

# Clinicodemographical assessment of colorectal cancer with emphasis on B3GALNT2, MUC1, P53 and Ki67-related risk of metastasis

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## Research article

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# Abstract

**Background:** The incidence of colorectal cancer (CRC) is rising, worldwide, and is attributed to genetics and epigenetic factors. We aimed to evaluate the key clinicodemographical/epidemiological-and molecular impacts on metastatic CRC in CRC patients from a highly populated area in northeastern Iran. Retrospective clinical materials-based cohort design and patients were analyzed with respect to age, sex, colorectum anatomy, metastasis, mortality as well as to expression of molecular markers B3GALNT2, mucin I (MUC1), P53 and Ki67. **Methods:** Patients, 6260 registered CRC with 3829 underwent surgery, from three medical university hospitals in the study area, during 2006-2016, were analyzed for the clinicodemographic aspects of age, sex, stage of CRC, history of smoking, familial/occupational status and post-surgery survival period as well as mRNA/protein expression of B3GALNT2, MUC1, P53 and Ki67. Factors were set to estimate mortality and risk of metastatic CRC. **Results:** ~61%, ~33% and ~6% of adenocarcinomatous CRC were located in colon, rectum and rectosigmoid, respectively, of which younger and older than 50 was mainly in transvers colon-and colorectum, respectively. Post-surgery survival period of metastatic CRC patients was remarkably longer in patients aged > 50 than those < 50 years old, and worse in females than males. B3GALNT2 high, MUC1 high, P53 low and Ki67 high mRNA/protein expression in metastatic stage III CRC were highly associated with increased metastasis and mortality. B3GALNT2 high, MUC1 high, P53 low and Ki67 high mRNA/protein expression correlated with increased risk of a progressed CRC state and mortality. The risk to develop metastatic CRC was higher in males, younger, urban residing-and employed individuals, indicative of a plausible non-genetics/epigenetics contribution to CRC. **Conclusions:** The role of possible diagnostic biomarkers, B3GALNT2, MUC1 and Ki67, but not P53, in the etiology/early detection of metastatic CRC is promising. Epigenetic contribution to metastatic CRC risk is predominant.

## Background

Colorectal cancer (CRC) is normally triggered by the effects of environmental (epigenetic) and genetic factors [1]. The frequency/distribution of age, sex and anatomical sites of cancer in different parts of the colon and rectum differ, which can be attributable to different effects of etiologic factors at the site of CRC. Different molecular/genetic mechanisms are involved in the biological pathway of CRC. In this pathway, the conversion of a normal epithelium to a proliferous one and thus adenoma/adenocarcinoma occurs through DNA methylation-associated mutation in tumor suppressors (e.g. APC, SMAD4, and P53) [2]. Nuclear protein, P53, is a key pro-apoptotic and anti-proliferative molecule in healthy individuals, and its mutation occurs in various cancer; changes in its mRNA/protein expression is one of the underlying reasons of resistance to chemotherapy [3]. Conversely, the 345-395 kDa Ki67 is a crucial molecule for cell proliferation in healthy men/women. The overexpression of Ki67 mRNA/protein has been reported in various tumors [5]. Both P53 and Ki67 are highly expressed in large intestine of CRC patients [4,5].

Mucin 1 (MUC1) is a transmembrane glycoprotein that is over-expressed in various types of human carcinoma, including breast, colon, lung and prostate cancer [6]; it is synthesized as a single polypeptide that undergoes auto-cleavage into MUC1-N and MUC1-C subunits, which form a stable heterodimer at the

cell surface [7]. The MUC1-N terminal subunit contains variable numbers of glycosylated tandem repeats [6], while the MUC1-C terminal subunit consists of -extracellular, transmembrane and cytoplasmic domains with 58, 28 and 72 amino acids, respectively [8].

As a precursor of mucins family, B3GALNT2 has first been identified as a novel glycosyltransferase having  $\beta$ 1,3-glycosyltransferase motifs, which are highly conserved in  $\beta$ 1,3-galactosyltransferase and  $\beta$ 1,3-N-acetylglucosaminyltransferase families [9]. The purified putative catalytic domain of B3GALNT2 possess of N-Acetylgalactosaminyltransferase activity to form  $\beta$ 1,3-linkage [7]. Nevertheless, there is little study on the role of B3GALNT2 and related mucin family in metastasis of CRC.

Globally, the worringly rising cancer has claimed millions of lives yearly; though lung cancer claims most lives of all cancers, globally, CRC is nonetheless the second fatal factor in America, while the GIT cancer is considered to emerge as the first with predominantly CRC [10-12], which is considerably demanding critical measures, worldwide. Various studies reveal that about 60% of the cases with CRC have been detected in developed countries, though early detection/percussion measures have downturned the rate [13]. For example, increased annual colonoscopy checkups for people of both sexes aged 50-75 have substantially prevented the CRC and its annual death rate in the USA [11]. Conversely, annual CRC prevalence/rate and its mortality have been reported to be increasing in developing countries like Iran [11,14,15]. To proceed with the current situation in the Middle Eastern countries like Iran regarding the CRC prevalence, it should be noted that the rise in the emergence of cancer, particularly afflicting the patients older or younger than 50 in last 25 years, has alarmingly made this region highly susceptible to cancers, especially CRC [16,17].

Currently, due to the decline in birth rate and the relative life expectancy, the world population is tended towards older. Iran is also experiencing a remarkable change in its age pyramid [18], likely towards older [(i.e., ~22% of the Iranians will be older than 60 by 2050) [19]. So, elder males and females are looming [20]. Due to the huge emergence of CRC and its mortality even in youngers, it is expected that its prevalence turns out to be a challenging dilemma regarding cancer management system in Iran. The present study is concentrated on scrutinizing the CRC patients' medical files classified within the last 10 years, post-surgery survival with some IHC assays and evaluation of P53, Ki67, B3GALNT2 and MUC1 mRNA/protein levels in (non)metastatic CRC. Here we also aimed to highlight the specific relationship between the changes in age pyramid and the CRC prevalence, the percentage of CRC emergence in various parts of the colorectum, along with determining the efficiency of preventing early CRC.

## Methods

### Collection of CRC patient samples

This study was approved by the ethical committee of Mashhad University of medical science with ID IR.FUBS.REC.1395.42456 with the commitment of the principles of the declaration of Helsinki. As a cross-sectional research, the present study was conducted in 3 medical university hospitals (Ghaem, Omid and Imam Reza) in the study area, Mashhad, Iran, during 2006-2016 (Fig. 1), where they received 6260 CRC

patients, with 3829 with stages I-IV CRC, undergoing surgery. Figure 1 also emphasizes the clinicopathological importance, precise detection of CRC and histopathological sampling site and how best to relate clinical aspect of CRC to further cellular-and-molecular assays; it would also help governments/NGO/medical scientists and non-clinicians/molecular biologists to provide more appropriate CRC-preventive protocols to lower the CRC incidence.

The details of the selection of patients for the clinicodemographical and molecular analyses are shown as a flow chart (Fig. 2). Retrospectively and prospectively, further clonicoepidemiological along with molecular analyses with medical records (i.e., colonoscopy, CT scan, pathology report, anatomical subsites, location/type of CRC/stages and metastasis, age, gender, occupation, familial, place of living, smoking/alcohol/adict and educational status, post-surgery survival in the surgery ward during hospitalization and treatment of the patients) were analyzed (n=3829). All subjects signed their informed consents before participation and then with signed consent forms and questionnaires, colonoscopy and CT scan. Indeed, before immunostaining and molecular assays, to select/include stage III CRC patients (n=315), TNM method was carefully selected to classify the CRC patients as metastatic stage III. Only 60 (metastatic stage III CRC [not-yet being under chemotherapy but scheduled for surgery, n=60] and non-metastatic stage III CRC/control individuals, n=60) with an identical numbers (30 men and 30 women) and ages ( $57.21 \pm 8.57$  years old) of their control counterparts ( $55.69 \pm 9.34$  years old) [i.e. a total of 120 samples (60 men and 60 women)] were selected for further experimental design and molecular analyses. The targeted colorectal tissue samples were also used for performing IHC analysis (n=315).

### **Immunohistochemical and epidemiological trends**

Selected colorectal tissues samples from stages III and IV metastatic CRC patients (n=315, age=30-80 years) were immunostained with anti-P53 (clone: DO-7), anti-Ki67 (clone: MIB-1) and Anti-Human MUC1 (CD227) antibodies, Clone 214D4 (all with dilution of 1/100 and from Dako, Carpinteria, CA; prediluted) accordingly [20]. Briefly, with EXPOSE human Specific HRP/DAB Detection IHC kit (Abcam, USA) the sections were incubated with the primary antibodies for 60 min, and IHC was carried out using biotinylated secondary antibody and streptavidin-alkaline phosphatase conjugate. The antigens were demonstrated by using diaminobenzidine as the chromogen. For negative controls, the primary antiserum step was omitted. All examinations on samples were performed in a blinded manner. The percentage of positively immunostained cells for P53 and Ki67 was counted in 10 different microscopic fields for every section (magnification  $\times 400$ ). Results obtained from the image analyzer and software (NIS Elements imaging software (Nikon Instruments NY, Melville, I) were subjected to statistical analyses. Further, the Kaplan-Meier test/estimator was used to estimate the survival rate of the CRC patients.

### **qPCR assays**

For qPCR assays of P53 and Ki67 mRNA expression, extracted RNA (with kit, Roche Diagnostics, Indianapolis, IN) was purified from metastatic and non-metastatic tissues (n=60) and routinely reverse transcribed for cDNA synthesis kit (Fermentas, Finland). Exon junction or intron-spanning primers were designed for 3 pairs of primers (P53, Ki67 and  $\beta$ -actin, as the reference gene, (designed primer sequences

are available upon request). The qPCR conditions for all genes were carried out (in duplicate) with a cycling program including holding for 10 min at 95°C, followed by cycling 45 times at 94°C, annealing temperature of 58°C, and 72°C (20 sec for each temperature), accompanied by melting curve analyses and agarose gel electrophoresis to ascertain the specificity of the reactions or the absence of non-specific PCR products (data not shown). Using GenEX Version 6 software (MultiD, Göteborg, Sweden) and Relative Expression Software Tool (REST; QIAGEN, Hilden, Germany), the normalization and analyses of the qPCR data were calculated accordingly [21]. Comparative qPCR results of P53 and Ki67 mRNA expression in metastatic and non-metastatic CRC tissues along with comparison of full heat-map between Ki67 and P53 genes in CRC data were evaluated to see the possible correlation of the mRNA expressions of Ki67 and P53 with CRC metastasis. All experiments were carried out in triplicates. Data were analyzed according to the comparative Ct ( $2^{-\Delta\Delta Ct}$ ) method [21].

Newly designed primers used herein were as follows: MUC1, F: 5'-CCCTATGAGAAGGTTTCTG-3', R: 5'-CCTACAAGTTGGCAGAAG-3', B3GALNT2, F: 5'-CCACAGTTTTCTTAGCTC-3', R: 5'-CTCAGTGACATCCTACTC-3', P53, F: 5'-CGATGGTGTACTTCCTGATA-3', R: 5'-CAGCTCTCGGAACATCTC-3', Ki67, F: 5'-TCCTTTGGTGGGCACCTAAGACCTG-3', R: 5'-TGATGGTTGAGGTCGTTCTTGATG-3',  $\beta$ -actin: F: 5'-CTACCTTCAACTCCATCA-3', R: 5'-GAGCAATGTCTTGATCTTC-3'.

### **Western blot (WB) analysis**

WB analysis was performed as previously described [22]. Briefly, large intestinal cancerous and non-cancerous tissues (0.1 gm) were lysed in 0.9ml lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Nonidet P-40, 0.5% CHAPS] including 0.1% protease inhibitor cocktail III (Calbiochem, San Diego, CA, USA). The tissue lysates were kept on ice for 30 min and centrifuged at 16000  $\times g$  for 10 min to remove tissue debris. Total protein level of tissue lysates was measured using bicinchoninic acid assay (BCA) kit (Thermo Scientific). Samples were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (PVDF, Roche, Germany). Membranes were then blocked overnight by 5% skimmed milk in TBS 0.1% Tween-20, at 4°C. After incubation of membranes with primary antibody, rabbit anti-human B3GALNT2 pAb (ab228993, Abcam) and anti- $\beta$ -actin (AC-15, Sigma-Aldrich, A-5441) at a dilution of 1:1000 and anti-rabbit HRP-conjugated secondary antibody (cell signaling technology, USA) at a dilution of 1:5000; visualization was performed using enhanced CL-based Clarity Western ECL Substrate (Bio-Rad) in combination with an ECL imaging system (Uvitec, Germany). Signals on WB were quantified by Image J 1.42q software (Wayne Rasband, NIH, Bethesda, MA) and the results were eventually normalized to the B-actin band intensity which was considered as an internal control for loading variations.

### **Statistical analyses**

The collected data were analyzed via one way *t*-test, ANOVA and Tukey tests. The significance level was 0.05. All results were presented as mean  $\pm$  SD.  $\chi^2$  tests were used to determine the association between P53, Ki67 B3GALNT2 and MUC1 expression with clinicopathological characteristics (especially

metastasis), along with analysis of receiver operating characteristics (ROC) [to calculate the predictive values and compare diagnostic efficacy of the studied molecules using the MedCalc statistical program package], whereas the Kaplan-Meier method and log-rank test were used for univariate survival analysis. Also, comparative multivariate ROC analysis with t-test was used to predict any significant relation between P53, Ki67 B3GALNT2 and MUC1 with metastasis. Cox proportional hazards regression was carried out for multivariable survival analysis. Odds ratio (OR) and 95% confidence intervals (CI) were obtained (MedCalc). All analyses were carried out using GraphPad Prism 6.

## Results

### Demographical, anatomical location and histological trends

Table 1 shows the detailed demographic variables with sample and clinical characteristics of CRC patients at diagnosis stage/site. Of the 6260 CRC registered patients studied during the 11-year period, 3829 underwent surgery (with a mean age of  $55.81 \pm 24.53$  years). The lowest mean age was in 2014 ( $52.36 \pm 0.03$  years). Figure 1, reveals the differences between the number of metastatic CRC patients during the cross-sectional retrospective study period to highlight the trend/incidence of metastatic CRC during 2006-2016 in the study area, and potentially broader region.

Of all cases of surgically treated CRC, 60.16 % (2303 cases) were colon cancer, 32.77 % (1255 cases) rectal cancer and 7.07 % (271 cases) rectosigmoid cancer; among those older than 50, most CRC was in the rectum (Fig. 3). Further, 71.37 % (2733 cases) and 28.59 % (1095 cases) were men and women, respectively. With minimal and maximal metastatic CRC cases appeared in 2006 and 2013, respectively (Fig. 1E), the most common histological types with the frequencies of 91.53 % and 7.31 %, respectively, were adenocarcinoma, not otherwise specified, and mucinous producing adenocarcinoma (Figs. 1A-D). There was a significant relationship between the risk of CRC and anatomical subsites, where the highest and lowest OR appeared to be the transverse colon (OR = 1.17, 95% CI 1-1.2) and sigmoid (OR=0.07, 95% CI 0.06-0.07) with P = 0.0001 (Table 1).

### Metastasis trends

Of all cases, 2.84 % (109 cases), 36.04 % (1380 cases), 57.61 % (2206 cases) and 3.49 % (134 cases) related to stages I, II, III and IV, respectively, (Fig. 3A). During the eleven-year of this study, 315 cases of stage III metastatic CRC were registered and metastasis seemed also to be increasing in the region under study over the past 10 years (Fig. 4E). All patients underwent surgery, of which 8.64% (331 cases) died in hospital after surgery. The overall post-CRC surgery survival period (months) was  $42.72 \pm 20.04$  months; post-surgery survival period of metastatic CRC patients younger and older than 50 (Fig. 3C) and female and males (Fig. 3D), with significantly higher post-CRC surgery survival periods in patients aged  $\geq 50$  than those  $< 50$  years old (Fig. 3C) and in males than females (Fig. 3E).

### Immunohistochemical trends

Of 315 metastatic stage III CRC patients, 218 (69.2%) and 239 (75.87%) showed over-expressed and Ki67, B3GALNT2 and MUC1 proteins, but downregulated P53 respectively (see Table 2 and Fig. 4). Further, there was a significantly positive correlation between overexpressed P53 and Ki67 with clinicopathological signs [(specially metastasis in liver (122), lungs (109), omentum (84), bone (58), brain (2) and other organs (8)], though the correlations between over-expressed P53 and Ki67 with age and sex were insignificant (see Table 2). Further, Kaplan-Meier test was used to estimate the survival rate of metastatic CRC patients (n=315) revealed that the post-surgery survival rates in those <50 years old (n=86) and  $\geq 50$  (n=229) years old were 24 (mean=13.16) months and 24-48 (mean= 33.09) months, respectively (Fig. 3E). Significant correlation were observed between the increased Ki67 (OR = 3.1, 95% CI 2.3-4.2, P < 0.0001), MUC1 (OR = 2.2, 95% CI 1.6-2.9, P = < 0.0001) P53 (OR = 1.1, 99% CI 0.8-1.5, P = 0.25) and the likelihood of metastatic CRC (Table 1 and Figs 4). Figure 4 also shows the percent of metastatic and non-metastatic CRC patients expressing Ki67 (Fig. 4C), with number of those metastatic (n=315) and non-metastatic (n=3514) CRC patients expressing P53 (Fig. 4B). The protein level of Ki67, but not P53, strongly correlated with metastasis (P < 0.0001). MUC1 (with IHC) and B3GALNT2 (with WB) showed significant overexpression (P < 0.0001 and P=0.0077, respectively) in metastatic CRC compared to non-metastatic ones (see Figs 4D and 4H). Figures 4B, 4C, 4D and 4H show the overall protein expression of P53, Ki67, MUC1, (immunohistochemistry) and B3GALNT2 (WB), respectively. Further, comparative ROC analysis of IHC of colorectal tissues in metastasis and non-metastasis CRC P53, Ki67 and MUC1 in metastasis CRC patients and CRC revealed the optimal thresholds for, P53 (AUC = 0.65), Ki67 (AUC = 0.94) and MUC1 (AUC = 0.65) (Fig. 4E-G).

### qPCR trends

Results of globally comparative qPCR assays on P53 and Ki67 and related heat-map analyses, depicted (Figs 4L and 4M), revealed a strong correlation between the mRNA levels of Ki67, P53, B3GALNT2 and MUC1 and metastasis. mRNA expression of Ki67, B3GALNT2 and MUC1 in stage III CRC patients with metastasis were significantly higher than those without metastasis, (see Figs 4I and 4J) P<0.001). Conversely, the mRNA expression of P53 in stage III CRC patients with metastasis was significantly lower than those of metastasis (P<0.01, Fig. 4I).

## Discussion

Here we reported a survey on >6000 CRC patients, but 3829 were being surgically operated and used for the related clinicodemographic correlation, and of these only ~8.3% were transcriptionally/proteomically evaluated for well-known key cancer markers (P53 and ki67) along with B3GALNT2 and MUC1. Though we did not experimentally assess the epigenetic changes, but the clinicodemographic analyses unequivocally exclude genetic contributing factors (i.e., relevant contribution of environmental/non-genetic/epigenetics factors). Based on the results of this study and supported by others, the incidence of CRC is increasing every year, in a way that from 2006 to 2016, a total of 6260 admitted CRC patients ~two-third of them underwent surgery for the affected subsites of colorectum in three specialized hospitals in Mashhad, Iran. According to the previous studies in Iran, CRC is the fourth most common

cancer in Iranian men after gastric, bladder, and prostate cancers, and the second most common cancer in Iranian women. The existing reports unravel >4000 new cases of CRC are diagnosed every year in Iran [8, 19]. Though the incidence of CRC in Iran is relatively low compared to many countries, it is worryingly increasing [18]. The mortality rate of CRC varies among different racial groups and in various geographical parts, with the lowest incidence in Asia and Africa and the highest in North America and Europe [23]. This is probably due to the predominantly epigenetics/environmental factors, such as stress, low physical activity, life style, age pyramid etc. with lesser extent to genetics [8,24,25]. Our study herein showed that the incidence of CRC in men was higher than that of women; this is consistent with the results of others [20]. The mean age of patients with CRC in the study area was ~55 years [8], and more serious pattern with rising risk of CRC-related mortality was observed in northwestern neighboring countries (like Turkey, Armenia...) [26]. The results of previous studies in Iran and other countries indicate that CRC in men and women are different in each country and region [8, 26, 27]. To our knowledge, it seems likely that deficient in some key trace elements [8] and the microbial flora [28] of the colon at older than 50 could be a significant attributable factor for the development of CRC. Nonetheless, the worrying rise of metastatic CRC incidence in the study area (Iran and even Mideast-wide) emphasizes the notion that governments/NGO/medical scientists must focus more on the debatable CRC preventive protocols to lower the CRC risk in the region.

Our results also indicated that the CRC in patients older than 50 occurred in the rectum, and in those aged 20 to 50 it normally occurred in the colon. Consistent with another study [29], the majority of CRC cases (3986 patients, 63.67%) were related to colon. Also, the incidence of rectum, sigmoid and descending colon cancers were more frequent among men. In line with others [30], carcinoma in cecum, ascending colon and right part of transverse colon seemed to be more prevalent in females. We also found adenocarcinoma (91.53%) appeared to be the most common pathology followed by mucinosis (7.31%), which occurred in the second place. This finding is in line with surgery reference books [31]; indeed, 94% of tumors in the colon are predominantly adenocarcinoma [32,33]. The results of the present study suggest that most hospitalized stages III/IV CRC patients' post-surgery's 5-year survival rate typically ranged from a 90%, 70% and 10% for those encountered with localized, mild regional and sever distant metastasis, respectively. [i.e., much more shortened life expectancy in sever metastatic CRC patients, and the lower post-surgery survival rate is attributed to delayed detection and lack of follow-up to complete treatment]. Indeed, the earlier the stage of diagnosis the higher the chance of survival [34], emphasizing the urgent need to find more definitive oncomarkers for early stage of CRC.

The results here also showed that the survival rates of 1, 3, 5 and 10 years old patients were 78.79%, 54.06%, 28.4% and 10.39%, respectively, regardless of others (i.e., gender, education, occupation, clinical features of colorectal cancer before diagnosis, tumor familial, place of residence, smoking, alcohol and addict). This survival rate is lower compared to Europeans and North-Americans, in which delayed detection and lack of follow-up to complete treatment can be attributed to the lower post-surgery survival rate [35]. In line with others' [36], we also found that only age is associated with the survival rate of CRC patients.

Although the prognosis of metastasis and post-surgery relapse in stage III CRC patients is unclear, herein we could see some promising aspects of P53, Ki67, MUC1 and B3GALNT2 on severity of CRC metastasis. Indeed, with IHC-based over-expression of particularly Ki67, MUC1 and overproduction B3GALNT2 proteins (but not P53) along with overexpressed Ki67, MUC1 and B3GALNT2 mRNA (but not P53 mRNA) in metastatic stage III CRC patients and a strong correlation between mRNA of Ki67, MUC1 and B3GALNT2 (but not P53 mRNA) and metastasis emphasize the potential involved of these four proteins in pathogenesis, prognosis and survival rate of CRC. This result is consistent with others [35,37]. To generate clinically useful molecular guidelines for CRC preventive measures, detection of P53, Ki67, MUC1 -and-B3GALNT2 related expressing signals should be precisely implemented.

In this study, 40.97% of CRC patients (94.77% of the males and 5.22% of the females) were smokers. However, the relationship between smoking/smoking habits and the incidence of CRC was not confirmed [38]. Similarly, the results of a case study in the US surprisingly showed little relationship between long-term smoking and increased risk of CRC [39]. Nonetheless, smoking exacerbates many health issues in today's society, and its involvement should not be ignored.

Mucins have an important function as protective layer for epithelial tissues in the gut and elsewhere in the body. It is well known that during carcinogenesis mucins can be lost or aberrantly expressed in locations where they are not present constitutively. They might be involved in tumor progression and spread. However, the prognostic value of aberrant mucin expression in CRC is controversial [40,41]. Previous publications have reported the impact of MUC1 expression on tumor progression and also on survival [42]. For instance, MUC1 overexpression in CRC patients worsens TNM-based CRC stage, recurrent metastasis and overall survival rate [43].

Glycosylation is a post-translational modification and is associated with various physiologic events. In different cancers, the expression of glycosyltransferase in the ER and Golgi apparatus can vary and result in different glycolipid or glycoprotein structures. Polypeptide N acetylgalactosaminyl transferase (ppGalNAc-T) has been found to be a biomarker and prognostic indicator for breast, gastric and ovarian cancers [44, 45]. N-acetylglucosamine transferases (GlcNAcT) have been proposed to have a role in invasion or metastasis in gastric and breast cancer as well as serving as biomarkers [46]. Multiple sialyltransferases have been associated with enhanced breast and colorectal cancer with effects on prognostic indicators [47].

The aberrant expression of glycosyltransferase and the immature glycan structure of proteins and lipids are observed in many cancers. These phenomena are also involved in the development and progression of cancers [48,49]. Abnormalities of the glycan structure and thus oncogenic roles of a cancer-specific glycosyltransferase, UDP-N-acetyl- $\alpha$ -D-galactosamine (GalNAc): polypeptide GALNT6 of glycoproteins are frequently observed in cancer cells [6], regulated cell proliferation and cytoskeleton structure through aberrant O-glycosylation and stabilization of an oncoprotein MUC1 [50].

Indeed, B3GALNT2 was indicated to be the member of the  $\beta$ 1,3-glycosyltransferase ( $\beta$ 3GT) family by having three  $\beta$ 3GT motifs and its function was shown by *in vitro* analyses to be a synthesis of

GalNAc $\beta$ 1–3GlcNAc $\beta$ 1-R structure on both N-glycans and O-glycans of proteins [51]. However, the biological and biochemical functions of B3GALNT2 have not been clarified in mammalian cells, including human cancer cells, primarily because the GalNAc  $\beta$ 1–3GlcNAc $\beta$ 1-R structure has been reported only in  $\alpha$ -dystroglycan in mammalian cells [52]. Recently, mutations in the B3GALNT2 gene were identified in individuals with dystroglycanopathy by whole-exome and Sanger sequencing technologies, suggesting that  $\alpha$ -dystroglycan is the potential substrate of B3GALNT2 [53].

We examined herein the contribution of P53, Ki67, MUC1 -and B3GALNT2 mRNA to pinpoint the role of examined molecules as biomarkers and also in the biology of the CRC. Since we tested little on those molecules in a healthy population, or in GIT diseased states other than CRC, [i.e., risk factors diseases leading to CRC (e.g., inflammatory bowel disease etc.), it is worth assessing the impacts of P53-and Ki67 on those diseases for further potential clinical value. The observed impact of P53, Ki67, MUC1-and B3GALNT2 mRNA's potentially dynamic effects on cycle/proliferation/spread of large intestinal epithelial cells at the organelle and protein levels might occur [54,55], and could open new doors to understanding the clinicopathological behavior of P53, Ki67, MUC1-and B3GALNT2 molecules as well as the molecular mechanisms that might translate to the body of CRC patients (see schematic Fig. 5). Since mRNA and protein testing of P53, Ki67, MUC1 and B3GALNT2 is not so relevant as expected, it would be worth assessing more samples for more molecules/markers from metastatic colorectal tissues at the levels of high throughput protein assays. Although IHC and WB is useful assay for assessing on protein level, which gives relevant accumulation of P53, Ki67, MUC1 -and B3GALNT2 proteins following mutations. Nevertheless, IHC (which was not done on so many samples herein) and performing additional P53, Ki67, MUC1 -and B3GALNT2 structural and mutational analyses are warranted.

## Conclusions

We reported herein retrospectively on a cohort of 3829 CRC patients in one of the highly populated cities in northeastern Iran underwent surgery along with some clinical and demographic risk factors, and B3GALNT2, MUC1, P53 and Ki67 P53 and Ki-67 IHC or WB and transcription in 315 of those CRC patients as well. The worrying rise of metastatic CRC incidence in the study area (Iran and Mideast-wide), especially in youngsters, males, urbans, employed, familial unrelated and transvers colon alarmingly outweighs the contribution of environment/epigenetic to CRC risk. Ki67, B3GALNT2 and MUC1, but not P53, could be suitable biomarkers for CRC detection, due mainly to correlation of B3GALNT2<sup>high</sup>, MUC1<sup>high</sup> and Ki67<sup>high</sup>, mRNA/protein, with CRC metastasis/spread. Nonetheless, the role of possible diagnostic biomarkers, P53 and especially B3GALNT2, MUC1 and Ki67, in the etiology, prognosis and early detection of CRC is promising.

## Abbreviations

*B3GALNT2*:  $\beta$ -1,3-N-Acetylgalactosaminyltransferase 2, CI: Confidence intervals, CL: chemiluminescence, CRC: colorectal cancer, EGFR, Epidermal growth factor receptor, *GalNAc*: N-Acetylgalactosamine, *GALNT6*: Polypeptide N Acetylgalactosaminyltransferase 6, GIT: Gastrointestinal tract, *GlcNAc*: GT O-

linked *N-Acetylglucosaminetransferase*, HIF1- $\alpha$ : Hypoxia-inducible factor 1- $\alpha$ , ICAM-1: Intercellular adhesion molecule-1, IHC: Immunohistochemistry, Ki-67: Marker of proliferation (MKI) 67 protein, *MUC1*: Mucin 1, NGO: non-governmental organization, OR: Odd ratios, P53: Tumor protein p53, qPCR: quantitative polymerase chain reaction, ROC: Receiver operating characteristic, Scr: Steroid receptor coactivator, STAT: signal transducer and activator of transcription, *TNM*: tumor-nodes-metastases, WB: Western blot.

## Declarations

### Ethics approval and consent to participate

This study was approved by the Ethical Committee of Mashhad University of medical science with ID IR.FUBS.REC.1395.42456 with the commitment of the principles of the declaration of Helsinki. All subjects signed their informed consents before participation.

### Consent for publication

Not applicable.

### Availability of data and materials

All data generated or analyzed during this study are included in this published article.

### Competing interests

The authors declare that they have no competing interest.

### Funding

Not applicable.

### Authors' contributions

AGR and JM collected the data, performed the treatments, designed, wrote and finalized the study. JM was also responsible for paper and for its supervision. AA, SH, AT and HD reviewed statistical analyses, supported the data collection and critically revised the manuscript. All authors read and approved the final manuscript.

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## Tables

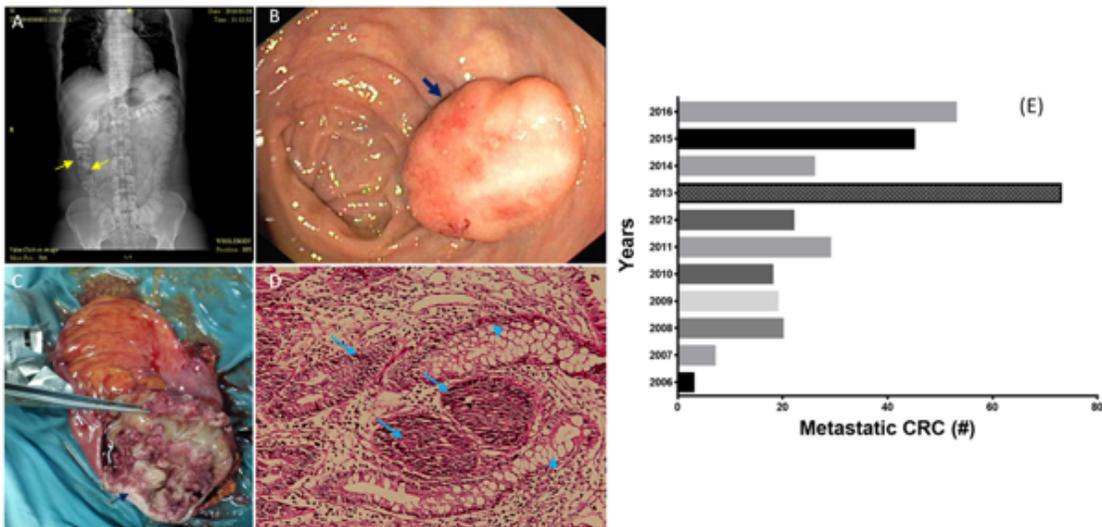
**Table 1** Odds ratios (OR) and corresponding 95% confidence intervals (CI) analyses in relation to overall demographic and risk variables, sample characteristics, various levels of participants and clinical characteristics of CRC patients/participants at diagnosis stage/site (n=3829).

Variable	Parameters, n (%)	OR (95% CI)	<i>p</i>
Age group	≥ 50 years, 2839 (74.14)	2.8 (2.6,3.1)	< 0.0001
	< 50 years, 990 (25.85)		
Education	Illiterate, 1015 (35)	0.36 (0.33,0.39)	
	Diploma, 1593 (37.5)	0.71 (0.65,0.77)	< 0.0001
	Academic, 1221 (27.5)	0.46 (0.43,0.5)	
Gender	♂, 2733 (57.5)	2.4, (2.2,2.7)	0.0001
	♀,1096 (42.5)		
Job	Unemployed, 930 (27.5)	0.3 (0.2,0.3)	< 0.0001
	Employed, 2899 (72.5)		
Colorectal tumor location	Ascending, 559 (15.64)	0.17 (0.15,0.18)	
	Descending, 364 (9.5)	0.1 (0.09,0.11)	
	Transverse, 2068 (54.5)	1.17 (1,1.2)	0.001
	Rectum, 1255 (33.65)	0.48 (0.44,0.52)	
	Sigmoid, 271 (7.26)	0.07 (0.06,0.07)	
Metastasis	Ki67 <sup>high</sup> , 239 (75.8 )	3.1 (2.3,4.2)	0.0015
	Ki67 <sup>low</sup> , 76 (24.1 )	1.1 (0.8,1.5)	
	P53 <sup>+</sup> , 170 (53.9 )	2.2 (1.6,2.9)	0.0061
	P53 <sup>-</sup> , 145 (46.0)		
	MUC1 <sup>high</sup> , 218(69.2)		
	MUC1 <sup>low</sup> , 97 (30.7)		
Tumor familiar	Yes, 102 (2)	36.5 (29.8,44.7)	
	No, 3727 (98)		< 0.0001
Place of residence	Urban, 2963 (77.3)	3.4 (3.1,3.7)	0.02
	Rural, 866 (22.6)		
Clinical features before CRC diagnosis	Constipation,1100 (28.7)	0.41 (0.37,0.41)	
	Colonoscopy, 809 (21.1)	0.26 (0.24,0.29)	
	Diarrhea, 985 (25.7)	0.34 (0.31,0.37)	
	Abdominal pain, 2097 (54.7)	1.2 (1.12,1.3)	< 0.0001
	Rectal bleeding, 799 (20.8)	0.26 (0.24,0.28)	
	Weight loss, 102 (2.6)	0.02 (0.02,0.03)	
	Rectal abnormality, 1201 (31.3)	0.45 (0.42,0.49)	
	Anemia, 637 (16.6)	0.19 (0.18,0.21)	
	Nausea & vomiting, 310 (8)	0.08 (0.08,0.09)	
	Weakness, 254 (6.6)	0.07 (0.06,0.08)	
Others	Smoking, 1111 (29)	0.4 (0.37,0.44)	< 0.0001
	Alcohol, 450 (11.7)	0.16 (0.13,0.14)	
	Addict, 213 (5.5)	0.05 (0.05,0.06)	

**Table 2** Chi- $\chi^2$  analyses in relation to correlation of MUC1, Ki67 and P53 expression with clinicopathologic features in metastatic colorectal cancer (CRC, n=315).  $\chi^2$  is the relation between oncoprotein's expression and clinicopathological features (when  $\chi^2 \leq 0.5$  is significant).

Clinicopathologic features (n)	MUC1 <sup>high</sup> n (%)	MUC1 <sup>low</sup> n (%)	$\chi^2$ P	Ki67 <sup>high</sup> (%)	Ki67 <sup>low</sup> (%)	$\chi^2$ P	P53 <sup>+</sup> (%)	P53 <sup>-</sup> (%)	$\chi^2$ P
<b>Age, y</b>			0.85			0.719			0.81
≤50 (195)	141 (72.3)	54 (27.7)		153 (78.46)	43 (21.54)		104 (53.33)	91 (46.67)	
≥50 (120)	77 (64.16)	43 (35.84)		86 (71.66)	33 (28.34)		66 (55)	54 (45)	
<b>Gender</b>			0.99			0.852			0.0004
♂ (219)	179 (81.73)	40 (18.27)		184 (84) 55 (57.29)	35(16) 41(42.71)		135 (61.64)	84 (38.35)	
♀ (96)	39 (40.62)	57 (59.38)					38 (39.58)	58 (60.41)	
<b>Location</b>			0.108			0.137			0.04
Colon (284)	198 (69.71)	86 (30.29)		217 (76.4)	67 (23.6) 8 (28.58)		160 (56.33)	124 (43.67)	
Rectum (28)	18 (64.28)	10(35.72)		20 (71.42)	1 (33.34)		10 (35.71)	18 (64.28)	
Sigmoid (3)	2 (66.66)	1 (33.34)		2 (66.66)			-	3 (100)	
<b>Total Metastasis</b> (315)	218 (69.2)	97 (30.8)	-	239 (75.87)	76 (24.13)	-	76 (24.13)	145 (46.03)	-

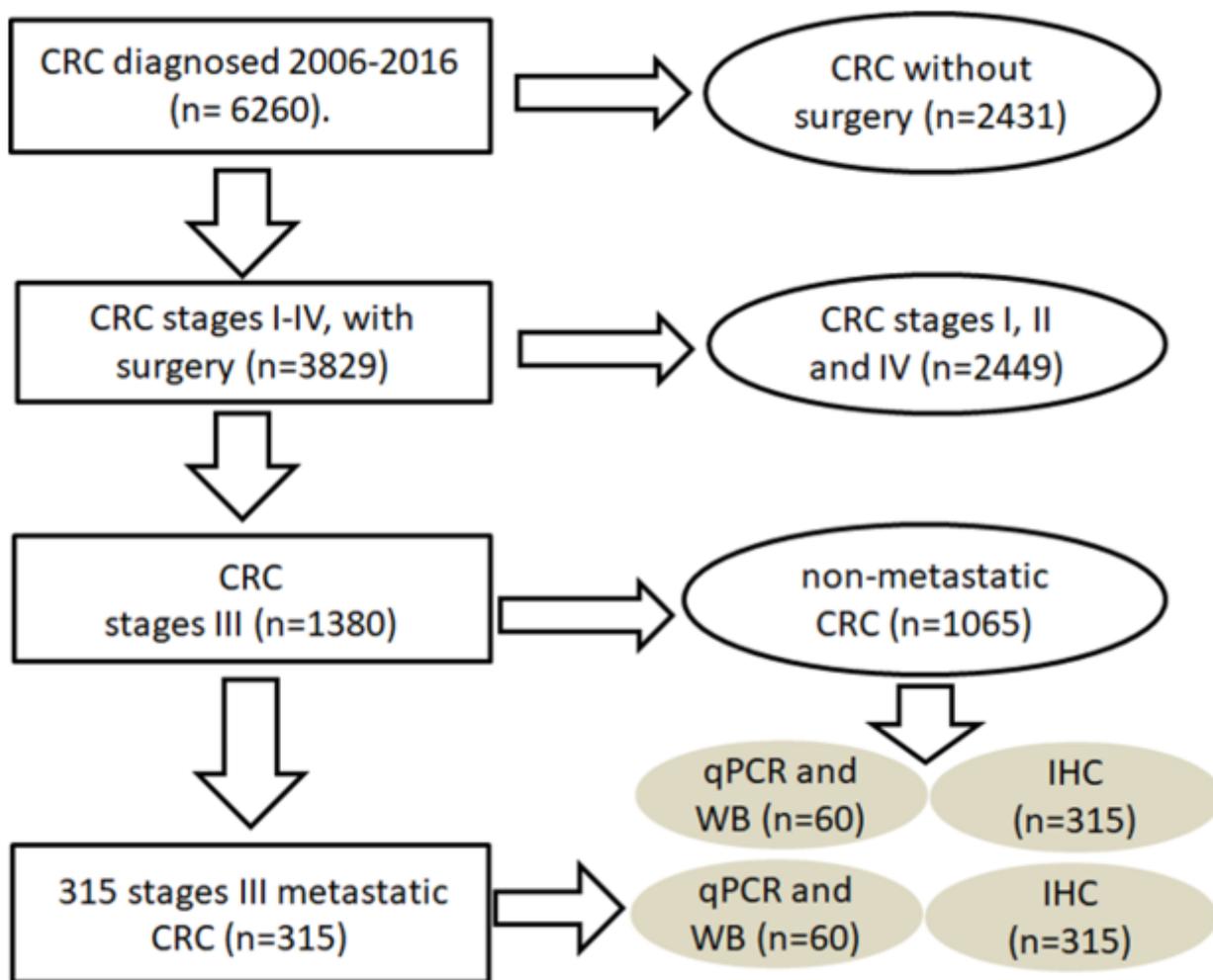
## Figures



**Figure 1**

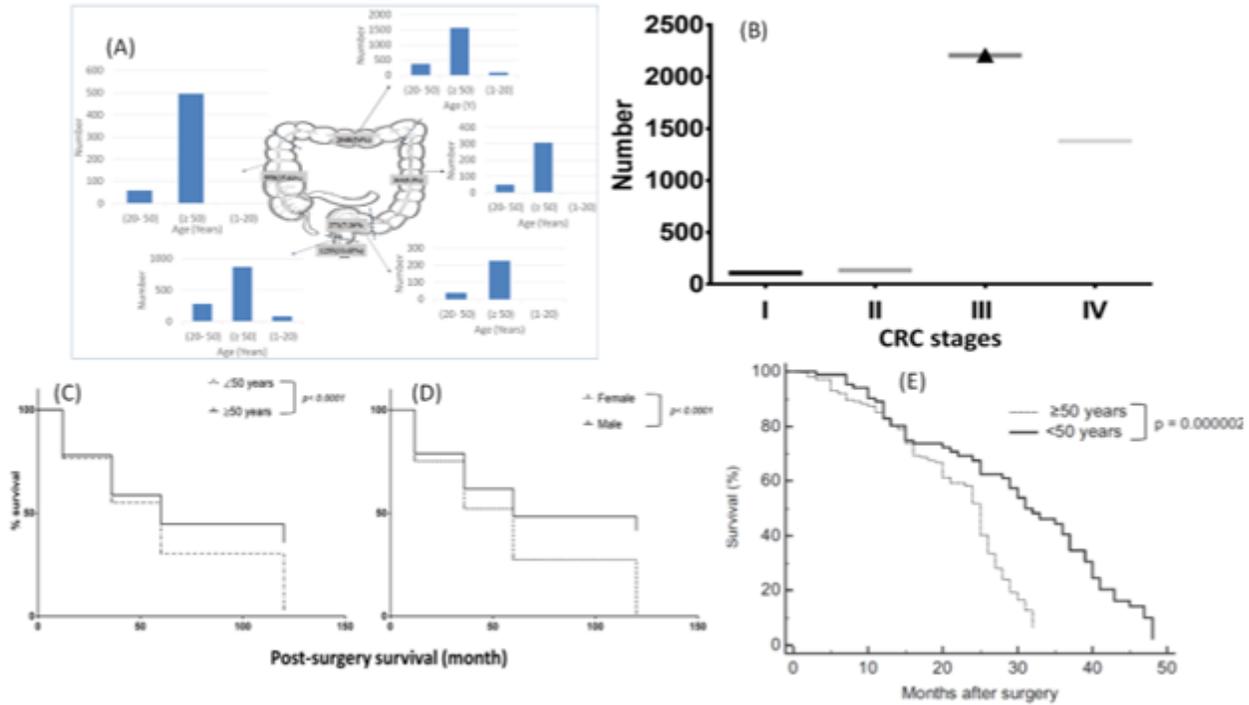
Upper panel, the study area (Northeastern Iran with comparative countrywide information related to colorectal cancer (CRC) rate per 100000 in male (♂) and female (♀), referred to hospitals in 2014. [other than the study area are referred to (26)]. The [https://en.wikipedia.org/wiki/Module:Location\\_map/data/Iran/doc-linked\\_map](https://en.wikipedia.org/wiki/Module:Location_map/data/Iran/doc-linked_map) was created/modified using Arc.GIS software (version 10.2.0.3348) and 2013© Microsoft Windows, Paint (version 6.3). Lower

panel, subjects in the staging category of CRC. A) CT scan, B) colonoscopy of cancerous part of descending colon, arrows C) removed cancerous parts of descending colon for further molecular analyses and D) pathologic characteristics, i.e., adenocarcinoma, a predominant form of CRC (H&E,  $\times 100$ ) along with huge sub-mucosal hyperplasia/proliferation of enterocytes (arrows) and goblet cells (arrows heads) and lymphocytes proliferation. Differences between the number of metastatic CRC patients during the cross-sectional retrospective study period in the region with maximal metastasis in 2013 (E) importantly highlight the worrying rise of metastatic CRC incidence with the trend is increasing between 2006-2016 in the region (Iran and potentially broader Mideast-wide). The figure also gives further information to (non)clinicians-molecular biologists about the clinical importance, precise detection/sampling location and how best to relate clinical aspect of CRC to cellular/molecular assays; it would also help governments/NGO/medical scientists to focus more in depth on the debatable CRC's multifactorial preventive protocols to lower cancer risk.



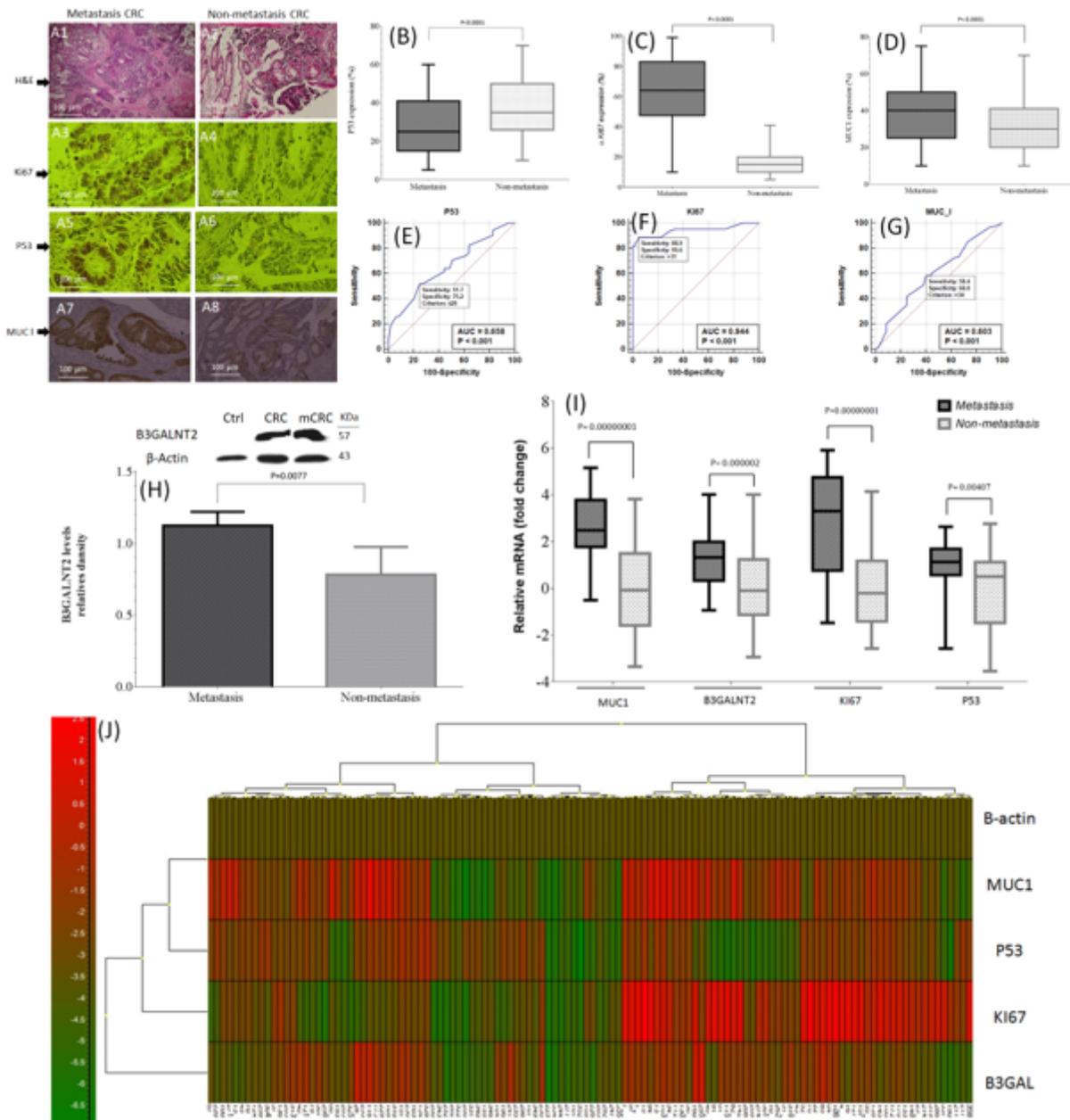
**Figure 2**

Flow chart depicting the selection of colorectal cancer (CRC) patients.



**Figure 3**

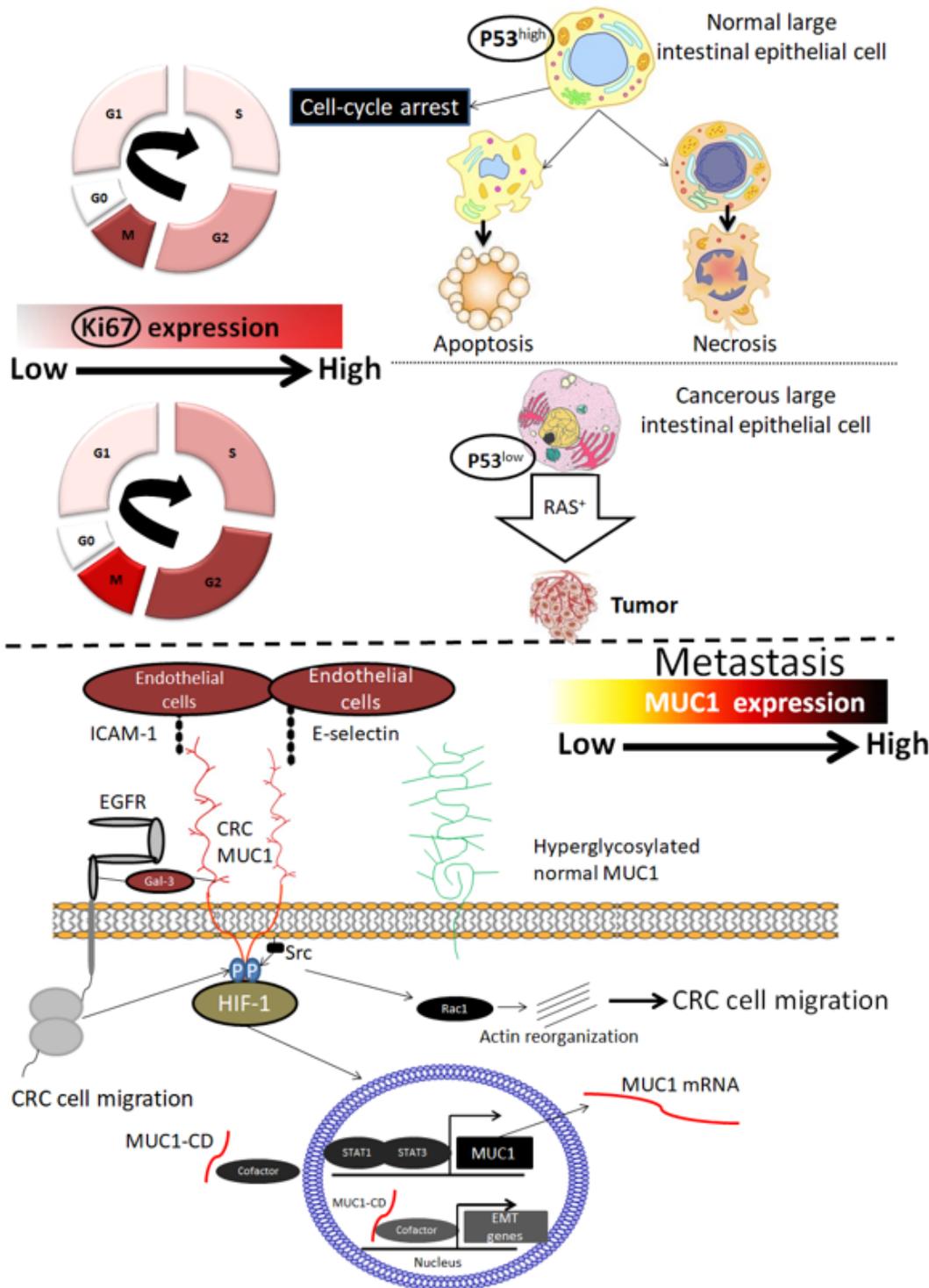
Distribution of various subsites of colorectum with relation to the age affected by CRC observed in Mashhad, Iran, 2006-2016 (A). Number of colorectal cancer (CRC) patients with different stages of CRC (B). Post-surgery survival period (months) of CRC patients males (n=2733, 57.5%) and females (n=1096, 42.5%) (C), and younger (n=990, 28.85%) and older (n=2839, 74.14%) than 50 (D). Results of Kaplan-Meier test to estimate the survival rate of the metastatic CRC patients (n=315, of which n=86 <50 and n=229  $\geq 50$ ) and p-value was calculated through a log rank test (E). The A, B, C and D (but not E) are from the same cohort and contain similar numbers of subjects. [For details please also see the methods and Fig. 2].



**Figure 4**

Representative light microscopic with H&E staining images of colorectal cancer (CRC) stage III with metastasis (A1) and non-metastasis (A2). Immunohistochemical reactions of the CRC stage III tissues, with high (A3) and low (A4) Ki67, high (A5) and low (A6) P53, high (A7) and low (A8) MUC1 expression/labelling index with/without metastasis. Mean values of protein expression of P53 (B), Ki67 (C) and MUC1 (D). Comparative ROC analysis showing the percent of non-metastatic and metastatic CRC patients expressing P53 (E), Ki67 (F) and MUC1 (G) along with WB analyses of B3GALNT2 with CL-based image [insert] (H). Comparative qPCR results of MUC1, B3GALNT2, Ki67 and P53 mRNA expression in metastatic and non-metastatic CRC tissues (I, n=60, with various p-values done with t-test). Comparison of full heat-map between Ki67 and P53 genes in CRC data showing the correlation of the mRNA

expressions of MUC1, B3GALNT2, Ki67 and P53 with metastasis subtypes (J). A1-A8 along with B-G (n=315) and H-J (n=60) are originated from the same cohort. [For details see the methods and Fig. 2].



**Figure 5**

Schematic diagram depicting how noticeably  $P53^{low}$ ,  $Ki67^{high}$ ,  $B3GALNT2^{high}$ ,  $MUC1^{high}$ , mRNA/protein levels are involved in cell cycle/proliferation/spread. As such, the mRNA/protein levels of Ki67, B3GALNT2 and MUC1, but not P53, are strongly correlated with CRC metastasis, observed herein;

though Ki-67 is present in all, but not resting or the G0 phase, of cell cycle. The intensity of Ki-67 expression in each phase of cell cycle, nonetheless, remains elusive. The intensity of mRNA expression of Ki-67 and P53 increases/changes, nevertheless the protein levels of those molecules could be evaluated for future prospective, and Ki-67 protein accumulation along with its mRNA worth further analyses. Compared to noncancerous/normal colorectal cells, MUC1 undergoes post-modification (glycosylation) changes in CRC cells and exacerbates metastasis condition. Cell-surface disruption, including binding of MUC1 to E-selectin and ICAM-1, induces cancer cells to enter the blood stream. MUC1 cytoplasmic fraction is phosphorylated by EGFR and Src. Src phosphorylation can increase Rac1 activity, skeletal alteration and cellular motility. EGFR phosphorylation by cell motility, and interaction with HIF1- $\alpha$ . PDGF-A transcription has a positive effect on  $\beta$ -catenin transcriptional activity. The cytoplasmic domain of MUC1 interacts with transcription factors, such as  $\beta$ -catenin, p120-catenin and the estrogen receptor  $\beta$ , causing nuclear translocation of MUC1. Epithelial stimuli and expression are regulated by mesenchymal transition genes (EMTs), and MUC1 expression is regulated following STAT1/STAT3 binding to MUC1 and increase the production of MUC1 and thus metastasis.