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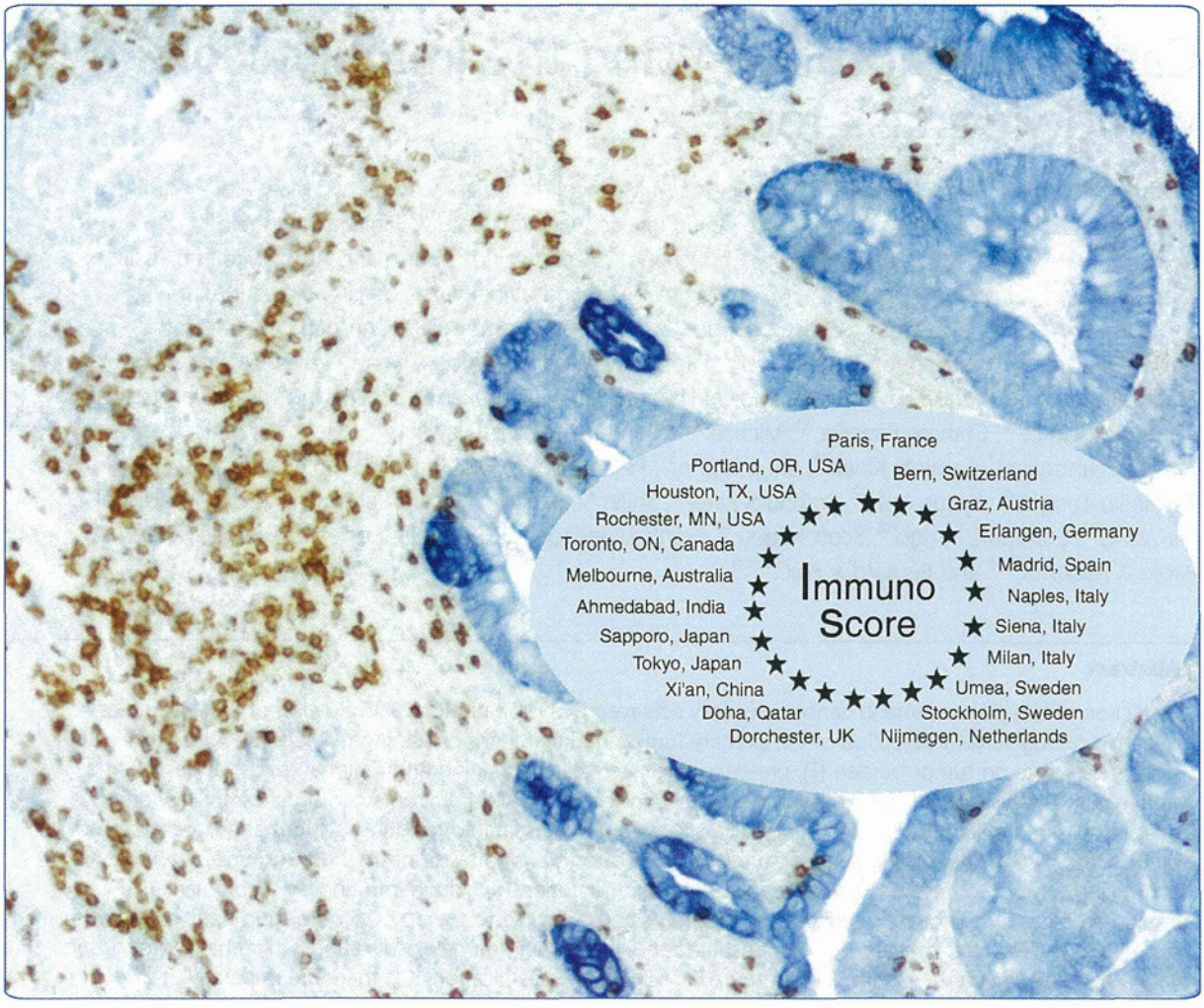

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REVIEW Open Access

Cancer classification using the Immunoscore: a worldwide task force

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Abstract

Prediction of clinical outcome in cancer is usually achieved by histopathological evaluation of tissue samples obtained during surgical resection of the primary tumor. Traditional tumor staging (AJCC/UICC-TNM classification) summarizes data on tumor burden (T), presence of cancer cells in draining and regional lymph nodes (N) and evidence for metastases (M). However, it is now recognized that clinical outcome can significantly vary among patients within the same stage. The current classification provides limited prognostic information, and does not predict response to therapy. Recent literature has alluded to the importance of the host immune system in controlling tumor progression. Thus, evidence supports the notion to include immunological biomarkers, implemented as a tool for the prediction of prognosis and response to therapy. Accumulating data, collected from large cohorts of human cancers, has demonstrated the impact of immune-classification, which has a prognostic value that may add to the significance of the AJCC/UICC TNM-classification. It is therefore imperative to begin to incorporate the 'Immunoscore' into traditional classification, thus providing an essential prognostic and potentially predictive tool. Introduction of this parameter as a biomarker to classify cancers, as part of routine diagnostic and prognostic assessment of tumors, will facilitate clinical decision-making including rational stratification of patient treatment. Equally, the inherent complexity of quantitative immunohistochemistry, in conjunction with protocol variation across laboratories, analysis of different immune cell types, inconsistent region selection criteria, and variable ways to quantify immune infiltration, all underline the urgent requirement to reach assay harmonization. In an effort to promote the Immunoscore in routine clinical settings, an international task force was initiated. This review represents a follow-up of the announcement of this initiative, and of the J Transl Med. editorial from January
(Continued on next page)

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2012. Immunophenotyping of tumors may provide crucial novel prognostic information. The results of this international validation may result in the implementation of the Immunoscore as a new component for the classification of cancer, designated TNM-I (TNM-Immune).

Background

Conventional clinical and pathological risk prediction in cancer patients is usually achieved by histopathological evaluation of tissue samples obtained during surgical removal of the primary tumor. The histopathological characteristics used can include: the size of the tumor; tissue integrity; atypical cell morphology; histological grade; aberrant expression of protein and genetic markers; evidence of malignant transformation, senescence and proliferation; characteristics of the invasive margin (IM); depth of invasion; and the extent of vascularization. In addition, histological or radiological analyzes of tumor-draining and regional lymph nodes, as well as of distant organs, are carried out looking to identify evidence of metastases. In accordance with this classification system, the evaluation of cancer progression is performed longitudinally and then applied to estimate patient prognosis. The parameters used to predict disease-free (DFS), disease-specific (DSS) and overall (OS) survival are taken from statistical analysis of patients with similar disease progression characteristics and corresponding clinical outcome. Tumor staging (AJCC/UICC-TNM classification) summarizes data on the extent of the tumor burden (T), presence of cancer cells in draining and regional lymph nodes (N) and evidence of metastases (M). This classification, based only on tumor invasion parameters, has been shown to be valuable in estimating the outcome of patients with a variety of cancers [1-3].

However, these traditional classification tools provide limited information in estimating patient post-operative outcome. It is well known that clinical outcome can significantly vary among patients within the same histological tumor stage [4]. In some patients, advanced stage cancer can remain stable for years, and although rare, partial or full regression of metastatic tumors can occur spontaneously [5]. In contrast, relapse, rapid tumor progression and patient death is associated with approximately 20-25% of TNM I/II stage patients, despite complete surgical resection and no evidence of residual tumor burden or distant metastasis [5].

The predictive accuracy of this traditional staging system relies on the assumption that tumor progression is largely a cell-autonomous process. The focus of this classification is solely on the tumor cells and fails to consider and incorporate the effects of the host immune response [6]. Histopathological analysis of tumors has revealed the infiltration of inflammatory and lymphocytic cells [7]. Detailed intra-tumor analysis illustrates that these immune infiltrates are not randomly distributed. Tumor-infiltrating immune cells appear to be localized and organized within dense infiltrates in the center of the tumor (CT), at the IM of tumoral nests and in adjacent tertiary lymphoid structures (TLS). The presence of immune cells may reflect a distinct underlying biology of the tumor, as gene expression profiling and other assays have revealed the presence of a broad signature of inflammation. This signature includes evidence for innate immune activation, chemokines for innate and adaptive cell recruitment, immune effector molecules, and expression of immunoregulatory factors [8-10]. Immune infiltrates are heterogeneous between tumor types, and are diverse from patient to patient. All immune cell types may be found in a tumor, including macrophages, dendritic cells (DC), mast cells, natural killer (NK) cells, naïve and memory lymphocytes, B cells and T lymphocytes (which include various subsets of T cell: T_H1, T_H2, T_H17, regulatory T cells (T_{REGS}), T follicular helper cells (T_{FH}) and cytotoxic T cells). The analysis of the location, density and functional orientation of different immune cell populations (termed the immune contexture [11,12]) in large collections of annotated human tumors has allowed the identification of components that are beneficial for patients and those that are deleterious [6,9,12-14]. Nonetheless, to implement any new tumor biomarker including immune infiltrates for routine clinical use, careful evaluation of its laboratory validity and clinical utility is essential [15].

Since tumor molecular features and immune reactions are inter-related, a comprehensive assessment of these factors is critical [16]. Examining the effects of tumor-host interactions on clinical outcome and prognosis clearly represents an evolving interdisciplinary field of molecular pathological epidemiology, the paradigm of which has recently been established [6,11,17,18]. Pathological immunity evaluation may provide novel information on prognosis and help identify patient cohorts more likely to benefit from immunotherapy.

A new classification of cancer based on the tumor microenvironment

Increasing literature [9,11,13,14,19] and meeting reports [20-22] support the hypothesis that cancer development is influenced by the host immune system. A common theme has emerged, emphasizing the critical need to evaluate systemic and local immunological biomarkers. It is in agreement that this may offer powerful

prognostic information and facilitate clinical decision-making regarding the need for systemic therapy [6,23]. Numerous data collected from large cohorts of human cancers (with sample sizes n = 415, 599 and 602, [9,13,14], respectively) demonstrated that the number, type and location of tumor immune infiltrates in primary tumors, are prognostic for DFS and OS. Altogether these immune parameters are designated as the immune contexture [11,12]. Notably, two large studies (with sample sizes n = 843 and 768, [24,25], respectively) have shown that tumor immune infiltrate patterns and subsets in colorectal cancer are significant prognostic biomarkers, even after adjusting for stage, lymph node count, and well-established prognostic tumor molecular biomarkers including microsatellite instability (MSI), *BRAF* mutation, and LINE- hypomethylation.

A potential clinical translation of these observations is the establishment of an Immunoscore, based on the numeration of two lymphocyte populations (CD3/CD45RO, CD3/CD8 or CD8/CD45RO), both in the CT and in the IM of tumors, as a clinically useful prognostic marker [14]. For instance, colorectal cancer (CRC) patients with local tumor, no detectable lymph node or distant metastasis are usually treated by surgery alone. However, 20-25% of these patients will have recurrence of their disease indicating that occult metastases were already present at the time of curative surgery. No tumor-associated marker predicts recurrence in these patients. The Immunoscore ("I") utilizes the numeration of CD8 and CD45RO cells in the CT and the IM of resected tumors to provide a score ranging from Immunoscore 0 ("I"0), when low densities of both cell types are found in both regions, to Immunoscore 4 ("I"4), when high densities are found in both regions. This Immunoscore approach was applied to 2 large independent cohorts (n = 602). Only 4.8% of patients with a high "I"4, relapsed after 5 years and 86.2% were alive. In comparison, 72% of patients with a low score ("I"0 and "I"1) experience tumor recurrence and only 27.5% were alive at five years. These "I"0 and "I"1 patients potentially could have benefited from adjuvant therapy, had the Immunoscore been incorporated into the tumor staging [14].

The Immunoscore classification, demonstrating the prevalence of immune infiltrates, potentially has 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. In contrast, the histopathologic staging system (T stage, N stage, and tumor differentiation) was no longer significant [13]. Tumor invasion was shown to be statistically dependent on the nature of the host-immune reaction. Indeed, the immune pattern remained the only significant criteria 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 and Broussard et al. in the Journal of Clinical Oncology [13,26]. It has thus been suggested that the prevalence of post-surgical immune infiltrates, and not tumor status, is the key indicator for reoccurrence, metastasis and therefore clinical outcome.

These results suggest that once human cancer becomes clinically detectable, the adaptive immune response may play a critical role in preventing tumor 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 [27]. The trafficking properties and the long-lasting antitumor capacity of memory T cells could result in long-term immunity in human cancer.

Although first described in CRC, the impact of the immune cytotoxic and memory T cell phenotype has been demonstrated in many other human tumors and appears to be a general phenomenon [23,28]. It is interesting to note that the implications of this immune phenotype apply not only to various organs of cancer origin (such as breast, colon, lung, head and neck, kidney, bladder, ovary, prostate), but also to various cancer cell types (adenocarcinoma, squamous cell carcinoma, large cell cancer, melanoma, etc).

A recent Nature Cancer Review meta-analysis [12] summarizes the impact of immune cells including B cells, NK cells, myeloid derived suppressor clls MDSC, macrophages, and all subsets of T cells on clinical outcome from more than 120 published articles. Beyond colorectal cancer, a strong T cell infiltration associated with good clinical outcome has been reported in many different tumours, including melanoma, head and neck, breast, bladder, urothelial, ovarian, esophageal, renal, prostatic, pancreatic, cervical, medulloblastoma, merkel cell carcinoma, hepatocellular, gastric, and lung cancers [12]. Thus, high densities of T cells (CD3+), of cytotoxic T cells (CD8+), and of memory T cells (CD45RO+) were clearly associated with a longer DFS (after surgical resection of the primary tumour) and/or OS.

The prognostic impact of other immune cells such as B cells, NK cells, MDSC, macrophages, and subset of T-helper populations, (T_H2, T_H17, T_{REG} cells) may differ depending on the type of cancer, and on the cancer stage [12]. In contrast, T cells, cytotoxic T cells, T_H1 cells, and memory T cells were strongly associated with good clinical outcome for all cancer types [12]. Thus, general characteristics emerge in which cytotoxic T cells, memory T cells, and T_H1 cells are associated with prolonged survival.

The Immunoscore as a new approach for the classification of cancer

Considering the important role of the host immune signature in controlling tumor progression, it is now imperative to initiate the incorporation of the Immunoscore as a component of cancer classification [13,14] and a prognostic tool [23]. This strategy has a dual advantage: firstly, it appears to be the strongest prognostic factor for DFS and OS, particularly in early stage cancers and secondly, it could allude to potential targets for novel therapeutic approaches, including immunotherapy. Current immunohistochemical technologies allow the application of such analyses by laboratories concerned with routine diagnostic and prognostic assessment of tumors.

The inherent complexity of immunohistochemistry, in conjunction with protocol variability, analysis of different immune cell types, inconsistent tissue region selection criteria, combined with differences in conjunction with qualitative and semi-quantitative criteria to measure immune infiltration, all contribute to the variability of the results obtained, and raise the concern that specialized protocols and training may be required. It is therefore essential to pursue assay uniformity to reduce these limitations. Many markers, signatures, and methods have been described to evaluate the prognosis of cancer patients. Yet, very few such markers and laboratory assays are used in clinical practice. Thus, we believe that harmonization of an assay evaluating the "inflammation", i.e. the Immunoscore of the tumor is essential. Analytical and clinical validation of the assay is required before the Immunoscore will reach clinical applicability for individual patients. However, current immunohistochemical technologies allow the application and cross-validation of such analysis in laboratories performing routine diagnostic and prognostic assessment of tumors. In order to be able to compare results in the future, and for the development of more effective prognostic and predictive markers to improve clinical decision-making, it is important to perform a standardized set of experiments. Assay harmonization should minimize data variability and allow worldwide correlations of Immunoscore results with clinical outcomes. Harmonization guidelines resulting from this process are expected to be simple to implement and will improve assay performance. Effective large-scale assay harmonization efforts have already been conducted for commonly used immunological assays of peripheral blood immune cell populations [29,30].

A fundamental parameter to determine the Immunoscore will include the immune cell density, calculated by numerical quantification of two lymphocyte populations, cytotoxic and memory T cells at the CT and the IM of tumors. This core criterion will establish prognosis of patient clinical outcome, regardless of the absence of other cancer associated prognostic markers, such as in early tumor stage (I/II) patients [14]. In human cancers, a high density of T_H1/cytotoxic memory T lymphocytes, located both in the CT and IM of the primary tumor, is associated with long DFS and OS, in addition to low risk of relapse and metastasis. This was particularly illustrated in CRC [5,9,13,14,19], and should be applicable to most human tumors [23]. Thus, this Immunoscore classification may help identify the high-risk patients who would benefit the most from adjuvant therapy.

Impact on response to cancer therapies

Whether the immune contexture of the primary tumor predicts therapeutic responses is of paramount importance for patient clinical management. Data based on immune signatures have established that a strong immune component is predictive of good response to chemotherapy in breast cancer [31-33], a tumor in which a high lymphocyte infiltrate is associated with higher response rate in neo-adjuvant therapy [34,35]. In hepatic metastases of CRC, high CD8 infiltrates in the IM predicts better response to chemotherapy and prolonged survival [36]. In melanoma, an immune signature displaying high expression of T_H1 and cytotoxicity-associated genes, correlates with favorable clinical outcome to several different therapeutic vaccines [8]. In addition, high numbers of CD8 T cell infiltrates within metastatic melanoma correlated with prolonged survival [37]. However, the high T_H1 and cytotoxic immune response associated with prolonged survival in patients receiving adjuvant therapies may not be a prediction of response to the therapy, but rather the fact that the host-immune response within the tumor protects the patient and prolongs patient life. To assess the impact of the Immunoscore as a predictive marker, it should be evaluated prospectively in randomized clinical trials.

An open access call for a broad participation to the development of a task force dedicated to the evaluation of the Immunoscore in cancer patients

Over the past few years, the area of immune regulation at the level of the tumor microenvironment has gained a forefront position in cancer research, in CRC [9,12-14], in melanoma [38] and all other cancer types [6]. The Immunoscore was initially described several years ago [9], and more recently advances have been made in the development of the Immunoscore as a prognostic factor [13,14] that could be used in routine testing [39]. In an effort to promote the utilization of such Immunoscore in routine clinical settings worldwide, the Society for Immunotherapy of Cancer (SITC), the European Academy of Tumor Immunology (EATI), and "La Fondazione Melanoma Onlus", initiated a task force on "Immunoscoring as a New Possible Approach for the Classification of Cancer" that took place in Naples, Italy, February 13th, 2012 [39]. This perspective represents a follow-up on this initiative,

originally announced in a J Transl Med. editorial in January 2012 [39]. The working group, composed of international expert pathologists and immunologists, identified a strategy for the organization of worldwide participation by various groups for the validation of the Immunoscore. The objectives of the meeting included discussing: the role of immune system in cancer; a review of the AJCC/UICC-TNM classification of CRC; the role of the microenvironment in melanoma biology; the review of the AJCC classification of melanoma; the relevance of HLA-A2 in cancer prognosis and tumor malignancy; data utilizing the Immunoscore and a proposal for standardizing the operating procedures for the Immunoscore quantification. Furthermore, the international working group evaluated the feasibility of using the Immunoscore for the classification of cancer. Evidence-based selection of specific markers and their combinations for the Immunoscore was discussed including biological rationale, clinical use, synthetic meta-analysis of the Immunoscore, analytical performance, reagents availability and testing, metrics for decision making, cross-laboratory validation of methodology and identification of potential problems during development of other markers. Practical aspects of the validation of the assay by participating centers were proposed including consideration of cancer types, cancer stages, and the definition of a working group of pathologists for the validation phase.

CRC has been most comprehensively studied and the prognostic significance of immunologic parameters has been best validated, thus special emphasis will be placed in this disease for this formal validation. As neo-adjuvant treatments are nowadays recommended for rectal cancer, it may be advisable to separate the validation of colon cancers and rectal cancers. Other cancer types, including melanoma and breast cancers were additionally discussed and their validation will follow. An independent international consensus panel of expert laboratories discussed cross-laboratory assay validation for the development of an Immunoscore prognostic method. As evaluation of cytotoxic memory $CD8^+$ T cells ($CD3^+$, $CD8^+$, $CD45RO^+$, Granzyme B^+ (GZMB)) provides the best method to discriminate patient outcome, any combination of two of these aforementioned markers should have similar statistical power. Because of technical difficulties including background noise (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 will be performed on whole slide sections (Figure 1). For harmonization of the assay and reproducibility of the method, all laboratories agreed to test the prognostic value of specific immune cell infiltration following the recommended initial guidelines. The inherent complexity of quantitative immunohistochemistry underscored the urgent need to reach assay harmonization. The components of the Immunoscore are listed in Table 1. Additional markers could be added subsequently to refine the methodology even further if required. After worldwide validation, a consensus detailed protocol will be available.

To be used globally in a routine manner, evaluation of a novel marker should have the following characteristics: pathology-based, feasible in routine settings, simple, inexpensive, rapid, robust, reproducible, quantitative, standardized, and powerful. The Immunoscore fulfills all these keys aspects summarized in Table 2.

The purpose of the Immunoscore worldwide task force is to validate these points.

The goals of the first ongoing initiative are the following:

1) to demonstrate the feasibility and reproducibility of the Immunoscore.
2) to validate the major prognostic power of the Immunoscore in routine settings for patients with colon cancer stage I/II/III.
3) to demonstrate the utility of the Immunoscore to predict stage II colon cancer patients with high risk of recurrence.

Thus, the benefit of the Immunoscore worldwide study would be to validate the feasibility, reproducibility,

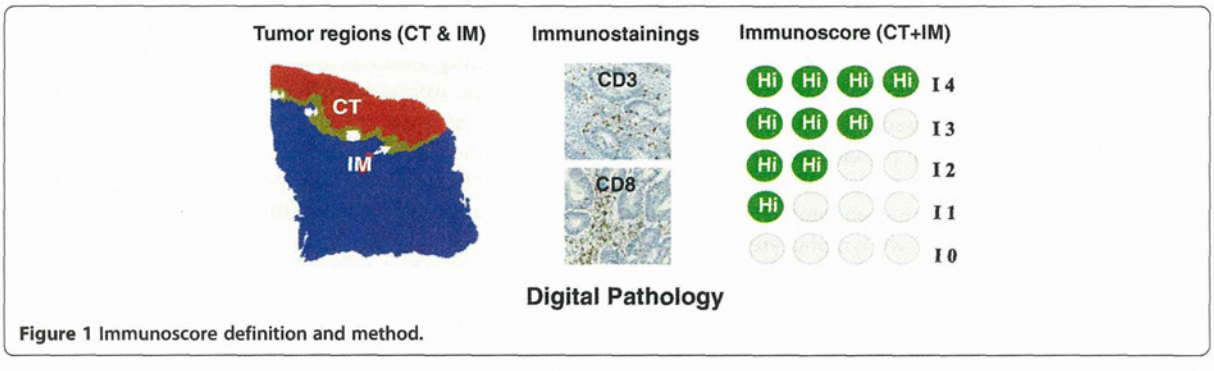

Figure 1 Immunoscore definition and method.

Table 1 Current Immunoscore procedure and reagents

Procedure	Current recommended steps
Tumor selection	Block which is the most infiltrated by the immune cells and containing the core of the tumor (CT) and the invasive margin (IM)
Sample preparation	2 paraffin sections of 4-microns of the tumor block deposited in deionized water on Superfrost-plus slides
Immuno-histochemistry (IHC)	2 single stainings using IVD certified antibodies
Antigen retrieval	CC1 tris-based buffer pH8
Primary antibody	CD3 (2GV6, Ventana) and CD8 (C8/144, Dako)
Primary antibody diluant	K 004 (Clinisciences) for CD8
Secondary reagents	Ultraview TM DAB (Ventana)
Counterstaining	Hematoxillin II (Ventana)
Autostrainer	Benchmark XT (Ventana)
Scanner	NanoZoomer 2.0-HT (Hammamatsu)
Digital pathology	Architect XD software (Definiens)
Immunoscore quantification	Immunoscore Plug-in (INSERM / AP-HP)

and prognostic value of the routine Immunoscore on colon cancer patients.

The goals of the next initiatives will be the following:

1) promote the worldwide use of the Immunoscore as a routine testing for cancer classification.
2) to validate the major prognostic power of the Immunoscore for patients with other cancer types (melanoma, breast, ovarian, endometrial, etc...).
3) to demonstrate the utility of the Immunoscore to predict response to treatments in clinical trials.

In the inaugural World Immunotherapy Council meeting (February 21st - 24th 2012, Curacao), the Immunoscore task force, led by the Society for Immunotherapy of Cancer (SITC), received the support from several additional cancer immunology societies including; Biotherapy Development Association (BDA); Canadian Cancer Immunotherapy Consortium (CCIC); Cancer Immunotherapy Consortium (CIC) of the Cancer Research Institute (CRI); Association for Cancer Immunotherapy (CIMT); Committee for Tumor Immunology and Bio-therapy (TIBT); European Academy of Tumor Immunology (EATI); European Society for Cancer Immunology and Immunotherapy (ESCII); Italian Network for Tumor Biotherapy (NIBIT); Japanese Association of Cancer Immunology (JACI); Nordic Center for Development of Antitumor Vaccines (NCV-network); Progress in Vaccination Against Cancer (PIVAC); Adoptive engineered T cell Targeting to Activate Cancer Killing (ATTACK) and the Tumor Vaccine and Cell Therapy Working Group (TVACT). These groups share a clinical or basic interest in the immunobiology of the tumor microenvironment and will collaborate with worldwide expert pathologists to assess the validity of this new approach. Following the Immunoscore Workshop and the World Immunotherapy Council meeting, 22 international expert centers agreed to participate in this visionary enterprise. These participants represent 22 Centers Worldwide from 16 countries including Asia, India, Europe, North America, Australia, and Middle East (Figure 2). Additionally, pathologist associations and other medical specialty groups have been invited to participate.

A preliminary summary of this effort will be presented during the "Workshop on Tumor Microenvironment" prior to the SITC annual meeting (October 24th - 25th 2012, Maryland, USA). Finally a "Workshop on Immunoscore" (December 5th 2012, Naples, Italy), will lead to the preparation of a summary document providing recommendations for the harmonization and implementation of the Immunoscore as a new component for the classification of cancer TNM-I (Immune).

Table 2 Characteristics of a good marker and of the Immunoscore

Must be	Immunoscore	Characteristics
Routine	YES	Technic to be performed by pathologist using bright field and precise cell evaluation
Feasible	YES	Established pathology technics, using 2 regular whole slide FFPE section
Inexpensive	YES	Automatized immunohistochemistry
Rapid	YES	2 simple staining less costly than complicated molecular techniccs
Robust	YES	Autostainers, scanner, and digital pathology reduce the time to perform an Immunoscore
Reproducible	YES	Two strong membrane staining, with no background, allowing the numeration of individual cells
Quantitative	YES	Inter-observers variability is removed by the use of digital pathology, taking into account cell location and counts
Standardized	YES	Standardized operating procedure should be performed to insure reproducibility and worldwide comparisons
Pathology-base	YES	Necessity of pathologist expertise to validate cell type, cell location, and cell counts performed by digital pathology
Powerful	YES	The immunoscore has a prognostic value highly significant even in Cox multivariate including TNM classification[13]

Figure 2 Worldwide expert centers participating in the Immunoscore task force.

Conclusion

Prediction of clinical outcome in cancer is usually achieved by histopathological evaluation (AJCC/UICC-TNM classification) of tissue samples obtained during surgical resection of the primary tumor. However, it is now recognized that clinical outcome can significantly vary among patients within the same stage. The current classification provides limited prognostic information, and does not predict response to therapy. Recent literature demonstrated the importance of the host immune system in controlling tumor progression. Accumulating data, collected from large cohorts of human cancers, has demonstrated the impact of immune-classification, which has a prognostic value that may add to the significance of the current classification, and that has been demonstrated to be superior to the AJCC/UICC TNM-classification in colorectal cancer. It is therefore imperative to begin to incorporate the 'Immunoscore' into traditional classification, thus providing an essential prognostic and potentially predictive tool. Given the power of a proper immune evaluation of cancer patients, the Immunoscore is likely to be important for the field of cancer, beyond the field of tumor-immunology. In an effort to promote the Immunoscore in routine clinical settings, an international task force was initiated. The results of this international validation may result in the implementation of the Immunoscore as a new component for the classification of cancer, designated TNM-I (TNM-Immune). It is hoped that this effort will better define the prognosis of cancer patients, better identify patients at high-risk of tumor recurrence, to improve the quality of life by predicting and stratifying patients who will benefit from adjuvant therapies and, ultimately, to help save the lives of patients with cancer.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JG is coordinating this Immunoscore initiative, conceived the study, and wrote the manuscript. JG, FP initiated the Immunoscore project. FP, CL, AB, JG performed the initial experiments related to the Immunoscore. HKA participated in the drafting of the manuscript. FMM, TAG, BAF, JG from the SITC, initiated a task force and organized meetings on Immunoscore. PAA, from La Fondazione Melanoma Onlus organized initial meetings on Immunoscore. AL, CB, GB, FT, PD, AH, MA, LL, MM, FG, FP, FMM, BAF, JG were experts involved in the design of the immunoscore study, and expert pathologists participating to the inaugural Immunoscore workshop. MT, JPA, SO, GT, with their expertise, supported the Immunoscore initiative. GVM, SG, LH, CH, HSJ, CO, HZ, PSO, JODT, GP, MIN, RH, RL, AL, SNK, TF, BAF, JG, were experts participating to the WIC meeting and supporting the Immunoscore initiative. FP, AL, IZ, AB, CB, GB, FT, LC, PD, AH, MA, MM, FVV, LL, FG, PSO, PAS, BAC, BGW, YK, SH, CL, PG, PW, NS, TT, KI, RP, IDN, YW, CDA, SK, FAS, PAA, BAF, JG are expert participants of the initial worldwide Immunoscore task force study. All authors read and approved the final manuscript.

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ORIGINAL RESEARCH

High expression of epithelial cellular adhesion molecule in peritoneal metastasis of gastric cancer

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Abstract Intraperitoneally administrated epithelial cellular adhesion molecule (EpCAM) monoclonal antibody is a therapeutic agent in patients with malignant effusion in several types of carcinoma. However, the role of EpCAM in peritoneal metastasis (PM) lesions and primary lesions of gastric cancer (GC) is still unclear. Therefore, in this study, we investigated EpCAM expression in GC patients with PM. We investigated the expression of EpCAM in 35PM lesions and 104 biopsy samples as primary lesions. Immunohistochemical staining was performed using the Ventana Benchmark XT (Roche Diagnostics) system. EpCAM expression was evaluated by calculating the total immunostaining score, which is the product of the proportion score and the intensity score. Overexpression was defined as a total score greater than 4. All PM specimens showed overexpression of EpCAM, and GC cells in both the surface layer and the deep layer of the PM showed a high expression of EpCAM. Meanwhile, in the biopsy sample, the expression of EpCAM ranged from none to strong. The EpCAM score results for PM specimens and biopsy samples were 11.0±2.0 and 6.9±3.9, respectively. The difference between the scores was statistically significant ($P<0.05$). The intraperitoneally administrated EpCAM antibody might have a anti-cancer effect in PM lesions of GC. Additionally, it can be assumed that only GC cells which express a high level of EpCAM might metastasize to the peritoneum.

Keywords Gastric cancer · Peritoneal metastasis · Epithelial cellular adhesion molecule (EpCAM) · Target therapy

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Introduction

Gastric cancer (GC) is the second most common cause of cancer-related death worldwide [1]. Although surgery is the only curative procedure for localized advanced GC, for metastatic or recurrent GC patients, chemotherapy is the only therapeutic approach.

Recently, a number of new drugs to treat GC have become available. Unfortunately, these agents are not particularly effective, resulting in a high recurrence rate, a low survival rate, and a poor prognosis for metastatic or recurrent GC patients [2]. Additionally, GC patients with peritoneal metastasis (PM) have lower survival rates than other GC patients. In a multicenter prospective study, the median survival time was only 3.1 months for GC patients with PM [3]. Thus, another type of treatment for GC patients, particularly those with PM, is required. For example, target therapies that are associated with the expression of a particular gene may open up a new avenue for cancer treatments.

Table 1 Clinicopathological features of patients

Clinicopathological factors	No. of cases
Gender	
Males	25
Females	10
Average age (range), years	58.6 (22–75)
Borrmann type	
I	0
II	1
III	14
IV	20
Laurens system	
Intestinal type	8
Diffuse type	27
Number of biopsy samples	104

For histopathology typing, gastric cancers were classified as being intestinal or diffuse on the basis of the Laurens system

The epithelial cellular adhesion molecule (EpCAM) is a 39–42-kDa, 314-amino-acid type I transmembrane glycoprotein [4]. EpCAM is detected in the basolateral membrane of the majority of epithelial tissues, and overexpression of EpCAM has been demonstrated in a variety of epithelial cancers [5, 6].

EpCAM has been reported to have effects on cell adhesion, signaling, migration, proliferation, and differentiation, each of which are properties related to metastasis of several types of cancer [7]. In addition, an EpCAM monoclonal antibody, catumaxomab, has been licensed for clinical use in the European Union since 2009 for the intraperitoneal treatment of malignant effusion in patients with EpCAM-positive cells where standard therapy is not available or no longer feasible. Heiss et al. have reported that catumaxomab conferred a puncture-free survival in a prospective randomized phase II/III trial [8]. Furthermore, a subsequent analysis of the report by Heiss et al. revealed that catumaxomab had a significant overall survival benefit to GC patients [9]. However, the expression of EpCAM on the primary lesions and PM lesions of GC is still unclear. Therefore, in this study, we investigated EpCAM expression in GC patients with PM.

Materials and methods

Surgical specimens

Biopsy samples and specimens of PM were obtained from 35 GC patients during upper gastrointestinal endoscopy and staging laparoscopy conducted in our department between 2008 and 2011. All GC patients lacked non-curative factors, such as distant metastasis to liver, lung, or lymph nodes except for PM. In accordance with the Department of Surgery Kinki University Faculty of Medicine policy, written informed consent was obtained from the patients at the time of initial treatment.

Initial treatment

The initial treatment of these patients consisted of single intraperitoneal administration of paclitaxel followed by sequential systemic chemotherapy with S-1 plus paclitaxel. The details of the treatment regimen were described previously [10].

Immunohistochemical study

All sections were placed on the Ventana Benchmark XT (Roche Diagnostics) for detection of the EpCAM oncoprotein. The sections were dewaxed and then subjected to pretreatment with cell conditioning 1 solution (Roche Diagnostics) for 30 min. Sections were then washed with reaction buffer followed by incubation with the mouse monoclonal primary antibody EpCAM (0.1 μg/mL, Vu1D9, Cell Signaling Technology, USA) for 32 min. On-board detection using ultraView Universal DAB kit (Roche Diagnostics), used in accordance with the manufacturer's instructions, was used to detect the location of the primary antibody EpCAM.

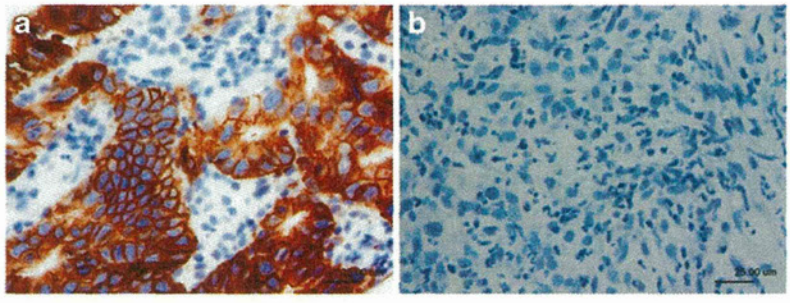

Fig. 1 EpCAM expression in a biopsy sample of gastric cancer. **a** Strong reactivity of EpCAM was visible in most gastric cancer cell membranes in biopsy samples. A representative samples with a score of 12 is shown. **b** Representative sample of gastric cancer cells in a biopsy sample with no reactivity of EpCAM (scored as 0). *EpCAM* epithelial cellular adhesion molecule

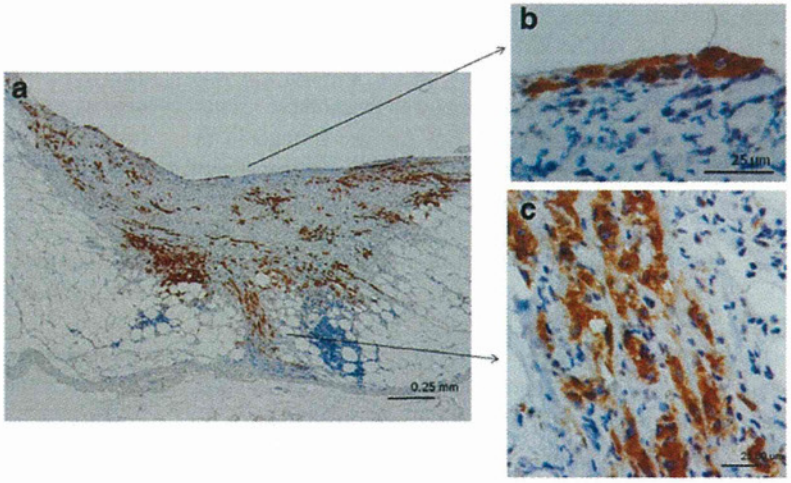

Fig. 2 EpCAM expression of gastric cancer cells in a peritoneal metastasis lesion. **a** High expression of EpCAM is observed in most gastric cancer cells in the peritoneum, scored as 12. **b** Gastric cancer cells show a high expression of EpCAM in the surface layer of the peritoneum. **c** Gastric cancer cells also show a high EpCAM expression in the deep layer of the peritoneum. EpCAM reactivity shows the membrane and cytoplasm of tumor cells. *EpCAM* epithelial cellular adhesion molecule

Immunohistochemical analysis

EpCAM expression was evaluated by calculating the total immunostaining score, which was defined as the product of the proportion score and the intensity score. EpCAM expression was evaluated by the following formula [11]: the proportion score described the estimated fraction of positively stained tumor cells (0, none; 1, <10 %; 2, 10–50 %; 3, 50–80 %; 4, >80 %). The intensity score represented the estimated staining intensity (0, no staining; 1, weak; 2, moderate; 3, strong). The total score ranged from 0 to 12. EpCAM overexpression was defined as a total score greater than 4 [12].

Statistical analyses

The statistical software GraphPad Prism 5 (GraphPad Software Inc, USA) was used to analyze data by Fisher's exact test. A difference of $P<0.05$ was considered as significant.

Results

Patient characteristics

The patients had a median age of 58.6 years (range 22–75 years). There were ten female and 25 male patients. Borrmann III and IV types accounted for the majority. The details of the main clinicopathological features of patients are presented in Table 1. The median survival time of the 35 patients was 23.4 months.

Expression of EpCAM in biopsy samples of gastric cancer

EpCAM expression in 104 biopsy samples from 35 GC patients was determined with immunohistochemical staining. On average, we investigated 2.97 biopsy samples per patient. EpCAM was located on the membrane of GC cells. We observed a diverse range of EpCAM expression intensities. The staining scores of EpCAM ranged from 0 to 12, with an average score of 6.9±3.9. Eighty samples showed overexpression of EpCAM. Figure 1a, b shows representative cases.

Expression of EpCAM in PM of gastric cancer

EpCAM expression in 35PM specimens from 35 GC patients was determined with immunohistochemical staining. EpCAM was located not only on the membrane; diffuse staining was also found in the cytoplasm. Strongly positive-staining tumor cells were found in both the surface layer and the deep layer of the peritoneum. The resulting staining scores of EpCAM ranged from 8 to 12, with an average score of 11.0±2.0. All PM specimens were classified as having EpCAM-overexpressing tumors. Figure 2 shows a representative case.

A significant difference in immunoreactive intensity and average staining score of EpCAM was found between the PM specimens and the biopsy samples ($P<0.05$; Table 2).

Discussion

Between 70 and 100 % of tumor cells in malignant effusions from gastric, ovarian, breast, and colorectal cancer have

Table 2 Overexpression of EpCAM in PM lesions and biopsy samples

	EpCAM overexpression		*P*
	Positive	Negative	
PM lesions	35	0	0.004
Biopsy samples	80	24	

EpCAM epithelial cellular adhesion molecule, *PM* peritoneal metastasis

been found to express EpCAM [13–15]. However, the expression of EpCAM in PM lesions has not been defined. In our study, all specimens of PM with GC showed EpCAM overexpression. This is the first report to reveal these results.

In our study, the expression of EpCAM was stronger in the PM lesions than in the primary lesions. The expression of EpCAM in primary lesions was investigated in biopsy samples. The biopsy samples showed a wide range of EpCAM expression. Conversely, in the PM lesions, almost all GC cells showed a strong EpCAM expression. Furthermore, in vitro studies of EpCAM showed enhanced cell proliferation independent of c-myc and cyclin D_1/E [16, 17].

Additionally, it was reported that EpCAM negatively modulated cadherin-mediated cell adhesion by disruption of the link between α-catenin and F-actin [18]. Furthermore, EpCAM loosened the tight junctions between cells and modulated proliferation, differentiation, and tissue maintenance [19]. Similar phenomena have already been confirmed in breast and renal cancer [19]. In gastric cancer, overexpression of EpCAM might also disrupt cell–cell contact, enabling the cellular migration that is required for metastasis [19]. Thus, only GC cells whose proliferation was enhanced by EpCAM might metastasize to the peritoneum, as this is one of the most frequent metastatic sites of GC.

GC patients with PM have poorer survival outcomes than other GC patients [3]. To improve the survival rate of GC patients with PM, multidisciplinary methods, including intraperitoneal chemotherapy, hyperthermia, and aggressive surgery, have been used to treat PM [20] [21]. However, these trials did not result in a satisfactory clinical outcome. One of the reasons that PM resists multidisciplinary therapy is due to the stem cell characteristics of the cancer cells. Cancer stem cells are responsible for cancer relapse as they are resistant to conventional cancer therapy, such as chemotherapy and radiation [22, 23]. In our results, all PM specimens showed EpCAM overexpression. EpCAM expression is a biologically and clinically relevant characteristic of cancer stem cells from primary GC tissue [24].Therefore, GC cells in PM lesions may have stem cell-like characteristics. The very poor clinical outcomes in GC patients with PM are consistent with these findings.

To improve treatment outcomes of GC with PM, antibody-based cancer therapies are required. Catumaxomab, which is specific for the EpCAM target antigen, is used to treat cancer patients with malignant ascites in the European Union. The clinical benefit of catumaxomab administered by the intraperitoneal route was demonstrated by prospective randomized phase II/ III trials [8]. The antibody can deliver a deadly signal to the cancer cell only by binding to the surface target. However, it seems that the unsatisfactory antitumor effect of catumaxomab on disseminated lesions in the peritoneum is due to the limited penetration of intraperitoneal catumaxomab into the peritoneal surfaces. Additionally, in our study, GC cells in PMs that expressed EpCAM were present not only in the surface layer but also in the deep layer of the peritoneum. Therefore, intraperitoneally administered catumaxomab may only be effective to treat cancer cells in malignant ascites and in the surface layer of the peritoneum.

To further improve treatment outcomes, the investigation of combination therapies comprising systemic chemotherapy plus intraperitoneal catumaxomab and/or intravenously administered catumaxomab may be necessary. Further investigations are required in the future.

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ORIGINAL RESEARCH

Peritoneal metastatic lesions of gastric cancer exhibit low expression of human epidermal growth factor receptor 2

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Abstract The prognosis of gastric cancer patients with peritoneal metastasis is very poor. Recent findings suggest that use of trastuzumab, a monoclonal antibody-based agent that targets human epidermal growth factor receptor 2 (HER2), may improve the prognosis of gastric cancer patients with HER2 overexpression and/or gene amplification. However, whether these mechanisms of HER2 upregulation are present in gastric cancer patients with peritoneal metastasis is unclear. The status of HER2 expression in a cohort of samples obtained from 35 gastric cancer patients with peritoneal metastasis was investigated using immunohistochemistry and fluorescence in situ hybridization. In 18 cases, we also investigated the influence of induction chemotherapy on HER2 overexpression. The frequency of HER2 overexpression and gene amplification was 2.9 % (1/35) in peritoneal metastatic lesions. There was concurrence in HER2 status in the samples examined prior to and following induction of chemotherapy. Most samples from the gastric cancer patients with peritoneal metastasis did not show HER2 amplification and/or overexpression. Although our study size was small, these results suggest that trastuzumab, which is critically dependent on HER2 expression, might not be an effective agent for these patients. Consequently, other therapeutic approaches for these patients must be developed.

Keywords Gastric cancer · Peritoneal metastasis · Human epidermal growth factor receptor 2 · Trastuzumab

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Introduction

Gastric cancer is the second most common cause of cancer death worldwide. About 1 million people will be diagnosed with gastric cancer per year, and around 700,000 people annually die from their illness [1]. One of the most frequent causes of death from gastric cancer is peritoneal metastasis [2]. Based on the findings of a multicenter prospective study, the median survival time for gastric cancer patients with peritoneal metastasis is around 3.1 months [2].

In a recent randomized, prospective, multicenter clinical Phase III trial, the ToGA trial, efficacy and safety of trastuzumab (a humanized monoclonal anti-human epidermal growth factor receptor 2 (HER2) antibody) for the treatment of HER2-positive gastric cancer patients were evaluated [3]. The findings of this trial showed that trastuzumab conferred an overall survival benefit and was considered a well-tolerated treatment for HER2-positive gastric cancer patients. However, the ToGA trial included patients with peritoneal metastasis, in addition to patients with inoperable locally advanced, recurrent, or metastatic gastric cancer or gastroesophageal cancer. Therefore, the benefits of trastuzumab for gastric cancer patients with peritoneal metastasis remained unclear.

The clinical efficacy of trastuzumab is crucially dependent upon the expression of HER2. Therefore, in this study, we investigated HER2 overexpression and gene amplification in peritoneal metastatic samples obtained from gastric cancer patients.

Materials and methods

Surgical specimens

Peritoneal metastatic samples were obtained from 35 gastric cancer patients at the time of staging laparoscopy between 2008 and 2011. The patients did not have metastases at any other sites (e.g., liver, lung, and lymph nodes). We also investigated 18 primary gastric lesions that were obtained at the time of surgery after the initiation of S-1 (an oral fluoropyrimidine derivative consisting of tegafur, gimestat (which has dihydropyrimidine dehydrogenase-inhibiting activity), and otastat potassium)-based induction chemotherapy. Primary gastric lesions were compared with biopsy samples obtained before induction chemotherapy. In accordance with the policies of the Department of Surgery at Kinki University Faculty of Medicine, written informed consent was obtained from the patients at the time of surgery.

Immunohistochemistry

Freshly resected tissues were fixed overnight at 4 °C in 4 % paraformaldehyde diluted in 0.1 M PBS. The samples were then dehydrated in a series of graded alcohol solutions and embedded in paraffin. Finally, 4-μm thick serial sections were processed for immunohistochemistry, in addition to routine H&E staining.

All sections were placed on the Ventana Benchmark XT (Roche Diagnostics, Tokyo, Japan) system for detection of the HER2 oncoprotein. The sections were dewaxed and then subjected to pretreatment with cell conditioning 1 solution (Roche Diagnostics) for 30 min. The sections were then washed with a reaction buffer followed by digestion with a Protease 1 (Roche Diagnostics) solution for 8 min. The sections were washed again with the reaction buffer and incubated with HER2 mouse monoclonal primary antibody (3.3 μg/ml, Clone SV2-61γ; Nichirei, Tokyo, Japan) for 28 min. On board detection using the ultraView Universal DAB kit (Roche Diagnostics) in accordance with the manufacturer's recommendations was employed to visualize HER2 expression.

Immunohistochemical analysis

Analysis of immunohistochemical findings included evaluation of intensity and staining pattern of HER2 within the tumor cells. As in the ToGA trial [3], scoring for HER2 staining was based on four categories: no staining, or weak staining in fewer than 10 % of the tumor cells (0); weak staining in part of the membrane in more than 10 % of the tumor cells (1+); complete staining of the membrane with weak or moderate intensity in more than 10 % of the cells (2+); and strong staining in more than 10 % of the cells (3+).

Table 1 Clinicopathological information

		Number
Sex	Male	25
	Female	10
Average age (range; years)		58.6 (22–75)
Borrmann type	I	0
	II	1
	III	14
	IV	20
Laurens system type	Intestinal	8
	Gastric	27

Fluorescence in situ hybridization

On the basis of the methodology used in the ToGA trial, the cases that scored 2+ in the immunohistochemical analysis were also examined with fluorescence in situ hybridization (FISH). The HER2 gene was amplified with dual-color FISH probe using a Passvision HER2 DNA probe kit (Vysis, Inc.; Downers Grove, IL, USA) in accordance with the manufacturer's instructions.

Fluorescence in situ hybridization analysis

An image of the region of interest was captured using a CCD camera (ACT-2U; Nikon Corporation, Tokyo, Japan).

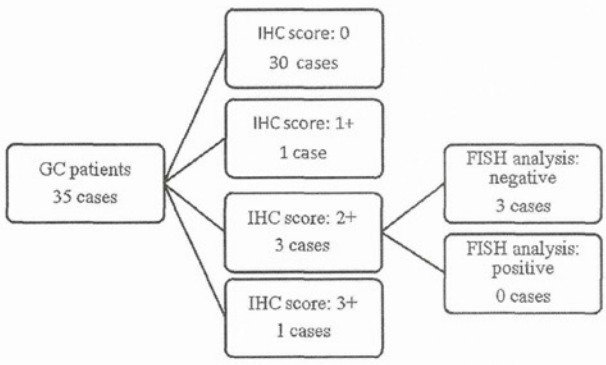

Fig. 1 HER2 overexpression patterns in samples of peritoneal metastatic lesions. HER2 overexpression and gene amplification overexpression were observed in the peritoneal metastatic lesions of only one patient (2.9 %). *HER2* human epidermal growth factor receptor, *GC* gastric cancer, *IHC* immunohistochemistry, *FISH* fluorescence in situ hybridization

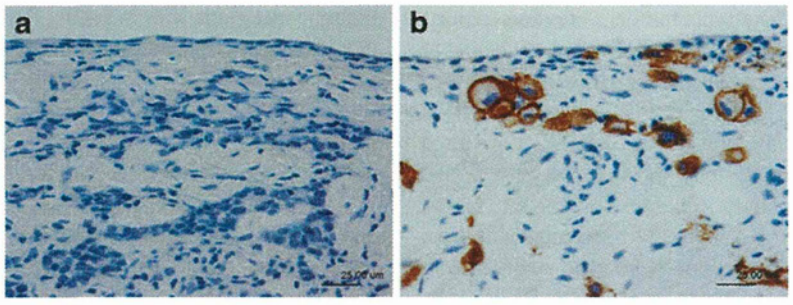

Fig. 2 Representative images demonstrating HER2 expression in peritoneal metastatic specimens from patients with gastric cancer. **a** Tumor rated 0, which shows no HER2 expression in the tumor cells. **b** Tumor rated 3+, showing strong HER2 expression at the tumor cell membrane. No reactivity in activated mesothelial cells was reported. *HER2* human epidermal growth factor receptor 2

A cell was considered to be amplified when a definite cluster or more than ten signals for HER2 was found. Known positive and negative cells were used as controls. Gene amplification was scored when a minimum of 20 cancer cell nuclei exhibited a HER2/CEP17 ratio of greater than 2, or when a HER2 signal cluster was observed.

Results

Patient characteristics

The main clinicopathological features of the patients are presented in Table 1. Borrmann type IV and diffuse forms of gastric cancer accounted for the majority.

Expression of HER2 protein in gastric cancer

HER2 expression status in 71 gastric cancer specimens (35 peritoneal metastatic lesions, 18 primary gastric lesions, and 18 biopsy samples) was determined using immunohistochemistry. The peritoneal metastatic lesions from gastric cancer patients were characterized as follows: 30 cases (85.7 %) were rated 0, one case (2.9 %) was rated 1+, three cases (8.6 %) were rated 2+, and one case (2.9 %) was rated 3+ (Fig. 1). A representative case is shown in Fig. 2.

With respect to the 18 primary gastric lesions that were obtained at surgery after induction chemotherapy, the HER2 expression status in this group was classified according to the following: 15 cases (83.3 %) were rated 0 and the three remaining cases were rated 1+, 2+, and 3+, respectively (Fig. 3). These results were totally consistent with the HER2 expression status of the biopsy samples.

HER2 gene amplification in gastric cancer

As a follow-up, specimens that rated 2+ for HER2 expression were also analyzed by FISH. Three specimens were analyzed. No sample showed HER2 gene amplification. Finally, the frequency of HER2 overexpression and gene amplification in the peritoneal metastatic lesions was 2.9 % (1/35); this lesion was diffuse type.

Discussion

According to our data, the incidence of HER2 of peritoneal metastatic lesions was very low, with 77.1 % (27/35) of the

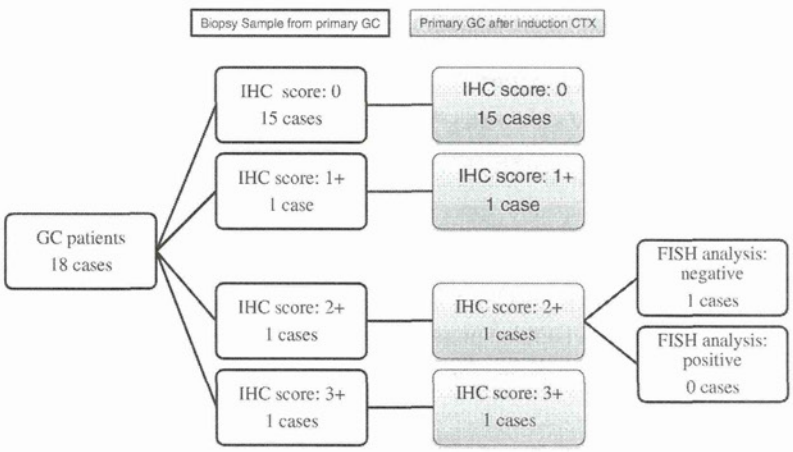

Fig. 3 Influence of HER2 overexpression after induction of chemotherapy. HER2 overexpression and gene amplification overexpression were consistent before and after chemotherapy, as assessed by FISH analysis. *HER2* human epidermal growth factor receptor, *GC* gastric cancer, *IHC* immunohistochemistry, *FISH* fluorescence in situ hybridization

patients exhibiting diffuse-type tumors. Previous reports have indicated that gastric cancers classified as being of the intestinal type are more likely to be HER2-positive than diffuse-type tumors [4] [5]. Our results are consistent with these data. In addition, the prevalence of HER2 expression in diffuse type in the ToGA study was found to be 6 %, and therefore similar to our study [6].

With respect to the ToGA study, 27 % patients who were rated 2+ for HER2 overexpression were gene amplification-positive. In our study, no patients rated 2+ for HER2 overexpression were also found to be positive for HER2 gene amplification. The differences between the ToGA trial findings and the results of our study may be attributed to the histological subtypes reviewed in each study. In the ToGA trial, 75 % of patients had intestinal-type tumors; in contrast, in our study, only 22.9 % of patients had intestinal-type tumors. It has been noted that diffuse-type gastric cancers frequently metastasize to the peritoneum. Thus, on the basis of our findings, it seems likely that gastric cancer with peritoneal metastasis is not associated with HER2 overexpression and/or gene amplification.

The effects of targeted therapy on HER2 expression have been explored in other settings and in other tumor types. For instance, Taucher et al. reported that epirubicin and docetaxel administration as a neoadjuvant therapy for primary breast cancer is not associated with significant changes in HER2 expression [7]. In contrast, in a study of ovarian cancer, Nijman et al. reported an increase in HER2 expression following platinum-based chemotherapy, although their findings were not statistically significant [8]. In the present study, we examined HER2 overexpression in biopsy samples that were obtained before induction chemotherapy and in primary gastric lesions obtained during surgery of 18 gastric cancer patients with peritoneal metastasis that had undergone induction chemotherapy. Our data showed a strong concordance in HER2 expression both before and after chemotherapy.

The findings of the ToGA trial suggested that a monoclonal antibody that targets HER2 may improve the prognosis of advanced and/or recurrent gastric cancer patients with HER2 overexpression and/or gene amplification [3]. Although our study size was small, few gastric cancer patients with peritoneal metastasis showed HER2 overexpression and/or gene amplification. These results suggested that trastuzumab may not be the most effective treatment strategy for gastric cancer patients with peritoneal metastasis. On the basis of these findings, other therapeutic approaches for gastric cancer patients with peritoneal metastasis must be developed and investigated. However, our presented data for the subgroup of gastric cancer with peritoneal metastasis alone indicates that the prevalence of HER2 expression is very low. This does not preclude a higher HER2 expression rate in cases with multiple metastatic sites including the peritoneum. Thus, further investigations are required in the future.

Conclusions

In conclusion, this is the first study to report that almost all gastric cancer patients with peritoneal metastasis examined did not exhibit HER2 amplification and/or overexpression. Therefore, another type of target therapy should be considered for treatment of gastric cancer patients with peritoneal metastasis.

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Conflict of interest The authors declare that they have no conflict of interest.

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