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# Tetraspanin co029 expression as a tumor biomarker for monoclonal antibodies preparation: antigenic assessment in colorectal cancer cells

## Expressão de tetraspanina co029 como biomarcador tumoral para preparação de anticorpos monoclonais: avaliação antigênica em células de câncer colorretal

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## ABSTRACT

**Introduction:** The identification of innovative cancer biomarkers is a very relevant ongoing quest. Moreover, their role in cancer diagnosis and clinical management has been radically changed in the last few years with the major emphasis on cancer molecular classification, therapeutic target identification, and therapeutic protocol responsivity. tetraspanins are a family of transmembrane proteins correlated with tumor stage, tumor type and patient outcome affecting cell growth, morphology, invasion, and metastasis. **Methods:** We expressed the 31kDa transmembrane human tetraspanin co029 antigen in *Escherichia coli* expression host cells using Gateway® platform. Western blotting and ELISA techniques, together with gene sequencing, confirmed the identity of TSP co029 recombinant protein. Forty clones producing antibodies against TSP co029 were obtained. These antibodies were incubated with human colorectal cancer cells in different conditions. ELISA and immunohistochemistry were also performed. **Results:** The expressed tetraspanin had an appropriate conformation and antigenic integrity to produce antibodies with affinity to the native TSP co029 biomarker. The affinity of the purified recombinant protein and antibodies were confirmed by western blotting, florescent staining of human colorectal cancer cells in fluorescence and confocal microscopies and by ELISA and immunohistochemistry. **Conclusion:** Our data showed that the recombinant protein and antibodies produced in this study allowed the confirmation of tetraspanin co029 protein presented on the surface of human colorectal cancer cells.

**Keywords:** Tetraspanins; Recombinant proteins; Antibodies, Monoclonal; Biomarkers, Tumor.

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## RESUMO

**Introdução:** A identificação de biomarcadores de câncer inovadores é uma busca contínua muito relevante. Além disso, seu papel no diagnóstico do câncer e no manejo clínico mudou radicalmente nos últimos anos, com grande ênfase na classificação molecular do câncer, na identificação do alvo t *Western blotting* erapêutico e na responsividade do protocolo terapêutico. As tetraspaninas são uma família de proteínas transmembrana correlacionadas com o estágio do tumor, tipo de tumor e evolução do paciente, que afetam o crescimento celular, morfologia, invasão e metástase. **Métodos:** Expressamos o antígeno de tetraspanina co029 humana transmembrana de 31kDa em células hospedeiras de expressão de *Escherichia coli* usando a plataforma Gateway®. As técnicas de *Western blotting* e ELISA, juntamente com o sequenciamento de genes, confirmaram a identidade da proteína recombinante TSP co029. Obtiveram-se quarenta clones produtores de anticorpos contra TSP co029. Esses anticorpos foram incubados com células de câncer colorretal humano em diferentes condições. ELISA e imunohistoquímica também foram realizados. **Resultados:** A tetraspanina expressa tinha uma conformação adequada e integridade antigênica para produzir anticorpos com afinidade para o biomarcador TSP co029 nativo. A afinidade da proteína recombinante purificada e dos anticorpos foram confirmados por *western blotting*, coloração fluorescente de células de câncer colorretal humano em microscopias fluorescentes e confocais, e por ELISA e imunohistoquímica. **Conclusão:** Nossos dados mostraram que a proteína recombinante e os anticorpos produzidos neste estudo permitiram a confirmação da proteína tetraspanina co029 apresentada na superfície de células de câncer colorretal humano.


**Descritores:** Tetraspaninas; Proteínas recombinantes; Anticorpos monoclonais; Biomarcadores tumorais.

## INTRODUCTION

Biomarker is a term originated from "biological marker" and refers to a broad category of medical signs – that is, objective indications of medical state observed from outside the patient – which can be measured accurately and reproducibly.[1] A biomarker was once defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological or pathogenic process or a pharmacologic response to an intervention, according to the definition of National Institutes of Health Biomarkers Definitions Working Group.[2] Although this definition was made in 1998, practical use of biomarkers in clinical research is somewhat newer, and the best approaches to this practice are still being developed and refined. The distinction between the tumor- and non-tumor-initiating cells is based on the super expression or subexpression of biomarkers. A biomarker can be of different origins. It can be a cell surface molecule such as a protein or a glycoprotein, it can also be a differential expression or activation level of a transcription regulator or a chemokine, a gene expression specific profile, or even an enzyme or a miRNA.[3,4]

In the last years prognostic biomarkers have acquired a further relevance given they can contribute to a better identification and characterization of cancer staging, relation between treatment information and patient outcomes, such as overall survival, metastasis, and therapy decisions via treatment response information. Thus, increasing the chance of an accurate diagnosis and prognosis with the final goal of the individual tailoring of treatments.[5,6]

Tetraspanins (TSP) are a family of four-span transmembrane proteins, known as major plasma membrane organizers. They form TSP-enriched microdomains (TEMs or TERMs) through lateral association with one another and other membrane proteins.[7] If multiple microdomains associate with each other, larger platforms can form.[8,9] TSP are usually small proteins[10] that play important roles on cell migration, intracellular trafficking and signal-transduction.[11,12] Some TSP are widely expressed, as is the case of CD81 and CD151, while others are restricted. TSP co029, specifically, was initially reported as a tumor-associated antigen expressed in gastric, colorectal, and pancreatic cancer cells associated to a tumor progression- promoting activity.[13] Alterations in TSP co029 expression are associated with tumor progression and increased occurrence of metastasis. A reduced cell migratory ability is seen when TSP co029 expression is silenced in HT29 colon cancer cells.[14] TSP co029 appears to promote cell movement by altering cell-matrix and cell-cell adhesions. It is well established that cell-cell and cell-matrix adhesion directly determines cell motility.[14,15]

Expression of recombinant proteins is a powerful tool broadly used in research for the elucidation of structure-function relationships of biomarkers, the identification of components mediating intracellular traffic, and moreover for the production of polyclonal and monoclonal antibodies specific for selected biomarkers.[3] The development of new potential products and tools in research begins with the choice of a production host.

While one single perfect host for every protein does not exist, different expression systems including bacteria, yeast and mammalian cells have been well established.[16] Bacterial hosts are the most used for the production of recombinant proteins, reaching approximately 30% of current biopharmaceuticals on the market.[17] The production of recombinant protein in bacterial expression system implies in an economic platform allowing large scale production.[3,17]

Hybridoma technology features effective usage of innate functions of B-lymphocytes. This platform promotes the fusion of B-lymphocytes previously stimulated with the biomarker and myeloma cells, generating hybridoma cells with the inherent ability to continuously produce monoclonal antibodies specific to antigens of interest. Monoclonal antibodies, indispensable tools in research, diagnostics and therapy, are universal binding molecules with a high specificity for their target. The biotechnological generation of monoclonal antibodies was enabled by the hybridoma technology published in 1975 by Köhler and Milstein.[18] Currently, monoclonal antibodies are used in a broad range of applications as flow cytometry, magnetic cell sorting, immunoassays or therapeutic approaches, as immunotherapies.[19,20]

In this study, we expressed human TSP co029 protein in prokaryotic expression host cells with appropriate antigenic characteristic for further polyclonal and monoclonal antibodies production. Final analysis of the integrity and affinity of recombinant TSP co029 and its related antibodies were measured by ELISA, western blotting, florescent staining of colorectal cancer cells in fluorescence, and confocal microscopies and immunohistochemistry.

## METHODS

### Cloning, expression and purification of TSP co029

The coding sequence of human TSP co029 gene (NCBI Reference Sequence, NM_001369760.1, NP_001356689) for a protein of 237 amino acids was obtained from HT29 human colorectal cancer cells (BCRJ).

CCDS Sequence Data

Blue highlighting indicates alternating exons.

(a)

Nucleotide Sequence (714 nt)

ATGGCAGGTGTGAGTGCCTGTATAAAATATTCTATGTTTACCTTCAACTTCTTGTTCTGGCTATGTGGTA
TCTTGATCCTAGCATTAGCAATATGGGTACGAGTAAGCAATGACTCTCAAGCAATTTTTGGTTCTGAAGA
TGTAGGCTCTAGCTCCTACGTTGCTGTGGACATATTGATTGCTGTAGGTGCCATCATCATGATTCTGGGC
TTCCTGGGATGCTGCGGTGCTATAAAAGAAAGTCGCTGCATGCTTCTGTTGTTTTTCATAGGCTTGCTTC
TGATCCTGCTCCTGCAGGTGGCGACAGGTATCCTAGGAGCTGTTTTCAAATCTAAGTCTGATCGCATTGT
GAATGAAACTCTCTATGAAAACACAAAGCTTTTGAGCGCCACAGGGGAAAGTGAAAAACAATTCCAGGAA
GCCATAATTGTGTTTCAAGAAGAGTTTAAATGCTGCGGTTTGGTCAATGGAGCTGCTGATTGGGGAAATA
ATTTTCAACACTATCCTGAATTATGTGCCTGTCTAGATAAGCAGAGACCATGCCAAAGCTATAATGGAAA
ACAAGTTTACAAAGAGACCTGTATTTCTTTCATAAAAGACTTCTTGGCAAAAAATTTGATTATAGTTATT
GGAATATCATTTGGACTGGCAGTTATTGAGATACTGGGTTTGGTGTTTTCTATGGTCCTGTATTGCCAGA
TCGGGAACAAATGA

(b)

Translation (237 aa)

MAGVSACIKYSMFTFNFLFWLCGILILALAIWVRVSNDSQAIFGSEDVGSSSYVAVDILIAVGAIIMILG
FLGCCGAIKESRCMLLLFFIGLLLILLLQVATGILGAVFKSKSDRIVNETLYENTKLLSATGESEKQFQE
AIIVFQEEFKCCGLVNGAADWGNNFQHYPELCACLDKQRPCQSYNGKQVYKETCISFIKDFLAKNLIIVI
GISFGLAVIEILGLVFSMVLYCQIGNK

Figure 1. The nucleotide (a) and amino acid (b) sequences of TSP co029. https://www.ncbi.nlm.nih.gov/CCDS/CcdsBrowse.cgi?REQUEST=CCDS&DATA=CCDS8999. Blue highlighting indicates alternating exons.

Primers were designed with *att*B sites for cloning on Gateway® system for a N-terminal histidine Tag, according to the manufacturer instructions (Figure 1).

The entry clone was obtained by inserting the gene of interest in the pDNOR plasmid by a BP recombination reaction at 25ºC for 16h. *Escherichia coli* TOP10 (Novagen) bacteria was transformed by thermal shock. Positive colonies selected with specific primers for the gene of interest by PCR were cultured in Luria-Bertani (LB) medium supplemented with 50µg/mL of kanamycin, and the plasmid was purified with plasmid DNA Miniprep Kits (invitrogen), following the instructions. Subsequently, the entry clone containing the gene of interest flanked by the attL sites was combined with pEXP1-DEST vector by LR recombination reaction at 25ºC for 16h, generating the final expression clone. Competent *E. coli* BL21AI (Novagen) bacteria was transformed by thermal shock and cultured in LB medium supplemented with 100µg/ml ampicillin at 37ºC for 12h. Gene sequencing was performed after each stage using Sanger sequencing technique in order to confirm the correct insertion of the gene.

One liter of bacteria was monitored by a 600nm spectrophotometer for 0.4-0.6 optimum optical density (OD) and the protein synthesis was induced by the addition of 1mM of isopropyl β-D-thiogalactoside (IPTG) at 37°C for 4h under orbital rotation (225rpm). The bacterial pellet was centrifuged at 12,000g for 45min at 4°C when pellet resuspended in 40mL of lysis buffer [50mM Tris, 0.5M NaCl, 0.2mM EDTA, 3% sucrose, Triton-X 1% and 10mM imidazole] plus protease inhibitors [200ug/ml lysozyme, 1mM PMSF, 20ug/ml DNAse]. Cells were sonicated on ice (3 cycles of 30s, 1min interval), and then centrifuged at 12,000g for 45min at 4°C. The supernatant and pellet were filtered through a 0.22µm filter. Recombinant protein was purified on a HisPur Ni-NTA chromatography cartridge nickel column, concentrated on a 10kDa tube (Vivaspin Millipore) and quantified by a BCA kit (ThermoFisher) according to the manufacturer's instructions.

### SDS PAGE and western blotting for TSP co029 recombinant protein confirmation

Recombinant TSP co029 protein production and purification were confirmed by SDS PAGE and western blotting methods. Five ug of the purified TSP co029 protein were add to 12% polyacrylamide gels for Coomassie R-250 (Biorad) staining and PVDF membrane transfer. PVDF membrane was then incubated with a his-tag primary monoclonal antibody (Invitrogen) (1:2,000) and consequently with anti-IgG HRP antibody (Santa Cruz) (1:5,000). Immunoreactive bands were visualized using western blotting ECL kit (GE Healthcare Life Science) according to the manufacturer's instructions and image was captured by Digital Image System LAS 4000 (GE Healthcare Life Science).

## Production of polyclonal and monoclonal anti-TSP co029 recombinant protein

Based on Kohler and Milstein (1975) methodology,[18] two nine-week-old female BALB/c mice were subcutaneously immunized with 20ug of recombinant TSP co029 protein diluted on 200ul of Vac-SIM® as adjuvant (EnduraVax).[21] A protocol of six inoculations two weeks apart was concluded. Sera were collected immediately after each inoculation for the determination of specific antibodies titers by ELISA. A final boost was intraperitoneally performed and, three days later, the spleen cells were fused with Sp2/0 IL-6 myeloma cells (ATCC) using 37ºC PEG/DMSO solution with a 1min incubation. The fused cells were cultured and selected with hypoxanthine-aminopterin-thymidine (HAT) medium and hypoxanthine-thymidine (HT) medium (Sigma) for 7 days on a 37ºC 5% $CO_2$ incubator. The initial screen of positive growth wells was done by ELISA. TSP co029 recombinant protein was diluted 10µg/ml in coating buffer [0.05M carbonate-bicarbonate, pH 9.6] and microtiter plates MaxiSorp Surface (NUNC Thermo Scientific) were coated at 4ºC for 16h. After blocking, 100µl of culture supernatants of the HAT/HT-selected hybridomas were added and incubated for 1h. The presence of specific antibodies was detected with peroxidase-conjugated anti-mouse IgG (1:5,000) (Santa Cruz) and TMB (Sigma) under a 450nm spectrophotometer (ThermoScientific). ELISA positive hybridomas were selected for expansion. The selected clones (3.E211B) for mAbs and (3.E2) for pAbs were grown in DMEM (Invitrogen) supplemented with 10% FBS, penicillin (100U/ml) and streptomycin (100mg/ml) (Invitrogen) and insulin-transferrine-sodium selenite (ITS). Culture supernatants were harvested and used for ammonium sulfate precipitation).[22] Precipitated proteins were dialyzed against PBS at 4ºC. mAbs were purified by two extra protocols including a G protein purification column (Sigma) and an anionic exchange chromatography column HiTrap Q HP (GE Healthcare). At the end, mAbs were concentrated by a 50kDa tube (Vivaspin Millipore), quantified by a BCA kit (ThermoFhisher) and stored at 4ºC until use.

Aliquots of the mAbs were conjugated to Alexa Fluor 647 with Fluorochrome Protein Labeling Kit (Invitrogen), according to the manufacture instructions. As described before, pAbs and mAbs production and purification was confirmed by SDS PAGE. Antibodies were added on a 10% polyacrylamide gel stained by coomassie blue.

## Fluorescent and confocal microscopy analyses of TSP co029 mAbs

For the evaluation of the effects of mAbs, human colorectal cancer HT29 cells were plated at a density of $2x10^5$ cells/well in 96-well flat-bottom plates together with 0.10 and 0.20mg/ml of 3.E211B anti-TSP co029-Alexa Fluor 647 conjugated mAb. Incubation was performed for 6h and 16h at 37ºC. Cells were examined on fluorescent microscope (Karl Zeiss AxioStar Plus) and on laser confocal microscope (Nikon C2) at 640-642nm, emission filter LP590. Photographic records were taken with a digital camera and analyzed by Software NIS-Elements (Nikon). Z- Stack Capture mode was used for immunolocalization of staining. Control cultures without mAbs were observed in parallel to exclude possible effects of the antibodies on the cells.

## ELISA analyses of TSP co029 mAbs

Crude extract of human colon adenocarcinoma HT29 cells was initially prepared. Cells were cultivated in supplemented DMEM medium (20% FCS and 1% penicillin) and after reaching confluence; they were released by trypsin, centrifuged at 200xg for 4min, and resuspended in non-supplemented medium. The cell suspension was sonicated at 40% amplitude, in 5 cycles of 15sec of sonication and 30sec interval, on ice. Subsequently, the cell lysate was centrifuged for 10min to cell debris removal and the cells extract were resuspended in PBS or DMEM.

Basically, 96-well microtiter plates MaxiSorpTM Surface (NUNC) were sensitized with 10ug of recombinant TSP co029, HT cells extract in PBS or HT29 extract in DMEM diluted in buffer 0.05M carbonate-bicarbonate pH 9.6 in triplicate for 16 hours at 4ºC. The plates were washed three times with 0.15M phosphate buffer saline pH 7.2 with 0.05% of Tween 20 (LGC Biotechnbology) (washing buffer) and, the non-specific sites were blocked with 2.5% casein protein in washing buffer at 37ºC for an hour. After new washing steps, 100µl of mAbs anti-TSP co029 (containing 10ug of antibodies) were added into each well and the plates were incubated at room temperature for an hour. Following, the plates were submitted to washing steps and incubated at room temperature for an hour with conjugated anti-IgG murine antibody (Santa Cruz Biotechnology) diluted 1:10,000 in washing buffer. The plates were washed again and 100ul of substrate TMB (Thermo Fisher Scientific) were added to each well. The reaction was stopped after 20 minutes of incubation in the dark by addition of 50ul/well of 2N sulfuric acid. The results were obtained as absorbance values at 450nm in microplate reader (BioRad Laboratories 3550). Negative controls were done by using recombinant proteins from *Schistosoma mansoni* parasite with no similarity to TSP co029: Circulating Cathodic Antigen (CCA, PubMed accession number O02197.1) and Major Egg Antigen (MEA, PubMed accession number AAA29903.1).

## Immunohistochemistry analyses of TSP co029 mAbs

Four µm thick histological sections were prepared for immunohistochemical reactions. Novolink Polymer Detection System (Leica Biosystems) anti-mouse/anti-rabbit detection kit was used according to the manufacturer's instructions. For the recovery of TSP co029 receptor antigens, steam heat (Pascal®) with Dako Cytomation Target Retrieval Solution (Dako) pH 6.0 citrate was used.

Briefly, the slides with histological sections were incubated with the appropriate primary antibody (1:100) for 16h in a humid chamber at 4ºC. The immunoreactivity was visualized with the chromogen 3'-diaminobenzidine (DAB) substrate system (Dako) and contrasted with the Mayer's hematoxylin. Colorectal cancer positive tissue fragment samples were used as positive controls for the reactions. For negative controls, the primary antibody was replaced with phosphate-buffered saline (PBS). The slides were comparatively analyzed by AxioVision and ImageJ software, for morphometric characterization and classification of protein expression.

### Data analyses

The absorbance values were analyzed with Minitab software by the Kolmogorov-Smirnov normality test. Normally distributed data were analyzed by Student's t-test using $p<0.05$ as the significance level.

## RESULTS

### Cloning and expression of TSP co029 gene

The TSP Co029 gene was expressed in *E. coli* BL21AI, with 6x his tag at 5' ends. The optimum condition for expression was achieved after 4 h induction by IPTG (1mM), at 37ºC and OD 600 of 0.6. Purification of the recombinant protein was carried out, and SDS PAGE analysis revealed as a major band (~31kDa) in the induced fractions (Figure 2). The approximate yield of purification was approximately 1.63mg/ml culture.

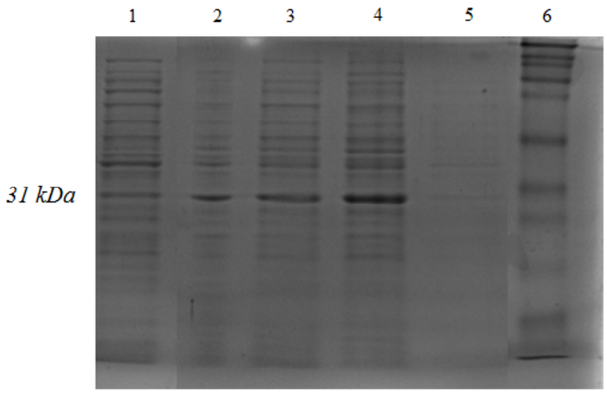


**Figure 2.** SDS PAGE analysis of expression of TSP co029 recombinant protein. Lane 1 to 4: 1, 2, 3 and 4h induced bacterial extract, respectively; Lane 5: uninduced bacterial extract. Lane 6: ladder.

Western blotting analyzes using anti-His-Tag monoclonal antibody confirmed the recombinant protein production (Figure 3).

### pAbs and mAbs anti-TSP co029 production

The titers of antibodies against TSP co029 recombinant protein in the sera of immunized mice showed that all mice were immunized against the antigen, but in one mouse (number 1) increase of antibody titer was more than other and this mouse had higher anti-TSP co029 antibody (OD>2.0) (Figure 4) and was selected for the fusion.

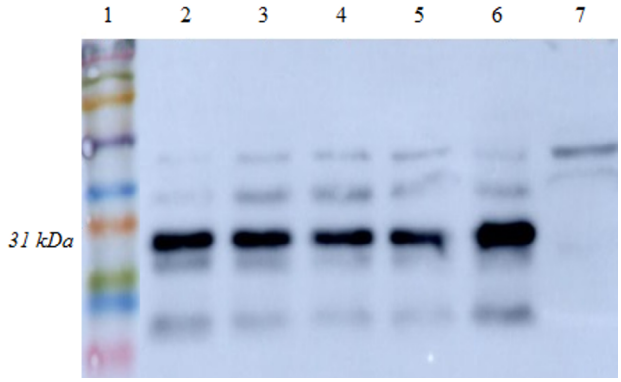


**Figure 3.** Western blot analysis of TSP co029 using anti-His-Tag monoclonal antibody. Lane 1: ladder; Lane 2 to 6: purified TSP co029 recombinant protein from supernatant; Lane 7: pellet with no protein.


**Figure 4.** Enhancement of immune responses in mice by all injections (p-value<0.01) of TSP co029 in sera of two BALB/c mice evaluated by ELISA assay using 1:100 dilution of immune mice sera.

The fusion of immunized mice splenocytes and myelomas yielded 105 HAT-resistant hybridoma pre clones producing specific pAbs. Ten pre clones with high TSP co029 binding, determined by ELISA, were stored in liquid nitrogen and 1 (3.E2) was submitted to cloning step. A new ELISA determined 40 positive clones. Seven clones secreting specific mAbs were also expanded and stored in liquid nitrogen. mAbs were then isotyped and the characterization of the selected pAbs and mAbs specifically reacting with TSP co029 recombinant protein is summarized in Table 1 (supplemental file). Final yield of 4.6mg/ml was obtained for pAbs and 1.1mg/ml was obtained for mAbs.

### In vitro fluorescent analysis of TSP co029 mAbs

Human colorectal cancer HT29 cells incubated with different concentrations of 3.E211B anti-TSP co029-Alexa Fluor 647 conjugated mAb showed membrane staining of all viable cells after 6h (Figure 5) and 16h on fluorescent microscopy. Results from both incubation times were the same. Furthermore, cellular localization of TSP co029 was visualized by confocal fluorescence microscopy (Figure 6). The HT29 cells treated with 3.E211B anti-TSP co029-Alexa Fluor 647 conjugated mAb demonstrated cell membrane labeling.

**Table 1.** Characterization of pAbs and mAbs produced against TSP Co029 protein.

| Pre clones and clones | Isotype | TSP Co029 specificity |
|---|---|---|
| 3.B2 pAb | ND | +++ |
| 3.B3 pAb | ND | ++ |
| 3.C2 pAb | ND | +++ |
| 3.C3 pAb | ND | ++ |
| 3.C4 pAb | ND | ++ |
| 3.D2 pAb | ND | ++ |
| 3.D3 pAb | ND | ++ |
| 3.E2 pAb | ND | ++++ |
| 3.E3 pAb | ND | +++ |
| 3.E4 pAb | ND | +++ |
| 10.F2 pAb | ND | +++ |
| 3E2.B11 inAb | IgGl | ++++ |
| 3E2.C11 inAb | IgGl | ++ |
| 3E2.G4 inAb | IgGl | +++ |
| 3E2.B8 niAb | IgGl | +++ |
| 3E2.B9 niAb | IgGl | ++ |
| 3E2.G3 inAb | IgGl | + |
| 3E2.C11 inAb | IgGl | +++ |

+/++/+++/++++ = positive reaction grade; ND = not determined.

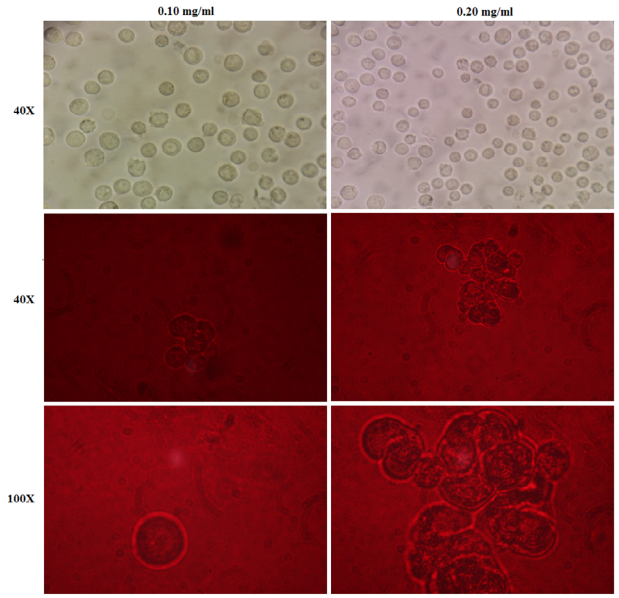


**Figure 5.** Fluorescence imaging of HT29 cells incubated with of 3.E211B anti-TSP co029-Alexa Fluor 647 conjugated mAb. Incubation was performed with 0.10 and 0.20mg/ml of conjugated mAb for 6h at 37°C. Analyses were done under visible illumination and under 642nm, emission filter LP 590 on a Zeiss AxioStar Plus fluorescence microscope.

Z-Stack Series mode analysis confirmed the exclusive membrane staining with no fluorescent moieties in the cytoplasm of the cells, as can be confirmed in supplementary material section.

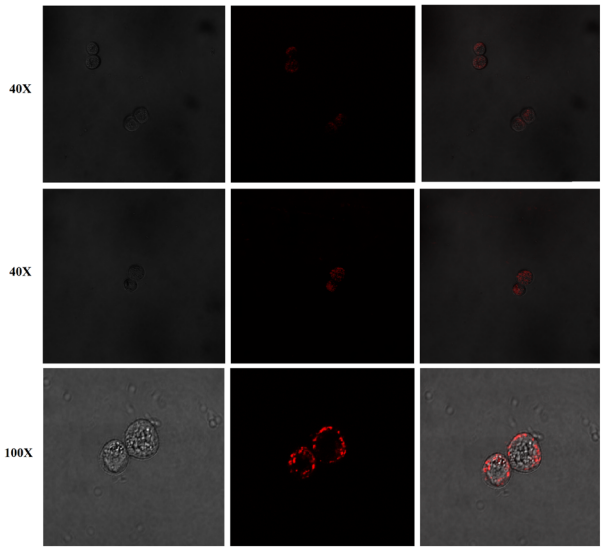


**Figure 6.** Confocal fluorescence imaging of HT29 cells incubated with 0.20mg/ml of 3.E211B anti-TSP Co029-Alexa Fluor 647 conjugated mAb for 6h at 37°C. HT29 cells under white light, cells under 640nm emission filter LP 590 and both overlapping images on a Nikon C2 confocal microscope, respectively.

## ELISA and immunohistochemistry analyses of TSP co029 mAbs

Figures 7 and 8 present the specificity of produced mAbs anti-TSP co029 respectively by ELISA and immunohistochemistry assays. mAbs were able to detect the recombinant TSP co029 and TSP co029 presented in HT29 cells extracts by ELISA ($p$<0.05) when comparing to the blank and negative controls.

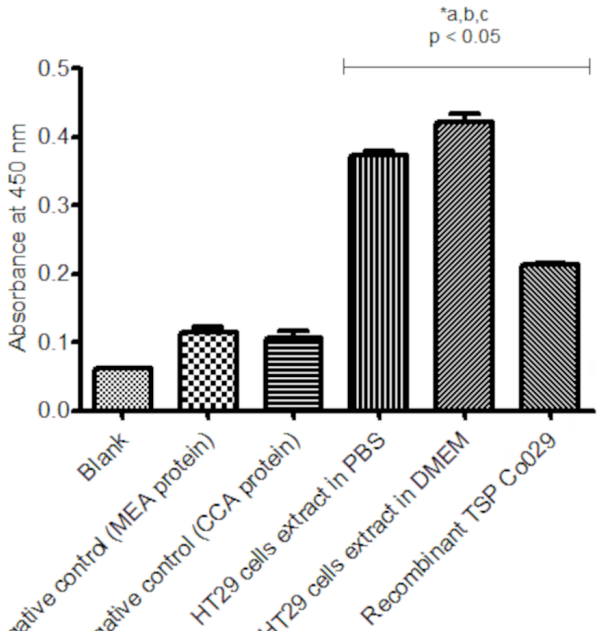


**Figure 7.** Reactivity of recombinant TSP co029 and HT29 cells extracts after incubation with the produced mAbs in ELISA. Statistical results are represented by *a,b,c when comparison was made for groups HT29 cells extract in PBS, HT29 cells extract in DMEM and recombinant TSP co029 to a) blank ($p$-values=0.0004, 0.0010, and 0.0002, respectively), b) negative control (CCA protein) ($p$-values=0.0017, 0.0021, and 0.0076, respectively), and c) negative control (MEA protein) ($p$-values=0.0017, 0.0021, and 0.0075, respectively).

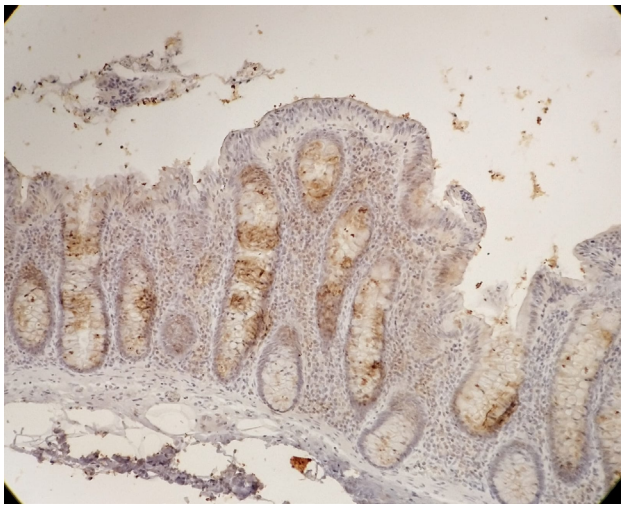

**Figure 8.** Representative staining of colorectal cancer cells from a patient tissue (4µm) stained by the mAb anti-TSP co029 (brown) at 10x magnification. The slides with histological sections were incubated with primary antibody (1:100) and immunoreactivity was visualized with DAB substrate. Analyses were done by AxioVision and ImageJ software.

Immunohistochemistry analyses using fragments of tissue extracted from a colorectal cancer patient showed the stained tumor cells when mAbs anti-TSP co029 were used.

## DISCUSSION

Tetraspanins are known to effect adhesion, growth or cell movement and share the common feature of spanning the membrane four times.[10-12,14,23] However, the role of TSP co029 in cancer remains unexplored. In contrast to other metastasis-suppressive tetraspanins, such as CD9 and CD82, which have been reported to be downregulated in cancerous tissue, TSP co029 was noted to be overexpressed in gastric, hepatic and colorectal tumor cells.[24,25] Together, it has been particularly related to metastasis and cell migration.[13-15,26-28] For this reason, TSP co029 might play and important role in disease progression and present relation with a bad prognosis and enhanced metastasis in different tissues.

In this study, the transmembrane TSP co029 protein (237 amino acids) was expressed in *E. coli* prokaryotic expression system using Gateway® platform. A 31kDa-recombinant protein was successfully expressed. Western blotting and ELISA techniques, together with gene sequencing, confirmed the identity of TSP co029 recombinant protein. Mice humoral immunity was stimulated by the purified TSP co029 to raise specific antibodies. After in vitro polyclonal and monoclonal antibodies production, 40 hybridoma clones were obtained and 7 presenting high affinity were selected for the study. Final analysis confirmed that TSP co029 antigenic integrity was achieved in both fluorescent and confocal microscopy analyses, and also in ELISA and immunohistochemistry assays. Human colorectal cancer cells presented florescent staining on the membrane after 6h and 16h incubation. mAbs also recognized the recombinant TSP co029 produced here and the protein presented in HT29 cells extracts on ELISA incubation. Finally, immunohistochemistry performed with a colorectal tumor tissue fragment incubated with produced mAbs revealed the presence of TSP co029. These data confirm the affinity of mAbs anti-TSP co029 recombinant protein to the native TSP co029 biomarker on the tumor cells. It is important to keep in mind that some antibodies can inhibit or activate functions of their target molecules and could influence the behavior of the cells to be stained. By confronting cells with or without mAbs conjugated to Alexa Fluor 647, no alterations were observed.

## CONCLUSION

As a conclusion, the expressed TSP co029 had an appropriate conformation and antigenic integrity to produce antibodies with affinity to the native TSP co029 biomarker. Hence, the recombinant protein and antibodies produced in this study allowed the confirmation of TSP co029 protein presented on the surface of human colorectal cancer cells. Further *ex vivo* studies should elucidate the TSP co029 expression and functions by comparing tumor tissues and non- affected tissues from the same individuals with different types and stages of solid tumors.

### Impact statement

Research has endeavored to identify innovative biomarkers to initiate their use on cancer diagnosis and clinical management, in order to help physicians and the health team in choosing the best individual option of chemotherapy treatment. In this work, we identified a transmembrane protein called tetraspanin co029 by producing specific antibodies in the laboratory. Using high technology microscopies, we were able to bind our specific antibodies to this protein on the surface of colorectal cancer cells extracted from adult patients.

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## CONFLICTS OF INTEREST

The authors have declared that no competing interests exist.

### Research ethics committee

This project was approved by the Ethical Research Committee of Fiocruz for animal use (CEUA-LM1/18) according to the International Guiding Principles for Biomedical Research Involving Animals developed by the Council for International Organizations of Medical Sciences and by the Ethical Committee on Research with Human Beings 34778, TTMB 0002/2019 additional term.

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## AUTHORSHIP

RFQG, LAC and JVA drafted the manuscript and carried out the assays, and analysis and interpretation of the data. RFQG, ANR and MMDAC conceived the study, designed the study protocol and critically revised the manuscript for intellectual content. LAC, CAC, JVA, MLCP, NGC, WJJ, VCFS, ASSM and MSMV carried out the experiments. All authors read and approved the final manuscript.

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