The Epithelioid Variant of Angiomyolipoma in Kidney Expresses a Higher Level of Epidermal Growth Factor Receptor Than Its Conventional Type

Frank Chen, MD, PhD, David G. Hicks, MD, Christopher A. D'Angelis, MD, PhD, Haiying Chen, MD, Sheila Sait, PhD, Shaozeng Zhang, MD, PhD

Abstract

The epidermal growth factor receptor (EGFR) is a transmembrane tyrosine kinase that plays an important role in tumor growth, invasion and metastasis. EGFR Positive immunoreactivity ranged from focal to diffuse in overexpression has been shown in many different tumors and multiple EGFR-targeted therapies are currently being investigated. Renal epithelioid angiomyolipoma (epiAML) is an aggressive and potentially malignant variant of conventional angiomyolipoma (cAML). Currently, the status of EGFR expression in epiAML is unknown. We compared EGFR protein expression, as well as expression levels of several other tumor regulatory proteins including c-Kit, p53, alpha-catenin, beta-catenin and osteopontin in 6 cases of cAML and 10 cases of epiAML by semiquantitative immunohistochemistry (IHC). EGFR gene amplification was analyzed by fluorescent in situ hybridization (FISH). Within epiAML, EGFR immunostaining was significantly higher when compared to cAML (90% vs. 16.7%, p<0.01). epiAML, whereas no diffuse reactivity was found in cAML. EGFR gene amplification was not identified in either cAML or epiAML as illustrated by FISH. Expression levels of c-Kit, osteopontin, p53, alpha-catenin and beta-catenin were similar between cAML and epiAML. In conclusion, the expression of EGFR protein is significantly higher in epiAML than that in cAML and this increase is not related to increased gene copy number. The increased expression of EGFR in epiAML may be responsible for its aggressive behavior relative to cAML and could represent a potential therapeutic target.
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Key Words: *angiomyolipoma, epithelioid angiomyolipoma, epidermal growth factor receptor (EGFR)*

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Introduction

Angiomyolipoma (AML) is the most common benign mesenchymal tumor of the kidney and is often associated with tuberous sclerosis. AML belongs to the family of vessels, adipose tissue, and smooth muscle-like cells.[1,2] The presence of fat enables most of AML to be diagnosed with a great accuracy using current imaging techniques.[3] Large or symptomatic tumors are indicated for resection, nephrectomy or local ablation.[12] Similar to all the PEComas, AML demonstrates a characteristic immunophenotype, with co-expression of smooth muscle markers (muscle specific actin and smooth muscle actin) and melanogenesis markers (HMB-45, melan-A, and tyrosinase). These tumors are negative for cytokeratin. Recently, an epithelioid variant of AML (epiAML) has been described,[1,4-7] composed of either pure epithelioid cells, or a mixture of epithelioid cells and smooth muscle fibers. Adipose tissue is often absent or scant in epiAML. cAML has histologic and clinical features of an essentially benign lesion, whereas epiAML is usually highly cellular and can closely simulate a high-grade sarcomatoid renal carcinoma. EpiAML is considered to be potentially malignant due to its metastatic capability.[5,7]

The aim of the present study was to investigate possible mechanisms responsible for the aggressive behavior of epiAML. We compared the expression levels of multiple oncogenic proteins, adhesion molecules, and signaling pathway molecules in cAML and epiAML. The protein markers tested include epidermal growth factor receptor (EGFR), p53, c-Kit, alpha-catenin, beta-catenin and osteopontin. EGFR is a 170,000-kDa transmembrane glycoprotein involved in cellular growth, differentiation, and proliferation.[8,9] Abnormal expression of EGFR has been

described in many human tumors and implicated in the development and prognosis of malignancies. In addition EGFR has been used as a prognostic marker and as a molecular target for anticancer agents.[10] The tumor suppressor gene p53 encodes a 53-kDa nuclear protein that is involved in the regulation of cell growth.[11] Various p53 mutations have been reported in malignant tumors, and these mutations are considered important for tumorigenesis and/or tumor progression.[12] C-Kit is a transmembrane receptor tyrosine kinase, linked to many different malignant tumors, including gastrointestinal stromal tumors and mast cell diseases.[13,14] However, c-Kit expression in AML has only rarely been studied.[15] Alpha-catenin and beta-catinin are actin-binding proteins that participate in adhesion complexes with important functions in the E-cadherin-mediated cell-cell adhesion system.[16,17] Alterations in alpha-catenin and beta-catenin are related to the pathogenesis of different tumors.[18-20] Osteopontin is an integrin adhesion molecule and its overexpression in tumors has been associated with enhanced dissemination of malignant neoplasms.[21] To our knowledge, osteopontin expression in AML has not been characterized previously. We sought to examine the immunophenotypical expression of aforementioned markers in 16 cases of AML (10 epiAML and 6 cAML). In addition we examined the amplification status of EGFR by FISH to determine whether this was the mechanism for increased EGFR expression in epiAML.

Materials and Methods

Ten cases of epiAML and 6 cases of cAML from 1996 to 2005 were retrieved from the archive of the Department of Pathology at Roswell Park Cancer Institute, Buffalo, NY. All specimens had been routinely fixed in formalin and processed in paraffin. All cases were positive for HMB45 (performed at time of diagnosis). These cases were further reviewed by two urologic pathologists who independently confirmed the diagnoses. A single block from each case was selected for immunohistochemical (IHC) studies for EGFR, p53, c-Kit, alpha-catenin, beta-catenin, and osteopontin. The primary antibodies together with their sources, dilutions, and methods of pretreatment are shown in **Table 1**. IHC studies were performed by standard protocol. Briefly, Sections of 5 μm in thickness were cut from the formalin-fixed, paraffin embedded blocks, put on positive charged slides, and dried overnight at room temperature before deparaffinization in xylene and rehydration through-graded ethanols. Immunostains were performed using an automated stainer (Dako, Carpinteria, CA) and a HRP detection kit (Dako, Carpinteria, CA). Diaminobenzidine was used as the chromogen and sections were counterstained with hematoxylin. Positive controls were included in each run of immunostaining. Negative controls consisted of replacement of the primary antibodies with the matching immunoglobulin control (Dako, Carpinteria, CA) at an equivalent concentration.

The IHC stains were evaluated by one urologic pathologist and one surgical pathologist. For EGFR, alpha-catenin, and beta-catenin, only membranous immunoreactivity was recorded as positive. For p53, only nuclear staining was considered as positive. Cytoplasmic immunoreactivity was evaluated for c-Kit and osteopontin. For all antibodies, IHC staining intensity was scored as negative (<5% positive cells), focally positive (5-50% positive cells) and diffusely positive (>50% positive cells). Fisher's exact test was used to compare the expression of each of the immunomarkers between cAML and epiAML. A p value of <0.05 was considered to be statistically significant.

FISH was performed using a previously described method utilizing formalin-fixed paraffin embedded 4 μM tissue sections.[22] A commercially available probe set, LSI EGFR spectrum orange/CEP 7 Spectrum Green Probe set (Vysis/Abbott Molecular Inc., Des Plains, IL) was used. The EGFR locus was labeled in orange and the centromere of chromosome 7 was labeled with green, allowing for simultaneously evaluation of the EGFR copy number relative to total chromosome 7 copy numbers (internal control). Briefly, tissue sections were deparaffinized in a xylene substitute (Hemo-De) (Fisher Scientific, Pittsburgh, PA), and prepared for FISH assay using a paraffin pretreatment kit (Vysis/Abbott Molecular Inc., Des Plains, IL) as per manufacturer's instruction. The probe DNA and target DNA were hybridized at 37 C for 18-20 hours. After washing, the sections were counterstained with 4', 6-Diamidino-2-phenylindole dihydrochloride (DAPI II) (Vysis/Abbott Molecular Inc., Des Plains, IL) and analyzed on an epifluorescence microscope. At least 50 nuclei with strong and well-defined signals in each case were examined. The copy numbers of EGFR gene (orange signals) and total chromosome 7 (green signals) in cAML and epiAML were analyzed.

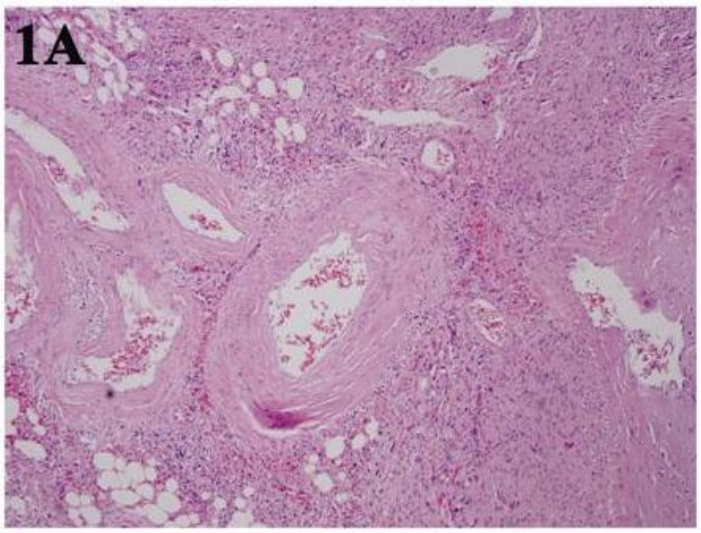
Results

The age of the cAML patient group ranged from 33 to 76 years old (median age 60). The age range of the epiAML group was 15 to 70 years old (median age 47.5). Both groups showed a marked female predominance, with the female to male ratio in cAML and epiAML being 5:1 and 4:1, respectively.

Histologically, cAML consisted of a mixture of adipose tissue, smooth muscle, and thick-walled blood vessels with characteristic smooth muscle cells emanating from the vessel walls, forming fascicles of spindle cells (**Figure 1**). In contrast, cases of EpiAML were predominantly solid, highly cellular, composed of medium to large epithelioid cells and some giant multinucleated cells. The epithelioid cells showed abundant amphophilic or eosinophilic granular cytoplasm often containing eccentric nuclei with prominent nucleoli. The presence of such pleomorphic epitheloid cells with so-called "ganglion cell features" strongly supports a diagnosis of epiAML (**Figure 2**). Extensive necrosis was observed in 40% of epiAML cases. Areas with features of typical cAML were observed in 70% of epiAML cases.

Table 1. Antibodies, Sources, Dilutions, and Methods of Pretreatment.

Antibody	Source	Dilution	Pretreatment
EGFR	Dako, Carpinteria, CA	1:200	Proteinase K, 5 min
P53	Dako, Carpinteria, CA	1:50	High PH buffer, 30 min
c-Kit	Dako, Carpinteria, CA	1:50	Vector pretreatment, 30 min
Alpha-catinin	Zymed, S San Francisco, CA	1:50	High PH buffer, 30 min
Beta-catinin	Zymed, S San Francisco, CA	1:200	Citrate buffer, 60 min
Osteopoitin	Zymed, S San Francisco, CA	1:250	Citrate buffer, 10 min



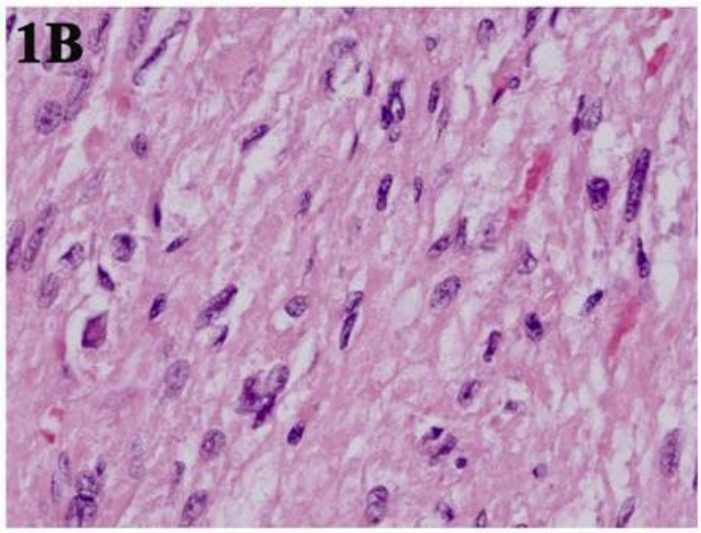
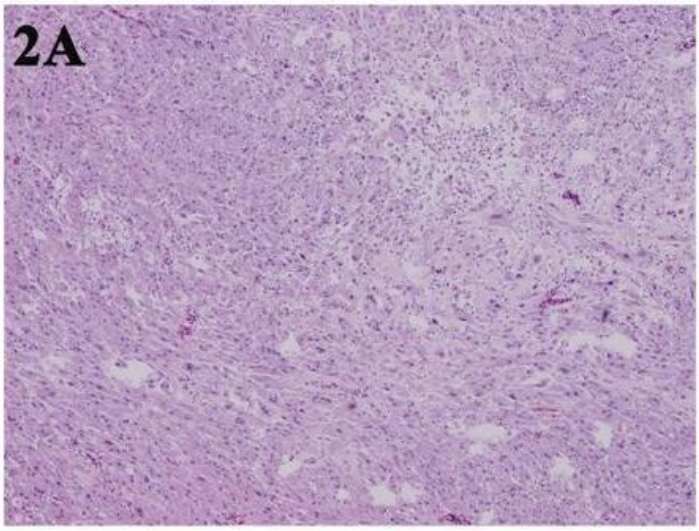


Figure 1. Conventional angiomyolipoma. **1A.** H&E stain at low power (100X). **1B.** H&E Stain at high power (400X).



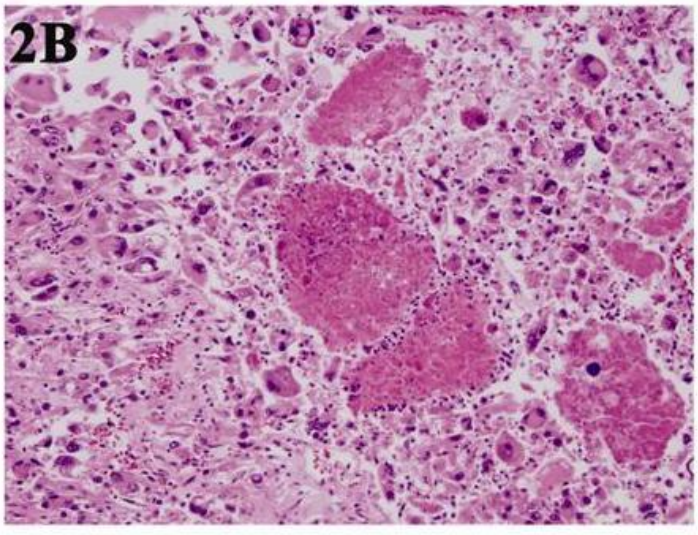


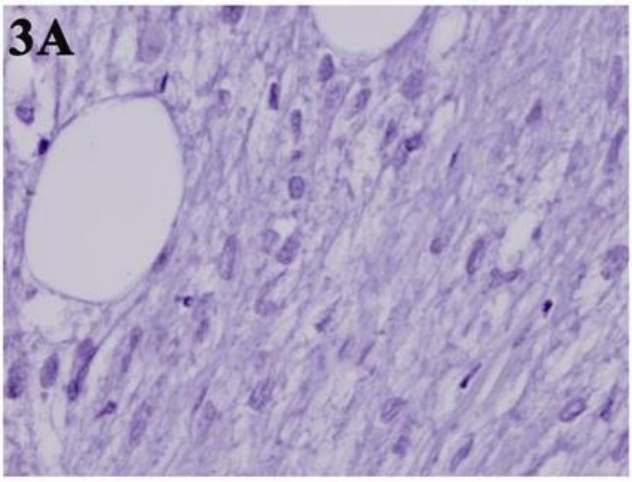
Figure 2. Epithelioid angiomyolipoma. **2A.** H&E stain at low power (100X). **2B.** H&E stain at high power (400X).

A statistically significant increase in EGFR expression was present in epiAML as compared with cAML ($p < 0.01$) (**Table 2**). Nine of 10 (90%) epiAML exhibited diffuse strong (2 cases), focal moderate (3 cases) or focal weak (4 cases) EGFR staining, whereas only one out of six (16.7%) cAML cases showed focal weak positivity (**Figure 3**). The expression of p53 and c-Kit was both weak and focal, and there was no significant difference of expression between cAML and epiAML. While both cAML and epiAML expressed osteopontin (83.3% and 100%, respectively), only focal staining was present in cAML. In contrast, 50% of epiAML expressed osteopontin diffusely, and the rest expressed focally (**Figure 4**). The alpha-catenin and beta-catenin expressions appeared higher in epiAML than that in cAML (90% vs. 50% and 50% vs. 16.7%, respectively). However, due to the small sample size, no statistical significance was found between the two groups.

Table 2. Immunohistochemical Analysis of Epithelioid AML and Conventional AML.

	EpiAML			cAML		
	Diff Pos.	Focally Pos.	Total Pos.	Diff Pos.	Focally Pos.	Total Pos.
EGFR	20% (2/10)	70% (7/10)	90%(9/10)*	0 (0/6)	16.7% (1/6)	16.7% (1/6)*
P53	0% (0/10)	30% (3/10)	30%(3/10)	0 (0/6)	33.3% (2/6)	33.3% (2/6)
C-Kit	10% (1/10)	50% (5/10)	60%(6/10)	0 (0/6)	33.3% (2/6)	33.3% (2/6)
Alpha-catenin	40% (4/10)	50% (5/10)	90%(9/10)	0 (0/6)	50% (3/6)	50% (3/6)
Beta-catenin	20% (2/10)	30% (3/10)	50%(5/10)	0 (06)	16.7% (1/6)	16.7% (1/6)
Osteopontin	50% (5/10)	50% (5/10)	100%(10/10)	0 (0/6)	83.3% (5/6)	83.3% (5/6)

* p <0.01 with Fisher exact test
cAML: Conventional Angiomyolipoma
EpiAML: Epithelial Angiomyolipoma



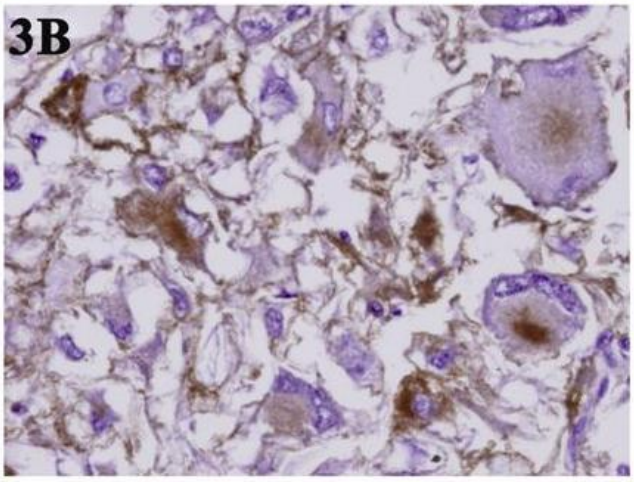
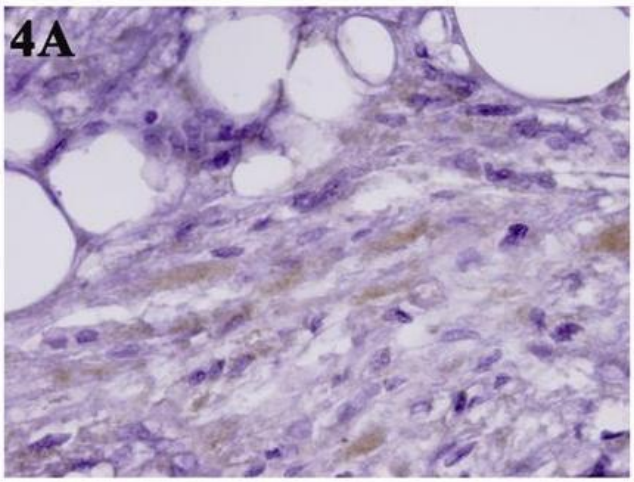


Figure 3. EGFR Immunostain in conventional angiomyolipoma and epithelial angiomyolipoma. **3A.** Conventional angiomyolipoma showing no EGFR expression. **3B.** An example of epithelial angiomyolipoma with EGFR expression.



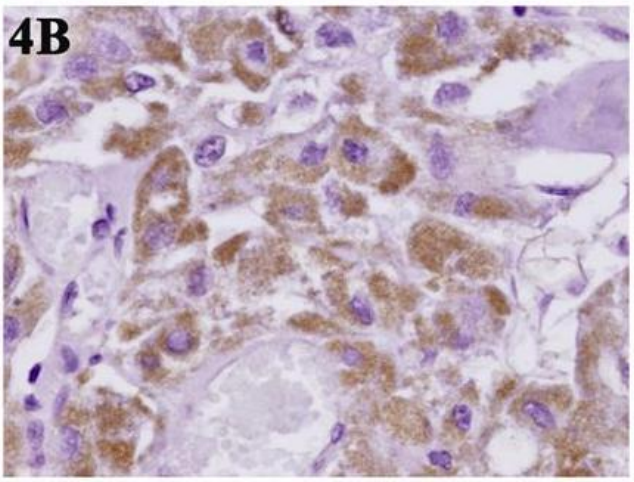


Figure 4. Osteopontin immunostain in conventional angiomyolipoma and epithelial angiomyolipoma. **4A.** Conventional angiomyolipoma with focal osteopontin expression. **4B.** An example of epithelial angiomyolipoma with diffuse osteopontin expression.

To evaluate a possible mechanism responsible for our finding of increased EGFR protein expression, we examined EGFR gene copy number by FISH in both epiAML and cAML. Most cells in both EpiAML and cAML showed 2 copies of EGFR per cell. The average ratio of EGFR copy number to the number of chromosome 7 in cAML and in epiAML were 1.085:1, and 1.003:1 respectively, indicating that EGFR genes were not amplified in either cAML or epiAML.

Discussion

Angiomyolipoma is the most common mesenchymal tumor of kidney. It is composed of variable proportions of thick-walled blood vessels, adipose tissue, and smooth muscle-like cells[1]. The smooth muscle-like cells of AML are believed to arise from the perivascular epithelioid cells. Tumors with predominant perivascular epithelioid cell components arranged around vascular spaces are known as PEComas.[1,2] This group of tumors includes angiomyolipoma, lymphangioleiomyomatosis, clear cell 'sugar' tumor of the lung, and a group of rare, morphologically and immunophenotypically similar lesions arising at a variety of visceral and soft tissue sites. The coexpression of myogenic (muscle specific actin and smooth muscle actin) and melanogenic (HMB-45, melan-A, and tyrosinase) markers is the distinctive feature of PEComas.[1,2]

In cAML, the smooth muscle-like cells form mainly fascicles of spindle cells. EpiAML is a recently described variant of AML[3] in which the smooth muscle-like cells are characterized by a predominantly epithelioid morphology. These epithelioid cells are reactive with HMB-45 and smooth muscle actin antibodies. EpiAML potentially display aggressive behavior.[5,7,23] Currently, the only acceptable criterion for malignancy in AML is distant metastases (mainly lung and liver). To date, 26 cases of renal malignant AML have been reported, 2 of which do not have epithelioid cells.[7,23]

EGFR is a cellular transmembrane receptor with tyrosine kinase activity.[8,10] The EGFR gene is located on the short arm of chromosome 7 and encodes a 170 kDa transmembrane protein consisting of an extracellular EGF-binding domain, a short transmembrane region, and an intracellular domain with ligand-activated tyrosine kinase activity.[9,10] Two ligands can activate EGFR: epidermal growth factor (EGF) and transforming growth factor-alpha. EGFR protein overexpression has been reported to occur in different human cancers, including glioblastoma multiforme, non-small cell carcinoma of lung, adenocarcinoma of colon, and small percentage of breast cancers.[8,10] Because EGFR-dependent signaling plays an important role in cancer cell proliferation, apoptosis, angiogenesis, invasion and metastasis[9,10], EGFR has been investigated extensively as a potential molecular target for cancer therapies. The use of EGFR kinase inhibitors including gefitinib has received FDA approval for use in cancer therapy.[8] In our study, epiAML expressed significantly higher levels of EGFR than cAML, suggesting a link between EGFR and the aggressive behavior of epiAML. The result also raises a possibility that EGFR-targeted therapy may be potentially beneficial for epiAML patients with metastatic disease.

We have shown that despite a marked increase in EGFR immunostaining in epiAML, a corresponding increase in EGFR gene copy number was not identified by FISH. Similar to our observations, Bhargava *et al*., found that EGFR gene amplification was an infrequent event in breast cancer, occurring in only 6% of breast carcinomas with over-expressed EGFR proteins.[24] Similar findings have been reported in squamous cell carcinoma of the head and neck and phyllodes tumor of the prostate.[25,26] These findings indicate that mechanisms other than gene amplification, such as post-translational regulation, may be responsible for EGFR protein over-expression. However these potential mechanisms have yet to be characterized.

Various p53 mutations have been reported in malignant tumors, and these mutations are considered important for tumorigenesis and tumor progression.[12] Kawaguchi and colleagues recently reported a case of renal epiAML associated with p53 overexpression and a missense p53 mutation in the epithelioid cell population, and suggested that p53 mutation plays an important role in the malignant transformation of renal epiAML.[23] However, in our study, p53 expression was demonstrated only focally in both cAML (33% of cases) and epiAML (30% of cases). This result does not support the hypothesis that p53 plays an important role in the tumorigenesis of epiAML. Similar to our findings, Park *et al.* reported that a p53-positive uterine PEComa does not harbor p53 gene mutation.[27] Genetic study performed by Martignoni *et al* on 3 cases of renal epiAML showed a loss of heterozygosity at the TSC2-containing region on 16p in one case, and on 3p in two cases, suggesting that multiple genetic alterations are taking place in these tumors. Additional studies in a larger population of patients, particularly those having epiAML, may help to further elucidate the role of p53 and loss of heterozygosity in the malignant transformation of AML.

C-Kit is a transmembrane signaling transduction receptor tyrosine kinase.[14] Expression of c-KIT in AML has only rarely been studied. C-Kit expression in AML was reported as 100%[15] and 79%[28] respectively. In our study, c-Kit expression was detected in only 33.3% of cAML and 60% of epiAML, and no statistically significant difference was identified between cAML and epiAML. A more recent report[15] proposed that the tyrosine kinase inhibitor imatinib might be of therapeutic value in cases of epiAML that exhibit significant c-Kit positivity, however current evidence is lacking.

Beta-catenin is a 92 kDa protein with important functions in the E-cadherin-mediated cell adhesion system and also acts as a downstream signaling molecule in the Wnt pathway.[16,17] Normally, beta-catenin is found in the cell membrane, where it is believed to be pro-apoptotic. However when beta-catinin is over-expressed and translocated into the nucleus, the cells acquire resistance to apoptosis.[16] It has been found that beta-catenin plays a key role in the development of colorectal cancer and is mutated in colorectal cancer cell lines.[29] Beta-catenin aberration has also been linked to the pathogenesis of other tumors, including prostate cancer, hepatocellular carcinoma, ovarian carcinoma, medulloblastoma, as well as melanoma.[16,20] The role of beta-catenin was not investigated in AML until recently. In 2005, Mak *et al*. showed that beta-catenin and its effectors were up-regulated in tuberous sclerosis complex (TSC)-related angiomyolipoma, indicating

that beta-catenin signaling may play a role in TSC pathogenesis.[30] In keeping with these findings, our study also showed that beta-catenin expression was present in 50% of epiAML and 16.7% of cAML, however a larger sample size will be needed to further clarify the statistical significance of beta-catenin expression between these two types of AML.

Alpha-catenin is an actin-binding protein[31] that attaches microfilaments and associated proteins to trans-membranous cell adhesion molecules via beta-catenin. Mutations involving alpha-catenin genes are known to produce proteins that break important links between cell adhesion molecules and components of the cell signaling Wnt pathway.[29] Disruption of these adhesions releases beta-catenin into the nucleus to switch on genes, leaving tumor cells free to invade.[16,31] So far there is no report investigating the relationship between alpha-catenin and AML. Immunostaining for alpha-catenin in the current study found 40% of diffuse reactivity and 50% of focal reactivity of alpha-catenin in epiAML compared with 0% diffuse reactivity and 50% of focal reactivity in cAML. Involvement of alpha-catenin in Osteopontin is an alpha(v)beta3 integrin adhesion molecule[21] whose overexpression in tumors appears to enhance dissemination.[21] Here we report for the first time that osteopontin is present in both cAML and epiAML, with 50% of epiAML showing diffuse cytoplasmic staining in contrast to no diffuse reactivity in cAML. The findings imply a possible link between osteopontin and tumor dissemination of epiAML.

In summary, we have shown that epiAML express significantly higher levels of EGFR than their less aggressive counterparts cAML. These differences may explain the differences in clinical behavior between these two types. In addition, it is possible that expression of EGFR and/or other proteins in epiAML may become candidate molecules for targeted therapy. More studies on the mechanism of EGFR overexpression might provide additional insight into the biologic behavior of this aggressive variant of AML.

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