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Immunoscore as a Predictor of Disease Recurrences and Patients' Survival in Colon Cancer: A Clinicopathologic Study

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Abstract

BACKGROUND: For years, the American Joint Committee of Cancer/International Union against Cancer TNM staging system was the only accepted staging system for colorectal cancer. Different studies highlighted limitations in this staging system with the need to another staging approach that takes into consideration the individual patient immune response. Recently, the immunoscore was introduced; however, no accurate data regarding its sensitivity and specificity over the routinely used TNM staging system.

AIM: We aimed to provide definite sensitivity, septicity, and predictive values for both IS and TNM staging system in prognosis prediction, as evidence-based statistical documentation of its validity to clinical use.

METHODS: Fifty-three slides of colon cancer cases were stained for CD3 and CD8 immunohistochemical stains. The density of the stained cells was measured used an image analysis system in the core of the tumor and invasive margin. Immunoscore was calculated and results were compared with TNM in the recurrence-free survival of the patients. The sensitivity and specificity for each test were calculated.

RESULTS: High IS was correlated with a good prognosis in the studied cases. IS sensitivity reached 85.7% compared to 28.6% in TNM staging system and the specificity was 78.1% compared to 37.5% in TNM system.

CONCLUSION: IS is a promising prognostic estimation tool in colon cancer with better sensitivity and specificity than TNM staging system. The routine use of IS is now becoming a mandatory step.

Introduction

Colorectal cancer (CRC) is now considered the second position in cancer-related mortality worldwide and is recognized to be a heterogeneous disease, with diverse prognostic outcome probabilities [1].

Staging of CRC is one of the most useful strategies for proper treatment planning. For decades, the American Joint Committee of Cancer/International Union against Cancer TNM staging system was highly recommended and the mostly accepted worldwide, with a high compliance rate in all medical centers and institutions [2].

However, despite its popularity, recent reviews have highlighted several limitations in this staging system [2], proved by that the prognosis can significantly vary among patients within the same stage. As well, tumor recurrences rate for cancer reaches up to 50% during the follow-up period regardless the stage, despite the optimal management, the facts that point to the potential influence of the individual tumor cells' biology [3].

The concept of this traditional staging system is based fundamentally on the assumption that tumor development and progression are basically cell-autonomous processes which depend on the properties of the tumor cell itself and its degree of anaplasia. The prognosis, therefore, depends on the tumor cells progress inside the body at the time of surgery, the fact that ignore the variable and fascinating role of the host immune response and the active dynamic role of the tumor microenvironment [4].

In the past 10 years, the focus of cancer research has notably changed and the study of tumor microenvironment as a whole and tumor-associated inflammation specifically has taken the spotlight [5]. The comprehensive studies in tumor-associated inflammation throughout these years were extremely beneficial for cancer researches progress in different aspects and drive to the discovery of immunotherapy which proofed great results in different cancers as leukemia and cancer breast [6].

T lymphocytes, basically CD3 and CD8 positive cells, are considered the maestro of the body adaptive immune response, both cells play a fundamental role in the stage of immunosurveillance of the immunoediting [7].

In 2012, Galon *et al*. [8] proposed what called "immunoscore" IS and invited the scientific committees

to further study its validity in different cancers. From that time and then, research teams all over the world started to explore IS and test its possible advantages over the routinely used staging system [9].

According to Galon *et al*. [10], the evaluation of "immunoscore" is calculated by counting CD8 and CD3 T lymphocytes both in the tumor core stroma and in the invasive margin (IM) fronts. The immunoscore is proposed to add significant data and provides important information regarding tumor cross-talk within its microenvironment that may tell a lot about the tumor behavior, the step that is mandatory to overcome the downsides in the routinely applied TNM staging system [10].

Many studies had examined the validity of immunoscore in different cancers as cancer liver and stomach [11], [12].

In cancer colon, immunoscore has been studied in correlation with different pathologic prognostic parameters [13]; however, correlation of IS with tumor recurrences and patients' survival is not frequently studied, and until now, the accuracy of IS in terms of sensitivity and specificity is not clear.

In this study, we are comparing the accuracy of cancer colon versus TNM staging system in predicting tumor recurrences and patient survival and well as in their association with other known prognostic factors as perineural invasion and tumor grade; aiming to provide definite sensitivity, septicity, and predictive values for both IS and TNM staging system in prognosis prediction, as evidence-based statistical documentation of its validity to clinical use.

Material and Methods

Fifty-three cases of colectomy for colon cancer were included in the study, collected retrospectively from cases in El Sheikh Zaid specialized Hospital, and other private centers from cases between 2015 and 2016:

i. Histopathology and data collection
- The hematoxylin and eosin-stained sections from the tumor and dissected pericolic lymph nodes were examined for TNM staging. According to Edge and Compton, cases were staged as 1, 2, 3, and 4 [14]. For better data calculations, stages were grouped as two groups; low stage (Stages 1 and 2) and high stage (Stages 3 and 4)
- Nodal deposits were evaluated according to the TNM routine methods as follows: N0: No nodes affected, N1: Positive tumor deposit in up to 2 lymph nodes, and N2: Positive deposits in more than two lymph nodes [14]
- The presence of perineural invasion was evaluated as 1, the absence of perineural invasion in the examined tumor sections was expressed as 0
- Tumors' grades were evaluated as 1, 2, and 3 according to the routine guidelines.

ii. Prognostic data was represented by the recurrence-free survival RFS in 2 years' time follow-up period

The occurrence of recurrence or mortality within the 2 years' time of the study was expressed as 1, while the absence of recurrence in the 2 years was evaluated as 0.

iii. Immunohistochemistry
- Sections from the tumor paraffin block were cut at 3–5 microns and stained for CD3, CD8 immunohistochemical stains using Ventana semi-automated Autostainer (Ventana ES; Ventana) which apply the following technique
- Deparaffinization in xylene and rehydration in graded alcohol
- Blocking endogenous peroxidase activity using H2O2 in phosphate-buffered saline
- Antigen retrieval by TRIS-EDTA, PH9.9 in a microwave at 800W for 2 min and at 150 W for 15 min
- Incubation at room temperature with diluted primary antibodies for CD8 (C8/144B, 3 μg/ml; Dako, Glostrup, Denmark) and with an antibody against CD3 (2GV6, 0·4 μg/ml; Ventana, Tucson, AZ, USA)
- Detection using ultraView Universal DAB IHC Detection Kit (Ventana, Tucson, AZ, USA)
- Counterstaining using Meyer's hematoxylin.

iv. Image analysis morphometric study

The morphometric analysis was performed at the pathology laboratory, at the Medical Research Centre of Excellence Unit, National Research Centre using the image analysis system Leica QWin DW3000 (LEICA Imaging Systems Ltd., Cambridge, England), which consists of Leica DM-LB microscope with JVC color video camera attached to a computer system.

The slide to be examined was placed on the stage of the microscope. The light source was set to the required level. Successful adjustment of illumination was checked for the video monitor. The morphometric analysis was carried out on tumor tissue core and the invasion front in each slide stained with CD3 and CD8 using an objective lens with a magnification of ×20 [15].

The most representative five ×200 fields were assessed for each tumor section. Any areas of necrosis were as well as processing related artifacts were neglected [16].

v. Immunoscore calculation

Immunoscore was calculated according to Zhu *et al*. [17] considered the median of the values obtained from counting the CD3 and CD8 values as their cutoff

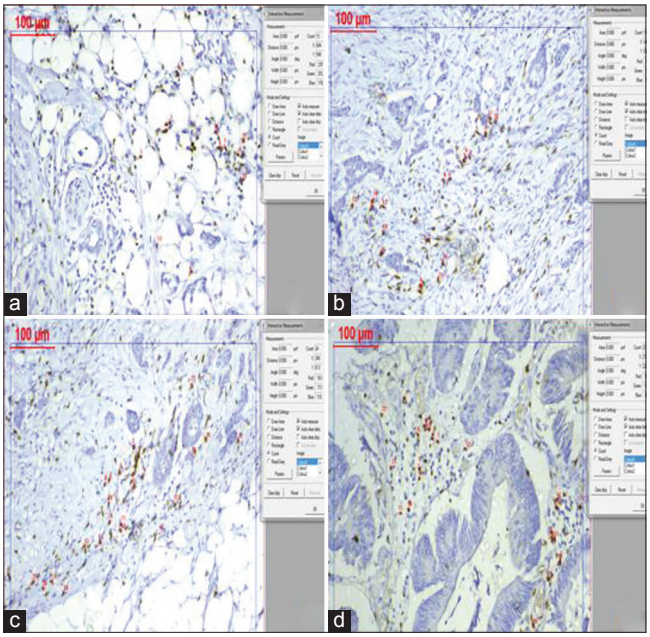

Figure 1: Immunoscore calculation using image analysis system by counting the stained nuclei on the monitor in high power fields in the tumor core and invading front from a case of low immunoscore; (a) invading front of CD3 stained tumor section, (b) tumor center of CD3 stained tumor section, (c) invading front in CD8 stained tumor section, and (d) tumor center of CD8 stained tumor section

value in segregation the low (0) and high (1) individual case immunoscore, and similarly, scores from 0 to 4 (by submission of the score of each marker both in tumors' cores and their invasion margins) were used (Figures 1 and 2).

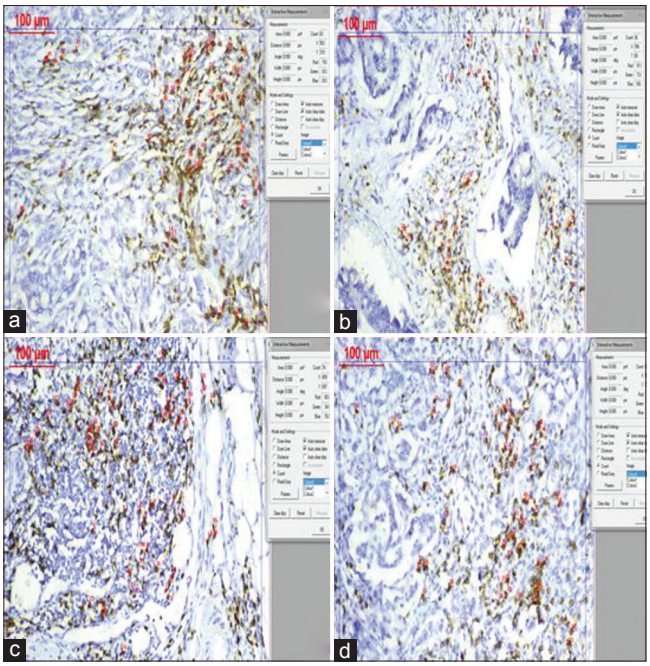

Figure 2: Immunoscore calculation using an image analysis system by counting the stained nuclei on the monitor in high power fields in the tumor core and invading front. A case of high immunoscore, (a) tumor invading front of CD3 stained tumor section, (b) center of CD3 stained tumor section, (c) tumor invading front of CD8 stained tumor section, and (d) center in CD8 stained tumor section

For better data calculations, IS was grouped as two groups; low IS (0, 1, and 2) and high IS (3 and 4).

vi. Statistical methods.

- Microsoft excel 2013 was used for data entry and the Statistical Package for the Social Science version 24 was used for data analysis
- Simple descriptive values (arithmetic mean and standard deviation) were used to summarize the quantitative data and frequencies used
- Bivariate relationship was displayed in cross-tabulations and comparison of proportions was performed using the Chi-square and Fisher's exact tests where appropriate
- Accuracy was represented using terms of sensitivity and specificity. Receiver operator characteristic (ROC) analysis was used to determine the optimal cutoff values
- $p < 0.05$ was considered statistically "significant."

Results

i. Data description: Table 1.

- The study included 53 cases of colon cancer
- The age of the cases ranged from 29 years to 78 years, with a mean of 56 years and median 55 years
- The study included 31 male (58.5%) and 22 females (41.5%).
- Regarding the TNM stage, the cases were distributed as follows:
  - Stage 1: 7 cases (13.2071%)
  - Stage 2: 19 cases (35.8490)
  - Stage 3: 22 cases (41.5094%)
  - Stage 4: 5 cases (9.4339%).
- The stage groups results were as follows:
  - Low stages 26 cases (49.1%)
  - High stages 27 cases (50.9%).
- Nodal examination revealed
  - N0: 26 cases (49.1%)
  - N1: 14 cases (26.4%)
  - N2: 13 cases (24.5%).
- Perineural invasion: Was detected in 23 cases (43.4%) and was absent in 30 cases (56.6%)
- Tumor grades were as follows
  - Grade I: 0
  - Grade II: 40 cases (75.5%)
  - Grade III: 13 cases (24.5%).
- RFS data were as follows
  - Positive for recurrence or mortality from the disease in 32 cases (60.4%)
  - Negative for recurrence in 2 years survival in 21 cases (39.6%).

Table 1: Detailed results of IS within all of the studied groups

Cases' data	Immunoscore									
	Score 0		Score 1		Score 2		Score 3		Score 4	
	No.	%	No.	%	No.	%	No.	%	No.	%
Sex										
Male	3	60.0	5	62.5	10	83.3	7	38.9	6	60.0
Female	2	40.0	3	37.5	2	16.7	11	61.1	4	40.0
Stage group										
Low stage	1	20.0	2	25.0	6	50.0	7	38.9	10	100.0
High stage	4	80.0	6	75.0	6	50.0	11	61.1	0	0.0
Stage										
Stage 1	0	0.0	0	0.0	2	16.7	3	16.7	2	20.0
Stage 2	1	20.0	2	25.0	4	33.3	4	22.2	8	80.0
Stage 3	2	40.0	5	62.5	5	41.7	10	55.6	0	0.0
Stage 4	2	40.0	1	12.5	1	8.3	1	5.6	0	0.0
RFS										
No	5	100.0	6	75.0	7	58.3	3	16.7	0	0.0
Yes	0	0.0	2	25.0	5	41.7	15	83.3	10	100.0
PNI										
No	4	80.0	5	62.5	6	50.0	10	55.6	5	50.0
Yes	1	20.0	3	37.5	6	50.0	8	44.4	5	50.0
Nodal										
N0	1	20.0	2	25.0	6	50.0	7	38.9	10	100.0
N1	1	20.0	3	37.5	2	16.7	8	44.4	0	0.0
N2	3	60.0	3	37.5	4	33.3	3	16.7	0	0.0
Grade										
Grade 2	2	40.0	3	37.5	10	83.3	15	83.3	10	100.0
Grade 3	3	60.0	5	62.5	2	16.7	3	16.7	0	0.0

PNI: Perineural invasion, RFS: Recurrence-free survival.

- The results of the immunoscore were as follows
  - IS 0: In 5 cases (9.4%)
  - IS 1: In 8 cases (15.1%)
  - IS 2: In 12 cases (22.6%)
  - IS 3: In 18 cases (34%)
  - IS 4: In 10 cases (18.9%).
- The IS groups were: Low IS was seen in 25 of all studied cases (47.2%) while high IS was present in 28 cases (52.8%).

ii. The results of IS study according to the other variables, Table 2.

Table 2: Association of IS of the included cases in relation to all variables (sex, stage category, stages, RFS, PNI, nodal status, and tumor grade)

Cases' data	Immuno Score category				p-value
	Low score (0, 1 and 2)		High score (3 and 4)		
	Count	%	Count	%	
Sex					
Male	18	58.1	13	41.9	0.059
Female	7	31.8	15	68.2	
Stage category					
Low stage (1 and 2)	9	34.6	17	65.4	0.072
High stage (3 and 4)	16	59.3	11	40.7	
Stage					
Stage 1	2	28.6	5	71.4	0.219
Stage 2	7	36.8	12	63.2	
Stage 3	12	54.5	10	45.5	
Stage 4	4	80.0	1	20.0	
RFS					
No	18	85.7	3	14.3	<0.001*
Yes	7	21.9	25	78.1	
PNI					
No	15	50.0	15	50.0	0.637
Yes	10	43.5	13	56.5	
Nodal					
N0	9	34.6	17	65.4	0.041*
N1	6	42.9	8	57.1	
N2	10	76.9	3	23.1	
Grade					
Grade 2	15	37.5	25	62.5	0.013*
Grade 3	10	76.9	3	23.1	

*Significant *P* value, PNI: Perineural invasion, RFS: Recurrence-free survival.

According to the statistical studies of this data, most of the cases of Stage I showed high IS (71.4%). In Stage II, high IS was seen in 63.2%. The percentage of high IS decreased to be 45.5% in cases of Stage III and 20% in cases of Stage IV. However, statistical analysis showed an insignificant correlation between IS and TNM stages (p = 0.072).

- Among the cases showing positive perineural invasion, 56.5% of them showed high IS and 44.5% showed low IS (statistical insignificance p = 0.637)
- IS showed significant statistical association with nodal status, high IS was seen in 65.4% of cases with no nodal metastasis (N0), in contrast to 57.1% and 23.1% of cases of N1 and N2, respectively (p = 0.041)
- Regarding tumor grade, high IS was detected in most of the cases of Grade II (62.5%), in contrast to 23.1% of cases with Grade III (positive statistical significance p = 0.013)
- Correlation of the results of IS with the clinical data represented by RFS in 2 years' time of the study showed low IS in 85.7% of cases with no recurrence, with high IS in 78.1% of cases with positive recurrences (strong statistical association p < 0.001).

In terms of accuracy, ROC curve analysis revealed that immune score was a significant discriminator for RFS (p < 0.05) where immune score showed an area under the curve = 0.879 with (95% confidence interval [CI] 0.788–0.971) (Figure 3).

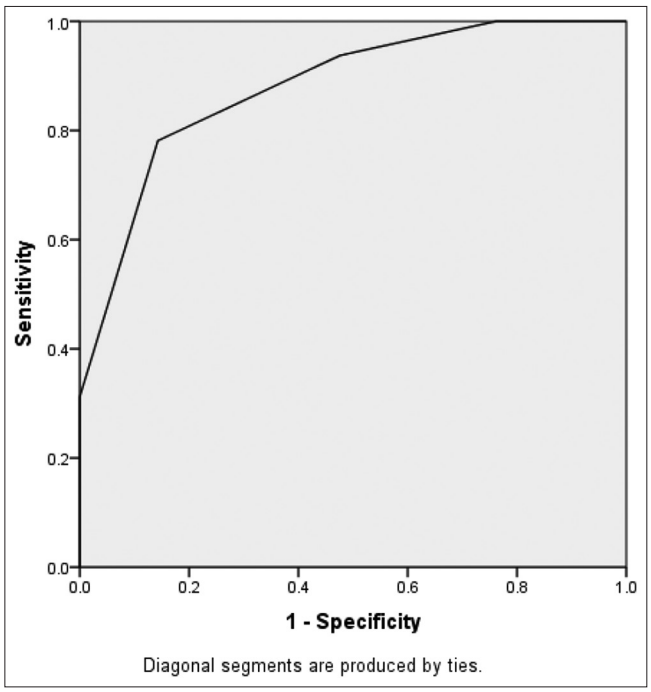

Figure 3: Receiver operator characteristic curve analysis revealed that immune score was a significant discriminator for RFS (p < 0.05) where immune score showed an area under the curve = 0.879 with (95% confidence interval 0.788–0.971). The best cutoff point of RFS was 2.50 with 78.1% sensitivity and 85.7% specificity

IS can predict RFS with sensitivity = 85.7%, specificity = 78.1%, PPV = 72.0%, and NPV = 89.3%, in comparison with the TNM staging system which could

predict the patient prognosis (represented by RFS) with sensitivity = 28.6%, specificity = 37.5%, PPV = 23.1%, and NPV = 44.4%.

Discussion

Tumor microenvironment has become an important aspect in oncology research, focusing on the tumor cells interaction with its surroundings and considering the dynamic nature of the tumor cells-host immune system relationship [5].

The studies in tumor microenvironment lead eventually to the emergence of the what is called "Immunoscore" which was proposed to delineate the nature of the host immune reaction and represent an auspicious added value to the commonly used staging systems [13].

According to Galon *et al*., the immunoscore ("I") utilizes the counting of CD8 and CD3 T lymphocytes in the CT and the IM of the tumors to calculate a score value that ranges from (0), when low densities of both cell types are found in two examined regions, to (4), when high densities of both markers are found in both locations [8]. However, the exact determination of the thresholds (cutoff value) for low and high values differed in the subsequent studies [18].

The values' median and "best P value" were the most frequently used cutoff strategies, with variable overall potential advantages and disadvantages [9].

In this study, we calculated the IS of 53 cases of cancer colon by counting the CD3 and CD8 positive T lymphocytes in the tumor IM and its core using the image analysis system. The median value of the measures was used as the cutoff for determining the low and high scores for each marker before the final immunoscore calculation for each case. Immunoscore was compared with TNM staging in relation to tumor grade, perineural invasion, nodal status, and the most important RFS. Accuracy of the test was evaluated in terms of sensitivity, specificity, and predictive values for each test of them in (both immunoscore and TNM staging system) in respect to patient prognosis, represented by RFS in 2 years of follow-up.

According to our results, higher IS values were observed in low TNM stages and vice versa. However, despite the higher values of IS in lower TNM stages yet, the association showed no statistical significance.

Regarding the other prognostic factors, IS was strongly related to the nodal status as well as to the tumor grades ($p = 0.041$ and 0.013, respectively). However, no statistical association was seen between IS and the perineural invasion.

The association between the IS and the tumor stages was observed as well in some previous studies; Sinicrope *et al*. noticed higher IS in the low TNM stage category, with 91% of patients of low stages showed high IS, versus 68% of the patients of high stages. In their study, the values showed a significant statistical association [19].

In the present study, a strong association between IS of the cases and their RFS was observed ($p < 0.001$), as 85.7% of cases with no recurrences or disease-related mortality were of high IS category in comparison to 14.3% of low IS group.

In similar regards, Anitei *et al*. studied IS in a patient with and without pre-operative chemotherapy CT. They noticed the IS was associated with disease-free survival and overall survival data. They suggested IS as an important risk factor and recommended its evaluation in an international multicenter study, although they advised against the measurement of IS after CT, as the later may cause significant changes in the immune cells' distribution within the tumor [2].

Galon *et al*. noticed that only 13% of cases that showed recurrences in their study was of high IS category. They related the results of IS with mismatch repair genes MSI and used both tests to define patients eligible for immunotherapy (immune checkpoint inhibitors, ICI), based on that patients with high T-cell infiltration, hence high IS, will eventually show a higher expression of PD-1 and PDL1, and therefore, they will be more likely to respond to ICI. They recommend more studies of IS accuracy and predictive values [20].

Galon *et al*. tested IS in patients of Stage II colon cancer with high-risk factors; in their results, they demonstrated that up to 69% of these patients showed a low rate of recurrences and that the recurrence was associated with higher IS values. They, therefore, concluded that IS may be a valuable test in neoadjuvant chemotherapy decision in Stage II patients [21].

The results of the international immunoscore project were concordant to our results regarding the association between IS values and patients' recurrences and survival. For example, in patients of Stage II tumors in their study (n = 1434), high IS was significantly associated with a low risk of recurrence and high overall survival rates ($p < 0{\cdot}05$). Recurrence within 3 years' time was seen in 23 (6%) patients with high IS, 73 (11%) patients with intermediate IS and 77 (20%) patients with low IS [22].

Sun *et al*. provided a more simplified interpretation of the results of Pages *et al*. study; they correlated IS values with the restricted mean survival time (RMST). They got RMST of 6.85 years (95% CI 6.54–7.17) in the high-immunoscore group and 5.25 years (4.79–5.71) in the low-immunoscore group. They, therefore, concluded that IS correlation with RMST provides clinically valid measurement of recurrence

time for each immunoscore category and highlight the role of IS as a prognostic test [11].

Similarly, Kirilovsky *et al*. discussed the possible rationale basis of IS. From their study, they recommended IS as a predictor for CT and radiotherapy response as a predictor for immunotherapy response and a pivotal factor in recurrence and survival prediction [23].

Using the provided data in our results, ROC curve was used to define novel intrinsic values for IS in recurrence risk estimation and clinical prognosis prediction. According to our results, IS could effectively predict RFS of the 2 years follow-up of this study; the sensitivity was 85.7% compared with 28.6% in TNM staging system. The specificity was 78.1% versus 37.5% specificity of the TNM staging system. The positive predictive value PPV of IS was up to 72% in contrast to TNM PPV which was 23.1% and finally the negative predictive value of IS was 89.3% compared with 44.4% for TNM. According to these values, IS is superior as a screening and confirmatory test over TNM staging system.

Although many studies had evaluated the accuracy of IS and TNM in the prediction of the clinical prognosis; yet, none of them – to the best of our knowledge – provided quantitative data about this point in terms of sensitivity, specificity, PPV and NPV.

The high sensitivity of IS in this study recommended it as a strong screening test for prognosis in colon cancer so that low IS should, therefore, consider as a poor prognostic factor. On the other hand, the high specificity of IS recommended it as a good confirmatory test that can confirm the data suggested by other risk factors profile.

Many studies recently recommended the approval of IS testing for all cases of colon cancer as prognostic profiling of the cases. In 2016, Kirilovsky *et al*. suggested what they call “TNMI” to integrate the use of IS as part of tumor staging [23].

New studies pointed to the importance of IS not only as a prognosis predictor but moreover as a predictor of the possible role of immunotherapy in colon cancer [24]. Moreover, similarly, Ogino and Giannakis pointed to the significance of tumor immune response study. They suggest that what they called “Tumor Immunity in the Micro Environment (TIME) classification” may become a new creditable trend incorporating the host immune response in cancer classification system. However, they found that the IS has not taken its proposed role in clinical use until now [25].

We agree with Donnem *et al*. that the validation of the IS measure methodology and pre-definition of the cutoff value used in its calculation is the most important and still missing points in the routine clinical application of IS [9].

Despite the effort provided by the international immunoscore team to validate IS as an important test in colon cancer patients, yet, still, the unavailability of a defined independent cutoff value to be used internationally in all centers and be internationally accepted, still a handicapping point against the routine use of IS in daily work. Collaborative work is needed to initiate robust predefined organ-specific cutoff values similar to the values used in Ki67 evaluation, for example.

Conclusion

Our study demonstrated the promising role of IS in prognostication of colon cancer, presented its association with different risk factors and quantitatively measured its accuracy as a predictor of survival in colon cancer.

Accordingly, we, therefore, recommend to integrate the computed technologies of image analysis and digital pathology in the individual patient’s care and transfer the IS from the research zone to the clinical daily use, so that IS may be a proposed part of the pathology report, to enrich the clinical management with the immune system profile data that will dramatically influence the management in colon cancer and all other tumors.

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