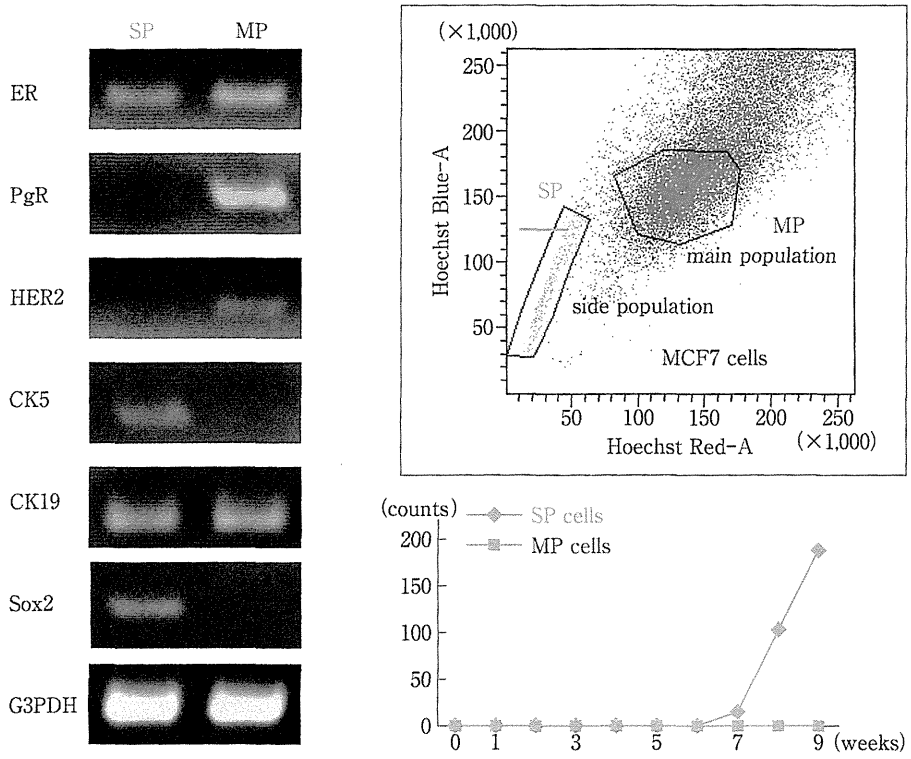

図2 乳がん幹細胞の分離と遺伝子発現

乳がん細胞株MCF7細胞をHoechst染色し，side population（SP）とmain population（MP）を分離した（右上パネル）．SP細胞をNOD/SCIDマウスに移植すると，MP細胞と比較して高い造腫瘍能を示した（右下パネル）．SP細胞とMP細胞の遺伝子発現プロファイルをRT-PCR法にて比較解析した．SP細胞は，HER2（−），PgR（−），ER（+/−），CK5（+）のbasal cell形質を示す．また，Sox2のような幹細胞遺伝子を発現している（左パネル）．

染色性細胞群はside population（SP）と呼ばれ，この中にがん幹細胞も濃縮されることが知られている．2004年，このSP法を用いて乳がん細胞株SK-BR-3からがん幹細胞が分離された[6]．著者らもMCF7細胞からSP細胞を分離し，cancer initiating cellとしての形質を示し，遺伝子発現プロファイルを比較した（**図2**）．

3）ALDH1活性

ALDH1（aldehyde dehydrogenase 1）はアルデヒドを酸化する解毒酵素の一種であるが，レチノールの酸化を介して幹細胞の分化にも関与している．正常造血幹細胞や前駆細胞は高いALDH1活性をもつことが知られていた．ALDH1活性に応じて蛍光を発する試薬としてALDEFLUOR試薬が開発され，これによってALDH1活性の高い幹細胞をソーティングすることが可能となった．2007年，Ginestierらは培養細胞株ではなく，ヒト乳がん組織から分離した乳がん細胞を用いて，ALDEFLUOR（+）細胞群が自己複製能と多分化能をもつ乳がん幹細胞を含み，500個の細胞移植によって腫瘍を作ることを示し，更にALDH1（+）CD44（+）CD24（−）細胞群はわずか20個の細胞移植によって腫瘍を形成することを示した[7]．

4）Sphere形成能

幹細胞は足場非依存性に生存・増殖する能力をもち，適切な条件のもと，低付着性プレート上で培養すると，細胞浮遊塊（sphere）を形成する．乳腺幹細胞が形成するsphereはmammosphereと呼ばれ，幹細胞形質の一つの指標

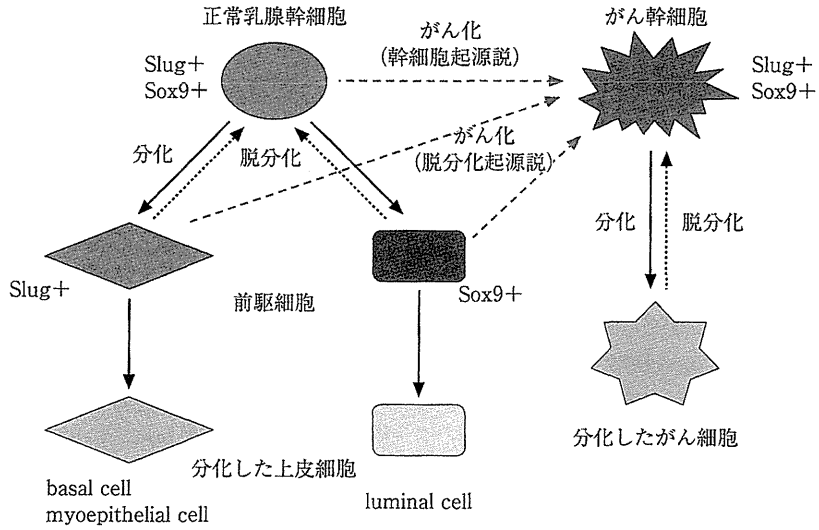

図3 正常乳腺細胞の分化・脱分化と乳がん幹細胞

乳がん幹細胞の起源は，①乳腺幹細胞ががん化した可能性(幹細胞起源説)，②分化した乳管上皮細胞あるいは基底細胞ががん化して脱分化を起こした可能性(脱分化起源説)，の2つの可能性が考えられる．

分化した乳管上皮細胞にSox9とSlugを共発現させると幹細胞の形質を獲得することから，分化した細胞がマスター転写因子の発現によって脱分化を起こし，幹細胞形質を獲得しうることが証明されている．

CD44(−)の分化した乳がん細胞のシングルクローンを培養すると，20％以上の細胞がCD44(+)がん幹細胞に戻ることから，がん幹細胞は高度の可塑性をもっていると推察されている．

とされる．このsphere形成能を指標として乳がん幹細胞を同定する試みも行われている．Pontiらは，sphereを形成する乳がん細胞を分析し，CD44(+)CD24(−/low)分画を豊富に含み，1,000個の細胞移植で腫瘍を形成したことを報告している[8]．

3 乳がん幹細胞の起源

幹細胞様形質と高い造腫瘍能を有する乳がん幹細胞の起源についてはいまだ明らかになっていない．乳腺は乳管上皮細胞(luminal cell)と，それを取り囲む筋上皮細胞および基底細胞(basal cell)によって構成され，これらは乳腺幹細胞から分化する[9]．乳がん幹細胞が乳腺幹細胞と同様の形質をもっているという理由から，乳がん幹細胞の起源を乳腺幹細胞に求める説はあるが，その直接的な証明はなされていない．現在のところ，乳がん幹細胞の起源については，①乳腺幹細胞に遺伝子変異またはエピゲノム変化が蓄積してがん化した可能性(幹細胞起源説)，②分化した乳管上皮細胞あるいは基底細胞に遺伝子変異またはエピゲノム変化が蓄積してがん化し，脱分化を起こして幹細胞様形質を獲得した可能性(脱分化起源説)，の2つの可能性が考えられる(**図3**)．例えば，若い頃に妊娠を経験すると乳がんのリスクが下がることが知られている．これは妊娠により乳腺幹細胞がさかんに分化することにより，若い段階で乳腺幹細胞の数が減少しがんの発生母地が減少するためと考えられ，幹細胞起源説を支持する．また，Sox2，Oct4などの幹細胞遺伝子を分化した細胞に導入すると多能性幹細胞(iPS細胞)が発生すること，また最近Weinbergらが報告したように，分化した乳管上皮細胞にSox9とSlugを共発現させると幹細胞の形質を獲得することか

らも[18]，分化した細胞がわずか1-2種類のマスター転写因子の発現によって幹細胞形質を獲得しうることは明らかである．これらの事実は脱分化起源説を支持している(**図3**)．

乳がんは遺伝子発現パターンによって，①luminal type A，②luminal type B，③basal type，④ER陰性/HER2陽性，⑤normal-likeの5つのサブタイプに分類されるが，それぞれのサブタイプに特有のがん幹細胞が存在するのか，それらのがん幹細胞は異なる起源をもっているのか，それとも共通の起源をもち異なる分化を遂げているのか，今後の解明が待たれるが[10]，恐らくいずれの説も正しいのではないかと思われる．

もう一つの大きな課題は，がん幹細胞から分化したがん細胞は，脱分化してがん幹細胞形質を再獲得するかどうかという問題である．Weinbergらは，CD44(−)の分化乳がん細胞のシングルクローンを培養すると，20％以上の細胞がCD44(＋)がん幹細胞に戻ると報告し，がん幹細胞は高度の可塑性をもっていると推察されている．著者らの研究では，がん細胞の種類によって脱分化しやすい細胞と可塑性の低い細胞とがあることがわかりつつある．問題は，どのような刺激や遺伝子転写因子の発現がその可塑性を制御しているのか，その分子機序を解明しなければならない．低酸素環境ではより多くの幹細胞様細胞が誘導される．また著者らは，がん細胞にある種の物理的ストレスを加えるとSP細胞が増加し，造腫瘍能が高まることを確認している．恐らく，ストレス刺激はがん幹細胞への脱分化を促進する刺激の一つとなっていると思われる．脱分化の分子機構もがん幹細胞治療の標的として期待される．

4 乳がん幹細胞性維持の分子メカニズムと分子標的

正常幹細胞の維持・分化メカニズムの解明と連動して，がん幹細胞の幹細胞性維持メカニズムも明らかになりつつある．重要なのは，がん幹細胞と正常幹細胞との違いを明らかにし，それをもとにがん幹細胞を選択的に障害する治療戦略を立てることであろう．乳がん幹細胞には，Wnt/β-cateninシグナルや，Hedgehogシグナルなど，他臓器由来のがん幹細胞(大腸癌，造血器腫瘍など)と共通した幹細胞性維持機構のほかに，上皮間葉移行(EMT)のような乳がん幹細胞に特徴的な分子メカニズムも明らかになってきた(**図4**)．

1) Akt/β-cateninシグナル

Korkayaらのグループは，多くのがんでPTENがん抑制遺伝子に変異がみられることからPTENが制御するAkt/β-cateninシグナルに着目した[11]．彼らはPTENを抑制することにより，Aktの活性化およびその下流のGSK-3βのリン酸化，β-cateninの活性化を誘導し，幹細胞マーカーであるALDH1の発現上昇を認めた．Akt阻害薬(perifosine)によって，その下流のβ-catenin活性化を抑制すると，乳がん幹細胞の造腫瘍能が抑制された．Akt/β-cateninシグナルが乳がん幹細胞性維持に重要な役割を担うことを示す結果と考えられ，乳がん幹細胞標的治療として期待される．

このことは，これまで乳がんの有効な治療法の一つとして知られているハーセプチンの有効性にも関与すると考えられる．ハーセプチンは受容体型チロシンキナーゼHER2を標的とする分子標的治療薬であり，HER2の下流で活性化されるPI3KおよびAktを抑制する．Akt/β-cateninが乳がん幹細胞性に重要な役割を担う分子であるならば，ハーセプチンも乳がん幹細胞標的治療に有効である可能性がある．実際に，HER2の過剰発現がないluminal typeの乳がんに対してアジュバントセラピーとしてハーセプチンを用いた場合，骨転移の頻度を抑制したとの報告がある．

Akt/β-cateninシグナルに関しては，血管新生阻害薬と乳がん幹細胞との関連性において興味深い報告がなされている[12]．がんが増殖するためには新生血管からの栄養および酸素供給が必要であるとの考え方から，血管新生阻害薬が誕生した．この治療薬は，がんに特有の腫瘍内血管新生を標的にしているため，がん特異性が

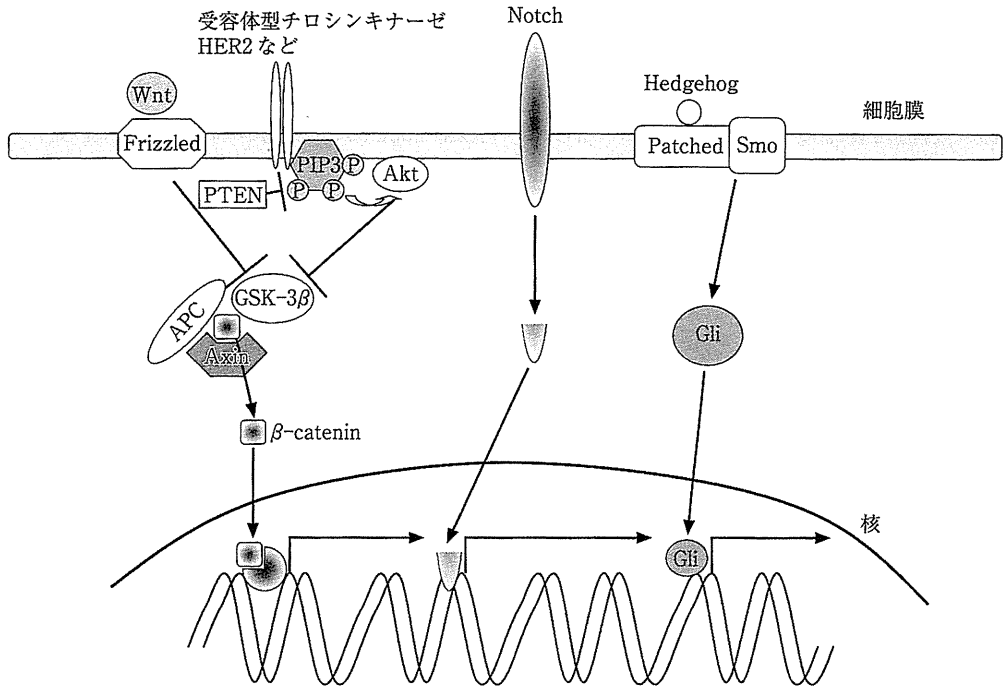

図4 乳がん幹細胞を支持するシグナル伝達

乳がん幹細胞では様々なシグナル伝達が活性化している．Wnt 刺激は Wnt レセプター(Frizzled)を介して GSK-3β を不活化し，β-catenin/TCF の転写を活性化する．HER2 などの受容体型チロシンキナーゼは Akt 活性化を介して β-catenin を活性化する．

リガンド(DLL など)に結合した Notch レセプターはその一部が切断され，核内に移行し，転写を活性化する．

Hedgehog がレセプター(Patched)に結合すると，Gli が核内に移行し転写を活性化する．

これらのシグナルによる遺伝子転写因子の活性化によって，乳がん幹細胞の幹細胞性に重要な遺伝子の転写が誘導されていると考えられる．

高いと期待されている．スニチニブやベバシズマブなどの血管新生阻害薬を投与されたマウスの腫瘍局所では，低酸素反応性転写因子 HIF1α が活性化し，HIF1α 依存性に ALDH1(+)乳がん幹細胞が増加することが示された．また同時に，HIF1α 依存性に PI3K/Akt/β-catenin シグナルも活性化していた．この結果は，前述したようにストレス刺激とがん幹細胞の可塑性を示唆しており，血管新生阻害治療の問題点を提起していると考えられる．血管新生阻害薬と，Akt/β-catenin シグナル阻害薬との併用が抗腫瘍効果を高める可能性がある．

2) Notch シグナル

ショウジョウバエの翅に切れ込み(Notch)のある変異体の研究から Notch 受容体が発見された．Notch 受容体のヒトホモログは 4 種類知られており，その中でも Notch4 が乳がん幹細胞において高発現することが示されている[13]．乳がん細胞における Notch4 を short hairpin RNA (shRNA)で抑制すると，乳がん幹細胞の減少および造腫瘍能の抑制が観察された．このことは，Notch シグナルが乳がん幹細胞維持に重要な役割を担うことを示唆する．

最近の報告では，乳がん幹細胞において micro RNA の一つである miR-34c のプロモーター領域がメチル化され，miR-34c 発現が抑制されていることが示されている[14]．miR-34c は Notch4 を抑制する micro RNA で，乳がん幹細胞における miR-34c 発現低下が Notch4 発現上昇を誘導している可能性が考えられている．

3）Hedgehogシグナル

Liuらのグループは，乳腺幹細胞でHedgehogシグナル関連分子が高発現し，その下流で幹細胞の自己複製能に重要な分子として知られるBmi-1が発現することを観察している[15]．同様のシグナル活性化とBmi-1の過剰発現は乳がん幹細胞においても観察されており，がん幹細胞の幹細胞性維持にこれらが重要な役割を果たしていると思われる．現在では，Hedgehogシグナルを標的とする分子標的治療薬（cyclopamine）の臨床試験が行われている[16]．

4）上皮間葉移行（EMT）と乳がん幹細胞

上皮間葉移行（epithelial mesenchymal transition：EMT）は，細胞が上皮細胞形質から間葉系細胞形質に変化する現象で，胚形成や創傷治癒過程でみられる重要な生理現象である．上皮系のがん細胞においても，EMTによって間葉系細胞に類似した紡錘形細胞に変化し，遊走能亢進や浸潤能の亢進などの間葉系細胞形質を獲得する現象が知られおり，がんの浸潤や転移と密接にかかわるとされている．

2008年にWeinbergらにより，ヒト不死化乳腺細胞にEMTを誘導すると，vimentinを発現して間葉系細胞形質の獲得とともに幹細胞マーカーの発現が確認され，mammosphereを形成することが示された．乳がん細胞株において，EMTを誘導すると乳がん幹細胞様の形質を獲得することを報告し，乳がんにおけるEMTとがん幹細胞の関係がクローズアップされた[17]．この結果は，EMTと乳がん幹細胞には共通の制御機構が存在していることを示唆している．更に前述したように，同グループはEMTを誘導する転写因子の一つであるSlugを転写因子Sox9と共発現させることによって，分化した乳管上皮細胞を乳腺幹細胞に形質転換させることに成功した．また，乳がん幹細胞に発現しているSlugまたはSox9のいずれかをノックダウンすると幹細胞形質や造腫瘍能が失われることを証明した[18]．このことは，乳腺においてはEMTと幹細胞には共通の分子メカニズムが存在し，がん幹細胞治療における重要な標的となりうることを示している．

Shimonoらは，ヒト乳がん幹細胞の網羅的miRNA発現解析により，miR-200 familyが特異的に低下していることを報告している[19]．miR-200cはEMT誘導因子であるZEB1を抑制する作用をもっていることから[20]，乳がん幹細胞におけるmiR-200cの発現低下はEMTおよび乳がん幹細胞の幹細胞形質を誘導している可能性が考えられる．

このように乳がんにおいてはがん幹細胞とEMTとの間に密接な関連が証明されつつあるが，著者らの検討では大腸癌や肺癌では必ずしも関連性は見いだされなかった．乳腺組織に特有の現象ではないかと推察している．

5）微小環境と乳がん幹細胞

がん幹細胞の維持に腫瘍細胞周囲の微小環境が重要であることが知られている．幹細胞周囲の微小環境はニッチ（niche）と呼ばれ，微小血管，線維芽細胞などの間葉系成分，膠原線維のような細胞外基質，単球・マクロファージなどの免疫細胞から構成されている．

最近，ニッチを構成する間葉系細胞および免疫細胞から分泌されるケモカイン（IL-8，CXCL7）やサイトカイン（IL-6）が，乳がん幹細胞のSTAT3のシグナルや，Aktシグナルを活性化し，自己複製やストレス耐性などの幹細胞形質や造腫瘍能を亢進させる働きをしているとの報告がなされている[21]．これらがん幹細胞を維持する微小環境を標的とする治療法や薬剤も開発されつつある．

おわりに

がん幹細胞は，高い腫瘍形成能や抗がん剤耐性などの悪性形質を備えたがんの根源細胞と考えられ，がんの根治を目指すためにはがん幹細胞の正体を知りそれを克服することが重要な課題である．がん幹細胞性を維持する分子メカニズムが徐々に明らかとなりつつあり，乳がん幹細胞を標的とした分子標的治療などの治療法の開発が進みつつある．しかしながら，正常幹細胞の分子メカニズムとの共通点も多く，がん幹細胞を特異的に標的とする治療法の確立にはいまだ時間がかかるかもしれない．著者らのグル

ープは，正常細胞には発現がなくがん幹細胞にのみ発現するがん幹細胞特異抗原を同定し，細胞傷害性T細胞(CTL)によってがん幹細胞をねらい撃ちする免疫療法の基礎研究を行っており，期待できる結果を得つつある[22,23]．近い将来，他の分子標的治療も含めてがん幹細胞標的治療が現実になることを期待する．

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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

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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,

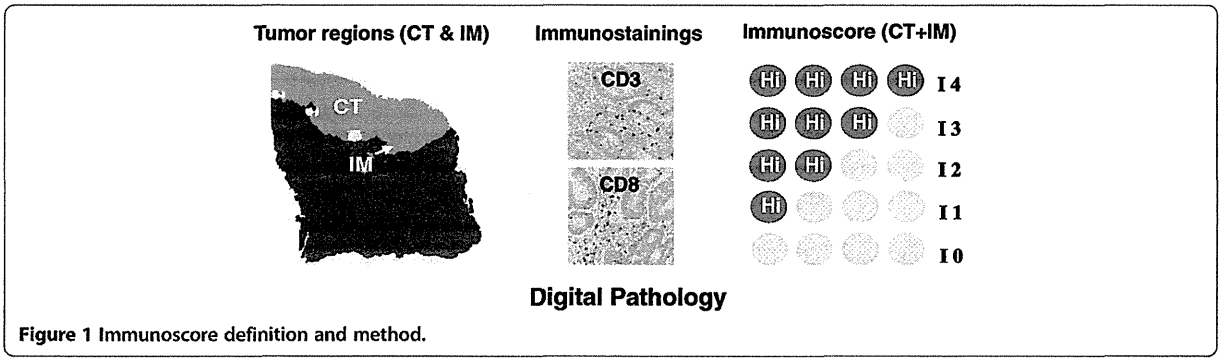

Figure 1 Immunoscore definition and method.

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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Cytotoxic T lymphocytes
Sniping cancer stem cells

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Keywords: cancer stem cell, immunotherapy, tumor antigen, CTL, antigenic peptide

Abbreviations: CSC, cancer stem-like cell; CIC, cancer-initiating cell; CTL, cytotoxic T lymphocyte; TAA, tumor-associated antigen

Cancer stem cells (CSCs)/cancer-initiating cells (CICs) are characterized as a small population of cancer cells that have high tumor-initiating ability. CSCs/CICs are resistant to several cancer therapies, and eradication of CSCs/CICs is essential to cure cancer. How can we eradicate CSCs/CICs? Cytotoxic T lymphocytes (CTLs) might be a promising answer.

Cancer stem-like cells (CSCs)/cancer-initiating cells (CICs) are defined as a small population of cancer cells that have (1) high tumor-initiating ability, (2) self-renewal ability and (3) differentiation ability (**Fig. 1A**).[1] In recent studies, CSCs/CICs have shown to be resistant to cancer therapies by their senescence state, high expression of transporters to efflux anti-cancer drugs, high expression of apoptosis inhibitors, low expression of reactive oxygen species.[2] Thus, the action of CSCs/CICs are regarded as major mechanisms of cancer recurrence, distant metastasis and treatment resistance. However, effective cancer treatment targeting CSCs/CICs effectively have not been reported so far.

The prominent nature of the acquired immune system is its antigen specificity due to antigen-specific receptors including T cell receptors and B cell receptors, and isolation of human tumor-associated antigens (TAAs) has enabled us to target caner cells specifically in an antigen-specific manner.[3] Cancer immunotherapy trials using TAAs have recently been performed in several facilities and significant results have been obtained.[4] However, it is still not clear whether the immune system can recognize therapy-resistant CSCs/CICs or not. Some reports on immunity and CSCs/CICs have recently been published, and natural killer (NK) cells and γδT cells have been shown to recognize CSCs/CICs derived from human colon cancer and gliomas; however CTLs, which are a major component of the acquired immune system, have not been characterized yet.[5]

We analyzed the relation between CTLs and CSCs/CICs.[6] We isolated CSCs/CICs from human colon cancer cells using a side population (SP) technique. Since CTLs recognize antigenic peptides derived from TAAs, we evaluated the expression of TAAs in colon CSCs/CICs and non-CSCs/CICs. Colon CSCs/CICs expressed *CEP55*, one of the TAAs, at the same level as did non-CSCs/CICs. In a further study, we evaluated the expression of several TAAs in both CSCs/CICs and non-CSCs/CICs, and we found that the expression pattern can be classified into the following groups (**Fig. 1B**, unpublished data): (1) **CSC/CIC antigens**, which are expressed in CSCs/CICs but not in non-CSCs/CICs (e.g., MAGE-A3 and MAGE-A4); (2) **shared antigens**, which are expressed in both CSCs/CICs and non-CSCs/CICs (e.g., CEP55, SURVIVIN); and (3) **non-CSC/CIC antigens**, which are expressed in only non-CSCs/CICs but not in CSCs/CICs (e.g., AMACR, HIFPH3). Therefore, CEP55 is one of the (2) shared antigens.

Since we have established CTL clone #41 which is specific for CEP55-derived antigenic peptide,[7,8] we evaluated the reactivity of CTL clone #41 for colon CSCs/CICs and non-CSCs/CICs. Interestingly, CTL clone #41 recognized both colon CSCs/CICs and non-CSCs/CICs at the same level in vitro. Furthermore, CTL clone #41 inhibited the tumor-initiating ability of colon CSCs/CICs in vivo. These findings clearly indicate that treatment-resistant colon CSCs/CICs, as well as non-CSCs/CICs are sensitive to CTLs. Therefore, CTL-based immunotherapy is a promising approach to target CSCs/CICs.

In the next stage, another question has emerged. Which are the best TAAs for CSC/CIC-targeting cancer immunotherapy: (1) CSC/CIC antigens, (2) shared antigens or (3) non-CSC/CIC antigens? Non-CSC/CIC antigens do not seem to be suitable for targeting CSCs/CICs since they are not expressed in CSCs/CICs. Further analyses are under way to address thes questions, and we have found that targeting CSC/CIC antigens was more effective than targeting shared antigens in a CTL adoptive transfer model and a DNA vaccination model (unpublished data). Both CSC/CIC antigens and shared antigens are expressed in CSCs/CICs; however, the anti-tumor effects are

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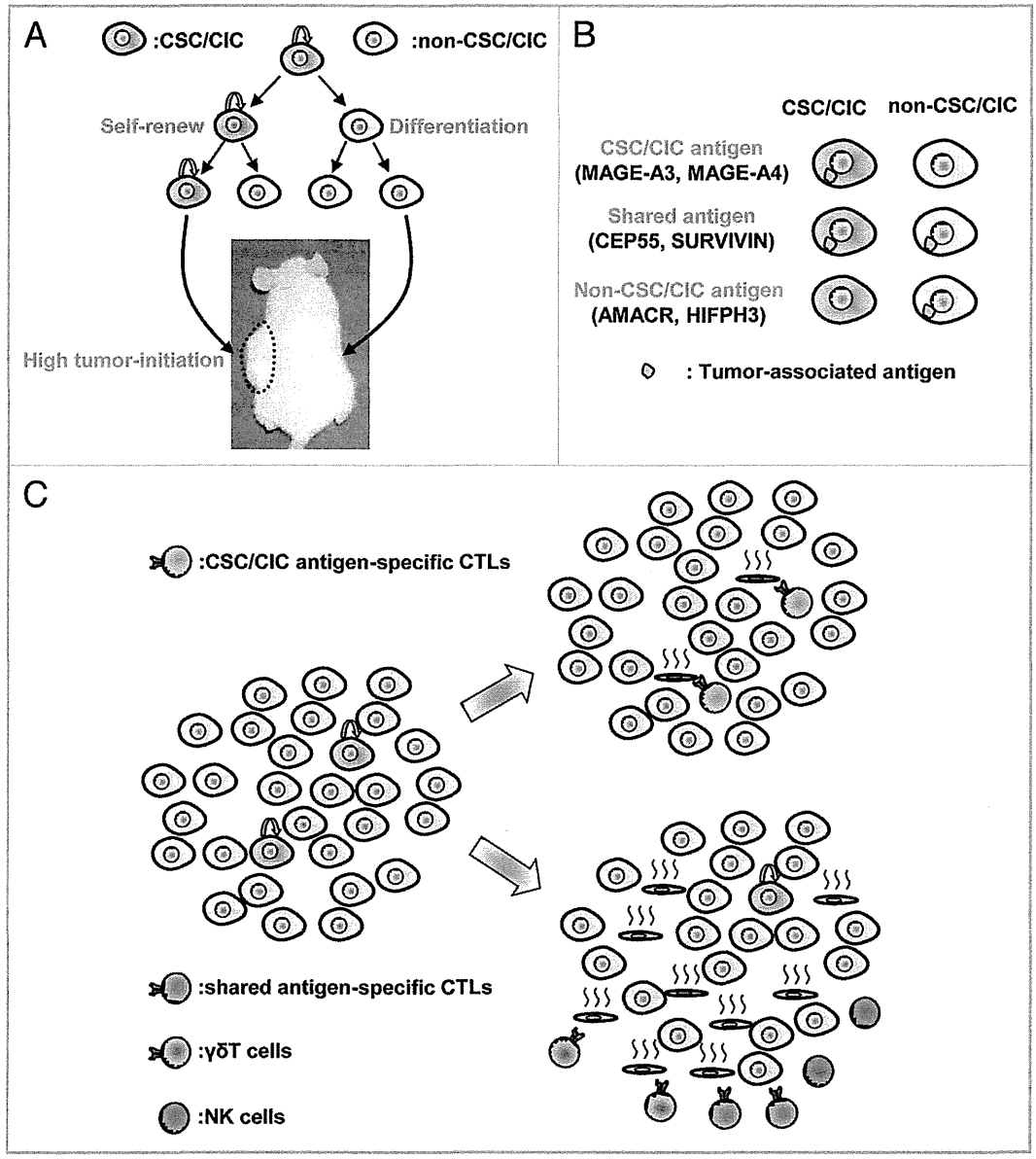

Figure 1. CSC/CIC targeting immunotherapy. (A) Characters of CSC/CIC. CSC/CIC has three distinct characteristics: (1) high tumor-initiating ability, (2) self-renewal ability and (3) differentiation ability. (B) Three groups of tumor-associated antigens. Tumor-associated antigens can be classified into 3 groups according to the expression in CSC/CIC and non-CSC/CIC: (1) CSC/CIC antigens, which are expressed in CSCs/CICs but not in non-CSCs/CICs (e.g., MAGE-A3 and MAGE-A4); (2) shared antigens, which are expressed in both CSCs/CICs and non-CSCs/CICs (e.g., CEP55, SURVIVIN); and (3) non-CSC/CIC antigens, which are expressed in only non-CSCs/CICs but not in CSCs/CICs (e.g., AMACR, HIFPH3). (C) CSC/CIC-targeting immunotherapy. CSC/CIC antigen specific CTLs recognize only higher tumorigenic CSCs/CICs, whereas shared antigen specific CTLs, NK cells and γδT cells recognize both CSCs/CICs and non-CSCs/CICs. CSCs/CICs might be eliminated most efficiently by CSC/CIC antigen-specific CTLs.

different. We are not sure about the exact mechanisms and we are now analyzing; however, these data indicate that targeting CSC/CIC specific antigens is more effective than targeting shared antigens.

The numbers of CTL clones are very restricted and limited in vivo, and the maximum numbers of one CTL clone might be about 10^7 to 10^8 cells in the whole body. On the other hand, cancer tissues contain 5×10^8 cancer cells per gram,[9] and advanced cancer tissues may therefore contain more than 10^{10} cancer cells. It is easy to imagine the difficulty in eliminating all cancer cells with such a limited number of CTLs (Estimated effector/target ratio is about 0.001 in the case of 10^7 CTL and 10^{10} cancer cells.). On the other hand, if we focus on just CSCs/CICs targeting CSC/CIC antigens, the situation will be improved (Estimated

effector/target ratio is about 0.1 in the case of 10^7 CTL, 10^{10} cancer cells and 1% frequency of CSCs/CICs.). Therefore, targeting CSC/CIC antigens might be a more effective approach to eradicate higher tumorigenic CSCs/CICs and may bring about greater anti-tumor effects (**Fig. 1C**).

As stated above, NK cells and γδT cells have been reported to recognize CSSs/CICs. However, these immune cells belong to the innate immune system and do not recognize target cells in an antigen-specific manner. Thus, activation of these cells in vivo may not be more effective than CSC/CIC antigen-specific CTLs (**Fig. 1C**). CTL adoptive transfer therapy has recently been described in detail,[10] and huge numbers of CTLs can be obtained by in vitro culture. Therefore, (2) shared antigens may also be suitable candidates for CTL adoptive transfer therapy using high numbers of CTLs.

In summary, CTLs can recognize CSCs/CICs as well as non-CSCs/CICs, and targeting CSC/CIC antigens with CTLs may be a reasonable approach for CSC/CIC targeting therapy.

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N-propionyl-4-*S*-cysteaminylphenol induces apoptosis in B16F1 cells and mediates tumor-specific T-cell immune responses in a mouse melanoma model

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ABSTRACT

Background: *N*-propionyl-4-*S*-cysteaminylphenol (NPr-4-*S*-CAP) is selectively incorporated into melanoma cells and degrades them. However, it remains unclear whether NPr-4-*S*-CAP can induce cell death associated with the induction of host immune responses and tumor suppression in vivo.
Objective: To examine the molecular mechanism of NPr-4-*S*-CAP-mediated cytotoxicity toward melanoma cells and to test whether NPr-4-*S*-CAP can suppress transplanted primary and secondary B16F1 melanomas.
Methods: Cytotoxicity and apoptosis of melanoma cells were assessed by cell counting, flow cytometry, and detection of reactive oxygen species (ROS) and apoptotic molecules. NPr-4-*S*-CAP-associated host immunity was studied using a B16F1 mouse melanoma model through the application of CD4- and CD8-specific antibodies and tetramer assay.
Results: NPr-4-*S*-CAP suppressed growth of pigmented melanoma cells associated with an increase of intracellular ROS, activation of caspase 3 and DNA fragmentation, suggesting that NPr-4-*S*-CAP mediated ROS production, eliciting apoptosis of melanoma cells. Growth of transplanted B16F1 melanomas was inhibited after the consecutive intratumoral injections of NPr-4-*S*-CAP, and the tumor growth after rechallenge of B16F1 was significantly suppressed in the treated mice. This suppression occurred when the treated mice were given the anti-CD4 antibody, but not the anti-CD8 antibody. Tetramer assay demonstrated increased TYRP-2-specific $CD8^+$ T cells in the lymph node and spleen cells prepared from NPr-4-*S*-CAP-treated B16F1-bearing mice.
Conclusions: These suggest that NPr-4-*S*-CAP induces apoptosis in melanoma cells through ROS production and generates $CD8^+$ cell immunity resulting in the suppression of rechallenged B16F1 melanoma.

1. Introduction

Although early lesions of primary melanoma are curable by excision, treatment of metastatic melanoma is significantly more difficult and the current systemic therapies have little effect on the overall survival rate or period [1,2]. Melanin pigment is synthesized in cytoplasmic organelles called melanosomes, specifically present in differentiated melanocytes. Melanin precursors possess strong cytotoxicity toward various kinds of cells, including melanoma cells, when tyrosinase, a key enzyme of melanin biogenesis, is ectopically expressed [3]. Thus, therapy targeting melanogenesis and enhancing cytotoxicity can be highly effective for growth suppression and possible induction of systemic immune responses against malignant melanoma without significant systemic side effects.

We have employed chemical agents biologically unique to melanoma cells such as the sulfur-amine analogs of tyrosine,

Abbreviations: NPr-4-*S*-CAP, *N*-propionyl-4-*S*-cysteaminylphenol; NPr-2-*S*-CAP, *N*-propionyl-2-*S*-cysteaminylphenol; AMF, alternating magnetic field; ROS, reactive oxygen species; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; RPMI, RPMI1640 medium; PBS, phosphate-buffered saline; HSP, heat shock protein; TYRP-1, tyrosinase-related protein-1; TYRP-2, tyrosinase-related protein-2.

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N-acetyl-4-*S*-cysteaminylphenol (NAc-4-*S*-CAP) and *N*-propionyl-4-*S*-cysteaminylphenol (NPr-4-*S*-CAP). They have been shown to be good substrates for tyrosinase [4–6] and to be selectively incorporated into melanoma cells, causing cytotoxicity against them and melanocytes [7–11]. However, it remains unclear whether NPr-4-*S*-CAP can induce cell death associated with the induction of host immune responses resulting in the tumor suppression in vivo.

In this study, we examined the molecular mechanism of NPr-4-*S*-CAP-mediated cytotoxicity toward melanoma cells by focusing on intracellular reactive oxygen species (ROS) and tested whether NPr-4-*S*-CAP could suppress transplanted primary and secondary B16F1 melanomas. We analyzed the molecular basis of B16F1-specific host immunity through the application of CD4- and CD8-specific antibodies. The relations between apoptosis of melanoma cells and ROS production by NPr-4-*S*-CAP and clinical implications for melanoma therapy with this compound are discussed.

2. Materials and methods

2.1. Cell lines and culture

Murine fibroblast NIH3T3, murine melanoma B16F1, human pigmented melanoma 70W and G361, human non-pigmented melanoma TXM18 and SK-mel-24 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Gaithersburg, MD, USA) supplemented with 5% fetal bovine serum (FBS) [12]. Human pigmented melanoma cell line M-1 was established from a surgical specimen of a Japanese patient [13] and maintained in RPMI1640 medium (RPMI, Gibco BRL) supplemented with 5% FBS. Murine lymphoma cell line RMA was cultured in RPMI1640 medium supplemented with 5% FBS [12]. Cells were incubated at 37 °C in a 5% CO_2 atmosphere.

2.2. Chemicals

NPr-4-*S*-CAP (MW = 225 Da) was kindly provided by Department of Dermatology, University of Alberta (Canada). The compound was synthesized as described by Tandon et al. [9]. NPr-2-*S*-CAP, an inactive form of cysteaminylphenol used as a negative control [5], was synthesized as follows: 195 mg of 2-*S*-cysteaminylphenol, which was prepared by the reaction of phenol with cystamine in 47% HBr [14], was reacted with 1.5 ml of pyridine and 1 ml of propionic anhydride for 30 min at room temperature. After evaporation of the reaction mixture under reduced pressure in a vacuum pump, colorless crystals of NPr-2-*S*-CAP were obtained in a quantitative yield. For the study, NPr-4-*S*-CAP and NPr-2-*S*-CAP were dissolved in propylene glycol (Wako, Osaka, Japan) at a concentration of 244 mM and sterilized by filtration.

2.3. Animal models for tumor formation

Female C57BL/6J mice (4 weeks old, approximately 10.0 g) were obtained from Hokudo (Sapporo, Japan). For the experiment, 3.0×10^5 B16F1 melanoma cells in 0.1 ml of phosphate-buffered saline (PBS) were injected subcutaneously into the right flank in C57BL/6J mice on day 0. On day 8, 24 mice were randomly divided into four treatment groups. From the 8th day, tumor-bearing mice were injected with 0.1 ml of NPr-4-*S*-CAP (24.4 mmol, 5.5 mg) in propylene glycol directly into the tumor with a 26-gauge microsyringe. Group I mice received s.c. administration of NPr-4-*S*-CAP every other day for a total of three days (days 8, 10, and 12) and administration of 0.1 ml of propylene glycol on days 14 and 16. Group II mice received NPr-4-*S*-CAP every other day for a total of five days (days 8, 10, 12, 14, and 16), and in group III NPr-4-*S*-CAP was administered every day for five days (from day 8 to day 12). Control mice were injected with 0.1 ml of propylene glycol every day for five days (Protocol 1). To compare the anti-melanoma effect of NPr-4-*S*-CAP with that of NPr-2-*S*-CAP, mice were divided into another three groups on day 8, and injected with 0.1 ml of NPr-4-*S*-CAP, NPr-2-*S*-CAP or propylene glycol every day for five days (Protocol 2). RMA lymphoma cells (5.0×10^5) were injected subcutaneously into the right flank on day 0 as a control for B16F1 melanoma and, from day 8, mice received NPr-4-*S*-CAP or propylene glycol every day for five days (Protocol 3). Tumor diameters were measured every other day and tumor volumes were calculated using the following formula: long axis × (short axis)^2 × 0.5. The right tumors of treatment and control groups in Protocol 1 were resected surgically on day 22. On day 36, mice were re-challenged with 1.5×10^5 B16F1 cells that were injected into the left flank and tumor diameters were measured every other day from the 37th to 120th days. As a control for B16F1 rechallenge, 2.5×10^5 RMA lymphoma cells in 0.1 ml of PBS were injected subcutaneously into the left flank in group III on day 36.

Animal experiments were carried out according to the principles described in the "Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology" of Japan.

2.4. Histopathological study of tumor sections

The tumors were removed and fixed in 10% formalin in PBS. Paraffin-embedded sections were then prepared and processed for hematoxylin–eosin (HE)-staining. For immunohistochemical analysis, the frozen tissues were stained with anti-mouse CD4 mAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-mouse CD8 mAb (Acris Antibodies, ACR, Herford, Germany).

2.5. In vivo T-cell depletion assay

Mice that received NPr-4-*S*-CAP every day for five days in Protocol 1 were injected with 0.2 mg of an anti-CD4 antibody (anti-mouse CD4, eBioscience, San Diego, CA, USA), anti-CD8 antibody (anti-mouse CD8, eBioscience) or rat IgG (Rat IgG1, aBd Serotec, Kidlington, UK) i.p. on days 29 and 43, i.e., one week before and after the rechallenge test on day 36. The growing tumor diameters were measured and tumor volumes were calculated.

2.6. In vitro cytotoxicity assay

After mice were treated with NPr-4-*S*-CAP every day for five times as described above, the spleen cells were harvested from a mouse which was completely cured by day 36 by NPr-4-*S*-CAP. 5×10^6 spleen cells were then re-stimulated with irradiated B16F1 cells in 2 ml of RPMI1640 supplemented with 50 μM β-mercaptoethanol (Invitrogen, Carlsbad, CA, USA) and 5% FBS for five days. For the control, spleen cells were prepared from a naïve mouse and a mouse transplanted with RMA lymphoma cells and treated similarly with NPr-4-*S*-CAP. Cytotoxic activity of spleen effector cells against target B16F1 or RMA cells was determined by standard ^{51}Cr release assay.

2.7. Tetramer assay

Spleens and regional lymph nodes were removed on day 40 after primary melanoma resection. Lymphocytes prepared from regional lymph nodes were incubated at 37 °C for 30 min in the staining buffer (PBS with 0.1% BSA and 0.1% sodium azide) containing 10 μl of APC-labeled TYRP-2-specific tetramer (Medical & Biological Laboratories Co. Ltd., Nagoya, Japan). Cells were washed once, then incubated at 4 °C in the staining buffer containing 3 μg of anti-CD8a mAb conjugated to FITC (Becton,