

# MUC1 cytoplasmic tail detection using CT33 polyclonal and CT2 monoclonal antibodies in breast and colorectal tissue

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**Summary.** The immunohistochemical detection (IHC) of MUC1-CT employing a polyclonal antibody (CT33) in relation to CT2 monoclonal antibody (MAb) was analyzed. Western blot (WB) was used to determine the molecular mass of CT. Materials and methods: we studied 163 breast and 89 colorectal cancer specimens, 10 breast and 14 colorectal benign conditions, and 12 breast and 20 colorectal normal samples. From each tumor sample, subcellular fractions were obtained and analyzed by SDS-PAGE and WB. A nonparametric statistical analysis was employed; data were standardized and a Kendall-Tau correlation was applied. Results: by IHC, 146/163 (90%) and 151/163 (93%) of breast cancer were positive with CT33 and CT2, respectively; a statistically significant correlation was obtained ( $t=0.5199$ ). Seven out of ten (70%) benign breast specimens were positive with CT33 while all samples stained with CT2; in normal breast sample tissues, all were positive with both Abs. In colorectal cancer samples, both antibodies stained 47/89 (53%) samples; CT2 reacted in 13/14 (93%) of benign samples while CT33 showed a positive reaction in 9/14 (64%) of benign specimens. In normal samples, CT2 showed staining in 17/20 (85%) of samples and CT33 was reactive in 12/20 (60%). By WB, in breast and colorectal cancer samples, similar results were obtained with both antibodies: a main band at about 30kDa which represents the smaller subunit.

**Conclusion:** CT33 polyclonal antibody has demonstrated its efficacy to detect MUC1 in breast and colorectal cancer tissues with similar reactivity to CT2. It is worthwhile to affirm that CT33 is a good indicator of MUC1 expression.

**Key words:** MUC1 cytoplasmic tail, Breast cancer, CT33 polyclonal antibody, CT2 monoclonal antibody

## Introduction

MUC1 is a large, type I transmembrane glycoprotein expressed on the apical surface of normal secretory epithelial cells and at high levels over the entire surface of carcinoma cells. Full-length MUC1 is synthesized as a single polypeptide chain, which undergoes an early proteolytic cleavage, creating two subunits that remain associated during its post-translational processing and transport to the cell surface (Ligtenberg et al., 1990). The large fragment contains most of the extracellular domain while the smaller subunit consists of a short extracellular domain, a transmembrane domain and a tyrosine-phosphorylated cytoplasmic tail (CT) of 72 amino acids (Gendler, 1990; Ligtenberg, 1990; Gendler et al., 2001). Indirect evidence suggests that the CT is involved in signal transduction, since it contains potential docking sites for Grb2/Sos and  $\beta$ -catenin and can be phosphorylated by GSK-3 $\beta$ , c-Src, EGFR and PKC- $\delta$  (Gendler, 2001; Pandey et al., 1995; Yamamoto et al., 1997; Agrawal et al., 1998; Li et al., 1998, 2001a-c; Schroeder et al., 2001; Ren et al., 2002). Furthermore, Meerzaman et al. (2001) demonstrated that tyrosine phosphorylation of MUC1 led to activation of the Ras-Raf-MEK-Erk2 MAP kinase pathway. As Wang et al. (2003) speculated, it is probable that individual phosphorylations may be related to distinct functional roles played by MUC1, since it has been implicated in diverse physiological processes such as cell-cell and cell-matrix adhesion, cell growth and differentiation, oncogenesis and both natural as well as specific immunity.

Some of the immunological events associated with

benign breast disease can also be detected in cancer samples and, hence, comparing the antigenic differences between non-malignant and malignant lesions could lead to the identification of epitopes involved in cancer development.

Different authors have detected MUC1 in normal and neoplastic breast tissue employing anti-MUC1 protein core MAbs such as C595, HMFG1, HMFG2, SM3 (Griffiths et al., 1987, Croce et al., 1997, Luna-Moré et al., 2001, Rahn et al., 2001). In a previous report (Croce et al., 2003a), we have proven an increase in MUC1 detection employing an anti-CT MAb (CT2) in comparison with different anti-extracellular monoclonal antibodies in breast cancer. In the present study, we investigated the subcellular localization of MUC1 CT in human breast and colorectal cancer tissues by means of two antibodies: CT2 MAb and CT33 polyclonal antibody. Our results indicated that CT was distributed on the plasma membrane, in the cytoplasm and nucleus. In addition, Western blot analysis revealed that both antibodies reacted with a main band at the same MW (approximately 30kD).

CT33 polyclonal antibody has demonstrated its efficacy to detect MUC1 in breast and colorectal cancer tissues with reactivity similar to CT2 MAb. It is worthwhile to speculate that CT33 is a good indicator of MUC1 expression.

## Materials and methods

### Materials

#### Tumor samples

A total of 163 breast cancer specimens and 89 colorectal cancer samples were studied which were clinically categorized and staged according to the UICC TNM classification system; 10 breast and 14 colorectal benign conditions as well as 12 breast and 20 colorectal normal samples were also assayed.

A tumor fraction was fixed in methacarn (methanol 60%, chloroform 30% and acetic acid 10%) for two hours for histopathological diagnosis and immunohistochemical analysis while another was rinsed with fresh sterile Hank's balanced salt solution (HBSS) and subsequently processed for preparation of subcellular fractions.

Experiments were done according to the Helsinki Declaration. Informed consent was obtained from all patients included in this study. This research was approved by the local Human Investigation Committee, Faculty of Medical Sciences, University of La Plata, Argentina.

#### Antibodies

Two anti-MUC1 cytoplasmic tail (CT) antibodies were assayed: a polyclonal antibody (CT33) and a monoclonal antibody (CT2 MAb) developed at Prof.

Sandra Gendler Department, in Armenian hamster, directed against the last 17 amino acids (SSLSYNTPAVAATSANL) of the cytoplasmic tail of MUC1 (Schroeder et al., 2001).

An anti-MUC1 extracellular protein core MAb was also assayed, C595 MAb (IgG3) which was gently provided by Prof. Mike Price, Cancer Research Laboratory, University of Nottingham, Nottingham, UK; it defines the tetrameric epitope RPAP (Price et al., 1990).

### Methods

#### Preparation of CT33 Antiserum

A synthetic peptide was commercially prepared (Quality Biologicals, Gaithersburg, MD) corresponding to the COOH-terminal 17 amino acids of the MUC1 cytoplasmic tail (NH<sub>2</sub>-SSLSYTNPAVAATSANL-COOH). The peptide was coupled to keyhole limpet hemocyanin (KLH) (Sigma, St. Louis, MO) using glutaraldehyde as described by Harlow and Lane (1988). Briefly, 5.0 mg of KLH in 1.0 ml of PBS, pH 7.0 was mixed with 5.0 mg of peptide in 1.0 ml of PBS, to which was added in a drop-wise fashion, while vortexing, 2.0 ml of 0.2% glutaraldehyde (Sigma). The mixture was incubated for 1 hr at room temperature with constant mixing, dialyzed against 4x1 L PBS at 4°C, passed through a 0.2 µm syringe filter, and stored in 1.0 ml aliquots at -80°C until immunization. The MUC1 CT-KLH conjugate was mixed with an equal volume of Freund's complete adjuvant (Sigma) and 1.0 mg injected intramuscularly into New Zealand rabbits. At 2, 4, and 6 weeks post-immunization, rabbits were boosted with 0.5 mg of the MUC1 CT-KLH conjugate emulsified in incomplete Freund's adjuvant. Rabbits were bled from the marginal ear vein at 2 week intervals following the last booster immunization and serum stored at -80°C.

#### Immunohistochemical analysis

The technique was performed following standard procedures (Croce et al., 1997); before immunostaining with MAbs, tissues were treated with 10 mM sodium citrate buffer at 100°C for 5 minutes for antigenic retrieval.

Dewaxed sections were placed in methanol with hydrogen peroxide (3%) for 15 minutes to block endogenous peroxidase activity. After three washes in phosphate buffered saline (PBS), sections were blocked for non-specific binding with normal horse serum diluted 1:10 in 1% bovine serum albumin (BSA)/PBS for 15 minutes and rinsed. Then, sections were incubated with Abs overnight at 4°C; Abs were diluted as follows: 1/100, CT33 and 1/1000, CT2. After three washes with PBS, either biotin conjugated anti-mouse Igs (Sigma, St. Louis, Mo., USA) or anti-rabbit Igs (dilution 1/400) was added, incubated for 60 min and washed in PBS; secondly, peroxidase-conjugated streptavidin was added

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and developed with 3', 3'-diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, Mo., USA); slides were counterstained with hematoxylin and coverslipped with mounting media. Negative controls were incubated with PBS instead of MAbs.

Specimens were examined by light microscope and the antibody staining patterns were scored in a semi-quantitative manner (Feickert et al, 1990). Staining intensity was graded as negative (-), low (+), moderate (++) , strong (+++) and very strong (++++). The number of low power (x100) optical fields in a specimen that were positively stained was expressed as a percentage of the total number of optical fields containing tissue. The staining of cytoplasm, plasma membranes and nucleus was evaluated; cells were considered positive when at least one of these components was stained.

The pattern of reaction was classified following other authors (Renkonen et al., 1997; Luna-Moré et al., 2001) as the membrane, cytoplasmic and mixed pattern (cytoplasmic mixed with plasma membrane staining), and the positive reaction of the lumen content identified as cellular debris or secretion.

### Preparation of extranuclear membrane fractions

Fractions were prepared from human tumor tissues according to Price et al. (1985). Briefly, tissues were homogenized in 0.01 M TRIS, pH 7.2 and 0.01 M phenylmethylsulfonyl fluoride (PMSF, Sigma, St. Louis, Mo., USA); homogenates were centrifuged at 600xg and at 105000xg at 4°C. Supernatant (cytoplasmic fraction) and the second precipitate were resuspended in PBS (extranuclear membrane fraction) and stored at -20°C.

### SDS PAGE and Immunoblotting

Electrophoretic analysis was conducted following standard procedures (Laemmli, 1970). Subcellular fractions were mixed with SDS-PAGE sample buffer to a final concentration of 2 mg of protein per ml; then, samples were heated at 90°C for 5 minutes. Forty µl of sample (80 µg) were loaded per well into a discontinuous 4-10% acrylamide mini-gel (Gibco-BRL, Gaithersburg, Maryland, USA); after electrophoresis, gels

were transferred to nitrocellulose membranes (Towbin et al., 1979), which were incubated with the MAbs above mentioned.

### Statistical analysis

A nonparametric statistical analysis was performed; data was standardized and a Kendall-Tau correlation was applied. Spearman correlation ( $p < 0.05$ ) was also run.

## Results

### Immunohistochemical results

#### Breast cancer

We investigated MUC1 CT expression employing two antibodies: CT2 MAb and CT33 polyclonal antibody. Most breast samples reacted with anti MUC1 CT antibodies, since 151/163 (93%) stained with CT2 while 146/163 (90%) stained with CT33; they stained a number of tumor cells in most malignant samples investigated (Fig.1 A,B). In Table 1, the percentages of positive samples are listed in relation to tumor stage.

The pattern of reaction was classified as membrane (linear), cytoplasmic and mixed. A similar percentage of cancer specimens reacted with the mixed pattern observed most frequently (46% with CT2 and 45% with CT33); between the two antibodies a Spearman significant positive correlation was found (0.4408,  $p < 0.05$ ). A linear pattern was detected in 30% with CT2 and 31% with CT33 (0.1970,  $p < 0.05$ ) while both antibodies showed a cytoplasmic pattern in 24% (0.2891,  $p < 0.05$ ). Several samples also showed a nuclear staining.

Since apical staining is considered a normal feature, apical and non-apical reactivity were also investigated; a very high correlation between CT33 linear pattern versus apical staining was found (0.9882,  $p < 0.05$ ) while with CT2 MAb, a Spearman significant positive correlation was also detected (0.5858,  $p < 0.05$ ).

Intensity was graded in 4 positive grades; interestingly, with both antibodies, samples exhibited a high staining (Table 2); this observation is in keeping with the assumption of a strong biosynthesis of MUC1 in malignant samples.

**Table 1.** Immunohistochemical results obtained in breast tissues.

	STAGE	CT33	CT2	C595
Malignant	I	35/37 (95)	37/37 (100)	24/37 (65)
	II	60/70 ( 85.7)	61/69 (88.7)	47/70 (67)
	III	45/48 (94)	43/47 (91.5)	25/48 (52)
	IV	6/8 (75)	8/8 (100)	7/8 (87.5)
Benign		7/10 (70)	10/10 (100)	7/10 (70)
Normal		12/12 (100)	12/12 (100)	8/12 (67)

Results are expressed (%).

**Table 2.** Intensity of malignant positive breast samples expressed in percentages.

INTENSITY	CT2 MAb	CT33 Ab
Low (+)	14	18
Moderate (++)	29	34
Strong (+++)	35	29
Very strong (++++)	23	19

### Breast benign and normal specimens

All benign samples reacted with CT2 MAb while 77% specimens stained with CT33. The intensity was mainly moderate and restricted to some areas while the pattern of expression was apical, either linear or mixed.

All normal samples reacted with CT2 and CT33 antibodies, mostly with a moderately intense lineal pattern (66% with both antibodies) that was restricted to the apical part of the cell. One sample showed a strong staining.

### Colorectal cancer

An accurate immunohistochemical analysis was performed in colorectal cancer, which showed that both antibodies stained 47/89 (53%) of samples; with CT2 MAb, in 26 samples, the linear reaction was concentrated in the apical part of the cell, a positive correlation between apical versus linear was detected. CT33 polyclonal antibody reactivity showed a diffuse cytoplasmic staining; staining was found at the apical part of the membrane (linear pattern) in only one case.

In most samples, the intensity of the reaction varied from low to moderate although, in some specimens, an intense reactivity was detected. In figure 1 C and D examples of CT2 and CT33 staining are depicted.

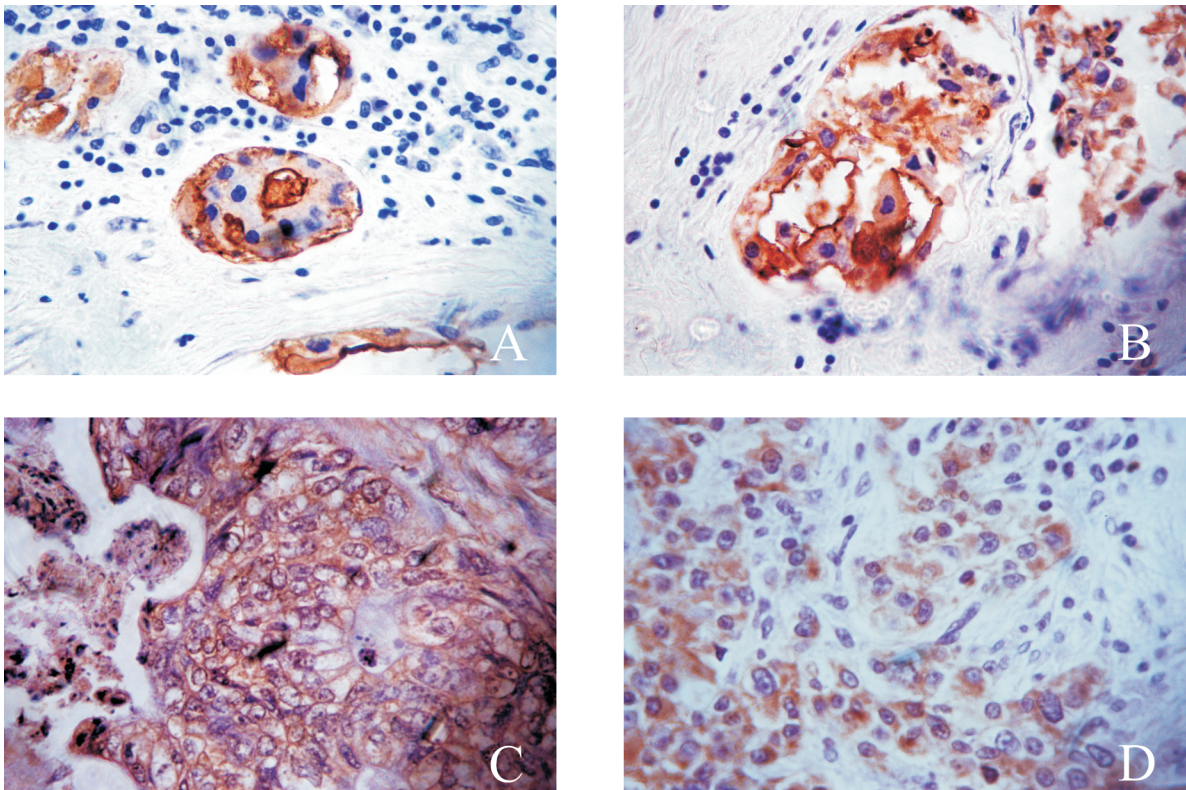
### Colorectal benign and normal specimens

CT2 MAb stained 13/14 (93%) benign samples; goblet and columnar cells showed reactivity in secretory vesicles, microvilli and in the cytoplasmic remnants; it was expressed most strongly on the apical membrane surface. There was also evidence of staining of

**Table 3.** Immunohistochemical results obtained in colorectal tissues.

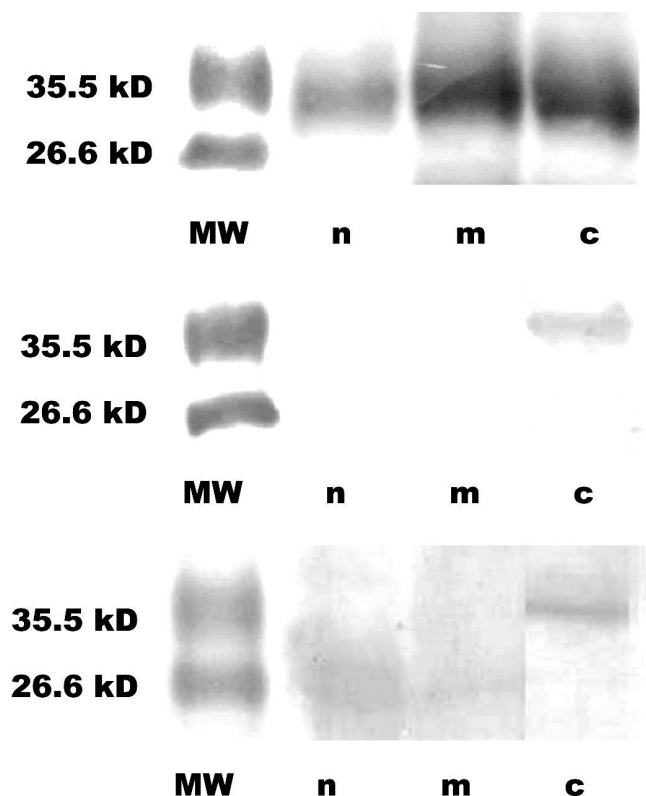
SPECIMENS	CT2 MAb	CT33 Ab	C595 MAb
Malignant	47/89 (53)	47/89 (53)	51/89 (57)
Benign	13/14 (93)	9/14 (64)	3/14 (21)
Normal	17/20 (85)	12/20 (60)	13/20 (65)

Results are expressed (%).



**Fig. 1.** Immunohistochemical findings obtained in cancer tissues incubated with anti-MUC1 CT2 MAb (A, C) and CT33 Ab (B, D). A and B. Breast carcinoma sections from not otherwise specified (NOS) ductal type. C and D. Poorly differentiated colorectal adenocarcinoma sections. A and B. malignant glands show a strong staining at cytoplasmic as well as plasmatic membrane level. C. Staining is observed mainly at plasmatic membrane level while some cells show cytoplasmic reaction. D. Reaction is found at cytoplasm, although some nuclei are also stained. Original magnifications: A, x 400; B, C and D, x 630

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**Fig. 2.** Western blot of subcellular fractions belonging to breast and colorectal cancer samples: n, nuclear fraction, m, membrane fraction and c, cytoplasmic fraction. Both anti-MUC1 CT Abs detect a band at approximately 30 kDa. Standard molecular weights are indicated at left. Top: breast cancer subcellular fractions incubated with CT2. Middle: breast cancer subcellular fractions incubated with CT33. Bottom: colorectal cancer subcellular fractions incubated with CT33; the band in the nuclear fraction exhibits a faster mobility.

flocculent material within goblet cells. In columnar cells, expression also frequently comprised perinuclear staining and, in many cases, nuclei were also reactive. On the other hand, CT33 showed a positive reaction in 9/14 (64%) benign specimens with a cytoplasmic and basal staining of columnar and goblet cells.

In the case of normal samples, CT2 MAb showed an apical positive staining in 17/20 (85%) of samples. A cytoplasmic pattern was most frequently found with variable intensity. CT33 was reactive in 12/20 (60%) of specimens, showing a mixed pattern and a low level of reactivity observed mainly at the basal part of columnar cells.

### Staining of MUC1 extracellular domain

Immunohistochemical analysis was also performed with an anti-MUC1 VNTR MAb (C595 MAb); of breast cancer samples, 63% showed a positive reaction, while 70% of benign specimens and 67% of normal specimens were positive. On the other hand, colorectal samples

showed a positive staining in 57% of malignant samples, 21% of benign specimens and 65% of normal samples.

### Western blot analysis of subcellular fractions (Fig.2)

To further characterize the MUC1 CT fragment reactive with the Abs, we examined the membrane, cytoplasmic and nuclear subcellular fraction distribution using SDS-PAGE and Western blotting analysis. In breast cancer samples, both Abs detected a band of about 30 kDa. CT2 MAb detected MUC1 CT fragment in all three subcellular fractions while CT33 Ab identified a band only in the cytoplasmic fraction and the reaction was weaker compared to CT2 MAb staining. Colorectal cancer specimens showed a band at 30 kDa in the cytoplasmic fractions while in membranes a band of slightly faster mobility was detected.

### Discussion

MUC1 is an integral membrane glycoprotein expressed on the apical surface of epithelial cells where it acts as a signaling receptor. Its cytoplasmic tail (CT) contains seven, highly conserved tyrosine residues, some of which are constitutively phosphorylated and serve as recognition sites for SH2 domain proteins involved in intracellular signal transduction.

In this report, we compared the reactivity of two antibodies against MUC1 CT. CT2 MAb has been previously proven useful to detect MUC1 in comparison with anti-VNTR MABs (Croce et al., 2003a,b) while CT33 polyclonal antibody has not been employed until now. We analyzed malignant and control breast and colorectal tissue samples; in cancer specimens, MUC1 CT was detected at the plasma membrane level, the cytoplasm and also at the nuclei.

There are few reports about the cytoplasmic localization of MUC1 CT in cancer; Wen et al. (2003) investigated intracellular trafficking of MUC1 CT in human pancreatic cancer cell lines S2-013 and Panc-1 and detected MUC1 CT at the inner cell surface, in the cytosol and in the nucleus. They hypothesized that the association between  $\beta$ -catenin and fragments of the MUC1 CT facilitated the cytosol-to-nuclear translocation of  $\beta$ -catenin, and contributed to its nuclear accumulation.

Since overexpression is known to be a malignant characteristic, we evaluated the pattern of expression, the percentage of reactivity and also the intensity; tumor samples reacted in a high percentage of cases, showing strong reactivity with both antibodies. These anti-MUC1 CT antibodies are capable of detecting mature and immature mucin, since they stained cytoplasmic as well as membrane MUC1 and also the secreted molecule.

It is well recognized that mucins expressed by colorectal neoplasms differ from those of normal epithelium. Hence, it has been reported (Carrato et al., 1994; Aijoka et al., 1996; Winterford et al., 1999) that in normal samples, MUC1 synthesis is greatest in the lower

crypts and diminishes with cell maturation. In the present study, both CT2 and CT33 recognized MUC1 mucin expressed by normal and benign samples in either basal or columnar cells.

With immunohistochemical analysis and Western blotting, we found reactivity at the nuclear level; MUC1 CT localization in the nucleus is of interest given its sequence conservation across species (Spicer et al, 1995) and because it contains putative docking kinases and proteins involved in signal transduction (Spicer et al., 1995, Gendler, 2001).

In concordance with other authors and a previous work (Schroeder et al., 2001; Croce et al., 2003a), Western blotting analysis of subcellular fractions showed a band at about 30 kDa. In colorectal cancer, with CT33 MAb, we found that nuclear fractions showed a band of a lower MW (Fig. 2). Wen et al. (2003) found that the nuclear fragments of MUC1 CT showed faster mobility in SDS-PAGE than those from cell/cytoplasm. They hypothesized that a proteolytic cleavage event releases a fragment of the MUC1 CT that traffics to the nucleus.

In agreement with a previous work (Croce et al., 2003a), in breast cancer, we found that antibodies against MUC1 CT detected a higher number of positive results than the anti-MUC1 tandem repeat MAb here employed (C595 MAb): CT2 MAb reacted with 93% of samples and CT33 antibody with 92%, whereas C595 MAb reacted with 63%. In colorectal cancer, similar percentages of positive reaction were found: 53%, 53% and 57%, respectively. In breast cancer, MUC1 extracellular domain may be secreted and released to serum as well as ascites fluid (Devine et al., 1993; Linsley et al., 1998, Croce et al., 2001).

Wen et al. (2003) argued that taking into account that the tandem repeat binds to different adhesion molecules, the fact that the deletion of the tandem repeat significantly reduces detectable association with  $\beta$ -catenin suggests that there is a functional link between the extracellular tandem repeat and signalling through the cytoplasmic tail. They also pointed out that the cytosol-to-nuclear translocation of the fragment of MUC1 CT and  $\beta$ -catenin represents one of multiple pathways of signal transduction for these two fragments and that MUC1 CT may be associated with other factors in the cytoplasm and the nucleus.

The present investigation has demonstrated that the CT33 polyclonal antibody has efficacy to detect MUC1 in breast and colorectal cancer tissues with similar reactivity to CT2 MAb. It is tempting to affirm that CT33 is a good indicator of MUC1 expression. Since MUC1 CT has been implicated in important roles, we consider it crucial to have accurate antibodies which can be employed in different aspects of research.

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