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DOTTORATO DI RICERCA IN BIOPATOLOGIA
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Possible new targets in cancer: the example of CD162

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CHAPTER 1
Role of the glycoprotein CD162 (PSGL-1) as a potential target for immunotherapy in T cell lymphomas

Introduction

Lymphomas are a heterogeneous array of cancers affecting the lymphoid tissue and deriving from the uncontrolled proliferation of lymphocytes, cells belonging to the immune system. Lymphomas are classically divided into Hodgkin's Lymphoma (HL) and Non-Hodgkin's Lymphomas (NHLs). HLs are characterized by the the presence of a peculiar type of cancer cell named Reed Sternberg cell. NHLs are caused by the clonal expansion of B cells, T cells or Natural Killer (NK) cells which lose the capability to perform their main functions and begin to progressively localize and accumulate into lymphoid organs. Lymph nodes, bone marrow and spleen are mainly involved during lymphomagenesis, however, neoplastic clones can often disseminate and colonize also other extranodal sites such as the central nervous system, skin or liver.

Even if the basis underlying the development of lymphoid neoplasms still remain partially unknown, multiple risk factors have been linked to the occurance of lymphomas. Indeed, chromosomal alterations, autoimmune disorders featured by a chronic antigenic stimulation, immunosuppression (driven by virus infections or by immunosuppressive therapy regimens) can play a pivotal role in lymphomagenesis.

About the 80-90% of NHLs involve B-cells and just a minority of cases are of T-cells origin (e.g. Peripheral T-cell Lymphomas), as a consequence of their low incidence T-cell lymphomas are not well characterized yet and often represent a real challenge both for the diagnostic approach and for their clinical management. Such features make T-cell NHLs an interesting field of investigation aimed to better clarify their pathogensis and to develop new therapeutic strategies.

Peripheral T cell lymphomas (PTCLs) represent a miscellaneous group of rare hematological neoplasms,

accounting almost for the 10-15 % of all non-Hodgkin lymphomas [1], that commonly share an aggressive behaviour and poor prognosis. Such values of incidence may follow a pattern of geographical distribution since the majority of cases are registered in Asia compared to western countries [2]

PTCLs arise from post-thymic T lymphocytes or mature NK which after the occurance of genetic alterations head towards anomaly in their proliferate rate. According to the most recent WHO classification of hematological and lymphoid malignancies, PTCLs are broadly divided in two categories: the branch of specified forms and the one of not otherwise specified (NOS) entities [3]. While the specified group is further subdivided into defined lymphoproliferative disorders showing characteristic genetic aberrations, typical clinical presentation and immunophenotype, PTCLs-NOS lack of distinctive features and represent the majority of PTCLs for which no peculiar immunophenotipic or molecular profile has been typified yet. PTCLs comprise forms such as anaplastic large T-cell lymphoma (ALCL) in its ALK+ and ALK- variants [4] , angioimmunoblastic T- cell lymphoma (AITL) [5], extranodal forms like the hepatosplenic γδ-T cell lymphoma (HSTL) [6] and the NK/T-cell lymphoma – nasal type [7], the Adult T-cell leukemia/lymphoma (ATLL) associated to HTLV-1 infection [8].

Despite the overmentioned classification, peripheral T cell lymphomas can be characterized by: infrequency, poor prognosis, unresponsiveness to conventional chemotherapic treatments. Clinical approaches to PTCL have so far provided similar treatments to those used for B cell lymphomas (anthracyclines-based regimens as CHOP or CHOP-like), however, no encouraging results have been achieved and the 5 years overall survival rate usually do not exceed the 40% of cases [9, 10].

A valid and promising therapeutic option may come from targeted therapies based on the use/subministration of monoclonal antibodies (mAbs). Once engaged their specific target, mAbs are able to activate immunological mediators such as the complement system and in turn mediate citotoxic effects on cancer cells. Targeting cancer cells through the use of monoclonal antibodies able to bind tumor associated antigens has contributed, in the later years, to change the approach for the treatment of both solid and hematological neoplasms. To date, several mabs have been introduced in clinical practice, examples are: the anti-HER2 receptor Trastuzumab for the treatment of HER2 positive breast cancer [11], the anti-VEGF-A Bevacizumab for treatment of colorectal cancer [12], or the anti-CD20 Rituximab for the treatment of B-cell lymphomas [13]. Administration of mabs alone or in association with chemoterapic regimens have so far demonstrated a more accurated ability to specifically eradicate cancer clones sparing the normal cellular counterpart from elimination. mAbs can exert anti-tumor effects by mediating the activation of immune effectors recognizing the Fc fragment of mabs adsorbed on target cells, or through direct cytoxic effects.

The immune effectors involved in tumor cell lysis are: the complement system which is able to mediate the so-called complement-dependent cytotoxicity (CDC), and cytotoxic effector cells including: dendritic cells [14], monocytes [15], natural killer (NK) cells [16], and T- cells [17; 18] mediating the antibody-dependent cell-mediated cytotoxicity (ADCC).

The classical complement cascade becomes active after that its first component, the C1q protein, recognizes and binds to the Fc portion of the antibody adsorbed on target cell surface. Such event initiates a signaling pathway which brings, through the cleavege of the components C4 and C2, to the formation of the protein complex C3 convertase. The C3 convertase is further

involved in the cleavege of C3 and formation of C5 convertase that cleaves C5. C5 finally complexes with C6, C7, C8 and C9, thus forming the membrane attacking complex (MAC) and elicitating the CDC.

On the contrary, ADCC does not involve soluble proteins to kill target cells but requires effector cells to recognize and bind, through their Fcγ receptors (FcγRs), the Fc portion of mabs attached on target cell surface. The interaction between FcγRs and Fc causes the release of cytotoxic granules responsible for cell lysis.

One of the first suitable target proposed for PTCLs treatment was the membrane antigen CD52, target of the mAb Campath (Alemtuzumab). However, due to its variable and heterogeneous expression on cancer cells, use of Alemtuzumab did not prove to be effective and the relapse rate after treatment is high [19; 20]. Another mAb which is currently tested in several clinical trials is the chimeric anti-CD30 conjugated with an anti-tubulin chemoterapeutic agent, the brentuximab-vedotin (SGN-35). PTCLs cells can else be CD30+ and share this feature with Hodgkin lymphoma cells. Treatments with SGN-35 have been shown to obtain good results also in relapsed cases and in patients refractory to conventional chemotherapeutic administration [21,22]

In 2009, our group investigated the cell adhesion molecule P-Selectin Glycoprotein Ligand-1 (PSGL-1 , CD162) as a suitable candidate of immunotherapy of multiple myeloma [23].

PSGL-1 is a disulfide-linked homodimeric glycoprotein of 240 kDa carrying two *Sialyl-Lewis*[X] groups, it's expressed on all myeloid and lymphoid lineages and it acts as the ligand for all three selectins [24]. If opportunely sulfated on its tyrosines (Tyr13 and Tyr 15), PSGL-1 can drive tethering and rolling as well as cell extravasation [25]. Although PSGL-1 pathway has

not been completely described, its engagement induces the activation of Src family kinases (SFKs), spleen tyrosine kinases (Syk) and p38 (belonging to the MAP kinases group) [26], its triggering is moreover involved in T cell homeostasis regulating CD8+ cells proliferation [27].

In light of the known expression of PSGL-1 on activated T cells and according to the capability of anti-PSGL-1 monoclonal antibody to induce murine T cell death in vitro [28], we sought to determine if PSGL-1 could act as a potential target for humoral immunotherapy in PTCLs.

Aim of this study was to evaluate PSGL-1 expression in PTCLs cases and the *in vitro* effects elicited by treatment with two anti-PSGL-1 mAbs on human ALCL cell lines. We also investigated the role of immunological effectors able to mediate tumor cell lysis after the binding of the antibody to its target by ADCC and CDC assays.
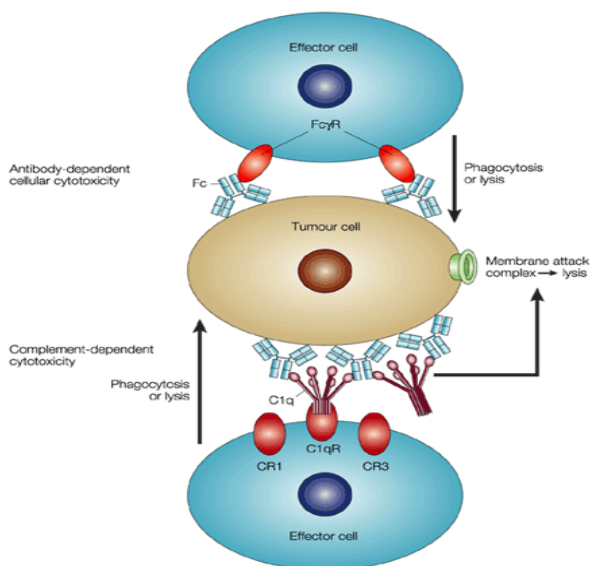
Materials and Methods

Gene Expression Profiling Analysis

Total RNA was extracted with the use of the TRIzol reagent (Invitrogen and Life Technologies), purified with the use of the RNeasy Kit (QIAGEN), and processed according to the Affymetrix Expression Analysis Technical Manual. Fragmented cRNA (15 µg) was hybridized to HG-U133 2.0 Plus microarrays (Affymetrix). The gene expression values were determined by MAS 5 algorithm in Expression Console (Affymetrix), and normalization was performed by scaling to a target intensity of 500. Normalization quality control was performed by box-plot and MA plot consistency.

Cell lines and Tissue Specimens

The human ALCL cell lines SU-DHL, TS, JB6, KI-JK, SUP-M2, KARPAS-299 and the PTCL-NOS cell line MAC were kindly provided by Prof G. Inghirami (Università di Torino). The cells were grown in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS) (Hyclone Laboratories, Logan, United Kingdom), 2mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and incubated at 37C° in 5% CO_2 humidified atmosphere.
Formalin-fixed, paraffin-embedded tissue samples from 110 cases of ALCL and 50 cases of PTCL-NOS cases were collected in tissue microarrays whose sections were gently provided by Prof. PP Piccaluga (Università di Bologna)

Tissue Microarrays
For tissue microarray (TMA) construction, a slide stained with hematoxylin and eosin was prepared from each paraffin block,

and representative tumor regions were morphologically identified and marked on each slide. Tissue cylinders with a diameter of 1.0 mm were punched from the marked areas of each block and brought into a recipient paraffin block using a precision instrument. Four-μm thick sections were cut from each recipient block and used immunohistochemical stains.

Antibodies
The following antibodies were used in our experiments: LEAF™ purified anti-human CD162 Antibody (Biolegend); anti-human CD162 azide-free (Immunotools); anti-human CD46 (Immunotools); anti-human CD55 (IBGRL Reserch products, Bristol, UK); anti-human CD59 (isolated by the Department of Bacteriology and Immunology of the S.Junnikkala Haartman Institute); Alexa 488-conjugated goat anti-mouse (Invitrogen Molecular Probes).

Immunohistochemistry

For in situ single-marker immunohistochemical analysis sections of tissue microarrays made of ALCL and PTCL-NOS cases were deparaffinized with xylene and rehydrated to water through a graded alcohol series. Antigen unmasking was performed using a microwave epitope retrieval technique with 10 mmol citrate buffer (pH 9.0) at high temperature for 20 min. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide. Sections were incubated with anti-human CD162 (Biolegend) at room temperature for 1 hour. Staining was performed with the Novolink Max Polymer Detection System (Leica Microsystems) and with DAB (3,3' Diaminobenzidine) substrate chromogen (Leica Microsystems). Finally, sections were countestained with hematoxilyn.
Slides were evaluated using a Leica DM2000 equipped with a

Leica DFC320 digital camera.

Flow Citometry

Flow cytometry analysis was performed on PTCL cells using indirect immunofluorescent staining Samples were analysed on a Becton Dickinson FACScalibur (BD Biosciences, NJ, USA)

CDC

$2x10^5$ cells were incubated with TB5 with or without blocking antibodies against CD46, CD55 and CD59 (2.5μg/mL) diluited in PBS-BSA 2% Ca^{2+}-Mg^{2+} to a final volume of 100 μl for 10 minutes at room temperature prior to addition of 25% normal human serum (NHS). After further incubation at 37°C for 1hour, the number of residual viable cells was estimated using the MTT assay and the percentage of dead cells was calculated with the TECAN Infinite-200 microplate reader (TECAN).

ADCC

$5x10^4$ cells were washed and resuspended in 100μL of PBS and incubated with the lipophilic tracer FAstDil (Invitrogen) for 15 minutes at 37°C. Not-bound dye was eliminated by 3 washes in PBS and cells were pelleted by centrifugation (1 minute at 250g) and resuspended in 200 μL of RPMI-1640. Labeled cells were incubated with human effector cells (PBMCs, effector-to-target ratio of 50:1) and anti-human PSGL-1 antibodies (2.5μg/mL) for 48 hours at 37°C.
Non-viable cells count was obtained by calculating the release of FAstDil in solution with the TECAN Infinite-200 microplate reader (TECAN).

Direct Cytotoxicity

$2x10^5$ cells were incubated with anti-human PSGL-1 antibodies (2.5μg/mL) in 100μL RPMI-1640 for 48 hours at 37°C. The number of residual viable cells was estimated using the MTT assay and the percentage of dead cells was calculated with the TECAN Infinite-200 microplate reader (TECAN).

Confocal Analysis

10^5 were spotted on a cytospin slide and fixed in 3-4% paraformaldehyde in PBS for 15 minutes at room temperature. Cells were sequentially incubated with 1%BSA in PBS for 30 minutes to block unspecific binding of the antibodies and with anti-human PSGL-1 antibodies (1mg/ml) for 1 hr at room temperature in a humidified chamber.
Finally, cells were incubated with the Alexa 488-conjugated goat anti-mouse secondary antibody for 1 hr at room temperature in dark.
Slides were evaluated using the Leica TCS SP5 Laser Scanning Confocal Microscope.

Results

Gene Expression Profiling Analysis reveals increasing levels of PSGL-1 mRNA in Anaplastic Large T cell lymphomas.

T-cell lymphomagenesis is often followed by the aberrant expression of immunophenotipic markers which are tipically expressed by normal T cells. Consequently, T-cell lymphoproliferative disorders may be associated to loss of CD3, CD4 and CD8, reduction of CD45 and incoherent CD79a and CD20 expression [29; 30; 31] On the ground of such evidences, we sought to determine if PSGL-1 mRNA may vary its level of expression in peripheral T cell neoplasms through a gene expression profile analysis (GEP). We collected PSGL-1 transcripts from AITL, ALCL, ATLL, PTCL-NOS, reactive and normal specimens. The analysis of variance between the different groups of lymphoproliferative disorders demonstrated an overall increase of PSGL-1 mRNA in PTCLs, in particular, Anaplastic Large T-cell Lymphoma was found to show the highest expression of the transcript. GEP data prompted us to choose the ALCL as a prototypical setting for the evaluation of PSGL-1 expression in tissue samples.
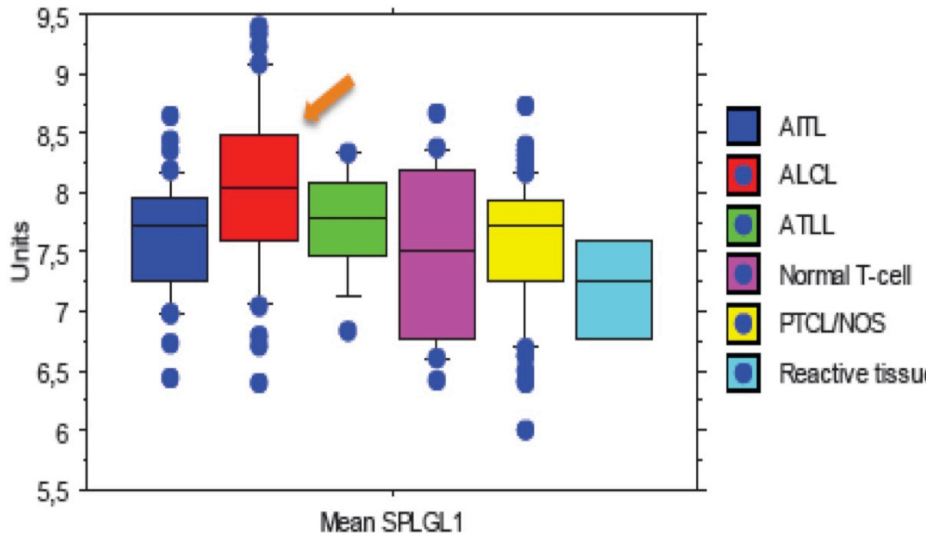
GEP Analysis of PSGL-1 mRNA PTCL Vs Control

PSGL-1 is widely expressed in ALCL cases

Given to the fact that a high level of tissue expression is an essential requirement to choose a molecule for targeted immunotherapy, we attempted to assess PSGL-1 expression in ALCL cases versus PTCL-NOS cases, chosen as our control. We, moreover, assessed a semi-quantitative analysis, assigning to each case a value ranging from score 0 to score 3+ , to classify all cases according to the intensity of the staining.

110 cases of ALCL and 50 of PTCL-NOS were collected using the Tissue Microarray technique and immunohistochemical staining was performed. Taking into account the totality of PTCLs collected (160), we described a median score of intensity of 3+ (3.1% score 0, 11% score 1+; 28% score 2+, and 52,5% score 3+) in the 92% of positive cases. However, ALCL and PTCL-NOS cases did not show the same median score of PSGL-1 expression that was 3+ in ALCL cases (1.8% score 0, 11.8% score 1+, 23.6% score 2+, and 59% score 3+) and 2+ in PTCL-NOS histotype (6% score 0, 10% score 1+, 38% score 2+, and 38% score 3+). Such evidence demonstrated an homogeneous expression of PSGL-1 in ALCL cases (the 94% of cases) the majority of whom displayed the highest intensity of positivity for PSGL-1. Immunohistochemical data not only confirmed the previous results obtained by GEP analysis but also convinced us to investigate the expression of PSGL-1 in ALCL human cell lines.

Cases	ALCL 110	PTCL-NOS 50	ALCL/PTCL-NOS 160
Positive Cases	104 (94%)	43 (86%)	92%
Negative Cases	2 (1.8%)	3 (6%)	3.1%
Not Evaluable Cases	4 (3.6%)	4 (8%)	5%
Intensity +1	13 (11.8%)	5 (10%)	11%
Intensity +2	26 (23.6%)	19 (38%)	28%
Intensity +3	65 (59%)	19 (38%)	52.5%
Median Value of Intensity	+3	+2	+3

PSGL-1

Negativo

Grado 1

Grado 2

Grado 3
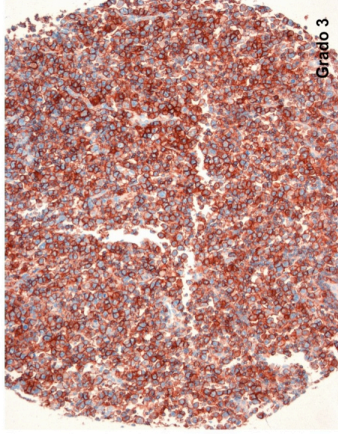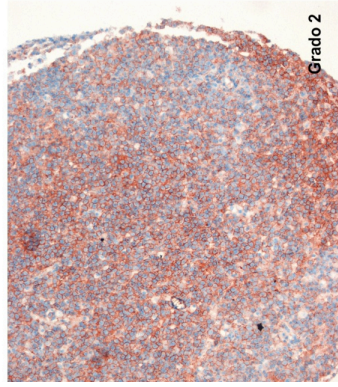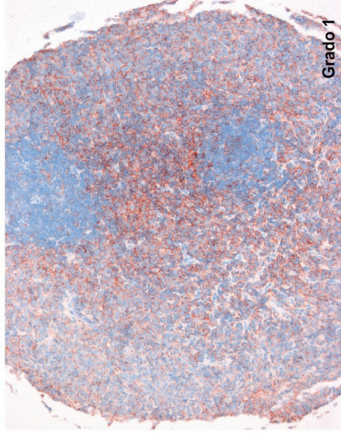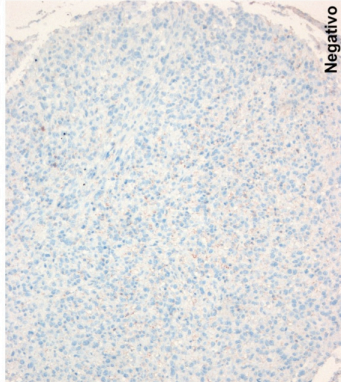
quantitative analysis of PSGL-1 expression

Anaplastic Large T Cell Lymphoma cell lines are positive for PSGL-1

To further elucidate the role of PSGL-1 and to transfer our evidences to an "in vitro" setting, we analyzed PSGL-1 distribution on human ALCL cell lines surface by cytofluorimetry. Six human ALCL cell lines bearing the t(2,5) and its fusion product NPM-ALK, together with a PTCL-NOS cell line chosen as control, were assessed for PSGL-1 expression using two different anti-human PSGL-1 monoclonal antibodies: the KPL-1 and the TB5 clones. Both mabs were selected according to their blocking activity (KPL1) or agonist effect (TB5) towards PSGL-1 and their application was aimed to obtain insights about the effects that may arise from the block or activation of PSGL-1.

In Cytofluorimetric analysis, KPL1 and TB5 clones revealed a comparable capability to bind their target which was overall expressed by each cell line analyzed. In particular, the expression of PSGL-1 assessed with KPL-1 and TB5 was respectively: 98.5% and 98.4% in TS cell line; 90.9% and 95.9% in JB6; 91.7% and 71% in SUDHL-1; 76.2% and 44.7% in KARPAS-299; 55.5% and 31.9% in KI-JK; 29.2% and 13.9% in SUP M2; 83.8% and 57.9% in our control cell line MAC-1 (data not shown).

In general, KPL1 was showed to be more capable to bind PSGL-1 than TB5, such event was probably due to a more easy access for KPL1 to its binding epitope. On the basis of the results obtained by cytofluorimetric analysis, we set up three different assays to study the antibody-mediated lysis of our ALCL cell lines: the complement-dependent cytotoxicity (CDC), the antibody dependent cell-mediated cytotoxicity (ADCC), and the direct cytotoxicity.

Anti-PSGL-1 mabs are able to induce antibody mediated cell lysis

In light of the mabs ability to induce cell death, we set up three lines of experiments in vitro to evaluate the capacity of anti-PSGL1 mabs to mediate human ALCL cell lines killing by means of CDC, ADCC and direct citotoxicity.

Overall, both KPL1 and TB5 did not show an elevated capacity to induce a pronounced complement activation followed by a conspicuous CDC. In particular, KPL-1 mediated a lysis equal to the 8.7% in our control cell line MAC-1 (with a standard deviation of 4.5%), a CDC equal to the 16.5% in SUDHL-1 (with a standard deviation if 4.4%), the 11.8% in JB6 cells (with a standard deviation of 6.5%), the 10.6% in TS cells (with a standard deviation of 4.6%), almost the 1% in KIJK cells (with a standard deviation of 2.16%), the 5% in SUPM2 cells (with a standard deviation of 3.1%). No CDC was mediated by KPL-1 in Karpas-299 cell line.

Alike the clone KPL-1, the binding of TB5 to PSGL-1 elicitated a weak complement dependent citotoxicity that was equivalent to 19.9% in MAC-1 cell line (with a standard deviation of 7.1%), 15% in SUDHL-1 cells (with a standard deviation of 4.4%), the 3.8% in JB6 (with a standard deviation of 3.9%), 7.8% in TS (with a standard deviation of 4.6%), the 9.8% in Karpas-299 (with a standard deviation of 6.4%), the 8.6% in KIJK (with a standard deviation of 6.1%), the 7.5% in SUPM2 (with a standard deviation of 3.1%).

Our results demonstrated the low ability of both mabs to fix complement; a partial explanation of such evidences may be similar to the one which underlies on the feeble complement dependent citoxicity mediated by rituximab which action has been proved to be counteracted by the presence of complement inhibitory proteins on the surface of CD20+ cells [32]. Membrane-bound complement regulatory proteins (mCRPs)

CD46, CD55 and CD59 are capable to interfere with complement deposition on target cells so as to prevent the formation of the membrane attack complex and inhibiting cell lysis, tumor cells frequently express mCRPs and the use of new bispecific monoclonal antibodies neutralizing both mCRPs and tumor-associated antigens has been proposed [33; 34]. In light of these findings, we sought to determine if human ALCL cell lines express mCRPs by flow citometry, demonstrating a shared expression of mCRPs between the ALCL cell lines SUDHL-1 and TS (data not shown). Owing to the results obtained by FACS analysis, we repeated the CDC experiments using the anti-PSGL-1 clone TB5, together with antibodies able to neutralize the activity of mCRPs. The concurrent block of CD46, CD55, and CD59 produced a remarkable increase of cell lysis in respect to the one obtained in CDC experiments using the anti-PSGL-1 mab alone. Indeed, the percentage of tumor cells lysis ranged from 15% (obtained with the TB5 alone) to the 24.1% in the presence of TB5 in association with the anti-CD46, anti-CD55 and anti-CD59 (with a standard deviation of 1.6%) for SUDHL-1 cells; from 7.8% in the presence of TB5 alone to 51.8% in the presence of TB5 in association with anti-CD46, anti-CD55 and anti-CD59 (with a standard deviation of 5.1%) for TS cells.

These data suggest that targeting PSGL-1 can mediate tumor cell lysis through the activation of the complement cascade, however, the action of anti-PSGL-1 mabs is limited by the presence of mCRP on cancer cell surface.

The second group of experiments was set up to study the ability of anti-PSGL-1 mabs to prime ADCC in the presence of effector cells with citotoxic activity.

ADCC assays showed a different capability of anti-PSGL-1 mabs to induce cell lysis, while treatment with mab KPL-1 was associated with low levels of cell death, treatment with the mab

TB5 was associated to moderate levels of cell killing. More specifically, KPL-1 did not induce cell death neither in our control cells MAC-1, nor in Karpas-299 and in SUPM2. Conversely, KPL-1 induced an ADCC equal to the 17.1% in SUDHL-1 cells (with a standard deviation of 3.8%), the 11% in JB6 cells (with a standard deviation of 3.9%), the 6.8% (with a standard deviation if 3.9%) in TS cells, the 13.5% (with a standard deviation of 2.6%), the 0.3% (with a standard deviation of 0.44%). Otherwise than KPL-1, treatment with the mab TB5 led to higher levels of effector cell-mediated citotoxicity. TB5 produced the 7.5% of ADCC in MAC-1 (with a standard deviation of 4.3%), the 47.2% in SUDHL-1 (with a standard deviation of 11.4%), the 14.6% in JB6 (with a standard deviation of 11.4%), the 29.7% in TS (with a standard deviation of 9.8%), the 21.8% in Karpas-299 (with a standard deviation of 1.4%), the 31.8% in KIJK (with a standard deviation of 2.5%), and the 20% in SUPM2 (with a standard deviation of 1.4%).

The third and last series of experiment was designed to investigate the role of mabs KPL-1 and TB5 to induce a direct citotoxic effect in the absence of immune effectors such complement or cells with cytotoxic activity.

The results of our experiments, validated analyzing the fraction of viable cells after treatment with both KPL-1 and TB5 mAbs, demonstrated that TB5 was more effective in inducing cell lysis than KPL-1. KPL-1 determined the 12.3% of cell lysis in MAC-1 (with a standard deviation of 4.3 %), the 8.8% in SUDHL-1 (with a standard deviation of 4.4%), the 10.5% in JB6 (with a standard deviation of 4%), the 7.7% in TS (with a standard deviation of 4.7%), the 3% in KIJK (with a standard deviation of 3%), the 3% in SUPM2 (with a standard deviation of 2.7%), no lysis was promoted in Karpas-299 by KPL-1.

TB5 clone was able to trigger the 14.9% of cell killing in

MAC-1 (with a standard deviation of 4.5%), the 65% in SUDHL-1 (with a standard deviation of 10.7%), the 92% in JB6 (with a standard deviation of 12.4%), the 37% in TS (with a standard deviation of 7.6%), the 36.8% in Karpas-299 (with a standard deviation of 8.2%), the 24.6% in KIJK (with a standard deviation of 3.9%), the 27.5% in SUPM2 (with a standard deviation of 4.6%).

Taken together, our data show a significant ability of TB5 to induce direct cytotoxic effects on tumor cell lines compared to KPL-1. The explanation of such relevant difference between KPL-1 and TB5 in inducing cell killing may be the result of a cross-linking of PSGL-1 on cell surface after treatment with the agonist TB5. The use of high titers of TB5 would lead to a supraliminal activation of PSGL-1 signaling pathway and could hesitate in the activation of the intrinsic apoptotic pathway.
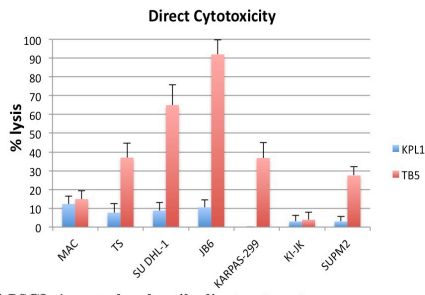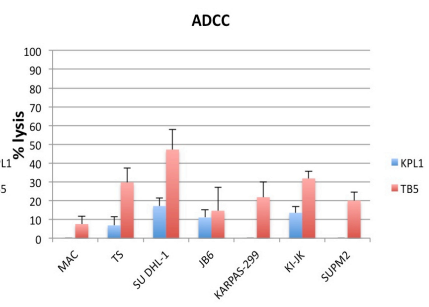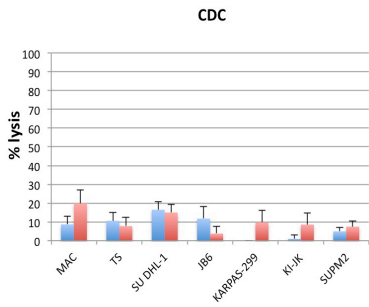
**Cytotoxic effects of anti-PSGL-1 monoclonal antibodies treatment**
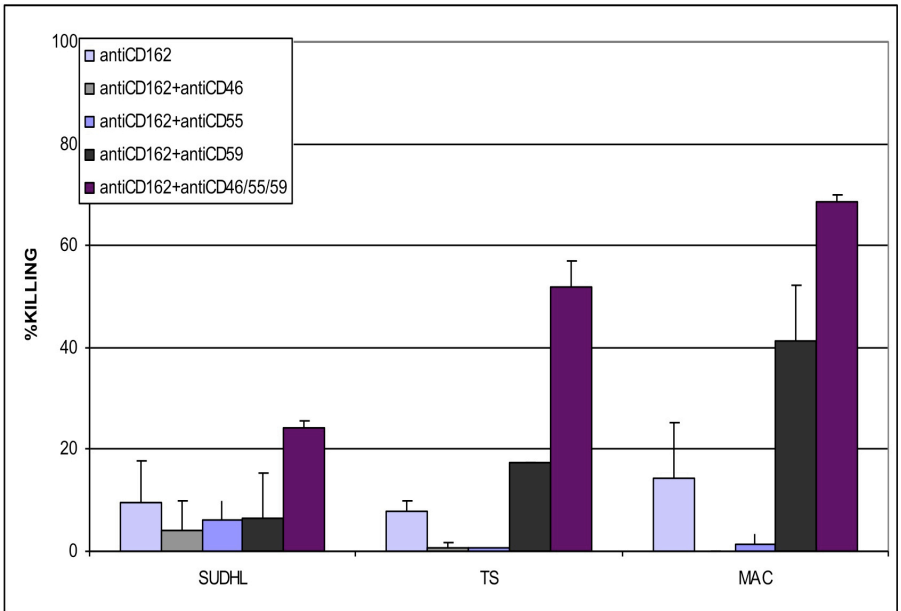
**mCRPs neutralization mediate higher CDC**

KPL-1 and TB5 mediate different localization of PSGL-1 on cell surface

In order to demonstrate a diverse distribution of PSGL-1 induced by KPL-1 or TB5 on cell membrane, ALCL cell lines were stained with both mAbs conjugated with a fluorescent dye. The confocal microscope analysis of the mAbs binding showed a noticeable discrepancy of PSGL-1 localization on cell surface. PSGL-1 was homogeneously deployed on cells after KPL-1 binding, on the contrary, TB5 mediated the localization of our target in specific regions of cell membrane thus forming a peculiar phenomenon termed as capping. The formation of caps is usually induced by mabs bearing agonist activity and can precede the initiation of a signaling pathway. The TB5-dependent cross-linking of PSGL-1 further substantiates our hypothesis according to which a more effective cell killing, obtained after TB5 binding, could be due to a pronounced activation of the PSGL-1 molecular cascade and in mAb driven cell apoptosis.

KPL1
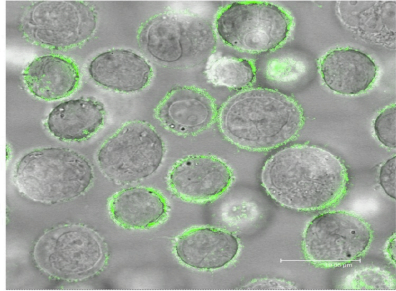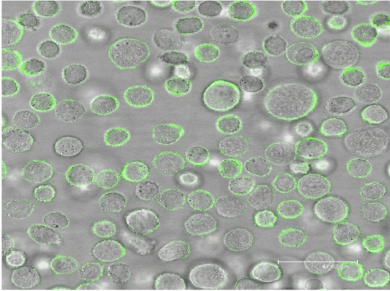
TB5
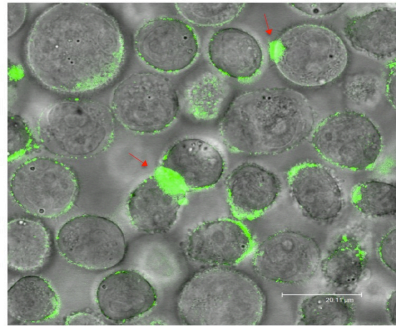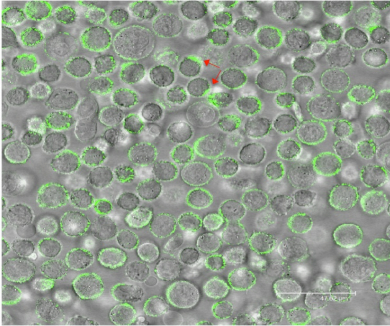
Confocal analysis of PSGL-1 distribution after treatment with mabs

Discussion

The greater understanding of the biological mechanisms and genetic aberrations that lie behind cancer transformation, have provided us a clearer picture of the intratumour and intertumour complexity. The most recent studies have demonstrated that rarely malignant tumours originate from a single cell and that they rather are the result of a clonal selective pressure which brings to the collection of neoplastic clones characterized by an epigenetic plasticity. Indeed, both primary tumour and metastasis are formed by cellular subpopulations which play different but complementary roles aimed to neoplastic progression [35]. On the other hand, a deep diversity can be observed between neoplasms belonging to the same class, for instance, breast neoplasms [36] or peripheral T-cell lymphomas [37]. PTCLs are characterized by tumour cells displaying not only functional and morphologic differences but also various immunophenotypical profiles. In light of such tumour heterogeneity, the development of new "tailored" therapies able to specifically target malignant clones and to spare the cellular healthy counterpart, represents a primary goal. In recent years, monoclonal antibodies were adopted as adjuvants to improve the efficacy of conventional cancer chemotherapy. According to the cancer immunoediting hypothesis tumoral spread begins as a consequence of the escape of neoplastic cells from immunological surveillance, monoclonal antibodies act as a booster of host immune system and mediate the specific elimination of clones by activation of immune effectors. Mabs mediate cell killing through the activation of the complement system or the stimulation of NK/T-cell citotocity, both immune effectors recognize the Fc portion of mabs attached on the surface of their target via an Fc receptor and promote cancer cell elimination. Beside a

complement-dependent citotoxicity (CDC) and an antibody-dependent cell-mediated citoxicity (ADCC), mabs can elicitate a direct citotoxicity which is triggered simply by the binding of mabs on target cell membrane. On the ground of the encouraging results obtained by the use of monoclonal antibodies for the treatment of hematological malignancies [38; 39], we sought to determine the *in vitro* effects of mabs treatment on human PTCLs cultures. In particular, we investigated the efficacy of two monoclonal antibodies able to target the P-selectin glycoprotein ligand 1 (PSGL-1), the principal binding partner of selectins which is expressed on activated T-lymphocytes, platelets, plasmacells and monocytes. PSGL-1 primarily drives cell tethering and rolling but its activity is also implied in cell survival [19], moreover, PSGL-1 could act as a potential target for humoral immunotherapy in multiple myeloma [15] .

We first analyzed and compared the expression of PSGL-1 mRNA between some peripheral T-cell lymphomas and normal tissue by gene expression profiling, finding an overall increased expression of our target in PTCLs especially in the anaplastic large cell lymphoma group (ALCL). Basing on our preliminary results, we chose ALCL as a prototypical model of investigation to test the tissue expression of PSGL-1 by immunohistochemistry. Immunohistochemistry was performed on 110 cases of ALCL and 50 of PTCL-NOS (chosen as control), collected with the tissue microarray technology, and demostrated a constant and strong expression of the marker in nearly all cases of ALCL examined.

Finally, we showed a considerable ability of anti-PSGL-1 monoclonal antibodies to induce CDC, ADCC and direct cytotoxicity on PTCLs cell lines.

Taken together our experimental data demostrate that PSGL-1 may be eligible as a potential immunotherapic target for the

treatment of the anaplastic large cell lymphoma, however, the use of animal models should be auspicable since it could give us a deeper insight regarding the physiological effects that could be obtained from PSGL-1 targeting.

CHAPTER 2
Published Articles

2.1 Monoclonal antibodies as carriers

Introduction

Beside the capability to induce a direct cytotoxic effect, monoclonal antibodies can be exploited to serve as a drug carrier to selectively kill tumour cells. Indeed, mabs can be chemically conjugated both with chemotherapeutic agents, that's the case of Brentuximab Vedotin [13], or with chemotherapy-loaded nanoparticles able to fuse with cancer cell membrane and specifically deliver drugs into it [40].

PLOS ONE

New Potential Therapeutic Approach for the Treatment of B-Cell Malignancies Using Chlorambucil/ Hydroxychloroquine-Loaded Anti-CD20 Nanoparticles

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Abstract

Current B-cell disorder treatments take advantage of dose-intensive chemotherapy regimens and immunotherapy via use of monoclonal antibodies. Unfortunately, they may lead to insufficient tumor distribution of therapeutic agents, and often cause adverse effects on patients. In this contribution, we propose a novel therapeutic approach in which relatively high doses of Hydroxychloroquine and Chlorambucil were loaded into biodegradable nanoparticles coated with an anti-CD20 antibody. We demonstrate their ability to effectively target and internalize in tumor B-cells. Moreover, these nanoparticles were able to kill not only p53 mutated/deleted lymphoma cell lines expressing a low amount of CD20, but also circulating primary cells purified from chronic lymphocitic leukemia patients. Their safety was demonstrated in healthy mice, and their therapeutic effects in a new model of Burkitt's lymphoma. The latter serves as a prototype of an aggressive lympho-proliferative disease. In vitro and in vivo data showed the ability of anti-CD20 nanoparticles loaded with Hydroxychloroquine and Chlorambucil to increase tumor cell killing in comparison to free cytotoxic agents or Rituximab. These results shed light on the potential of anti-CD20 nanoparticles carrying Hydroxychloroquine and Chlorambucil for controlling a disseminated model of aggressive lymphoma, and lend credence to the idea of adopting this therapeutic approach for the treatment of B-cell disorders.

Introduction

B-cell malignancies are a heterogeneous group of clinical conditions with highly variable clinical courses that span between indolent diseases like the chronic lymphocytic leukemia (CLL) and highly aggressive lymphoproliferative disorders, like Burkitt lymphoma (BL) [1,2,3,4]. B-cell tumor treatments include dose-intensive chemotherapy regimens and immunotherapy via monoclonal antibodies (mAbs) [5]. Despite the promising survival rates, these intensive multi-agent treatments display a high degree of toxicity, and a significant percentage of patients are also unresponsive [6,7,8]. Several limitations have been described to explain refractory/relapse patients. In particular, genetic modification in specific onco- or oncosuppressor genes, such as p53 [9], is associated

with unsuccessful chemotherapeutic regimens. In contrast, antibody-based immunotherapy has little side effects but its efficacy is mainly driven by the expression of sufficient amounts of tumor-associated antigen on the neoplastic cell surface [10].

In recent years, nanotechnology has attracted significant interest from oncologists given its potential to offer a new paradigm to overcome complex therapeutic targeting [11,12,13]. Nanoparticles made with biodegradable biopolymers (BNPs) as carrier material have been extensively investigated for sustained and controlled delivery of imaging and therapeutic agents with high efficacy and minor side effects [14,15,16,17,18,19]. Targeted delivery of nanoparticles can be achieved by attaching specific ligands or antibodies onto the nanoparticle surface [20,21,22,23,24,25].

2.2 Microenvironmental Role in Cancer Progression

Introduction

The role played by the microenvironment in supporting cancer progression was neglected in the past years and a greater attention was focused on tumour cell biology. In recent years many evidences demonstrated that both solid tumours and hematologic neoplasms develop in an environment enriched by inflammatory cells (macrophages, mast cells, T and B cells) and bioactive molecules (TGFβ, IL10, IFNγ) that through a constant crosstalk with cancer cells foster the clonal expansion. The study of such dynamics could provide us a clearer picture of cancer development.

Review Article

Microenvironment-Centred Dynamics in Aggressive B-Cell Lymphomas

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Aggressive B-cell lymphomas share high proliferative and invasive attitudes and dismal prognosis despite heterogeneous biological features. In the interchained sequence of events leading to cancer progression, neoplastic clone-intrinsic molecular events play a major role. Nevertheless, microenvironment-related cues have progressively come into focus as true determinants for this process. The cancer-associated microenvironment is a complex network of nonneoplastic immune and stromal cells embedded in extracellular components, giving rise to a multifarious crosstalk with neoplastic cells towards the induction of a supportive milieu. The immunological and stromal microenvironments have been classically regarded as essential partners of indolent lymphomas, while considered mainly negligible in the setting of aggressive B-cell lymphomas that, by their nature, are less reliant on external stimuli. By this paper we try to delineate the cardinal microenvironment-centred dynamics exerting an influence over lymphoid clone progression in aggressive B-cell lymphomas.

1. Introduction

B-cell malignancies represent a heterogeneous group of diseases characterized by different biological features and clinical behaviour, the latter ranging from indolent to highly aggressive. As for most neoplasms, the natural course of B-cell malignancies is characterized by tumour progression, featured by a flow of events leading to the enhancement of proliferative and invasive capabilities, towards the establishment of a more aggressive phenotype. Even if most of the processes involved in cancer progression are inherent to the neoplastic clone, this event is, actually, the result of an articulated mechanism, which seems to require the constant crosstalk between neoplastic cells and the faulty surrounding microenvironment. An ever-increasing amount of evidences suggest that this bijective relationship is a prime determinant of cancer natural history and evolution. Much

has been so far discovered about the role of tumour intrinsic mechanisms of neoplastic progression, and the focus of research has been progressively shifting toward the study of microenvironment-centred dynamics. Cancer-associated microenvironment represents a multifaceted entity, which not only provides structural support to neoplastic cells (proper stroma) but also acts as a "fertile soil" that, through humoral factors (bioactive molecules such as cytokines, chemokines, and adhesion molecules), nonmalignant cellular elements of the stroma (fibroblasts and endothelial cells) and the immune system (macrophages, mast cells, B and T lymphocytes) fosters tumour clone survival and expansion, local invasion/spreading, and escape from the immunological response.

The relative contribution of these branches of the tumour microenvironment may vary in the diverse tissues and organs in which lymphomas arise as well as in different lymphoma

The Aryl Hydrocarbon Receptor Modulates Acute and Late Mast Cell Responses

Riccardo Sibilano, Barbara Frossi, Marco Calvaruso, Luca Danelli, Elena Betto, Alessandra Dall'Agnese, Claudio Tripodo, Mario P. Colombo, Carlo E. Pucillo and Giorgia Gri

Anti-tumor activity of CpG-ODN aerosol in mouse lung metastases

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Studies in preclinical models have demonstrated the superior anti-tumor effect of CpG oligodeoxynucleotides (CpG-ODN) when administered at the tumor site rather than systemically. We evaluated the effect of aerosolized CpG-ODN on lung metastases in mice injected with immunogenic N202.1A mammary carcinoma cells or weakly immunogenic B16 melanoma cells. Upon reaching the bronchoalveolar space, aerosolized CpG-ODN activated a local immune response, as indicated by production of IL-12p40, IFN-γ and IL-1β and by recruitment and maturation of DC cells in bronchoalveolar lavage fluid of mice. Treatment with aerosolized CpG-ODN induced an expansion of CD4+ cells in lung and was more efficacious than systemic i.p. administration against experimental lung metastasis of immunogenic N202.1A mammary carcinoma cells, whereas only i.p. delivery of CpG-ODN provided anti-tumor activity, which correlated with NK cell expansion in the lung, against lung metastases of the poorly immunogenic B16 melanoma. The inefficacy of aerosol therapy to induce NK expansion was related to the presence of immunosuppressive macrophages in B16 tumor-bearing lungs, as mice depleted of these cells by clodronate treatment responded to aerosol CpG-ODN through expansion of the NK cell population and significantly reduced numbers of lung metastases. Our results indicate that tumor immunogenicity and the tumor-induced immunosuppressive environment are critical factors to the success of CpG therapy in the lung, and point to the value of routine sampling of the lung immune environment in defining an optimal immunotherapeutic strategy.

Synthetic oligodeoxynucleotides containing dinucleotides with unmethylated CpG motifs (CpG-ODN), agonists of TLR9, represent a novel approach to stimulating an effective anti-tumor response as demonstrated in preclinical models[1] and in patients with malignant melanoma, renal carcinoma and recurrent or refractory lymphoma.[1,2] However, efficacy of CpG in the lung, where virtually continuous exposure to environmental antigens dictates local immunological homeo-

stasis and the meticulous control of immune responses, has been limited to the control of lung metastatic growth only when administered before tumor cell injection or immediately after when disease is minimal in various preclinical tumor models.[3–5] Although a randomized Phase II trial with non-small lung cancer patients showed promising results and led to two large randomized Phase III trials comparing CpG-ODN plus chemotherapy to chemotherapy alone, interim analysis of those trials did not support the promise of CpG-ODN in enhancing the efficacy of chemotherapy and caused the suspension of the trials.[2] Several studies have suggested that the route of administration is a critical factor in influencing CpG activity. The anti-tumor effect of CpG has been optimized by direct injection into the tumor to enable increased local concentrations in the tumor microenvironment and to ensure effective local activation of both innate and adaptive immune responses.[6–9]

Thus, local instead of systemic administration of the TLR9 agonist might represent an option to improve the efficacy of CpG as adjuvant in protecting against lung cancer or in treating and/or preventing lung metastases derived from different primary tumors. Bronchial and bronchoalveolar

2.3 MicroRNAs and Cancer

Introduction

MicroRNAs (miRNAs) are small noncoding RNAs not longer than 22 nucleotides. Despite miRNAs were initially considered devoid of function, an increasing number of studies demonstrate their capability to finely regulate: proliferation, apoptosis, differentiation, transcriptional and post transcriptional events; moreover, they were proved to drive cancer initiation, progression and metastasis. Hence, miRNAs are able to play a protumorigenic role (oncomiR) or act as tumor suppressors [41].

The abrogation of the HOXB7/PBX2 complex induces apoptosis in melanoma through the miR-221&222-c-FOS pathway

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Cutaneous melanoma is the fastest increasing cancer worldwide. Although several molecular abnormalities have been associated with melanoma progression, the underlying mechanisms are still largely unknown and few targeted therapies are under evaluation. Here we show that the HOXB7/PBX2 dimer acts as a positive transcriptional regulator of the oncogenic microRNA-221 and -222. In addition, demonstrating c-FOS as a direct target of miR-221&222, we identify a HOXB7/PBX2→miR-221&222 →c-FOS regulatory link, whereby the abrogation of functional HOXB7/PBX2 dimers leads to reduced miR-221&222 transcription and elevated c-FOS expression with consequent cell death. Taking advantage of the treatment with the peptide HXR9, an antagonist of HOX/PBX dimerization, we recognize miR-221&222 as effectors of its action, in turn confirming the HXR9 efficacy in the treatment of human melanoma malignancy, whilst sparing normal human melanocytes. Our findings, besides suggesting the potential therapeutic of HXR9 or its derivatives in malignant melanoma, suggest the disruption of the HOXB7/PBX2 complexes, miR-221&222 inhibition or even better their combination, as innovative therapeutic approaches.

Malignant melanoma is the most aggressive form of skin cancer whose incidence has more than tripled in the white population during the last 20 years. Although surgical excision is mostly a definitive treatment at the early stages of the disease, at present standard treatments are ineffective after metastatic dissemination and patients with advanced disease have a grim prognosis, with a 5-year survival rate of less than 20%.[1] The homeobox (*HOX*) genes are a family of homeodomain containing transcription factors that define the identity of cells and tissues during early development.[2] Most cases of aberrant *HOX* gene expression include *HOX* genes that are normally silenced in adult cells and re-expressed in a wide variety of neoplasias suggesting the *HOX* family as another class of oncofetal genes.[3] A number of studies have shown the contribution of some HOX genes in cancer. These include HOXA5 and HOXA10, playing tumor suppressor functions in breast carcinoma, or HOXC11 inducing S100β, an established marker of melanoma progression.[4-6] HOXB7 has been reported as a master regulator in the oncogenic hierarchy.[7] HOX proteins bind to DNA through a highly conserved 60 amino acid sequence called the homeodomain. The specificity and stability of HOX binding to DNA are achieved when it forms complexes with cofactors such as PBX and MEIS in humans. In previous studies, we demonstrated *HOXB7* constitutive expression in melanoma primary lesions and cell lines whereby it is able to bind to the promoter and activate the transcription of *bFGF*.[8] Also, by using a dominant-negative PBX mutant (PBXNT), we showed that HOXB7 requires PBX as a co-factor for its oncogenic activity.[9] The HOX/PBX binding interaction, mediated through a specific and highly conserved hydrophobic hexapeptide,[10,11] strongly increases HOX/DNA affinity. Synthetic peptides mimicking this hexapeptide motif can interfere with HOX/PBX binding, including the small cell permeable peptide HXR9, which specifically antagonizes the interaction between HOX and PBX interfering with the binding of these proteins to the "HOX/PBX" DNA consensus sites. This disruption in turn triggers apoptosis in cancer cells both *in vitro* and *in vivo*.[12] Considering the significant role played by HXR9 in renal, ovarian and non small cell lung cancer (NSCLC) as well as in murine B16 melanoma

CHAPTER 3
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