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Rearrangements and Increased Expression of Cyclin D1 (CCND1) in Neuroblastoma

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Abstract
Cyclin D1 regulates G1 cell cycle progression by controlling the phosphorylation of the retinoblastoma protein (pRb). This pathway is frequently deregulated in many malignancies. In neuroblastoma, however, no consistent G1 cell cycle checkpoint aberrations have been found. We examined the possible deregulation of Cyclin D1 (CCND1) in this tumour. mRNA expression profiles of neuroblastoma generated by SAGE (Serial Analysis of Gene Expression) revealed a high expression of CCND1 in a subset of neuroblastoma cell lines and tumours. The CCND1 expression level can equal to 0.3% of the total cellular mRNA. Northern blot analysis of CCND1 expression showed a relative over-expression in 16 of 23 neuroblastoma cell lines and 10 of 15 tumour samples. In the majority of cases, the high CCND1 mRNA levels also lead to high CCND1 protein levels. In search for mechanisms causing this relative over-expression we screened for amplifications and rearrangements of CCND1. Five amplifications were found in 202 neuroblastoma tumours and cell lines. Analysis of the 3’UTR of CCND1 showed a rearrangement in 1 of 96 tumours. These clonal aberrations of CCND1 together with the high expression suggest a role for deregulated CCND1 activity in neuroblastoma tumorigenesis.

Introduction
D type Cyclins play a critical role in cell cycle progression by activating their Cyclin Dependent Kinase partners CDK4 and CDK6. This leads to phosphorylation of the pocket protein pRb which then releases the E2F transcription factors that promote expression of several key regulators of G1 to S cell cycle progression. This cascade causes irreversible passage through the restriction point (1-3).
Genetic aberrations and over-expression of the Cyclin D1 gene (CCND1) have been reported for several human neoplasms (4). CCND1 aberrations were first identified in parathyroid adenomas, where the PTH promoter is juxtaposed to CCND1 on chromosome 11, causing over-expression of CCND1 (2). In mantle cell lymphoma, juxtapositioning of cis-acting sequences by translocations as well as rearrangements in the 3’ untranslated region (UTR) of CCND1 can cause over-expression (5-7). Amplification of CCND1 is reported with high frequency in head and neck squamous cell carcinoma, oesophageal cancers, squamous cell carcinoma of the lung and mammary carcinoma. Several human cancers exhibit CCND1 over-expression without these genetic aberrations (4). Furthermore, transgenic mice with tissue specific CCND1 over-expression in mammary epithelial cells, develop mammary hyperplasia and carcinoma (8).
Neuroblastoma is a childhood tumour with an extreme diversity in clinical behaviour, ranging from spontaneous regression in most stage 4s and some stage 1 or 2 tumours, to fast and fatal progression despite aggressive treatment in many stage 3 and 4 tumours (9;10). Several genetic aberrations, like MYCN amplification, 1p deletion, and 17q gain have been associated with poor prognosis (11-13). Analysis of cell cycle checkpoint aberrations has not yielded many clues for neuroblastoma tumour development (14;15). A mutation in CDK6 that disrupts the CDKN2A inhibiting function and two CDK4 amplifications have been described in neuroblastoma cell lines (16;17).
In this study we report a high expression of CCND1 both at the RNA and protein level, in approximately two thirds of neuroblastoma cell lines and tumours. Amplification of
CCND1 was found in 1 of 24 neuroblastoma cell lines and 4 out of 179 tumour samples. In one tumour we found a rearrangement in the 3’UTR of CCND1. Together, these data indicate that aberrant expression of CCND1 might play a role in neuroblastoma tumorigenesis.

**Materials and methods:**

*Sample Collection and Clinical Data.*

Neuroblastoma cell line culture conditions were as described (18). Tumour samples and normal tissue were obtained in the period 1990 - 2001 from patients treated in the Emma Kinderziekenhuis-Academic Medical Center in Amsterdam, Sophia Kinderziekenhuis in Rotterdam and the University Hospital of Gent. Tumour samples were snap frozen and stored at -80°C. All patients were classified according to the INSS staging system. Additional clinical and genetic diagnostics were performed as described (12).

*SAGE and Data Analysis.*

SAGE libraries of 2 neuroblastoma cell lines and 3 neuroblastoma tumours were generated as described (19). The other SAGE library data were obtained from NCBI SAGEmap (http://www.ncbi.nlm.nih.gov/SAGE) (Lal et al., 1999). Combined tissue type SAGE libraries from the various tumours were each derived from at least 4 individual SAGE libraries of tissue and cell lines of a specific tumour. Each tissue type library consisted of a minimal 96,494 tags. The normal combined tissue SAGE library consisted of 18 different SAGE libraries of 7 different non-cultured primary tissue samples (brain, breast, colon, kidney, lung, pancreas and prostate). The total tag number in this compiled library was 879,252. The normal cell line SAGE library consisted of 13 different libraries from short term cultures of 8 different tissues (brain, breast, kidney, ovary, pancreas, prostate, skin and vascular tissue). The total tag number of this compiled library was 386,014. Data were analysed using the human transcriptome map web application (http://bioinfo.amc.uva.nl/htm/) (20).

*Northern Blotting.*

Total cellular RNA was isolated using standard LiCl method (21)), quantified spectrophotometrically and 1μg of RNA was loaded on a test gel for adjustment of variations in RNA concentration. 15 μg of RNA was then electrophoresed through a 1% agarose gel containing 6.7% formaldehyde and blotted on Hybond N membrane (Amersham, Piscataway, USA) in 16.9x SSC and 5.7 % formaldehyde.

*Southern Blotting.*

DNA isolation was performed according to standard procedures (12). For amplification and breakpoint analysis, tumour and normal tissue samples were digested with TaqI and BamHI restriction enzymes (Roche, New Jersey, USA). Electrophoreses was carried out on a 1% agarose gel in 0.5% TBE. DNA fragments were transferred to a Hybond N+ membrane (Amersham, Piscataway, USA) by the alkaline blotting method.
Hybridisations.

Probes were generated by RT-PCR. For CCND1 we used the following primers: CCND1 exon 3-4 probe: 5’-tcattgaacacttcctctcc-3’ and 5’-gtcacacttgatcactctgg-3’; CCND1 exon 5 3’UTR centromeric probe: 5’-tatccctgccccctcct-3’and 5’-tcctcaaatacgagcaa-3’; CCND1 exon 5 3’UTR telomeric probe: 5’-acaagttggtagctgcc-3’ and 5’-atgccggttatctgtttg-3’; COX8 probe: 5’-aagcttggtgatcagtaa-3’and 5’-ttatattgatcagcaagggg-3’. Probes were sequence verified. Probes were 32P labelled by random priming. We hybridised Southern and Northern blot filters for 16 hours at 65°C in 0.5 M Na2HPO4, 7% SDS, 1mM EDTA and 50 μg/ml herring sperm DNA. Measurement of signal intensity was performed with a Fuji phosphor imager and Aida 2.41 software.

Western Blotting.

The neuroblastoma cell lines were harvested during exponential growth. From tumours 5 consecutive 20 μm sections were used for protein isolation. Cells were lysed in 50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS and protease inhibitors (protease cocktail, Roche, New Jersey, USA). Protein was quantified with RC-DC protein assay (Bio-Rad, Hercules, USA). Loading was controlled by Bio-Rad Coomassie staining of a reference SDS-Page gel. Lysates were separated on a 12.5 % SDS-Page gel and electro

![Figure 1. CCND1 expression levels in SAGE libraries normalised to 20,000 tags per library. The CCND1 expression levels for the neuroblastoma (NB) libraries were derived from our SAGE libraries. The expression levels of CCND1 in other tumour libraries, the normal tissue library and normal cell line library were derived from NCBI SAGEmap (http://www.ncbi.nlm.nih.gov/SAGE) (Lal et al., 1999). The normal tissue library was compiled from SAGE libraries of normal brain, breast, colon, kidney, lung, pancreas and prostate tissues. The normal cell line library was compiled from SAGE libraries of short term cultures of brain, breast, kidney, ovary, pancreas, prostate, skin and vascular cells.](image-url)
blotted on a transfer membrane (Millipore, Gloucestershire, UK). Blocking and incubation were performed using standard procedures. DCS-6 mouse monoclonal CCND1 (Neomarkers, Fremont, USA) and Actin C2 mouse monoclonal (Santa Cruz, Santa Cruz, USA) antibodies were used as primary antibodies. After incubation with a secondary sheep anti-mouse or anti-rabbit horseradish peroxidase linked antibody (Amersham, Piscataway, USA), proteins were visualised using an ECL detection kit (Amersham, Piscataway, USA). Antibodies were stripped from the membrane using a 2% SDS, 100 mM β-mercapto-ethanol, 62.5 mM Tris-HCL pH 6.7 buffer.

**Results**

*SAGE Expression Profiling Reveals High CCND1 Expression in Neuroblastoma.*

We constructed SAGE mRNA expression libraries from two neuroblastoma cell lines and three neuroblastoma tumours (22). SAGE can quantitatively identify all transcripts expressed in these tissues and cell lines. In total, we sequenced >300,000 SAGE mRNA transcript tags, each representing a mRNA expressed in these neuroblastomas. Analysis of mRNA expression profiles from the 11q chromosomal region with the Human Transcriptome Map web application (20) revealed a high expression of CCND1 in a subset of neuroblastomas (Fig 1). For the cell line SK-N-FI and the tumour N165, the levels of CCND1 expression were 63.0 / 20,000 and 36.9 / 20,000 tags respectively. Some neuroblastomas therefore reach a CCND1 mRNA expression level of over 0.3% of all mRNAs. Only in a subset of the SAGE libraries of mammary carcinoma, were comparable CCND1 expression levels found. In all other SAGE libraries from, e.g., colon tumours and brain

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<th>Gene</th>
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<th>N165</th>
<th>N159</th>
<th>NS2</th>
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Table 1. Expression of cell cycle regulating genes in SAGE libraries. The expression of cell cycle regulating genes normalised to 20,000 tags in each library is shown. SAGE libraries are derived from neuroblastoma cell lines (SK-N-FI, IMR-32) and neuroblastoma tumours (NS2, N159 and N165). Also the expression in a compiled normal tissue library is given. This library is derived from 31 different SAGE libraries from normal tissue and short term cultured cell lines from normal tissue (Lal et al, 1999).
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tumours CCND1 expression ranged from 2-8 tags / 20,000 tags. Combined SAGE libraries of normal tissues or of short term cultures of normal tissues both showed a much lower expression of CCND1 (2.2 and 4.0 per 20,000 tags respectively) compared to neuroblastoma libraries. We also compared the expression of CCND1 with the expression of other cell cycle regulating genes (Table 1). Only Cyclin B1 was expressed to a fairly high level (12.9 per 20,000 tags) in the IMR-32 SAGE library. The other Cyclins, Cyclin Dependent Kinases and Cyclin Dependent Kinase Inhibitors were expressed at relatively low levels.

High CCND1 Expression Levels in a Panel of Cell Lines and Tumours.
In order to verify the high CCND1 expression in neuroblastoma we hybridised a Northern blot of 23 neuroblastoma cell lines with a CCND1 exon 3-4 probe. This confirmed the high expression levels measured by SAGE in the cell lines SK-N-FI and IMR-32 (Fig 2A). Furthermore, it showed comparable or even higher CCND1 expression in 16 of 23 (70%) cell lines (Fig 2A). As neuroblastoma cell lines represent only an aggressive subset of the whole clinical spectrum, we also analysed CCND1 expression in a series of 15...
neuroblastoma tumours. CCND1 was expressed in a SAGE library of tumour N52, to a level of 8.9 per 20,000 tags. On Northern blot N52 showed only a weak CCND1 expression (Fig 2C). CCND1 mRNA expression in the tumour samples was elevated compared to N52 CCND1 expression in 10 of 15 (67%) tumour samples (Fig 2C). To analyse CCND1 protein levels, Western blots with 15 neuroblastoma tumours and 9 neuroblastoma cell lines were probed with the DCS-6 monoclonal antibody. For the cell lines the protein levels correlated quite well with the observed mRNA levels (Fig 2B). The 15 tumour samples also showed a correlation between mRNA and protein levels (Fig 2C). All tumours with low CCND1 mRNA expression had low levels of CCND1 protein. The majority of tumours with high mRNA expression also had readily detectable levels of CCND1 protein. Tumour N442 with high CCND1 mRNA but weak CCND1 protein expression was a clear exception. This suggests a posttranscriptional regulation of CCND1 levels in this tumour. The relatively low CCND1 level in tumour N249 may have been caused by a lower amount of protein loaded on the gel, as suggested by a control hybridisation of the filter with an anti-Actin antibody.

Amplification of CCND1 in Neuroblastoma.

We analysed the CCND1 copy number in our panel of 179 tumour samples and 23 cell lines by Southern blot. We performed double hybridisation with a COX8 probe for loading control. The COX8 gene maps 8.1 Mb downstream of CCND1 in chromosome band 11q13. In the panel of 179 tumour samples, 4 cases showed amplification ranging from 4 to 18 fold (Fig 3). In our cell line panel we found one amplification of CCND1 in Lan6 (data not shown). For 2 of these 5 neuroblastomas with CCND1 amplification we could also analyse the level of mRNA expression. Cell line Lan6 and the tumour N194 both showed high CCND1 mRNA expression (Fig 2A and Fig 2C). No RNA or protein was available for the other samples.
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We screened 96 neuroblastoma tumours by hybridisation of a Southern blot with a 3’ UTR CCND1 probe (Fig 4C). In tumour N508 we detected an abnormal CCND1 fragment, both with BamHI and a TaqI digests (Fig 4A). This indicated a breakpoint between the two TaqI sites in the 3’ UTR of CCND1. We then analysed whether this rearrangement occurred 3’ or 5’ of probe A. We hybridised the Exon 3-4 probe to the filter. In the BamHI digest this probe recognised the same rearranged band as probe A. This showed that the rearrangement occurred 3’ of probe A. We then hybridised the filter for loading control. COX8 maps to chromosomal band 11q13.

**Figure 3.** CCND1 amplifications in neuroblastoma tumours. Southern blot filter with TaqI digested pairs of tumour (t) and constitutional DNA (c) were hybridised with a CCND1 probe and a COX8 probe for loading control. COX8 maps to chromosomal band 11q13.

**Figure 4.** Rearrangement in the CCND1 3’ UTR of tumour N508. (A) Southern blot of the tumour N508 (t) and constitutional tissue (c) of the same patient, hybridised with Probe A. The abnormal sized bands in the tumour DNA digested with TaqI and BamHI are indicated by arrows. (B) Northern Blot of the tumour N508 (t) and the SK-N-AS cell line as control (c). (C) A schematic presentation of CCND1 with the location of the restriction sites for BamHI and TaqI endonucleases. The untranslated regions are indicated as light grey and the coding sequence as dark grey. Probe A was used for the screening of 98 patients for 3’ UTR rearrangements. Probe B and the exon 3-4 probe were used to determine the breakpoint in tumour N508.
with probe B, which is located 3’ of probe A. Unexpectedly, probe B showed no aberrant band in the TaqI and BamHI digest. This indicated that the breakpoint in the TaqI and BamHI fragment had occurred in between probes A and probe B (Fig 4C). Probe B only recognised the un-rearranged allele of the 3.1 kb TaqI CCND1 fragment, implying that the 3’ end of the TaqI fragment of the rearranged CCND1 allele was missing. This indicates that there was either an interstitial deletion in the aberrant CCND1 copy or an unbalanced translocation. To determine whether there was indeed mRNA transcribed from the rearranged copy of CCND1, we hybridised a Northern blot of N508 with the exon 3-4 probe. This probe recognizes the normal 4.3 Kb CCND1 transcript in control RNA (Fig 4B). In N508, in addition an aberrant transcript of 7 Kb was present (Fig 4B), indicating that the rearranged CCND1 gene was indeed transcribed.

**Correlations with Other Genetic Alterations and Clinical Parameters.**

Since CCND1 RNA was differentially expressed in neuroblastoma tumours, we searched for correlations with other well known diagnostic and prognostic parameters. Expression levels were scored as absent/low for tumour N36, N52, N106, N225 and N406 and moderate/high for the other tumours. No significant correlations were found with tumour stage, age, ferritin levels, LDH levels or any of the genetic characteristics (MYCN amplification, 1p deletion and 17q gain) (Table 2). Although there was a tendency that high CCND1 expression is more frequently found in tumours with LOH of 1p, gain of 17q and higher tumour stages, the number of analysed tumours was clearly to small to support a statistically significant correlation.

**Discussion**

The analysis of our SAGE libraries showed that CCND1 was highly expressed in a large subset of neuroblastomas. Expression levels in cell line SK-N-FI were about 0.3% of all cellular mRNAs and the Northern blot analysis of the cell line panel showed even higher levels in some cell lines. A major question is whether these high levels play a role in neuroblastoma pathogenesis, or whether they represent an unusually high expression level that is normal for neuroblasts. The finding of chromosomal rearrangements or amplifications of CCND1 in some neuroblastoma tumours suggests that these...
cells emerged from clonal selection of CCND1 over-expressing cells. This implies that increased CCND1 levels in the tumours with CCND1 amplification contribute to tumourigenesis. The expression levels in the tumours with CCND1 aberrations were well comparable with the expression levels found in two third of the neuroblastomas with germline CCND1. This indicates that also in neuroblastoma without CCND1 amplification, the high CCND1 expression levels might be involved in tumorigenesis. This pattern is similar to other malignancies like colorectal carcinoma, where aberrations of CCND1 are rare, but over-expression is found in 30-46% of cases (23). Since only a small percentage of neuroblastomas with high CCND1 expression had rearrangements, also regulating mechanisms are likely to be involved in causing the high CCND1 expression. CCND1 expression and translation are regulated by several cell signalling pathways. The Wnt-beta catenin pathway, RAS-MAPK pathways, the Neu pathway and PI-3-kinase/Akt dependent pathway are all regulating CCND1 at the transcriptional level and are thus candidates for aberrations (24-26). These pathways are known to be involved in several malignancies, but as yet they are not reported to play a role in neuroblastoma.

Aberrations in cell cycle regulation have been reported in neuroblastoma cell lines at low frequency. Two CDK4 amplifications and one mutation in the Cyclin Dependent Kinase Inhibitor (CDKI) binding site of CDK6 have been found (16;17). Alterations of CDKIs are very rare in neuroblastoma. The well known alterations in the CDKI CDKN2A gene are not found. Surprisingly, elevated CDKN2A expression has been reported in neuroblastoma together with high CDK4 kinase activity in the same cells leading to Rb protein hyperphosphorylation (14-16;27). Integrating these data suggests that the high expression of CCND1 in neuroblastoma could stimulate the CDK4/6 kinase activity. The levels of CDKN2A could be insufficient to inhibit this increased kinase activity and thus pRb remains hyperphosphorylated, allowing G1 progression. An interesting finding was the observed CCND1 rearrangement in one neuroblastoma. Rearrangements in the 3’UTR of CCND1 are frequently found in other tumours. E.g., mantle cell lymphoma show rearrangements in the 3’UTR which are thought to result in more stable transcripts (5;6). We showed that the rearranged CCND1 in this tumour expressed an aberrant transcript of 7 Kb due to alterations in the 3’end of the transcript. This transcript could have an altered half life as has been observed for the transcript of rearranged CCND1 genes in mantle cell lymphoma (Rimokh et al., 1994). It has been shown that CCND1 deficient mice have only minor developmental abnormalities (28). Cell cycle regulation in these mice is thought to be rescued by Cyclin D2 and D3. A recent report indicates that CCND1 ablation in mice protects against the development of mammary carcinoma induced by by Neu and Ras oncogenes. This indicates that, in mammary epithelial cells, the Neu and Ras pathways are connected to the cell cycle machinery solely through CCND1 (29). Together, these two findings suggest that CCND1 ablation could be highly selective in preventing tumour proliferation dependent on the activated oncogenic pathway. New CDK 4/6 specific inhibitors are generated by protein based drug design (30;31). The high CCND1 expression in a substantial part of neuroblastoma tumours combined with these recent advances in intervention design warrant further studies to evaluate CCND1-CDK4/6 as therapeutic targets in neuroblastoma.
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Reference List

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