*Brief communication***Cyclin I is expressed in human breast cancer and closely associated with VEGF and KDR expression**

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Summary

In the present study, cyclin I protein expression in 114 invasive human breast cancers was correlated with cell cycle and angiogenesis-related proteins and clinico-pathological data. A strong association was found between cytoplasmic cyclin I staining and VEGF ($p = 0.001$) as well as the VEGF receptor KDR ($p = 0.001$), suggesting a link between cyclin I and angiogenesis.

In addition to the BRCA1 and BRCA2 genes that have been linked to hereditary forms of breast cancer it is obvious that there are genes that play important role(s) in the aetiology of the 95% of breast cancers that are not inherited. New screening approaches are necessary to reveal potential key gene products in breast cancer formation. We cloned 950 breast cancer associated cDNAs by subtractive hybridization and differential display methods using matched breast tumor and normal breast cell line RNAs [1]. One of the novel cDNAs, now known as cyclin I, is weakly expressed in normal human breast epithelium suggesting that its presence in breast tumors could be linked to the development and/or maintenance of breast cancer [2, 3].

A previous publication suggested that a testis-specific variant of cyclin I exists, and we examined its relationship to the cyclin I gene by determining the full mRNA and gene structure for cyclin I (Figure 1a) discovering that the cyclin I gene consists of 7 exons spanning 27,983 nucleotides of chromosome 4 in the human genome sequence (Figure 1a). Transcribed as a single 1912 bp transcript our sequence represents an extension of the original mRNA sequence by 652 nucleotides [2]. No splice variants appear to exist as no GenBank cDNA sequences lack any of the exons. The mRNA sequence for the putative testis-specific isoform ends at nucleotide 76 of exon 6, an unlikely position for a splicing variant. This 'isoform' sequence was published after sequencing of a pBluescript cDNA insert, and in our opinion represents an incomplete clone of the full mRNA [4]. Thus, the cyclin I gene is most likely expressed as a single transcript and this is supported by the presence of a single band in Northern blots from earlier work [2–4].

Structurally, the cyclin I protein is most closely related to cyclin G, which is independent of the cell cycle. Cyclin G is a transcriptional target of the p53 tumor suppressor protein in irradiated cells, suggesting that it might be involved in p53-mediated apoptosis [5]. Some other cyclins have additional roles in non-cycling cells [6]. Cyclin I regulation during cell-cycle progression *in vivo* has been studied in the synchronized wave of hepatocyte proliferation observed after partial hepatectomy [7] and using a synchronized human fibroblast cell line [2]. In both model systems, no significant change in the cyclin I mRNA expression level was observed. This is consistent with the previously reported lack of induction of cyclin I by actinomycin D in human cell lines [8].

Cyclin I may be post-transcriptionally regulated by ubiquitination as we reported elsewhere [3]. Ubiquitin-mediated degradation plays a central role in the regulation of cell growth and cell proliferation by controlling the abundance of key cell cycle proteins. In addition, unscheduled proteolysis of many cell cycle regulators is found in multiple types of human cancers, contributing significantly to tumorigenesis [9,10]. Dysregulation of ubiquitin-proteasome mediated degradation of cyclin I could therefore be related to aberrant cell cycle regulation and/or resistance to apoptosis, which are characteristic of invasive breast and other tumors.

Clinical implications of cyclin I protein expression in primary breast tumors were investigated here by high-throughput tumor tissue microarray and immunohistochemistry (Figure 1b). The protein content of important cell cycle regulatory proteins (retinoblastoma protein, cyclin E, cyclin D1, p16, p21, p27 and p53) was also determined as well as key molecules in the transformation and tumor angiogenesis process (c-erbB2,

was possible to evaluate the cyclin I staining. The cyclin I antibody produced cytoplasmic staining of variable intensity in the majority of the breast cancer samples with low staining in 30.2%, intermediate in 43.0% and

strong staining in 19.8% whereas only 7% lacked cytoplasmic cyclin I staining (Table 1). This result is consistent with earlier work where the subcellular localization of GFP–cyclin I fusion proteins was examined, revealing that cyclin I does not associate with a particular cellular compartment, but is essentially uniformly distributed throughout the cells [11].

In order to obtain as much information as possible, the cyclin I staining intensities in the cytoplasm and nuclei as well as the fraction of positive nuclei were recorded for each tumor (Table 2). These results were compared with clinico-pathologic data associated with each patient sample (Table 2). From this data we generated *p*-values describing how probable it is that we have observed false

positive results. *p*-Values less than 5% are considered to be of significant confidence and are asterisked in Table 2. We have also included *r*-values for this data, a correlation coefficient that measures the connection between two variables such that if the *r*-value is 1 it is a perfect correlation between the two values (Table 2). The intensity of staining in both nuclear and cytoplasmic regions of each tumor sample was found to have a strong inverse association with stage and tumor size (Table 2). However there was no association between cyclin I and tumor grade, histologic type, estrogen receptor status or proliferation. Eighty-four of the patients with unilateral and non-metastatic disease were included in a survival analysis with a median follow-up time of 8.7 years. There were no significant associations between the cyclin I staining parameters and breast cancer specific survival.

The G1/S checkpoint is frequently deregulated in breast cancer resulting in alterations in the protein content of important cell cycle regulatory proteins which we hypothesized might affect or be affected by changes in cyclin I status. Due to a potential function for cyclin I in tumorigenesis either linked to cell cycle functions or to other pathways, we screened for possible associations

Table 1. Distribution of staining intensity in primary breast samples

Staining intensity	Percent of samples
None = 0	7
Low = 1	30.2
Intermediate = 2	43.0
Strong = 3	19.8

Table 2. Associations between cyclin I and clinico-pathologic variables, cyclin I, oncogenic proteins, and cell cycle regulatory protein expression (*p*-values, *r*-values in parenthesis)

Clinical parameters & cell cycle proteins	Nuclear fraction <i>p</i> -values (<i>r</i> -values)	Cyclin I staining intensity	
		Nuclear <i>p</i> -values (<i>r</i> -values)	Cytoplasmic <i>p</i> -values (<i>r</i> -values)
Tumor type	0.095	0.997	0.696
Grade	0.486 (0.077)	0.122	0.539
Axillar lymph node	0.031* (−0.248)	0.287	0.165
Stage	0.026* (−0.252)	0.232	0.04*
Menopausal status	0.175 (0.149)	0.240	0.411
Estrogen receptor	0.992 (−0.001)	0.371	0.794
Progesteron receptor	0.961 (0.005)	0.752	0.203
Tumor size	0.001* (−0.352)	0.026* (−0.247)	0.775 (−0.032)
Proliferation	0.636 (−0.053)	0.790 (0.030)	0.372 (−0.099)
Cyclin E	0.196 (0.141)	0.388 (0.094)	0.909 (−0.012)
Cyclin D1	0.644 (0.051)	0.619 (−0.054)	0.650 (0.050)
p16	0.132 (0.164)	0.838 (0.022)	0.126 (0.166)
p21	0.940 (0.009)	0.663 (−0.049)	0.670 (−0.048)
p27	0.614 (0.058)	0.521 (0.074)	0.306 (−0.118)
pRb	0.871 (−0.019)	0.51	0.244
p53	0.284 (0.119)	0.021*	0.384
p53M	0.700 (0.042)	0.316	0.436
c-erbB2	0.520 (0.072)	0.982	0.875
VEGF	0.995 (0.001)	0.094 (0.184)	< 0.001* (0.431)
KDR	0.015* (0.263)	0.011* (0.274)	< 0.001* (0.499)

Diagnostic tumor samples were collected from 114 patients with breast carcinoma, immunohistochemically stained and analyzed for variables as described [15]. Cyclin I immunohistochemistry was done using the antibody described in [3] and the sections from the tissue microarray described in [12–14, 16]. Microarrayed tumor sections were examined by two separate investigators and a classification into four grades (0–3) was applied representing lack of staining, weak staining, intermediate staining and strong staining, respectively [15]. Associations between linear parameters were calculated using Spearman's two-tail significance test. The Kruskal–Wallis non-parametric significance test was applied when comparing a linear and a grouped parameter. Comparisons between groups were performed with the log-rank test and cross table analysis with the chi-square test. All calculations were performed using SPSS version 9.0 (SPSS Inc., IL, USA).

between cyclin I and known molecular effectors of cellular transformation. This was possible because tissue sections were derived from microarrayed breast tumors that we had earlier used to determine protein levels of retinoblastoma protein, cyclin E, cyclin D1, p16, p21, p27 and p53 as well as c-erbB2, VEGF, and KDR [12–14]. Cyclin I was not associated with proliferation or the majority of cell cycle regulatory proteins including Rb-phosphorylation, cyclin E associated kinase activity or p53 mutations (Table 2). There was nevertheless a strong link between cytoplasmic cyclin I and tumor-specific VEGF as well as between all three cyclin I staining parameters and the VEGF receptor KDR (Figure 1b, Table 2). This association is interesting and may indicate that cyclin I is involved in angiogenesis, a potentially separate function from cell cycle regulation.

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