Published in final edited form as:

Genes Chromosomes Cancer. 2014 May ; 53(5): 381–391. doi:10.1002/gcc.22149.

Clear Cell Sarcoma of the Kidney Demonstrates an Embryonic Signature Indicative of a Primitive Nephrogenic Origin

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Abstract

Clear cell sarcoma of the kidney (CCSK) is a tumor affecting children with a median age of 3 years at diagnosis. The cell of origin of CCSK is unknown and data on the molecular changes giving rise to CCSK is scarce. This has hindered the identification of positive diagnostic markers and development of molecularly targeted treatment protocols for CCSK. We have characterized a panel of CCSK to gain information regarding its molecular profile and possible origin. High-resolution genomic analysis with single nucleotide polymorphism array of 37 tumors did not reveal any clues to the mechanisms behind tumor development as remarkably few genetic imbalances were found. Gene expression analysis revealed a highly characteristic gene signature, enriched for pathways involved in embryonic development, including kidney formation. The presence of markers for two different developmental lineages in the embryonic kidney was therefore investigated in the tumor cells. *FOXD1* which identifies cells giving rise to stromal elements, and *CITED1*, a marker for cells primed for nephrogenic epithelial differentiation, were both highly expressed in CCSK. In addition, the early embryonic marker *OSR1* was expressed at higher levels in CCSK than in Wilms tumor, normal fetal kidney or adult kidney. As this marker discriminates the intermediate mesoderm from other mesodermal structures, our study could suggest that CCSK arises from a mesodermal cell type that retains the capacity to initiate differentiation towards both nephrons and stroma, but remains locked in a primitive state.

INTRODUCTION

Clear cell sarcoma of the kidney (CCSK) is a rare pediatric tumor affecting infants and children at a median age of 36 months at diagnosis (Argani et al., 2000). CCSK was for a long time associated with poor prognosis and the occurrence of late metastases, but the

overall survival has improved over the last decades and has now reached 86%, partly because of the addition of doxorubicin to the treatment protocol (Argani et al., 2000; Seibel et al., 2004). The classic microscopic pattern of CCSK includes cords of round or spindle-shaped cells with clear cytoplasm and ovoid to rounded vesicular nuclei with inconspicuous nucleoli. The cells are surrounded by fibrovascular septa ranging from a thin “chicken-wire” like arrangement to broad sheets containing an arborizing capillary vasculature (Argani et al., 2000). This classic pattern is found at least focally in the majority of the tumors, but is often mixed with other histological components such as (in descending order of frequency) myxoid, sclerosing, cellular, epithelioid, palisading, spindle-cell, storiform, and anaplastic elements (Argani et al., 2000).

Little is known about the pathogenesis of CCSK. Regarding potentially causative somatic genetic rearrangements, two comparative genomic hybridization studies of CCSK have been performed; one found imbalances of 1q and 11q in one case out of four (Barnard et al., 2000) and another study including 30 CCSKs revealed genomic imbalances in only four cases, including gains of 1q and 19p, and losses of 4p, 10q, and chromosome 19 (Schuster et al., 2003). A recurrent finding in CCSK is a balanced translocation, t(10;17)(q22;p13) (Douglass et al., 1985; Punnett et al., 1989; Sheng et al., 1990; Kaneko et al., 1991; Rakheja et al., 2004; Brownlee et al., 2007) and recently a fusion transcript of *FAM22* in 10q22 and *YWHAE* in 17p13.3 was found in 7 of 51 CCSKs analyzed (O’Meara et al., 2012). For the majority of CCSKs, no somatic mutations potentially contributing to their pathogenesis have been found. Previous studies of genomic imbalances in CCSK were performed on platforms unable to detect copy number neutral imbalances (CNNI) and could not reveal the putative presence of pathogenetically significant allelic imbalances of this type. In comparison, such imbalances are known for Wilms tumor, as exemplified by CNNI 11p (Rivera and Haber, 2005).

The cellular origin of CCSK is furthermore puzzling, as it has a histological appearance absolutely nonreminiscent of kidney. A global gene expression analysis of CCSK compared to Wilms tumor and fetal kidney revealed that enriched genes in CCSK were mainly involved in neuronal processes and in the AKT-signaling pathway (Cutcliffe et al., 2005). In addition, the sonic hedgehog pathway was highly represented, which could indicate a primitive embryonic origin of this tumor. The embryonic kidney develops from two structures derived from the intermediate mesoderm: the nephric duct and the metanephric mesenchyme (Dressler, 2009). The nephric duct grows caudally in the developing fetus, and in the proximity of the metanephric mesenchyme, a ureteric bud is formed that grows into the metanephric mesenchyme and starts to branch. Cells in the metanephric mesenchyme condense around the tips of the ureteric bud and form the cap mesenchyme, a self-renewing nephron progenitor population (Kobayashi et al., 2008). A portion of the cells in the cap mesenchyme forms pretubular aggregates, which undergo mesenchymal to epithelial transition and form the nephrons via transient comma- and s-shaped structures. Another cell population in the metanephric mesenchyme constitutes the progenitors of the stromal lineage forming for example the renal capsule, the medullary interstitium, and the mesangial cells (Hendry et al., 2011). Markers for nephrogenic progenitor populations have mainly been identified from studies in mice. For example, *Osr1* specifies the intermediate mesoderm

from the other structures derived from the lateral mesoderm and is essential for formation of the metanephric kidney (Wang et al., 2005; James et al., 2006; Mugford et al., 2008). The self-renewal capacity of the cap mesenchyme is, in turn, dependent on the expression of Six2 (Self et al., 2006; Kobayashi et al., 2008) and cells positive for Six2 and Cited1 define the nephron progenitor compartment (Mugford et al., 2009). When cells are differentiating into nephrons, the expression of Cited1 is down regulated while Six2 persists in primitive tubules (Mugford et al., 2009). Foxd1 defines the niche of the metanephric mesenchyme that gives rise to the stromal lineage (Hatini et al., 1996; Levinson et al., 2005).

By SNP-array, gene expression array, and immunofluorescence analyses we have characterized a panel of CCSKs to gain information regarding the genetic background and possible embryonic origin of this tumor type. Taken together we suggest that CCSK is derived from a mesodermal cell type, which in a nephrogenic context appears to retain the potential to differentiate into both nephrons and stroma, but remains arrested at a primitive developmental stage.

MATERIALS AND METHODS

Tumors and Kidney Tissue

The study was reviewed and approved by the regional ethics committee (L119-03) and by the review boards of the participating institutes. Parental written informed consent was obtained prior to the study. Frozen tissue from CCSKs were obtained from Children's Oncology Group (COG) of North America (35 cases) and from Skåne University Hospital, Lund, Sweden (2 cases) after histopathological review. The age at diagnosis ranged from 3 months to 9 years, with a median age of 1 year, and a male to female ratio of 3.8:1. Paraffin-embedded material was available from the two CCSKs from Department of Pathology, Lund, and from six additional CCSKs received from Our Lady's Children's Hospital, Crumlin, Ireland. Five mixed type Wilms tumors (Skåne University Hospital, Lund, Sweden) were used as comparison for quantitative PCR analyses. Information regarding age and sex of the patients is listed in Supporting Information Table 1. Sections from paraffin-embedded anonymized fetal kidneys (gestational weeks 12-13, 16, and 19), and frozen non-neoplastic kidney tissue (six adult nephrectomy patients used for RNA extraction), were obtained from the biobank at Department of Pathology at Lund University (Ethics Approval L2012-405). Total RNA from normal fetal kidneys was included: samples from two different female fetuses, gestational week 30 (BioChain, Hayward, CA), pooled kidney RNA from two male fetuses, at week 18 and week 22 (Stratagene/Agilent Technologies, Santa Clara, CA) and pooled RNA from 34 male and female fetal kidneys, at weeks 12-31 (Clontech, Mountain View, CA). An external gene expression dataset (GSE30946), analyzed with Affymetrix U133A (Gadd et al., 2012), was used for validation of CCSK gene expression. It contained 16 CCSKs, 15 Wilms tumors, 10 rhabdoid tumors, 12 cellular congenital mesoblastic nephromas, and two infantile fibrosarcomas.

Whole Genome Genotyping by SNP Arrays

DNA was extracted from fresh frozen CCSKs with the DNeasy Blood & Tissue Kit, (Qiagen, Valencia, CA), according to the manufacturer's recommendations. To investigate

genomic imbalances at a high level of resolution, DNA was analyzed on a 1M Genotyping BeadChip SNP-array (Illumina, San Diego, CA) containing approximately 1 million genomic markers, with a median spacing of 1.5 kb. Data were analyzed according to standard methods, detailed in Supplementary Materials and Methods.

RNA Extraction and Gene Expression Array

RNA was extracted from CCSKs, Wilms tumors and adult kidney tissue with the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA) followed by On-column DNase Digestion (Qiagen). RNA quality was assessed with an Agilent Bioanalyzer and RNA with an RNA integrity number (RIN) above six was hybridized to HumanHT-12 v4 Expression BeadChips (Illumina). Data quality control and quantile normalization was performed with the GenomeStudio software (Illumina Inc.). Gene expression data are available in the Gene Expression Omnibus database, <http://www.ncbi.nlm.nih.gov/geo/>, accession number GSE49972.

Statistical Analyses

Gene expression data were investigated by principal component analysis (PCA) plots and hierarchical clustering (HCL) (Qlucore Omics Explorer Software v2.3, Qlucore AB, Lund, Sweden). Data were log₂-transformed and normalized by setting the mean to 0 and σ to 1. Variables contributing to less than 5% of the variance of the most differentially expressed variable were removed by filtering the data on the basis of $\sigma/\sigma_{\max}$. HCL was done using the Euclidean metric and the Average linkage criteria. Gene set enrichment analysis (GSEA) and analysis with the Database for Annotation, Visualization and Integrated Discovery (DAVID), are described in Supplementary Materials and Methods.

A list with totally 54 genes of established importance in the development of nephrogenic structures (intermediate mesoderm, metanephric mesenchyme, cap mesenchyme, ureteric bud, renal vesicles, pretubular aggregates, stromal cells, comma, and s-shaped structures) was generated from previous reports (Dressler, 2009; Hendry et al., 2011; Faa et al., 2012) (Supporting Information Table 2). The interaction of these gene sets with the CCSK datasets was evaluated by HCL in Qlucore Omics Explorer Software. Reporters for all 54 genes were present in the Illumina expression array used, while these were lacking for seven of the genes (*DLL1*, *FGFR1*, *HOXB4*, *OSR1*, *PCSK9*, *ROBO2* and *WNT9B*) in the external GSE30946 data set (Gadd et al., 2012).

Quantitative Real-Time PCR

High quality total RNA could be extracted from 15 tumors (Supporting Information Table 1). RNA was converted to cDNA with random hexamers and Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA). TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad, CA) was used to determine gene expression (Supporting Information Table 3). The expression levels were normalized to the expression of three housekeeping genes (*SDHA*, *ALAS1*, and *ACTB*) (Mengelbier et al., 2010). The comparative Ct method was used to determine relative gene expression (Vandesompele et al., 2002). The expression of *OSR1*, *CITED1*, and *FOXD1* were visualized as a heat map generated with Multi Experiment Viewer, v.4.8.1 (Saeed et al., 2003).

Immunofluorescence

Immunofluorescence on tissue sections was performed according to standard methods, as detailed in Supplementary Materials and Methods.

RESULTS

CCSK Displays Few Allelic Imbalances

To detect allelic imbalances of potential importance to CCSK pathogenesis, high resolution whole genome genotyping SNP-array was performed on 37 CCSKs. Except for known copy number variants, eleven cases (30%) showed no imbalances. The majority of the remaining tumors only displayed minor segmental changes (Fig. 1 and Supporting Information Table 4). The most frequent allelic imbalances found in CCSK were partial or complete gains or losses of chromosome arms 1q, 10q, or 17p, present in six cases (CCSK3, CCSK9, CCK19, CCSK29, CCSK30, and CCSK37). No tumor showed aberrations in all of these three chromosome arms (Table 1). The aberrations in CCSK29 overlapped with the previously described breakpoints for the t(10;17)(q22;p13) translocation, and this case has been shown to contain a *YWHAЕ-FAM22* fusion transcript (O'Meara et al., 2012). All patients with tumors showing an absence of 1q, 10q or 17p aberrations ($n = 24$) were alive after five years, whereas two out of six with aberrations on these chromosomes were deceased, indicating a correlation between these aberrations and short-term survival ($P < 0.05$, Fisher's exact test). However, no statistically significant difference between the groups was found when analyzing 10 years' progression-free and overall survival, as two out of 24 patients without aberrations died during this time period.

CCSK Displays a Uniform Gene Expression Profile

In order to distinguish CCSK specific gene expression profiles, a gene expression array was performed on the 22 out of the 37 tumors yielding RNA with sufficient quality (Supporting Information Table 1). In comparison with the gene expression profile of six normal adult kidneys and four fetal kidneys, the CCSKs formed a distinct cluster by PCA. The gene expression profile for CCSK was more similar to fetal kidney than adult kidney (Fig. 2A). The above-mentioned imbalances in 1q, 10q, and 17p had some impact on the global gene expression pattern (Fig. 2B), in part through a direct effect of these aberrations on the expression of genes located in the affected genomic segments (data not shown). However, cases with these segmental aberrations did not form a distinct subgroup.

CCSKs are Enriched for Embryonic Signaling Pathways

CCSK displayed a significantly distinct gene expression signature compared with normal fetal and adult kidney, as shown by 195 up-regulated and 162 down-regulated gene sets ($FDR < 0.25$), as demonstrated by GSEA (Supporting Information Table 5). Seven out of 13 up-regulated gene sets in CCSK ($FDR < 0.05$) were genes involved in neural pathways (Fig. 3A), which confirm results from previous studies (Cutcliffe et al., 2005). However, the CCSK gene expression signature was also enriched for signaling pathways correlated to embryogenesis in general (Fig. 3B), together with pathways characteristic of neoplasia such as cell cycle associated signaling. Similar results were obtained when the CCSK signature

was compared to either fetal or adult kidney signatures (results not shown). Differentially expressed genes in CCSK compared to normal kidney were also analyzed with the functional annotation clustering analysis tool in the DAVID-database. The most enriched biological signaling pathway groups in CCSK were “embryonic development” and “cell morphogenesis involved in differentiation and axonogenesis” (Table 2).

CCSKs are Positive for Markers of Primitive Nephrogenic Compartments

The enrichment for gene expression profiles of early fetal development in CCSK prompted us to investigate the CCSK phenotype from the perspective of nephrogenesis. Surprisingly, we found that expression array reporters corresponding to markers of the intermediate mesoderm (*OSR1*), the nephrogenic lineage (*CITED1*) and the stromal lineage (*FOXD1*) were concomitantly up-regulated in CCSK in comparison with normal fetal and adult kidney (Fig. 3C–3E). To compare the gene expression of these markers in CCSK to a childhood tumor that has previously been extensively correlated to nephrogenic differentiation, we also quantified, by PCR, the RNA expression of *OSR1*, *CITED1* and *FOXD1* in five mixed type Wilms tumors. Especially the *CITED1* expression was considerably higher in CCSK compared to Wilms tumor and normal kidney (Fig. 3F and Supporting Information Fig. 1).

To explore the impact of increased RNA levels of *CITED1*, *FOXD1*, and *OSR1*, the expression of the corresponding proteins was investigated in fetal kidney and in CCSK. In fetal kidney, *CITED1* was expressed in the metanephric blastema, with the strongest expression observed in the cap-mesenchyme surrounding the ureteric bud (Fig. 4A and 4B). *FOXD1*-staining was most prominent in renal capsular stromal cells (Fig. 4C), while the metanephric blastema, glomeruli, and stroma showed weaker staining (Fig. 4D). *OSR1* was faintly positive in the metanephric mesenchyme, but stronger expression was observed in occasional cells in the stroma and the capsule (Fig. 4E and 4F). Immature epithelial structures such as renal vesicles, comma-shaped, and s-shaped bodies together with more differentiated tubules, were negative for all three proteins. Among CCSKs, the majority of tested tumors were positive for *CITED1* and *OSR1* (5/7 and 6/6 cases, respectively). The most prominent expression of both *OSR1* and *CITED1* was found in the nuclei of epithelioid cells surrounding a jagged lumen, a structure previously described in CCSK as pseudotubular structures or tumor tubules (Fig. 4G and 4I), but the protein was also detected focally in nonepithelioid cells. All tested CCSKs (8/8) were positive for *FOXD1*. This protein was typically expressed in a widespread reticular cytoplasmic pattern, but in two tumors (CCSK38 and CCSK43) round or spindle-shaped cells were also positive (Fig. 4H). Co-stainings with *CITED1* and *FOXD1* were then done to investigate the proportion of tumor cells corresponding to the two different nephrogenic lineages. Dual positivity was detected focally, typically in cells with a spindle-shaped morphology (Fig. 4N–4Q). In contrast, the epithelioid cells in pseudotubular structures were exclusively positive for *CITED1* (Fig. 4J–4M), whereas cells surrounding these structures were exclusively positive for *FOXD1* (Fig. 4M and 4Q). Taken together, this indicates that CCSK mimics a spectrum of differentiation spanning from intermediate mesoderm to its initial differentiation into the lineages of stroma and cap mesenchyme.

Genes with Importance for Nephrogenesis Display Distinct Expression Profiles in CCSK and Other Pediatric Kidney Tumors

To further validate whether the CCSK phenotype could be understood in a context of embryonic kidney formation, the expression of genes with established importance for specific functions or developmental stages during nephrogenesis was investigated in our panel of CCSKs, in relation to normal adult and fetal kidney (Supporting Information Table 2), and in comparison to other pediatric kidney tumors in an external data set GSE30946 (Gadd et al., 2012) (Fig. 5). The expression of genes correlated to nephrogenesis clearly differentiated CCSK from other tumors as well as from normal kidney (Fig. 5A). Overall, nephrogenesis genes showed the highest expression in CCSK and in Wilms tumor, in contrast to the rhabdoid tumors that displayed high expression of only *MYC* and *TCF21* (Fig. 5B).

Nephrogenesis genes were then grouped according to the structure in which they are expressed during kidney formation (Supporting Information Table 2). Hierarchical clustering analyses were done to visualize the impact of these groups of genes on the clustering of the tumor types. CCSK showed the highest degree of separation from the other tumor types when applying the gene set correlated to pretubular aggregate formation. CCSK and Wilms tumor displayed inverse gene expression patterns; *SIX2*, *PAX8*, *FGF8*, and *ETV4* were up-regulated in WT in comparison to CCSK, whereas *LEF1*, *CDH4*, *WNT4*, and *LHX1* showed higher expression in CCSK (Fig. 5C).

By GSEA, the external data set, GSE30946 (Gadd et al., 2012) was then used to correlate CCSK enriched signaling pathways (in comparison with other pediatric kidney tumors), to oncogenic expression signatures (Supporting Information Table 6). Supporting the high expression of *LEF1* and the mesenchymal nature of CCSKs, the gene expression profile of CCSK in comparison to other pediatric renal tumors, correlated strongly to an oncogenic signature (GSE3229/LEF1_UP.V1_UP) obtained in a colon carcinoma cell line where epithelial to mesenchymal transition was induced by *LEF1* (Fig. 5D). The gene expression signature of CCSK also correlated positively to an expression profile obtained when Notch signaling was inhibited in a T-ALL cell line with the gamma-secretase inhibitor DAPT (GSE6495/NOTCH_DN.V1_UP; Fig. 5E). Given the importance of Notch signaling for tubular differentiation (Cheng et al., 2007) this finding is in line with a perturbed or blocked mesenchymal to epithelial differentiation in CCSK.

DISCUSSION

CCSK is often described as an enigmatic tumor type because of the fact that its morphologic appearance does not resemble that of the kidney, such that the cellular origin is unknown. We characterized a panel of CCSKs with the aim of gaining information regarding the mechanisms behind tumor derivation. High-resolution SNP-array uncovered little information about the mechanisms behind CCSK tumor formation. This is in contrast to Wilms tumor, for which segmental aberrations such as CNNI 11p and 16q deletions can be linked to tumor pathogenesis (Grundy et al., 2005; Rivera and Haber, 2005). A balanced translocation such as the t(10;17)(q22;p13) found in a proportion of CCSKs (Douglass et al., 1985; Punnett et al., 1989; Sheng et al., 1990; Kaneko et al., 1991; Rakheja et al., 2004;

Brownlee et al., 2007) would not be detected by SNP-array analyses. However, the few recurrent aberrations we did detect were events on chromosome arms 10q and 17p together with 1q-gains, also a finding previously described in CCSKs (Barnard et al., 2000; Schuster et al., 2003). A trend towards poorer short-term survival was found for patients with aberrations in 1q, 10q, and/or 17p, but the significance was not retained for long-term survival. Of note, gain of 1q is an adverse prognostic factor in patients with Wilms tumor even if the tumors display a favorable histology (Hing et al., 2001; Grundy et al., 2005).

Although the presence of segmental aberrations did influence the gene expression profile of the tumors and creates some diversity within the group, CCSKs still displayed a highly characteristic expression profile, in which genes important for early nephrogenesis were key elements. The global gene expression profile for CCSKs resembled that of fetal kidney more than adult kidney, reflecting the immature morphology of this tumor type. Because of the unknown cellular origin of CCSK, it is difficult to choose a biological comparison group for investigating differentially expressed genes. Cutcliffe et al. compared the gene-expression profile of CCSK to Wilms tumor and fetal kidney (Cutcliffe et al., 2005) but, to avoid subtracting genes expressed during the development of the fetal kidney we also included RNA from adult kidney in our experiments (Cutcliffe et al., 2005). However, we obtained similar results irrespective of whether we compared CCSK to fetal or adult kidney (results not shown), or to a combination of both normal kidney types. In line with Cutcliffe et al., CCSK signatures were enriched for pathways correlated to diverse neural processes (Cutcliffe et al., 2005), together with pathways involved in embryogenesis in general. There are several examples of genes annotated to both neuronal and embryonic gene ontologies, such as genes involved in sonic hedgehog signaling. Thus we speculate that the puzzling enrichment of neural processes in CCSK could reflect an over-representation of genes annotated to neurogenic gene ontologies despite having a general importance for embryonic signaling. In addition, when analyzing the 3000 most highly expressed genes in CCSK compared to non-neoplastic kidney with the DAVID annotation tool, gene ontologies correlated to embryogenesis received a slightly higher enrichment score than ontologies correlated to neurogenesis.

To evaluate whether CCSK could be linked to a specific kidney developmental lineage, gene expression of markers for stromal differentiation (*FOXD1*) and nephric differentiation (*CITED1*) was analyzed. High RNA levels of both *CITED1* and *FOXD1* were detected in CCSK cells, as well as expression of *OSR1*, a gene distinguishing the intermediate mesoderm from other mesodermal structures. All three genes exhibited higher expression levels in CCSK than in normal kidney and in Wilms tumors. The diverse protein expression of *CITED1* and *FOXD1* in different cells of CCSK indicates that these cells were arrested after commitment towards the stromal (*FOXD1*+) and nephric (*CITED1*+) lineages, respectively. Tumor tubules or pseudo tubular structures have previously been described in CCSK, and have been demonstrated to be cytokeratin negative despite their epithelioid appearance (Argani et al., 2000). We found that these cells were positive for both *CITED1* and *OSR1* and negative for *FOXD1*, probably reflecting a differentiation towards nephrons abrogated at an early developmental stage.

Interestingly, the expression of genes important in kidney development was sufficient to separate pediatric kidney tumors into their biological groups by nonsupervised hierarchical clustering. The most prominent separation of CCSK from other childhood renal tumors was obtained when investigating genes with importance for the pretubular aggregates, in line with the absence of epithelial differentiation in CCSK. CCSK and Wilms tumor showed almost an inverse expression pattern of these genes, in accordance with the true epithelial/tubular differentiation seen in the majority of Wilms tumor. A lack of, or weak expression of genes crucial for the transition from mesenchymal to epithelial structures, such as *PAX2* and *WT1* (Hendry et al., 2011), might explain the block in nephronic differentiation in CCSK (Fig. 5B). In addition, inhibition of nephron formation prior to the s-shaped stage has been demonstrated in cells lacking *FGF8* (Grieshammer et al., 2005), in agreement with the low expression of *FGF8* in CCSK compared to Wilms tumor (Fig. 5B and 5C). One of the significantly enriched oncogenic pathways found in CCSK in comparison to other pediatric kidney tumors, was a signature achieved when over-expressing *LEF1*, implicating that the high expression of *LEF1* in CCSK might play a role for CCSK tumor biology by maintaining the mesenchymal phenotype. The importance of Notch signaling for nephrogenic differentiation is well established (Cheng et al., 2007). Although some of the key genes in this pathway are expressed in CCSK such as *NOTCH1* and *DLL1*, the gene expression profile of the CCSKs largely displayed enrichment of genes associated with inhibition of Notch signaling, in line with a failure of epithelial differentiation. The majority of the CCSK cells were *FOXD1* positive, reflecting stromal differentiation. Retinoic acid signaling is crucial for kidney formation and *RARα* is also expressed in the stromal cells of the fetal kidney (Mendelsohn et al., 1999; Batourina et al., 2001). However, the majority of the CCSKs analyzed in this study expressed low levels of *RARα* (Fig. 5A and 5B), which might explain the lack of terminal stromal differentiation in this tumor type.

It should be noted that many genes involved in nephrogenesis are also expressed in other embryonic processes. Our findings do therefore not single handedly prove that CCSK emerges from the nephrogenic lineage. However, its expression of early nephrogenic markers together with the fact that CCSK is typically an intrarenal tumor makes this a plausible assumption. In that context, the present study shows that the biology of CCSK is likely to reflect an earlier stage of development than Wilms tumor, but that CCSK nevertheless retains characteristic nephrogenic markers, placing its phenotype in the window of differentiation between intermediate mesoderm and pretubular aggregates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We would like to acknowledge the COG Renal Tumor Study Group and the Tissue Bank for contributing with CCSK samples and clinical data. We would also like to thank Dr Martin Johansson at the Center for Molecular Pathology, Lund University, SUS Malmö, Sweden, for providing kidney tissue and Dr David Lindgren at Translational Cancer Research, Lund University, Sweden, for valuable discussions. We are grateful for the assistance from the Swegene Centre for Integrative Biology at Lund University (SCI-BLU) and for technical assistance from Margareth Isaksson.

Supported by: The Swedish Childhood Cancer Foundation, the Swedish Cancer Society, the Swedish Research Council, the Crafoord Foundation, the Gunnar Nilsson Cancer Foundation, the Royal Physiographic Society, the Medical Faculty at Lund University, and National Institutes of Health (NIH) Grant numbers: U10 CA98413 (COG Statistics and Data Center grant) and U10 CA98543 (COG Chair's grant).

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Figure 1.

CCSK displays few segmental genomic aberrations. Summary of all aberrations detected by SNP-array analysis of 37 CCSKs. Deletions (hemizygous) are depicted in red, gains in green and copy number neutral imbalances (CNIs) in blue. All aberrations are summarized in Supporting Information Table 4. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 2.

CCSK displays a uniform gene expression profile. Principal component analysis of gene expression data demonstrating (A) the clustering of 22 CCSK, six adult kidneys (AK) and four fetal kidneys (FK) and (B) the impact of segmental aberrations on the clustering of the CCSK. Reporters with less variance than 5% of the maximal variance were removed leaving 20,852 variables in (A) and 31,259 variables in (B). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 3.

CCSK retains the expression of markers for primitive nephrogenic compartments and developmental lineages. (A) Pathway analysis of CCSK in comparison with fetal and adult kidney reveals an enrichment of neuronal signaling ($q = 0.007$, $NES = 1.87$) and (B) embryonic development ($q = 0.095$, $NES = 1.56$). The expression of markers for developmental lineages in the fetal kidney is evaluated in our in house gene expression data set: (C) *OSR1* for intermediate mesoderm, (D) *CITED1* for the nephronic lineage, and (E) *FOXD1* for the stromal lineage. (F) Results in (C–E) are confirmed by quantitative PCR in CCSK in comparison with mixed type Wilms tumor (WT), fetal kidney (FK), (gestational week 12–31) and adult kidney (AK). Expression of the genes of interest was correlated to three house-keeping genes: *SDHA*, *ALAS1*, and *ACTB*. High expression is depicted in red while green indicates low expression as demonstrated by the scale bare. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 4.

Protein expression, in fetal kidney and CCSK, of markers for primitive nephrogenic compartments and lineages. Immunofluorescent staining of CITED1, FOXD1 and OSR1 in fetal kidney (gestational week 16 - 19) and CCSK. Nuclei were counterstained with DAPI (blue). (A-I) stained proteins are visualized in red and the autofluorescence from the tissue in green. (A and B) CITED1 is expressed in the cap mesenchyme (cpm) in the fetal kidney. (C) FOXD1 is most abundant in the capsule (ca); however, focal expression is seen in the condensed metanephric blastema (cm), glomeruli and stroma. (E and F) OSR1 is only expressed in a few cells in the capsule and the stroma (s). The lower panel demonstrates co-stainings with FOXD1 (red) and CITED1 (green). (G and L) In CCSK, as exemplified by CCSK1, the strongest CITED1 expression is detected in epithelioid cells surrounding a lumen (lu) (P), but areas with CITED1 expression in cells with spindle shaped morphology

are also found and (Q) those cells are also positive for FOXD1. (H, K, and O) FOXD1 is positive in the majority of the tumor cells with the exception of the CITED1 positive epithelioid (ep) cells (H, K, and M). (I) The strongest OSR1 positivity in the tumor is detected in the epithelioid cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 5.

Expression of key nephrogenesis genes in childhood renal tumors. A list of totally 54 genes expressed during nephrogenesis was created as described in Materials and Methods. The mean expression value was calculated when more than one reporter was present for individual genes. High expression is depicted in red while low expression is shown in green. (A–B) Reporters with less variance than 1% of the maximal variance were removed leaving 40 of 54 variables in (A), and 42 of 47 variables in (B). (C) Genes expressed in pretubular aggregates most efficiently separates CCSK from the other tumor types. (D and E) The association between enriched pathways in CCSK compared to other pediatric kidney tumors (GSE30946) (Gadd et al., 2012) and c6 oncogenic signatures, was investigated by GSEA. (D) CCSK significantly ($q = 0.048$, NES = 1.71) correlated to a signature obtained in a colon carcinoma cell line where epithelial to mesenchymal transition was induced by *LEF1* (GSE3229/ LEF1_UP.V1_UP) and (E) to a signature obtained when inhibiting Notch signaling in a T-ALL cell line with the gamma-secretase inhibitor DAPT (GSE6495/

NOTCH_DN. V1_UP; $q = 0.051$, NES = 1.72). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE 1

Overview of Segmental Aberrations in CCSK on Chromosome Arms 1q, 10q, and 17p

Case	1q	10q	17p
CCSK3	Gain		Loss
CCSK9	Gain	Loss	
CCSK19		Loss	Loss
CCSK29		Gain	Loss
CCSK30	Gain	Loss	
CCSK37			Loss

TABLE 2

Enriched Pathways in CCSK Compared to Fetal and Adult Kidney, Analyzed by the Annotation Tool DAVID

Annotation cluster	Enrichment score	Term
1	34.8	Nuclear lumen
2	31.8	DNA binding and transcription
3	25.8	Non-membrane-bounded organelles
4	23.8	Chromosomes
5	21.1	Cell cycle
14	8.8	Embryonic development (skeletal and organ morphogenesis)
20	6.1	Cell morphogenesis involved in differentiation and axonogenesis