Research Article

Cyclin A2 Protein Overexpression Is Not Caused by Gene Amplification in Colon Cancer

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1. Introduction

In the quest to illuminate the complex nature of malignancy, the regulation of cell growth through the family of cyclins has received attention [1]. Cyclin D1 more than cyclin A2 has previously been shown to be implicated in human cancers [2, 3]. In breast cancer, overexpression of cyclin A2 protein has shown an unfavourable impact on patient prognosis [4–10]. In colon cancer, however, the prognostic value varies among different studies, showing a favourable prognosis [11, 12] and an unfavourable prognosis [13, 14]. The mechanism behind the increased protein expression of cyclin A2 is unknown. One might speculate if the overexpression is a result of increased synthesis of cyclin A2, the clinical outcome of the patient might be different from another whose overexpression is caused by impaired degradation. It has been questioned whether increased expression is a contributing factor to tumourigenesis or only a consequence of increased cell proliferation [15]. One of the possible mechanisms behind overexpression of cyclin A2 is gene amplification. Several studies have been performed regarding gene amplification of cyclin A2 [4, 11, 16]. However, most of these studies have analysed tissue that include both tumour cells and stromal cells and tissue, while protein expression...
has been evaluated in the tumour cells only. Depending on the amount of tumour cells in the tissue analyzed, correlation may vary. Hence, evaluation of CCNA2 amplification solely in the tumour cells could provide important information that might clarify the mechanisms behind cyclin A2 protein overexpression.

2. Materials and Methods

2.1. Patients and Tissue Specimens. From a consecutive series of 219 patients with colonic adenocarcinoma and 80 patients with breast carcinoma, formalin-fixed, paraffin-embedded tissue samples from 23 colonic adenocarcinomas and 11 breast carcinomas contained more than 60% cyclin A2 positive cells (Figures 1 and 2), as shown in two previously studies [4, 11]. Tumour tissue samples from 22 colon and eight breast tumours were accessible to this study. Normal tissue was available in 21/22 colon cancer patients and in 5/8 breast cancer patients.

Of the patients included in the colonic adenocarcinoma group, one was classified as Dukes A, 17 as Dukes B, three as Dukes C, and one as Dukes D. Nineteen of the tumours were moderately differentiated, whereas three were poorly differentiated carcinomas. There were ten females and 12 males with a mean age of 68 ± 11 years (range 44–87) at surgery. Breast carcinomas included in this study were classified as invasive ductal carcinomas. Six tumours were classified as histology grade 3 and two as histology grade 2.

As a positive control for the ability of our methods to detect gene amplification, we analyzed breast cancer tissue samples where CCNA2 amplification was previously detected by others and correlated significantly with the overexpressing of cyclin A2 protein [4]. Tumour cells were isolated with laser microdissection and gene amplification analyses were performed with both real-time PCR and fluorescence in situ hybridization (FISH).

2.2. Processing of Tissue. For immunohistochemistry, four μm sections from formalin-fixed, paraffin-embedded tumour tissue were applied on coated slides. Deparaffinization, rehydration, and epitope retrieval were performed in a Dako PT Link (Dako, Glostrup, Denmark) at 97°C for 20 min. The immunostaining procedure was carried out in a Dako Autostainer Plus applying the Envision Flex, High pH system (Dako).

For genetic analysis of selected tumour cells, formalin-fixed, paraffin-embedded biopsies were cooled down at 4°C for two hours. They were cut using a conventional microtome and five μm sections were mounted on a slide with polyethylene naphthalate membrane according to the manufacturer’s instructions. Slides used for breast cancer tissue were additionally coated with a 0.1% solution of Poly-L-Lysine (Sigma-Aldrich, St. Louis, MO, USA) at 60°C for one hour to increase the adherence of the sections to the slides. The slides were incubated at 70°C overnight to improve adhesiveness. Prior to laser microdissection, the sections were deparaffinized and rehydrated using xylene and ethanol according to the following procedure: twice with xylene for five minutes, then twice each with 100% and 96% ethanol and once with 70% ethanol for two minutes, respectively.

For whole tumour analysis, approximately three 10μm sections were put into 2 mL reaction tubes. In addition, sections from separate paraffin-embedded blocks containing adjacent normal tissue from the patients were put into 2 mL reaction tubes for further processing. Preliminary removal of paraffin was performed on tumour and normal sections by extraction with xylene as follows: once with xylene, twice with ethanol, evaporate ethanol at 37°C for 15 minutes. The sections were washed twice with PBS to remove the fixative before DNA extraction.

2.3. Laser Microdissection. The sections for laser microdissection were processed following the manufacturer’s recommendations including five minutes staining with Hagens
Hematoxylin solution and a quick dehydration through increasing ethanol series followed by air-drying at room temperature. Microdissection was performed by using the PALM Laser-MicroBeam System (PALM, Bernried, Germany) which enables the contact-free isolation of selected cells by means of the laser pressure catapulting technique into the lid of a 0.5 mL reaction tube. At first, areas were pointed out by a pathologist under microscopic guidance. Subsequently, the pathologist was consulted during uncertainty. Approximately 5000 cells were captured per specimen.

2.4. DNA Extraction. DNA extraction of laser microdissected cells was performed according to Lehmann & Kreipe [17]. The cells were lysed in 200 μL of proteinase K digestion buffer (50 mM Tris–HCl, pH 8.1, 1 mM EDTA, 0.5% Tween 20, 0.1 mg/mL proteinase K) in an inverted position in a hybridization oven at 56°C overnight. The lysed samples were centrifuged and incubated at 95°C for ten minutes in a thermo block with heated lid for inactivation of proteinase K. The lysate was transferred to a new tube, and the DNA was precipitated by adding 0.1 vol of sodium acetate (pH 7.0) containing 100 μg/mL Dextran (Sigma) as a carrier (100 μg/mL) and 2.5 vol of ethanol. This precipitation step removes stains and other impurities and concentrates the DNA. After incubation at −20°C for at least 24 h, the samples were centrifuged (20 min, 14,000 g, 4°C), washed once with 70% ethanol, air dried, and dissolved in 50 μL nuclease-free water. An aliquot of this was used for subsequent real-time PCR analysis. DNA isolation of total tumour and normal tissue was performed according to DNeasy Blood & Tissue Kit (Qiagen, Düsseldorf, Germany).

2.5. Quantitative Real-Time PCR. Quantitative real-time PCR analysis was performed using the ABI Prism 7900HT Real-Time PCR System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) with the software program SDS 2.3 (Applied Biosystems). Due to the low number of cells analysed (approximately 500 cells/PCR reaction) and to the assumable small differences in gene copy numbers, three endogenous controls were included in every experiment: HSA (human serum albumin), RPL32 (ribosomal protein L32), and ACTB (β-actin). Primer and probe assays for HSA and CCNA2 were ordered from Applied Biosystems using the Custom TaqMan Gene Expression Assay Service (order 2400949). The primer and probe sequences are as follows (the first oligonucleotide is the forward PCR primer, the second one is the TaqMan probe, and the third one is the reverse PCR primer): CCNA2; 5’-GCCACAGTAGGAGTTCTCCCCATAT, FAM5’-CCCCGCCAACACTG-3’NFQ, 5’-CAGACCGGCAGCATACACA. HSA; 5’-CCCCGAACTCTTTTTCTTGTGCTAAA, FAM5’-AAGCTGCTTTTACAG-3’NFQ, 5’-ACAGGCGAGGCGAGTATTACAG. The primers and probes had a concentration of 18 μM and five μM, respectively. Primer and probe assays for RPL32 (Hs00851655_g1) and ACTB (Probe part nr. 4316033. Primers part nr. 4304971) were commercially designed and were ordered from Applied Biosystems. The PCR amplification was performed using a 96-well tray with a 20 μL final reaction mixture containing ten μL TaqMan Universal PCR Master Mix, No AmpErase UNG (2X), one μL 20X Assay Mix, four μL dH2O, and five μL DNA (5 ng/μL). DNA from normal tissue was used as a calibrator sample. In one colon cancer patient and three breast cancer patients, normal tissue was not available. A mixture of DNA extracted from all normal tissue in the corresponding cancer group was utilized as calibrator sample for these patients. Commercial DNA from human placenta (Sigma) was used as positive control and dH2O was used as negative control. Experiments were performed in triplicate for each sample. Default thermal cycling conditions were used in the real-time PCR analysis (Applied Biosystems).

2.6. Fluorescence In Situ Hybridization. Gene copy numbers were detected by FISH. 2–2.5 μm thick paraffin sections were cut, deparaffinized, and pretreated using an in-house method which includes microwave treatment for 20 minutes in TRIS-EDTA buffer pH 9.0 and subsequent incubation with ISH protease 3 (Ventana Medical Systems, Inc., Tucson, AZ, USA) at 37°C for 15–20 minutes. Cyclin A2-probes were selected by means of the UCSC human genome browser Human February 2009 (hg19) assembly (http://genome.ucsc.edu/) and produced as fosmid probes. Cy3-dUTP-labeling of fosmids was performed essentially as described by Lichter et al. [18]. A commercial Spectrum Green-labelled probe specific for centromer 4 (Abbott Molecular Inc., Des Plaines, IL, USA) was applied for reference. The slides were washed, dehydrated and incubated with a mixture of the two probes. DNA was denatured for ten minutes at 80°C and hybridization was carried out overnight at 37°C. After washing, nuclei were counterstained with Dapi (VectaDish Mounting Medium with Dapi) (Vector Laboratories, Inc., Burlingame, CA, USA) and results were read in a Zeiss fluorescence microscope (Carl Zeiss, Oberkochen, Germany) counting Cyclin A2 and centromere 4 signals in at least 300 nuclei within tumor areas and in 200 nuclei within normal mucosa/glands aside the tumour (internal control). For each sample, the Cyclin A2/centromere 4 signal ratio and the average number of Cyclin A2 and centromer 4 signals per nucleus were calculated both in tumour areas and normal epithelial cells.

2.7. Data Analysis. To determine the CCNA2 amplification we used the 2−ΔΔCt method [19]. According to this method the relative gene copy number is calculated by the following equation:

\[ 2^{-\Delta\Delta C_t} = \frac{\left( C_{t, CCNA2} - C_{t, endogenous\ controls} \right)}{\left( C_{t, CCNA2} - C_{t, endogenous\ controls} \right)}_{tumour\ cells/total\ tumour} - \frac{\left( C_{t, CCNA2} - C_{t, endogenous\ controls} \right)}{\left( C_{t, CCNA2} - C_{t, endogenous\ controls} \right)}_{normal} \]
The data provided from this equation represents the fold change in gene number in selected tumour cells or total tumour relative to normal tissue when normalized to the three endogenous controls. The value of $$2^{-\Delta \Delta Ct} > 2$$ was considered CCNA2 amplification. Approximately equal efficiencies of target (CCNA2) and reference genes (HSA, RPL32 and ACTB) amplification were confirmed, meaning that the $$2^{-\Delta \Delta Ct}$$ method is suitable (data not shown).

2.8. Statistical Analysis. The number of tumours selected was based on the assumption that nearly 30% of breast cancer show CCNA amplification [4] thus we expected our study to verify gene amplifications in some of the chosen tumours. For colonic tumours, the portion with gene amplification is unknown. The cutoff value for gene amplification was set to 2.0 for statistical analyses. Copy numbers below 2.0 were defined to represent samples with nonamplified CCNA2, whereas values above 2.0 were considered to reflect samples with CCNA2 amplification. A value of $$P < 0.05$$ was considered statistically significant. Statistical analyses were carried out using SPSS software (version 13 for Windows).

2.9. Research Ethics. This project was approved by the Regional Committee for Medical Research Ethics, Eastern Norway. The Norwegian Social Science Data Service has approved the collection and analysis of data.

3. Results

Of the colon cancer samples available for laser capture microdissection and gene amplification analyses, none showed CCNA2 amplification (Table 1). The mean value of the gene copy numbers of CCNA2 in the microdissected tumour cells in colon was 0.88 ± 0.28. This value was significantly lower than for the total tumour, which was calculated to be 1.02 ± 0.30 ($$P = 0.008$$). However, the values indicate that there is no CCNA2 amplification in the colon cancer samples, in either the tumour cells or the total tumour.

When studying the available breast cancer samples, the gene amplification analyses revealed CCNA2 amplification in 3/8 total tumour samples ($$2^{-\Delta \Delta Ct} > 2$$) (Table 2). Additionally, in one of these 3 samples, gene amplification was detected in the isolated tumour cells as well. The mean value of the gene copy numbers of CCNA2 in the breast cancer samples was 1.24 ± 0.60 and 1.76 ± 0.66 in the isolated tumour cells and total tumour, respectively. The difference in gene copy number between tumour cells and total tumour was significant and calculated to be 0.52 ($$P = 0.004$$).

Due to small differences in copy numbers between different tumour samples, the real-time PCR data were verified on five colon and five breast cancer patients using FISH. FISH demonstrated no CCNA2 amplification in any of the patients examined (Tables 1 and 2). However, in breast cancer cases 1, 2, and 4 the FISH data indicated polysomy of chromosome 4 or segments of chromosome 4 in the tumour tissue when compared to the patient’s normal tissue (>2.0 signals per nucleus) (Table 2, Figure 3). Noticeably, these breast cancer patients were poorly differentiated, invasive ductal carcinomas, and thus may be aneuploid and contain complex karyotypic abnormalities. Two of these three patients showed increased copy number in real-time PCR.

4. Discussion

In the present study, CCNA2 amplification was investigated in isolated tumour cells from colon cancer tissue in order to elucidate the mechanism behind high cyclin A2 protein expression. CCNA2 amplification was not detected in either the whole tumour tissue sections or the isolated tumour cells in the colon cancer patients using real-time PCR and FISH. Thus, isolating and analyzing tumour cells in colonic tumour tissue in order to examine the mechanism behind the increased cyclin A2 protein expression did not explain the cause of the protein expression in this tissue. However, with real-time PCR we detected gene amplification of CCNA2 in tumour cells and in total tumour in high cyclin A2-expressing breast cancer tissue. Here, statistical significant correlation between gene amplification and protein expression has previously been identified [4]. In the breast cancer cases, CCNA2 amplification was detected in three out of eight total tumour tissue samples using real-time PCR. In isolated tumour cells, CCNA2 amplification was detected in one of these three samples. In this tissue amplification in approximately 30% of cases was detected as shown by others [4]. However, FISH did not detect any CCNA2 amplification, but showed polysomy of chromosome 4 or segments of chromosome 4 in three tumour tissue samples, suggesting an alternative mechanism than gene amplification behind the high protein expression of cyclin A2 in breast cancer tissue. So, the assumed gene amplification in breast cancer tissue could be explained by chromosomal aberrations, underlining the importance of verifying experimental results with an alternative method.
Table 1: No CCNA2 amplification was detected in either the isolated tumour cells or the total tumour in the colon cancer patients. Fluorescence in situ hybridization data in tumour tissue and in normal tissue confirm this.

| Case | Fold change in isolated tumour cells | Fold change in total tumour | Tumour tissue: cyclin A2 pr nucleus/centromere 4 pr nucleus | Normal tissue: cyclin A2 pr nucleus/centromere 4 pr nucleus |
|------|-------------------------------------|-----------------------------|----------------------------------------------------------|----------------------------------------------------------|
| 1    | 0.77                                | 1.09                        | 1.5/2.0                                                  | 1.3/1.6                                                  |
| 2    | 0.59                                | 0.65                        | 1.14                                                    |                                                          |
| 3    | 0.80                                | 1.16                        | 2.0/2.0                                                  | 1.5/1.6                                                  |
| 4    | 1.18                                | 1.14                        |                                                          |                                                          |
| 5    | 0.68                                | 0.91                        |                                                          |                                                          |
| 6    | 0.89                                | 1.07                        |                                                          |                                                          |
| 7    | 1.17                                | 1.32                        |                                                          |                                                          |
| 8    | 1.46                                | 1.22                        |                                                          |                                                          |
| 9    | 1.23                                | 1.85                        |                                                          |                                                          |
| 10   | 0.66                                | 0.58                        |                                                          |                                                          |
| 11   | 1.08                                | 1.50                        |                                                          |                                                          |
| 12   | 0.38                                | 0.80                        |                                                          |                                                          |
| 13   | 0.72                                | 0.77                        |                                                          |                                                          |
| 14   | 1.25                                | 0.97                        |                                                          |                                                          |
| 15   | 1.06                                | 1.17                        |                                                          |                                                          |
| 16   | 1.07                                | 1.24                        | 1.9/1.9                                                  |                                                          |
| 17   | 0.50                                | 0.84                        |                                                          |                                                          |
| 18   | 0.82                                | 0.91                        | 1.4/1.8                                                  | 1.5/1.6                                                  |
| 19   | 0.66                                | 0.86                        |                                                          |                                                          |
| 20   | 0.92                                | 0.92                        |                                                          |                                                          |
| 21   | 0.77                                | 0.85                        |                                                          |                                                          |
| 22   | 0.67                                | 0.64                        | 1.7/1.8                                                  | 1.2/1.4                                                  |

$^{a2-\Delta\Delta\text{Ct}}$, where $\Delta\Delta\text{Ct} = (\text{Ct}_{\text{CCNA2}} - \text{Ct}_{\text{endogenous controls}})_{\text{Tumour cells}} - (\text{Ct}_{\text{CCNA2}} - \text{Ct}_{\text{endogenous controls}})_{\text{Normal}}$.

$^{b}$Chromosome aberration was measured by fluorescence in situ hybridization counting cyclin A2 and centromere 4 signals in nuclei within tumour areas and normal mucosa. The average number of cyclin A2 and centromere 4 signals per nucleus is indicated both in tumour and normal tissue.

Our results on CCNA2 amplification in colon tumours are in agreement with other studies claiming that protein overexpression is not necessarily caused by gene amplification [4, 11, 16, 20]. These results indicate that cyclin A2 protein overexpression, as seen with immunohistochemistry in colon carcinomas, is neither associated with CCNA2 amplification nor polysomy of chromosome 4. Even though tumour cells frequently display genomic and chromosomal instability which may lead to aberrant protein expression, several posttranscriptional mechanisms also influence the protein expression, including epigenetics, and these may influence the cyclin A2 protein expression [1, 21].

Overexpression of cyclin A2 is often detected in the colonic adenocarcinomas and the impact of cyclin A2 protein expression on clinical outcome of colon cancer patients is not clear. Some studies have shown that cyclin A2 overexpression is associated with reduced overall survival [13, 14], while others have reported an association with improved survival [11]. A recent study on rectal carcinomas claimed that overexpression of cyclin A2 was associated with lower local recurrence rate [22]. In this paper, it is indicated that variations in protein expression of cyclin A2 could partly be explained by gene amplification in rectal carcinomas. It is possible that mechanisms behind cyclin A2 overexpression vary between different tumour types and depending on what mechanisms cause the protein overexpression, the impact on patient prognosis may also vary.

Prior to the isolation process by laser microdissection the cells were stained with hematoxylin and eosin to reveal the morphology. Ideally, the tumour sections should have been stained for cyclin A2 in order to isolate and analyse the cyclin A2-expressing tumour cells. Unfortunately, immunohistochemical staining for cyclin A2 resulted in low-quality morphology, and together with the dehydrated sections and the lack of cover slip, the identification of tumour cells became difficult. More than 60% of the tumour cells displayed cyclin A2 protein, most of them nearly 90%. Isolating as many as approximately 5000 tumour cells from every tissue sample may have contributed to cyclin A2-positive cells dominated the total number of isolated cells.

Combining laser microdissection with real-time PCR to achieve the quantitative analysis of DNA from FFPE samples alleviates several difficulties. The DNA from FFPE samples is to a different extent fragmented in individual samples.
Furthermore, when analysing a minute number of cells small variations in gene copy number may be difficult to detect [17]. For these reasons we have performed a relative quantification of gene copy numbers by real-time PCR. Our design includes three endogenous controls which are not amplified in the tumour tissue examined, and we have minimized the amplicon size because of DNA fragmentation. DNA amplification involves a stretch of DNA much larger than the selected gene that is amplified [23]. Thus, verifying with an alternative method is important in order to confirm or disprove the PCR results.

Others have found that combining laser microdissection with sensitive molecular methods such as real-time PCR, previously unanswerable questions about the development and progression of human cancer can be explored [24–26]. Notwithstanding, in our study, the increased cyclin A2 protein expression could not be explained by CCNA2 amplification analyses in isolated colonic tumour cells. We thus conclude that the study of single-tumour cell, does not reveal additional information to the analysis of the complex tumour.

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Table 2: CCNA2 amplification in tumour cells and in total tumour in breast cancer tissue compared to normal tissue, as well as fluorescence in situ hybridization data in tumour tissue and in normal tissue.

| Case | Tumour type | Histological grade | Fold change in isolated tumour cells | Fold change in total tumour | Tumour tissue: cyclin A2 pr nucleus/centromere 4 pr nucleus | Normal tissue: cyclin A2 pr nucleus/centromere 4 pr nucleus |
|------|-------------|--------------------|-------------------------------------|-----------------------------|------------------------------------------------------------|------------------------------------------------------------|
| 1    | Ductal      | 3                  | 2.11                                | 2.70                        | 2.1/2.2                                                   | 1.7/1.8                                                   |
| 2    | Ductal      | 3                  | 1.83                                | 2.04                        | 2.0/2.2                                                   | 1.7/1.8                                                   |
| 3    | Ductal      | 2                  | 1.04                                | 2.14                        | 1.5/1.6                                                   | 1.7/1.8                                                   |
| 4    | Ductal      | 2                  | 1.65                                | 1.81                        | 3.4/3.7                                                   | 1.7/1.8                                                   |
| 5    | Ductal      | 2                  | 1.37                                | 1.99                        | 1.8/1.7                                                   | 1.8/1.9                                                   |
| 6    | Ductal      | 3                  | 0.39                                | 0.60                        | —                                                         | —                                                         |
| 7    | Ductal      | 3                  | 0.88                                | 1.79                        | —                                                         | —                                                         |
| 8    | Ductal      | 3                  | 0.67                                | 0.99                        | —                                                         | —                                                         |

\[ \Delta \Delta Ct = (Ct_{CCNA2} - Ct_{endogenous controls})_{tumour cells/Tumour} - (Ct_{CCNA2} - Ct_{endogenous controls})_{Normal}. \]

\[ \text{Chromosome aberration was measured by fluorescence in situ hybridization counting cyclin A2 and centromere 4 signals in nuclei within tumour areas and normal mucosa. The average number of cyclin A2 and centromere 4 signals per nucleus are indicated both in tumour and normal tissue.} \]

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