Towards the introduction of the 'Immunoscore' in the classification of malignant tumours

Jérôme Galon,1,2,3* Bernhard Mlecnik,1,2 Gabriela Bindea,1,2 Helen K Angell,1,2 Anne Berger,1 Christine Lagorce,1 Alessandro Lugli,6 Inti Zlobec,6 Arndt Hartmann,7 Carlo Bifulco,8 Iris D Nagtegaal,9 Richard Palmqvist,10 Giuseppe V Masucci,11 Gerardo Botti,12 Fabiana Tatangelo,12 Paolo Delrio,12 Michele Maio,12 Luigi Laghi,13 Fabio Grizzi,15 Martin Asslaber,16 Corrado D’Arrigo,17 Fernando Vidal-Vanaclocha,18 Eva Zavadova,19 Lotfi Chouchane,20 Pamela S Ohashi,21 Sara Hafezi-Bakhtian,22 Bradly G Wouters,22 Michael Roehrl,22 Linh Nguyen,24 Yutaka Kawakami,25 Sghi Chi Hazama,26 Kyotaka Okuno,27 Shuji Ogino,28 Peter Gibbs,29 Paul Waring,30 Noriyuki Sato,31 Toshihiko Torigoe,31 Kyogo Itoh,32 Prabhu S Patil,32 Shilin N Shukla,33 Yili Wang,34 Scott Kopetz,35 Frank A Sinicrope,36 Vioirel Scripcariu,37 Paolo A Ascierto,38 Francesco M Marincola,39 Bernard A Fox,40,41 and Franck Pagès1,2,3,42

1 INSERM, U872, Laboratory of Integrative Cancer Immunology, Paris, France
2 Université Paris Descartes, Paris, France
3 Centre de Recherche des Cordeliers, Université Pierre et Marie Curie Paris 6, France
4 Digestive Surgery Department, Georges Pompidou European Hospital, Paris, France
5 Department of Pathology, Avicenne Hospital, Bobigny, France
6 Institute of Pathology, University of Bern, Bern, Switzerland
7 Department of Pathology, University of Erlangen, Germany
8 Department of Pathology, Providence Portland Medical Center, Portland, OR, USA
9 Pathology Department, Radboud University Nijmegen Medical Centre, The Netherlands
10 Department of Medical Biosciences, Pathology, Umea University, Sweden
11 Department of Oncology—Pathology, Karolinska Institute, Stockholm, Sweden
12 Department of Pathology, Istituto Nazionale per lo Studio e la Cura dei Tumori, Fondazione G Pascale, Naples, Italy
13 Colorectal Surgery Department, Istituto Nazionale per lo Studio e la Cura dei Tumori, Fondazione G Pascale, Naples, Italy
14 Division of Medical Oncology and Immunotherapy, University Hospital of Siena, Istituto Toscana Tumori, Siena, Italy
15 Molecular Gastroenterology and Department of Gastroenterology, Humanitas Clinical and Research Centre, Rozzano, Milan, Italy
16 Institute of Pathology, Medical University of Graz, Austria
17 Department of Histopathology, Dorset County Hospital, Dorchester, UK
18 CEU-San Pablo University School of Medicine and HM Hospital of Madrid Scientific Foundation, Institute of Applied Molecular Medicine (IMMA), Madrid, Spain
19 Department of Oncology, Charles University in Prague, First Faculty of Medicine, Department of Oncology of the First Faculty of Medicine and General Hospital, Prague, Czech Republic
20 Weill Cornell Medical College, Doha, Qatar
21 Ontario Cancer Institute and Campbell Family Institute for Cancer Research, Princess Margaret Hospital, University Health Network, Toronto, ON, Canada
22 Department of Pathology and Laboratory Medicine, University Health Network, Toronto, ON, Canada
23 LHN Program in BioSpecimen Sciences, University Health Network, Toronto, ON, Canada
24 Campbell Family Institute for Breast Cancer Research, Princess Margaret Cancer Centre, University Health Network, Toronto, ON, Canada
25 Division of Cellular Signaling, Institute for Advanced Medical Research, Keio University School of Medicine, Tokyo, Japan
26 Department of Digestive Surgery and Surgical Oncology, Yamaguchi University Graduate School of Medicine, Japan
27 Department of Surgery, Kirki University School of Medicine, Osaka-sayama, Japan
28 Department of Pathology, Brigham and Women’s Hospital, Harvard Medical School and Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA
29 Department of Medical Oncology, Royal Melbourne Hospital, Australia
30 Department of Pathology, University of Melbourne, Australia
31 Department of Pathology, Sapporo Medical University School of Medicine, Japan
32 Department of Immunology and Immunotherapy, Kurume University School of Medicine, Japan
33 The Gujarat Cancer and Research Institute, Asarwa, Ahmedabad, India
34 Institute for Cancer Research, Centre for Translational Medicine, Xi’an jiaotong University, Xian, People’s Republic of China
35 MD Anderson Cancer Center, Houston, TX, USA
36 Mayo Clinic and Mayo College of Medicine, Rochester, MN 55905, USA
37 Department of Surgery, University of Medicine and Pharmacy ‘Gr T Popa’, Department of Surgical Oncology, Regional Institute of Oncology, Iași, Romania
38 Medical Oncology and Innovative Therapies Unit, Istituto Nazionale per lo Studio e la Cura dei Tumori, Fondazione ‘G Pascale’, Napoli, Italy
39 Research Branch, Sidra Medical and Research Centre, Doha, Qatar
40 Laboratory of Molecular and Tumour Immunology, Earle A. Chiles Research Institute, Robert W. Franz Cancer Center, Providence Portland Medical Center, Portland, OR, USA
41 Department of Molecular Microbiology and Immunology, Oregon Health and Science University, Portland, OR, USA
42 Immunomonitoring Platform, Laboratory of Immunology, Georges Pompidou European Hospital, Paris, France

© 2013 The Authors. Journal of Pathology published by John Wiley & Sons Ltd on behalf of Pathological Society of Great Britain and Ireland. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

INVITED REVIEW

Towards the introduction of the 'Immunoscore' in the classification of malignant tumours

Jérôme Galon,1,2,3* Bernhard Mlecnik,1,2 Gabriela Bindea,1,2 Helen K Angell,1,2 Anne Berger,1 Christine Lagorce,1 Alessandro Lugli,6 Inti Zlobec,6 Arndt Hartmann,7 Carlo Bifulco,8 Iris D Nagtegaal,9 Richard Palmqvist,10 Giuseppe V Masucci,11 Gerardo Botti,12 Fabiana Tatangelo,12 Paolo Delrio,12 Michele Maio,12 Luigi Laghi,13 Fabio Grizzi,15 Martin Asslaber,16 Corrado D’Arrigo,17 Fernando Vidal-Vanaclocha,18 Eva Zavadova,19 Lotfi Chouchane,20 Pamela S Ohashi,21 Sara Hafezi-Bakhtian,22 Bradly G Wouters,22 Michael Roehrl,22 Linh Nguyen,24 Yutaka Kawakami,25 Sghi Chi Hazama,26 Kyotaka Okuno,27 Shuji Ogino,28 Peter Gibbs,29 Paul Waring,30 Noriyuki Sato,31 Toshihiko Torigoe,31 Kyogo Itoh,32 Prabhu S Patil,32 Shilin N Shukla,33 Yili Wang,34 Scott Kopetz,35 Frank A Sinicrope,36 Vioirel Scripcariu,37 Paolo A Ascierto,38 Francesco M Marincola,39 Bernard A Fox,40,41 and Franck Pagès1,2,3,42

1 INSERM, U872, Laboratory of Integrative Cancer Immunology, Paris, France
2 Université Paris Descartes, Paris, France
3 Centre de Recherche des Cordeliers, Université Pierre et Marie Curie Paris 6, France
4 Digestive Surgery Department, Georges Pompidou European Hospital, Paris, France
5 Department of Pathology, Avicenne Hospital, Bobigny, France
6 Institute of Pathology, University of Bern, Bern, Switzerland
7 Department of Pathology, University of Erlangen, Germany
8 Department of Pathology, Providence Portland Medical Center, Portland, OR, USA
9 Pathology Department, Radboud University Nijmegen Medical Centre, The Netherlands
10 Department of Medical Biosciences, Pathology, Umea University, Sweden
11 Department of Oncology—Pathology, Karolinska Institute, Stockholm, Sweden
12 Department of Pathology, Istituto Nazionale per lo Studio e la Cura dei Tumori, Fondazione G Pascale, Naples, Italy
13 Colorectal Surgery Department, Istituto Nazionale per lo Studio e la Cura dei Tumori, Fondazione G Pascale, Naples, Italy
14 Division of Medical Oncology and Immunotherapy, University Hospital of Siena, Istituto Toscana Tumori, Siena, Italy
15 Molecular Gastroenterology and Department of Gastroenterology, Humanitas Clinical and Research Centre, Rozzano, Milan, Italy
16 Institute of Pathology, Medical University of Graz, Austria
17 Department of Histopathology, Dorset County Hospital, Dorchester, UK
18 CEU-San Pablo University School of Medicine and HM Hospital of Madrid Scientific Foundation, Institute of Applied Molecular Medicine (IMMA), Madrid, Spain
19 Department of Oncology, Charles University in Prague, First Faculty of Medicine, Department of Oncology of the First Faculty of Medicine and General Hospital, Prague, Czech Republic
20 Weill Cornell Medical College, Doha, Qatar
21 Ontario Cancer Institute and Campbell Family Institute for Cancer Research, Princess Margaret Hospital, University Health Network, Toronto, ON, Canada
22 Department of Pathology and Laboratory Medicine, University Health Network, Toronto, ON, Canada
23 LHN Program in BioSpecimen Sciences, University Health Network, Toronto, ON, Canada
24 Campbell Family Institute for Breast Cancer Research, Princess Margaret Cancer Centre, University Health Network, Toronto, ON, Canada
25 Division of Cellular Signaling, Institute for Advanced Medical Research, Keio University School of Medicine, Tokyo, Japan
26 Department of Digestive Surgery and Surgical Oncology, Yamaguchi University Graduate School of Medicine, Japan
27 Department of Surgery, Kirki University School of Medicine, Osaka-sayama, Japan
28 Department of Pathology, Brigham and Women’s Hospital, Harvard Medical School and Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA
29 Department of Medical Oncology, Royal Melbourne Hospital, Australia
30 Department of Pathology, University of Melbourne, Australia
31 Department of Pathology, Sapporo Medical University School of Medicine, Japan
32 Department of Immunology and Immunotherapy, Kurume University School of Medicine, Japan
33 The Gujarat Cancer and Research Institute, Asarwa, Ahmedabad, India
34 Institute for Cancer Research, Centre for Translational Medicine, Xi’an jiaotong University, Xian, People’s Republic of China
35 MD Anderson Cancer Center, Houston, TX, USA
36 Mayo Clinic and Mayo College of Medicine, Rochester, MN 55905, USA
37 Department of Surgery, University of Medicine and Pharmacy ‘Gr T Popa’, Department of Surgical Oncology, Regional Institute of Oncology, Iași, Romania
38 Medical Oncology and Innovative Therapies Unit, Istituto Nazionale per lo Studio e la Cura dei Tumori, Fondazione ‘G Pascale’, Napoli, Italy
39 Research Branch, Sidra Medical and Research Centre, Doha, Qatar
40 Laboratory of Molecular and Tumour Immunology, Earle A. Chiles Research Institute, Robert W. Franz Cancer Center, Providence Portland Medical Center, Portland, OR, USA
41 Department of Molecular Microbiology and Immunology, Oregon Health and Science University, Portland, OR, USA
42 Immunomonitoring Platform, Laboratory of Immunology, Georges Pompidou European Hospital, Paris, France

© 2013 The Authors. Journal of Pathology published by John Wiley & Sons Ltd on behalf of Pathological Society of Great Britain and Ireland. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
Abstract

The American Joint Committee on Cancer/Union Internationale Contre le Cancer (AJCC/UICC) TNM staging system provides the most reliable guidelines for the routine prognostication and treatment of colorectal carcinoma. This traditional tumour staging summarizes data on tumour burden (T), the presence of cancer cells in draining and regional lymph nodes (N) and evidence for distant metastases (M). However, it is now recognized that the clinical outcome can vary significantly among patients within the same stage. The current classification provides limited prognostic information and does not predict response to therapy. Multiple ways to classify cancer and to distinguish different subtypes of colorectal cancer have been proposed, including morphology, cell origin, molecular pathways, mutation status and gene expression-based stratification. These parameters rely on tumour-cell characteristics. Extensive literature has investigated the host immune response against cancer and demonstrated the prognostic impact of the in situ immune cell infiltrate in tumours. A methodology named ‘Immunoscore’ has been defined to quantify the in situ immune infiltrate. In colorectal cancer, the Immunoscore may add to the significance of the current AJCC/UICC TNM classification, since it has been demonstrated to be a prognostic factor superior to the AJCC/UICC TNM classification. An international consortium has been initiated to validate and promote the Immunoscore in routine clinical settings. The results of this international consortium may result in the implementation of the Immunoscore as a new component for the classification of cancer, designated TNM-I (TNM-Immune).

Keywords: Immunoscore; colorectal cancer; colon carcinoma; tumour microenvironment; immune response; classification; prognostic markers; predictive markers; T cells

Introduction

Cancer is a complex and dynamic disease characterized by major hallmarks. They include sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming of energy metabolism and evading immune destruction [1]. The most common system for classifying the extent of spread of cancer is the American Joint Committee on Cancer/Union Internationale Contre le Cancer (AJCC/UICC) TNM classification [2–4]. The tumour staging gives an estimation of the degree of tumour progression at the time of the surgical resection. The higher the degree of tumour progression, the greater the chance that the tumour will have undergone clonal evolution and acquired a set of unfavourable characteristics, such as the ability to invade lymphatic or blood vessels or to metastasize to distant sites. Furthermore, multiple tumour-cell parameters are an indication of the intrinsic biology of the tumour.

The TNM classification has been used for over 80 years and is valuable in estimating the outcome of patients for a variety of cancers [2–4]. There has been a continual refinement of the staging system. In 2009, the Union for International Cancer Control issued the seventh edition of the TNM classification guidelines. The pathologists generate data from a pathological snapshot. This static measurement integrating the tumour grade and stage is correlated with a dynamic process, such as the time to occurrence or death. The system is used in clinical trials to select patients who are eligible for inclusion, and in cancer registries to compare outcomes between different series, across different countries and over different time periods [5]. An accurate, stable internationally agreed staging system is essential to global progress in this disease. Its main aim should be to provide prognostic information and, based on this information, individual treatment decisions can then be made. In daily practice and in guidelines, the TNM category is directly linked to treatment strategies and, as such, changes in the TNM staging system have a considerable and direct impact on the cancer care that patients receive.

This TNM staging system has stood the test of time but provides incomplete prognostic information. Clinical outcome can significantly vary among patients within the same histological tumour stage [5]. In some patients, advanced-stage cancer can remain stable for years, and although rare, partial or full regression of metastatic tumours can occur spontaneously [6]. In contrast, relapse, rapid tumour progression and patient death is associated with approximately 25% of TNM II stage colorectal cancer (CRC) patients, despite complete surgical resection and no evidence of residual tumour burden or distant metastasis [6].

Unfortunately, the predictive accuracy of the traditional staging system relies on the assumption that disease progression is a tumour cell-autonomous process. The focus of this classification is solely on the
tumour cells and fails to incorporate the effects of
the host immune response [7]. The phenotype of a
tumour is not governed only by the epithelial compo-
nent but also by the tumour environment, that is, other
cells in contact with the tumour, the mesenchyme and
the inflammatory infiltrate. These components deter-
mine the net inputs to the cell, which include ligands,
cell–cell adhesion molecules, metabolites, oxygen and
multiple soluble factors [1]. Relatively little informa-
tion is available on the spatial organization of key
proteins and cells, although new imaging techniques
offer the potential for high-resolution measurements of
the spatio-temporal dynamics of large numbers of pro-
teins [8].

The TNM classification was never surpassed in
multivariate analysis by alternative methods such as
immunohistochemistry for tumour biomarkers, flow-
cytometry for DNA content, molecular signatures or
genetic features. However, it was shown that the analy-
sis of a specific type of intratumoural immune response
was indeed surpassing the TNM classification in mul-
ivariate analysis [9,10]. Thus, tumour progression has
now to be considered as the result of a balance between
an invasive tumour process and a defence system
whose major component is constituted by the host
immune response.

Molecular subtypes of colorectal cancer

Several ways to classify cancer have been proposed
(Figure 1). These rely on tumour cell characteristics,
including morphology, molecular pathways, mutation
status, cell origin and gene expression-based methods,
and allow the distinction of multiple, often overlapping,
subtypes.

A morphology-based classification allows the dis-
tinction between five major types of CRC. More
than 90% of CRCs are adenocarcinomas originat-
ing from epithelial cells. A number of histolog-
ical variants are described: mucinous, signet ring
cell, medullary, micropapillary, serrated, cribriform
comedo-type, adenosquamous, spindle cell and undif-
ferentiated. These histopathological criteria have mod-
est prognostic value.

A second class is based on molecular pathways. CRC
is molecularly defined into three groups: chromosomal
unstable (CIN), microsatellite unstable (MSI) and
CpG island methylator phenotype (CIMP). Most cases
arise through the CIN pathway, with imbalances in
chromosome number and loss of heterozygosity. These
cancers have accumulation of mutations in specific
tumour suppressor genes and oncogenes that activate
pathways critical for CRC initiation and progression.
A high degree of MSI (MSI-high) is present in 15% of
CRCs and represents a specific type of genomic insta-
BRAF

ability characterized by frequent microsatellite length
mutations. Frameshift mutations in microsatellite
instability high (MSI-High) colorectal cancers are
Most MSI-high cancers are caused by epigenetic
silencing of a mismatch-repair gene (MLH1). This
silencing typically occurs in tumours of the CpG
island methylator phenotype (CIMP-high) [12,13].
MSI-high cancers overlap with those of CIMP-high
cancers [13,14]. CIMP-high represents a specific
type of epigenomic instability that is characterized
by widespread promoter CpG island methylation and
epigenetic gene silencing [15]. Nonetheless, differ-
ent from the MSI subgroup, CIMP-high patients
have similar characteristics to MSI, and have been
associated with old age, female gender, proximal
tumour location, poor tumour differentiation, BRAF
mutation, wild-type TP53, high-level of global DNA
methylation and stable chromosomes (CIN) [12,13].
MSI tumours have a more favourable prognosis and
are less prone to lymph node or distant metastasis [16].
CIMP-high might be a prognostic marker independent
of the presence of MSI and BRAF mutation [17].

A third method to classify CRCs is based on
mutation analysis. Following the discoveries from
Bert Vogelstein et al that tumours result from the
sequential accumulation of alterations in oncogenes
and tumour suppressor genes and the appearance of
driver mutations, several mutations are now regularly
tested in CRCs. These include the APC, KRAS, TP53,
BRAF, NRAS, PI3KCA and CTNNB1 genes. Somatic
mutations in codons 12, 13 and 61 of KRAS, and
more recently NRAS, predict innate resistance to mAbs
targeting epidermal growth factor receptor [18–20].
The BRAF V600E mutation is an adverse prognostic
factor in stage IV colorectal cancer but does not have
clinical utility at present [21].

The fourth and fifth methods, assessing the cell of
origin and gene expression, respectively, to classify
CRCs are molecular based techniques. Using this
approach, six groups of CRCs were recently defined,
based on similarities with distinct cell types within
the normal colon crypt and the response to classic
chemotherapy and targeted therapies [22]. Other gene
expression-based analysis described three groups of
CRC patients [23], which correlated with two of the
known molecularly defined groups, namely the MSI
renamed CCS2) and the CIN (CCS1) groups, whereas
the third group corresponded partly with the CIMP
group (CCS3). Further analysis of precursor lesions
(serrated polyps) suggested that this latter group could
be derived from the serrated pathway. Some partial
overlap exists between both proposed gene expression-
based classifications, especially with respect to the
group that is defined by the MSI-high phenotype. Other
gene signatures were also reported and, strikingly,
most of the genes were unique to each signature.
These signatures showed low prediction accuracy and
moderate clinical usefulness [24].

Interestingly, both profiles emphasize the impor-
tance of ZEB1, a transcription factor regulating the
epithelial–mesenchymal transition (EMT). However,
EMT in CRC is generally not a feature of the whole
tumour but is seen at the invasive margin (IM). The stroma of human colorectal tumours was shown to contain TWIST1-positive cancer cells with mesenchymal phenotypes [25]. EMT is characterized by tumour cell budding, nuclear expression of β-catenin, loss of CDH1 (E-cadherin), gain of CDH2 (N-cadherin) and alteration of other epithelial cell adhesion molecules [26]. These features are often not molecularly defined but the result of interplay between the tumour and the microenvironment. Such local features lead to heterogeneity of the gene expression profile. Thus, gene expression classification may be prone to bias, due to different percentages of tumour cells in the sample, but also due to lack or uncontrolled presence of the IM in the sample used for RNA extraction. These issues were not addressed in either study [22,23]. Because of the limitation of gene expression signatures, and to facilitate implementation and testing in large patient series, both studies developed an immunohistochemistry approach based on gene expression data. Unfortunately, half of the patients turned out to be unclassifiable [22] and the absence of CDX2 positivity in all CCS3 samples (25% of CRCs) [23] does not correspond with extensive literature data (98% positivity in CRCs) [27].

Importantly, neoplastic cells interact with host non-neoplastic cells (including immune cells) and extracellular matrix in the tumour microenvironment, and those components influence each other and modify an integral phenotype of any given tumour [28]. Many markers, signatures and methods have been described to evaluate the prognosis of cancer patients, yet very few such markers and laboratory assays translate into clinical practice or reach the statistical power of the TNM classification.

New ways to classify cancers using tumour microenvironment-related information

Modern classification of tumours is based on the recognition of disease entities that are characterized by morphological, phenotypical and genetic markers. Each classification system needs to be reliable, reproducible, clinically relevant and biologically meaningful. Many hurdles have to be taken into consideration. First, the inevitable presence of non-neoplastic cells, including immune cells, in ‘tumour areas’ means that DNA (or RNA) from the tumour area is not ‘pure’ DNA (or RNA) from neoplastic cells. These ‘contaminants’, often > 50% of non-tumour cells, may in fact have a profound biological meaning. Thus, the degree of immune cell infiltration may correlate with tumour molecular changes or may mask a correlation between
Immunoscore classification of malignant tumours

203

a tumour marker and the outcome, because ‘contaminating’ non-neoplastic cells can influence the results of a tumour molecular assay. For sensitive mutation detection, sequencing technologies allow the detection of approximately 5% of mutant alleles. For quantitative DNA methylation assays, a careful assessment of a potential influence of contaminating normal cells is necessary. This is even more problematic with gene expression signatures, where a different degree of non-neoplastic cells is observed among the assays.

Second, a precise characterization of specific tumour-infiltrating cells requires the use of an immunohistochemical technique to detect in the tissue the presence and localization of specific antigens expressed by subsets of immune cells. The evaluation of immune cells in haematoxylin and eosin (H&E)-stained sections can be done at a low cost compared to an immunohistochemical technique. However, an evaluation of a specific subset of immune cells is generally not possible with H&E-stained sections. For example, lymphocytes with opposite functions, such as CD4+ T cells with Th1 orientation versus Th2 orientation versus immune cells with regulatory functions (Treg cells), or NK cells, NKT cells, B cells or cytotoxic CD8+ T cells, are indistinguishable without proper marker evaluation and require antibody labelling by immunohistochemistry. Nowadays this is simple, as specific antibodies with high affinity and a high signal:noise ratio allow the detection of these immune cell subpopulations with a single staining.

Third, it is known that immune cells are scattered in the core of the tumour (CT) within the tumour stroma and the tumour glands, in the invasive margin (IM) and in organized lymphoid follicles distant from the tumour. A statistically significant correlation between immune cell density in each tumour region (CT and IM) and patient outcome has been shown in colorectal cancer [9]. Further, the combined analysis of tumour regions (CT plus IM) improved the accuracy of prediction of survival for the different patient groups compared with single-region analysis [9]. Given the major clinical importance of distinct tumour regions, it is appropriate to conduct immune cell infiltration evaluation systematically in the two separate areas, the core of the tumour and the invasive margin [9,10]. For routine practice, this requires immune cell evaluation on whole-tissue sections, taking into account their location.

Fourth, objective ways of counting immune cells need to be achieved in order to remove the subjectivity of field selection and imprecise semi-quantitative evaluation. Most of the tumour markers are generally more complicated to quantify than immune cells, since only a fraction of the tumour cells express the antigen and the staining intensity has often been taken into consideration. Standardized and reproducible measurement of the intensity of staining, and hence quantitation of protein expression, is intrinsically difficult using immunohistochemistry. In contrast, immune cell types are easier to quantify because well-characterized markers exist, giving complete membrane staining for immune cell subpopulations (eg CD3+ for T cells), allowing counting cells as individual cells. Nowadays, digital pathology and image analysis software can detect stained immune cells and determine their densities (n cells/mm2) in histological sections. Validation studies demonstrated the high concordance of these automated systems in comparison to optical counts [9,29]. This is particularly important to facilitate routine pathology and to speed up the process of quantification. In particular, given the huge number of infiltrating immune cells within a tumour (eg a mean of 75 000 CD3+ cells present on a 4-μm-thick section of tumour slide from a CRC stage II/II patient), it would be unrealistic to ask pathologists to count them all. In addition, immune cells such as CD3+ lymphocytes are often aggregated in complex cell clusters. Algorithms for segmenting clusters of densely packed cells permit a precise counting of cells. Given the major importance of the determination of the density of immune cells [9,30] to predict a patient’s outcome, it is now required to take advantage of the digital pathology to determine the exact count of stained cells and the surface of the tissue analysed.

Fifth, the heterogeneity of a tumour applies to tumour cells but to the microenvironment as well. Upon evaluation of whole-tissue sections, a pathologist often needs to choose specific fields to perform detailed image analysis. The selection of the tissue areas to study may depend on subjective interpretation, and the evaluation needs to be validated by a second independent observer blinded for the other results. Inter-observer variability can reach very high levels [31]. The use of computer-assisted image analysis provides important advantages, as all the fields are analysed in the whole tumour section [32]. For example, the determination of the mean density of stained cells in a tumour region is supported by an objective computer-based cell-counting method, leading to a good level of reproducibility between users.

Sixth, specific markers need to be selected. They should be robust, well-established and have high predictive value. A growing body of literature [9,10,30–33–36] supports the hypothesis that cancer development is influenced by the host immune system. This may offer powerful prognostic information and facilitate clinical decision making regarding the need for systemic therapy [7]. Accumulating evidence suggests that CD3+ [9,10,30,34–37–43], CD8+ [9,10,34,38,43–52] and CD45RO+ [9,10,34,40,47,49,53,54] cells have roles in antitumour immune responses. Numerous data collected from large cohorts of human CRCs (with sample sizes n = 415, 599 and 602, respectively) demonstrated that the number, type and location of tumour immune infiltrates in primary tumours are prognostic for disease-free survival (DFS) and overall survival (OS) [9,10,30]. Altogether, these immune parameters are designated the ‘immune contexture’ [33,35,55]. Notably, several large studies of CRCs (with sample...
sizes $n = 843$ and $n = 768$, respectively) have shown that tumour lymphocytic reaction and T cell sub-populations (CD8, CD45RO, FOXP3) are significant prognostic biomarkers, even after adjusting for stage, lymph node count and well-established prognostic tumour molecular biomarkers, including microsatellite instability (MSI), $BRAF$ mutation and LINE-1 hypomethylation [54,56]. A possible association exists between MSI status and immune cell infiltrates [38]. MSI tumours often contain intra-epithelial T cells in response to the expression of neo-antigens on the cell surface [42], in particular those that do not undergo non-sense medicated decay [11]. This probably contributes to the better prognosis of patients with MSI tumours. Furthermore, CRCs from a large cohort ($n = 1197$) and external validation ($n = 209$) confirmed the prognostic value associated with CD3$^+$, CD8$^+$ and CD45RO$^+$ (PTPRC) T cell infiltration in CRCs [43].

A recent meta-analysis [55] summarized the impact of immune cells, including B cells, NK cells, MDSC, macrophages and all subsets of T cells on clinical outcome from more than 120 published articles. Importantly, the beneficial impact of the immune infiltrate with cytotoxic and memory T cell phenotype has been demonstrated in cancers from diverse anatomical sites; including colorectal cancer but also malignant melanoma, lung, gastric, oesophageal, head and neck, breast, bladder, urothelial, ovarian, cervical, prostatic and pancreatic cancer, hepatocellular carcinoma, medulloblastoma and Merkel cell carcinoma [55]. It is interesting to note that the implications of this immune phenotype apply not only to various organs of cancer origin, but also to various cancer cell types, i.e. adenocarcinoma, squamous cell carcinoma, large cell cancer and melanoma. Thus, general characteristics emerge in which cytotoxic T cells, memory T cells and $T_{H1}$ cells are associated with prolonged survival [35,57,58]. In contrast, the prognostic impact of other immune cells, such as B cells, NK cells, MDSCs, macrophages and a subset of T-helper populations ($T_{H2}$, $T_{H17}$, $T_{REG}$ cells), differ depending on the type of cancer and on the cancer stage [55]. Altogether, the publications indicate that a precise analysis of the immune component of the tumour microenvironment by computer-based analysis may be essential to managing patients better. Thus, precise analysis of the tumour microenvironment by pathologists may be essential for future clinical implementation and better patient management. An expertise in this new emerging field is now warranted to translate it into the clinical practice.

‘Immunoscore’ as a new approach for the classification of cancer

A potential clinical translation of these observations is the establishment of a scoring system designated ‘Immunoscore’ (Figure 2), derived from the immune contexture (Figure 3) [33,35,55], and based on the numeration of two lymphocyte populations (CD3/CD45RO, CD3/CD8 or CD8/CD45RO), both in the core of the tumour (CT) and in the invasive margin (IM) of tumours, as a clinically useful prognostic marker in colorectal cancer [34]. Detailed description of the immune contexture in comparison to the Immunoscore has been described previously [35,36]. The Immunoscore provides a score ranging from Immunoscore 0 (I0) when low densities of both cell types are found in both regions, to Immunoscore 4 (I4) when high densities are found in both regions. This test has a dual advantage: first, it appears to be the strongest prognostic factor for DFS, disease-specific (DSS) and
OS, including at early-stage colorectal cancers; and second, it has biological meaning (adaptive immune response to tumours) and provides a tool or a target for novel therapeutic approaches, including immunotherapy (as recently illustrated in clinical trials boosting T cell responses with anti-CTLA4, anti-PDCD1 (PD-1) and anti-CD274 (PD-L1) [59–62]. Current immunohistochemical technologies allow the application of such analyses in routine diagnostic pathology. Thus, considering the probable universal character of the immune control of tumours, it is essential for patients to take into account the immune parameter as a prognostic factor and to introduce the Immunoscore as a component of cancer classification [6,7,35,63,64].

The Immunoscore was shown to be very powerful, for instance, in CRC patients with clinically localized colorectal cancer, with no detectable tumour spreading to lymph nodes or distant organs. These patients are usually treated only by a surgical removal of the tumour; however, approximately 25% of these patients will have recurrence of their disease, indicating that occult metastases were already present at the time of curative surgery. No tumour-associated marker predicts the recurrence of this subgroup of patients that could have a benefit for an adjuvant therapy. The Immunoscore (I) approach (with an enumeration of CD8⁺ and CD45RO⁺ cells in the CT and the IM) was applied to two large independent cohorts (n = 602). Only 4.8% of patients with a high I4 relapsed after 5 years and 86.2% were still alive. In contrast, 72% of patients with a low score (I0 and I1) experience tumour recurrence and only 27.5% were alive at 5 years. These I0 and I1 patients could potentially have benefited from adjuvant therapy if the Immunoscore had been incorporated into the tumour staging [34].

The Immunoscore classification, demonstrating the prevalence of immune infiltrates, was shown to have a prognostic significance superior to that of the AJCC/UICC TNM classification system. For all patients with CRC stages I/II/III, multivariate Cox analysis revealed that the immune criteria remained highly significantly associated with prognosis (DFS, DSS, OS). In contrast, the histopathological staging system (T stage, N stage and tumour differentiation) was no longer significant [10]. Tumour invasion was shown to be statistically dependent on the host immune reaction. Indeed, the immune pattern remained the only significant criterion over the classical AJCC/UICC TNM classification for DFS and OS, and led to an editorial entitled ‘TNM staging in colorectal cancer: T is for T cell and M is for memory’ accompanying the publication by Mlecnik et al [10,65]. It has thus been suggested that the prevalence of tumour immune infiltrates, more than the tumour status, could be a key indicator for recurrence, metastasis and therefore clinical outcome (Figure 4). These results suggest that once human cancer becomes clinically detectable, the adaptive immune response plays a critical role in preventing tumour recurrence. The ability of effector-memory T cells to recall previously encountered antigens leads to a protective response. Following primary exposure to antigen, memory T cells disseminate and are maintained for long periods of time [66]. The trafficking properties and the long-lasting antitumour capacity of memory T cells could result in long-term immunity in human cancer. Over the past few years, the area of immune regulation at the level of the tumour microenvironment has gained a forefront position in cancer research, in CRC [9,10,30,34,55], melanoma [67] and all other cancer types [7]. The Immunoscore, initially described several years ago [9] as a prognostic factor [10,34], could also play a role as a marker to predict the response to biotherapies targeting the immune checkpoints [63,64].

The inherent complexity of immunohistochemistry, in conjunction with protocol variability, contributes to the variability of the results obtained. A standardized consensus method is required. Large-scale assay harmonization efforts have already been witnessed, conducted for commonly used immunological assays of peripheral blood immune cell populations [68,69]. It is therefore essential to pursue assay uniformity to reduce these limitations. A clinical validation of the Immunoscore with standardized procedures is necessary to reach clinical applicability for individual patients.

We performed multiple Immunoscore quality controls to test the accuracy and repeatability of the method. We first observed that the automated cell-counting method achieved a very good level of correlation with the optical counting for CD3 and CD8 immunostainings and an excellent reproducibility of
the count of stained cells. In addition, the variability between users for the determination with the software of the immune cell densities in tumour regions was 5.2% and 2.5% for CD3 and CD8, respectively (unpublished data). All the tests indicated a reliable assessment of the Immunoscore. To be used globally in a routine manner, evaluation of a novel marker should have the following characteristics: do-able in routine, feasible, simple, inexpensive, rapid, robust, reproducible, quantitative, standardized, pathology-based and powerful. The Immunoscore has a potential to fulfil these key aspects (Figure 5). Importantly, for patients with rectal cancer treated by chemoradiotherapy (pCRT) before surgery, preoperative chemoradiation therapy induces histological reactions precluding the realization of an Immunoscore, since the delineation of the analysed tumour regions (CT and IM) is often no longer practicable. Also, assessment of anti-tumour immunity by the Immunoscore is inappropriate in biopsies, since the limitation of the material precludes a precise delimitation of the tumour and the invasive margin. However, immune cell quantification remains an interesting possibility in tumour biopsies.

To assess the Immunoscore in clinical practice and measure its prognostic value, we are conducting a prospective multicentre study (French PHRC programme) for 600 patients from seven hospitals bearing a colorectal cancer treated by primary surgery. In an effort to promote the utilization of the Immunoscore in routine clinical settings, we initiated a worldwide Immunoscore consortium, with the support of the Society for Immunotherapy of Cancer (SITC) [64]. Several thousands of tumours from patients will be Immunoscore-tested by the 23 centres from the worldwide consortium. The worldwide Immunoscore consortium, composed of international expert pathologists and immunologists, identified a strategy for the organization of worldwide retrospective study for the validation of the Immunoscore in colon cancer. Evidence-based selection of specific markers for the Immunoscore was discussed. Because of background staining and loss of antigenicity in stored sections (CD45RO) and granular staining (GZMB), it was decided to employ the two easiest membrane stains, CD3 and CD8. Thus, the combination of two markers (CD3+ and CD8+) in two regions (CT and IM) was agreed for validation in standard clinical practice. Precise quantification is currently performed on whole slide sections (Figure 2), following the recommended initial guidelines.

The purpose of the ongoing Immunoscore worldwide consortium is to validate the following points: first, to demonstrate the feasibility and reproducibility of the Immunoscore; second, to validate the major prognostic power of the Immunoscore in routine for patients with colon cancer stages I/II/III; and third, to demonstrate the utility of the Immunoscore to predict stage II colon cancer patients with high risk of recurrence. Twenty-three international pathology expert centres are now participating in the Immunoscore enterprise. These participants represent 23 centres from 17 countries, including Asia, Europe, North America, Australia and the Middle East (Australia, Austria, Canada, China, Czech Republic, France, Germany, India, Italy, Japan, The Netherlands, Qatar, Spain, Sweden, Switzerland, the UK and the USA). It is hoped that this initiative will result in the implementation of the Immunoscore as a new component for the classification of cancer, TNM-I (Immune). The Immunoscore should better define the prognosis of cancer patients, better identify patients at high risk of tumour recurrence [70], help to predict and stratify patients who will benefit from therapies [35] and, ultimately, help save the lives of patients with cancer.

Acknowledgements

We acknowledge all the scientists who made contributions to the area of research reviewed here that were not cited due to space constraints. The work performed in

<table>
<thead>
<tr>
<th>Good biomarker</th>
<th>Immunoscore characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Routine</td>
<td>Technique to be performed by pathologist using bright field and precise cell evaluation</td>
</tr>
<tr>
<td>Feasible</td>
<td>Established pathology techniques, using 2 regular whole slide FFPE sections</td>
</tr>
<tr>
<td>Simple</td>
<td>Automated immunochemistry</td>
</tr>
<tr>
<td>Inexpensive</td>
<td>Two simple stainings less costly than complicated molecular techniques</td>
</tr>
<tr>
<td>Rapid</td>
<td>Two strong membrane stainings, with no background, allowing the enumeration of individual cells</td>
</tr>
<tr>
<td>Robust</td>
<td>Autostainers, scanner, digital pathology reduce the time to perform an Immunoscore</td>
</tr>
<tr>
<td>Reproducible</td>
<td>Inter-observers variability is removed by the use of digital pathology, taking into account cell location and counts</td>
</tr>
<tr>
<td>Quantitative</td>
<td>Precise cell density (cell number / surface area)</td>
</tr>
<tr>
<td>Standardized</td>
<td>Standardized operating procedure should be performed to ensure reproducibility and worldwide comparisons</td>
</tr>
<tr>
<td>Pathology-based</td>
<td>Necessity of pathologist expertise to validate cell type, cell location, and cell counts perform by digital pathology</td>
</tr>
<tr>
<td>Powerful</td>
<td>The Immunoscore has a prognostic value highly significant even in Cox multivariate including TNM classification 9, 10</td>
</tr>
</tbody>
</table>

![Figure 5. Characteristics of a good biomarker and of the Immunoscore.](image-url)
the Laboratory of Integrative Cancer Immunology and the immunomonitoring platform of the HEGP hospital was supported by grants from the Institut National du Cancer, France (INCa), Assistance Pubbile-Hôpitaux de Paris, the national PHRC programme IMMUCOL, the Canceropole Ile de France, INSERM, MedImmune, Qatar National Research Fund under its National Priorities Research Programme (Award No. NPRP09-1174-3-291), the European Commission (7FP, Geninca Consortium; Grant No. 202230), Cancer Research for Personalized Medicine (CARPEM) and the LabEx Immuno-oncology. The authors wish to acknowledge the following organizations for supporting the Immunoscore following the WIC meeting: Society for Immunotherapy of Cancer (SITC); the European Academy of Tumour Immunology (EATI); La Fondazione Melanoma Onlus; National Cancer Institute, USA (NCI); Biotherapy Development Association (BDA); Canadian Cancer Immunotherapy Consortium (CCIC); Cancer Immunotherapy Consortium (CIC); Cancer Research Institute (CRI); Association for Cancer Immunotherapy (CIMT); Committee for Tumour Immunology and Biotherapy (TIBT); European Society for Cancer Immunology and Immunotherapy (ESCI); Italian Network for Tumour Biotherapy (NIBIT); Japanese Association of Cancer Immunology (JACI); Nordic Centre for Development of Antitumour Vaccines (NCV-Network); Progress in Vaccination Against Cancer (PVAC); Adoptive therapeutic T cell targeting to Active Cancer Killing (ATTACK); Tumour Vaccine and Cell Therapy Working Group (TVACT); and Institut National du Cancer, France (INCa).

Author contributions

JG is coordinating the Worldwide Immunoscore initiative, conceiving the study and wrote the manuscript; JG and FP initiated the Immunoscore project; BM, GB, FP, CL, AB and JG performed the initial experiments related to the Immunoscore; FP and HKA participated in the drafting of the manuscript. AL, CB, GB, FT, PD, MM, FH, MB, FMM, BAF and JG were experts involved in the design of the Immunoscore study and expert pathologists participating to the inaugural Immunoscore workshop. All authors, JG, BM, GB, HKA, AB, CL, AL, IZ, AH, CB, IDN, RP, GVM, GB, FT, PD, MM, LL, FG, MA, CDA, FVV, EZ, LC, PSO, SHB, BGW, MR, LN, YK, SH, KO, SO, PG, PW, NS, TT, KY, PSP, SNS, YW, SK, FAS, PAA, FMM, BAF, VS, FP are participants of the initial worldwide Immunoscore task force study and have read and approved the manuscript.

References


---

**2012 Annual Review Issue in the Journal of Pathology...**

**Silencing disease genes in the laboratory and the clinic**
Jonathan K Watts and David R Corey

**Anoikis: an emerging hallmark in health and diseases**
ML Taddel, E Giannoni, T Fiaschi and P Chiarugi

**Notch in the kidney: development and disease**
Yasemin Sirin and Katalin Susztak

**To view these articles, and more, please visit:** [www.thejournalofpathology.com](http://www.thejournalofpathology.com)

Click ‘ALL ISSUES (1892 - 2011)’, to read articles going right back to Volume 1, Issue 1.

**The Journal of Pathology**

**Understanding Disease**

© 2013 The Authors. *Journal of Pathology* published by John Wiley & Sons Ltd on behalf of Pathological Society of Great Britain and Ireland. www.pathsoc.org.uk

*J Pathol* 2014; 232: 199–209

[www.thejournalofpathology.com](http://www.thejournalofpathology.com)