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PIK3CA AND PTEN MUTATIONS IN ADENOID CYSTIC CARCINOMA OF THE BREAST METASTATIC TO KIDNEY

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ABSTRACT

Adenoid cystic carcinoma (ACC) of the breast rarely metastasizes and has been associated with excellent prognosis. We describe a patient with renal metastasis of primary breast ACC five years after the mastectomy. A detailed molecular genetic analysis of the primary and metastatic tumors demonstrated somatic mutations in two well known cancer genes associated with regulation of PI3K/AKT signaling pathway: 1) PIK3CA which encodes the catalytic alpha subunit of the phosphoinositide-3-kinase and 2) PTEN, which encodes phosphatase and tensin homolog. The mutation identified in PIK3CA (Ex1+169 A>C) predicts an amino acid change from isoleucine to methionine at codon 31 (I31M) and resides in the p85-binding domain of exon 1. The mutation identified in PTEN (IVS4-3 C>T) resides in intron 4 near the splice acceptor site of exon 5 and was associated with an aberrant PTEN transcript lacking exon 5, which is necessary for protein tyrosine phosphatase function and tumor suppressor properties of PTEN. Increased promoter methylation of PTEN was present in renal metastasis, coinciding with the decrease in the level of normal PTEN transcript.

These coexistent mutations/epigenetic inactivations in PI3K/AKT pathway may be responsible for the unusually aggressive course of adenoid cystic carcinoma..

INTRODUCTION

Adenoid cystic carcinoma (ACC) of the breast is a rare histologic type of breast carcinoma, comprising less than 1% of all mammary carcinomas.¹ ACC is a basal-like breast carcinoma characterized by a distinct morphology and immunohistochemical profile.² ACC is usually associated with a very good prognosis.³ Distant metastases are very rare and usually affect the lungs¹. Only several cases with high grade tumors and distant metastases have been published, and only one case with symptomatic metastasis to the kidney has previously been reported.⁴ We report here a case of ACC of the breast with symptomatic renal metastasis, and for the first time show coexistent mutations in genes encoding phosphoinositide-3-kinase catalytic alpha subunit (PIK3CA) and phosphatase and tensin homolog (PTEN). Both PIK3CA and PTEN are integral part of the mammalian target of rapamycin (mTOR) signaling pathway.⁵ MTOR, a protein kinase of the PIK3/Akt signaling pathway, plays a central role in controlling malignant growth, cell cycle progression and proliferation.⁵

CASE HISTORY

76-year-old woman presented with abdominal pain, painless hematuria and urinary frequency. CT scan showed a 6 cm expansive, poorly circumscribed tumor of the right kidney that was considered a primary renal carcinoma. Radical nephrectomy was performed and subsequent pathologic examination revealed a 90x48x40 mm, gray-white, hemorrhagic tumor involving pelvicalyceal system and cortex and medulla of the upper part of the kidney. Review of patient's medical records showed that she underwent a mastectomy at a different institution for 1.8 cm primary mammary adenoid cystic carcinoma (pT1c N0 MX R0) 5 years prior to current surgery. The patient did not receive any adjuvant therapy. Original formalin fixed paraffin embedded (FFPE) tissue blocks were obtained and morphologic, immunohistochemical and molecular analyses were then performed to compare these 2 tumors.

METHODS

Immunohistochemistry

Immunohistochemistry was performed using the standard avidin-biotin complex (ABC) detection system and diaminobenzidine tetrahydrochloride (DAB) chromagen. Table 1 lists antibodies used and results of staining in both primary and metastatic tumors. The expression levels of cyclooxygenase-2 (COX-2) and PTEN were measured on immunohistochemically stained tissue slides using the Automated Cellular Imaging System (ACIS, ChromaVision Medical Systems, Inc., San Juan Capistrano, CA).

Molecular genetic analysis

Patient samples and DNA and RNA preparation. DNA extractions from cancer and normal tissues were performed following manual microdissection to differentiate somatic from germ-line mutations. Tissue samples were digested with Proteinase K at 55°C overnight with continuous agitation (120 rpm). DNA was purified and concentrated using an Amicon Microcon YM-30 column (Millipore Corp., Billerica, MA). RNA was extracted from FFPE tissues using the Paraffin Block RNA Isolation kit according to the manufacturer's instructions (Ambion, Austin, TX). Any trace gDNA was removed from each aliquot of extracted RNA with DNase I using a commercial DNA-free Kit (Ambion).

PCR amplification of selected cancer genes. Primer pairs were designed to amplify selected exons of the following genes: KIT (ex 8-17); PDGFRA (ex 12, 14, 18); EGFR exons 18-21, ERBB2 (ex 18-23); KRAS (ex 2-3); NRAS (ex 2-3); BRAF (ex 11, 15); PIK3CA (ex 1, 9, 20); PTEN (ex 2-8); P53 (ex 5-8); and APC (ex 15). Amplicons included 50 bp of intronic sequence to allow for detection of mutations residing in/near splice junction regions. Primer selection was performed with MutationDiscovery.com, a web-based Software (Transgenomic, Inc., Omaha, NE) which incorporates Primer 3 oligonucleotide selection and design criteria. Specificity and yield of each PCR product was routinely assessed by gel electrophoresis (2% agarose, 1X TAE buffer).

Mutation scanning by Surveyor nuclease analysis and fragment analysis.

Heteroduplexed PCR products were combined with 15 U of Surveyor Nuclease and 1µl Enhancer (Transgenomic), then incubated at 42°C for 20 min. Digestions were terminated with 2 µl Stop solution (0.5 M EDTA at pH 8.0) and analyzed on a Transgenomic WAVE System equipped with a High Sensitivity Detection module (WAVE-HS) for fluorescent detection of ds-DNA fragments. This scanning methodology has shown a limit of detection of 1 mutant copy in 100-200 total copies.

Mutation identification by DNA sequencing. PCR products were purified and cycle sequenced with amplicon specific primers. Sequencing products were run on an ABI 3100 Genetic Analyzer. Data were analyzed using Sequencher (GeneCodes, Ann Arbor, MI) and by manual review of chromatograms.

PTEN cDNA analysis by RT-PCR. The nucleotide sequences and exon-intron boundaries of PTEN and GAPDH were derived from NCBI (<http://www3.ncbi.nlm.nih.gov/>). Primers (SigmaGenosys, The Woodlands, TX) spanning exonic junctions were designed to amplify fragments representing selected regions of the PTEN coding sequence (Table 2). RT-PCR was performed using the One-Step kit (Invitrogen, Carlsbad, CA) and end products were analyzed on the WAVE-HS and by DNA sequencing.

PTEN promoter methylation analysis by DHPLC and DNA sequencing.

The methylation status of the PTEN promoter was examined by analyzing the sequence context of PCR products obtained from sodium bisulfite modified genomic DNA. The sulfonated DNA was recovered using the Wizard DNA clean-up system (Promega, Madison, WI) and the conversion reaction was completed by desulfonating in 0.3 M NaOH for 10 minutes at room temperature. The DNA was ethanol precipitated and resuspended in water. PCR was done using specific primers designed outside of any CpG islands to amplify both methylated and unmethylated promoter sequences. Final PCR products were analyzed by DHPLC on a WAVE System in which allelic differences in methylation are detected due to the sequence-dependent separation characteristics of the DNASep DHPLC column. To obtain information regarding differential methylation across the PTEN promoter region, PCR products were also bi-directionally sequenced.

RESULTS

Microscopically, the renal tumor was composed of small epithelial cells that formed microcysts with centrally located PAS positive substances. The tumor was organized in both cribriform and solid patterns, but no sarcomatoid areas were noted. Differential diagnostic considerations included primary renal carcinoma (renal medullary carcinoma and mucinous tubular and spindle cell carcinoma) and metastatic adenoid cystic carcinoma. Both mammary and renal tumors (Figure 1A) showed strong positivity for c-kit, p63, CK5/6, CK18, SMA, Vimentin, E-cadherin and B-catenin. ER, PR and ERBB2 were not detected. These results are consistent with basaloid type of mammary carcinoma and confirmed the morphologic diagnosis of metastatic ACC to the kidney.

COX2 expression was stronger in the metastasis than in the primary tumor. In primary ACC 35% of cells were positive with the relative intensity 60.5 ± 1.9 (in the most intensely stained part of the tumor) while in the renal metastasis COX2 was detected in nearly all cells with relative intensity 73.3 ± 2.2 . PTEN expression in primary and metastatic cancer was detected in the similar proportion of cells (41% and 37%, respectively), but the relative intensity was lower in metastatic cancer (75.3 vs. 92).

Molecular analyses revealed the following gene mutations in both primary adenoid cystic carcinoma of the breast and renal metastasis: 1. PIK3CA: Ex1+169 A>G predicting an amino acid substitution of isoleucine with methionine at codon 31(I31M) and 2. PTEN (IVS4-3 C>T near the splice acceptor site of exon 5 (Figures 1B and 1C, respectively). No mutations were found in normal breast and kidney tissues. In addition, neither primary nor metastatic tumors showed mutations in KIT, PDGFRA, EGFR, ERBB2, KRAS, NRAS, BRAF, P53 and APC

genes. Analysis of PTEN cDNA amplified by RT-PCR showed aberrant transcript that lacked exon 5 in both the primary and metastatic cancers (Figure 1D). This would suggest that the PTEN splice acceptor mutation present in both samples is affecting processing of PTEN mRNA, with exon 5 not being included. The deleted region encodes PTEN amino acids 85-164 and is essential for protein tyrosine phosphatase function and overall tumor suppression. Normal PTEN transcript, presumably derived from the non-mutated allele was also detected in both specimens. The amount of normal PTEN in renal metastasis appeared to be reduced relative to levels detected in primary breast tumor. Additional molecular evidence supporting the reduction of PTEN transcript in the metastasis comes from detailed analysis of promoter methylation. A distinct difference in the degree of methylation across the 3 regions of the PTEN promoter that contains the CpG residues was observed between the primary and metastatic ACC (data not shown). Of twenty seven CpG islands evaluated, 63 % were methylated (fully or partially) in the primary tumor while 93% were methylated in the renal metastasis.

DISCUSSION

Although ACC shows a distinct morphological pattern, its diagnosis, particularly in the case of a remote, isolated renal metastasis, presents a significant diagnostic challenge. Only one case with symptomatic renal and lung metastases has previously been reported.⁴ Furthermore, renal medullary carcinoma and mucinous tubular and spindle cell carcinoma may exhibit morphologic features similar to high grade adenoid cystic carcinoma. ACC is usually composed of two population of the cells (myoepithelial and epithelial) that form circumscribed clusters of cells arranged in solid, cribriform, tubular and trabecular arrangements although one pattern can predominate². Immunohistochemical documentation of two cell populations in our case was extremely helpful in distinguishing metastatic ACC from rare primary renal carcinomas with ACC-like growth patterns.

ACC of the breast fulfills the criteria for the diagnosis of basal-like breast carcinoma as recently proposed (ER, PR and HER2/neu negative and p63, CK5/6, SMA and KIT positive).^{6,7} Classification of breast carcinoma based on gene expression patterns identified five different subtypes of breast cancer including: luminal type (A and B), ERBB2 type, basal-like and normal-like type. Basal-like carcinomas are distinct clinical and pathologic entity that exhibits significantly worse outcome than luminal type carcinomas.^{6,8} It is then paradoxical that ACC of the breast which is immunophenotypically classified as basal cell type cancer, have been particularly associated with favorable outcome.³ Therefore, only

detailed molecular genetic analysis such as the one performed in our study could provide additional important prognostic and potentially useful therapeutic information in an individual case.

To our knowledge, the mutations in PTEN (IVS4-3 C>T) and PIK3CA (I31M) have not been previously detected neither in salivary gland nor breast ACC, and other cancer types. Our case is the first case that demonstrated mutations of these two genes in both primary and metastatic ACC. Furthermore, our case of ACC demonstrated two coexistent mutations in mTOR signaling pathway which rarely occurs. Several studies have pointed out that PIK3CA mutations were mutually exclusive with the mutations of PTEN, suggesting that oncogenic signaling occur either through activation of PIK3CA or inactivation of PTEN.⁹ However, recently published studies in endometrial carcinoma demonstrated that coexistent mutations of PIK3CA and PTEN genes are indeed possible.¹⁰ Our case also showed that such combination can also occur in mammary ACC. Furthermore, we showed that reduced expression of PTEN can also occur through increases in promoter methylation. PTEN and PIK3CA are two important tumor suppressor genes and oncogenes in the mammalian target of rapamycin (mTOR) signaling pathway.⁵ MTOR is a protein kinase that has a central role in controlling cancer growth and progression. It integrates signals from growth factors, energy stores and hypoxia.⁵

Phosphoinositide kinase (PIK) is a lipid kinase that phosphorylates the inositol ring of phosphoinositides, acting as signal transducers. Mutational activation of PIK3CA is essential for carcinogenesis in some cancers. PIK3CA mutations are common in solid tumors and several have been shown to activate AKT, leading to growth-factor independent growth, inhibition of apoptosis and increased cell invasion and metastasis.¹¹ PIK3CA mutations have been detected in 8-40% of breast carcinomas and are associated with poor outcome.¹² *Saal et al*⁹ identified PIK3CA mutations in 26% of 342 breast carcinoma specimens at about equal frequency irrespective of the tumor stage.

PIK3 proteins consist of the family of proteins divided into three classes that differ in its structure, tissue distribution, mechanism of action and its function. The most important PIK3 proteins are those related to proliferation and carcinogenesis including class IA – the catalytic subunit p110 α and its associated regulatory subunit p85. Activation of class IA proteins might activate several signaling pathways including AKT and Rictor-mTOR complex.^{5,11}

PI3-AKT signals govern several transcription factors including forkhead box (FOXO) proteins and nuclear factor κ B (NF κ B).¹³ NF κ B acts as a positive regulator of cell survival, proliferation, cellular invasion and angiogenesis. Several studies demonstrated that Akt

signals through NF κ B in order to induce cyclooxygenase-2 (COX-2) expression in mutated PTEN tumor cells.¹⁴ COX-2 significantly contributes to tumorigenesis through increased angiogenesis, invasiveness and inhibition of apoptosis.¹⁴ Our case demonstrated higher COX-2 expression in the metastasis than in the primary tumor (Figure 1A).

Although PTEN gene is one of the most commonly mutated genes in human tumors,¹⁵ its importance in ACC has not been described. Approximately 50% of breast carcinomas carry a mutation or loss of at least one copy of the PTEN gene which results in PIK3 activation.¹⁶ PTEN mutations are often associated with an aggressive metastatic tumor phenotype since PTEN might interfere with focal adhesion kinase (FAK) and Shc functions enabling increased cell motility and spreading.¹⁵ Inactivation of PTEN might also result in increased cell cycle progression through the Akt-dependent phosphorylation and inactivation of glycogen synthase kinase-3 (GSK-3) which, in turn, leads to cyclin D1 stabilization.¹⁵ It also has a pivotal role in apoptosis since the loss of PTEN results in an increased PIP3 concentration and in Akt activation, leading to protection from various apoptotic stimuli.¹⁵ In our case we demonstrated increased methylation of PTEN gene promoter associated with reduced expression of PTEN protein in the metastasis compared to the primary tumor. A complete loss of PTEN is more frequent in metastatic than in primary tumors¹⁶ and in cases of breast carcinomas where decreased PTEN expression was associated with increased neoangiogenesis and poor clinical outcome.

Results of such in-depth molecular analysis may have additional therapeutic implications. No mutations were observed in genes encoding the receptor tyrosine kinases, KIT and PDGFRA, known targets of imatinib mesylate inhibition (Gleevec) suggesting a limited response to this targeted therapy based upon clinical data of other cancer types (e.g. gastrointestinal stromal tumors, small cell lung carcinoma, melanoma). Despite consistent expression of c-kit in ACC³, recent clinical trials showed no clinical benefit from Gleevec therapy in advanced head and neck ACC.¹⁷ No mutations were observed in the gene encoding the receptor tyrosine kinase, EGFR, a known target of both gefitinib and erlotinib, suggesting a limited response to this targeted therapy based upon clinical data from other cancer types (e.g. non-small cell carcinoma of the lung).

In vitro and clinical studies confirmed that deregulation of the PI3K/PTEN/Akt pathway was associated with resistance to doxorubicin and tamoxifen, drugs commonly used in metastatic breast cancer therapy.¹⁸ This may have significant implications since chemotherapy and hormonal therapy are basic modalities for the treatment of metastatic breast carcinoma. On the other side, specific mutations we detected in our case, give new insights in both breast

carcinogenesis and novel therapeutic modalities. Thus, rapamycin, which acts as mTOR inhibitor, might have promising antitumor activity in subset of tumors with upregulated PIK3/AKT/mTOR signaling pathway. Also, three rapamycin analogs, temsirolimus (CCI-779, Wyeth), everolimus (RAD001, Novartis Pharma AG) and AP23573 (Ariad Pharmaceuticals) have been included in clinical trials for the treatment of cancer.⁵ To date, clinical trials results showed that mTOR inhibitors were well tolerated and might induce prolonged stable disease and tumor regressions in cancer patients.⁵

Other components of this vital signaling pathway might also become the potential targets for specific small molecules. Thus, the mutated p110alpha proteins are ideal targets for such inhibitors that discriminate between the oncogenic and the wild-type forms of the enzyme. In summary, we report a case of metastatic ACC of the breast characterized by coexistent mutations in two important genes of mTOR signaling pathway (PIK3CA and PTEN) which might be responsible for the aggressive clinical course. Breast ACC is a triple negative (ER/PR/HER2), basal-like carcinoma that responds poorly to the conventional and even some targeted chemotherapies. Novel molecular targets identified in this case, may provide potential for therapeutic intervention(s).

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Figure Legend

Figure 1. A) COX-2 expression (IHC stain, 20x) in kidney metastasis of ACC from the breast. Inset - Photomicrograph of the primary ACC in the breast (H&E, 20x). B) PIK3CA mutation detection and sequencing data. C) PTEN mutation detection and sequencing data. D) RT-PCR of PTEN cDNA transcript showing exon 5 deletion and aberrant transcript that lacks exon 5 in both the primary and metastatic cancers.

Table 1. Antibodies and staining results in primary and metastatic ACC. ¹DAKO, Glostrup, Denmark; ²Cell Marque, Hot Spring, AR; ³Cayman Chemical, Ann Arbor, MI; ^aACIS relative stain intensity: 92.7 (in primary tumor) vs. 75.3 (in metastasis); ^b ACIS intensity: 60.5 (in primary tumor) vs. 73.3 (metastasis)

| Immunohistochemical marker | Clone | Dilution | Primary ACC | Metastatic ACC |
|-----------------------------------|---|-----------------|--------------------|-----------------------|
| Vimentin | V9, monoclonal ¹ | | +++ | +++ |
| C-kit (CD117) | A-4502, polyclonal ¹ | | +++ | +++ |
| E-cadherin | NHC-38, monoclonal ¹ | | +++ | +++ |
| B-catenin | B-catenin-1 ¹ | | +++ | +++ |
| CK5/6 | D5/16B4 ¹ | | -/+ | +++ |
| Estrogen Receptor | 1D5, monoclonal ¹ | | - | - |
| Progesterone Receptor | pgR636, monoclonal ¹ | | - | - |
| HER2/neu | Polyclonal LOT000255667 ¹ | | - | - |
| p63 | 4A4, monoclonal ¹ | | +++ | +++ |
| Smooth Muscle Actin | 1A4, monoclonal ¹ | | ++ | ++ |
| PTEN | Polyclonal ² | | ++ ^a | + ^a |
| Cox-2 | polyclonal rabbit IgG ³ | | + ^b | ++ ^b |

Table 2. Oligonucleotide primers used for amplification of PTEN cDNA. The selected primers were used to PCR amplify cDNA fragments representing selected regions (exons 3 through 7) of the PTEN coding sequence.

| Primer ID | Primer sequence (5' > 3') | Tm (°C) | Length (nt) |
|--------------------------------------|-----------------------------|---------|-------------|
| <i>forward primers</i> | | | |
| cPTEN-ex3f | GGATTCAAAGCATAAAAACCATTAC | 62.3 | 25 |
| cPTEN-ex4f | GCTGAAAGACATTATGACACCGC | 66.5 | 23 |
| cPTEN-ex5f | GATCTTGACCAATGGCTAAGTGA | 64.1 | 23 |
| <i>reverse primers</i> | | | |
| cPTEN-ex5r | TACAGTGAATTGCTGCAACATGA | 65.2 | 23 |
| cPTEN-ex6r | TTCCGCCACTGAACATTGGAA | 69.6 | 21 |
| <i>exon junction reverse primers</i> | | | |
| cPTEN-ex4-5r | GTCTTCAAAGGATATTGTGCAACTCTG | 67.3 | 28 |
| cPTEN-ex5-6r | GGAATAGTTACTCCCTTTTGTCTCTG | 63.9 | 27 |
| cPTEN-ex6-7r | CAAAGTGGAGGATCTGCAGTTCC | 64.9 | 22 |