

Elevated cyclin E level in human clear cell renal cell carcinoma: possible causes and consequences

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The expression of cyclin E gene (*CCNE*) in relation to the expression of its major regulatory protein, E2F1, was examined in clear cell renal cell carcinomas (ccRCC). We show that the overexpression of E2F1 is accompanied by the significant increase of the mean amounts of cyclin E mRNA, as well as of total cyclin E protein and its low molecular weight forms in cancer tissues as compared to peritumoral controls. A significant increase of the mean amount of total cyclin E was found in peritumoral tissues compared to cancer-free kidneys, suggesting that cancer cells might secrete factors having a profound influence on the metabolism of neighbouring tissues. A significant, positive correlations between E2F1 protein and total cyclin E mRNA, as well as between E2F1 protein and full length cyclin E protein were found in cancer-free kidneys and in peritumoral tissues, but not in ccRCCs. The overexpression of cyclin E positively correlated with the decreasing degree of tumor differentiation, implicating a role for cyclin E in the promotion of tumorigenesis.

Keywords: human clear cell renal cell carcinoma (ccRCC), gene for cyclin E (*CCNE*), E2F1 transcription factor, cyclin E, low molecular weight forms of cyclin E (LMW)

INTRODUCTION

Oncogenesis results from deregulation of the cell cycle and alteration of apoptosis (Evan *et al.*, 2001). The “R” restriction checkpoint located between G₁ and S phase of the cell cycle, is controlled by retinoblastoma protein (pRB). pRB is not phosphorylated until early G₁ and in this form it binds and inhibits certain E2F proteins. Its sequential phosphorylation by cyclin D-dependent cdk4/cdk6 kinases and by cyclin E-dependent cdk2 kinase results in its dissociation from the E2F–pRB complex, and the release of E2F1, 2 and 3 activatory members of the E2F transcription factor family (Weintraub *et*

al., 1995; Moroy & Geisen, 2004). E2F1, the best described member of the E2F family, then activates a number of genes encoding proteins involved in cell cycle progression, including cyclin E gene (*CCNE*) (Ohtani *et al.*, 1995; Muller *et al.*, 2001). Overexpression of E2F1, but neither its mutations nor gene amplification, has been found in many different cancers (Tsanotulis & Gorgoulis, 2005; Fang & Han, 2006).

The gene for cyclin E, one of the key targets of E2F1, contains six putative E2F binding sites, but only the three located most distantly from the transcription start site seem to be involved in gene activation (Geng *et al.*, 1996). A large complex containing E2F4 (inhibitory member of E2F family), among

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Abbreviations: *CCNE*, homo sapiens gene for cyclin E; ccRCC, clear cell renal cell carcinoma; cdk, cyclin-dependent kinase; EMSA, electrophoretic mobility shift assay; HPRT, hypoxanthine phosphoribosyltransferase; LMW, low molecular weight forms of cyclin E; phenylmethylsulfonyl fluoride; PMSF, phenylmethylsulfonyl; pRB, retinoblastoma protein; TR, thyroid hormone nuclear receptor.

others, represses transcription of this gene until the G₁ phase is reached (Polanowska *et al.*, 2001). Once activatory members of the E2F family dissociate from the phosphorylated pRB, they bind to the *CCNE* promoter, strongly activating its transcription (Ohtani *et al.*, 1995; Geng *et al.*, 1996). *CCNE* encodes a 395 amino-acid polypeptide, its shorter splice variants, and an isoform containing additional 15 amino acids at the N terminus (Porter & Keyomarsi, 2000). The protein accumulates periodically with a maximum at the end of G₁ phase (Hwang & Clurman, 2005) and undergoes degradation during the S phase. The cyclin E-cdk2 complex phosphorylates pRB and interacts with a number of other proteins playing a role not only in the promotion of the cell cycle transition, but also in DNA replication (Jackson *et al.*, 1995) and in apoptosis (Mazumder *et al.*, 2004).

Deregulated cyclin E expression is very common in different cancers (Donnellan & Chetty, 1999; Schraml *et al.*, 2003; Hwang & Clurman, 2005). The full length protein, as well as its shorter forms (low molecular weight forms, LMW) that are generated by cyclin E N-terminal proteolysis, accumulate (Porter *et al.*, 2001; Akli & Keyomarsi, 2003). It might be a result of gene amplification or its excessive activation, mutation, or a result of increased stability of LMW (Schraml *et al.*, 2003; Akli & Keyomarsi, 2003). LMW proteins seem to be functionally hyperactive and their presence is associated with acceleration of the cell cycle and genomic instability (Spruck *et al.*, 1999; Porter *et al.*, 2001). Accumulation of cyclin E is associated with more advanced stage of the disease, poorer prognosis and shorter survival rates (Muller-Tidow *et al.*, 2001; Bales *et al.*, 2005).

In this study we used human clear cell renal cell cancer (ccRCC) (Cohen & McGovern, 2005) as a model system. A number of factors involved in proliferation control are deregulated in this type of cancer including p21, p27, pRB (Haitel *et al.*, 2001), VHL and NFκB (An & Rettig, 2005), as well as thyroid hormone receptors (TRs) (Puzianowska-Kuznicka *et al.*, 2000; 2006). In this paper we show that the correlation between the expression of E2F1 and its target gene encoding cyclin E observed in control tissues is lost in ccRCC. In addition, we show that the expression of cyclin E on both mRNA and protein levels is significantly increased in ccRCCs in comparison to peritumoral tissues and cancer-free kidneys, and this increase correlates with the decreasing degree of tumor differentiation, suggesting a role for this protein in the pathogenesis of ccRCC.

MATERIALS AND METHODS

Tissues. Tissue samples were obtained with the permission of the Ethical Committee of Hu-

man Studies from 34 patients during nephrectomy performed due to kidney cancer. Fragments of the tumors as well as of peritumoral tissues (respective controls) were excised and immediately frozen on dry ice. Tissues were also obtained from cancer-free kidneys, excised due to trauma or ectopic position (15, cancer-free controls). ccRCC was diagnosed upon histological examination using WHO criteria. Tumors were divided into three groups depending on the grade of differentiation: G1 – well differentiated (n=11), G2 – intermediate grade of differentiation (n=14), and G3 – poorly differentiated (n=9).

RNA isolation. Frozen tissue (100 mg) was homogenized in a glass-teflon homogenizer in 1 ml of TRIZOL Reagent (Invitrogen). RNA was isolated according to manufacturer's protocol. The RNA concentration was calculated from spectrophotometry measurements.

Real-time PCR. Synthesis of cDNA was performed with RNA isolated from tumor-respective control tissues. Total RNA (7.5 µg) was reverse-transcribed with oligo-dT primer in a total volume of 15 µl using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas). Next, cDNA samples were diluted with sterile, deionized water to a volume of 40 µl. Each PCR reaction was performed with 1.3 µl of cDNA solution (corresponding to 250 ng of total RNA). Real-time PCR was performed using a Chromo4™ Four-Color Real-Time Detector (Bio-Rad). The sequences of the primers were as follows: for *CCNE* 5'-GTGGTGCGACATAGAGAACTG-3' and 5'-CGCTGCTCTGCTTCTTACC-3' (PCR product length 257 bp); for control gene *HPRT* 5'-TTTGGAT-TATACTGCCTGAC-3' and 5'-GGACTAATTATGGACAGGAC-3' (350 bp); *HPRT* is a housekeeping gene encoding the ubiquitously expressed enzyme that catalyses an early step in the purine salvage pathway). The cycles were as follows: 95°C for 15 min, then 38 cycles of 95°C for 20 s, 59°C for 20 s, 72°C for 20 s. PCR products were detected with SYBR-Green I using the QuantiTect™ SYBR®Green PCR Kit (Qiagen). Expression level of *CCNE* was normalized against that of *HPRT* gene.

Isolation of nuclear proteins. The method used for isolation was previously described by Madej *et al.* (2003). All buffers were supplemented with pepstatin A (1 µg/ml), aprotinin (2 µg/ml) and PMSF (0.5 mM). The amount of nuclear proteins in the supernatant was measured by Bradford's method. Aliquots (30 µl) were stored at -70°C.

Western blotting. Nuclear extracts (40 µg) were resolved on 10% polyacrylamide gel and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% nonfat milk in TBS-T buffer (20 mM Tris/HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20), washed twice with TBS-T for 10 min at room temp., then incubated with rabbit polyclonal

anti-cyclin E primary antibodies (1:100 in TBS-T, Ab-3 corresponding to amino acids 381–395 of human cyclin E; Calbiochem), rabbit polyclonal anti-E2F1 antibody (1:100 in TBS-T; C20, Santa Cruz Biotechnology), and with monoclonal anti- β -actin antibody (1:5000 in TBS-T; Sigma) for 1 h at room temp., washed 3 times for 10 min in TBS-T, then incubated for 1 h at room temp. with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (1:10000 in TBS-T; Dako), respectively, and washed again as described above. The specific proteins were visualized with an enhanced-chemiluminescence detection system (Supersignal West Pico chemiluminescent substrate; Pierce) according to a standard procedure. The amount of the specific protein was estimated from the densitometry measurements after normalization against the β -actin band.

Electrophoretic mobility shift assays. The assays were performed with nuclear extracts isolated from ccRCCs and their respective controls. The probes were identical to *CCNE* promoter fragments containing E2F binding sites: the first binding site 1-E2F 5'-ACGCGGCTTTTGGCCGCTCCAGCGCCGCTC-3', and both the second and the third binding sites 2,3-E2F 5'-GCGCCGCGCGCCAGACTTCTCCCGCTCCCGC-3' (Geng *et al.*, 1996). The probes were labeled by fill-in reaction with Klenow fragment and [α - 32 P]dCTP. Five micrograms of each nuclear extract were incubated at room temp. for 20 min in 25 μ l of binding buffer consisting of 20 mM Hepes, pH 7.5, 40 mM KCl, 10 mM MgCl₂, 1 mM EGTA, 0.5 mM dithiothreitol, 0.1% Nonidet P-40, 10% glycerol (Cobrinik *et al.*, 1993) supplemented with 250 ng–1 μ g of dIdC, 0.5 ng of the probe, and protease inhibitors. If required, the samples were supplemented with 25-fold molar excess of specific competitor or non-specific competitor 5'-CCTGCTGATCTATCAGCACAGATTAG-3'. The products of the reaction were resolved on a 5% native gel. Gels were then dried and exposed against Kodak film for 24 h.

Statistical analysis. Data were presented as mean values \pm S.E.M. Statistical analysis was performed using one-way ANOVA, Tukey test, and the nonparametric Spearman correlation test (95% confidence intervals) with $P < 0.05$ considered statistically significant.

RESULTS

The amount of E2F1 protein in ccRCC and in control tissues

The amounts of E2F1 protein in ccRCCs and in peritumoral tissues were determined by Western blot (Fig. 1A). Overexpression of this protein was

seen in 89% of cancer samples compared to their respective controls (Fig. 1B). In 44.5% of the cases the amount of E2F1 was higher by more than 50% of that in the respective control, in 44.5% of the cases it was up to 50% higher than that in the respective control, while in the remaining 11% the amount of E2F1 was lower in cancers than in peritumoral tissues. The difference between the mean amounts of E2F1 in the respective controls pooled together and in G1 cases was not significant ($P = 0.4574$), but it reached significance in G2 and G3 cases ($P = 0.00314$ and $P < 0.0001$) (Fig. 1C).

The binding of E2F proteins to the binding sites from cyclin E promoter

Electrophoretic mobility shift assays of E2F transcription factors binding to *CCNE* promoter-like sequences were performed under previously established conditions (Cobrinik *et al.*, 1993; Geng *et al.*, 1996). It has previously been shown that the first three out of the six putative E2F binding sites in human *CCNE* promoter are involved in the regulation of *CCNE* activity (Geng *et al.*, 1996). Therefore, the probes used by us were identical to the first (1-E2F), or the second and the third (2,3-E2F) E2F binding sites. The band pattern we observed was identical to the one described before by other authors. The full set of EMSA performed with both E2F probes and nuclear extracts from ccRCC-respective control pairs revealed that the binding was slightly more efficient in 44.5%, equal in 33.3%, and weaker in 22.2% of cancer cases compared to the respective controls as shown by densitometric measurements of the specific bands (Fig. 1D). It should be noted that the majority of the ccRCC cases with unchanged or lower protein binding to the E2F recognition sites from *CCNE* promoter were characterized by E2F1 overexpression. This suggests that in ccRCC tissues other factors might be present that either sequester E2F1 or interfere with its binding to the probe.

The amount of cyclin E mRNA in ccRCCs, in peritumoral tissues, and in cancer-free kidneys

The amount of the specific cyclin E mRNA was checked by means of a real-time PCR (Fig. 2). In all cases the amount of mRNA was higher in ccRCC than in the respective control. The mean amount of cyclin E mRNA in G1, G2, and G3 cases was significantly higher than the mean amount of this mRNA in their respective controls C1, C2, and C3 ($P = 0.03687$, $P = 0.00071$, $P = 0.0028$, respectively). Similarly, the mean amount of cyclin E mRNA in G1, G2 and G3 cases was significantly higher than in cancer-free kidneys ($P = 0.00588$, $P < 0.0001$ and $P = 0.00286$, respectively). It is noteworthy that in comparison to

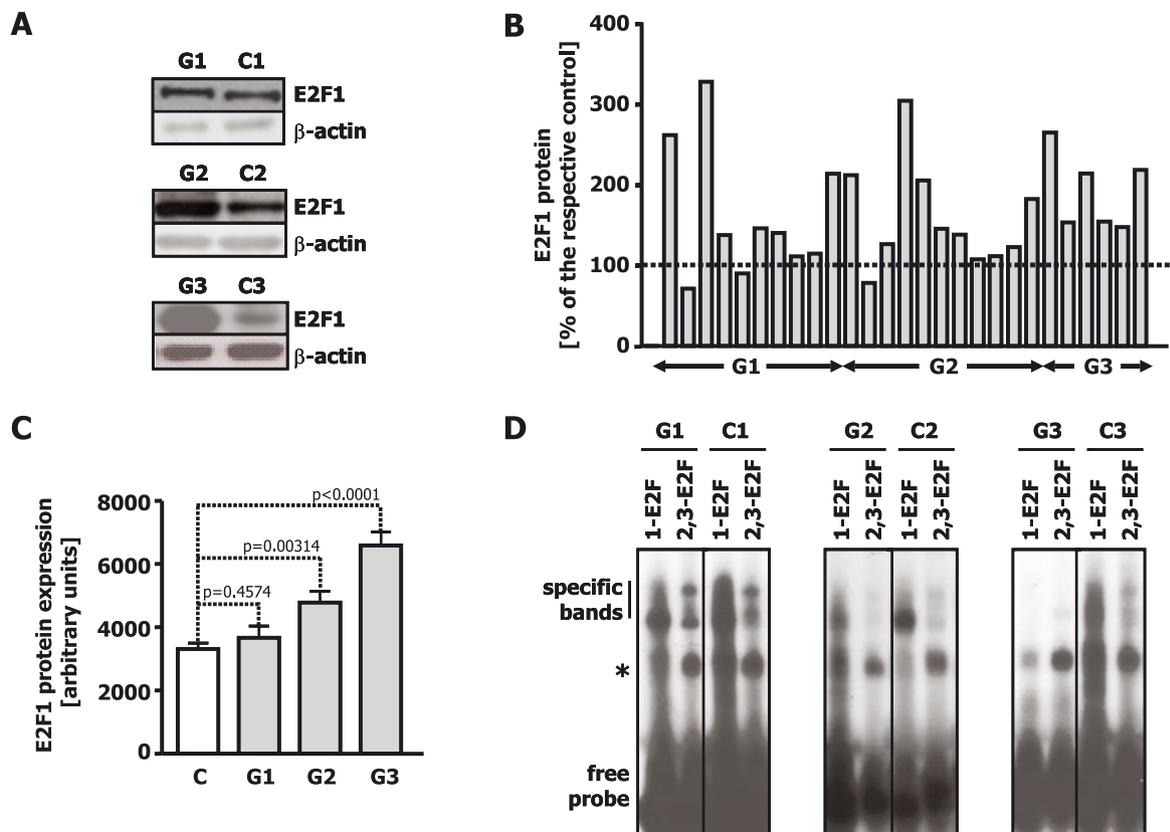


Figure 1. Amount of E2F1 protein in representative ccRCCs and in their respective controls, and the binding of E2Fs to CCNE promoter.

A. Representative immunoblots of nuclear extracts isolated from ccRCCs and their respective controls, probed with anti-E2F1 antibody. The membranes were re-probed with anti- β -actin antibody. **B.** The amount of E2F1 protein in ccRCCs as percentage of its amount in the respective controls. **C.** The mean amounts of E2F1 protein in ccRCCs and in the respective controls pooled together. **D.** Electrophoretic mobility shift assay of the same ccRCC-respective control nuclear extract pairs performed with the probes resembling functionally active E2F binding sites from CCNE promoter. 1-E2F: first E2F binding site; 2,3-E2F: third binding sites. G1, G2, G3: grades of ccRCC differentiation; C: respective controls pooled together; C1, C2, C3: controls for G1, G2, G3, respectively; *non-specific binding.

the mean amount of cyclin E mRNA in cancer-free kidneys, the mean amount of this mRNA in C1 and C2 controls was significantly higher ($P=0.02509$ and 0.00233 , respectively). The difference between cancer-free controls and C3 controls was not significant.

Correlations between E2F1 protein and cyclin E mRNA

A significant positive correlation between E2F1 protein and total cyclin E mRNA was found in cancer-free kidneys ($r=0.8652$, $P=0.0003$) (Fig. 3A) and in peritumoral control tissues ($r=0.5390$, $P=0.0096$) (Fig. 3B), but not in clear cell renal cell carcinomas ($r=0.1346$, $P=0.5212$) (Fig. 3C).

The expression of total cyclin E protein and its low molecular weight forms

Total amounts of cyclin E and of LMW were evaluated in human ccRCC-respective control pairs

and in cancer-free controls. Blots were probed with antibodies generated against a synthetic peptide corresponding to amino acids 381–395 of human cyclin E that detect full length protein and smaller proteins corresponding to different forms of cyclin E. Bands of 52 and 51 kDa representing large forms of cyclin E, as well as predominant 42, 36, and 30 kDa bands representing LMW forms of cyclin E were detected in ccRCC tissues. In addition to the full length forms, two bands, 42 and 36 kDa, were observed in cancer-free controls, while the 42, 36, and in some cases – 30 kDa LMW forms were present in tumor-surrounding tissues (Fig. 4A). Densitometric analysis showed that the mean total cyclin E content in cancer-free tissues expressed in arbitrary units was 3717 ± 274 , and in peritumoral controls the value was 4685 ± 226 . It is noteworthy that this difference was significant ($P=0.02326$). The difference between the total cyclin E amount in cancer-free controls and C1 controls (3878 ± 464) was not significant ($P=0.77569$), but it was highly significant for C2 (4897 ± 289) and

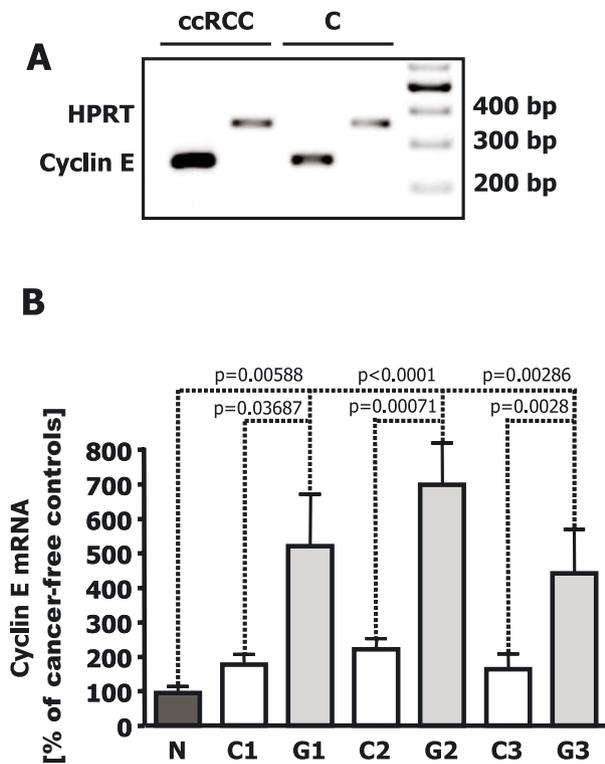


Figure 2. Amount of cyclin E mRNA in ccRCCs, in their respective controls, and in cancer-free kidneys.

cDNA used in classic PCR and in real-time PCR reactions was made on the template of total RNA isolated from ccRCCs and from their respective controls. **A.** Ethidium bromide staining of real-time PCR-amplified CCNE product and HPRT control gene product (28 cycles). **B.** The mean amount of cyclin E mRNA in ccRCCs and in their respective controls as percentage of the mean amount of this mRNA in cancer-free kidneys. N: cancer-free controls; G1, G2, G3: grades of ccRCC differentiation; C1, C2, C3: controls for G1, G2, G3, respectively.

C3 (5115±408) controls ($P=0.00735$ and $P=0.0084$, respectively). The mean amount of cyclin E in G1 tumors was 5514±407, in G2 6183±510, and in G3 10122±1627. The differences between cancer-free controls and G1, G2, and G3 tumors were significant ($P=0.00122$, $P=0.00047$, and 0.00026 , respectively), as were the differences between C1 and G1, C2 and G2, as well as C3 and G3 ($P=0.01748$, $P=0.04123$, and $P=0.00605$, respectively) (Fig. 4B).

Analysis of LMW forms of cyclin E showed that their mean amount in cancer-free controls was 1305±337, in C1 controls it was 1448±286, in C2 1725±281, and in C3 2072±468. The differences between cancer-free controls and C1, C2, C3 were not significant ($P=0.77055$, $P=0.36174$, $P=0.23256$, respectively). The differences between cancer-free controls and G1 (2768±352), G2 (3788±603), and G3 (4480±896) tumors were significant ($P=0.00843$, $P=0.00081$, and $P=0.00062$, respectively). The differences between C1 and G2, C2 and G2, as well as C3

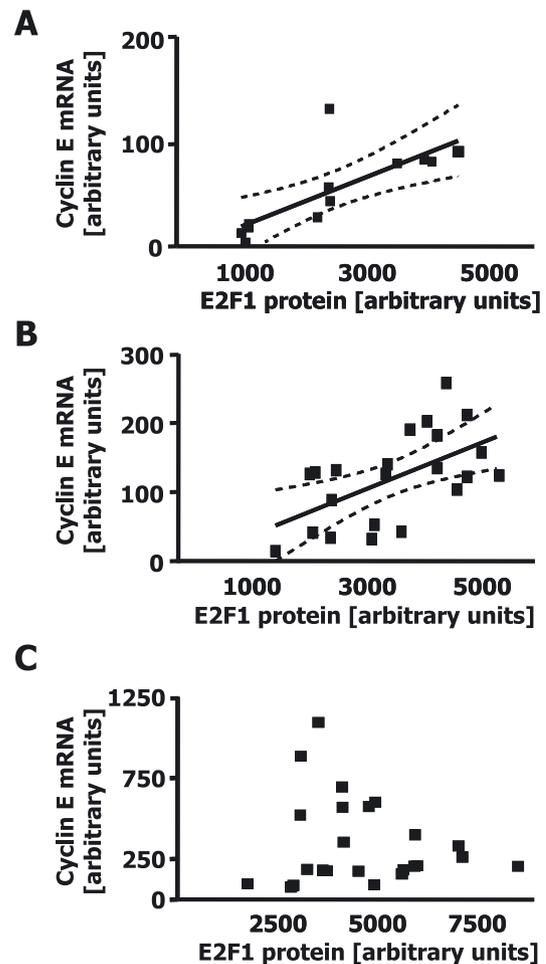


Figure 3. Correlation between E2F1 protein and cyclin E mRNA amounts in ccRCCs, in their respective controls, and in cancer-free kidneys.

A. A significant positive correlation between E2F1 protein and cyclin E mRNA levels in cancer-free kidneys ($r=0.8652$, $P=0.0003$). **B.** A significant positive correlation between E2F1 protein and cyclin E mRNA levels in peritumoral tissues ($r=0.5390$, $P=0.0096$). **C.** Lack of such correlation in ccRCCs ($r=0.1346$, $P=0.5212$).

and G3 were also significant ($P=0.00931$, $P=0.00491$, $P=0.0017$, respectively) (Fig. 4C). In cancer-free tissues the LMW forms constituted 24.33±4.92% of total cyclin E, in the respective controls pooled together they represented 31±2.95%, while in G1 tumors 56.67±4.50%, in G2 52.56±6.21%, and in G3 50.31±3.07%. These differences were statistically significant (cancer-free controls vs. G1, G2, and G3: $P<0.0001$, $P=0.00183$, and 0.00018 , respectively; the respective controls vs. G1, G2, and G3: $P<0.0001$, $P=0.00208$, and 0.00047 , respectively).

Correlations between E2F1 protein and cyclin E protein

On the basis of the available data, it is justified to presume that the amount of full length cyclin

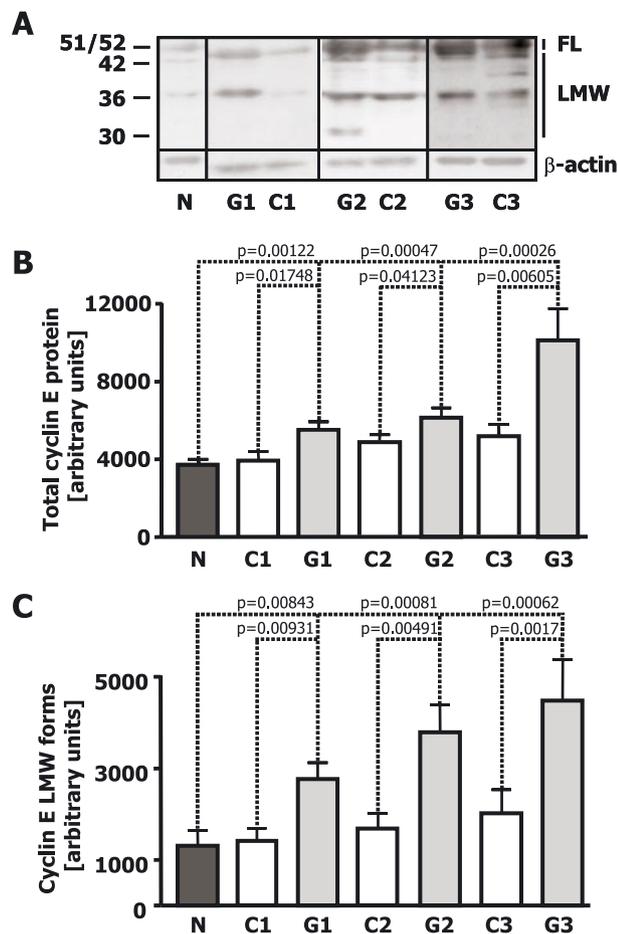


Figure 4. Amount of total cyclin E and its low molecular weight forms in ccRCCs, in their respective controls, and in cancer-free kidneys.

A. Typical expression of full length cyclin E (52, 51 kDa) and its LMW forms (42, 36, 30 kDa) as analyzed by immunoblotting. FL: full length; LMW: low molecular weight forms. **B.** Mean total cyclin E expression in cancer-free, peritumoral control, and cancer tissues. **C.** Expression of LMW forms of cyclin E in cancer-free, peritumoral control, and cancer tissues. N: cancer-free kidneys; G1, G2, G3: grades of ccRCC differentiation; C1, C2, C3: controls for G1, G2, G3, respectively.

E protein corresponds to the transcriptional activity of *CCNE* gene. Therefore, the correlation between E2F1 protein and full length cyclin E was analyzed. Such correlation was significant in cancer-free kidneys ($r=0.5919$, $P=0.0426$) (Fig. 5A) and in peritumoral tissues ($r=0.4708$, $P=0.0152$) (Fig. 5B), but not in ccRCCs ($r=0.1887$, $P=0.3772$) (Fig. 5C).

Correlations between cyclin E mRNA and different forms of cyclin E protein

A significant positive correlation between total cyclin E mRNA and the full length protein forms was found in cancer-free kidneys ($r=0.6294$, $P=0.0283$) (Fig. 6A) and in peritumoral tissues

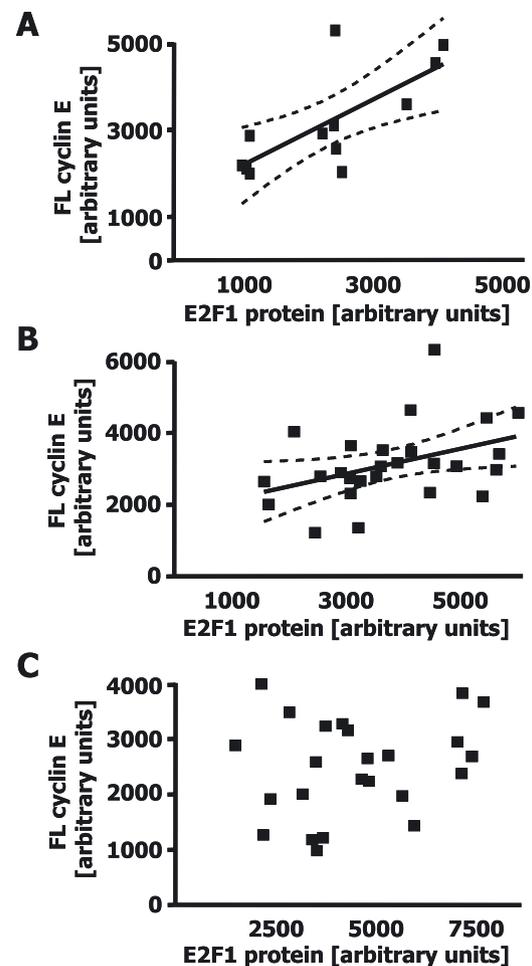


Figure 5. Correlation between E2F1 protein and full length cyclin E protein amounts in ccRCCs, in their respective controls, and in cancer-free kidneys.

A. A significant positive correlation between E2F1 protein and full length cyclin E protein amounts in cancer-free kidneys ($r=0.5919$, $P=0.0426$). **B.** A significant correlation in peritumoral tissues ($r=0.4708$, $P=0.0152$). **C.** Lack of such correlation in ccRCCs ($r=0.1887$, $P=0.3772$).

($r=0.5851$, $P=0.0107$) (Fig. 6B), but not in ccRCCs ($r=0.3353$, $P=0.1484$) (Fig. 6C). There was a significant correlation between the amount of total cyclin E mRNA and the amount of total (full length and LMW) cyclin E proteins in cancer-free kidneys ($r=0.6853$, $P=0.0139$), but not in peritumoral tissues or in cancer tissues. There was no correlation between the amount of total cyclin E mRNA and the amount of cyclin E LMW forms in any type of tissues analyzed.

A significant positive correlation between total cyclin E and full length cyclin E was found in cancer-free kidneys ($r=0.8571$, $P<0.0001$), in peritumoral tissues ($r=0.7684$, $P<0.0001$), and in ccRCCs ($r=0.6093$, $P=0.0007$). The correlation between total cyclin E and LMW forms was also significant in all tissue types analyzed ($r=0.5783$, $P=0.0239$ in cancer-free kidneys, $r=0.8734$, $P<0.0001$ in the respective

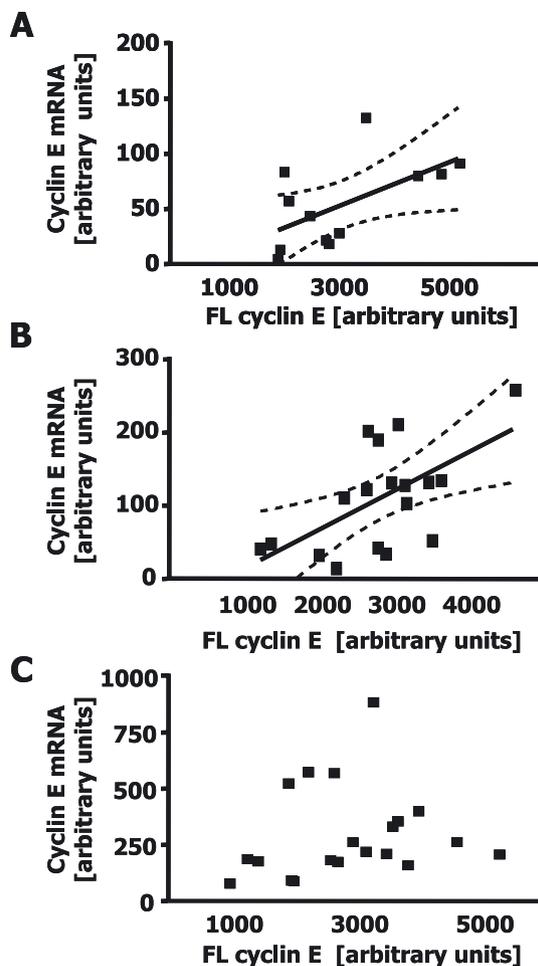


Figure 6. Correlation between cyclin E mRNA and full length protein amounts in ccRCCs, in their respective controls, and in cancer-free kidneys.

A. A significant positive correlation between cyclin E mRNA and full length cyclin E protein amounts in cancer-free kidneys ($r=0.6294$, $P=0.0283$). **B.** A significant correlation in the respective control tissues ($r=0.5851$, $P=0.0107$). **C.** Lack of such correlation in ccRCCs ($r=0.3353$, $P=0.1484$).

controls, $r=0.8480$, $P<0.0001$ in ccRCCs). A statistically significant correlation between the amount of full length cyclin E and the amount of LMW forms was found in cancer-free kidneys ($r=0.8303$, $P=0.0047$). However, once cancer-free cases without LMW forms detected were added to this analysis, the correlation became non-significant ($r=0.1928$, $P=0.4913$). Such a correlation was significant in the respective controls ($r=0.4429$, $P=0.039$), but not in ccRCC tissues ($r=0.1374$, $P=0.4945$).

DISCUSSION

Two major mechanisms might lead to cyclin E overexpression in tumors: abnormally high

transcription rate, and slower protein degradation. In this work we examined the pathway which potentially increases cyclin E abundance as a result of the undue activation of its gene by an excess of E2F1 transcription factor. We found a significant positive correlation between E2F1 protein and total cyclin E mRNA levels in cancer-free kidneys and in peritumoral tissues, but not in ccRCCs. Furthermore, there was no correlation in the cancers between *CCNE* promoter binding by E2F factors and the level of cyclin E mRNA. At least two mechanisms come to mind to explain these results. First, the increase in the amount of E2F1 protein might not translate into its increased transcriptional activity. Indeed, such activity of E2F1 depends not only on the level of this protein, but also on the amount and function of other proteins: pRB, whose activity is significantly disturbed in the majority of tumors, other members of the E2F family that compete with E2F1 for binding to DNA, or altered function of other proteins. Second, factors other than E2F1 could be also involved in direct regulation of *CCNE* promoter in ccRCC. It is noteworthy that our data are consistent with the results obtained by Pajalunga and Crescenzi (2004) who showed that forced overexpression of E2F factors did not increase *CCNE* transcription, but specifically stabilized cyclin E by reducing its conjugation with ubiquitin and, consequently, its degradation.

In cancer tissues, there was no correlation between the amount of total cyclin E mRNA and the amount of total cyclin E or LMW forms of this protein that are products of proteolysis of full length cyclin E. This was an expected result since it has previously been shown that the increase in the amount of cyclin E in cancers is dependent not only on the increase of gene transcription, but also on the presence and accumulation of LMW forms that are both more stable and more active than the full length protein (Porter *et al.*, 2001; Akli & Keyomarsi, 2003; Pajalunga & Crescenzi, 2004; Bedrosian *et al.*, 2004). However, we observed a lack of such correlations also in peritumoral tissues. It is possible that cancer cells secrete factors having a profound influence on the metabolism of the adjacent tissues, and that the LMW cyclin E forms observed in the respective controls arise not only as a result of the increased transcription of *CCNE*, but also due to proteolysis of full length protein (as in the cancer cells themselves) as suggested by the finding that in these tissues the amount of LMW forms correlated with the amount of full length cyclin E.

Taken together, our data show that the expression of cyclin E and its LMW forms was excessive in ccRCC tissues both in well differentiat-

ed and in poorly differentiated cases. This overexpression correlated with the decreasing degree of tumor differentiation suggesting a role for cyclin E both in the initiation, and in the promotion of kidney tumorigenesis.

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