Abstract

Background/aim: Mucins such as MUC1 and MUC5AC are known for their protective and moisturizing role of the intestinal epithelium. Their expression is tightly controlled given their essential role in the normal tissue homeostasis, whereas their deregulation leads to chronic inflammation and even cancer. This study aims to assess the expression profiles of MUC1 and MUC5AC and their implications in colorectal carcinogenesis.

Materials and methods: We conducted a retrospective study of 202 patients who underwent colorectal cancer surgery. The expression of MUC1 and MUC5AC was investigated by immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR). Statistical analysis of mucins expression pattern along with the patients’ clinicopathological criteria was performed by the chi-square test, survival curves were plotted using the Kaplan-Meier product-limit method, and differences between the survival curves were tested using the log-rank test.

Results: Expression of both mucins was abnormally high in tumor tissues for either mRNA or protein. MUC1 expression correlated with advanced cancer stages and lymph node metastases either for mRNA (p < 0.016 and p < 0.002 respectively) and protein level (p < 0.006 and p < 0.001 respectively). Whereas MUC5AC expression didn’t pinpoint any significant association between the clinicopathological criteria, but patients who expressed MUC5AC showed an increase in overall survival (p < 0.009).

Conclusion: The expression of MUC1 might be a poor prognostic biomarker in colorectal cancer and could play a role in tumor transformation and metastasis. However, MUC5AC expression might be a good prognostic in the Tunisian cohort.

Keywords: Colorectal cancer, MUC1, MUC5AC, immunohistochemistry, RT-PCR.
1. Introduction

The mucosal surface of the gastrointestinal tract is covered with mucus secreted by specialized epithelial cells known as goblet cells. This mucus serves to lubricate, hydrate and protect the epithelium against potential hazards [1,2]. As major components of mucus, mucins are high molecular weight glycoproteins, that heavily contain a large number of O-linked oligosaccharides and few N-glycan chains, linked to a protein backbone also known as mucin core protein or MUC. Over the years, many reports highlighted the involvement of MUC expression in the invasion and metastasis formation of numerous malignancies such as breast [3], gastric [4] and pancreatic carcinomas [5]. To date, a total of 22 mucins have been identified and classified into two subgroups; secreted and transmembrane [6]. Some of these mucins were deemed as reliable prognostic biomarkers in many types of carcinoma prognosis. Nevertheless, findings are sometime inconclusive especially in colorectal cancer (CRC) [7,8]. Two mucin genes seem to be among the most studied in colorectal carcinoma: MUC1 and MUC5AC [9–12]. The transmembrane mucin MUC1, located on chromosome 1q21-24, can be found on the apical surfaces of most epithelial cells, including breast, digestive, respiratory and genitourinary tract [6]. The secreted mucin MUC5AC, situated on chromosome 11p15.5, is usually expressed in the stomach and respiratory tract [13]. This mucin is normally absent in healthy colon but frequently present in colorectal adenomas and colon cancers [14].
Several researches reported an increase of both *MUC1* and *MUC5AC* expression compared to the normal mucosa [2,15]. Consequently, *MUC1* could be a marker of poor prognosis indicating that its up-regulation may be involved in colorectal cancer (CRC) progression [6,10]. In contrast, *MUC5AC* expression could be a good marker of better prognosis and increases the overall survival of patients [16]. Therefore, we aim to evaluate the expression pattern of both *MUC1* and *MUC5AC* in colorectal carcinoma by immunohistochemical and molecular methods, to establish the possible links between their expressions and colorectal carcinoma prognosis.

2. Materials and methods

2.1. Patients and tumor samples

We conducted a retrospective study of 202 Tunisian patients who underwent colorectal cancer surgery between 2000 and 2017. The study protocol was approved by the Ethics Committee of Mongi Slim Hospital. The collected data included sex, age, tumor localization, histological type, differentiation and TNM staging (stages I, II, III, and IV). Tumor/Node/Metastasis (TNM) staging was based on the American Joint Committee on Cancer (AJCC) eighth edition [17].

2.2. Immunohistochemical study

The two protein expression was analyzed on 4 µm thick formalin-fixed paraffin-embedded samples. Slides were incubated in an oven at 37 °C overnight, deparaffinized in Toluene, rehydrated in descending concentrations of ethanol and finally in double-distilled water. Later, they were immersed in citrate buffer (pH = 6) preheated in a
microwave for 5 minutes to unmask the epitopes and then kept at room temperature for
20 minutes, followed by a Tris-washing.

Peroxidase block was used to inhibit endogenous peroxidase activity. After washing
two times in Tris, **MUC1** (monoclonal mouse anti Muc1 protein antibody, catalog NCL-
MUC1, clone Ma695, dilution 1:100, Novocastra, Newcastle Upon Tyne, UK) and
**MUC5AC** (monoclonal mouse anti Muc5AC protein antibody, catalog NCL-MUC5AC,
clone CLH2, dilution 1:50, Novocastra, London, UK) were incubated at room
temperature for one hour. After washing with Tris, the sections were incubated with a
post-primary block for 30 minutes. The revelation was conducted using 3,3’-
Diaminobenzidine (DAB) chromogen (Liquid DAB+ Substrate Chromogen System,
Novocastra, Newcastle Upon Tyne, UK) followed by nuclear staining using
hematoxylin.

### 2.3. RNA extraction and reverse transcriptase-polymerase chain reaction (RT-
PCR)

RNA extraction was performed on 34 frozen specimens taken from patients who
underwent colorectal tumor resection at the Department of Surgery, and on 168 archival
formalin-fixed, paraffin-embedded (FFPE) blocks, preserved at the Department of
Pathology and Cytology of the Mongi Slim Hospital, La Marsa, Tunisia.

Regarding frozen specimens, samples were kept at –80°C in RNAlater Stabilization
Solution (Invitrogen™, Carlsbad, CA, USA). To start, we homogenized the fragments
using a sterile, RNase-free mortar and pestle, then we immediately transferred the
homogenates into sterile, RNase-free microcentrifuge tubes containing Trizol Reagent
(Invitrogen™, Carlsbad, CA, USA). The total RNA isolation was conducted according
to the procedure provided by the manufacturer using PureLink® RNA Mini Kit (Invitrogen™, Carlsbad, CA, USA).

As for the FFPE samples, we placed 3-8 pieces of 10 μm sections into a sterile, RNase-free microcentrifuge tube and proceeded to a total RNA isolation with PureLink™ FFPE Total RNA Isolation Kit (Invitrogen™, Carlsbad, CA, USA) according to the manufacturer's instructions. Following RNA purification from both frozen and FFPE specimens, we performed a final DNase I digestion (Invitrogen™, Carlsbad, CA, USA) to remove genomic DNA contamination.

RNA was stored at -80 °C until used. RNA concentration was determined using Qubit® RNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), and the quality was verified using Agilent 2100 Bioanalyzer. It analyzes 12 samples simultaneously using RNA Nano Chips (Agilent Technologies, Inc., Santa Clara, CA, USA). The 2100 Expert software allows the automatic calculation of RNA Integrity Number (RIN). Its value is between 1 and 10, and the higher this value is, the better the quality of RNA. According to RIN scores, we chose the samples with the highest value for both frozen and FFPE samples.

The cDNA was synthesized by M-MLV Reverse Transcriptase (Invitrogen™, Carlsbad, CA, USA, in a 25 μL total volume reaction containing 200 ng of total RNA for each sample.

The 35 cycle amplification included 94 °C/30 s; 63 °C/30 s for MUC1 (58 °C/30s for MUC5AC); 72 °C/30 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal standard. PCR primers for MUC1, MUC5AC, and GAPDH are detailed in Table 1.
The RT-PCR-products were verified on a 2% agarose gel, stained with 0.1 mg/mL ethidium bromide, to check the amplification of the different genes under UV light. After that, MUC1 and MUC5AC PCR products were analyzed by an on chip-electrophoresis, for that, we used Agilent DNA 1000 Chip kit. All chips were prepared according to the manufacturer’s instructions. Note that both the size and the concentration of each separated band were automatically calculated by the 2100 bioanalyzer software.

2.4. Statistical analysis

All the clinicopathological criteria were analyzed with the SPSS 20.0 software. Descriptive analysis and the categorical variables were evaluated with chi-square test. For the overall survival, we analyzed the expression profiles of MUC1 and MUC5AC simultaneously and four phenotypes were established: MUC1+MUC5AC+ / MUC1-MUC5AC- / MUC1+MUC5AC- and MUC1-MUC5AC+, aside from each gene separately. Survival curves were plotted using the Kaplan-Meier product-limit method, and differences between the survival curves were tested using the log-rank test. The results were considered to be significant when P values were less than 0.05.

3. Results

The 202 patients included 110 males (54%) and 92 females (46%), with a sex ratio M:F of 1.19. The average age was 60 years (ranging from 29 to 92 years). Tumors were more frequently located in the left colon (76%). The immunohistochemical study was performed on samples of paraffin-embedded tissues from 202 colorectal cancer patients.
It consisted of 54 cases of mucinous adenocarcinoma (MA) (27%), 28 cases of adenocarcinoma with mucinous component (MC, mucin formation < 50%) (14%) and 120 cases of non-mucinous carcinomas (NMC) (59%). Normal colorectal mucosa from tissue margins served as controls. The same samples were studied by RT-PCR either from frozen (34 cases) or paraffin-embedded tissues (168 cases).

Histologically, our series consisted of 164 well-differentiated cases (81%), 30 moderately (15%) and 8 poorly differentiated (4%). TNM staging system showed that 20 cases were stage I (10%), 80 cases were stage II (40%), 92 cases were stage III (45%) and 10 cases were stage IV (5%).

3.1. Immunohistochemistry expression of MUC1

Out of 202 samples of normal mucosa, 18 (9%) showed positive staining for MUC1 in both cytoplasm and apical membrane (Figure 1a), whereas the remaining samples reacted weakly or not at all (Figure 1b).

MUC1 expression was remarkably increased in the tumoral tissue compared to the normal mucosa, 88 out of 202 cases (44%) were positively stained in the apical membrane and cytoplasm (Figure 2).

54% of stage III (50 cases among 92) and 60% of stages IV (6 cases among 10) were positives for MUC1 protein expression; in contrast only 20% and 35% respectively for stage I (4 cases among 20) and II (28 cases among 80) were positive.

Only 30% of patients with no lymph node metastases (N0) show a positive signal for MUC1, whereas those with lymph nodes N1, N2 and N3 showcase a positive staining in 62%, 47% and 60% of the cases, respectively.
Statistical analysis of MUC1 expression pattern along with the patients’ clinicopathological criteria showed that the signal positivity was strongly associated with the advanced cancer stages (p < 0.006) and lymph node metastasis (p < 0.001).

The results were summarized in table 2.

### 3.2. Immunohistochemistry expression of MUC5AC

Since normal colorectal mucosa does not express MUC5AC, we used a gastric mucosa as a positive control. The later showed a strong signal in the cytoplasm and the apical membrane.

MUC5AC exhibited a similar staining pattern to MUC1 in terms of the membrane and cytoplasm expression. It was observed in 30 out of 202 colorectal carcinomas cases (15%) (Figure 3).

Statistical analysis of MUC5AC immunohistochemical results in comparison with the different clinicopathological criteria did not show any significant association. The results were summarized in table 2.

### 3.3. mRNA expression of MUC1 and MUC5AC in colorectal carcinomas

MUC1 gene expression was assessed using RT-PCR, revealing an amplicon of 188 bp in 48% of the cases (96/202).

For MUC5AC gene, amplification demonstrated that 40 out of 202 cases (20%) presented an amplicon of 111 bp.

GAPDH, our reference gene, was successfully amplified in all cases (Figure 4).

A detailed analysis of all clinicopathological and immunohistochemical criteria in comparison with the genes molecular expression was carried out in order to identify any particularities (Table 3).
MUC1 was found to be highly involved in the advanced stages (p < 0.016) and lymph node metastasis (p < 0.002). Moreover, gene expression showed a significant correlation with immunohistochemical results (p < 0.000).

MUC5AC statistical analysis highlighted only an association between molecular expression and immunohistochemical profile (p < 0.000) (Table 3).

3.4. Overall survival statistical analysis

Among the 202 patients, 24 patients were lost to follow up, so these cases were not included in the survival curve analysis. The average overall survival rate of the 178 patients was 27 months (from 1 to 167 months).

Among the 178 patients 78 cases were MUC1 positive (44%) and 26 were MUC5AC positives (15%).

35% of the studied cases died during follow-up (62 patients).

16 cases were MUC1+MUC5AC+, 90 cases MUC1-MUC5AC-, 62 cases MUC1+ MUC5AC- and 10 cases MUC1-MUC5AC+. The death rates were 62.5%, 27%, 35.5% and 60% respectively.

Based on the expression profiles of MUC1 and MUC5AC simultaneously, the test didn’t pinpoint any significant association (p < 0.062).

To further evaluate whether the expression status of MUC1 or MUC5AC affects the prognosis we studied each gene separately.

Among the 62 deceased cases 32 expressed MUC1 (52%) and 16 expressed MUC5AC (26%). This percentage might suggest that MUC5AC expression could be a good prognostic biomarker unlike MUC1, but statistically we only found an association between MUC5AC expression and overall survival (p < 0.009) (Figure 5). Patients who
expressed *MUC5AC* showed an increase in overall survival as compared to those who did not express the protein.

4. **Discussion**

As the third most common cancer, colorectal cancer is tagged a preeminent public health concern and a main cause of the exponential morbidity rate throughout the world [18,19].

Mucins are large O-glycoproteins expressed on the epithelia, providing a protective barrier against mechanical, chemical, enzymatic, and microbial damage in the aerodigestive and genitourinary systems [20,21].

Mucins expression is tightly controlled given their essential role in the normal tissue homeostasis, whereas their deregulation leads to chronic inflammation and even cancer. It has been accepted that quantitative and qualitative changes in mucins were not only a consequence but also potential contributors to inflammation and cancer [15,22].

The current study focused on the evaluation of *MUC1* and *MUC5AC* implication in CRC, and the exploration of their possible use as markers in the detection and treatment of this neoplasm.

Our results highlighted that *MUC1* and *MUC5AC* mRNA and protein levels were notably up-regulated in tumor tissues when compared with the normal mucosa. A similar pattern was found in many other studies [2,10,15,23].

The immunohistochemical analysis showed *MUC1* presence in 9% of the studied normal mucosa. It should be noted that this percentage is variable since some studies reported a lack of *MUC1* expression in normal mucosa [10]; and others have mentioned expression in some or all of the studied cases [15,24].
As for adenocarcinomas, many studies detected a slight to moderate variation in \textit{MUC1} expression profile, in contrast with some other researches where the gene presence was noted in more than 80\% of the cases, ours was at 44\% [10,15,25].

This variability may be attributed to either ethnicity and geographical distribution or to the heterogeneous designs of these studies (number of samples, sampling methods, tumor sites, tumor stages…) [2].

In literature, the high expression of \textit{MUC1} positively correlates with stage, metastasis, poor tumor differentiation, and worse long-term survival [10,26–29].

The statistical analysis of immunohistochemical profiles and mRNA expression of our cases showed that \textit{MUC1} expression is relatively associated with advanced stages and lymph node metastasis [25,26].

Several studies pointed out \textit{MUC1} involvement in colorectal carcinomas progression and metastasis development. One of the possible mechanisms is that this mucin acts as a ligand for cell adhesion molecules which benefits \textit{MUC1}-expressing circulating tumor cells and helps them adhere to endothelial cells and therefore allowing their migration in secondary sites [2,27].

In normal physiological conditions, secretory mucin \textit{MUC5AC} is not expressed in the colonic mucosa, whereas its aberrant expression is observed during development of colon cancer [1,13,15,23,30].

Since \textit{MUC5AC} is not expressed in normal colon mucosa we used gastric mucosa as a positive control. However, \textit{MUC5AC} protein was found in 15\% of the colorectal carcinomas (30/202), similar results were published in other studies with varying percentages [10,13,15,21,31–33].
Although our results regarding *MUC5AC* are in accordance with the aforementioned findings, its expression did not serve as a statistically significant marker of tumor progression.

Regarding mRNA expression, most cases with positive RT-PCR amplification showed positive immunohistochemical staining of the studied gene.

Note that in some cases, the protein was not found despite the mRNA presence (8 cases for *MUC1* and 10 cases for *MUC5AC*). The discordance between transcript and protein expression levels would probably be due to post-transcriptional mechanisms [34,35].

In the current study, it was not possible to find statistically significant relationships between expression profiles of *MUC1* and *MUC5AC* simultaneously and survival of patients with CRC (p < 0.062).

The analysis of each gene separately showed that patients who expressed *MUC5AC* had a better survival rate (p < 0.009). For *MUC1* the evaluation was not significant (p < 0.889).

For *MUC5AC* many studies revealed that tumors with negative expression of *MUC5AC* showed a worse prognosis compared with tumors when *MUC5AC* was positive but others studies had an opposite result or didn’t pinpoint any correlations [1,9,30].

This advocates that *MUC5AC* presence can be a prognostic factor for non-aggressive colorectal carcinomas and a promising target in the treatment of colon cancer [23].

5. **Conclusions**

The expression of *MUC1* might be a biomarker for poor prognosis in colorectal cancer and could play a role in tumor transformation and metastasis. However, the expression of *MUC5AC* might have a better prognostic in the Tunisian cohort.
Given the importance of such analysis, future studies can be focused on the evaluation of other mucins like **MUC2** and **MUC4** to demonstrate the involvement of their expression and establish a combination between them in the colorectal carcinoma. Nonetheless, a further investigation, in larger patients and other techniques, is required to confirm our findings and predict the clinical usefulness of these molecular markers.

**Acknowledgments**

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All thanks to the staff of the department of pathology and cytology.

The authors declare that they have no conflict of interest.

**References**


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| **MUC1** | F: CCAGCCCGGGATACTACCAT  
R: GCGACGTGCCCCCTACAAGTT | 63                         | 188                 |
| **MUC5AC** | F: CCTTCGACGGACAGAGCTAC  
R: TCTCGGTGACAACACGAAAG | 58                         | 111                 |
| **GAPDH** | F: GGGTGTGAACCATGAGAAGT  
R: GACTGTGGTCATGAGTCCCT | 57                         | 136                 |
Table 2. MUC1 and MUC5AC protein expression profile with clinicopathological criteria of patients

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Table 3. MUC1 and MUC5AC mRNA expression profile with clinicopathological criteria of patients

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Figure 1. (a) Membranous and cytoplasmic positive staining with anti-Muc1 in healthy distant colorectal tissue (*MUC1* ×200); (b) Absence of anti-Muc1 staining in healthy colorectal tissue (*MUC1*, ×400)
Figure 2. Positive staining with anti-Muc1 in mucinous adenocarcinoma (MUC1 ×400)
Figure 3. (A) Positive staining with anti-muc5ac in mucinous adenocarcinoma (MUC5AC ×200)
Figure 4. Bioanalyzer gel image of *MUC1* and *MUC5AC* expression in colorectal cancer tissues. Ladder at 1500 bp; *MUC1* cDNA (188bp); *MUC5AC* cDNA (111 bp)
Figure 5: Patients survival curve