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Meta-analysis of adrenocortical tumour genomics data: novel pathogenic pathways revealed

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Sporadic adrenocortical tumours are common, but their pathogenesis is poorly elucidated. In this study, we present a meta-analysis and review of gene expression microarray and comparative genome hybridization (CGH) studies performed to date on these tumours, including our own data. Data of whole genome microarray studies from altogether 164 tumours (97 benign, 67 malignant) and 18 normal tissues were reclassified and reanalysed. Significant gene sets and cytogenetic changes from publications without available genomic data were also examined including 269 benign, 215 malignant tumour and 30 normal tissues. In our experimental study, 11 tumour and four normal samples were analysed by parallel mRNA and CGH profiling. Data were examined by an integrative bioinformatics approach (GeneSpring, Gene Set Enrichment Analysis and Ingenuity Pathway Analysis softwares) searching for common gene expression changes and paralleling chromosome aberrations. Both meta-analysis of available mRNA and CGH profiling data and our experimental study revealed three major pathogenetic pathways: (1) cell cycle, (2) retinoic acid signalling (including lipopolysaccharide/Toll like receptor 4 pathway), (3) complement system and antigen presentation. These pathways include novel, previously undescribed pathomechanisms of adrenocortical tumours, and associated gene products may serve as diagnostic markers of malignancy and therapeutic targets.
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Introduction

Adrenocortical tumours (ACT) are common; their prevalence may reach up to 5–7% in pathological series. Most of them are benign (adrenocortical adenoma, ACA), and the majority of these are hormonally inactive (IA); however, rare aldosterone- or cortisol-producing (APA or CPA) tumours are associated with significant morbidity and mortality. The prognosis of the rare adrenocortical cancer (ACC) is poor with an overall 5-year survival below 30% (Libè *et al*., 2007).

Several molecular pathways involved in adrenocortical malignancy have been revealed including overexpression of insulin-like growth factor 2 (IGF-2), mutations of tumour protein p53 (*TP53*) and alterations of Wnt/β-catenin and cAMP-mediated signalling (Soon *et al*., 2008). Despite these findings and the ongoing intensive investigation of adrenocortical tumorigenesis, we are far from an integrative view.

Studies on gene expression (mRNA profiling) and chromosome aberrations (comparative genome hybridization, CGH) by high-throughput bioinformatics-based approaches have revealed numerous pathomechanisms and molecular characteristics in several tumours. However, the overall interpretation of these findings and the selection of biologically relevant pathomechanisms is difficult (Hong and Breitling, 2008).

A number of mRNA and CGH (both classic and microarray-based) profiling studies have been performed on adrenocortical tumours to date that showed various alterations in gene expression and chromosome aberration patterns. Gene expression alterations may be used for the differentiation of benign and malignant tumours (de Fraipont *et al*., 2005; Soon *et al*., 2009) and for determining ACC prognosis (de Reyniès *et al*., 2009). Beside steady alterations observed in all settings (for example, overexpression of *IGF-2* mRNA in ACC), considerable differences can be found in different studies.

We hypothesized that by reclassifying and reanalyzing publicly available gene expression and CGH data then subjecting these to pathway analysis, biologically relevant and previously unknown pathogenic pathways may be identified. Common significant gene expression

changes between different microarray studies and corresponding mRNA expression and chromosomal alterations were searched for, that is, chromosome losses and gains paralleling under- or overexpression of genes by comparing Gene Set Enrichment Analysis (GSEA) results and data on cytogenetic changes (CGH and FISH (fluorescent in situ hybridization)).

To confirm the feasibility of this *in-silico* meta-analysis of mRNA and CGH profiling results, we performed an own pilot study including parallel mRNA and CGH profiling on 11 tumour samples and four normal adrenocortical tissues (NA) by microarray-based approaches. By applying the recently published methods for the comparison of simultaneous cDNA microarray and CGH data (Skawran *et al*., 2008), the implication of chromosomal aberrations in adrenocortical tumour pathogenesis could be revealed.

Results

Meta-analysis of gene expression data

The ACA–ACC comparison could be performed with the largest data sets, as malignancy was determined in all studies analysed. By statistical analysis of microarray studies performed by Giordano *et al*. (2009) and Tömböl *et al*. (2009), we have identified 554 and 450 significant gene expression changes between NAs and ACAs, respectively: 46 of these were common. In the same studies, 2509 and 2564 significant gene expression changes between NAs and ACCs were found and 1017 of these were common. Comparison of ACA and ACC groups was possible in three studies: 1929, 1251 and 1904 significant gene expression changes were found in the studies of de Reyniès *et al*. (2009), Giordano *et al*. (2009) and Tömböl *et al*. (2009), respectively: 402 expression changes were common in all (Supplementary Figure 1, Supplementary Table 1). As these microarray studies were performed on different microarray platforms using independent tumour sets, these common genes may be regarded as cross-validated.

Since the clinical profiles of samples were unavailable in one major study, the comparison of hormonal activity-related gene expression profiles between different studies was not feasible in all cases. Common significant gene expression changes in the studies of Giordano *et al*., (2009) and Tömböl *et al*., (2009) were identified: 15 between NAs and IAs, 17 between NAs and CPAs, and only 6 between IAs and CPAs. 305 significant gene expression changes between IAs and cortisol-secreting ACCs and 253 gene expression changes between CPA and cortisol-secreting ACC samples were found (Supplementary Table 2).

Correlation of GSEA results retrieved from publicly available microarray and cytogenetic data

GSEA was performed on NA–ACC and ACA–ACC comparisons. As potential adrenomedullary contamination may interfere with the analysis of normal adrenocortical samples and chromosomal aberrations are more infrequent in ACAs than in ACCs (Kjellman *et al*., 1996) (Supplementary Table 3), we focused on the ACA–ACC comparison. All chromosomal regions found to be differentially enriched by GSEA in the publicly available ACC sample sets have already been published previously (Table 1). All significantly enriched chromosomal regions in these studies were upregulated in ACCs suggesting the frequent gain of these chromosome regions. Chromosome region losses were often observed in ACCs by cytogenetic studies (Supplementary Table 3), however, our GSEA approach failed to reveal downregulated chromosomal regions. This observation highlights the limitations of indirect gene expression and cytogenetic data comparisons, and supports the necessity of simultaneous mRNA and CGH profiling approaches.

Analysis of parallel mRNA and CGH profiling in our own experimental study

With the direct comparison of GSEA and CGH results performed on the same samples, overlaps between chromosome aberrations revealed by CGH and significantly enriched chromosome regions identified by GSEA were established. CGH data are presented in Supplementary Table 4 and accessible at GEO under accession number: GSE17396. Among the 101 aberrant chromosomal regions detected in at least 75% of the investigated ACC samples by CGH, 46 regions were found to be significantly enriched in the same samples by GSEA (Figure 1a and Supplementary Table 5). Despite the low sensitivity (45.54%), the specificity of GSEA to identify aberrant chromosome regions was 84.86%. Among the 3815 genes harboured on these 46 aberrant chromosome regions, we found 1736 genes by applying Leading Edge Analysis and then loaded these genes onto IPA. About 15% of significant gene

Table 1 Comparison of the common GSEA results of the publicly available whole genome mRNA expression data sets and the literature findings on cytogenetic changes in ACCs

Gained chromosomal regions by GSEA	*Literature findings*
CHR5P12 CHR5P15 CHR5Q12 CHR5Q13 CHR5Q14 CHR5Q31 CHR5Q33 CHR5Q35	Kjellman *et al*., 1996; Figueiredo *et al*., 1999; Zhao *et al*., 1999; Dohna *et al*., 2000; Sidhu *et al*., 2002; Zhao *et al*., 2002; Stephan *et al*., 2008
CHR7P21 CHR7P22 CHR7Q22	Dohna *et al*., 2000; Sidhu *et al*., 2002; Zhao *et al*., 2002; Stephan *et al*., 2008
CHR12Q12 CHR12Q13 CHR12Q15 CHR12Q23 CHR12Q24	Kjellman *et al*., 1996; Figueiredo *et al*., 1999; Zhao *et al*., 1999; Dohna *et al*., 2000; Sidhu *et al*., 2002; Zhao *et al*., 2002; Stephan *et al*., 2008
CHR19Q12	Dohna *et al*., 2000; Sidhu *et al*., 2002

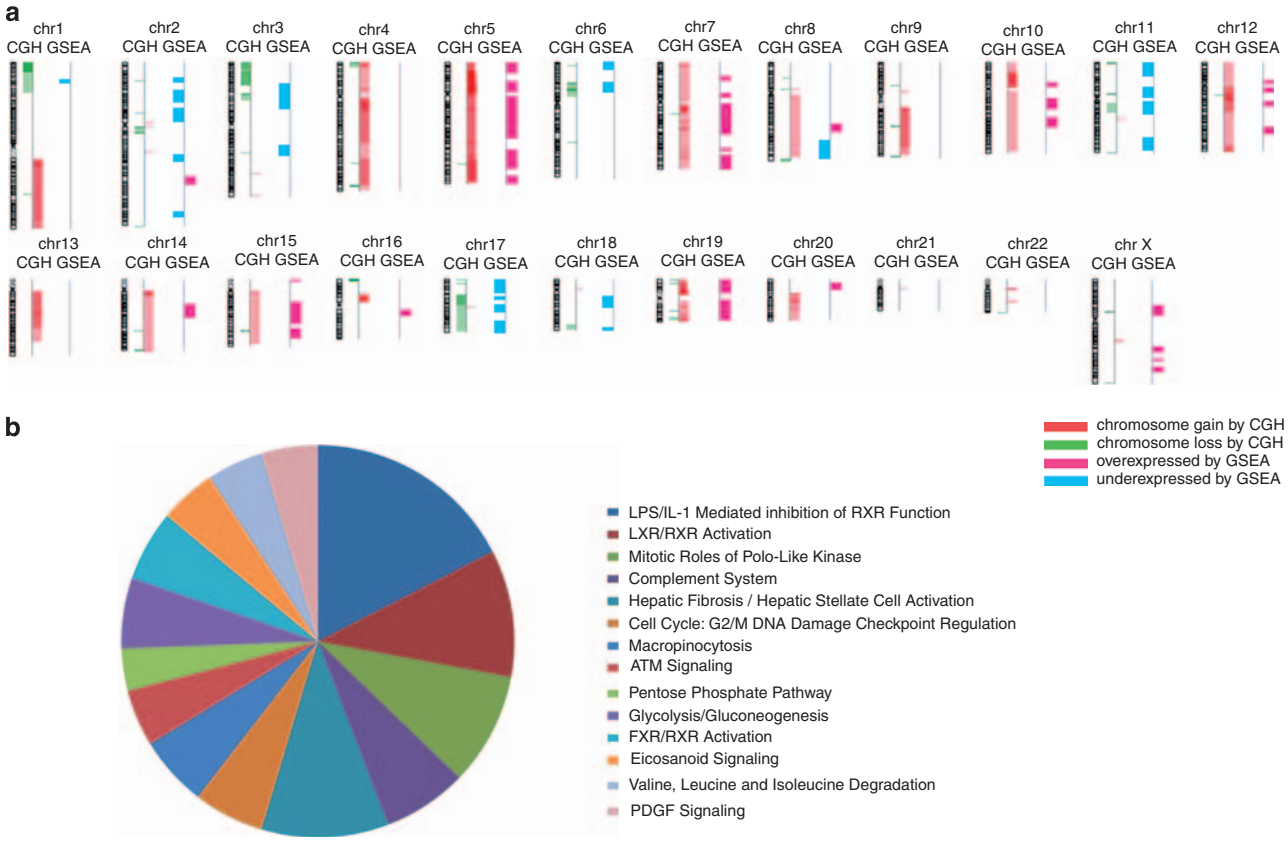

Figure 1 (**a**) Correlation of gene expression changes revealed by GSEA with CGH data in our experimental study. (**b**) Pie diagram representing the distribution of gene expression changes revealed by pathway analysis of publicly available microarray data sets.

expression changes could be related to chromosomal rearrangements.

Pathway analysis

Fifteen canonical pathways have been found to be significantly altered in at least two different microarray studies (Supplementary Table 6). Significant gene sets in NA–ACC and ACA–ACC comparisons overlapped with retinoic acid signalling, cell cycle, complement system and several metabolomic processes (Figure 1b).

IPA analysis of overlapping CGH and GSEA data sets in our experimental study using *in silico* identified leading edge subsets mostly revealed gene expression alterations involved in the regulation of cell cycle G2/M and G1/S checkpoints that were highly concordant with the results of microarray meta-analysis (including cyclins E1 and E2 (*CCNE1* and *CCNE2*), G2/mitotic-specific cyclin-B1 (*CCNB1*), cell division cycle 25 homologue C (*CDC25C*), myc proto-oncogene protein gene (*C-MYC*)).

Discussion

Pathway meta-analysis of available transcriptomics and CGH data as well as pathway analysis performed in our pilot study involving parallel CGH and transcriptomics profiling on the same samples revealed three major pathogenetic pathways that is, (1) damage of cell cycle, (2) retinoic acid signalling (3) antigen presentation and complement system.

Gene Ontology analysis has already been performed in a few microarray studies (Fernandez-Ranvier *et al*., 2008a, b), which identified 25 genes out of 314 at chromosome 11q13 that are mainly involved in the regulation of cellular growth and apoptosis. Giordano *et al*. (2009) associated the list of differentially expressed genes with chromosomal instability and 'functional aneuploidy' that underlines the relevance of parallel gene expression and CGH profiling. The gene expression profiles established by the large-scale study by de Reyniès *et al*. (2009) could be associated with pathways of DNA replication, mitotic cell cycle and immune response.

Whereas several findings support the relevance of cell cycle damage in adrenocortical tumorigenesis, no data have been published on the involvement of retinoic acid signalling to date. Here, we discuss the findings established by our meta-analysis and experimental CGH-transcriptomics study and present a review of these and previous observations.

Cell cycle

Damage of cell cycle has been described in the pathogenesis of several tumours, and there are some

reports describing expression changes of genes involved in G1/S and G2/M transition in ACCs. Overexpression G1 cyclins (cyclin E), and G1 cell division protein kinases (*CDK2, CDK4*) was reported in sporadic ACCs (Bourcigaux *et al*., 2000). Overexpression of *CCNE1* and *CCNE2* was found in four of the investigated microarray studies (Giordano *et al*., 2003, 2009; Tömböl *et al*., 2009; de Reyniès *et al*., 2009). GSEA of microarray studies revealed significant enrichment of overexpressed genes on chr19q12, where *CCNE1* is mapped (Giordano *et al*., 2009; Tömböl *et al*., 2009; de Reyniès *et al*., 2009). Gain of chr19q12 has already been described (Supplementary Table 3). GSEA of microarray studies by de Reyniès *et al*. (2009), Giordano *et al*. (2009) and Tömböl *et al*. (2009) revealed significant enrichment of overexpressed genes on chr12q13, where CDK2 is harboured. Gain of chr12q13 is often found in ACCs (Supplementary Table 3). Stephan *et al*. (2008) reported a 391 *kb*-long segment on chr12q13.2 that correlated with poor survival, *CDK2* is one of the ~25 genes mapped to this chromosome segment.

It is of interest to note that *C-MYC* overexpressed in several other tumours was found to be underexpressed in three microarray data sets analysed (Giordano *et al*., 2009; Tömböl *et al*., 2009; de Reyniès *et al*., 2009). In our direct comparison of CGH and GSEA results, its underexpression was correlated with loss of chr8q24, where *C-MYC* is harboured.

Overexpression of several genes involved in the regulation of G2/M phase transition was noted in ACCs and correlated with chromosome gains: *CCNB1* and cell division protein kinase 7 (*CDK7*) on chr5q13, ubiquitin C (*UBC*) on chr12q24, and Mdm2 p53-binding protein homologue (*MDM2*) on chr12q15 (Giordano *et al*., 2003, 2009; Tömböl *et al*., 2009; de Reyniès *et al*., 2009). Overexpression of *CCNB1* in ACCs was validated in the recent study by Soon *et al*. (2009) as well.

Several other members of G2/M transition were significantly overexpressed in ACCs without corresponding chromosomal alterations in at least three microarray studies, including *CCNB2*, *CDC2*, *CDC25B* and topoisomerase II alpha (*TOP2A*) (Giordano *et al*., 2003, 2009; Tömböl *et al*., 2009; Soon *et al*., 2009; de Reyniès *et al*., 2009). Overexpression of *TOP2A* has already been reported and considered as a diagnostic marker for ACCs (Giordano *et al*., 2003). TOP2A is a major target for a variety of antineoplastic agents, including etoposide and doxorubicin used in ACC chemotherapy (Patalano *et al*., 2009). Resistance to TOP2A targeting drugs in cell lines has been associated with reduced expression of the protein, mutations of binding sites and aberrant localization in the cytoplasm. Exportin (*XPO1*) mediates nuclear export of several proteins including TOP2A. In ovarian cancer, high cytoplasmic immunostaining of TOP2A was linked to *XPO1* overexpression and associated with borderline significance for decreased overall survival (Faggad *et al*., 2009). We have observed significant overexpression of *XPO1* in ACCs in two studies (Giordano *et al*., 2009; de Reyniès *et al*., 2009), however, aberrant cytoplasmic expression of TOP2A has not been reported yet in ACCs (Figure 2).

Retinoic acid signalling

Retinoids are specific ligands of retinoid acid receptors (RARs) and retinoid X receptors (RXR). RARs bind both all-trans retinoic acid and 9-*cis* retinoic acid while RXRs bind only 9-*cis* retinoic acid (Shimizu *et al*., 2009). During embryonic development, the adrenal gland may be the most important source of serum retinoic acid before liver maturation (Haselbeck *et al*., 1997). Retinoic acid concentration in the adult adrenal cortex is about half of that found in the liver, that may be by far sufficient for intraadrenal RAR and RXR activation (Haselbeck *et al*., 1997). Retinoids are involved in the pathogenesis of several tumours, and are used for cancer therapy and prevention. 9-*cis* retinoic acid treatment inhibited the growth of the human adrenocortical cancer cell line NCI-H295 (Ferruzzi *et al*., 2005).

The second oxidation step of retinoic acid synthesis catalysed by aldehyde dehydrogenase 1A enzyme family members (*ALDH1A1-3*) is rate-limiting (el Akawi and Napoli, 1994). We found decreased *ALDH1A1* and *ALDH1A3* mRNA expression in ACC relative to ACA in several microarray studies (Giordano *et al*., 2003, 2009; Velázquez-Fernández *et al*., 2005; Laurell *et al*., 2009; Soon *et al*., 2009; de Reyniès *et al*., 2009). Decreased *ALDH1A1* has been validated by QRT-PCR in two studies (Fernandez-Ranvier *et al*., 2008b; Laurell *et al*., 2009). Significant underexpression of RXR alpha (*RXRA*) was observed in ACCs in one study (Tömböl *et al*., 2009). By the direct comparison of CGH and GSEA results in our experimental study, decreased expression of RXR beta (*RXRB*) could be correlated with the loss of chr6p21 where *RXRB* is harboured. Based on these findings, retinoic acid production and action might be reduced in ACC, but this hypothesis awaits experimental confirmation.

Cholesterol and lipid metabolism: RXR/LXR and RXR/PPARG signalling

Retinoic acid bound RXRs may heterodimerize with other nuclear receptors including liver x receptors (LXR) and peroxisome proliferator-activated receptors (PPAR) (Shimizu *et al*., 2009). Since these receptors are involved in the regulation of drug metabolism and transport, changes in their concentrations may affect the effective drug dose during tumour therapy or may even be involved in adrenocortical carcinogenesis (Scripture *et al*., 2005). The adrenal gland expresses LXRA and LXR beta (LXRB, *NR1H2*). LXRs have pivotal roles in adrenal cholesterol metabolism, and influence steroid hormone production (Cummins *et al*., 2006). LXR alpha (LXRA, *NR1H3*) was significantly underexpressed in ACCs in three studies (Giordano *et al*., 2009; Tömböl *et al*., 2009; de Reyniès *et al*., 2009) that may be related to the already described loss of chr11p11 (Figure 3; Supplementary Table 3).

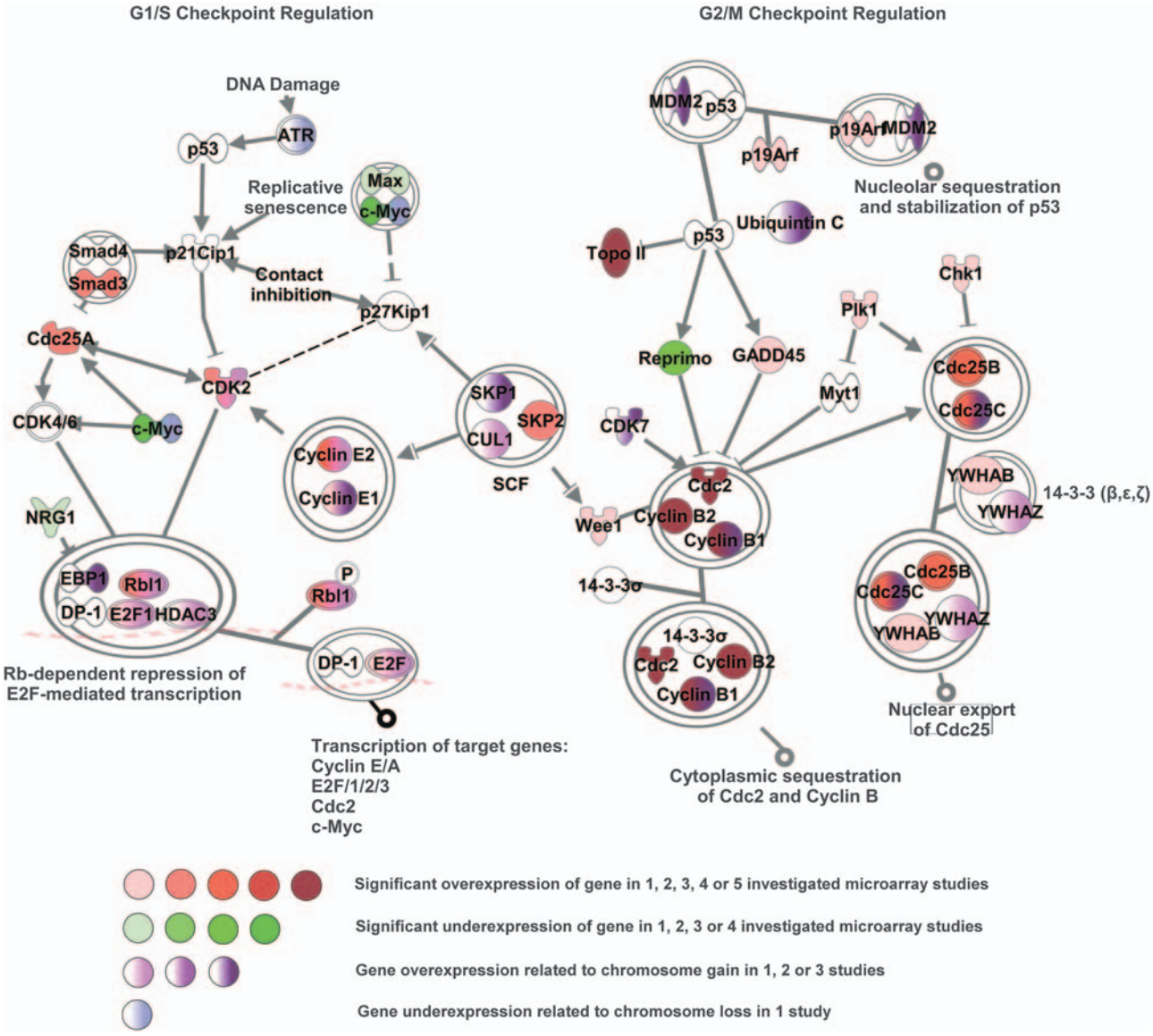

Figure 2 Pathways with altered gene expression patterns in the regulation G1/S and G2/M checkpoint in ACCs.

PPAR-γ (PPARG) regulates expression of genes involved in glucose and lipid homeostasis and is implicated in the pathogenesis of several tumours (Ondrey, 2009). PPARG is expressed in normal adrenals, benign and malignant ACTs (Betz *et al*., 2005), and in the NCI-H295 cell line (Ferruzzi *et al*., 2005). Treatment of NCI-H295 cells with PPARG activator thiazolidinediones decreased growth, invasiveness and induced their differentiation. Co-treatment with 9-*cis* retinoic acid and thiazolidinedione had additive effects *in vitro* (Betz *et al*., 2005; Ferruzzi *et al*., 2005). Expression of several genes targeted by LXRA and PPARG has been confirmed in the adrenal cortex, including ATP-binding cassette A1 and G1 (*ABCA1, ABCG1*), apolipoprotein E and C1 (*APOE, APOC1*), sterol regulatory element-binding transcription factor 1 (*SREBF1*), scavenger receptor-B1 (*SCARB1*) (Cummins *et al*., 2006).

We have observed the underexpression of all these target genes in ACC. Overexpression of the LXRA target *CD36* (thrombospondin receptor) gene was revealed in two studies (Tömböl *et al*., 2009; de Reyniès *et al*., 2009). Significant enrichment of overexpressed genes was found on chr7q21 by GSEA, where the *CD36* gene is located. The gain of chr7q21 has been described previously (Supplementary Table 3).

Overexpression of *CD36* may result in enhanced cholesterol influx from high and low density lipoprotein molecules (HDL and LDL) to adrenocortical cells, however, underexpression of SCARB1 that was observed by Slater *et al*., (2006) may interfere with cholesterol influx from HDL. Other genes involved in cholesterol efflux including *ABCA1* and *ABCG1* showed significant underexpression in several studies (Velázquez-Fernández *et al*., 2005; Giordano *et al*., 2009; Tömböl *et al*., 2009; Soon *et al*., 2009; de Reyniès *et al*., 2009).

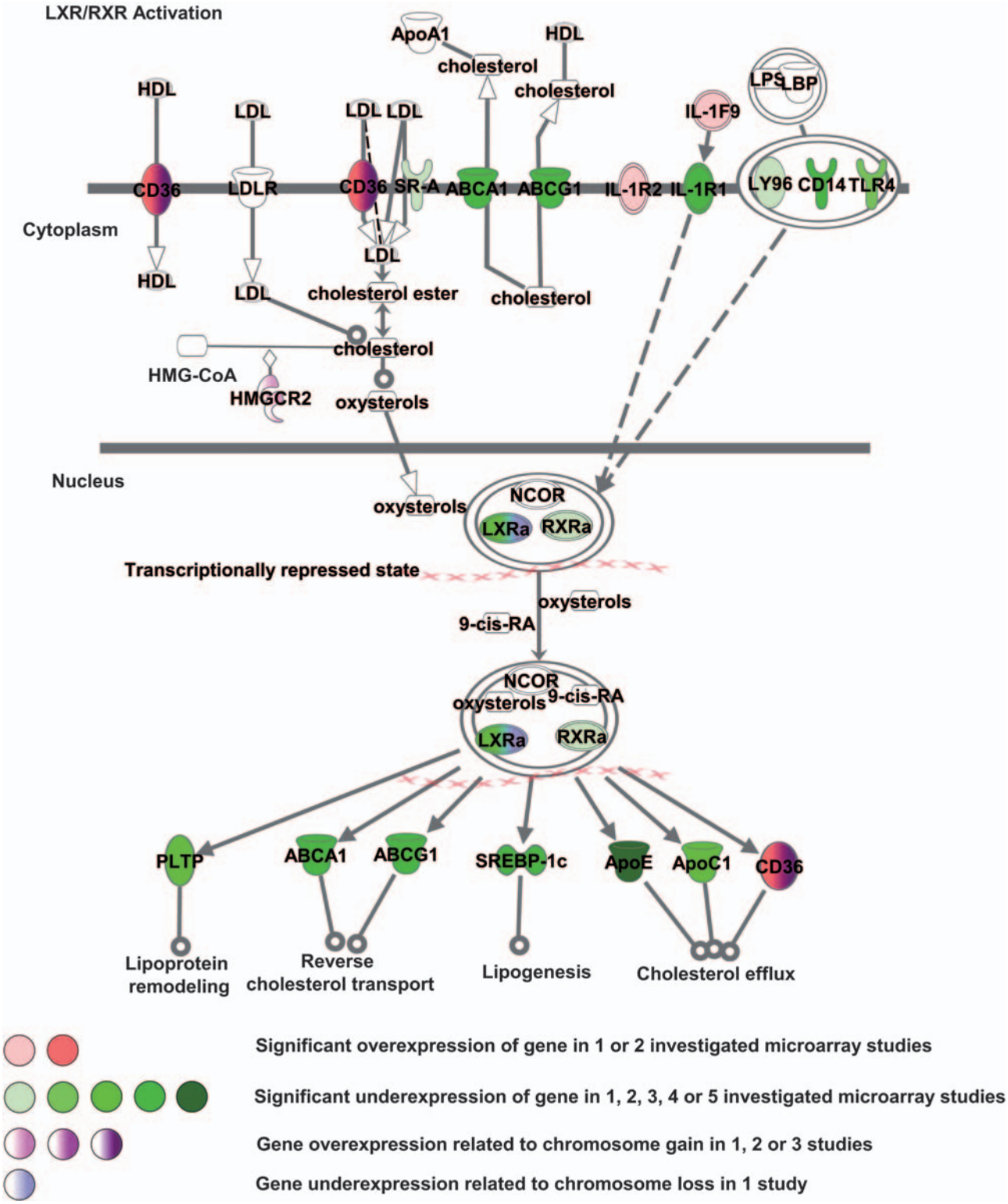

Figure 3 Pathways with altered gene expression patterns involved in LXR/RXR signalling in ACCs.

SREBF1 (a key regulator of genes involved in fatty acid synthesis and production of ester substrates) expression was found to be significantly lower in ACCs in four studies (Giordano *et al*., 2003, 2009; Tömböl *et al*., 2009; de Reyniès *et al*., 2009). APOE enhances cholesterol ester accumulation in adrenocortical cells (Thorngate *et al*., 2002). *APOE* was significantly underexpressed in ACCs in four adult microarray studies (Giordano *et al*., 2009; Tömböl *et al*., 2009; Soon *et al*., 2009; de Reyniès *et al*., 2009) and in childhood ACCs, as well (West *et al*.,

2007). These gene expression alterations affecting proteins are involved in the regulation of adrenocortical cholesterol and lipid metabolism may be related to the observation that ACCs are relatively lipid-poor in comparison with their benign counterparts (Figure 3).

Bacterial lipopolysaccharide (LPS) recognition via Toll-like receptor 4 (TLR4)

The TLR receptor family is related to interleukin 1 receptor (IL-1R) family. LPS-mediated TLR4 activation affects retinoic acid signalling, as it suppresses RXRA-mediated gene expression in the liver (Ghose *et al*., 2004). Several other molecules are required for the recognition of LPS by TLR4 including CD14 and lymphocyte antigen 96 (LY96) (Pålsson-McDermott and O'Neill 2004). TLR4 is expressed on human adrenocortical cells, and it is involved in immune-neuroendocrine crosstalk (Zacharowski *et al*., 2006). LPS directly stimulates cortisol secretion by NCI-H295 cells (Vakharia and Hinson, 2005), and IL-6 and IL-8 expression by human adrenocortical cells (Kanczkowski *et al*., 2009). We found significant underexpression of *TLR4* in ACCs in two studies (Giordano *et al*., 2009; Tömböl *et al*., 2009) and confirmed *TLR4* expression changes by QRT–PCR (data not shown). *CD14* and *LY96* expression was decreased in several studies.

The proinflammatory cytokine IL-1 may induce the activation of hypothalamic–pituitary–adrenal axis (Turnbull and Rivier, 1999). IL-1-mediated signalling is triggered by the binding of IL-1 to interleukin-1 receptor type 1 (IL1R1), whereas interleukin-1 receptor type 2 (IL1R2) is regarded as a decoy receptor (Subramaniam *et al*., 2004). We observed significant underexpression of *IL1R1* in ACCs in four studies (Giordano *et al*., 2003, 2009; Slater *et al*., 2006; de Reyniès *et al*., 2009), and the overexpression of *IL1R2* in one (Tömböl *et al*., 2009).

Complement system and antigen presentation

Alterations of complement system member gene expression were often observed in the investigated microarray studies. Significant underexpression of complement components involved in both classical and alternate routes of activation, for example, complement 1 q subcomponent, A and B chains (*C1QA*, *C1QB*), complement factors D and H (*CFD*, *CFH*) etc. was observed in three studies (Giordano *et al*., 2009; Tömböl *et al*., 2009; de Reyniès *et al*., 2009). *SERPING1* (serpin peptidase inhibitor, clade G (C1 inhibitor), member (1) underexpression was validated by QRT–PCR and it is considered as a potential prognostic marker of malignancy (Fernandez-Ranvier *et al*., 2008a, b) (Supplementary Figure 2).

Major histocompatibility complex (MHC) class II molecules are involved in the presentation exogenous antigens in the immune system, and are expressed by the zona reticularis of the human adrenal cortex (Wolkersdörfer *et al*., 2005). ACCs lack MHCII proteins and MHCII expression is regarded as a marker for benign lesions (Marx *et al*., 1996). We have observed underexpression of several MHCII antigens in ACCs (Supplementary Table 1) (Giordano *et al*., 2009; Tömböl *et al*., 2009; de Reyniès *et al*., 2009). By the direct comparison of GSEA and CGH results in our experimental study, we have found that MHCII underexpression correlated with the loss of chr6p21 where the cluster of these genes is harboured.

Significant underexpression of MHCII genes was observed in cortisol-producing ACTs compared with hormonally inactive ACTs. Beside the downregulation of MHCII antigens in CPA samples, overexpression of MHCI class I B (HLA-B) was observed in the study by Giordano *et al*. (2009). These expression changes might be related to a shift from MHCII to MHCI-mediated antigen presentation. The pathophysiological relevance of these findings is unclear.

It is hard to interpret these findings in the lack of data on the physiological or pathological relevance of these genes in adrenocortical functioning or tumours. Altogether, alterations of cytokine signalling, complement system and antigen presentation pathways suggest the involvement of immune mechanisms in ACT pathogenesis.

In conclusion, three major pathogenetic pathways were revealed both by the meta-analysis of available mRNA profiling and cytogenetic data and by our pilot study involving mRNA and CGH profiling on the same samples. The observation that the same pathways were found by both approaches supports the feasibility of *in silico* meta-analysis. As pathway analysis represents a more comprehensive form of analysis than the molecular studies of individual gene expression and cytogenetic alterations, this approach may reveal novel, previously unknown pathogenic pathways. As the therapeutic repertoire for the treatment of adrenocortical cancer is very limited, our results may pave the way for further *in vitro* studies aimed at the development of novel agents affecting these pathways.

Materials and methods

Meta-analysis of available gene expression data sets

Data sets. Whole genome raw mRNA expression data of 164 tumour (97 ACA and 67 ACC) and 18 NA samples from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) and ArrayExpress (http://www.ebi.ac.uk) were collected (Ye *et al*., 2007; de Reyniès *et al*., 2009, Giordano *et al*., 2009, Tömböl *et al*., 2009), reclassified and analysed (Table 2). Data on hormonal activity were unavailable in the study by de Reyniès *et al*., (2009).

Further significant gene sets from studies where raw gene expression data were unavailable have been obtained from Oncomine Research (http://www.oncomine.org) (Giordano *et al*., 2003) and seven papers found by literature search (PubMed, http://www.ncbi.nlm.nih.gov/pubmed) (Table 3). These included 30 NA, 182 ACA and 91 ACC samples. Characteristics of these gene sets are presented in Table 3. Cytogenetic (CGH and FISH) data on adrenocortical tumours were acquired from 10 publications found by literature search (Kjellman *et al*., 1996; Figueiredo *et al*., 1999, 2005; Russell *et al*., 1999; Zhao *et al*., 1999, 2002; Dohna *et al*., 2000; Sidhu

Table 2 Characteristics of the analyzed microarray data sets

Study	*Accession no.*	*Platform*	*Hormonal activity of samples*	*Tumour samples*
Ye *et al*., 2007	GEO: GDS2860	Affymetrix human genome U133 Plus 2.0 Array	5 NA	
			10 APA	10 ACA
de Reyniès *et al*., 2009	ArrayExpress: E-TABM-311	Affymetrix Human Genome U133 Plus 2.0 Array	Undefined	58 ACA
				34 ACC
Giordano *et al*., 2009	GEO: GSE10927	Affymetrix Human Genome U133 Plus 2.0 Array	9 NA	
			9 IA	21 ACA
			5 CPA	
			7 APA,	
			17 hormonally inactive ACC	29 ACC
			12 hormone-secreting ACC	
Tömböl *et al*., 2009	GEO: GSE14922	Agilent Whole Human Genome 4 × 44K Array	4 NA	
			4 IA	8 ACA
			4 CPA	
			4 hormone-secreting ACC	4 ACC

Table 3 Characteristics of significant gene sets in publications where raw whole genome microarray data were unavailable

Study	*Distribution of tumours*	*Description of gene set*	*No. genes*
Giordano *et al*., 2003	3 NA, 4 ACA, 11 ACC	List of significant ($P<0.05$ fold change 1.5 ×) gene expression changes between ACC and non-ACC (NAs and ACA) groups (http://oncomine.org)	192
Velázquez-Fernández *et al*., 2005	13 ACA, 7 ACC	The 15 most significant up- and down-regulated genes in ACC compared to ACA	30
Slater *et al*., 2006	10 NA, 10 ACA, 10 ACC	Genes found to be differentially expressed in ACA relative to NA	42
		Genes found to be differentially expressed in ACC relative to NA	148
		Genes found to be differentially expressed in ACC relative to ACA	74
West *et al*., 2007	7 NA, 5 ACA, 18 ACC	Genes found to be differentially expressed in pediatric ACTs (ACC and ACA) relative to NA	1019
		Genes found to be differentially expressed in pediatric ACCs relative to ACA	52
Fernandez-Ranvier *et al*., 2008a	43 ACA, 11 ACC	Genes of chr. 11q13 with significant differences in expression by cDNA microarray between ACC and ACA	25
Fernandez-Ranvier *et al*., 2008b	74 ACA, 11 ACC	Result of QRT-PCR validation of genes with significant differences in expression by microarray between ACC and ACA.	19
Laurell *et al*., 2009	4 NA, 17 ACA, 11 ACC	List of significant ($P<0.05$ fold change 2 ×) gene expression changes between non-ACC (NAs and ACAs) and ACC	262
		List of significant ($P<0.05$ fold change 2 ×) gene expression changes between IA and APA	378
		List of significant ($P<0.05$ fold change 2 ×) gene expression changes between CPA and APA	377
Soon *et al*., 2009	6 NA, 16 ACA, 12 ACC	Top ranking significant genes ($P<0.05$) between ACA and ACC (100 up- and 77 down-regulated)	177

et al., 2002; Bertherat *et al*., 2003; Stephan *et al*., 2008). These included 87 ACA and 124 ACC samples (Supplementary Table 3). The CGH data set presented by Stephan *et al*. (2008) was accessible at GEO, and these samples were reclassified and reanalysed by applying DNA Analytics software (version 4.0.85, Agilent Technologies, Santa Clara, CA, USA).

Altogether, gene expression and cytogenetic data from 42 NAs, 350 ACA and 270 ACC samples were available.

Statistical analysis of mRNA profiling studies. Identification of gene sets differentially expressed among NA, ACA and ACC groups and among NA, IA, CPA, APA, hormone-secreting and hormonally inactive ACC groups was carried out by one-way ANOVA. Comparison of hormone-secreting and hormonally inactive benign and malignant ACTs was performed by a two-way ANOVA. Each ANOVA was followed by Tukey's *post hoc* test for all pairwise multiple comparisons. An unpaired *t*-test was used for the NA–APA and ACA–ACC comparisons for two studies where only these two groups were examined (Ye *et al*., 2007; de Reyniès *et al*., 2009). Fold change filter was set to 2-fold. Furthermore, Benjamini–Hochberg multiple testing correction was performed in each analysis to minimize false-positive cases. All statistical analyses were performed by Genespring GX 10.0 Software (Agilent Tech Inc., Santa Clara, CA, USA) Supplementary Table 7 summarises the statistical tests applied.

Common significant expression changes between different studies were collected by an own programme written in C++ programme language by applying Code::Blocks software (Code::Blocks, Release 8.02, www.codeblock.org).

Gene set enrichment analysis (GSEA). GSEA is a novel computational approach developed for the analysis of fine gene expression alterations: it analyzes gene expression data by rank statistics, and determines whether a particular set of genes is over- or underrepresented in the samples compared (Subramanian *et al*., 2005). GSEA was performed by GSEA Software v2.0 (www.broad.mit.edu) in pairwise comparisons of NA vs ACA, NA vs ACC and ACA vs ACC. Gene expression results derived from microarray experiments were

correlated with chromosome gene sets. GSEA was performed using gene set permutation type as default and the number of permutation was set to 1000. Statistical significance levels were defined as nominal P-value <0.05 and false discovery rate (FDR) <0.25.

Correlation of GSEA results retrieved from publicly available microarray and cytogenetic data. To identify genes with the most relevant expression changes on an aberrant chromosome region, Leading Edge Analysis using GSEA Software v2.0 (www.broad.mit.edu) was performed. The leading edge subset of genes can be interpreted as the core that accounts for the significant chromosomal gene set's enrichment signal. By this approach, genes harboured on aberrant chromosome regions showing the most prominent expression changes could be identified. These gene sets were loaded onto Ingenuity Pathway Analysis (IPA) to identify pathways affected by chromosomal aberrations in ACTs.

Pilot study on ACTs involving parallel mRNA and CGH profiling

CGH was performed on 4 ACC and 7 ACA (4 IA and 3 CPA) samples that have already been studied by mRNA profiling by Tömböl *et al*. (2009) (GEO accession number: GSE14922). The study was approved by the Ethical Committee of the Hungarian Health Council, and informed consent was obtained from all patients involved. Tissue digestion, labelling, hybridization and data analysis of genomic DNA were performed according to the Agilent Technologies (Santa Clara, CA, USA) protocol version 2.0 for 105K arrays. The sample and reference (G1521 female DNA, Promega, Madison, WI, USA) CGH105A oligo microarray slides were scanned with Agilent Microarray scanner and data were extracted with Feature Extraction software version 9.5.1.1 including dye normalization (linear lowess). DNA Analytics software was used (version 4.0.85, Agilent Technologies, Santa Clara, CA, USA) for data analysis. The starting and ending points of the aberrations were confirmed by the ADM-2 algorithm with 6.0 threshold.

Gene expression alterations paralleling chromosome aberrations were searched for as previously described in the meta-analysis section. By applying Leading Edge Analysis, genes showing the highest gene expression alterations on aberrant chromosome regions could be selected and these were loaded onto IPA.

Pathway analysis

Ingenuity Pathways Analysis Knowledge Base is updated with current results published in journal articles and other data sources. By applying the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, www.ingenuity.com; Redwood City, CA, USA), gene sets can be functionally annotated and biologically relevant pathways may be identified.

Gene sets established both by meta-analysis of mRNA, GSEA and CGH data sets and by our pilot experimental study were subjected to IPA and results were compared with each other.

Conflict of interest

The authors declare no conflict of interest.

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