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The oncogenic mutation in the pleckstrin homology domain of AKT1 in endometrial carcinomas

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BACKGROUND: The phosphatidylinositol 3′-kinase (PI3K) – AKT pathway is activated in many human cancers and plays a key role in cell proliferation and survival. A mutation (E17K) in the pleckstrin homology domain of the *AKT1* results in constitutive AKT1 activation by means of localisation to the plasma membrane. The *AKT1* (E17K) mutation has been reported in some tumour types (breast, colorectal, ovarian and lung cancers), and it is of interest which tumour types other than those possess the *E17K* mutation.

METHODS: We analysed the presence of the *AKT1* (E17K) mutation in 89 endometrial cancer tissue specimens and in 12 endometrial cancer cell lines by PCR and direct sequencing.

RESULTS: We detected two *AKT1* (E17K) mutations in the tissue samples (2 out of 89) and no mutations in the cell lines. These two *AKT1* mutant tumours do not possess any mutations in *PIK3CA*, *PTEN* and *K-Ras*.

INTERPRETATION: Our results and earlier reports suggest that *AKT1* mutations might be mutually exclusive with other PI3K – AKT-activating alterations, although *PIK3CA* mutations frequently coexist with other alterations (such as *HER2*, *K-Ras* and *PTEN*) in several types of tumours.

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The AKT serine/threonine kinases regulate diverse cellular processes, including cell survival, proliferation, invasion and metabolism (Vivanco and Sawyers, 2002). The phosphatidylinositol 3′-kinases (PI3Ks) are widely expressed lipid kinases that catalyse the production of the second messenger phosphatidylinositol 3,4,5-triphosphate (PIP3), which activates AKT by recruitment to the plasma membrane through direct contact of its pleckstrin homology (PH) domain (Stokoe *et al*, 1997; Lemmon and Ferguson, 2000). Constitutive PI3K – AKT pathway activation can result from various types of alterations in this pathway, including mutation or amplification of receptor tyrosine kinases (such as *EGFR* and *HER2*), mutation of *Ras*, mutation or amplification of *PIK3CA* (the p110α catalytic subunit of PI3K) and inactivation of the tumour suppressor gene, *PTEN* (Yuan and Cantley, 2008). In addition to amplifications in multiple AKT isoforms in pancreatic, ovarian and head and neck cancers (Engelman *et al*, 2006), a somatic missense mutation in the PH domain of *AKT1* (E17K) was identified in breast, colorectal, ovarian and lung cancers and in melanoma (Carpten *et al*, 2007; Bleeker *et al*, 2008; Davies *et al*, 2008; Malanga *et al*, 2008). However, the *AKT1* mutation has not been identified in hepatocellular, gastric and pancreatic cancers, leukemia, as well as in glioblastoma multiforme (Bleeker *et al*, 2008; Cao *et al*, 2008; Kim *et al*, 2008; Mahmoud *et al*, 2008; Mohamedali *et al*, 2008; Riener *et al*, 2008; Zenz *et al*, 2008). Further study is required to fully understand which tumour types take advantage of *Akt1* (E17K) mutations to activate the PI3K – AKT pathway.

We reported earlier that *PIK3CA* mutations frequently coexist with other PI3K-activating alterations in breast (with *HER2* and *HER3*) and endometrial cancers (with *PTEN* and *K-Ras*), and that mutant p110α combined with mutant Ras efficiently transformed immortalised human mammary epithelial cells (Oda *et al*, 2005, 2008). Frequent overlapping mutations of *K-Ras* and *PIK3CA* were also reported in colorectal cancer (Parsons *et al*, 2005). Although coexistent mutations of *AKT1* and *PIK3CA* mutations are suggested to be infrequent in breast cancer (Carpten *et al*, 2007; Bleeker *et al*, 2008), it remains to be elucidated whether *AKT1* mutations are mutually exclusive with all the other PI3K – AKT-activating alterations in various tumour types.

Endometrial cancer is one of the tumour types in which the PI3K – AKT pathway is frequently activated by alterations of various genes. The frequency of mutations for *PTEN*, *PIK3CA* and *K-Ras* in endometrial cancer is reported as 54, 28 and 11%, respectively (Yuan and Cantley, 2008). In this study, we screened 89 endometrial carcinoma specimens and 12 endometrial carcinoma cell lines for mutations in *Akt1* (E17K) and analysed whether *AKT1* mutations coexist with any mutations in *PTEN*, *PIK3CA* and *K-Ras*.

MATERIALS AND METHODS

Tumour samples and genomic DNA

Surgical samples were obtained from 89 patients with primary endometrial carcinomas who underwent resection of their tumours

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at the University of Tokyo Hospital. All patients provided informed consent for the research use of their samples and the collection, and the use of tissues for this study was approved by the appropriate institutional ethics committees. Genomic DNA was extracted by a standard SDS-proteinase K procedure. Patient characteristics (histology, tumour grade and stage) are available in Supplementary Table 1. A detailed distribution of the histological subtypes was as follows; 81 (90%) endometrioid adenocarcinomas, 3 adenosquamous carcinomas, 1 clear cell carcinoma, 1 squamous cell carcinoma and 3 mixed carcinomas.

PCR and sequencing

The primer sequences and PCR conditions of exon 4 of the *AKT1* gene are forward: 5′-CACACCCAGTTCCTGCCT G-3′ and reverse: 5′-CCTGGTGGGCAAAGAGGGCT-3′. The PCR amplifications were with denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 60 s and final extension at 72°C for 10 min. The PCR conditions and the PCR primers for *PIK3CA* (exons 9 and 20), *PTEN* (exons 1–9) and *K-Ras* (exons 1 and 2) were described earlier (Minaguchi *et al*, 2001; Samuels *et al*, 2004; Oda *et al*, 2008). The PCR products were sequenced using the BigDye (Applied Biosystems, Foster City, CA, USA) terminator method on an autosequencer.

Cell lines

In this study, AN3CA, KLE, HEC-1B and RL95-2 were obtained from the American Type Culture Collection (Manassas, VA, USA) and HHUA was obtained from the RIKEN CELL BANK (Tsukuba, Japan). Ishikawa3-H-12 was a generous gift from Dr Masato Nishida (Kasumigaura Medical Center, Ibaraki, Japan). HEC-6, HEC-50B, HEC-59, HEC-88, HEC-108 and HEC-116 cell lines were also analysed in this study. The culture condition of all these cell lines was described earlier (Oda *et al*, 2008).

DNA methylation analysis

Bisulphite treatment was performed using the EZ DNA methylation kit (Zymo Research, Orange, CA, USA). As described earlier (Ehrich *et al*, 2006), we used Sequenom's MassARRAY platform to perform quantitative methylation analysis of multiple CpG sites for *PTEN* in 53 endometrial tumour specimens (Sequenom, San Diego, CA, USA). Chromosomal localisation of CpG islands for *PTEN* and the primer sequences in this study are shown in Supplementary Figure 1.

Immunohistochemistry (IHC)

Immunohistochemistry for PTEN on 4-μm tissue sections was performed and evaluated as described earlier (Minaguchi *et al*, 2007). In this study, the anti-PTEN Rabbit monoclonal antibody (138G6) (Cell Signaling, Beverly, MA, USA) was applied at a dilution of 1 : 100.

Single nucleotide polymorphism (SNP) array

Single nucleotide polymorphism array was performed in the two *AKT1* mutant tumours with tumour DNA. Experimental procedures for GeneChip were performed according to GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA, USA), using a Human mapping 50K Array Xba I (Affymetrix).

RESULTS AND DISCUSSIONS

The sequencing analysis for exon 4 of the *AKT1* gene in 89 tumour tissue samples of endometrial carcinomas showed the point mutation of G to A at nucleotide 49 (E17K) in two tissue samples (2.2%) (Figure 1). Both of the tumours were well-differentiated endometrioid adenocarcinomas with positive oestrogen receptor and progesterone receptor, suggesting that these two tumours are oestrogen dependent (corresponded to type I endometrial cancer). No mutations were detected in the 12 endometrial cancer cell lines.

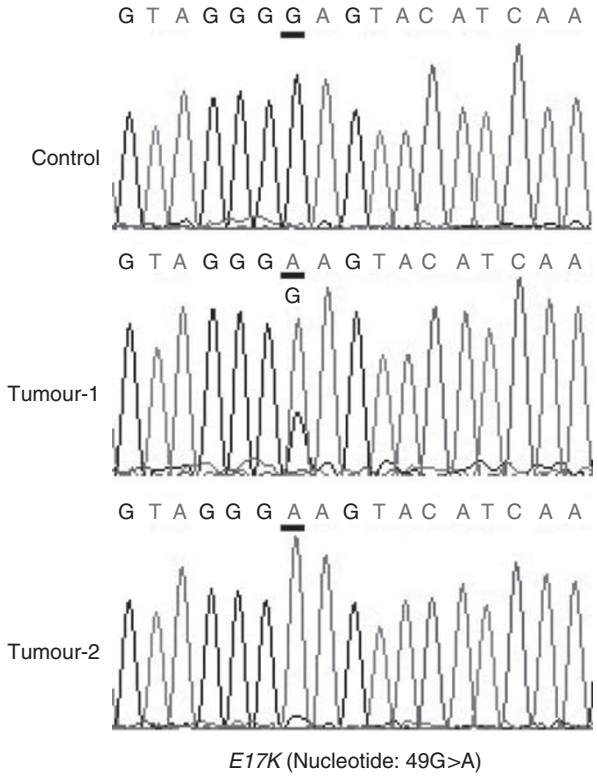

Figure 1 The sequence traces of two tumours and a normal control for exon 4 of *AKT1*. The *E17K* mutation is caused by a missense mutation (G to A) indicated. In tumour-2, the level of the mutant band (A) is much higher than that of the wild-type band (G). It is possible that this weak band is derived from DNA of normal cells and that the tumour might lose one allele at this locus.

Table 1 PI3K–AKT activating mutations and their coexistence in 97 endometrial cancers

	n **(%)**
Wild-type	24 (25)
AKT1 mutation alone	2 (2)
K-Ras mutation alone	4 (4)
PIK3CA mutation alone	6 (6)
PTEN mutation alone	30 (31)
Double mutations of *K-Ras* and *PIK3CA* (w/o *PTEN* mutation)	2 (2)
Double mutations of *K-Ras* and *PTEN* (w/o *PIK3CA* mutation)	3 (3)
Double mutations of *PIK3CA* and *PTEN* (w/o *K-Ras* mutation)	18 (19)
Triple mutations of *K-Ras*, *PIK3CA* and *PTEN*	8 (8)

PI3K, phosphatidylinositol 3′-kinase. Wild-type, no mutations in *PTEN*, *PIK3CA*, *K-Ras* and *AKT1*.

Thereafter, we attempted to figure out the exclusivity of *AKT1* mutations and other PI3K–AKT-activating mutations (Supplementary Table 1). The genotypic pattern of the four genes (*PTEN*, *PIK3CA*, *K-Ras* and *AKT1*) in 97 endometrial carcinomas (85 tumour tissue samples and 12 cell lines) was shown in Table 1. Coexistence with other mutations is frequently observed in the *PIK3CA* mutant (28 of 34; 82%) and in the *K-Ras* mutant (13 out of 17; 76%) tumours, but the two *AKT1* mutant tumours do not possess any mutations in *PTEN*, *PIK3CA* and *K-Ras*. As PI3K and

PTEN are competitive for PIP3 production, the *PIK3CA* mutation might require another upstream input or PTEN loss itself to fully activate the PI3K–AKT pathway. As AKT1 (E17K) functions downstream of PTEN and shows constitutive localisation to the plasma membrane in the absence of serum stimulation (Carpten *et al*, 2007), mutant *AKT1* (E17K) alone might be sufficient for complete activation of this pathway.

We also analysed DNA methylation and protein expression of PTEN, as hypermethylation and loss of heterozygosity (LOH) are other mechanisms to inactivate PTEN (Teng *et al*, 1997; Blanco-Aparicio *et al*, 2007). Quantitative analysis of DNA methylation using Sequenom's MassARRAY platform did not find promoter hypermethylation of *PTEN* in all the 53 samples that were examined (Supplementary Figure 2 and Supplementary Table 2), including the two *AKT1* mutant tumours. Although *PTEN* methylation had been reported in 18% of endometrial carcinomas (Salvesen *et al*, 2001), Zysman *et al* (2002) suggested that the pseudogene on chromosome 9 (Genbank accession number: AF040103), not *PTEN*, is predominantly methylated in endometrial carcinomas. In IHC, both tumours with the *AKT1* mutation were stained positively for PTEN in the cytoplasm, whereas all the four tumours with multiple frameshift mutations in *PTEN* were stained negatively (Supplementary Figure 3). We evaluated the chromosomal imbalances in the two *AKT1* mutant tumours, using SNP array (with more than 50 000 SNPs). Single nucleotide polymorphism array analysis showed that the two *AKT1* mutant tumours do not show copy number changes in the locus of *PTEN* (10q23.1) (data not shown). These data also support the fact that *AKT1* mutations are mutually exclusive with PTEN inactivation.

We found multiple *PTEN* mutations in 13 out of 85 clinical specimens and in 8 out of 12 endometrial cell lines (Supplementary Table 1), whereas LOH of *PTEN* was reported approximately at 30% in endometrial carcinomas (Toda *et al*, 2001). Thus, biallelic *PTEN* inactivation might be achieved through either biallelic mutations or monoallelic mutation with LOH in endometrial carcinomas. Considering the correlation between *PTEN* mutations and microsatellite instability (MSI) in endometrial carcinomas (Bilbao *et al*, 2006), it would be of interest to analyse whether *AKT1* and the other mutations in the PI3K pathway genes are also associated with MSI.

To date, *AKT1* (E17K) mutations have been reported in breast (25 out of 427; 5.9%), colorectal (4 out of 243; 1.6%), lung (4 out of 636; 0.6%) and ovarian cancers (1 out of 130; 0.8%) and in melanoma (1 out of 202; 0.5%). Breast, colorectal and endometrial cancers are the tumour types that frequently possess *PIK3CA* mutations (Campbell *et al*, 2004; Samuels *et al*, 2004; Oda *et al*, 2005). In lung cancer, the *AKT1* mutation was detected only in squamous cell carcinomas and not in any adenocarcinomas, which is in agreement with the higher incidence of *PIK3CA* mutations or amplifications in squamous cell carcinomas than adenocarcinomas (Kawano *et al*, 2006, 2007; Malanga *et al*, 2008). These data suggest that the *AKT1* mutation might occur in a tissue-specific manner and is more associated with the tumour types with frequent *PIK3CA* alterations.

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