Age dependent switching role of cyclin D1 in breast cancer

Carmela Rinaldi, Natalia Maria Malarba, Rosalia D’Angelo, Antonina Sidoti, Attilio Leottac, Santo Leo, Basilio Caparellio, Alessia Ruggeria, Vincenzo Mollace and Aldo Amato

Faculty of Medicine, Department of Biomorphology and Biotechnologies, Division of Biology and Genetics, University of Messina, Messina, Italy
Laboratory of Toxicology, Faculty of Pharmacy, Department of Pharmaceutical Science, University of Magna Graecia, Catanzaro, Italy
Department of Pathology, Lamezia Terme Hospital, Lamezia Terme CZ, Italy

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Abstract. Background: Cyclin D1 gene (CCND1) plays pivotal roles in the development of several human cancers, including breast cancer, functioning as an oncogene. The aim of this study was to better understand the molecular dynamics of ductal carcinomas with regard to proliferation and the ageing process.

Methods: 130 cases of ductal breast cancer in postmenopausal women, aged 52–96 in 3 age classes were selected. Tumoral tissues preserved in formaldehyde solution and subsequently embedded in paraffin were subjected to analysis Fluorescence in situ Hybridization (FISH), Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and immuno-histochemical tests. The molecular variables studied were estimated in relation to the patients’ age.

Results: The results obtained suggest that the increment of the levels of cyclin D1 in intra-ductal breast tumors in older woman that we have examined is significantly associated with a lower proliferation rate.

Conclusion: Cyclin D1, which characterizes tumor in young women as molecular director involved in strengthening tumoral proliferation mechanisms, may be seen as a potential blocking molecular switch in corresponding tumours in old women.

Keywords: CCND1, cyclin D1, breast intra-ductal tumors, cell cycle, older women

1. Introduction

Breast cancer is the most frequent malignancy in women and ductal carcinoma in situ (DCIS) of the breast is a heterogeneous disease, clinically and biologically.

Cyclin D1 gene (CCND1) encodes the regulatory subunit of a holoenzyme promoting progression through the G1-S phase of the cell cycle in a manner dependent on cyclin-dependent kinases (CDKs). The deduced cyclin D1 protein contains 295 amino acids and has a calculated molecular mass of 33.7 kD. Amplification or overexpression of CCND1 plays pivotal roles in the development of several human cancers, including breast cancer, functioning as an oncogene [11–21]. In addition, CCND1 regulates transcription factors, coactivators and corepressors that govern histone acetylation and chromatin remodeling proteins. The upregulation occurs through several different pathways, including epidermal growth factor receptor (EGFR) family proteins, that act not only as signaling initiators on the cell membrane, but also as nuclear transcriptional cofactors [16]. EGFR associated with the promoter region of CCND1, activates
overexpression of cyclin D1, through transcriptional activation of the gene, giving a more aggressive tumor behavior [10].

CCND1 has roles also in cellular growth, metabolism, cellular differentiation and apoptosis. Particularly, deregulation of CCND1 expression is one of the most common genetic aberrations found in malignancies and in actively cycling cells significantly increased also migration [15]. A recent study suggests that CCND1 overexpression markedly increased the apoptotic response of cells to CDK inhibitors (CDI) in human myeloma cells (U266) [5] often accompanied by cell cycle arrest and down-regulation of nuclear factor kappa B (NF-kappa B) [18–20]. The enhanced expression of cyclin D1 in breast ductal carcinoma is associated also with an increase in both proliferative activity [23] and expression of oestrogen receptors (ER) [3]. The immunohistochemical detection of estrogen (ER) and progesterone (PR) receptors in breast cancer is routinely used for prognostic and predictive testing [13]. The contemporary presence of enhanced cyclin D1 expression, receptor positivity for oestrogens and increased expression of ki-67 antigen, a marker of cellular proliferative activity, was considered as a proliferative axis of the breast ductal carcinoma. Increased cyclin D1 expression may be caused by manifold molecular events [25]: oestrogen hormones, for example, may induce cyclin D1 expression, especially in breast ductal carcinoma in situ and negative erb 1 [14–24]. At the same time, increased cyclin D1 expression, in breast ductal carcinoma in situ, does not automatically lead to increased cellular proliferation [9]. Also the confirmation of CCND1 amplification does not always mean the presence of increased expression, in fact few authors have found a correlation between CCND1 amplification and an increase in the protein [6, 22–27].

Previous studies have shown that an enhanced expression of cyclin D1 in senescent fibroblasts in culture, is not random. It is not clear, however, whether the presence of higher levels of the protein, which arrests cell growth, is an epiphenomenon or a cause or a consequence of the cell aging process. Studies on senescent cell lines have highlighted how these cells, if stimulated to grow, are suitable for carrying out repetitive events but, on entering the S phase, collapse [7–17]. The mechanism for the collapse of the proliferation may be linked to the reduced expression of genes involved in the initial phase of the process. In senescent cells, the levels of cyclin D1 are increased about seven fold compared to normal young cells. The enhanced expression of cyclin D1 also leads to proliferation inhibition [2–8]. Therefore the overexpression of cyclin D1 could be a defense mechanism, which the senescent cell triggers in response to inappropriate stimulation regarding proliferation [28]. The presence of an enhanced expression of cyclin D1 concentration in senescent cell lines is however a signal that this cyclin is involved in the cell ageing process. These data suggest that in aged cells the cyclin D1 overexpression has the function to stop proliferation. The aim of this study is to investigate on the cyclin D1 proliferating role in aging breast cancer cases. We involved a group of old women affected by advanced breast cancer to clarify cyclin D1 role in this particular subset of breast cancer cases.

2. Materials and methods

2.1. Collection of clinical data

Clinical information was obtained by review of medical records in specific electronic registers. Before recruitment, each patient was asked to sign an informed consent form. 130 patients’ information included: date of birth, age at diagnosis, age at last menstrual period and number of lymph nodes involved and stage at diagnosis. The median age of these patients was 52–96 years. 104 patients (80%) underwent total mastectomy and 26 (20%) breast-conserving surgery. No adjuvant chemotherapy was administered. The size of the breast cancers at diagnosis (according to the criteria of the World Health Organisation) was: pT1–pT2 in 116 cases (89%) and pT3–pT4 in 14 cases (11%). These cases were classified according to nuclear grading (NG) and necrosis at the time of diagnosis. We have no data on the clinical outcomes of these patients.

Tumoral tissues, obtained after surgery, were preserved in formaldehyde solution for more than 24 hours, and subsequently embedded in paraffin. The slides, on which they were fixed tissue sections of 4 μm sections, were coloured with haematoxylin and eosin to identify the most representative areas, which were then pre-treated in preparation for Fluorescence in situ Hybridization (FISH) and immuno-histochemical tests.

40 μm sections of formalin-fixed, paraffin-embedded tumour biopsy samples were identified also to test and validate the gene expression analysis by Reverse Transcription-Polymerase Chain Reaction (RT-PCR).
2.2. Immunohistochemical staining

Our method of performing immunohistochemistry has been reported in literature [7–26]. Briefly, the sections were incubated with the appropriate mouse monoclonal antibodies: Anti-ER Clone 1D5 (DAKO Corporation); Anti-PgR Clone PgR 636 (DAKO Corporation); Anti-ki67 Clone Mb1 (ZYMED), used at dilution rate of 1 : 30; Anti-cyclin D1 Clone SP4 (NEOMARKERS), used at dilution rate of 1 : 100. Primary antibody detection was accomplished using the DAKO Biotin-Streptavidin Peroxidase Detection Kit. Sections were incubated with biotinylated goat anti-mouse antibody for 10 min and streptavidin peroxidase for 5 min with Tris-buffered saline washes between steps. The diaminobenzidine tetrachloride Substrate Kit was used to visualize the antibody-antigen complex. Appropriate negative controls, consisting of histological sections of each case processed without the addition of primary antibody, were prepared for each antigen retrieval method, along with a positive/negative multi-tissue control section.

Two independent observers performed a blind analysis. All requisite techniques involved were previously validated and results of scoring were found to be reproducible between the two independent operators, as reported previously [17].

2.3. Fluorescence in situ hybridization (FISH)

To determine gene and chromosome copy numbers, levels of gene amplification or deletion in situ, both a locus-specific DNA probe and a chromosome enumeration probe for 11q13 and centromere 11 were used. Dual-target, dual-colour FISH assays were performed using the Path Vysion Cyclin D1 DNA probe kit (Vysis, Rosedale House, Richmond Surrey, UK), including the LSI Cyclin D1 sequence labelled in Spectrum Orange and the chromosome 11 centromere sequence labelled in Spectrum Green. The probes were applied to 4 μm tissue sections of tumour specimens, according to procedure reported previously [17]. Microscopic analysis was performed on an Olympus PROVIS AX70 microscope. Fluorescence signals were scored using single-band pass filters for DAPI, FITC, Texas Red. Fluochromes signals were captured individually and representative images were generated via computer digitization with the CytoVision System and Cyto- Probe Software.

The evaluation was performed considering the cases with a normal disomic signal pattern for CCND1, and centromere 11 showed a ratio of gene copy number to centromere copy number 2/2 or 1/1, respectively. Increased copy number was assessed when at least 30% of nuclei showed increased centromere and/or gene FISH signals. Amplification was assessed when at least half of these nuclei (15% of total) showed an unbalanced increase of gene copy numbers (ratio of gene copy number to centromere copy number ≥1.5). Genes were considered to be gained (over-presented) with a ratio of >1.5 and lost (under-presented) with a ratio of <1.5.

2.4. Total RNA isolation

RNA was extracted using the RecoverAll ™ Total Nucleic Acid Isolation Kit for FFPE Tissues (Applied Biosystems, cat.# AM1975) [1–19]. First the paraffin was removed from the tissue through a series of washing with ethanol and xylene. Next, it was subjected to a rigorous digestion with Proteinase K and Digestion Buffer in an incubation time tailored for RNA. Binding Solution and ethanol were added to the lysate and the solution was loaded onto a column. RNA bound to the resin was then washed with Wash Solution to remove any impurities and then eluted with the Elution Buffer.

2.5. Reverse transcription – polymerase chain reaction (RT-PCR)

Total RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, cat.# 4368814) in a 20 μl reaction volume containing 3 μg of total RNA, 10X RT-PCR buffer, 10U RNase inhibitor, 50U Multiscribe Reverse Transcriptase, 25X mM Dntp mix, 10X Random primer and Nuclease-free H2O under the following thermal cycler conditions: 25° for 10 min, 37° for 120 min, 85° for 5 min and 4° forever. cDNA is then used in separate PCR assay using 50 μl volume containing 5 μl of cDNA as template, 2.5 U AmpliTaq Gold DNA Polymerase and 0.2 μM of each primer designed by us of mRNA sequences (Accession NCBI NM_053056.2 – Table 1). DNA amplification was performed conve-
Table 1

| Primer | Forward | Reverse | Length (bp) | Exon nt | Accession NCBI |
|--------|---------|---------|-------------|---------|----------------|
| CCND1  | 5'-ggataactccggagcagt-3' | 5'-gaggtctcccattcga-3' | 107 | 2 | NM-053056.2 |
| GAPDH  | 5'-acctgccttggaggg5' | 5'-gcagggacctgagggccc-3' | 340 | 8-9 | NM-002046.3 |

CCND1: Cyclin D1 gene; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; nt: nucleotides; Length: amplified fragment length.

Table 2

| Age group | Tumour size | Nuclear grading | Lymph nodes | Total cases |
|-----------|-------------|-----------------|-------------|-------------|
| ≤70       | pT1–pT2     | Low             | 44           | 60          |
| 71–76     | pT3–pT4     | 40              | 12           | 64          |
| ≥76       | High        | 8               | 13           | 13          |

2.6. Statistical analysis

Given the variability of the data and asymmetrical distribution, statistical analysis was performed considering 3 age classes determined by calculating quartiles. Age classes were: E1 = ≤70 years, E2 = 71–76 years, E3 = ≥76 years.

Spearman rank correlation was used to calculate interdependence between the variables studied. Relative Incidence (RI) and the confidence interval (CI) at 95% were estimated to evaluate the influence of each molecular variable considered in three classes of age (E1 = ≤70 years, E2 = 71–76 years, E3 = ≥76 years).

Comparing subjects belonging to the first age class (E1) against the other classes (E2-E3) for the ER expression, RI was 0.38, indicating a prevalence of a low ER expression values for the E1 class. Further comparisons (E2 → E1 + E3 and E3 → E1 + E2) showing a RI values of 1.24 and 1.41, respectively, confirmed a prevalence in the E2 and E3 classes of high ER expression (Table 3A).

3. Results

3.1. Characteristics of the study population

Tumour size, nuclear grading and lymph node positivity are outlined in Table 2.

Significant interdependences on application of the Spearman rank correlation was founded and included the relationship between the ER and the age of selected breast cancer cases (p = 0.019); moreover, the PR and ER expression (p = 0.000).

In particular, we studied the estimated risk index (RI) in relation to each molecular variable considered in three classes of age (E1 = ≤70 years, E2 = 71–76 years, E3 = ≥76 years).

Comparing subjects belonging to the first age class (E1) against the other classes (E2-E3) for the PR expression, RI was 0.38, indicating a prevalence of a low PR expression values for the E1 class. Further comparisons (E2 → E1 + E3 and E3 → E1 + E2) showing a RI values of 1.24 and 1.41, respectively, confirmed a prevalence in the E2 and E3 classes of high PR expression (Table 3A).

A strong prevalence of high values of ki-67 proliferation index was registered for the subjects from the E1 class (RI value of 1.89). In the second age class, on the other hand, IR = 0.62 indicates a prevalence of low
Table 3A

| Class | Age | ER High | ER Low | RI | CI | PR High | PR Low | RI | CI | Ki-67 High | Ki-67 Low | RI | CI |
|-------|-----|---------|--------|----|----|--------|--------|----|----|-----------|-----------|----|----|
| E1    | ≤70| 8       | 26     | 0.38 | 0.19–0.78 | 10 | 24 | 1.00 | 0.53–1.90 | 22 | 12 | 1.89 | 1.02–3.49 |
| E2    | 71–76 | 16    | 16 | 1.24 | 0.68–2.26 | 10 | 22 | 1.10 | 0.57–2.10 | 12 | 20 | 0.62 | 0.33–1.16 |
| E3    | ≥76 | 34    | 30 | 1.41 | 0.99–1.99 | 18 | 46 | 0.95 | 0.66–1.49 | 30 | 34 | 0.91 | 0.64–1.29 |

Estimated risk index regarding expression of ER, PR and Ki-67 antigen variable relative to the three age classes.

Table 3B

| Class | Age | CCND1 High | CCND1 Low | RI | CI | D1 High | D1 Low | RI | CI |
|-------|-----|------------|-----------|----|----|--------|--------|----|----|
| E1    | ≤70| 6         | 28        | 0.39 | 0.17–0.88 | 8 | 28 | 0.45 | 0.20–1.00 |
| E2    | 71–76 | 12    | 20 | 1.09 | 0.59–2.03 | 8 | 24 | 0.70 | 0.34–1.42 |
| E3    | ≥76 | 28    | 36 | 1.42 | 1.01–1.99 | 28 | 36 | 1.63 | 1.17–2.27 |

Estimated risk index regarding amplification of CCND1 and expression of cyclin D1 variable relative to the three age classes.

**Discussion**

Our study shows that there is, also in vivo, a higher demand for cyclin D1 expression and that the highest

![Fig. 1. 2% Agarose gel electrophorogram of RT PCR products amplified using human RNA as template, primer pairs of CCND1 and primer pairs of GAPDH as internal control. Lane 1-2 we ran an aliquot containing GAPDH and CCND1 coamplified RT-PCR products of section tumor specimens of young and old patients respectively. Lane 3 we ran a 100 bp ladder.](image-url)

Fig. 1. 2% Agarose gel electrophorogram of RT PCR products amplified using human RNA as template, primer pairs of CCND1 and primer pairs of GAPDH as internal control. Lane 1-2 we ran an aliquot containing GAPDH and CCND1 coamplified RT-PCR products of section tumor specimens of young and old patients respectively. Lane 3 we ran a 100 bp ladder.

incidence of CCND1 amplification is seen, especially, in older age group (Fig. 2B) than younger (Fig. 2A). Molecular study shows also there are higher levels of CCND1 expression in older age group, than younger (Fig. 1). We wanted to verify if there was amplification of CCND1 also in healthy breast tissue of the patients. With the exception of one case, where there was cytoplasmic immunohistochemical positivity (data not shown), not considered significant and classified as a negative finding by the anatomic-pathologists. There were no signs of amplification in the gene under study, nor any enhanced nuclear expression of the corresponding protein.
Hybridization in situ of CCND1 in tissue section of tumor specimens of older age group (B) shows the highest incidence of gene amplification than younger patients (A).

We can therefore state that enhanced cyclin D1 is exclusive to cells which have undergone a transformation process in an aged tissular microenvironment and then to underline how breast tumoral disease has emerged as an age-dependent disease. On the basis of this information we have reconsidered the molecular variables studied in relation with the patient’s age and we have seen that an increase in age increases the degree of receptor expression for estrogens. In effect, the highest value was recorded in the oldest group (E3), with RI = 1.41, followed by RI = 1.24 in E2, with the lowest level in the youngest group E1, RI 0.38.

It is known that the aging process is a gradual hyper-differentiation process. The breast tissue suffers this molecular and structural differentiation process; young tissue is, paradoxically, poorer in receptor expression compared to older tissue.

The degree of expression for progesterone receptors regarding age shows a decreasing trend, opposite to the trend for oestrogen receptors. The degree of expression for progesterone receptors shows a particular trend, with RI = 1.01, 1.10 and 0.95 in E1, E2 and E3 respectively (Table 3A), suggesting that progesterone receptor expression decreases with an increase in age albeit without non-drastic changes. These data suggest a possible protective role of PR expression in the breast cancerogenesis, as reported in other papers [4–12].

Also the proliferation rate, in our study, reflects the relationship with the age variable: in group E1 RI = 1.89, while E2 and E3 have RI values of 0.62 and 0.91 respectively. There is an obvious discrepancy between the value recorded in the youngest compared to the other two groups where there is an evident reduction in the cellular reproduction capacity highlighted by histotype, grading and stage of selected cases as being equal. These results would suggest that there is a biological aggressivity age dependent. It was obvious that, with an increase in age, there was a corresponding parallel between the state of CCND1 and cyclin D1. In particular, an increasing trend of gene amplification is associated with a correspondent protein expression.

In biological and prognostic terms, it should be underlined that the proliferative axis cannot be identified in post-menopausal women, as in the corresponding younger population, with receptoral positivity for oestrogens, high proliferation rate and high expression of cyclin D1. Indeed this population of women is affected by tumors with a low proliferation rate which correlated to an increased expression of cyclin D1 and shows increased expression of oestrogen receptors.

5. Conclusions

The results of this study confirm the important role of Cyclin D1 in tumoral proliferation mechanisms, in the young women tumors. And above all, we show a more determining role of cyclin D1 in the hyperdifferntiation process associated with the process of cell aging, in old women tumors.

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