in apocrine breast carcinomas than in non-apocrine types. However, the cause/function for the increased expression of AMACR, specifically in apocrine breast epithelium, remains to be elucidated. Similarly, the role of AMACR's organelles peroxisomes and mitochondria in cancer development and progression is still poorly characterized (22). Previous data on prostate cancer indicate that AMACR activity in peroxisomes induces the release of peroxides that promote DNA damage of prostate cells, causing a potentially oxidative environment (2).

By studying the expression of AMACR in the early proliferative and pre-malignant lesions, our study yields additional insights into the potential involvement of AMACR in cancer development. We observed a gradual increase in AMACR expression starting from normal, benign to atypical breast lesions, indicating a potential oncogenic role of AMACR in breast carcinogenesis. A small number of cases showing discordant results in the expression of AMACR between in-situ (positive) and invasive (negative) is similar to HER2 discordant cases (23), reported in ~1% of invasive breast carcinomas. A few previous studies reported a low AMACR expression in breast cancer, focusing exclusively on invasive breast carcinomas (5, 8). Wietkiewicz et al. (11) reported AMACR expression in 26% of invasive breast carcinomas, while the expression of AMACR in normal, benign (n=15) and in situ lesions (n=4) was not explicitly studied. In contrast to the study of Wietkiewicz et al., we found a good correlation between AMACR expression and molecular subtypes of breast cancer (using immunohistochemical

surrogate definitions). Like Wietkiewicz et al., we also found a positive correlation between AMACR and HER-2/neu protein expressions.

Although a relatively small sample size limits our study, it indicated that AMACR is overexpressed in various benign, atypical and invasive breast lesions. Consequently, its diagnostic utility in breast pathology remains limited. Further molecular studies are necessary to elucidate the exact role of AMACR in breast cancer pathogenesis.

Conflict of Interest

All authors declare no conflict of interest.

Author contributions (CRediT)

Conceptualization: ZG and SV; Data curation: ZG, PS, SV; Formal analysis: ZG, PS, SV; Investigation: ZG, PS, SV; Methodology: ZG and PS; Supervision: ZG and SV; Validation: ZG, PS, SV; Roles/Writing - original draft: ZG and SV; Writing - review & editing: ZG, PS, SV.

Ethics approval and consent to participate

The study complied with the Declaration of Helsinki. All samples were de-identified, and the patients' information was anonymized for study purposes. The Institutional Review Board of the University of Oklahoma approved the study (IRB#12866, approval date: May 21, 2021).

Consent for publication

All authors consent to the publication of this research/data.

Data availability

257 The data sets that formed the basis of this article can be obtained from the corresponding
258 author on a reasonable request.

259 ## Acknowledgement

260 Open access funding of this article was provided by the Qatar National Library (QNL).

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337 ## Tables

Morphologic subtype	AMACR expression		Total
	negative	positive	
Ductal NST	18 (36%)	32 (64%)	50 (63%)
Lobular	6 (54.5%)	5 (45.5%)	11 (14%)
Mucinous	4 (50%)	4 (50%)	8 (10%)
Apocrine	1 (12.5%)	7 (87.5%)	8 (10%)
Metaplastic	1 (100%)	0 (0%)	1 (1%)
Micropapillary	0 (0%)	2 (100%)	2 (3%)
Total	**30 (37%)**	**50 (63%)**	**80 (100%)**

338

339 **Table 1**. The frequency of AMACR expression in different morphologic subtypes of breast
340 cancer. The differences were not statistically significant (p=0.23).

The molecular subtype of breast cancer	AMACR expression		Total
	negative	positive	
Luminal A	19 (59%)	14 (41%)	33 (45%)
Luminal B	2 (14%)	12 (86%)	14 (19%)
Luminal B (HER2+)	0 (0%)	8 (100%)	8 (11%)
HER2+	1 (20%)	4 (80%)	5 (6%)
Triple-negative breast cancer	7 (50%)	7 (50%)	14 (19%)
Total	**29 (39%)**	**45 (61%)**	**74 (100%)***

341 *Six invasive carcinoma cases did not have all surrogate biomarkers for molecular classification.

342

343 **Table 2**. Significant differences in AMACR expression were observed among the different
344 molecular subtypes of breast cancer (p=0.005).

345

Histologic grade	AMACR expression		Total
	negative	positive	
1	12 (60%)	8 (40%)	20 (31%)
2	7 (20%)	27 (80%)	34 (52%)
3	6 (50%)	6 (50%)	12 (19%)
Total	**25 (38%)**	**41 (62%)**	**66 (100%)***

*The remaining cases had no provided Nottingham combined histologic score (n=6) or were metastatic cancers with limited tissue available for the grading (n=8).

Table 3. The relationship between AMACR expression and tumor grade in invasive carcinoma cohort (p=0.01).

Type of lesions (histology)	AMACR expression (n=70)
Normal breast (Terminal Duct Lobular Unit)	4/51 (8%)
UDH/papillomatosis/adenosis	6/22 (27%)
Atypical lesions (in situ)	
• Atypical ductal hyperplasia/Ductal Carcinoma in Situ (DCIS)	18/20 (90%)
• Lobular Carcinoma in Situ (LCIS)	3/6 (50%)
• Flat Epithelial Atypia (FEA)	4/6 (66%)
Apocrine lesions	
• Apocrine metaplasia	13/13 (100%)
• Apocrine hyperplasia	21/21 (100%)
• Apocrine Ductal Carcinoma in Situ	1/1 (100%)*

*The case is also included in the DCIS cohort above (n=20).

Table 4. The distribution of AMACR in normal breast tissue, benign and preinvasive (atypical and in-situ) lesions of the breast (benign and non-invasive cohort, n=70).

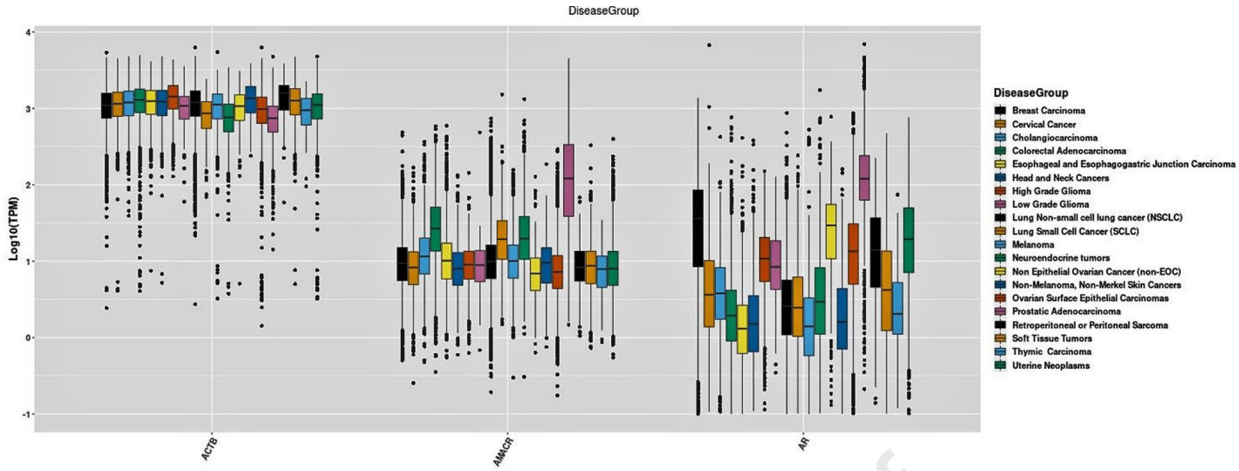
Figures

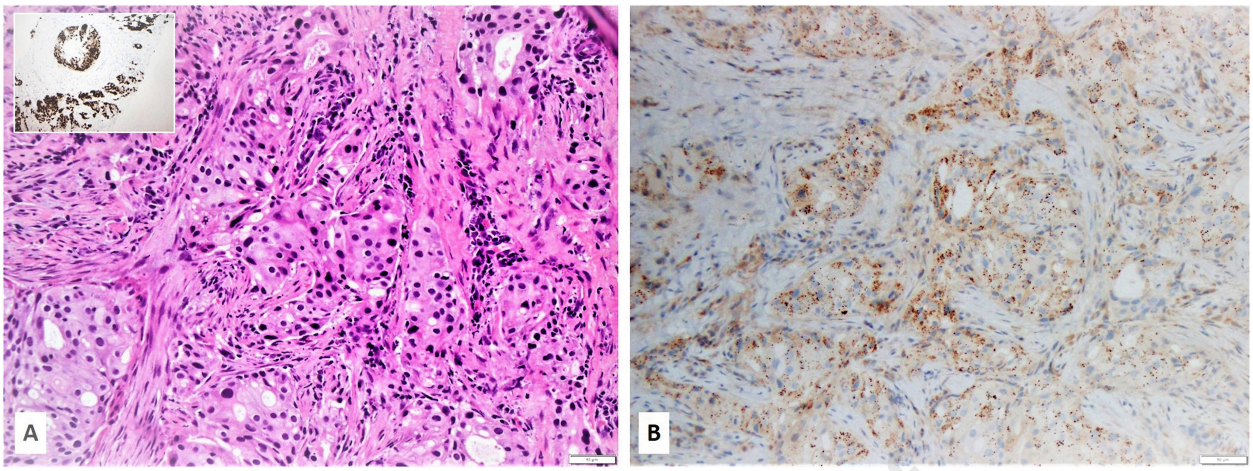
Figure 1. RNAseq analysis across the cancers. Bar charts show log10 expression (quantitative RNASeq in Transcripts per Million, TPM) on the Y-axis vs. cancer cohorts on the X-axis. AMACR expression has a less dynamic range in breast carcinoma vs. prostate, suggesting a growth-beneficial role in breast cancer. AMACR in the prostate shows a higher dynamic range and a higher median expression level than breast cancer, suggesting an opportunistically beneficial role. AR shows the reverse trend, highlighting the contrast between AMACR and AR in breast and prostate cancer, suggesting a possible antagonistic function.

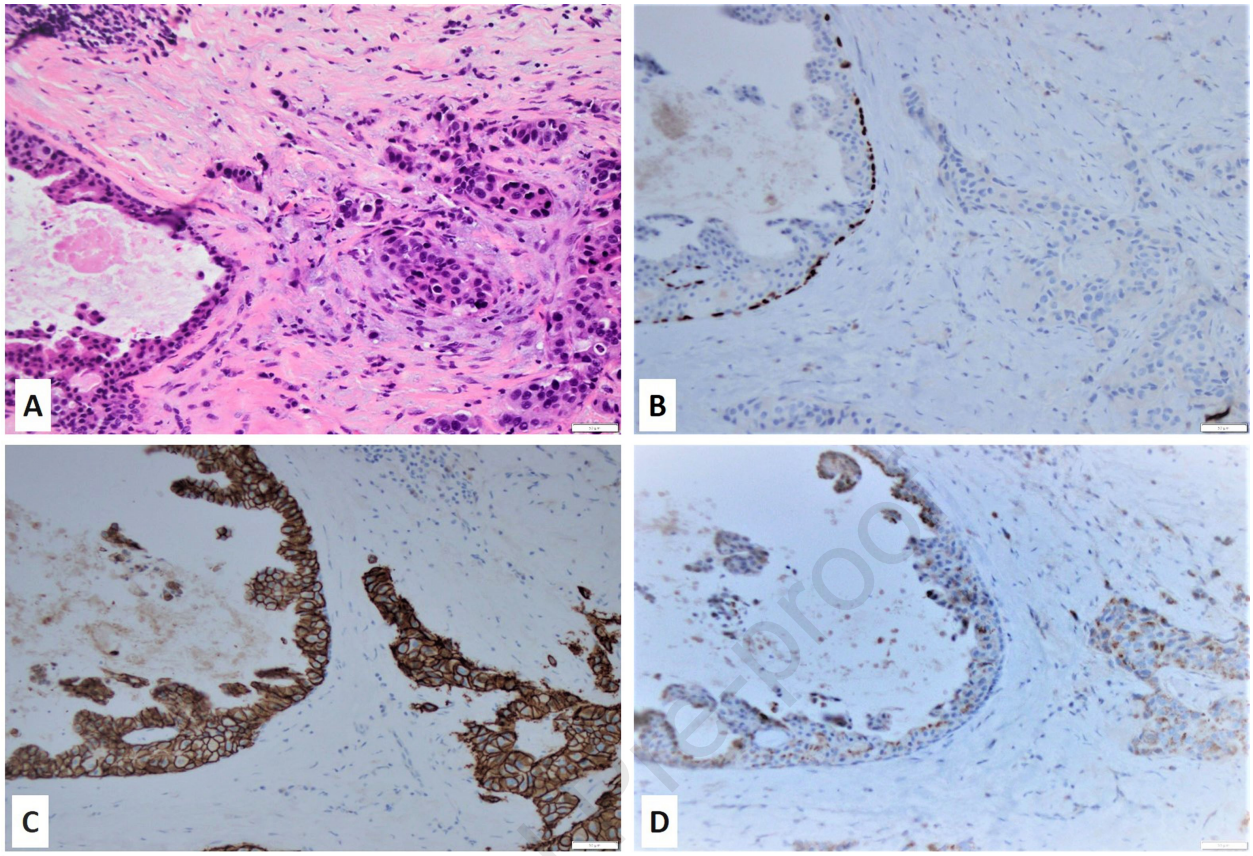
Figure 2A-B. A: H&E stained slide of invasive apocrine carcinoma with triple-negative phenotype and a strong Androgen receptor positivity (left upper image); B: Neoplastic cells were diffusely positive for AMAC (20x magnification).

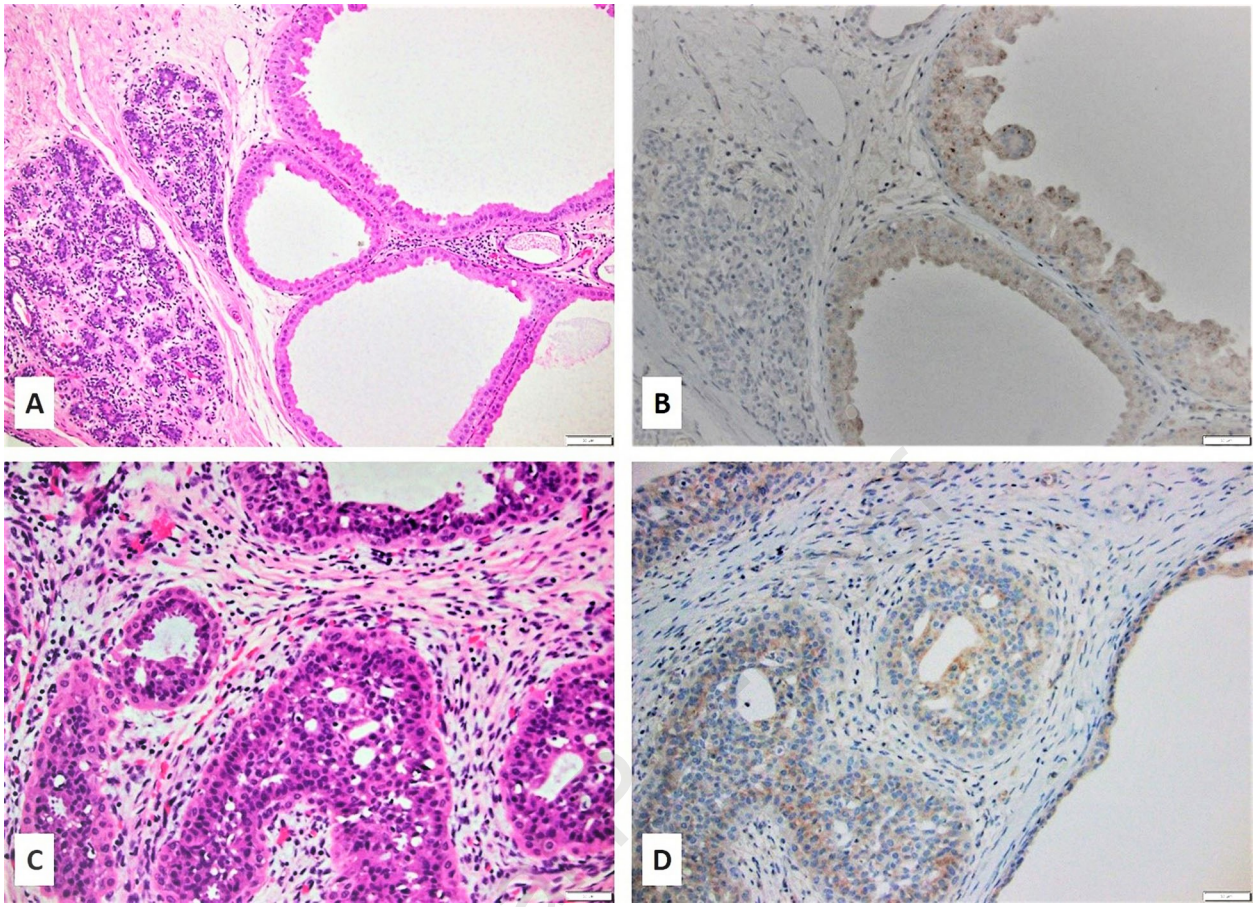
Figure 3A-D. AMACR expression in DCIS and invasive ductal carcinoma NST with HER2 expression. A: H&E stained image; B: IHC for p63 showing partially preserved basal cell layer of in-situ carcinoma; C: Strong HER2 expression in both in-situ and invasive carcinoma; D: cytoplasmic AMACR expression in epithelium of in-situ and invasive carcinoma (10x magnification).

Figure 4A-D. AMACR expression in early metaplastic and proliferative lesions. A: H&E stained slide of TDLU (left) and cystic apocrine lesion (right); B: AMACR is expressed in cystic apocrine lesions (metaplasia and hyperplasia), while TDLU is negative. C: H&E image of the usual ductal hyperplasia (UDH); D: UDH shows the expression of AMACR in most cells (A and B: 10x magnification; C-D: 20x magnification).









Highlights

- AMACR is a useful diagnostic biomarker for prostate carcinomas and several other malignancies.
- AMACR's role in breast cancer progression has not been fully investigated.
- We confirm a consistent AMACR expression in benign and malignant apocrine lesions of the breast.
- Our study revealed a common AMACR expression in various non-apocrine benign, atypical and invasive breast lesions.