Cancer Gene Therapy (2013) 20, 157–168

www.nature.com/cgt

REVIEW

New targets for the immunotherapy of colon cancer—does reactive disease hold the answer?

V Boncheva[1], SA Bonney[2], SE Brooks[2], M Tangney[3], G O'Sullivan [3], A Mirnezami[2] and B-A Guinn[1,2,4]

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers in both men and women, posing a serious demographic and economic burden worldwide. In the United Kingdom, CRC affects 1 in every 20 people and it is often detected once well established and after it has spread beyond the bowel (Stage IIA–C and Stage IIIA–C). A diagnosis at such advanced stages is associated with poor treatment response and survival. However, studies have identified two sub-groups of post-treatment CRC patients—those with good outcome (reactive disease) and those with poor outcome (non-reactive disease). We aim to review the state-of-the-art for CRC with respect to the expression of cancer-testis antigens (CTAs) and their identification, evaluation and correlation with disease progression, treatment response and survival. We will also discuss the relationship between CTA expression and regulatory T-cell (Treg) activity to tumorigenesis and tumor immune evasion in CRC and how this could account for the clinical presentation of CRC. Understanding the molecular basis of reactive CRC may help us identify more potent novel immunotherapeutic targets to aid the effective treatment of this disease. In this review, based on our presentation at the 2012 International Society for the Cell and Gene Therapy of Cancer annual meeting, we will summarize some of the most current advances in CTA and CRC research and their influence on the development of novel immunotherapeutic approaches for this common and at times difficult to treat disease.

Cancer Gene Therapy (2013) **20,** 157–168; doi:10.1038/cgt.2013.5; published online 15 March 2013

Keywords: colon cancer; SEREX; cancer-testis antigens; tumor antigens; reactive disease; immunotherapy targets

THE GLOBAL CHALLENGE

'You have cancer'—the diagnosis that millions of people around the world hear every year and also the second leading cause of death worldwide. The number of new cancer cases each year is gradually increasing and the mortality rate is expected to rise from 7.6 million in 2008 to over 17 million in 2030.[1,2] On average, one of every three people is expected to experience some type of cancer in the course of their lifetime and this frequency is expected to increase[3] as the population ages. The World Health Organization reports that an increasing percentage of all newly diagnosed cancer cases annually occurs in low- and middle-income countries and this is attributed to a wide range of behavioral, genetic and environmental risk factors.[4] Nevertheless, the increase in the average life expectancy and the adoption of an unhealthy lifestyle (such as smoking, physical inactivity and poor diet) worldwide have also contributed to the rise of a new trend in the cancer demography. Newly diagnosed cancers from the low- and middle-income countries today account for $>51\%$ of the total number and their share in the global burden is expected to continue to increase with the growth and aging of the population.[4] In addition, the lack of resources for early cancer detection and effective treatments in the developing world contributes to an increase in cancer-related deaths.

Today, some of the most commonly diagnosed cancers both in economically developing and developed countries include prostate, breast, lung and colorectal cancer (CRC)—'the big four'—accounting for nearly 50% of the total cases diagnosed.[5] Cancer is a global challenge, opposing a serious demographic and economic burden with worldwide economic costs estimated to be as high as £572 billion per year. These alarming statistics resulted in the development of national strategies and action plans for cancer control including prevention, early detection and effective treatment.[6] However, the demand for the development of new approaches to cancer screening and therapy is recognized globally and brings the research attention on these aims sharply into focus.

COLORECTAL CANCER

CRC is one of the most commonly diagnosed cancers worldwide. It affects the bowel and the rectum and is rare in people under 40, with almost 85% of cases being diagnosed in persons over 65 years of age.[6] Statistics show that men and women are affected equally, while it is the third most common type of cancer in men (after prostate and lung cancer) and the second most common cancer in women (after breast cancer).[6] One in every 20 people in the UK develops CRC with only half of them surviving beyond 5 years, mainly because it is often detected once well established and after it has spread beyond the bowel. The disease stage at the time of diagnosis governs both the choice of treatment and the prognosis. CRC is staged to reflect how far the cancer has spread and whether or not it has reached nearby structures such as lymph nodes or distant organs. The most commonly used staging system for CRC is that of the American Joint Committee on Cancer,

[1]Department of Life Sciences, University of Bedfordshire, Park Square, Luton, UK; [2]Cancer Sciences Unit, Somers Cancer Sciences Building, Southampton University Hospitals, University of Southampton, Southampton, UK; [3]Cork Cancer Research Centre, Mercy University Hospital and Leslie C. Quick Jnr. Laboratory, University College Cork, Cork, Ireland and [4]Department of Haematological Medicine, King's College London School of Medicine, London, UK. Correspondence: Dr B Guinn, Department of Life Sciences, University of Bedfordshire, Park Square, Room B303, Luton LU1 3JU, UK.
E-mail: barbara.guinn@beds.ac.uk
Received 14 October 2012; revised 20 December 2012; accepted 5 January 2013; published online 15 March 2013

also known as tumor nodes metastases system.[7] It describes three key pieces of information: 'T'—how far has the primary tumor grown; 'N'—the extent of spread to nearby lymph nodes; and 'M'—describes whether the cancer has metastasized. The information from the T, N and M is combined to determine the cancer stage grouping from Stage I (the least advanced) to Stage IV (the most advanced). Two of the older staging systems include Duke's[8] and Astler–Coller[9] but these are very rarely used today.

Stage I (A–C) (Duke's A–C) CRC is reported to be an asymptomatic malignancy, developing slowly by the progressive accumulation of genetic mutations within precancerous bowel lesions and polyps. Diagnosis at this stage reduces the risk of death from CRC, giving 90% chance of survival beyond 5 years[10,11] and significantly low levels of disease recurrence. However, most cases of CRC are detected once the cancerous cells have moved beyond the middle layers of the colon (Figure 1). This is classified as Stage IIB (Duke's B) and is one of the most commonly diagnosed forms of CRC. Currently, the course of treatment for CRC patients is fairly similar regardless of the significant differences in the biological features of each CRC case. Usually, the most effective approach is tumor resection, followed by chemo- or radiotherapy for Stage III and sometimes for Stage II CRC patients. However, recent studies suggest that not all Stage III patients benefit from these therapies and that 25% of Stage II cases are under treated.[12,13] Furthermore, a number of non-aggressive tumors are frequently overtreated, leading to the patient experience of unnecessary and severe side effects. Methods such as, fecal occult blood test, sigmoidoscopy, colonoscopy, virtual colonoscopy and double contrast barium enema offer improvements in the detection rates of CRCs.[14] However, their diagnostic value is limited with regards to costs, risks, lack of sensitivity especially in early stages and inconvenience to the patient.[15] Therefore, the focus remains on developing efficient methods for the early detection of CRC such as the identification of early disease biomarkers which could be used in non-invasive (urine and blood serum) tests. Such molecular biosensors for CRC would enable widespread screening alongside general health examinations and may further reduce the mortality rate associated with late stage detection of CRC.[16]

Murphy *et al*[17] suggested that there are two sub-groups of patients with Stage IIB CRC—those with good treatment outcome (reactive disease) and those with poor treatment outcomes (non-reactive disease). Whether the difference between these two groups of patients can be determined by differences in humoral responses warrants further investigation and provides the basis of our own current studies. Tumor biomarkers offer an opportunity to translate unique CRC biological features into diagnostically pertinent information and would enable personalized treatments, which could inform conventional and immunotherapeutic interventions. This would enable discerning treatment strategies for aggressive and non-aggressive cancers and the clear 'up front' distinction of reactive from non-reactive disease.

In part, to address this need, researchers are investigating immune responses in cancer patients to identify new immunotherapy targets and biomarkers. They are hoping to identify and evaluate tumor-associated antigens (TAAs) and cancer-testis antigen (CTAs) that could prove to be efficient diagnostic, prognostic or immunotherapeutic targets in CRC.[18] Recent developments in the fields of genomics and proteomics have greatly contributed to these studies, enabling the identification of multiple potential antigens within a single experiment.[19] Techniques, such as DNA microarray analysis, protein microarrays, peptide-major histocompatibility complex (pMHC) tetramers, serological identification of antigens by recombinant expression cloning (SEREX), serological proteome analysis (SERPA) and peptide elution from MHC for mass spectrometry analysis are now commonly used to evaluate the expression profiles of genes and proteins as well as antigen recognition within different types, sub-types and stages of cancer. Their application in a number of studies conducted on acute myeloid leukemia (AML),[20,21] diffuse large B-cell lymphoma,[22] lung cancer[23] and osteosarcoma[24] have already proven successful and have led to the discovery of a large panel of antigens with prognostic and immunotherapeutic relevance. It has also provided an insight into the biological complexity and individuality of each cancer case, demonstrating considerable heterogenicity among patients and within tumor types. Therefore, genomics, proteomics or a combination of multiple methods could aid the discovery of

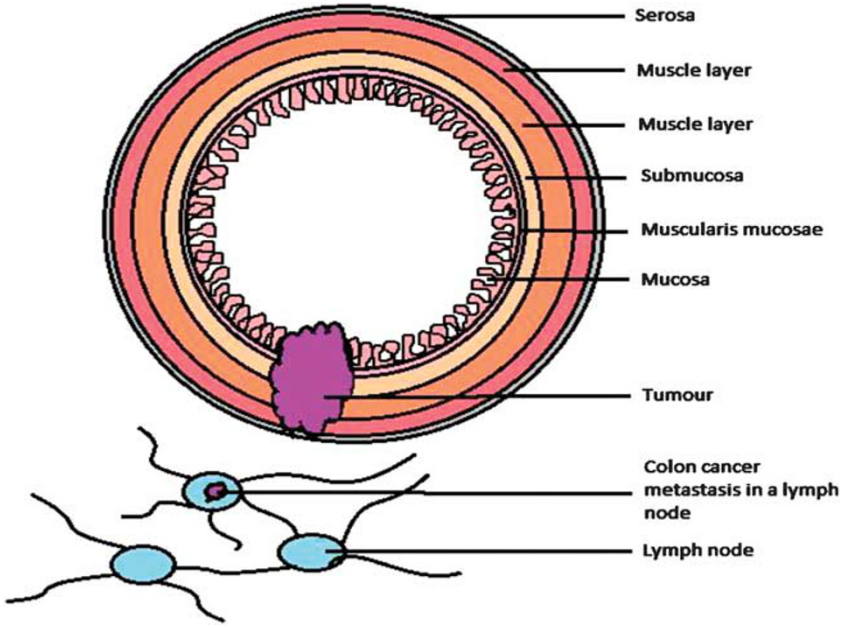

Figure 1. Shows a malignant primary tumor that has moved beyond the middle layers of the colon and has also metastasized to a nearby lymph node. These are stages IIB (Duke's B) onwards.

novel biomarkers specific for CRC and contribute to the development of personalized therapies, which would maximize efficiency and minimize side effects for the patient.

THE SEARCH FOR TUMOR ANTIGENS SPECIFIC FOR CRC

The desire to identify TAAs has inspired and attracted researchers for more than four decades. Most efforts were driven by the aim to uncover specific epitopes on cancer cells, which could elicit immune responses in the autologous host.[25,26] In the 1970s, a method known as 'autologous typing' was used successfully to identify a number of antigens including alpha fetoprotein (in hepatoma and germ cell tumors), carcinoembryonic antigen (CEA) (in gastrointestinal cancers), prostate-specific antigen (in prostate cancer), CA125 (in ovarian cancer) and AU (in melanomas).[27,28] This novel serological technique offered a substantial improvement over existing methods as it enabled the analysis of the cellular (T-cell defined) anti-tumor human immune responses in an autologous manner where only tumor-specific antigens could be recognized.[27] However, autologous typing did not completely fulfill the original hopes for its success because of several limitations. The major disadvantage arose from its reliance on cultured tumor cell lines and not all tumor types could be propagated *ex vivo* to allow autologous typing to be performed. Furthermore, only a small fraction of the total number of patients was found to have demonstrable levels of autologous antibody with specificity for cell surface antigens on their tumor.[25,26] In the cases when the technique was successful and a tumor antigen was identified, the low titer of antibodies that were often detected made any further biochemical or molecular characterization of the antigens almost impossible. However, the method of autologous typing contributed to the identification of a number of human tumor antigens, which were categorized into four classes: differentiation antigens—for example, gp-100; mutational antigens—for example, abnormal forms of p53; retroviral antigens—Epstein–Barr virus and human papilloma virus; and CT antigens.[26]

CT antigens

CTAs were classified as TAAs with restricted expression in testis, placenta and various types of cancer.[26] In the field of cancer serology they were quickly identified as the ideal targets for tumor-specific immunotherapeutic approaches.[29–33]

The selective expression pattern of CTAs is considered to be a consequence of gene activation by DNA demethylation[34,35] and histone post-translational modifications[36] both occurring constitutively in the testis, but also in tumor cells.[35] Some CTAs have been found to be expressed in healthy tissues such as liver, pancreas and spleen but at a significantly lower level ($<1\%$) when compared with their expression in testis.[31] Around 70 families of CTAs have been identified to date and further classified as X-CTAs and non-X-CTAs depending on the chromosomal location to which the genes are mapped.[37] The genes for distinct X-CTAs have been previously reported to encode for different antigenic peptides that are presented with HLA class I or HLA class II allo-specificities, eliciting both cell-mediated and humoral immune responses.[38] The blood–testis barrier and the lack of HLA class I expression on the surface of germ cells prevents the cells of the immune system from interacting with the CTAs expressed there.[39] It has been suggested that as the B and T cells have not been previously challenged by CTAs the immune response should be able to recognize CTAs as non-self structures when expressed on cancer cells[39] circumventing the need to break tolerance. These findings suggest that CTAs can be viewed as promising molecular targets for the development of immunotherapeutic interventions for specific cancer types without the possibility of triggering autoimmune responses. The identification of CTAs specific for a particular type of cancer is a promising approach for the development of peptide or recombinant full-length protein anti-cancer vaccines and for antigen-specific adoptive T-cell transfer as part of cancer therapy.[40] In that respect, NY-ESO-1 (a CTA with strong immunogenicity still detectable at sera dilutions of 1 in 40 000[41]) has been of interest in relation to the development of cancer vaccine trials. Several ongoing trials have been based on this antigen alone or its use in combination with an immune enhancer are currently ongoing.[42,43] However, the expression of other CTAs within various types of cancer and at different disease stages appears to be heterogeneous and further investigation is required for the identification of effective immunotherapeutic targets.

Although, CTA expression is generally correlated with tumor progression and immunogenicity in various types of cancer, little is known about this in relation to CRC. In addition, CTAs were often found to be poorly expressed (low level, heterogeneous expression) in CRC[44] highlighting the need for the identification of further immunogenic CTAs involved in CRC development. Such proteins would be candidates for cancer-specific therapy trials and may provide effective biomarkers for diagnosis, prognosis and monitoring of CRC disease progression.

Serological identification of antigens by recombinant expression cloning

The limited success of autologous typing demonstrated the need for a more comprehensive experimental approach for the identification of TAAs. It was not until the mid-1990s when a new autologous immunoscreening technique was shown to circumvent some of the limitations of autologous typing. This method was called SEREX.[45] It allowed the identification of numerous TAAs based on their recognition by antibodies in diluted pre-cleared autologous patient sera and in a small number of experiments. The advantages of SEREX were quickly recognized and a substantial pool of data encompassing serologically relevant antigens within cancer began to collect. SEREX was capable of identifying immunoglobulin G antibody responses to both highly (NY-ESO-1) and weakly immunogenic TAAs.[46,47] Approximately one-third of these antigens were novel and referred to as SEREX-defined antigens. In addition, sera was shown to be able to detect $CD8^+$ T-cell recognized TAAs, including NY-ESO-1,[41] MAGE-1 and tyrosinase[41,45,48] as well as TAAs recognized by immunoglobulin G antibodies, which are also known to require $CD4^+$ T-cell help (NY-ESO-1).[45,46,48–51] These findings demonstrate that the cellular and humoral immune system work in concert and are both stimulated by TAAs in the case of cancer. Furthermore, several antigens (NY-ESO-1, MAGE-1 and hMena)[52] that elicit humoral as well as cell-mediated immune responses have also been successfully identified through SEREX. Studies have investigated $CD8+$ and $CD4+$ T-cell recognition of SEREX-defined antigens and the data showed that co-immunization with such proteins in combination with a cytotoxic T lymphocyte (CTL) epitope enhanced $CD8^+$ induction in a $CD4^+$ T-cell-dependent manner.[53,54]

The continuously increasing number of SEREX antigens led to the creation of a Cancer Immunome Database, a free repository for the antigens identified by this serological approach.[55] The success of SEREX lies in its reliance on the construction of a cDNA library from human tissues (cell lines, primary tumor or normal donor testes) and their expression in a prokaryotic system.[45] Immunoscreening with SEREX permits quick and effective extraction, processing, sequencing and subsequent molecular analysis of the protein of interest as both the TAA and its coding cDNA are present in the same plaque. However, the use of SEREX has several limitations. It has been previously reported that SEREX-defined antigens are predominantly nuclear proteins which are often transcription factors[41] and/or ubiquitously expressed.[55,56] As such, they rarely show evidence of mutations or other structural

abnormalities and are often weakly immunogenic, incapable of eliciting and sustaining strong humoral immune responses.[25] In addition, phage can only express proteins in their primary structure, which may cause the failure to detect antigenic sequences consequent to eukaryotic post-translational modifications.[57] Regardless of the limitations, SEREX-defined antigens are already being investigated as diagnostic markers (testis-specific protease (TSP50) in ovarian and CRCs)[58] and as immunotherapeutic targets (OVA66 in ovarian cancer,[59] KIF20A in pancreatic cancer[60]).

Serological proteome analysis

SERPA is a powerful tool used for the identification and validation of immunogenic TAAs. Similar to SEREX, it uses the antibody repertoire contained within the sera of a cancer patient to successfully identify TAAs.[61] In comparison to SEREX, SERPA does not require the use of a cDNA library, making this method less time consuming and less labor intensive. Furthermore, SERPA is better suited for the detection of possible post-translational modification and protein isoforms as it relies on the separation of complex mixtures of proteins extracted from cell cultures or tumors. The separation is performed via the use of a two dimensional gel electrophoresis (2-DE).[62] A number of TAAs have been identified to date using SERPA in various types of cancer including lymphoma,[63] renal cell carcinoma,[64] ovarian cancer,[65] and CRC.[66] These studies have established the specificity of SERPA and its great potential in uncovering immunogenic TAAs and identifying tumor markers. However, similar to SEREX, most of the antigens identified by SERPA are predominantly weakly immunogenic intracellular proteins and very rarely membrane-associated ones.[67,68] This and the finding that a number of antigens found by T-cell cloning have also been found by SEREX suggests that most antigens induce B and T-cell responses,[69] although not necessarily to the same epitope(s). In addition, SERPA has demonstrated a drawback associated with the use of 2-DE: low abundance (such as regulatory and signal transduction proteins and receptors[70]), hydrophobic or insoluble proteins (such as membrane proteins[70]) are inherently difficult to detect.[67]

SEREX and SERPA are two methodologies that seem complementary to each other as they identify two different sets of antigens (SEREX for the identification of antigens with altered expression; SERPA for the identification of antigenic proteins resulting from post-translational modifications). Therefore, the identification and validation of CRC-related CTAs using SERPA and SEREX independently or as a combined approach is worthy of further consideration.

Recombinant antigen expression on yeast surface (RAYS)

RAYS is another serological strategy applied to the process of discovering immunogenic CTAs. RAYS permits the expression of immunogenic proteins on the surface of yeast allowing for a more natural folding of the protein and partial glycosylation (by virtue of it being a eukaryotic system).[57] This permits the analysis of proteins in their natural conformation when compared to the prokaryotic expression utilized by SEREX. This method has demonstrated specificity and sensitivity for the detection of an antibody response to a conformation-dependent epitope—the CRC antigen A33. Therefore, the CTAs that have escaped detection because of the fact that they elicit immune responses only after undergoing the appropriate post-translational modifications could potentially be identified via RAYS. To date, RAYS has allowed the confirmation of antigen immunogenicity through screening of eukaryotic cDNA expression libraries derived from pancreatic cancer[71] and prostate cancer.[72] RAYS offers a less time-consuming analysis of the serological autoreactivity in cancer patients and it has provided an effective anti-cancer vaccine platform (recognizing NY-ESO-1) in prostate cancer patients.[72] However, its further development is required to allow the detection of novel target antigens (such as is achieved with SEREX or SERPA) and its application in CRC requires further analysis.

Serum antibody detection array

To evaluate the seroreactivity of a number (or a panel) of SEREX-defined antigens in a particular type of cancer, a spot immunoassay, known as serum antibody detection array, has been successfully developed and utilized.[73] Although, several antigens have been evaluated in cases of colon cancer (MAGE-A3, SSX2 and NY-ESO-1[73]), additional improvements in the sensitivity of serum antibody detection array are still required.

Protein microarrays

Protein microarrays allow the rapid and easy detection of tumor antigens using patient sera.[74,75] Recent studies have demonstrated the efficiency of the technique in the rapid identification of immunogenic membrane-based TAAs with high reproducibility of the experimental analyses of lung and brain[74] and ovarian[76] cancers. In this context, protein microarrays has a great advantage as this methodology allows the construction and simultaneous analysis of a large panel of candidate tumor biomarkers ($\sim$9,000). However, it should be noted that although protein microarrays have vast screening potential they are limited to defined proteins from an albeit not insubstantive pool.

Understanding the molecular interactions between T-cell receptors on CTL and peptide/MHC class I complexes on tumor cells is an essential tool for the development of immunogenic vaccines.[77] Several such vaccines have already been designed based on TAA epitopes and have been implemented in Phase I and II clinical trials for different types of cancer (human papilloma virus,[78] WT1,[79] human telomerase reverse transcriptase[80] and HLA-A24$^+$HRPC[81] peptide vaccinations). The strength of vaccine-mediated immunological responses generally need to be enhanced and this will be much more feasible in patients in subsequent (Phase III) clinical trials who are likely to have less advanced disease. However, the results obtained from clinical trials to date warrants further investigation.

pMHC (tetramer) microarrays

The development of pMHC microarrays has allowed the rapid identification of antigen-specific populations of T cells in the peripheral blood of patients.[82] This approach has proven useful for epitope prioritization, and for the detection of multiple T-cell populations in cancer patients undergoing conventional treatments or tumor-associated peptide vaccine trials.[82,83] We are using the pMHC array to identify which epitopes are recognized by peripheral T cells from colon cancer patients during conventional treatment (Bonney *et al.*, in preparation).

TUMOR PROFILING IN PATIENTS WITH CRC

A number of studies have been conducted over the last decade aiming to identify novel biomarkers that would prove to be efficient in CRC profiling as prognostic, predictive or therapeutic biomarkers. The recent improvements in proteomics and genomics methods has greatly aided this aim and has led to the identification of a number of CTAs and TAAs relevant to CRC. However, the clinical significance of only a small fraction of these potential markers in CRC has been evaluated to date.

CTAs in CRC

NY-ESO-1 has been recently studied in relation to CRC. It was demonstrated that some CTAs are capable of eliciting strong humoral and cell-mediated immune responses in some patients

with CRC.[84] However, its expression in CRC is often highly heterogeneous, when present, which poses an obstacle in the development of a generalized immunotherapy. As discussed earlier in this review, NY-ESO-1 has also been targeted in several vaccine clinical trials worldwide involving CRC patients. Recently, a new Phase I trial of a fusion protein vaccine is being organized targeting solid tumors expressing NY-ESO-1 and this investigation includes Stage I–IV CRC.[85] In addition, NY-ESO-1 expression was shown to correlate with CRC stages and local lymph node metastasis[86] making it a potential prognostic biomarker for CRC.

TSP50 was originally identified as abnormally expressed in breast cancer cells[87] and has recently been studied for the first time in CRC patient samples. The expression of TSP50 was found to correlate with the clinicopathological characteristics and disease-specific survival for CRC patients.[88] Furthermore, the study demonstrated that TSP50 expression is highly specific for CRC as compared with colorectal adenomas and normal tissues, allowing the easy differentiation between them. TSP50 is an attractive predictive biomarker for poor survival in patients with early stages CRC (Stage I and II), but not in patients with advanced stage disease. As such, it is the only effective predictive biomarker reported to date for patients with early stage CRCs.[88]

CABYR is a calcium-binding tyrosine phosphorylation-regulated fibrous sheath protein and its expression was first identified in human spermatozoa ([89]; reviewed in Chiriva-Internati *et al.*).[90] Subsequent detection of CABYR in lung carcinoma and its absence in healthy tissues, led it to be considered as a novel CTA, which has been shown to have some immunogenic properties that could serve as a basis for the development of immunotherapy for cancer patients.[91] Additional studies on CABYR expression in brain,[92] hepatocellular[93] and other carcinomas[94] have shown that there are at least five different isoforms of this protein, which could have unique roles in the process of carcinogenesis. Following these discoveries a recent study has reported a frequent overexpression of CABYR a/b and c isoforms in CRC tumors when compared with adjacent normal tissues.[95] However, a more comprehensive investigation is required to determine whether CABYR expression correlates with tumor stages and is a suitable therapeutic vaccine candidate in CRC.

SPAG9 is another antigen found to be exclusively expressed in testis[96] that is a particularly attractive target for immunotherapy in epithelial ovarian cancer,[97] thyroid cancer[98] and in CML.[99] A recent study has investigated the expression of SPAG9 in CRC patients aiming to explore its possible role in colon cancer tumorigenesis and its effectiveness in eliciting a humoral immune response.[100] Interestingly, the study has reported a close relationship between SPAG9 expression and early stages of CRC development suggesting that it could serve as an early diagnostic biomarker for CRC patients. The investigation had also demonstrated that SPAG9 could have a key role in the tumor development and could also serve as a target for the development of immunotherapeutic methods.

Other CTAs, found to exhibit a strong correlation with CRC presentation are listed in Table 1.

TAAs in CRC

CD133 is a cell surface protein marker found on undifferentiated cancer cells that exhibit stem-like properties. These cells account for the propagation, growth and recurrence of AML[110,111] and CRC[112,113] and for the resistance of these cancers to current therapies. The functional importance of CD133 expression has been investigated in several studies in relation to the initiation and behavior of CRC.[113–115] The $CD133^+$ CRC stem cells are reported to have exhibited the ability to transfer cancer to a secondary recipient maintaining the same immunophenotype and the global gene expression profile of the primary tumor when compared with $CD133^-$ cells.[113,114] Furthermore, several studies have clearly identified the correlation between CD133 expression alone[114,115] or in combination with other protein markers[116] with CRC patient survival and have revealed it to be a reliable prognostic marker. In combination with CD44 and CD166 (cell surface protein markers), CD133 expression has also been linked to the presentation of low-, intermediate- or high-risk CRC cases with the ability to distinguish between them at an early stage of the disease (stage II).[114,116] CD133 is a promising predictive and prognostic marker in the diagnosis of CRC and particularly of interest as it is applicable to the early stages of the disease. However, the presence of such cell surface markers on the CRC stem cells has not been investigated in relation to the underlying cause of the non-reactive type of CRC.

CEA is a TAA whose expression levels are often monitored pre- and post-treatment in CRC patients as they have been found to be indicative of cancer recurrence[117] and poor disease prognosis.[118,119] Patients with elevated post-treatment CEA expression levels are often monitored more carefully for relapse of CRC and for local or distant recurrence.[117,119] To date, several studies have investigated the potential of CEA as an immunotherapeutic target in cases of CRC. Different research strategies have incorporated CEA peptides or CEA mRNAs in dendritic cell vaccines[120,121] and in plasmid DNA vaccines[122] demonstrating that these vaccines are well-tolerated and have immune-stimulatory capacity in patients with CRC. However, the overall outcome of these studies indicated that additional vaccine modulation is necessary to attain significant clinical impact. More recently, a study to investigate whether the vaccination of toll-like

Table 1. Antigens determined to have potential as diagnostic, prognostic or immunotherapeutic targets in CRC

CTA/panel of CTAs	*Chromosome location*	*Method of identification*	*First identified in*	*Potential uses in CRC*
BCP-20 (FBXO39)	17p13.1	SEREX	CRC[101]	Candidate diagnostic and immunotherapeutic target[101]
PAGE4	Xp11.23	Database mining	CRC[102]	Predictive panel for liver metastasis[102]
SCP-1	1p13-p12	SEREX		
SPANX	Xq27.1	Differential display		
MAGE-A4	Xq28	T-cell epitope cloning	Melanoma[103]	Colon cancer vaccine therapy with peptide of MAGE-A4[104]
STK31	7p15.3	Three-step microarray analysis	CRC[105]	Candidate target for immunotherapy[105]
SSX	Xp11.2	Reverse transcription-PCR	CRC[106]	Co-expression as predictive marker for metastasis
MAGE	Xq28			Candidate targets for immunotherapy[106]
SSX2	Xp11.22	SEREX	Melanoma[107]	Candidate target for immune therapy[108,109]

Abbreviations: CRC, colorectal cancer; CTA, cancer-testis antigen; SEREX, serological identification of antigens by recombinant expression cloning; SSX, synovial sarcoma X antigen.

receptor activated dendritic cells can induce more potent CTL responses and antitumour activity in CEA transgenic mouse tumor models was published.[123] It has demonstrated that the combined activation of TLRs can lead to better maturation status of dendritic cells and can also induce more effective antitumour immune responses against CRC. However, additional investigation is necessary to evaluate the effectiveness of this approach in human models.

CLINICAL SIGNIFICANCE OF CTAS IN CRC

The CTAs that have already been identified within different types of cancer could serve as biomarkers for discerning aggressive and non-aggressive cancers and for predicting treatment outcome and relapse. Their expression patterns and clinical significance is still under investigation, but there are very promising early results. Some of their clinical applications are described in the followings sections, and could expand the list of potential CTAs in CRC.

Potential biomarkers for discerning reactive from non-reactive disease

The expression profile of CTAs in relation to treatment outcome in particular types of solid cancers has been previously studied on several occasions[24,52,58–60] but further investigation is necessary to compare these findings with CRC cases. In this context, a recent study has demonstrated that γ-irradiation *de novo* upregulates the expression of various CTAs and MHC-I in a randomized fashion. Therefore, irradiation could be accounted responsible for the increased immunological response to certain tumors owing to the inflammation and cell damage it causes. This would be anticipated to cause the immune system to attend the site of damage, mop up cellular debris and present proteins including CTAs to the immune system.[124] These findings fit with demonstrations in leukemia that elevated tumor antigen expression at disease presentation is associated with improved survival.[125,126] Identifying and evaluating such TAA and CTAs would be beneficial for profiling individual tumors and for combining radiotherapy (or other cancer therapy approach) with immunization to maximize the effect of treatment in CRC. However, in order to design a combined treatment a thorough understanding about the mechanisms of initiating CTAs expression and the likely order of their expression is necessary.

The exact role of the numerous CTAs in relation to tumor response to various treatments still remains poorly defined. Particularly, in cases where adjuvant therapy is in order, it would be beneficial to have a panel of biomarkers to predict the likely success of the therapy. Several CTAs have been evaluated in gastrointestinal stromal tumor—with regards to recurrence, while levels of MAGE-A1, MAGE-A3, MAGE-A4, MAGE-C1 and NY-ESO-1 expression were investigated in response to imatinib adjuvant therapy.[127] This study demonstrated that CTA$^+$ gastrointestinal stromal tumors had a significantly shorter recurrence free survival compared with negative cases. Furthermore, the expression of NY-ESO-1 and MAGE-A3 was associated with elevated resistance to imatinib and therefore, with continuous tumor progression. Luetkens *et al.*[128] have also showed that PRAME expression remains stable under imatinib treatment and correlates with decreased overall survival in patients with CML.

Similar findings have been reported in several studies of prostate and lung cancer, multiple myeloma, AML, liposarcoma and others.[129–134] These suggest an important role of CTAs in the pathophysiological behavior of different tumors in response to treatments. Further investigation of a panel of antigens associated with particular types of cancer and their relationship to either reactive or non-reactive disease is yet to be attempted. In this relation, TSP50 is the only known CTA to have been characterized as a biomarker for disease prognosis in CRC,[88] but its association with treatment outcome is still to be analyzed. Furthermore, investigation of the interrelationships between groups of CTAs in CRC and their expression profiles could lead to significant discoveries about the underlying cause of a particular treatment response. This could benefit the design of a multivalent cancer vaccine targeting several antigens rather than just a single one.

Potential biomarkers for survival prognosis

Several studies have investigated the role of the CTAs as biomarkers of prognostic value regarding patient survival. Elevated levels of expression of particular CTAs have been shown to correlate with poor survival prognosis, particularly in solid tumors. In contrast to expectations some antigens have been shown to have above average levels and have better survival rates in patients with hematological malignancies. For example, expression of SSX2IP in the presentation of AML has been shown to predict good survival in patients with no detectable cytogenetic rearrangements.[125] The elevated levels of antigens provide targets for improved immune responses in patients post-conventional (chemotherapy, radiotherapy) treatment, when there is cancer cell damage and inflammation (danger) signals stimulating an immune response to clear up dead and dying cells. Furthermore, the CTAs expressed on non-solid tumors (such as AML) are more accessible and 'easier to see' by the immune system as they are not hidden within heterogeneous layers of cancer cells (as seen in solid tumors).

The prognostic value of CTAs has also been evaluated in the presentation of osteosarcoma by gene microarray where the high expression of MAGE-A could predict distant metastasis and poor survival. For patients with and without MAGE-A expressing tumors, the 5-year survival rates were found to be 39.6% and 80% respectively.[24] Similarly, increased levels of WT1 (another CTA) expression have been shown to correlate to poor prognosis and relapse in pediatric AML after induction therapy.[135] A number of products of translocations have been used in routine labs, which detect minimal residual disease and can indicate impending relapse with high accuracy.[136] The CTA expression in relation to patient survival in CRC cases has been the focus of few studies and further investigation is required to establish the clinical significance of this relationship.[88]

Potential biomarkers for discerning aggressive from non-aggressive disease

A recent study on prostate cancer has demonstrated that several CTAs are preferentially expressed in either aggressive or non-aggressive disease.[137] Such biomarkers could be particularly useful in preventing the overdetection and overtreatment of potentially indolent CRC or the undertreatment of a more aggressive type of disease.

Potential immunotherapeutic targets

In 2005, cancer patients expressing NY-ESO-1 and LAGE-1 antigens entered a Phase I clinical trial on a plasmid DNA (pPJV7611) cancer vaccine,[138] demonstrating the effectiveness of CTAs as immunotherapeutic targets. Recently, the first clinical trial of cancer vaccine therapy with artificially synthesized helper/killer-hybrid epitope long peptide of MAGE-A4 cancer antigen was initiated.[104] A patient with pulmonary metastasis of CRC was vaccinated and had shown a significant reduction in the tumor growth.

NON-REACTIVE DISEASE—OVERCOMING THE UNDERLYING ISSUES

CTAs are key molecules in the field of cancer serology. Their progressively expanding family is continuously providing potential novel targets for cancer immunotherapy or diagnostic/prognostic examinations. However, CTAs have also been evaluated for their role in oncogenesis, particularly their contribution to the

immortality, invasiveness, immune evasion, hypomethylation and metastatic capacity of the neoplasms.[139] Investigating the correlation between CTAs and the clinical presentation of CRC (reactive or non-reactive type) requires knowledge of the molecular mechanisms that govern their expression and physiological functions—mechanisms that still remain poorly understood. According to recent studies, genome wide hypomethylation has accounted for the aberrant expression of CTAs within CRC cells.[140–142] However, the epigenetic factors that dictate which panel of silenced genes to be reactivated and the physiological properties of these particular CTAs are still unknown. This is an area of actively ongoing research as it is believed that these factors are responsible for the heterogenicity in the CTA expression profiles of the individual CRC cases. A better understanding of the structural and serological properties of these antigens, their modes of expression and functions, should establish whether they are the ones governing the reactive and non-reactive CRC phenotypes.

CTAs in CRC have been associated with low immunogenicity under normal conditions, and only a few patients actually recognize the peptide epitopes and exhibit strong CTL or humoral immune responses to CTAs. This could be partially accounted to the structural stability of the proteins, encoded by the CT genes. It has been previously demonstrated that proteins must be cleaved to small peptides by intracellular proteinases prior to presentation to the immune system.[143] However, high stability proteins (α-helical secondary protein conformation known to provide the most optimal structural stability[141]) are less likely to undergo denaturation and polypeptide chain unfolding. Therefore, CTAs containing large proportions of α-secondary structure should be more resistant to protein unfolding and subsequent cleavage by the proteinases, thus resulting in an unsuccessful presentation to CTLs (low immunogenicity). For example, SCP-1 (a CTA of particularly low immunogenicity) has been shown to contain 76.6% α-helical structure from its total secondary conformation when compared with the highly immunogenic NY-ESO-1 CTA, containing only 20.7%.[141] The identification of such antigens that are predominantly expressed in non-reactive CRC patients could potentially be accounted for the therapy-resistant presentation of the disease. In such cases epigenetic modulation to induce expression of other CTAs may highly favor the immunotherapeutic approach to non-reactive CRC disease. In this respect, a study has recently reported a successful induction of NY-ESO-1 expression in CRC cells (but not in normal nontransformed cells) both *in vitro* and *in vivo* subject to hypomethylating agent 5-aza-2′-deoxycytidine (DAC) treatment.[144] The study reports that DAC-treated CRC cells are susceptible to MHC-restricted recognition by $CD8^+$ NY-ESO-1-specific T cells. A NY-ESO-$1_{157–165}$-specific T-cell receptor was successfully used to generate both $CD8^+$ and $CD4^+$ NY-ESO-$1_{157–165}$-specific T cells that selectively recognized DAC-treated CRC cells but not non-treated cells. These data reveal the great potential of combining epigenetic modulation and adoptive transfer of genetically engineered T lymphocytes targeting NY-ESO-1 or other CTAs for the development of a specific immunotherapy for CRC.

Another approach could also include the introduction of sequences capable of disrupting long α-helical stretches in the regions outside the potential epitopes.[141] Such approaches have the potential to improve the treatment response in patients with non-reactive type CRC. However, several challenges remain to be overcome, including the insufficient antitumour responses due to immunosuppression driven by T lymphocytes known as regulatory T cell (Tregs).

Infiltrating T lymphocytes

During the last decade, the search to understand the causes underlying presentation CRC has focused on Tregs. They have been shown to have a major role in cancer immunoevasion by directly inhibiting or even eradicating both CTLs and T-helper lymphocytes.[145] Tregs suppress autoreactive T cells without killing them through incompletely understood, contact-dependent mechanisms.[146] In healthy individuals, they represent 5–10% of the population of $CD4^+$ lymphocytes. However, in cancer patients, Tregs may increase up to 30% and are predominantly found among the tumor-infiltrating lymphocytes present.[145] Therefore, the failure of the T and B cells to recognize and eradicate immunogenic cancer cells could be accounted for by the ability of certain tumors to secrete chemokine CCL22, which recruits Tregs and immobilizes the function of the anti-cancer immune response. Indeed patients with ulcerative colitis and irritable bowel syndrome have been shown to have higher numbers of infiltrating T cells than healthy controls[147] which may have a role in controlling cancer-driven inflammation. Tregs in CRC have been shown to exhibit both pro- and anti-tumor activities governed by the level of inflammatory stimuli received and dependent of the phase of tumourigenesis (early or late).[148] Following treatment in a Phase II clinical trial for CRC patients, the level of circulating Tregs was reported to have almost reached normal levels accompanied by 70% increase in CTLs responses against CEA epitopes.[149] Therefore, it still remains to be determined whether presence of Tregs in CRC has a pro- or anti- tumor role, how this correlation changes with the stage of the disease and the clinical significance of these changes.

In addition, Tregs appeared to be highly specific for a distinct set of TAAs in CRC patients, suggesting that Tregs exert T-cell suppression in an antigen-selective manner.[150] Several key hallmarks of Tregs in CRC have been identified, highlighting their complex role in the progression of the disease, the survival prognosis and their potential as therapeutic targets. Overall, it was established that CRC patients develop multivalent and individual T-cell responses against a broad variety of different CRC-associated TAAs. Therefore, selecting a panel of antigens according to pre-existing T-cell responses as an intermediary could improve the efficacy of future immunotherapies for CRC and should be further investigated. Better understanding of the role and behavior of Tregs within CRC would improve tumor profiling on an individual basis and could aid the choice of the most adequate therapy for each individual case. It could also benefit the development of cancer vaccines and other immune-based therapies targeting particular CTAs in non-reactive type CRC.

Tumor-infiltrating CD45RO(+)-cell density is a prognostic biomarker associated with longer survival in CRC patients, independent of clinical, pathological and molecular features.[151] Similar findings have also been reported from another study, linking the density of CD45RO(+) memory T cells in the different regions of CRC with dissemination to lymphovascular and perineural structures and to regional lymph nodes in patients—low densities were associated with a very poor prognosis.[152] However, not only was the type and density of the infiltrating immune cells within human CRC predictive of clinical outcome but also their location within the tumor, that is, whether they are located in the center of the tumor or the invasive margin. A strong *in situ* immune reaction in both regions was shown to correlate with a favorable prognosis regardless of the stage (I, II and III CRC).[153] Conversely, a weak *in situ* immune reaction predicted a poor clinical outcome even in patients with stage I CRC. These data were further supported by Pagès *et al.*[154] who examined the relationship between the presence of CD8(+) and CD45RO(+) in two regions of the tumor in patients with early stage CRC with regards to tumor recurrence and overall patient survival. High densities of both CD8(+) and CD45RO(+) correlated significantly with lower rates of CRC recurrence and increased overall survival when compared with patients with low densities of both immune cell types within the primary tumors. In contrast, extramural

vascular invasion and high FOXP3$^+$ cell density in lymphoid follicles were independent factors for worse survival, whereas a high frequency of lymphoid follicles in histologically normal colonic mucosa was associated with better survival.[155] Therefore, the collective analysis of the type, density and the location of immune cells within the CRC could be used to predict patient survival and to identify the high-risk patients who would benefit most from adjuvant therapy.

Intrinsically disordered proteins (IDPs)

Following a bioinformatics approach that implements the application of two algorithms (FoldIndex and RONN) predicting the level of disorder within a sequence, the experimental outcome revealed that >90% of the examined 228 CTAs were IDPs.[156] The latter are proteins that lack the typical hydrophobic cores and therefore do not appear as having rigid 3D structures (along their entire length or in localized regions) under physiological conditions and instead, exist as dynamic ensembles.[156,157] However, IDPs can evade being detected as 'misfolded' and degraded by the cell's surveillance system through their ability to undergo 'disorder-to-order' transitions upon binding to biological targets—a paradox known as the 'order/disorder paradox'.[158] This is achieved as a segment of an IDP initially binds with the target, followed then by coalescing of the other protein segments facilitating the IDPs' folding.[159] However, recent studies have challenged this general view by revealing a phenomenon—uncoupled binding and folding of IDPs.[160] The complexity of the binding mechanisms of IDPs has been investigated by others[161,162] and a summary of possible pathways have been outlined in Figure 2.

The above mentioned properties associated with IDPs give an interesting angle of perception towards the expression, behavior and function of CTAs identified as IDPs. The lack of rigid 3D structures is believed to be responsible for the exposure of primary contact sites, which enables the faster, more effective and promiscuous binding at high concentrations to target molecules.[157] Together with the fact that intrinsic disorder has been identified as a determinant of genes that are harmful when overexpressed,[161] this could account for the correlation between CTA overexpression and disease prognosis. This is further supported by the fact that CTAs appear to occupy 'hub' positions (highly connected protein nodes) within the complex protein–protein interaction (PPI) network.[156,163] What was interesting in these findings was the fact that these networks are dynamic and grow incrementally by establishing new nodes. However, a desirable protein for recruitment to a hub position is a protein that is likely to participate in a large number of promiscuous interactions when overexpressed such as a CTA that is an IDP.[156] Eventually, the PPI network becomes dominated by such hubs leading to the overexpression of CTAs and to the creation of nodes with novel functions. This accounts for the poorer disease prognosis in later CRC stages and the phenotypic presentation of non-reactive CRC disease. This idea is further supported by the fact that a high abundance of IDPs is believed to result in undesirable interactions and potentially harmful effects of such interactions.[161] Perhaps the failure to respond to treatment in non-reactive type CRC could be due to targeting common nodes in a protein network rather than CTAs that occupy hub positions. Therefore, the search for such antigens could potentially take a turn towards more thorough investigation of the structure of the PPI networks established in cases of reactive disease.

CONCLUSIONS

Research over the last four decades has proven that CTAs hold a prominent role within the field of cancer serology and could be excellent diagnostic, prognostic and immunotherapeutic biomarkers in CRC. Furthermore, examination of their structural

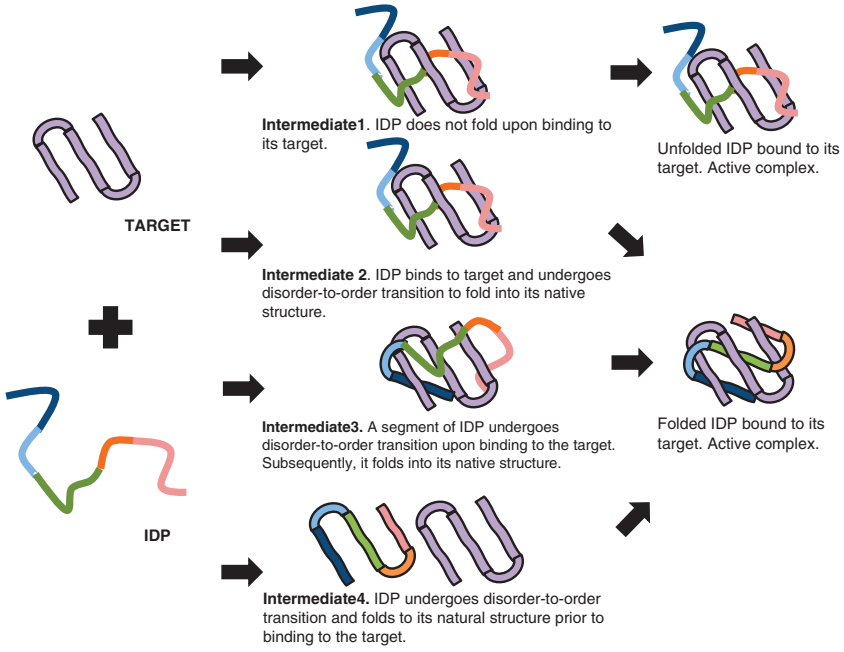

Figure 2. Diagrammatic representation of the different binding mechanisms and the disorder-to-order transition that the IDPs undergo before and upon binding to their targets (based on reference[160]). Some IDPs (particularly immune signalling-related IDPs) do not fold upon binding to their targets (for example, Intermediate 1). Other IDPs undergo partial or complete folding upon binding (Intermediate 2 and Intermediate 3) or fold before binding to their targets (Intermediate 4). The images shown are representative of the disorder-to-order transition that a hypothetical IDP would undergo.

properties and selective expression patterns may enable the understanding of the underlying molecular mechanisms that govern the reactive and non-reactive presentation of CRC. Nevertheless, the panel of CTAs with potential clinicopathological significance in relation to CRC still remains to be conclusively clarified. As this review pointed out, a large number of CTAs to date have been proven to be excellent prognostic biomarkers or targets for the development of peptide or protein vaccines in different solid tumors. However, the attempts to characterize such potent targets and to evaluate their significance in relation to CRC are still at their starting point. Up to date, the comprehensive analysis of the CTAs in relation to CRC have demonstrated that their secondary structural conformation and the presence of a large number of α-helical formations may be accounted for the reduced immunogenicity and treatment response of the CTAs associated with non-reactive type CRC. A solution to this potential problem has also been recently identified and evaluated—epigenetic modulation, which could induce the expression of highly immunogenic CTAs, that could subsequently be targeted by chemo- or immunotherapy. Particular attention should also be paid to the infiltration of Tregs within the tumor mass at different stages of the disease. Tregs have been associated with a rather complex and ambiguous role in the process of tumourigenesis in CRC patients as they have been demonstrated to exhibit both pro- and anti-tumor activities. Further study would be require to gain a better understanding on whether they play different roles in the presentation of reactive versus non-reactive CRC. Nevertheless, research on CTAs expression in CRC should follow the findings that these proteins are also exhibiting particular properties characteristic for IDPs.

In summary, the numerous studies and the significant data gathered regarding the expression pattern of CTAs and their role in carcinogenesis have not yet provided an explanation for the differences in the clinical presentation of CRC. However, they have demonstrated the complex variety of CTA expression mechanisms and their implications in hematological and solid cancers. The fact that certain CTA expressions could be indicative of poor prognosis in solid cancers but suggest good overall outcome in hematological malignancies brings to the fore a number of new challenges on the journey towards advancing clinical tumor immunotherapy. Still the main question remains unanswered: Can we transfer what triggers a good response in reactive disease to a patient with a poor anti-tumor response and improve the overall outcome? Identifying highly immunogenic peptides is a basic requirement for the development of immunotherapy vaccines. However, solid tumors, such as CRC pose additional obstacles on the road to mounting an immune response due to the fact that the antigens expressed within CRC cells are not readily accessible/easily recognized by the immune system. The heterogenicity in the nature of the tumor cells comprising CRCs and their multi-layered structure are the main reasons for evading immune recognition. However, understanding the underlying molecular basis of reactive disease could lead researchers to overcome these issues. Despite the amount of data gathered to date, the search for tumor-specific immunotherapy targets has remained elusive but as our understanding of how to induce effective immune responses grows so does our likelihood of improving patient outcomes.

FUTURE PERSPECTIVES

The identification of novel CTAs for CRC and evaluating the clinical significance of existing ones should be one of the main priorities in this field. As previous research has indicated, a number of CTAs could be effective prognostic biomarkers in CRC when evaluated as a panel of antigens rather than individually. The same approach could also be promising for the development of multivalent cancer vaccines that will target a panel of antigens in a particular tumor type.

It would also be beneficial to investigate the implications of high expression of novel CTAs on patient survival and treatment outcome. Furthermore, understanding the mechanisms of CTAs expression and epitope presentation to CTLs might provide new targets for epigenetic modulation or adjuvant therapies. Nevertheless, investigating the role of the known CRC-associated CTAs in relation to particular drug treatments or therapies should not be overlooked. As discussed earlier, a combined approach for the treatment of CRC might hold the key to the development of more successful therapies for CRC patients. Treatment with hypomethylating drugs on cancers with low CTA expression followed by immunotherapy has already shown promising results in both *in vitro* and *in vivo* studies. These findings should be further investigated in cases with CRC to provide a more conclusive idea of their effectiveness.

As suggested already, a more thorough investigation should enable the discovery of CTAs, which are also IDPs. Understanding the properties and the function of IDPs could assist a more comprehensive understanding of the physical and serological properties of CTAs and how they relate to CRC reactivity.

ABBREVIATIONS

AJCC, American Joint Committee on Cancer; AML, acute myeloid leukemia; CEA, carcinoembryonic antigen; CRC, colorectal cancer; CTA, cancer-testis antigen; CTL, cytotoxic T lymphocyte; DAC, 5-aza-2′-deoxycytidine; DC, dendritic cells; EBV, Epstein–Barr virus; GIST, gastrointestinal stromal tumor; IDP, intrinsically disordered protein; pMHC, peptide MHC; RAYS, recombinant antigen expression on yeast surface; SADA, serum antibody detection array; SEREX, serological identification of antigens by recombinant expression cloning; SERPA, serological proteome analysis; SSX, synovial sarcoma X antigen; SSX2, synovial sarcoma X breakpoint-2; TAA, tumor-associated antigen; TNM, tumor nodes metastases; Tregs, regulatory T cells; TSP50: testes-specific protease 50

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was funded in part by Cork Cancer Research Center. SAB and SEB were funded by Cancer Research UK.

REFERENCES

1 Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010; **127**: 2893–2917.
2 Bray F, Ren JS, Masuyer E, Ferlay J. Global estimates of cancer prevalence for 27 sites in the adult population in 2008. *Int J Cancer* 2013; **132**: 1133–1145.
3 Quaglia A, Vercelli M, Lillini R, Mugno E, Coebergh JW, Quinn M *et al.* Socio-economic factors and health care system characteristics related to cancer survival in the elderly. A population-based analysis in 16 European countries (ELDCARE project). *Crit Rev Oncol Hematol* 2005; **54**: 117–128.
4 Thun MJ, DeLancey JO, Center MM, Jemal A, Ward EM. The global burden of cancer: priorities for prevention. *Carcinogenesis* 2010; **31**: 100–110.
5 Jemal A, Center MM, De Santis C, Ward EM. Global patterns of cancer incidence and mortality rates and trends. *Cancer Epidemiol Biomarkers Prev* 2010; **19**: 1893–1907.
6 WHO. *WHO Library Cataloguing-in-Publication Data. 2008-2013 action plan for the global strategy for the prevention and control of noncommunicable diseases: prevent and control cardiovascular diseases, cancers, chronic respiratory diseases and diabetes*. World Health Organization 2008; Printed by the WHO Document Production Services: Geneva, Switzerland, ISBN 978 92 4 159741 8.
7 Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A. *AJCC Cancer Staging Manual*. 7th edn. Springer, New York, 2010.
8 Dukes CE. The classification of cancer of the rectum. *J Pathol Bacteriol* 1932; **35**: 323–332.

9 Astler VB, Coller FA. The prognostic significance of direct extension of carcinoma of the colon and rectum. *Annal Surg* 1954; **139**: 846–852.
10 Mayer J. Gastrointestinal tract cancer. In: Kasper D*Harrison's Principles of Internal Medicine*. 16th edn. McGraw-Hill, Medical Pub. Division, New York, 2005, pp 527–531.
11 O'Connell JB, Maggard MA, Ko CY. Colon cancer survival rates with the new America joint committee on cancer sixth edition staging. *J Natl Cancer Inst* 2004; **96**: 1420–1425.
12 Marshall JL, Haller DG, de Gramont A, Hochster H, Lenz HJ, Ajani JA *et al.* Adjuvant therapy for stage II and III colon cancer: consensus report of the international society of gastrointestinal oncology. *Gastrointest Cancer Res* 2007; **1**: 146–154.
13 Zaniboni A, Labianca R. Adjuvant therapy for stage II colon cancer: an elephant in the living room? *Ann Oncol* 2004; **15**: 1310–1318.
14 Strul H, Arber N. Screening techniques for prevention and early detection of colorectal cancer in the average risk population. *Gastrointest Cancer Res* 2007; **1**: 98–106.
15 National Cancer Institute Factsheets. Tests to detect colorectal cancer and polyps http://www.cancer.gov/cancertopics/factsheet/detection/colorectal-screening.
16 Kim HJ, Yu MH, Kim H, Byun J, Lee C. Noninvasive molecular biomarkers for the detection of colorectal cancer. *BMB reports* 2008; **41**: 685–692.
17 Murphy J, O'Sullivan GC, Lee G, Madden M, Shanahan F, Collins JK *et al.* The inflammatory response within Dukes' B colorectal cancers: implications for progression of micrometastasis and patient survival. *Am J Gastroenterol* 2000; **95**: 3607–3614.
18 Schlom J, Arlen PM, Gulley JL. Cancer vaccines: moving beyond current paradigms. *Clin Cancer Res* 2007; **13**: 1776–1782.
19 Wollscheid B, Watts JD, Aebersold R. Proteomics/genomics and signaling in lymphocytes. *Curr Opin Immunol* 2004; **16**: 337–344.
20 Guinn BA, Bland EA, Lodi U, Liggins AP, Tobal K, Petters S *et al.* Humoral detection of leukaemia-associated antigens in presentation acute myeloid leukaemia. *Biochem Biophys Res Commun* 2005; **335**: 1293–1304.
21 Knights AJ, Weinzierl AO, Flad T, Guinn BA, Mueller L, Mufti GJ *et al.* A novel MHC-associated proteinase 3 peptide isolated from primary chronic myeloid leukaemia cells further supports the significance of this antigen for the immunotherapy of myeloid leukaemias. *Leukemia* 2006; **20**: 1067–1072.
22 Liggins AP, Guinn BA, Hatton CS, Pulford K, Banham AH. Serologic detection of diffuse large B-cell lymphoma-associated antigens. *Int J Cancer* 2004; **110**: 563–569.
23 Hanafusa T, Mohamed AE, Kitaoka K, Ohue Y, Nakayama E, Ono T. Isolation and characterization of human lung cancer antigens by serological screening with autologous antibodies. *Cancer Lett* 2011; **301**: 57–62.
24 Zou C, Shen J, Tang Q, Yang Z, Yin J, Li Z *et al.* Cancer-testis antigens expressed in osteosarcoma identified by gene microarray correlate with a poor patient prognosis. *Cancer* 2012; **118**: 1845–1855.
25 Qiu J, Hanash S. Autoantibody profiling for cancer detection. *Clin Lab Med* 2009; **29**: 31–46.
26 Fratta E, Coral S, Covre A, Parisi G, Colizzi F, Danielli R *et al.* The biology of cancer testis antigens: putative function, regulation and therapeutic potential. *Mol Oncol* 2011; **5**: 164–182.
27 Old LJ. Cancer immunology: the search for specificity—G. H. A. Clowes Memorial Lecture. *Cancer Res* 1981; **41**: 361–375.
28 Thomas CM, Sweep CG. Serum tumor markers: past, state of the art, and future. *The Int J Biol Markers* 2001; **16**: 73–86.
29 Simpson AJ, Caballero OL, Jungbluth A, Chen YT, Old LJ. Cancer/testis antigens, gametogenesis and cancer. *Nat Rev Cancer* 2005; **5**: 615–625.
30 Lim SH, Zhang Y, Zhang J. Cancer-testis antigens: the current status on antigen regulation and potential clinical use. *Am J Blood Res* 2012; **2**: 29–35.
31 Caballero OL, Chen YT. Cancer/testis (CT) antigens: potential targets for immunotherapy. *Cancer Sci* 2009; **100**: 2014–2021.
32 Scanlan MJ, Gure AO, Jungbluth AA, Old LJ, Chen YT. Cancer/testis antigens: an expanding family of targets for cancer immunotherapy. *Immunol Rev* 2002; **188**: 22–32.
33 Cheng YH, Wong EW, Cheng CY. Cancer/testis (CT) antigens, carcinogenesis and spermatogenesis. *Spermatogenesis* 2011; **1**: 209–220.
34 De Smet C, Courtois SJ, Faraoni I, Lurquin C, Szikora JP, De Backer O *et al.* Involvement of two Ets binding sites in the transcriptional activation of the MAGE1 gene. *Immunogenetics* 1995; **42**: 282–290.
35 Ogawa K, Utsunomiya T, Mimori K, Yamashita K, Okamoto M, Tanaka F *et al.* Genomic screens for genes upregulated by demethylation in colorectal cancer: possible usefulness for clinical application. *Int J Oncol* 2005; **27**: 417–426.
36 Karpf AR. A potential role for epigenetic modulatory drugs in the enhancement of cancer/germ-line antigen vaccine efficacy. *Epigenetics* 2006; **1**: 116–120.
37 Old LJ. Cancer/testis (CT) antigens—a new link between gametogenesis and cancer. *Cancer Immun* 2001; **1**: 1.
38 Traversari C. Tumor-antigens recognized by T lymphocytes. *Minerva Biotechnologica* 1999; **11**: 243–253.
39 Medin J. *Experimental and applied immunotherapy*. Springer, 2010 p11–p17.
40 Slingluff C. The present and future of peptide vaccines for cancer: single or multiple, long or short, alone or in combination? *Cancer J* 2011; **17**: 343–350.
41 Jäger E, Chen YT, Drijfhout JW, Karbach J, Ringhoffer M, Jäger D *et al.* Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. *J Exp Med* 1998; **187**: 265–270.
42 Roswell Park Cancer Institute. Vaccine therapy with or without sirolimus in treating patients with NY-ESO-1 expressing solid tumors. US National Institutes of Health 2012. (Currently ongoing clinical trial).
43 Cancer Research Institute. Cancer vaccine collaborative: active clinical trials in FY 2009(http://www.cancerresearch.org/programs/research/Cancer-Vaccine-Collaborative/trials-2009.html).
44 Marits P, Karlsson M, Thörn M, Wanqvist O. Sentinel node-based immunotherapy of colon cancer. *In colorectal cancer: methods of cancer diagnosis, therapy and prognosis*. Hayat MA (Ed). Springer, New York. 2009, p293.
45 Sahin U, Türeci O, Schmitt H, Cochlovius B, Johannes T, Schmits R *et al.* Human neoplasms elicit multiple specific immune responses in the autologous host. *Proc Natl Acad Sci USA* 1995; **92**: 11810–11813.
46 Chen YT, Scanlan MJ, Sahin U, Türeci O, Gure AO, Tsang AO *et al.* A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc Natl Acad Sci USA* 1997; **94**: 1914–1918.
47 Old LJ, Chen Y-T. New paths in human cancer serology. *J Exp Med* 1998; **187**: 1163–1167.
48 Türeci Ö, Sahin U, Pfreundschuh M. Serological analysis of human tumor antigens: molecular definition and implications. *Molec Med Today* 1997; **3**: 342–349.
49 Zeng G, Wang X, Robbins PF, Rosenberg SA, Wang RF. CD4(+) T cell recognition of MHC class II-restricted epitopes from NY-ESO-1 presented by a prevalent HLA DP4 allele: association with NY-ESO-1 antibody production. *Proc Natl Acad Sci USA* 2001; **98**: 3964–3969.
50 Nakatsura T, Senju S, Yamada K, Jotsuka T, Ogawa M, Nashimura Y. Gene cloning of immunogenic antigens overexpressed in pancreatic cancer. *Biochem Biophys Res Commun* 2001; **281**: 936–944.
51 Nishikawa H, Tanida K, Ikeda H, Sakakura M, Miyahara Y, Aota T *et al.* Role of SEREX-defined immunogenic wild-type cellular molecules in the development of tumor-specific immunity. *Proc Natl Acad Sci USA* 2001; **98**: 14571–14576.
52 Modugno FD, Bronzi G, Scanlan MJ, Del Bello D, Cascioli S, Venturo I *et al.* Human mena protein, a SEREX-defined antigen overexpressed in breast cancer eliciting both humoral and CD8+ T-cell immune response. *Int J Cancer* 2004; **109**: 909–918.
53 Nishikawa H, Kato T, Tawara I, Saito K, Ikeda H, Kuribayashi K *et al.* Definition of target antigens for naturally occurring CD4+ CD25+ regulatory T cells. *J Exp Med* 2005; **201**: 681–686.
54 Neumann F, Wagner C, Preuss K-D, Kubuschok B, Schormann C, Stevanovic S *et al.* Identification of an epitope derived from the cancer testis antigen HOM-TES-14/SCP1 and presented by dendritic cells to circulating CD4+ T cells. *Blood* 2005; **106**: 3105–3113.
55 Cancer Immunome Database [http://ludwig-sun5.unil.ch/CancerImmunomeDB/].
56 Hartmann TB, Bazhin AV, Schadendorf D, Eichmüller SB. SEREX identification of new tumor antigens linked to melanoma-associated retinopathy. *Int J Cancer* 2005; **114**: 88–93.
57 Mischo A, Wadle A, Wätzig K, Jäger D, Stockert E, Santiago D *et al.* Recombinant antigen expression on yeast surface (RAYS) for the detection of serological immune response to cancer. *Cancer Immun* 2003; **3**: 5.
58 Shan J, Yuan L, Xiao Q, Chiorazzi N, Budman D, Teichberg S *et al.* TSP50, a possible protease in human testis, is activated in breast cancer epithelial cells. *Cancer Res* 2002; **62**: 290–294.
59 Jin S, Wang Y, Zhang Y, Zhang HZ, Wang SJ, Tang JQ *et al.* Humoral immune responses against tumor-associated antigen OVA66 originally defined by serological analysis of recombinant cDNA expression libraries and its potentiality in cellular immunity. *Cancer Sci* 2008; **99**: 1670–1678.
60 Imai K, Hirata S, Irie A, Senju S, Ikuta Y, Yokomine K *et al.* Identification of HLA-A2-restricted CTL epitopes of a novel tumour-associated antigen, KIF20A, overexpressed in pancreatic cancer. *Brit J Cancer* 2011; **104**: 300–307.
61 Klein-Scory S, Kübler S, Diehl H, Eilert-Micus C, Reinacher-Schick A, Stühler K *et al.* Immunoscreening of the extracellular proteome of colorectal cancer cells. *BMC Cancer* 2012; **10**: 70.
62 Forgber M, Trefzer U, Sterry W, Walden P. Proteome serological determination of tumor-associated antigens in melanoma. *PLoS ONE* 2009; **4**: e5199.
63 Forgber M, Gellrich S, Sharav T, Sterry W, Walden P. Proteome-based analysis of serologically defined tumor-associated antigens in cutaneous lymphoma. *PLoS ONE* 2009; **4**: e8376.
64 Klade CS, Voss T, Krystek E, Ahorn H, Zatloukal K, Pummer K *et al.* Identification of tumor antigens in renal cell carcinoma by serological proteome analysis. *Proteomics* 2001; **1**: 890–898.

65 Gagnon A, Kim J-H, Schorge JO, Ye B, Liu B, Hasselblatt K *et al.* Use of combination of approaches to identify and validate relevant tumor-associated antigens and their corresponding autoantibodies in ovarian cancer patients. *Clin Cancer Res* 2008; **14**: 764–771.
66 He Y, Wu Y, Mou Z, Li W, Zou L, Fu T *et al.* Proteomics-based identification of HSP60 as a tumor-associated antigen in colorectal cancer. *Proteomics Clin Appl* 2007; **1**: 336–342.
67 Suzuki A, Iizuka A, Komiyama M, Takikawa M, Kume A, Tai S *et al.* Identification of melanoma antigens using a serological proteome approach (SERPA). *Cancer Genomics Proteomics* 2010; **7**: 17–24.
68 Chen YT. Identification of human tumor antigens by serological expression cloning: an online review on SEREX. *Cancer Immun* 2004, [updated 2004; cited 1 April 2004]URL [http://www.cancerimmunity.org/serex].
69 Jäger E, Knuth A. The discovery of cancer/testis antigens by autologous typing with T cell clones and the evolution of cancer vaccines. *Cancer Immun* 2004; **12**: 6.
70 Chevalier F. Highlights on the capacities of ''Gel-based'' proteomics. *Proteome Sci* 2010; **8**: 23.
71 Wadle A, Kubuschok B, Imig J, Wuellner B, Wittig C, Zwick C *et al.* Serological immune response to cancer testis antigen in patients with pancreatic cancer. *Int J Cancer* 2006; **119**: 117–125.
72 Jung V, Fischer E, Imig J, Kleber S, Nuber N, Reinshagen F *et al.* Yeast-based identification of prostate tumor antigens provides an effective vaccine platform. *Anticancer Res* 2010; **30**: 895–902.
73 Scanlan M, Welt S, Gordon C, Chen YT, Gure A, Stockert E *et al.* Cancer-related serological recognition of human colon cancer. *Cancer Res* 2002; **62**: 4041–4047.
74 Stempfer R, Syed P, Vierlinger K, Pichler R, Meese E, Leidinger P *et al.* Tumour auto-antibody screening: performance of protein microarrays using SEREX derived antigens. *BMC Cancer* 2010; **10**: 627.
75 Gunawardana CG, Diamandis EP. High-throughput proteomic strategies for identifying tumour-associated antigens. *Cancer Lett* 2007; **249**: 110–119.
76 Gunawardana CG, Diamandis EP. Identifying novel antibody signatures in ovarian cancer using high-density protein microarrays. *Clin Biochem* 2008; **42**: 426–429.
77 Tsukahara T, Torigoe T, Tamura Y, Wada T, Kawaguchi S, Tsuruma T *et al.* Antigenic peptide vaccination: provoking immune response and clinical benefit for cancer. *Curr Immunol Rev* 2008; **4**: 235–241.
78 Muderspach L, Wilczynski S, Roman L, Bade L, Felix J, Small LA *et al.* A phase I trial of a human papillomavirus (HPV) peptide vaccine for women with high-grade cervical and vulvar intraepithelial neoplasia who are HPV 16 positive. *Clin Cancer Res* 2000; **6**: 3406–3416.
79 Oka Y, Tsuboi A, Taguchi T, Osaki T, Kyo T, Nakajima H *et al.* Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression. *Proc Natl Acad Sci USA* 2004; **101**: 13885–13890.
80 Bolonaki I, Kotsakis A, Papadimitraki E, Aggouraki D, Konsolakis G, Vagia A *et al.* Vaccination of patients with advanced non-small-cell lung cancer with an optimized cryptic human telomerase reverse transcriptase peptide. *J Clin Oncol* 2007; **25**: 2727–2734.
81 Noguchi M, Itoh K, Yao A, Mine T, Yamada A, Obata Y *et al.* Immunological evaluation of individualized peptide vaccination with a low dose of estramustine for HLA-A24 + HRPC patients. *Prostate* 2005; **63**: 1–12.
82 Soen Y, Chen DS, Kraft DL, Davis MM, Brown PO. Detection and characterization of cellular immune responses using peptide-MHC microarrays. *PLoS Biol* 2003; **1**: e65.
83 Chen DS, Soen Y, Stuge TB, Lee PP, Weber JS, Brown PO *et al.* Marked differences in human melanoma antigen-specific T cell responsiveness after vaccination using a functional microarray. *PLoS Med* 2005; **2**: e265.
84 Stockert E, Jäger E, Chen Y-T, Scanlan MJ, Gout I, Karbach J *et al.* A survey of the humoral immune response of cancer to a panel of human tumor antigens. *J Exp Med* 1998; **187**: 1349–1354.
85 US National Institutes of Health 2012, Vaccine therapy with or without sirolimus in treating patients with NY-ESO-1 expressing solid tumors (http://clinicaltrials.gov/ct2/show/NCT01522820).
86 Li M, Yuan YH, Han Y, Liu YX, Yan L, Wang Y *et al.* Expression profile of cancer-testis genes in 121 human colorectal cancer tissue and adjacent normal tissue. *Clin Cancer Res* 2005; **11**: 1809–1814.
87 Yuan L, Shan J, De Risi D, Broome J, Lovecchio J, Gal D *et al.* Isolation of novel gene, TSP50, by a hypomethylated DNA fragment in human breast cancer. *Cancer Res* 1999; **59**: 3215–3221.
88 Zheng L, Xie G, Duan G, Yan X, Li Q. High expression of testes-specific protease 50 is associated with poor prognosis in colorectal carcinoma. *PLoS ONE* 2011; **6**: e22203.
89 Naaby-Hansen S, Mandal A, Wolkowicz MJ, Sen B, Westbrook VA, Shetty J *et al.* CABYR, a novel calcium-binding tyrosine phosphorylation-regulated fibrous sheath protein involved in capacitation. *Dev Biol* 2002; **242**: 236–254.
90 Chiriva-Internati M, Cobos E, Da Silva DM, Kast WM. Sperm fibrous sheath proteins: a potential new class of target antigens for use in human therapeutic cancer vaccines. *Cancer Immun* 2008; **8**: 8.
91 Luo C, Xiao X, Liu D, Chen S, Li M, Xu A *et al.* CABYR is a novel cancer-testis antigen in lung cancer. *Clin Cancer Res* 2007; **13**: 1288–1297.
92 Hsu HC, Lee YL, Cheng TS, Howng SL, Chang LK, Lu PJ *et al.* Characterization of two non-testis-specific CABYR variants that bind to GSK3beta with a proline-rich extensin-like domain. *Biochem Biophys Res Commun* 2005; **329**: 1108–1117.
93 Li H, Fang L, Xiao X, Shen L. The expression and effects the CABYR-c transcript of CABYR gene in hepatocellular carcinoma. *Bull Cancer* 2012; **99**: E26–E33.
94 Tseng YT, Hsia JY, Chen CY, Lin NT, Chong PC, Yang CY. Expression of the sperm fibrous sheath protein CABYR in human cancers and identification of alpha-enolase as an interacting partner of CABYR-a. *Oncol rep* 2011; **25**: 1169–1175.
95 Shantha Kumara HMC, Caballero OL, Su T, Yan X, Ahmed A, Herath SAC *et al.* The cancer testis antigens CABYR a/b and CABY C are expressed in a subset of colorectal cancers and hold promise as targets for specific immunotherapy. *Gastroenterol* 2012; **142**: S–936Suppl 1. (Abstract).
96 Jagadish N, Rana R, Mishra D, Kumar M, Suri A. Sperm associated antigen 9 (SPAG9): a new member of c-Jun NH2-terminal kinase (JNK) interacting protein exclusively expressed in testis. *Keio J Med* 2005; **54**: 66–71.
97 Garg M, Chaurasiya D, Rana R, Jagadish N, Kanojia D, Dudha N *et al.* Sperm-associated antigen 9, a novel cancer testis antigen, is a potential target for immunotherapy in epithelial ovarian cancer. *Clin Cancer Res* 2007; **13**: 1421–1428.
98 Garg M, Kanojia D, Suri S, Gupta S, Gupta A, Suri A. Sperm-associated antigen 9: a novel diagnostic marker for thyroid cancer. *J Clin Endocrinol Metab* 2009; **94**: 4613–4618.
99 Kanojia D, Garg M, Saini S, Agarwal S, Kumar R, Suri A. Sperm associated antigen 9 expression and humoral response in chronic myeloid leukemia. *Leuk Res* 2010; **34**: 858–863.
100 Kanojia D, Garg M, Gupta S, Gupta A, Suri A. Sperm-associated antigen 9 is a novel biomarker for colorectal cancer and is involved in tumor growth and tumorigenicity. *Am J of Pathol* 2011; **178**: 1009–1020.
101 Song MH, Ha JC, Lee SM, Park YM, Lee SY. Identification of BCP-20 (FBXO39) as a cancer/testis antigen from colon cancer patients by SEREX. *Biochem Biophys Res Commun* 2011; **408**: 195–201.
102 Chen Z, Li M, Yuan Y, Wang Q, Yan L, Gu J. Cancer/testis antigens and clinical risk factors for liver metastasis of colorectal cancer: a predictive panel. *Dis Colon Rectum* 2010; **53**: 31–38.
103 De Plaen E, De Backer O, Arnaud D, Bonjean B, Chomez P, Martelange V *et al.* A new family of mouse genes homologous to the human MAGE genes. *Genomics* 1999; **55**: 176–184.
104 Takahashi N, Ohkuri T, Homma S, Ohtake J, Wakita D, Togashi Y *et al.* First clinical trial of cancer vaccine therapy with artificially synthesized helper/killer-hybrid epitope long peptide of MAGE-A4 cancer antigen. *Cancer Sci* 2012; **103**: 150–153.
105 Yokoe T, Tanaka F, Mimori K, Inoue H, Ohmachi T, Kusunoki M *et al.* Efficient identification of a novel cancer/testis antigen for immunotherapy using three-step microarray analysis. *Cancer Res* 2008; **68**: 1074–1082.
106 Choi J, Chang H. The expression of MAGE and SSX, and correlation of COX2, VEGF, and survivin in colorectal cancer. *Anticancer Res* 2012; **32**: 559–564.
107 Türeci O, Sahin U, Schobert I, Koslowski M, Schmitt H, Schild HJ *et al.* The SSX-2 gene, which is involved in the t(X;18) translocation of synovial sarcomas, codes for the human tumor antigen HOM-MEL-40. *Cancer Res* 1996; **56**: 4766–4772.
108 Ayyoub M, Hesdorffer CS, Montes M, Merlo A, Speiser D, Rimoldi D *et al.* An immunodominant SSX-2-derived epitope recognized by CD4 + T cells in association with HLA-DR. *J Clin Invest* 2004; **113**: 1225–1233.
109 Smith HA, McNeel DG. The SSX family of cancer-testis antigens as target proteins for tumor therapy. *Clin Dev Immunol* 2010; **2010**: 150591.
110 Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J *et al.* A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* 1994; **367**: 645–648.
111 Krivtsov A, Twomey D, Feng Z, Stubbs M, Wang Y, Faber J *et al.* Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. *Nature* 2006; **442**: 818–822.
112 Wang C, Xie J, Guo J, Manning HC, Gore JC, Guo N. Evaluation of CD44 and CD133 as cancer stem cell markers for colorectal cancer. *Oncol Rep* 2012; **28**: 1301–1308.
113 Ricci-Vitiani L, Lombardi D, Pilozzi E, Biffoni M, Todaro M, Peschle C *et al.* Identification and expansion of human colon-cancer-initiating cells. *Nature* 2007; **445**: 111–115.
114 Horst D, Kriegl L, Engel J, Kirchner T, Jung A. Prognostic significance of the cancer stem cell markers CD133, CD44, and CD166 in colorectal cancer. *Cancer Invest* 2009; **2**: 844–850.

115 Choi D, Lee H, Hur K, Kim J, Park G, Jang S *et al.* Cancer stem cell markers CD133 and CD24 correlate with invasiveness and differentiation in colorectal adenocarcinoma. *World J Gastroenterol* 2007; **15**: 2258–2264.
116 Horst D, Kriegl L, Engel J, Jung A, Kirchner T. CD133 and nuclear beta-catenin: the marker combination to detect high risk cases of low stage colorectal cancer. *Eur J Cancer* 2009; **45**: 2034–2040.
117 Lin J, Lin C, Yang S, Wang H, Jiang J, Lan Y *et al.* Early postoperative CEA level is a better prognostic indicator than is preoperative CEA level in predicting prognosis of patients with curable colorectal cancer. *Int J Colorectal Dis* 2011; **26**: 1135–1141.
118 Yeh C, Hsieh P, Chiang J, Lai C, Chen J, Wang J *et al.* Preoperative carcinoembryonic antigen elevation in colorectal cancer. *Hepatogastroenterology* 2011; **58**: 1171–1176.
119 Kirat H, Ozturk E, Lavery I, Kiran R. The predictive value of preoperative carcinoembryonic antigen level in the prognosis of colon cancer. *Am J Surg* 2012; **204**: 447–452.
120 Burgdorf S. Dendritic cell vaccination of patients with metastatic colorectal cancer. *Dan Med Bull* 2010; **57**: B4171.
121 Lesterhuis W, De Vries I, Schreibelt G, Schuurhuis D, Aarntzen E, De Boer A *et al.* Immunogenicity of dendritic cells pulsed with CEA peptide or transfected with CEA mRNA for vaccination of colorectal cancer patients. *Anticancer Res* 2010; **30**: 5091–5097.
122 Staff C, Mozaffari F, Haller BK, Wahren B, Liljefors M. A phase I safety study of plasmid DNA immunization targeting carcinoembryonic antigen in colorectal cancer patients. *Vaccine* 2011; **29**: 6817–6822.
123 Hong X, Dong T, Hu J, Yi T, Li W, Zhang Z *et al.* Synergistical toll-like receptors activated dendritic cells induce antitumor effects against carcinoembryonic antigen-expressing colon cancer. *Int J Colorectal Dis* 2012; **28**: 25–33(E-pub ahead of print).
124 Sharma A, Bode B, Wenger RH, Lehmann K, Sartori AA, Moch H *et al.* γ-radiation promotes immunological recognition of cancer cells through increased expression of cancer-testis antigens *in vitro* and *in vivo*. *PLoS ONE* 2011; **6**: e28217.
125 Guinn B, Greiner J, Schmitt M, Mills KI. Elevated expression of the leukemia-associated antigen SSX2IP predicts survival in acute myeloid leukemia patients who lack detectable cytogenetic rearrangements. *Blood* 2009; **113**: 1203–1204.
126 Greiner J, Bullinger L, Guinn BA, Döhner H, Schmitt M. Leukaemia-associated antigens are critical for the proliferation of acute myeloid leukaemia cells. *Clin Cancer Res* 2008; **14**: 7161–7166.
127 Perez D, Hauswirth F, Jäger D, Metzger U, Samartzis EP, Went P *et al.* Protein expression of cancer testis antigens predicts tumor recurrence and treatment response to imatinib in gastrointestinal stromal tumors. *Intl J Cancer* 2011; **128**: 2947–2952.
128 Luetkens T, Schafhausen P, Uhlich F, Stasche T, Akbulak R, Bartels BM *et al.* Expression, epigenetic regulation, and humoral immunogenicity of cancer-testis antigens in chronic myeloid leukemia. *Leuk Res* 2010; **34**: 1647–1655.
129 Smith HA, McNeel DG. Vaccines targeting the cancer-testis antigen SSX-2 elicit HLA-A2 epitope-specific cytolytic T cells. *J Immunother* 2011; **34**: 569–580.
130 Shiraishi T, Terada N, Zeng Y, Suyama T, Luo J, Trock B *et al.* Cancer/testis antigens as potential predictors of biochemical recurrence of prostate cancer following radical prostatectomy. *J Transl Med* 2011; **9**: 153.
131 Boehmer L, Keller L, Mortezavi A, Provenzano M, Sais G, Hermanns T *et al.* MAGE-C2/CT10 protein expression is an independent predictor of recurrence in prostate cancer. *PLoS ONE* 2011; **6**: e21366.
132 Tajima K, Obata Y, Tamaki H, Yoshida M, Chen YT, Scanlan MJ *et al.* Expression of cancer/testis (CT) antigens in lung cancer. *Lung Cancer* 2003; **42**: 23–33.
133 van Duin M, Broyl A, de Knegt Y, Goldschmidt H, Richardson PG, Hop WCJ *et al.* Cancer testis antigens in newly diagnosed and relapse multiple myeloma: prognostic markers and potential targets for immunotherapy. *Haematologica* 2011; **96**: 1662–1669.
134 Pollack SM, Jungbluth AA, Hoch BL, Farrar EA, Bleakley M, Schneider DJ *et al.* NY-ESO-1 is a ubiquitous immunotherapeutic target antigen for patients with myxoid/round cell liposarcoma. *Cancer* 2012; **118**: 4564–4570.
135 Lapillonne H, Renneville A, Auvrignon A, Flamant C, Blaise A, Perot C *et al.* High WT1 expression after induction therapy predicts high risk of relapse and death in acute myeloid leukemia. *J Clin Oncol* 2006; **24**: 1507–1515.
136 John A, Liu Y, Tobal K. Detection of minimal residual disease in acute myeloid leukemia: methodologies, clinical and biological significance. *Brit J Haematol* 2002; **106**: 578–590.
137 Shiraishi T, Getzenberg RH, Kulkarni P. Cancer/testis antigens: novel tools for discerning aggressive from non-aggressive prostate cancer. *Asian J Androl* 2012; **14**: 400–404.
138 Gnjatic S, Altorki NK, Tang DN, Tu SM, Kundra V, Ritter G *et al.* NY-ESO-1 DNA vaccine induces T-cell responses that are suppressed by regulatory T cells. *Clin Cancer Res* 2009; **15**: 2130–2139.
139 Zhang S, Zhou X, Yu H, Yu Y. Expression of tumour-specific antigen MAGE, GAGE and BAGE in ovarian cancer tissues and cell lines. *BMC Cancer* 2010; **10**: 163.
140 Mossman D, Kim K, Scott RJ. Demethylation by 5-aza-2′-deoxyxytidine in colorectal cancer cells target genomic DNA whilst promoter CpG island methylation persists. *BMC Cancer* 2010; **10**: 366.
141 Robins RA, Rees RC. Cancer/testis antigens and cancer immunology. In: *Immunology and Medicine Series* 2001; **30**: 28–37.
142 Glazer CA, Smith IM, Ochs MF, Begum S, Westra W, Chang SS *et al.* Integrative discovery of epigenetically depressed cancer testis antigens in NSCLC. *PLoS ONE* 2009; **4**: e8189.
143 Goldberg AL, Rock KL. Proteolysis, proteasomes and antigen presentation. *Nature* 1992; **357**: 375–379.
144 Chou J, Voong LN, Mortales CL, Towlerton AM, Pollack SM, Chen X *et al.* Epigenetic modulation to enable antigen-specific T-cell therapy of colorectal cancer. *J Immunother* 2012; **35**: 131–141.
145 Weinberg RA. *The Biology of Cancer*. Garland Science, 2007 p703–p707.
146 Curiel T. Tregs and rethinking cancer immunotherapy. *J Clin Invest* 2007; **117**: 1167–1174.
147 Holmén N, Lundgren A, Lundin S, Bergin AM, Rudin A, Sjövall H *et al.* Functional CD4 + CD25high regulatory T cells are enriched in the colonic mucosa of patients with active ulcerative colitis and increase with disease activity. *Inflamm Bowel Dis* 2006; **12**: 447–456.
148 Mougiakakos D. Regulatory T cells in colorectal cancer: from biology to prognostic significance. *Cancers* 2011; **3**: 1708–1731.
149 Correale P, Cusi MG, Tsang KY, Del Vecchio MD, Marsili S, Placa ML *et al.* Chemoimmunotherapy of metastatic colorectal carcinoma with gemcitabine plus FOLFOX 4 followed by subcutaneous granulocyte macrophage colony-stimulating factor and interleukin-2 induces strong immunologic and antitumor activity in metastatic colon cancer patients. *J Clin Oncol* 2005; **23**: 8950–8958.
150 Bonertz A, Weitz J, Pietsch D, Rahbari N, Schlude C, Ge Y *et al.* Antigen-specific tregs control T cell responses against a limited repertoire of tumor antigens in patients with colorectal carcinoma. *J Clin Invest* 2009; **119**: 3311–3321.
151 Nosho K, Baba Y, Tanaka N, Shima K, Hayashi M, Meyerhardt JA *et al.* Tumour-infiltrating T-cell subsets, molecular changes in colorectal cancer, and prognosis: cohort study and literature review. *J Pathol* 2010; **222**: 350–366.
152 Pagès F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molidor R *et al.* Effector memory T cells, early metastasis, and survival in colorectal cancer. *N Engl J Med* 2005; **353**: 2654–2666.
153 Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pagès C *et al.* Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 2006; **313**: 1960–1964.
154 Pagès F, Kirilovsky A, Mlecnik B, Asslaber M, Tosolini M, Bindea G *et al. In situ* cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer. *J Clin Oncol* 2009; **27**: 5944–5951.
155 Salama P, Stewart C, Forrest C, Platell C, Iacopetta B. FOXP3 + cell density in lymphoid follicles from histologically normal mucosa is a strong prognostic factor in early stage colon cancer. *Cancer Immunol Immunother* 2012; **61**: 1183–1190.
156 Rajagopalan K, Mooney S, Parekh N, Getzenberg R, Kulkarni P. A majority of the cancer/testis antigens are intrinsically disordered proteins. *J Cell Biochem* 2011; **112**: 3256–3267.
157 Marsh J, Dancheck B, Ragusa M, Allaire M, Forman-Kay D, Peti W. Structural diversity in free and bound states of intrinsically disordered protein phosphatase 1 regulators. *Structure* 2010; **18**: 1094–1103.
158 Kulkarni P, Rajagopalan K, Yeater D, Getzenberg RH. Protein folding and the order/disorder paradox. *J Cell Biochem* 2011; **112**: 1949–1952.
159 Zhou HX, Pang X, Lu C. Rate constants and mechanisms of intrinsically disordered proteins binding to structured targets. *Phys Chem Chem Phys* 2012; **14**: 10466–10476.
160 Sigalov A. Uncoupled binding and folding of immune signaling-related intrinsically disordered proteins. *Prog Biophys Mol Biol* 2011; **106**: 525–536.
161 Zhou HX. Intrinsic disorder: signaling via highly specific but short-lived association. *Trends Biochem Sci* 2012; **37**: 43–48.
162 Brown C, Johnson A, Dunker A, Daughdrill G. Evolution and disorder. *Curr Opinion Struct Biol* 2011; **21**: 441–446.
163 Midic U, Oldfield C, Dunker A, Obradovic Z, Uversky V. Protein disorder in the human diseasome: unfoldomics of human genetic diseases. *BMC Genomics* 2009; **10**: S12.