A Hot-spot of In-frame Duplications Activates the Oncoprotein AKT1 in Juvenile Granulosa Cell Tumors

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Original Article

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1. Introduction

Sex cord stromal tumors involve granulosa, theca or stromal cells, alone or combined. The most common ones are ovarian granulosa cell tumors (GCTs), which represent up to 5–8% of all ovarian tumors (Pectasides et al., 2008; Young and Scully, 1992). Two distinct subtypes have been described based on clinical presentation and histology: the juvenile and the adult forms. Five percent of GCTs occur in the prepubertal period and are often uncovered by a precocious pseudo-puberty and/or dysmenorrhea (Fleming et al., 2010). Although advanced-stage disease can be observed, most of the juvenile GCTs (JGCTs) are detectable at an early stage but recurrence and metastases are possible (Kalfa et al., 2007). The adult form (AGCTs) most commonly appears during the perimenopausal period. AGCTs are characterized by a tendency to late recurrence (Pectasides et al., 2008). The pathophysiological mechanisms underlying GCTs are still unclear. However, a recurring somatic mutation has been identified in the sequence of FOXL2, which encodes a transcription factor, in more than 95% of AGCTs (Shah et al., 2009). This mutation perturbs TGF-beta signaling in granulosa cells (Rosario et al., 2012). With regard to JGCTs, in a previous study we showed that FOXL2 expression was absent or reduced in the granulosa cells of a number of patients. Interestingly, the patients with absent/reduced FOXL2 expression displayed higher mitotic activity in the tumor and more advanced oncological stages. Furthermore, all recurring tumors displayed extinction of FOXL2 (Kalfa et al., 2007). In another study, we reported the occurrence of the activating somatic mutations...
R201C and R201H of G0s in one third of the analyzed JGCTs. This mutation may lead to a constitutive activation of mitogenic FSHR signaling in the latter (Kalfa et al., 2006). FSHR signaling also activates the oncoprotein AKT (Hunzicker-Dunn et al., 2012) that phosphorylates and inhibits transcription factors such as those belonging to the Forkhead box protein 0 family (FOXO) (Telikicherla et al., 2011). Because not only FSH but other mitogens such as IGFs also signal through PI3K and AKT in granulosa cells (Hunzicker-Dunn et al., 2012; Baumgarten et al., 2014; Reynaud et al., 2010), we hypothesized that alterations of this pathway might be involved in the molecular etiology of JGCTs. Thus, we searched for mutations in the genes encoding these proteins in a cohort of 16 tumors occurring in girls under 15 years of age. Recently, mutations in PIK3CA, which encodes the catalytic subunit of PI3K, were identified in various human cancers. Since 80% of these mutations cluster within exons 9, 18 and 20, we focused on them and found no alterations. With regard to AKT, we show here that more than 60% of the rest of the tumors often display several potentially damaging point mutations. Our study points to AKT1 as a major driver in the pathogenesis of JGCTs.

2. Materials and Methods

2.1. Patients

This study involves a cohort of 16 histologically proven JGCTs, occurring in girls under 15 years of age, collected between 1994 and 2014 (from the Necker-Enfants Malades Hospital, Paris and the University Hospital Montpellier tumor repositories). The available clinical data (from the Necker-Enfants Malades Hospital, Paris and the University Hospital Montpellier tumor repositories). The available clinical data

2.2. Nucleic Acid Extraction and Sequencing

Twelve samples were formalin-fixed paraffin-embedded (FFPE) (T1–T12). Four tumors were obtained as frozen samples (T13–T16). We isolated genomic DNA and RNA from FFPE tumors using the AllPrep DNA/RNA FFPE Kit (Qiagen) and the frozen ones were processed using standard procedures. To assess the somatic status of the mutations, we extracted DNA from manually isolated peritumoral tissue fragments (only available for 8 samples). We also analyzed the COV434 cell line, supposed to derive from a JGCT (Van den Berg-Bakker et al., 1993). Sequencing of exon 8 of the G0s gene, potentially harboring the activating mutations R201C and R201H, was performed as described (Kalfa et al., 2006). Exons 9, 18 and 20 of the PIK3CA (Li et al., 2005) and all of the exons of AKT1 were amplified from gDNA using the primers described below. For nested/semi-nested PCR's, when required, we used the primers F2 and/or R2 from the list below. Sanger sequencing was performed by MWG-Biotech AG according to their in-house procedures.

2.4. AKT1 Expression Constructs

The plasmids driving the expression of wild-type and mutated AKT1 fused to the mCherry protein were constructed by fusion PCR. Briefly, for the insertion mutations, two PCRs were performed to generate the 5’ and 3’ portions of the AKT1 coding sequence using, respectively, AKT1RED–EcorI–F primer and the corresponding mutagenic R primer and AKT1–F2 primer along with AKT1RED–BamHI–R. After purification of the PCR products, they were quantified, mixed in similar amounts and allowed to undergo eight cycles of PCR in the absence of primers, to generate the full-length mutated coding regions. Then, a final PCR reaction was performed using the EcoR1–BamHI primers. For E17K, we used the primers E17K F and AKT1RED–BamHI–R to generate the amplicon in a single PCR. The amplified EcoR1–BamHI were cloned (EcoR1–BamHI) into digested pDsRed vector to produce fusion proteins in frame with the mCherry. All constructs were sequenced to exclude the presence of PCR-induced mutations. The sequences of the primers used are the following:

1. Exon1-F1: 5′TGGCTTACTACATCGCTTCCCTT3′, Exon1-F2: 5′AGCGCCAGCCTAGAGGAGAAG3′,
2. Exon1-R1: 5′AGGGCAAGGCACCTCAGCAG3′,
3. Exon2-F1: 5′TGTCCTCGCAACCACTGCCAG3′, Exon2-F2: 5′AGGGTCTGACCCGTCCTAAG3′,
4. Exon2-R1: 5′ATGCACGCAGACAGAGGCT3′, Exon2-R2: 5′CAGCCAGCAGGACAGT3′,
5. Exon3-F1: 5′ATGCCTGAAGGGCGCGCTA3′, Exon3-F2: 5′AGGGTCTGACCCGTCCTAAG3′,
6. Exon3-R1: 5′GTGGGGTGGAGC3′, Exon3-R2: 5′GTGGGGTGGAGC3′,
7. Exon4-F1: 5′TGACCCAGGACCCCTCCTG3′, Exon4-F2: 5′TGGAAGAGAGTGGGGTGACCCG3′,
8. Exon4-R1: 5′TTTCTTACCCGCTGCTG3′, Exon4-R2: 5′AGGAAGGGGTGCTTCCGAG3′,
9. Exon5-F1: 5′CAGCAGCAGGACAGT3′,
10. Exon5-R1: 5′AGGAAGGGGTGCTTCCGAG3′,
11. Exon6-F1: 5′TTTCTTACCCGCTGCTG3′, Exon6-R1: 5′TTAAGCCCTACACATGACCAAA3′,
12. Exon6-R2: 5′AGGCAGCTGCTGCTG3′,
13. Exon7-F1: 5′TCAAGGCACTGGATATCAAG3′, Exon7-R1: 5′CCTTAACTCAAGGACAGAAGC3′,
14. Exon7-R2: 5′ACAGGCGACAAGTGCTAC3′,
15. Exon8-F1: 5′CACGGCTTGCCCTACAGGTT3′, Exon8-R1: 5′GTGATCTTCTAGTCCGCTGAGT3′,
16. Exon8-R2: 5′GTGGGGTGGAGC3′,
17. Exon9-F1: 5′ACTGACCTGAGGCCACCATCTT3′, Exon9-R1: 5′AGGATTGCGTGTCCCTCGAGA3′,
18. Exon9-R2: 5′GACGGAGACACTGATG3′,
19. Exon10-F1: 5′GCCAGCTCTCTCCATCTC3′, Exon10-R2: 5′AGGTTCGCTGGAGACAATTGACT3′,
20. Exon11-F1: 5′CGACACTGTGCGCTTGGTCTCCTT3′, Exon11-R1: 5′CTGATACTAGCTCTCCGCTCGAGA3′,
21. Exon11-R2: 5′ATGCCCTGCGTGGAGACAATTGACT3′,
22. Exon12-F1: 5′AAGCTCATGACTGTCCCTG3′, Exon12-R1: 5′ACTGCCCTCCACCTGATCATT3′,
23. Exon12-R2: 5′CTCTCTGAGTGTTGAGAGAAGGAG3′,
24. Exon13-F1: 5′GTGTGGCTCTTACTGAGGTCTG3′, Exon13-R2: 5′TGAGGTGAGCGAGGAGT3′,
25. Exon13-R1: 5′CTCTCTCCTCCTACAGGATGTT3′, Exon13-R2: 5′TGAGGTGAGCGAGGAGT3′,
26. gDNA-F1: 5′ATGCAAGGGGACACAGGAG3′, gDNA-R1: 5′CATGTCCTCCCTGGTGCAAG3′,
27. cDNA-F2: 5′CACCAAGATGGCAGGACAGGTA3′, cDNA-R2: 5′CTGGGATGGGATGGAGAG3′.
AKT1 RED – EcoR1-F: 5′AGCTTCCATTTGCGACCATCCGGCAGCTGGCTAATTGCTAGAC3′
AKT1-F2: GTGGACACTGTACCCG
AKT1 RED – BamH1-R: 5′ACCGGTGATCCGGGCGCCGGCATGAGTA
GAAGCTTGGCCGCCGGCGCCGGCATGAGTAAGGAC3′
InsertionT3R: 5′CATCATCCGCTGCCTGAAGCAGGCACCATCCGATGAG3′
InsertionT5: 5′CATCATCCGCTGCCTGCAGAGGACCACTGACTAGAT
AGCTTGGCCGCCGGCGCCGGCATGAGTAAGGAC3′
InsertionT9: 5′CATCATCCGCTGCCTGCAGAGGACCACTGACTAGAT
AGCTTGGCCGCCGGCGCCGGCATGAGTAAGGAC3′
InsertionT11R: 5′AGGTGTTGGGCCGGGGCCGCTCCAGGCGGATGATGAAGG3′
InsertionT12R: 5′AGGTGTTGGGCCGGGGCCGCTCCCTGCTTCATCAGCTGGCGGATGATGA3′
InsertionT15R: 5′AGGTGTTGGGCCGGGGCCGCTGGCAGGCGGATGATGAAGG3′
E1T7K: 5′GCCACCATGAGCGACGTGGCATTGTAGGAGGGTTGGCC
Q79K-F: 5′CATCATCCGCTGCCTGAAGCAGGCACCATCCGATGAG3′
Q79K-R: 5′CATCATCCGCTGCCTGAAGCAGGCACCATCCGATGAG3′
W80R-F: 5′CATCATCCGCTGCCTCGAGGACCATCATCTACG3′
W80R-R: 5′CATCATCCGCTGCCTGAAGCAGGCACCATCCGATGAG3′
Q79K-W80R-F: 5′CATCATCCGCTGCCTCGAGGACCATCATCTACG3′
Q79K-W80R-R: 5′CATCATCCGCTGCCTGAAGCAGGCACCATCCGATGAG3′

2.5. Protein Subcellular Localization, Western Blot and Luciferase Assays

HeLa cells were transfected with constructs driving the expression of AKT1 fused to the mCherry protein. Cells were seeded in 24-well plates containing sterile coverslips, 16 h before transfection to be confluent at the time of transfection and stained was obtained using the Pure Envision dual link kit (DAKO, CA, USA) (30 min of incubation).

Dual-Luciferase Reporter Assays (Promega, Madison, WI, USA) involved the reporter promoter 4×DBE-luc which contains 4 copies of the FOXP response element (DAF-16 family member-binding element or DBE) upstream of a minimal promoter driving the expression of the firefly luciferase gene (Furuya et al., 2000). Each experiment was performed in three replicates in 96-well plates. Cells were seeded 16 h before transfection to be at confluence at the time of transfection and transfected with 150 ng of total DNA per well (4×DBE-luc, AKT1 vector, FOXO3a (Addgen no 1787) or NLS-negative vector and renilla luciferase vector) using the calcium phosphate method and rinsed 24 h after transfection. At this point, cells were serum-starved or not for 24 h. Forty-eight hours after transfection, cells were washed with PBS before lysis and luciferase measurements were performed with a Tristar LB 941 luminometer (Berthold Technologies, Bad Wildbad, Germany). To monitor transfection efficiency, a Renilla luciferase vector (pRL-RSV, Promega, Madison, WI, USA) was co-transfected. Activity is expressed as relative luciferase units (RLU, i.e. the ratio of the firefly luciferase activity over the Renilla luciferase activity). Statistical significance was estimated by Student’s t-tests. Error bars represent the standard deviation between replicates.

3. Results

3.1. A Hotspot of In-frame Duplications and Point Mutations alter AKT1 in JGCTs

First of all, we performed a Sanger sequencing of the exon potentially harboring the previously reported activating mutations of Gos activating protein position 201 (Kalfa et al., 2006). The absence of this mutation suggested that these tumors were good candidates to harbor driver mutations elsewhere. A survey by direct sequencing of exons 9, 18 and 20 of PIK3CA in 7 tumors of the cohort showed the absence of mutations suggesting that PIK3CA mutations were not a frequent molecular lesion in JGCTs.

During the analysis of the AKT1 gene, the agarose gel electrophoresis of exon-3 PCR amplicons revealed the coexistence of two or three bands in 9 out of the 16 JGCT samples (Fig. 1A). One band had the expected length and the others were longer. One sample (T1) displayed only a slow-migrating fragment. These results pointed to the existence of insertion(s) in this exon. This was confirmed through exon-3 amplification with other pairs of primers. We performed a similar analysis using the cDNAs from 4 cryopreserved tumors (T13–T16, for which high-quality mRNA was available) and again the insertions were apparent, in agreement with the results obtained with the corresponding gDNAs (Fig. 1A). An analysis of gDNA extracted from the peritumoral tissue, available only for two of the samples bearing the insertions, formally proved the somatic status of these two mutations (Fig. 1B). Sanger sequencing of the isolated DNA bands showed the presence of 10 in-frame tandem duplications, which had never been reported either in the literature or in the databases (Fig. 1C and Table 1). The slowest-migrating bands were heteroduplexes of wild-type and mutated sequences. Interestingly, all duplications but two were different, which suggests that the mutational process affecting the underlying coding region is very dynamic and attributable to DNA-polymerase errors (Viguer et al., 2001). More details on the tandem duplications (and their official names) are available in the Supplementary material. A screening by PCR of the gDNA from 10 AGCTs (Benayou et al., 2015) colorectal carcinoma samples (Benayou et al., 2010) and 59 NCI cell lines provided no evidence for the existence of this type of insertion in such samples (Fig. 2). The latter result is in agreement with exome sequence data available for the NCI cell lines.

Given the high degree of identity between AKT1 and AKT2 in the region harboring the duplications, both at the DNA and protein
levels, we also analyzed exon 3 of AKT2 for the presence of duplications. No duplications could be observed. Full direct sequencing of the coding region of AKT1 uncovered an array of point mutations altering residues highly conserved among orthologs and even between the paralogs AKT1 and AKT2 (Fig. 3 and Table 1). These mutations were identified in 10 tumors and the status of somatic mutations was confirmed for seven of them (for which peri-tumoral gDNA was available). Interestingly, several tumors without in-frame duplications carried two or more potentially damaging point mutations. Two tumors harbored homozygous/hemizygous mutations (E91K and M458I in T3 and T21I and P348S in T7). Amplicon cloning and sequencing of individual clones showed that the somatic mutations underlying the substitutions Q79K and W80R appeared on the same allele in T2. Owing to the lack of high-quality AKT1 cDNAs for formalin-fixed paraffin-embedded (FFPE, T1–T12) samples, the allelic combinations (i.e. haplotypes) of the other co-occurring mutations could not be worked out, thus preventing their experimental exploration. In total, 14 out of 16 JGCTs harbor putative driver alterations of AKT1.

3.2. The In-frame Duplications Alter the Pleckstrin Homology Domain of AKT1 and Lead to Oncoprotein Activation

The tandem duplications described here alter the PHD of AKT1 (Gibson et al., 1994). The PHD binds to phosphatidylinositol-di/trisphosphates (PIP2 and PIP3) from the plasma membrane, which are produced by activated PI3K (Franke et al., 1997). This leads to the translocation of AKT to the plasmalemma. In such conditions, phosphoinositide-dependent kinases (among others) phosphorylate AKT1 on T308, leading to its partial activation. Full activation is achieved upon phosphorylation of S473 (Warfel et al., 2011; Mahajan and Mahajan, 2012). On structural grounds, the PHD involves two main beta sheets (Protein DataBase structures 1H10, 3O96 and 4EJN). The tandem duplications described here involve the 6th beta strand, according to the ribbon model displayed in Fig. 4. The co-occurring substitutions Q79K and W80R map right after the beta strand involved in the duplications. Interestingly, Q79K has already been shown to activate AKT1 probably by decreasing the interaction between the PHD and the kinase domain (Warfel et al., 2011; Yi et al., 2013) and W80 has been proposed to interact with F469 from the hydrophobic domain to keep AKT inactive (Calleja et al., 2009).

To determine whether the duplications within the PHD induced functional alterations of AKT1, we generated constructs driving the expression of wild-type and mutated AKT1 proteins (including a version carrying the activating mutation E17K Carpten et al., 2007) fused to the mCherry fluorescent protein. We observed that the wild-type AKT1-mCherry fusion protein displayed a rather diffuse localization and that E17K was enriched at the plasma membrane but was also present in the nucleus of transfected HeLa cells, irrespective

| Patient/tumor no. | Age (yr) | Estradiol (pg/ml) | Testosterone (ng/ml) | Delta-4 androstenedione (ng/ml) | LH (UI/l) | FSH (UI/l) | AKT1 exon 3 duplication length (bp) | AKT1 point mutations |
|-------------------|----------|-------------------|----------------------|---------------------------------|-----------|-----------|-----------------------------------|----------------------|
| 1                 | 6 y      | NA                | NA                   | NA                              | NA        | NA        | 36                                | –                    |
| 2                 | 4 y 3/12 | 44                | NA                   | NA                              | <0.2      | <0.2      | –                                 | Q79K*, W80R*         |
| 3                 | 4 y 2/12 | 19                | 0.19                 | NA                              | 0.3       | <0.2      | 30                                | D3Y, E91K, M458I     |
| 4                 | 13 y 5/12| 320               | 0.2                  | 1.6                             | NA        | NA        | –                                 | P348S*, Q232V, D274H*, S378F* |
| 5                 | 6 y      | 17                | 1.1                  | NA                              | NA        | NA        | 36                                | D3Y, G37D, R465C     |
| 6                 | 13 y 1/12| High              | Normal               | NA                              | NA        | NA        | –                                 | –                    |
| 7                 | 14 y 6/12| 23                | 3.69                 | 2.6                             | 4.2       | 2.72      | –                                 | T21I, P348S*         |
| 8                 | 6 y 9/12 | 157               | 2.4                  | 7.3                             | 1.3       | 0.3       | 36*                               | D3Y, C460S*          |
| 9                 | Neonatal | 760               | 1.1                  | 1.7                             | 0.34      | <0.2      | –                                 | D274H, L357F         |
| 10                | 1 y      | 10                | NA                   | NA                              | NA        | NA        | –                                 | –                    |
| 11                | 1 y 5/12 | <3                | <0.07                | NA                              | NA        | NA        | 39                                | K14I, K14N           |
| 12                | 10 y 8/12| 18                | 0.10                 | NA                              | 0.6       | 4.1       | 48*                               | A250F, G345S         |
| 13                | 8 y 6/12 | 100               | <0.07                | 1.10                            | 0.4       | <0.20     | 48                                | N199I                |
| 14                | 7 y 10/12| 103               | 0.82                 | 1.14                            | <0.4      | <0.5      | 36                                | –                    |
| 15                | 2 y 2/12 | 1116              | 2.2                  | NA                              | NA        | NA        | 24                                | –                    |
| 16                | 0 y 9/12 | NA                | NA                   | NA                              | NA        | NA        | 36                                | –                    |

NA: not available
* somatic status confirmed

Mutation names in red involve highly conserved residues. They are predicted to be damaging by PolyPhen 2 (http://genetics.bwh.harvard.edu/pph2/index.shtml). Only E91K, which alters a highly conserved residue was not predicted to be damaging by PolyPhen 2. SIFT (http://sift.jcvi.org) predicts all the mutations in bold to be damaging. Mutations E91N and M458I in T3 and T21I and P348S in T7 were found to be apparently homozygous.
of the presence of serum. The mutated proteins carrying the duplications were almost exclusively located under the plasma membrane region of transfected cells, in the presence or absence of serum (Fig. 5 and Supplementary material). One outstanding characteristic of the cells transfected with the mutated constructs was the abundance of filopodia-like cytoplasmic processes. The subcellular localization of the mutated proteins with the duplications was strikingly different from that of E17K AKT1 in our experimental setting. The pervasive presence at the plasmalemma of the elongated AKT1 proteins suggested the existence of a high level of activation. To test this hypothesis, we analyzed the phosphorylation status of seven AKT1 variants carrying the in-frame duplications, which is supposed to reflect protein activation (Warfel et al., 2011; Calleja et al., 2009). In agreement with our hypothesis, a Western-blot analysis using an antibody directed against phosphorylated S473 showed a dramatic difference between the wild-type and E17K variants with respect to the proteins carrying the duplications (Fig. 6A). This difference was obvious in low serum conditions as well as in the presence of serum. The presence of phosphorylated AKT1 was confirmed on histological tumor sections (Fig. 6B).

To obtain further functional insights, we studied the effect of the variants on a FOXO3a-based reporter system. Indeed, FOXO factors are negatively regulated by AKT in response to a series of growth factors and other signals. Phosphorylation of the FOXOs at three conserved sites by AKT causes their sequestration in the cytoplasm, preventing transactivation of their targets (Calnan and Brunet, 2008). Luciferase experiments were performed in HeLa cells co-transfected with the 4X-DBE-luc reporter, a FOXO3a expression vector (or a control vector) and various constructs driving the expression of wild-type or mutated AKT1 forms. Wild-type AKT1 elicited the expected response: in the presence of serum, FOXO3a was repressed by phosphorylated AKT1. In serum-starved cells, wild-type AKT1 repressed FOXO3a less strongly (Fig. 6C, D). Luciferase experiments demonstrated that the AKT1 proteins bearing the duplications were hyperactive and insensitive to serum-deprivation.
much like the E17K mutant (i.e. FOXO3a was repressed irrespective of the presence or absence of serum). Of note, the mutant Q79K-W80R AKT1 also displayed strong membrane localization and hyperactivation (Fig. 7).

4. Discussion

The mechanisms underlying GCT formation and progression remain largely unknown and their occurrence probably results from somatic mutations. Here, we contribute to elucidate the etiology of JGCTs by showing that more than 60% of the analyzed tumors, occurring in girls under 15, bear in-frame tandem duplications in AKT1 leading to the activation of the mutated proteins. Our functional data show that the mutated proteins display a marked membrane localization leading to AKT1 phosphorylation and to the appearance of filopodia-like processes. Of note, the tumors without AKT1 in-frame duplications often had two or more point mutations altering highly conserved residues.

The strong enrichment at the plasma membrane of the elongated AKT1 mutants is to be correlated with that of other natural or artificial mutants such as viral-Akt, myristoylated-Akt and Akt-E40K. Interestingly, the common trait behind their efficient cell transforming capability is their enhanced localization at the plasmalemma, achieved by a PHD with increased lipotropy (E40K mutation) or by the presence of a myristoylation signal, artificially introduced or provided by the viral Gag sequence fused to Akt in v-Akt (Aoki et al., 1998). Structural studies, including crystallography, have suggested that the E17K activating mutation directly alters the lipid binding pocket of the PHD (Carpten et al., 2007). In the case of the tandem duplications identified here, it is not clear what the basis of the membrane localization and activation is. However, if we assume that the first duplicated sequence “pairs” with the 5th beta strand of the PHD (as the former emerges from the ribosome during translation) then the second copy is left unpaired and might form a protrusion. Such a structural defect might alter the interaction with the plasma membrane and/or with the kinase domain (KD).
Fig. 3. Point mutations of AKT1 in JGCTs. Alignment of several AKT1 orthologous protein sequences from human (Homo sapiens, Hsa), mouse (Mus musculus, Mmu), pufferfish (Takifugu rubripes, Tru) and the frog Xenopus tropicalis (Xtr) as well as the AKT2 paralogs in the same species. Note the high degree of evolutionary conservation of the mutated residues highlighted in red in the human sequence. The corresponding substitutions are displayed at the bottom of the alignment.

Fig. 4. Ribbon representation of the AKT1 PHD domain and predicted effect of the duplications. A) Wild-type domain, according to the PDB Structures 1H10. The upper region mediates the interactions with the plasma membrane. The beta-strand involved in the duplications is highlighted in red. B) Predictions of the effects of several duplications on the 3D structure of the PHD domain (using Modeller Webb and Sali, 2014). We propose three main types of spatial arrangements, according to the insertions. These predictions do not take into account the presence of the rest of the protein. Structures (PDB: 3O96 and 4EJN) including the PHD and KD domains lack the segment linking both domains, which renders difficult the construction of realistic models. Only X-ray crystallography of both PHD and KD will allow us to work out the impact of the duplications on protein structure.
Mutations in AKT1 at the PHD–KD interface, as is the case here, that weaken their interaction have been previously reported in human cancers (Parikh et al., 2012). Such altered interactions in our mutants could explain the dramatic tropism for the plasmalemma and the concomitant increase of phosphorylation. A similar explanation would hold for the co-occurring mutations Q79K–W80R. However, further studies are required to completely elucidate the underlying mechanism(s).

Although JGCTs are of good prognosis, it would be interesting to study the properties of the filopodia-like processes induced by the mutated AKT1 carrying the duplications because of the known roles of filopodia/invadopodia in sensing, migration and intercellular interactions (Mattila and Lappalainen, 2008). These processes are similar to those observed upon eEF1A2 over-expression in BT549 human breast cancer cells and non-transformed Rat2 cells. Interestingly, eEF1A2 expression in BT549 cells stimulates filopodia formation, cell migration and invasion in a PI3K- and Akt-dependent manner (Amiri et al., 2007).

Along the same vein, squamous cell carcinoma lines engineered to express constitutively active AKT display down-regulation of E-cadherin, reduced cell–cell adhesion and increased motility in vivo (Grille et al., 2003).

One interesting question is raised by the apparent specificity of the AKT1 insertions. Several hypotheses can explain this fact. First, the tandem duplications only appear in the ovary because the underlying mutational process is favored in granulosa cells for as yet unknown reasons. Second, the selective advantage of tumor cells may involve interactions of the mutated AKT1 with ovarian-specific (signaling or interacting) partners. Finally, we cannot exclude that granulosa cells may need a strong level of AKT1 activation to become transformed, which would only be achieved with the observed duplications or with several AKT1 mutations per cell, such as Q79K and W80R. The fact that most of the JGCTs harbor AKT1 mutations and the involvement of the PI3K–AKT pathway in regulating cellular proliferation and survival suggests that the molecular lesions reported here are driver events.

Fig. 5. Subcellular localization of the AKT1 variants. Fluorescence microscopy of HeLa cells transfected with constructs driving the expression of different AKT1 variants fused to the mCherry fluorescent protein, in the presence of serum. Images were obtained with a Zeiss ApoTome microscope. This instrument allows the production of optical sections by using structured illumination. The left panels represent merged Z-stacks of typical cells (i.e. several images taken at different depths/focal planes within the cell). DNA was stained with Hoescht (in blue). The right panels represent optical sections, where DNA staining is not shown, to better appreciate the sub-cellular distribution of the AKT1 variants. Note the rather diffuse distribution of wild-type AKT1 and the membrane and the nuclear enrichment of E17K (activating mutation). The typical staining pattern of the mutated AKT1 carrying the duplications is strikingly different. Note the strong enrichment in the cortical sub-membrane regions. Moreover, the transfected cells displayed a profusion of filopodia-like processes (see insert). Similar results were obtained in the absence of serum. Further examples are provided in the Supplementary material.
Irrespective of the precise role of AKT1 in the origin and/or progression of the JGCTs, which is yet to be studied, the uncovering of the AKT1 insertions will facilitate the molecular diagnostics of JGCTs. Our findings open also targeted therapeutic perspectives because inhibitors of the PI3K–AKT–mTOR pathway(s) are being tested in clinical trials (Don and Zheng, 2011).

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For instance in tumor 4, we do not know the allelic combinations encoding the mutations because they co-occur with other mutations but we cannot tell apart their combinations.

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### Appendix A. Supplementary Data

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###Author Contributions

LB, ALT, AA and RAV conceived and designed the study. LB, ALT, AA, LG, BL, DF and RAV developed the methodology. LB, ALT, AA, LG, BL and RAV acquired data. LB, ALT, AA, SS, DF, CS, NK, LG and RAV analyzed and/or interpreted data. All authors wrote and provided final approval of the manuscript.

###Declarations of Interests

The authors declare no competing interests.

###Ethics Committee Approval

This project was approved by the ethics committees of the tumor repositories Tumorothèque Necker-Enfants Malades and Tumorothèque Montpellier.
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