Int J Clin Exp Pathol 2015;8(8):9446-9451
www.ijcep.com /ISSN:1936-2625/IJCEP0011861

Original Article

Immunohistochemistry of p53 and Ki-67 and *p53* mutation analysis in renal epithelioid angiomyolipoma

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Received June 24, 2015; Accepted July 27, 2015; Epub August 1, 2015; Published August 15, 2015

Abstract: Backgound: Renal epithelioid angiomyolipoma (EAML) is a rare variant of AML (angiomyolipoma) and is often associated with aggressive behaviors. The pathogenesis of EAML has been poorly understood. We analyzed the expression of p53 and Ki-67 by immunohistochemistry (IHC) and investigated p53 mutation analysis in 11 cases of EAML in comparison to classical AML. Methods: P53 and Ki-67 expression status were determined by IHC staining. P53 mutation analysis was performed using bi-directional sequencing. Results: Renal EAML tumors were significantly associated with more severe to moderate nuclear atypia (100% vs. 36.4%, $P = 0.004$) and mitotic activity (90.9% vs. 27.3%, $P = 0.008$) compared with AML tumors. Out of 11 cases of EAML, 8 were positive for p53. There was only 1 case with positive p53 expression in AML cases and expression of p53 protein showed significant difference between EAML and AML tumors (72.7% vs. 9.1%, $P = 0.008$). In addition, there were 7 AML and 6 EAML cases harbored P72R mutation (SNP) in exon 4 of p53. Compared with AML cases, 2 out of 11 cases of EMAL showed more than 10% positivity for ki-67. The finding of stronger p53 expression in renal EAML might have contributed to their malignant behavior. However, the abnormal p53 expression cannot be entirely explained by p53 mutations in the exons examined. Conclusions: Thus, the combination of immunohistochemical assessment of tumor antigens might improve our ability to predict the malignant outcome in EAML.

Keywords: EAML, p53, Ki-67, immunohistochemistry, mutation analysis

Introduction

Renal angiomyolipoma (AML) is a mesenchymal neoplasm composed of a variable proportion of adipose tissue, abnormal thick-walled blood vessels and spindle and epithelioid smooth-muscle like cells [1, 2]. Recent studies have documented that epithelioid angiomyolipoma (EAML), a rare variant of AML, shows local recurrence and/or distant metastases. The most prominent character of EAML is the large polygonal epithelioid cells instead of the spindle cells and these tumors always show large size and malignant phenotype, including sheet of necrosis and severe atypia [2-4]. However, the biological nature of this tumor is not fully understood because of its scarcity.

Inactivation of the tp53 (tumor p53) pathway by *tp53* mutations is one of the key genetic steps in many types of tumors [5]. *P53* missense mutations frequently lead to accumulation of abnormal p53 protein expression with the detection by immunohistochemistry (IHC) [6]. The p53 IHC staining and mutation analysis have been reported in a few case reports of AML, however, the results are conflicting [3, 7, 8]. In addition, clinical significance of Ki-67 as a proliferative cell and a prognostic marker has been investigated in human tumors [9], however, the expression of Ki-67 has not been systematically studied in AML. In the present study, we evaluated expression of p53 and Ki-67 by IHC and investigated *p53* mutation analysis in 11 cases of EAML in comparison to classical AML.

Methods

Study subjects

Eleven cases of renal EAML with corresponding paraffin-embedded material available for IHC and molecular analysis were retrospective-

Table 1. Primers used in this study

Prime	Primer Sequence 5'-3'
Exon4-F	GACCTGGTCCTCTGACTGCT
Exon4-R	GCATTGAAGTCTCATGGAAG
Exon5-F	TGTGCCCTGACTTTCAACTCT
Exon5-R	GGCAACCAGCCCTGTCGT
Exon6-F	GCTGGGGCTGGAGAGACGA
Exon6-R	CACCTGGAGGGCCACTGACAAC
Exon7-F	GGTCTCCCCAAGGCGCACTG
Exon7-R	GGGGATGTGATGAGAGGTGGAT
Exon8-F	GCCTCTTGCTTCTCTTTTCCTATC
Exon8-R	GGGAGAGGAGCTGGTGTTGTT
Exon9-F	AGCAGGACAAGAAGCGGTGG
Exon9-R	ACGGCATTTTGAGTGTTAGACTG

ly collected from the Department of Pathology, Cancer Hospital, Chinese Academy of Medical Sciences, Beijing, China from 2005 to 2012. For each case, hematoxylin and eosin stained sections as well as IHC stains for HMB-45, Melan-A, SMA and AE1/AE3, and CK18 were evaluated to confirm the original diagnosis. Another 11 cases of classic AML matched with sex, age and tumor size were selected for comparative study. The study was approved by the Institute Review Board of the Cancer Hospital, Chinese Academy of Medical Sciences (CAMS). Each participant signed an Institutional Review Board approved informed consent in accordance with current guidelines.

Immunohistochemistry of p53 and Ki-67

Immunohistochemistry was performed on 4-μm formalin-fixed, paraffin-embedded (FFPE) tissue sections using the fully automated Dako immunohistochemistry staining system (Autostainer Link 48, Dako, Denmark). Primary mouse monoclonal antibodies included p53 (DO-7, Dako) and Ki-67 (MIB-1, Dako).

The degree of immunoreactivity for p53 expression was evaluated semiquantitatively on the basis of staining intensity and the proportion of positive tumor cells. The staining intensity was graded as follows: 0 (no staining), 1 (light yellow), 2 (yellowish brown) and 3 (brown). The positive cells were graded according to the percentage of positive cells as follows: 0 (no positive tumor cells), 1 (< 10% positive tumor cells), 2 (11-50% positive tumor cells), 3 (51-80% positive tumor cells) and 4 (> 80% positive tumor cells). The percentage of positive cells and the staining intensity were then multiplied to generate the immunoreactivity score. Based on this score, the immunoreactivity was divided into three groups: negative immunoreactivity (a total score of 0), low immunoreactivity (a total score of 1-4), and high immunoreactivity (a total score of > 4).

The areas showing the representative Ki-67 expression in each section were chosen, and the minimum of 1,000 tumor cells were counted under light microscopic fields (× 400) using a computer-assisted image-analyzing system (Nuclear V9 from APERIO Company). The percentage of positive tumor cell nuclei was recorded as Ki-67 index. Cut-off value of 10% for Ki-67 was defined as high and low expression.

P53 mutation analysis

Assessment of *p53* mutational status was performed in the Molecular Pathology Laboratory of Department of Pathology, CAMS, using appropriate quality control procedures. Mutation status was determined using genomic DNA extracted from macrodissected formalin-fixed, paraffin-embedded tumor tissue. Genomic DNA was extracted using the QIAamp DNA Mini Tissue kit (Qiagen, Germany) following the manufacturer's standard protocol. *P53* mutation analysis of exons 4 to 9 was carried out using bi-directional Sanger sequencing analysis performed on an independent PCR reaction and primers were listed in **Table 1**. The sequencing was done on 3730XL with BigDye Taq FS Terminator version 3.1 with analysis done on an ABI Sequence Scanner version 1.0.

Statistical analysis

Differences of patient characteristics and clinicopathologic factors in the two-dimensional cross-comparison were evaluated statistically by Pearson's χ^2-test or Fischer's exact test. Statistical tests were two-sided, and $P < 0.05$ was considered significant. Statistics were carried out using SPSS software (version 16.0 of SPSS, Chicago, IL, USA).

Results

Clinicopathologic characteristics

The clinicopathologic characteristics of AML and EAML were summarized in **Table 2**. There were 11 patients with AML, including 3 men and 8 women and 11 patients with EAML, including 5 men and 6 women. Gender distribu-

Table 2. Clinicopathologic characteristics of EAML and AML

Characteristics	EAML (n = 11)	AML (n = 11)	*P*-value
Gender			0.66†
Male	5 (45.5%)	3 (27.3%)	
Female	6 (54.5%)	8 (72.7%)	
Nuclear atypia			0.004†
Severe-Moderate	11 (100%)	4 (36.4%)	
Mild-None	0 (0%)	7 (63.6%)	
Mitosis (/50 HPF)			0.008†
Positive	10 (90.9%)	3 (27.3%)	
Negative	1 (9.1%)	8 (72.7%)	
Necrosis			0.31†
Positive	4 (36.4%)	1 (9.1%)	
Negative	7 (63.6%)	10 (90.9%)	
Multinuclear giant cells			0.08†
Positive	7 (63.6%)	2 (18.2%)	
Negative	4 (36.4%)	9 (81.8%)	
Age, y			0.29§
Mean (SD)	43.4 ± 11.2	38.5 ± 9.6	
Median	46.0	38.0	
Range	25.0-58.0	25.0-57.0	
Tumor Size (cm)			0.87§
Mean (SD)	6.4 ± 4.87	6.72 ± 4.67	
Median	5.5	5.0	
Range	0.5-17.5	2.0-13.0	

Abbreviations: AML = Angiomyolipoma; EAML = Epithelioid Angiomyolipoma; HPF = High Power Fields. †Fischer's exact test. §Two-sided Kruskal Wallis test.

tion did not differ significantly between EAML and AML cases. The mean age at presentation for EAML patients was 43.4 ± 11.2 years, which showed no significantly difference to that for AML patients at 38.5 ± 9.6 years. In addition, tumor sizes did not vary between EAML and AML cases.

All the tumors were diagnosed according to the histological and immunohistochemical criteria in the current World Health Organization classification of renal tumors. All the cases expressed of one or two melanocytic markers (HMB-45, Melan-A), positive or negative for myoid markers (SMA) and negative immunoreaction for one or two epithelial markers (AE1/AE3, CK18). The clinicopathological features of EAML have been described in detail in another paper (unpublished data). EAML tumors were significantly associated with more severe to moderate nuclear atypia (100% *vs.* 36.4%, $P = 0.004$) and mitotic activity (90.9% *vs.* 27.3%, $P = 0.008$) compared with AML tumors (**Figure 1**). However, there were no significantly differences in necrosis and infiltration of multinuclear giant cells between EAML and AML tumors.

P53 and Ki-67 immunohistochemistry

Out of 11 cases of EAML, 8 were positive for p53 (**Figure 1**). There was only 1 case with positive p53 expression in AML cases and p53 expression demonstrated significant difference between EAML and AML tumors (72.7% *vs.* 9.1%, $P = 0.008$) (**Table 3**).

Ki-67 antigen labeling was localized to the nucleus with a fine brown granularity. Compared with AML tumors, the Ki-67 labeling index showed no significant difference with EAML tumors.

P53 mutation analysis

P53 mutation analysis was performed by bi-directional sequence analysis of exons 4 to 9 in 20 cases of EAML and AML tumors. One case for AML and 1 for EAML were not sequenced for mutation analysis because of poor DNA quality. We identified the P72R (c.215C > G) mutation in exon 4 of *p53* in both AML and EAML cases (**Figure 2**). There were 7 AML and 6 EAML cases harbored P72R mutation and we identified the SNP in additional normal tissues of 5 cases. Actually, the P72R mutation is at the site of a common SNP (single nucleotide polymorphisms), rs1042522 (minor allele frequency 23.3% in HapMap European ancestral samples (CEU)) and has not been previously reported as a somatic mutation. No additional mutation was identified in both cases.

Discussion

Angiomyolipoma is a type of tumor composed of epithelioid cells with clear and acidophilic cytoplasm, which belongs to the family of PEComas [2]. Pure epithelioid PEComa (so-called epithelioid variant of AML) is extremely rare and potentially malignant with metastasis in one third of cases in the literature. Although

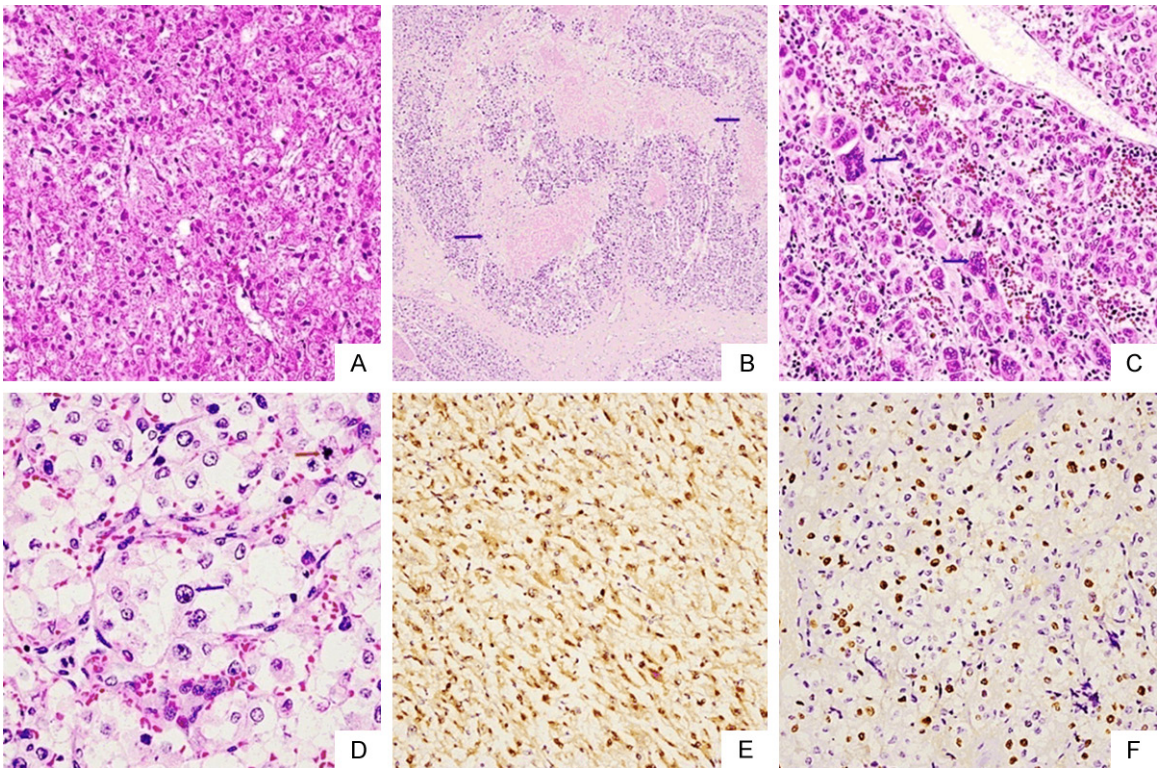

Figure 1. Histopathologic features of AML and EAML and the expression of P53 and Ki-67. A. AML (H&E, 200×), mild nuclear atypia and none nucleolus; B. EAML (H&E, 40×), blue array showed necrosis; C. EAML (H&E, 200×), blue array showed multinuclear giant cells; D. EAML, severe nuclear atypia, blue array showed obvious nucleolus and red array showed mitosis (H&E, 400×); E. Representative images of p53 expression (200×), positive expression in the nuclear; F. Representative images of Ki-67 index (200×), 30% in this case.

Table 3. Immunohistochemistry analysis of p53 and ki-67

Characteristics	EAML (n = 11)	AML (n = 11)	*P*-value
P53			0.008†
Positive	8 (72.7%)	1 (9.1%)	
Negative	3 (27.3%)	10 (90.9%)	
Ki-67			0.48†
≥ 10%	2 (18.2%)	0 (0%)	
< 10%	9 (81.8%)	11 (100%)	

Abbreviations: AML = Angiomyolipoma; EAML = Epithelioid Angiomyolipoma. †Fischer's exact test.

there were a lot of researches in the histopathologic features of EAML, the studies in molecular mechanism of this tumor in kidney were rare, only in case reports. Recent studies also suggested that p53 overexpression and mutations in EAML may indicate the malignant behavior of the tumor [10, 11]. To the best of our knowledge, this is the first study to investigate expression of p53 and Ki-67 by IHC and *p53* mutation analysis in a large series of Chinese patients.

The pathogenesis of EMAL is poorly understood. Mutation of *p53* gene has been involved in the tumorigenesis of multiple tumors [12]. *P53* functions as a transcription factor and can be activated in response to DNA damage, oncogene activation or hypoxia to induce apoptosis, cell-cycle arrest and modulation of autophagy [13-15]. There were several reports describing *p53* mutations in renal EAML. Direct sequencing identified 5 point mutations, 3 were silent mutation involving codon 154 [11], 218 [11] and 213 [8], and 2 were missense mutation involving codon 249 [7]. In our study, we investigated point mutations of exons 4 to 9 by bidirectional sequencing; however, there were no point mutations either in EAML cases or in AML cases except a SNP (P72R) in exon 4. A common genetic polymorphism occurs at codon 72 of human p53 with two alleles encoding either arginine (CGC) or proline (CCC). Most studies have identified that the polymorphism at codon

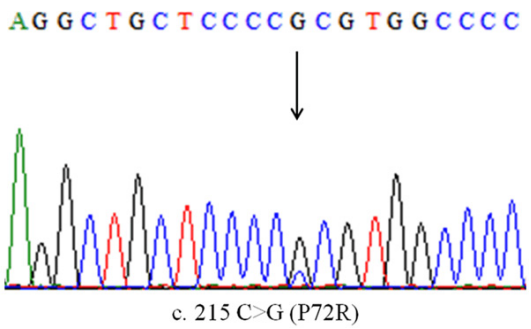

Figure 2. Detection of *p53* mutation in EAML and AML cases. P72R (c.215C > G) mutation in exon 4 of *p53*.

72 was associated with elevated risk of cervical [16], lung [17], breast [18], gastric [19] and thyroid cancer [20]. However, it has not been extensively studied in EAML. Our study has revealed that the P72R SNP was not associated with aggressive behavior of epithelioid angiomyolipoma.

Generally, the protein product of the normal *p53* gene has a very short half-life, which has hardly been detected in tissues. The product of the mutant gene is more stable and can be easily detected by IHC methods [6]. However, recent studies suggested that high levels of p53 protein are frequently detected in the absence of *p53* mutation in some types of tumors [21-23]. In addition, Chemeris and colleagues have also reported that 29 RCC (Renal cell cancer) samples were all positive for p53 protein expression while none of them had the *p53* point mutation [24]. Consequently, it is reasonable that p53 protein expression could be detected in the absence of *p53* mutation. Although *p53* mutation has been described in some case reports of renal EAML, no p53 mutation was found in two other cases reported by Ma et al. [8], Sato et al. [25] and our study. Thus, it seems possible that different mechanisms other than direct *p53* mutation may inactivate *p53* in renal EAML which may result in higher protein expression.

Ki-67 is considered to be an index of the proliferative activity of tumors [26]. Three other studies have reported the immune-reactivity of Ki-67 in EAML. Chandrasoma *et al.* found that greater than 50% of malignant EAML cells were positive for Ki-67 [27]. Another two cases of renal EAMLs also had greater than 10% positivity for Ki-67 and were negative for classic AML [28, 29]. This was in accordance with our results that 2 out of 11 cases of EMAL showed more than 10% positivity for Ki-67 index. However, there was no significant difference in Ki-67 index between AML and EAML cases due to small sample size in our study.

Conclusions

In summary, we found that EAML usually showed stronger p53 expression when compared with classic AMLs. Although no mutation in EAML was identified in our study, the stronger immunohistochemical positivity for p53 in majority of EAML cases might be attributed to different mechanisms other than *p53* mutations. Further studies need to investigate the alternative mutational events that might contribute to the abnormal p53 expression. And the combination of immunohistochemical assessment of tumor antigens might improve our ability to predict the malignant outcome in EAML.

Acknowledgements

We thank all study participants of the Department of Pathology for their contributions to this project. This work was supported by grants from Beijing hope run special fund (LC2011B35) and Basic scientific research fund of China central public welfare (JK2014B06 and 2013-JKB12).

Disclosure of conflict of interest

None.

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