

# Use of dissected paraffin block tissue as a source of mRNA for transcriptional profiling and biomarker identification: a review, with preliminary findings in adrenocortical carcinoma tissue

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**Cancer tissue obtained by biopsy or at surgery is usually handled and stored as formalin-fixed, paraffin-embedded (FFPE) blocks. While primarily prepared for histological examination, this material represents a valuable resource that is stable and transportable, and which is ideal for examination in 3 dimensions. It has been used for DNA and protein analysis but the potential for transcriptional profiling has been little exploited: the quality of the extracted RNA is uncertain and there are few reports of its efficient use. We review the background and practicalities of this approach and describe a pilot study as a worked example in which RNA of good yield and quality could be consistently obtained from differentiated areas of adrenocortical carcinoma (ACC) tissue.**

**Keywords:** FFPE, adrenocortical carcinoma, mRNA preparation, transcription profiling

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**Abbreviations:** ACA, Adrenocortical adenoma; ACC, adrenocortical carcinoma; FFPE, formalin-fixed, paraffin-embedded tissue

## INTRODUCTION

The generation being born today can be expected to live on average more than 100 years. A consequence is that humanity is going to increasingly struggle with ageing-related disease, with cancer among the most important. Study of mammals that are more resistant to ageing has determined that gene regulation rather than gene sequence is critical, with a crucial role of RNA-RNA cross-talk in mammalian ageing, oncogenesis, and pathogenesis of diverse cancers. This involves an intricate interplay among diverse RNA species such as lncRNA and miRNAs. Transcriptomics thus can be expected to be an important tool in the search for potential biomarkers and therapeutic targets for precision oncology (Huang, 2020).

Patient tumour material is routinely obtained by biopsy or after surgery and is stored in the form of FFPE blocks. This is the standard way that hospitals manage diagnostic procedures on tumour tissues, but these samples are maintained in long term storage and are thus

available for mutation profiling and precision oncology, with the developing prospect of using such findings for standard identification to aid the clinical decision-making process (Takeda *et al.*, 2018).

Cancer precision medicine based on tumour tissue analysis by next-generation sequencing and other methods is already beginning to provide diagnostic and prognostic information for immediate clinical application and is playing a crucial role in genotype-matching a tumour case with available clinical trials (Gerratana *et al.*, 2019).

Gene expression profiling from FFPE has been increasingly utilised in recent years. Applications have included classifying the tissue of origin of 32 different tumour classes (not including ACC) using a 92-gene panel, achieving 82% correct classification compared with 88% for preparations from frozen tissue (Ma *et al.*, 2006).

Subtyping of cancers using data from a variety of platforms for gene expression, both microarray and RNA-Seq methods, has been recently outlined, using a data-driven reference approach, aiming to eliminate platform-specific bias (Zhang *et al.*, 2019). Below we present an example of the use of FFPE as an investigative tool for the rare cancer ACC, a disorder that still presents major challenges for efficient diagnosis and prediction of potential for malignancy.

## FFPE RNA INTEGRITY

FFPE-origin RNA is known to be degraded in a specific manner. The most commonly used means to assess this has been formaldehyde gel electrophoresis with ethidium bromide staining, which requires visual assessment of the ribosomal bands. However, this is currently regarded as of limited reliability in comparison with modern genomic techniques due to the effects of the electrophoretic conditions, ethidium bromide fluorescence, an influence of the amount of RNA being loaded, and a lack of sensitivity and specificity (Fleige & Pfaffl, 2006). A newer method for RNA analysis is based on the Bioanalyser 7500 (Agilent). This assesses ribosomal bands and the entire electrophoretic track of the sample. It enables the actual presence or absence of degraded products to be automatically determined and expressed as the RNA Integrity Number (RIN). However, this in turn is claimed to have limitations (Chung *et al.*, 2008; Oberli *et al.*, 2008). A review by Zhang *et al.* (2017) concludes that both microarray and RNA-seq methods perform better with FFPE-derived RNA than predicted by the RIN. They cite published comparisons of paired FFPE-derived and frozen material giving similar results. RNA

chain lengths from FFPE-derived RNA are mostly in the 50–200 bp range.

FFPE material shows increasing degradation over time. A comparison of 10-year-old and recent samples showed lower RNA quantity in the older samples but of similar quality. However, while gene expression, using RT-PCR, of a 151 bp fragment was satisfactory in all samples, that of a 242 bp fragment was poor in the older material (Ribeiro-Silva *et al.*, 2007). Niesen and others (Niesen *et al.*, 2013) found that RNA yield decreased with storage time but, unexpectedly, the number of gene-present calls in their FFPE RNA tissue samples (using Affymetrix *Gene-Chips*) were comparable with freshly isolated human peripheral blood mononucleocyte cells. They suggested that transcripts are uniformly fragmented sequences across the full length, with the majority of the transcript being 75–100 bp and a small number of them retained at up to 3000–4000 bp in length. Other determinants of RNA quality are methods of fixation, storage temperature, and light. Base substitution by the fixative can diminish the accuracy of reads.

Tolerance to sample degradation has also been shown by Die & Román (2012), who scrupulously examined the influence of RNA quality on gene expression profiling by microarrays and found that amplification curves with exponential and plateau regions of properly sigmoidal shape could usually be acquired with qPCR, even from degraded templates. They studied the effects of varying amounts of deliberately induced RNA degradation on the expression profile of samples from human tumour and healthy tissue. Moderate degradation did not spoil microarray analyses and permitted meaningful results to be obtained if used carefully. They advised that with strongly degraded input RNA, small expression differences might give a false positive result. Accordingly, we have used in our example a normalisation method employing reference genes and have limited reporting of gene expression differences to 5-fold change or more.

## EXAMPLE: PILOT STUDY OF mRNA PREPARATION FROM ACC

### Background

ACC accounts for 2–11% of adrenal masses. It is responsible for approximately 0.2% of all cancer deaths in the US, with a mean survival time of only months. Available treatments are controversial; outcomes are poor, with only 35% survival at five years.

Imaging of adrenal tumours often reveals a large mass with a central area of necrosis or haemorrhage; heterogeneous enhancement favours malignancy (Moreira & Pow-Sang, 2002; Bharwani *et al.*, 2011). Histological diagnosis of ACC requires specialist expertise (Libe, 2019). The scoring system of Weiss is universal and may be improved using more criteria (Blanes & Diaz-Cano, 2007). It is difficult to predict malignancy based on clinical, biochemical or histologic grounds in the absence of capsular invasion and distant metastases (Fonseca *et al.*, 2012). These are also poor mortality indicators (Calissendorff *et al.*, 2016) but careful control of techniques offers some improvement (Jouinot & Bertherat, 2018). There is a high degree of intertumoural heterogeneity, both in terms of histology, biochemistry (Taylor *et al.*, 2017) and molecular biology (Libe, 2019), which may change with tumour progression (Vatrano *et al.*, 2018). This variability stands in strong contrast to the H295R ACC cell line model, used universally for *in vitro* study and also to the

relatively more common ACA, which shows a limited range of frequent mutations (Backman *et al.*, 2019).

Few studies have yet attempted to differentiate portions of these heterogeneous tumours. Separating zones within either fresh or frozen tissue would be problematic. Many method descriptions do not specify which was used, although Giordano used frozen tissue (Giordano *et al.*, 2003; Giordano *et al.*, 2009). For these rare carcinomas, gathering and processing of frozen tissue for gene expression studies on a large scale would not be feasible (Akerstrom *et al.*, 2015; Backman *et al.*, 2019). In contrast, long-term storage of tumour tissue as FFPE blocks is universal. If RNA of adequate quality and quantity for gene expression analyses could be isolated from this material, even blocks in long-term storage from a variety of centres could be studied. Since it is solid, direct comparison of histology and gene expression in tumour areas is theoretically possible, although limited by quantity of tissue available in some ACC cases. This has the potential to provide a much more comprehensive picture of the disruption of control pathways that lead to tumour development.

The pilot study described below aimed to efficiently isolate nucleic acids from differentiated portions of FFPE material of sufficient quality for gene expression profiling. We know of only one published study of FFPE from patients with ACC. This did not differentiate portions of the tumour and showed a poor success rate, with only 4 of 14 blocks producing useable material (Lombardi *et al.*, 2006). We first assessed the yield of RNA and then similarly used RT-PCR for  $\beta$ -actin to check the adequacy of the RNA template in some samples. We then compared performance of an ACC sample and normal tissue in an mTOR pathway array.

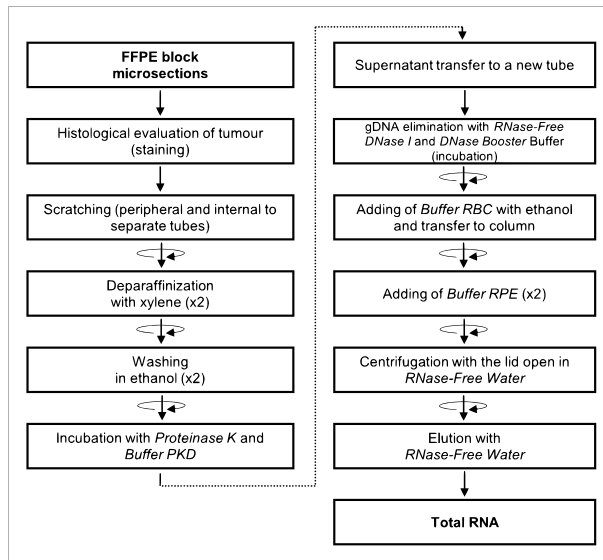
Although ACC tissue features accumulation of disturbances of several genes, increased expression of IGF-2 signalling is the most frequent change (Barlaskar *et al.*, 2009; Fonseca *et al.*, 2012). IGF-2 is an upstream promoter of the mTOR pathway (Fassnacht *et al.*, 2013; Szyszka *et al.*, 2016). Molecular biology has the potential to be a precision oncology tool which can identify alterations in different signalling pathways and offer single biomarkers or clusters that can be utilised in clinical practice (Durand *et al.*, 2011; Fonseca *et al.*, 2012; Fassnacht *et al.*, 2013; Assie *et al.*, 2014; Vatrano *et al.*, 2018).

### METHODS

Figure 1 summarises the preparation steps.

#### Preparation of portions of FFPE histopathology sections

The FFPE blocks used had been prepared 2–5 years previously from tissue being surgically removed from patients with ACC (n=10) or ACA (n=7). Microsections were prepared by an expert histopathologist; 9 slides per case were obtained, cut at 7  $\mu$ m thick sections. Only the first from each series was stained with hematoxylin–eosin (because staining of FFPE sections may enhance RNA degradation) and designated as *central* or *peripheral* by histopathology expert Dr Salvador Diaz-Cano. Regions of the tissue in the succeeding sections with morphological differences matching those in the stained section were identified using a single lens or binocular microscope at the lowest magnification that would enable distinction of areas while providing a wide enough field of view. The working space and tools were cleaned with RNase AWAY (Thermo Fisher Scientific). These sections (7 for RNA extraction, and one for DNA extraction) were dis-



**Figure 1. Workflow of Total RNA extraction from FFPE, using the RNeasy FFPE Kit.**

sected using a thin scalpel blade and the differentiated portions were pooled in two separate Eppendorf tubes for each extraction.

#### Isolation of RNA from FFPE tissue sections

The RNeasy FFPE Kit (Qiagen) was used, following the RNeasy FFPE Handbook protocol with modifications. It is specifically designed to, as much as possible, reverse the chemical modifications of RNA caused by formaldehyde, such as crosslinking, which cannot be detected by standard quality analysis, such as agarose gel electrophoresis and 'lab on-a-chip' (the Agilent Bioanalyzer system) and isolates RNA fragments longer than 70 nucleotides, which are suitable for gene expression analysis (Qiagen, 2013).

A maximum of 2 sections 5–20  $\mu\text{m}$  thick is recommended in the protocol. Our 7 sections on slides, divided into 2 zones, equated to the middle of this range. Each sample was first dewaxed in a fume cupboard using 1 ml xylene. After thorough vortex mixing and centrifugation at maximum speed for 5 min (2 min in the protocol), the xylene containing the paraffin was carefully poured off, taking care not to disturb the pellet remaining at the bottom of the tube. Deparaffinization was repeated, followed by a similar wash with 96% aqueous ethanol. The tubes were left in the fume cupboard until the pellet was completely dry. The next step, Proteinase K digestion, was extended from the time suggested in the Qiagen protocol from 15 min to 12 hours at 56°C, as it was observed that this had a very significant impact on the total RNA concentration and quality, in accordance with advice provided on the GenomeWeb site (Johnson, 2014). This was followed by 15 min at 80°C, according to the protocol, a step optimised to reverse formaldehyde-induced cross-linking. Cooling and then DNase treatment followed the protocol.

For RNA purification, the protocol recommends two options for the volumes of ethanol and Buffer RBC that are passed through the RNeasy MiniElute spin column. The higher volumes were found to give higher yield of the total RNA: Buffer RBC (500  $\mu\text{l}$ ) and ethanol (1200  $\mu\text{l}$ ). Buffer RPE  $\times 2$  was added to the column accord-

ing to the protocol. The RNA was eluted using 17  $\mu\text{l}$  RNase-Free Water (for use with RNAase-Free DNase I, provided by kit) with centrifugation at full speed for 3 min (1 min in protocol). The Qiagen columns used for FFPE RNA purification are claimed to remove fragments shorter than 200 bp.

After extraction, 2  $\mu\text{l}$  of the obtained RNA was immediately taken from the original tube and combined with 2  $\mu\text{l}$  RNase-free deionised water in a mini tube and kept on ice, ready for measurement of the concentration of total RNA using a NanoDrop 1000 Spectrophotometer (ThermoFisher). The parameters A260/A230 A260/A280 were in the range of 1.89–2.05; these values indicate satisfactory purity. Total RNA samples were stored in  $-80^{\circ}\text{C}$ .

#### RT-PCR for $\beta$ -actin

The TaqMan double-dye probe RT-PCR assay (Applied Biosystems) for  $\beta$ -actin was used to check for the presence of amplifiable RNA template. qRT-PCR fragment targets should ideally be 50–150 bp. The maximum amplicon size should not exceed 400 bp, and for probe assay, a study of the relationship between length and efficiency, has shown increase of Ct above a length of about 200 bp (Debode *et al.*, 2017). The  $\beta$ -actin TaqMan amplicon size is 171 bp.

The assay was carried out with the QuantiTect Reverse Transcription Kit (Qiagen), according to the QuantiTect Probe PCR Handbook (Qiagen, 2011a). This first eliminates genomic DNA contamination before RT-PCR. A Rotor-Gene 6000 real-time PCR cycler (ThermoFisher) was set, according to the protocol, to 50 repeats with: denaturation 10 s 94°C, annealing 57°C, hold 20°C. An amplification plot and table with Ct results was finally generated with the supplied software.

#### Pathway-targeted preamplification following cDNA synthesis

The extracted RNA was tested for performance in gene expression profiling using a pathway-specific Human mTOR Signalling RT<sup>2</sup> Profiler PCR Array (Qiagen 2015). One sample (peripheral ACC case 1) and control (normal cortex) were prepared in duplicate.

For the reverse transcription reaction, 200 ng total RNA for each sample was processed using the RT<sup>2</sup> PreAMP cDNA Synthesis Kit (Qiagen). This was less than advised in the protocol (500 ng) since we wanted to check whether it was possible to save RNA.

The cDNA synthesis and preamplification reaction procedures were carried out according to the RT<sup>2</sup> PreAMP cDNA Synthesis Handbook (Qiagen, 2011b). Incubations and cycling were processed with a Veriti Thermal Cycler (ThermoFisher). Lack of presence of genomic DNA is monitored by a quality control on the Qiagen PCR array, which also contains a reverse transcription (RT) control and a positive PCR control, which provides a check on cDNA synthesis efficiency. These checks were passed during our assessments.

#### PCR amplification on the Human mTOR Signalling RT<sup>2</sup> Profiler PCR Array (Qiagen 2015)

The main amplification reactions were carried using a ViiA 7 Real-Time PCR System (ThermoFisher) instrument equipped with ViiA™ 7 Software. The reaction on the RT<sup>2</sup> Profiler PCR Array 4 $\times$ 96 well array was initially prepared according to the RT<sup>2</sup> Profiler PCR Handbook. This was shown to give poor results for the amplifica-

tion plot obtained using the supplied software. The cycling program was then changed to an alternative FFPE ViiA 7 protocol specially obtained from Qiagen, which used the following cycling conditions: hold Stage, 50°C, 2.00 min; PCR Stage, 40 cycles 95°C, 0.15 min, 60°C, 1.00 min, melt curve stage with default settings. On completion, the Ct values obtained were much improved but with some delay, which indicated that it was better to use the amount of template specified in the protocol.

### Data normalisation and result calculation

Gene expression data was normalized using the arithmetic average of the three most stable of a panel of 5 housekeeping genes according to current practice (Pfaffl, 2007). The duplicates showed good agreement for each gene and the means were used for calculation. The  $\Delta\Delta CT$  method (to calculate expression differences between tumour tissue and normal) was applied using RT<sup>2</sup> PCR Array Data Analysis version 3.5 software. Fold changes relative to normal tissue higher than 5 were considered significant.

## RESULTS

### Extracted FFPE-derived total RNA

Total RNA obtained from the on-slide FFPE designated tumour areas was in the range of 30–380 ng/ $\mu$ l (Table 1); one area from a normal cortex was extracted, at a concentration of 280 ng/ $\mu$ l.

**Table 1. Concentration of total RNA extracted from FFPE.** This shows yield originating from the internal and peripheral parts of the tumour for each case. Ct values are for  $\beta$ -Actin in the probe RT-PCR assay.

Case No	Tumour type	Internal total RNA [ng/ $\mu$ l]	Ct from duplicates	Peripheral total RNA [ng/ $\mu$ l]	Ct from duplicates
1	ACA	30	18.95 20.27	170	17.49 17.32
2	ACC	63	21.61 22.39	108	23.04 21.89
3	ACA	114	21.02 21.28	40	17.67 17.58
4	ACC	131	19.30 19.54	184	18.97 20.22
5	ACC	54	21.66 22.32	88	20.95 21.11
6	ACA	256		115	
7	ACC	251		261	
8	ACC	105		42	
9	ACC	236		115	
10	ACC	102		242	
11	ACA	40		96	
12	ACC	380		90	
13	ACA	300		197	
14	ACA	81		184	
15	ACC	162		281	
16	ACC	114		100	
17	ACA	55		73	

The probe RT-PCR for the  $\beta$ -Actin gene was carried out on the first 5 pairs of samples to provide an assessment of whether the total RNA quality was adequate for amplification. All were found to give positive results. The amplification curves showed the expected shape and plateau regions, according to qPCR gold standards. Ct values for duplicates are shown in Table 1. These are all well below 29 cycles: higher values would have indicated ineffective nucleic acid isolation or PCR inhibition, inefficient RT-reaction, RNA/cDNA degradation, or too little template. The FFPE-originated templates were thus confirmed as sufficient for gene expression analysis with qPCR arrays.

Most samples provided a quantity of RNA that would be enough for 4 PCR array runs on a 94 times 4 well plate format (or two runs in duplicate) and dozens of qPCR validation reactions. The protocol recommends an RNA concentration of 500 ng per plate. Samples would need to be run in duplicate, which would require at least 68 ng/ $\mu$ l. For a few samples in which the concentration obtained was smaller than this, it would only be possible to run one PCR array.

Table 2 lists those genes in the mTOR pathway that were differently expressed. Appendix A (at <https://ojs.ptbioch.edu.pl/index.php/abp>) shows expression of all genes graphically. ACC peripheral tumour area versus normal adrenal tissue is expressed as fold change, together with brief detail on their role in the mTOR pathway, to help expand understanding of malignancy or biomarker potential.

### Discussion of the findings

The 84 genes targeted by the array are relevant to the mTOR pathway and so are related to the regulation of growth and differentiation and stress responses. Of these, 19 showed significant change in expression in the ACC tissue. Interpreting a given gene product as promoting or inhibiting tumour growth is not always straightforward, with, in some cases, opposite effects being reported for different malignant tumour types.

mTOR is a core component of two complexes, mTORC1 and mTORC2, each associated with several other components and subject to different stimulatory and inhibitory influences. In mTORC1, it acts as a serine-threonine protein kinase. Activation of this complex stimulates cell growth and proliferation in nutrient-rich conditions favourable to cell growth (Pópulo et al., 2012). In mTORC2, mTOR acts as a tyrosine protein kinase, controlling the actin cytoskeleton, with major effects on cell proliferation and survival. Under hypoxic conditions in a normal cell, mTORC1 activity is down-regulated by mechanisms which include activation of AMPK and by tuberous sclerosis complex 1 and 2 (TSC1/2). The latter deactivates Rheb (Ras homologue enriched in brain), a protein pivotal in the activation of mTORC1. The expression of hypoxia-modulated genes can differ between normal and tumour cells and may be affected by the tumour microenvironment (Cella et al., 2015).

Potential significance of these major changes is discussed in the following paragraphs, in the same sequence as in Table 2.

A marked downregulation of PRKAG3 and PRKAB2 is a feature of some tumour cells, which appear to be under selection pressure to downregulate AMPK; this would overcome cell growth being stopped in hypoxic conditions (Hardie, 2015). Normally, environmental stressors, such as heat shock, nutrient deprivation,

**Table 2. Differentially expressed genes between a sample from the periphery of an adrenocortical carcinoma and a sample of normal adrenocortical tissue.**

mRNA was extracted from formalin-fixed paraffin-embedded material and applied to a human mTOR Signalling RTC2 Profiler PCR Array (Qiagen). Brief information on the mTOR functions of each gene is listed, partly based on data provided by the company (Qiagen, 2015).

Gene name	Fold change	Function
<i>PRKAB2</i>	-8.9	These genes code for the protein kinase AMP-Activated non-catalytic subunit beta (PRKAB2) and non-catalytic subunit gamma (PRKAG3) of AMP-activated protein kinase (AMPK). This is an energy sensor which retards anabolic processes in response to metabolic stresses.
<i>PRKAG3</i>	-7.4	
<i>GSK3B</i>	-7.1	Glycogen synthase kinase 3 beta (GSK3B) takes part in a complex that phosphorylates beta-catenin, making it available for degradation by proteasomes. This can induce apoptosis in hypoxic conditions.
<i>MAPK3</i>	6.5	Mitogen-activated protein kinase 3 (MAPK3) plays a pivotal role in the MAPK signalling cascade, one of the major mTOR activators; it is the main downstream signal transducer of IGF-2; (IGF-2 is not on the array).
<i>INSR</i>	5.1	Insulin receptor (IR) binds IGF2 and is an important mTOR activator.
<i>IGF1</i>	-11.7	Insulin-Like Growth Factor 1 (IGF1) activates the protein kinase B (AKT) signalling pathway, which stimulates cell proliferation and inhibits apoptosis.
<i>MYO1C</i>	-20.6	Myosin-1c (MYO1C) suppresses AKT in the phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR signalling pathway.
<i>PIK3CD</i>	-36.6	PI3K catalytic subunit delta isoform (PI3K $\delta$ ). This promotes immune system activation.
<i>VEGFA</i>	6.7	Vascular endothelial growth factors A-C (VEGF-A to C) are mTOR effectors which promote vasculogenesis and angiogenesis.
<i>VEGFB</i>	14.8	
<i>VEGFC</i>	18.6	
<i>STK11</i>	5.5	Serine/threonine protein kinase 11 (STK11). It suppresses growth in conditions of low energy and nutrient levels <i>via</i> suppression of mTORC1. It relates to anchorage independent growth cell detachment and adhesion.
<i>EIF4E</i>	-8.8	The eukaryotic initiation factor 4E (eIF4E), directs ribosomes to the mRNA cap binding protein, enabling translation into protein. It is released by enhanced mTOR activity.
<i>PRKCG</i>	-8.3	Protein kinase C gamma type (PRKCG) is considered a tumour suppressor because of its pro-apoptotic effects and anti-proliferative effects.
<i>RHOA</i>	6.1	Ras Homolog Family Member A (RhoA) is an mTORC1 effector which promotes reorganization of the actin cytoskeleton to regulate cell shape and motility and plays a pivotal role in G1 cell cycle progression.
<i>PLD1</i>	5.0	Phospholipases D 1 & 2 (PLD) hydrolyze phosphatidylcholine (PC) to produce phosphatidic acid (PA) and choline in response to a variety of stimuli. PA is a second messenger and critical mediator of mitogenic activation of mTOR signalling.
<i>PLD2</i>	-8.8	
<i>RRAGA</i>	11.1	Ras-related GTP-binding protein A (RRAGA) is a guanine nucleotide-binding GTPase that is essential for response to amino acid availability by activation of mTORC1.

hypoxia, and ischemia inhibit the mTOR pathway via action of AMPK (Rubio *et al.*, 2013). This protein generally promotes glucose-sparing, oxidative metabolism, rather than the rapid glucose uptake and glycolysis used by quickly proliferating cells. Imaging of adrenal tumours often reveals a large mass with a central area of necrosis, likely indicating that they have outgrown their blood supply and so must have developed in a relatively hypoxic environment. Their patterns of steroidogenesis frequently indicate relative deficit of the enzyme activities that are mitochondrial, especially of 11 $\beta$ -hydroxylase, which also may reflect hypoxia (Moreira & Pow-Sang, 2002; Bharwani *et al.*, 2011; Taylor *et al.*, 2017).

Downregulation of GSK3B impairs phosphorylation of  $\beta$ -catenin, leading to the constitutive activation of the  $\beta$ -catenin-mediated Wnt pathway (Nault & Zucman-Rossi, 2014; Glibo *et al.*, 2021).  $\beta$ -Catenin accumulation in the nucleus promotes a large number of oncogenes and is a frequent characteristic of adrenocortical cancers. It is associated with a dismal prognosis. Study of candidate genes deregulated in  $\beta$ -catenin-mutated adrenocortical tumours may lead to a better understanding of the role of the pathway in adrenal cancer onset and maintenance (Durand *et al.*, 2011). Among other functions, GSK3B also acts on the mitochondria to decrease the threshold at which apoptosis occurs in response to stress stimuli

and to prevent the development of the Warburg effect, so that decreased expression would further the capacity of the tumour to survive hypoxic stress. Hypoxia is not effective in suppressing mTOR once other negative regulators are lost during malignant transformation (Cella *et al.*, 2015).

MAPK signalling was found to be upregulated, as was the insulin receptor. This pathway mediates direct cellular responses to many proliferative stimuli and transduces signal from IGF2 (El-Shewy *et al.*, 2007). The mTORC1 pathway is entwined with insulin signalling, with a regulatory feedback loop. IGF2 binding with the insulin receptor protects it from downregulation, which causes continuous mitogenic stimulation and thus sustained carcinogenic effects (Djiogue *et al.*, 2013; Fraenkel *et al.*, 2008). In chemoresistant Ewing's sarcoma, the IGF1/IGF receptor is switched to IGF2/insulin receptor dependency, which results in continuous activation of MAPK signalling, leading to proliferation and migration. This switch may also partially explain cases of chemoresistance to various anticancer drugs. Failure of efficacy of the mTOR suppressor everolimus in ACC was reported as related to the insulin signalling loop (Djiogue *et al.*, 2013). Thus, our present results appear to reflect this mechanism in ACC. In up to 90% of ACC, there is a loss of heterozygosity leading to bi-allelic ex-

pression of IGF2 (Barlaskar *et al.*, 2009; Fassnacht, *et al.*, 2013, Fonseca *et al.*, 2012). Dependence of recurrence-free survival on the level of expression of the IGF-II gene cluster has been shown (de Fraipont *et al.*, 2005). A comprehensive genomic study of ACC has differentiated three subtypes with divergent clinical outcomes, with all strongly expressing IGF-2 but with disparate regulation of miRNA (Mohan *et al.*, 2018).

In our study MYO1C was strongly downregulated in tumour cells *vs* normal cells. It is a tumour suppressor candidate, acting to suppress both expression and activation of AKT. Thus, a reduction results in activation of AKT and stimulation of the mTOR pathway. In transfection experiments in cultured HEK-293 cells, the levels of MYO1C and AKT were negatively correlated and also endometrial carcinoma grade was worst in those with lowest MYO1C expression (Visuttijai *et al.*, 2016). The PI3K pathway can be activated by diverse oncogenes or upstream growth factors that bind receptor tyrosine kinase (RTK), causing stimulation of protein translation and metabolic processes associated with cell proliferation, enhanced motility and, finally, inhibition of apoptosis. The molecular product of PI3K, AKT, activates mTOR. All the members of the PI3K/Akt/mTOR pathway, from the upstream RTK inducers to the final effectors, have been associated with cancer development and progression. Tian and colleagues (Tia *et al.*, 2019) quote that >70% of cancers have these increased.

PI3K $\delta$  expression was the most strongly downregulated. Although it is closely related to PI3K, it has a specific function in promotion of the immune system (Nunes-Santos *et al.*, 2019). Mutations are present in some cancers: this would be expected to diminish immune response and so enable tumour progression.

VEGFs were all markedly overexpressed in this study. Hypoxia plays a major role in increased vascular VEGF production, but activation of the PI3K/AKT/mTOR pathway in tumour cells can also increase VEGF secretion. VEGF A has been reported to be highly expressed in ACC (Fassnacht *et al.*, 2013), with correlation to the degree of malignancy (Foltyn *et al.*, 2012, Kolomecki *et al.*, 2001, Weddell *et al.*, 2018). VEGF B and C were especially strongly upregulated in our study. VEGFs interact with the VEGF receptors 1-3, which are RTKs. Several suppressive agents have been developed in prospective ACC treatment but with disappointing results (Fassnacht *et al.*, 2013). Inhibition of VEGFR 1 and 2 using a designed peptide diminished PI3K/AKT and MAPK/ERK1/2 signalling pathway activity and blocked angiogenesis, tumour growth, and metastasis in cultured cells (Sadremomtaz *et al.*, 2018). IGFs and insulin also interact with RTKs. Sorafenib, a multi-RTK inhibitor, is effective against some cancers but has a limited anti-proliferative effect in ACC. A recent study of Sorafenib incubation with the ACC cell line H295R confirmed its action but some cells survived (Cerquetti *et al.*, 2021).

Increased expression of STK11 is not easily interpreted. Although initially shown to be a tumour suppressor in some models, recent studies suggest that certain mutations of STK11 do lead to gain of oncogenic properties, such as in lung cancer, where mutations in exons 1 & 2 are associated with an aggressive subtype (Marcus & Zhou, 2010; Pécuchet *et al.*, 2017). Peutz-Jeghers syndrome features STK11 mutations and a high rate of cancer, especially those of breast and gastrointestinal tract. Activation of mTORC1 downstream targets has been shown in affected families (Li *et al.*, 2018).

Decreased expression of eIF4E is initially surprising since it is overexpressed in many cancers but underex-

pression does also occur, indicating it may have either an oncogenic or tumour suppressor function (Sobočan *et al.*, 2020).

Similarly, decreased expression of PRKCG is superficially unexpected since it is overexpressed in many cancers and had been thought to be tumour-promoting since its ligands, the phorbol esters, have unambiguous oncogenic properties. However, an analysis of PRKCG mutations in human cancers has shown that most were loss-of-function, and none were activating, indicating that it is a tumour suppressor. A depletion of its function promotes anchorage-independent growth (Antal *et al.*, 2015; Garg *et al.*, 2014).

RhoA is overexpressed in this study. It has also been associated with malignant cancer proliferation and metastasis. The latter may be enhanced by its action at the rear of migrating cells to promote detachment (Gordon *et al.*, 2014). It is seen as having a great potential as a drug target, as well as a candidate biomarker (Nam *et al.*, 2019).

It is interesting that PLD1 is overexpressed but PLD2 is underexpressed. PLD1 is activated by RhoA. Both PLDs are frequently overexpressed in cancers. Increased levels of choline-containing precursors are reported to be emerging markers of tumour progression (Glunde *et al.*, 2011). PLD1 integrates upstream signalling of mTOR, exerts control of cell size and is involved in amino acid availability sensing for mTOR (Fang *et al.*, 2003). PLD1 but not PLD2 is required for Rheb activation of the mTOR pathway. The TSC/Rheb pathway is an integrator at upstream signals such as cellular energy level and growth factors which regulate mTORC1 (Sun *et al.*, 2008). The extent to which these isozymes have separate roles remains uncertain; however, development of selective inhibitors is being actively pursued, both to elucidate this and as potential cancer therapeutic agents (Brown *et al.*, 2017).

RRAGA (RRAGa) was upregulated. This activates mTORC1 signalling *via* the GTPase Rheb. Rag GTPases are upstream effectors that are sensitive to nutrients (Hong-Brown *et al.*, 2012, Sancak *et al.*, 2010)

## CONCLUSIONS

We have shown that differentiated portions of FFPE sections of ACCs can reliably yield RNA of adequate quality for gene expression profiling. Our analysis of mTOR pathway gene expression using microarray analysis in one sample has shown changes that are already reported for this tissue or are plausible in the context of cancer, which further supports the potential of this material for clinical applications and research.

The yield is sufficient in most cases for running expression arrays in duplicate. However, this has been largely superseded by RNA-seq methods, which allow the use of much less material (Krogdams-Christensen 2019). This may enable finer differentiation within the block. An example is the use of single sections of archival breast cancer samples (Pennock *et al.*, 2019).

The RNA obtained from FFPE material using the method we have described or others that are commercially available should be suitable for all current approaches to gene expression studies. A comparison of nine mRNA profiling methods for FFPE *versus* frozen breast cancer material showed a generally good comparability (Turnbull *et al.*, 2020), with little influence on the ability to identify differentially expressed transcripts.

A painstaking approach to sample preparation, using dissection with a scalpel, appears worthwhile and also enables any normal tissue in the tumour sample to be eliminated or harvested separately. The single published study of ACC tissue (Lombardi *et al.*, 2006) discussed the potential for confusion caused by the presence of non-neoplastic tissue in the tumour samples and advised microdissection but considered that this could cause further degradation. A recent study of canine mammary tumours did use laser microdissection successfully, with the extracted RNA being analysed with NGS. These authors also achieved an up to tenfold improvement in yield with the use of focused ultrasonication with proteinase K to assist tissue disruption and perhaps also cleavage of RNA-protein crosslinks (Amini *et al.*, 2017). Simultaneous analysis of differentiated portions of the tumour using both metabolomic analysis by GC-MS and gene expression could be very powerful for improving the fundamental understanding of tumour progression, prognosis, or a new therapy option of this challenging disorder. Gene expression profiling may enable determination of tissue origin, with potential for clinical routine use.

We conclude that use of archival FFPEs offers a greatly expanded potential for transcriptomic studies of these rare adrenal carcinomas on a population-wide basis.

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