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ORAL PRESENTATIONS

O1
Revealing cancer initiating cells in metastatic melanomas by harnessing the host’s anti tumor humoral immune mechanisms

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Background: To determine whether the growth of tumors is sustained by the whole cancer cell population or it is maintained only by small fraction of cancer initiating cells, has crucial impact on the design of suitable therapies. That is why the research on defining the subpopulation of cancer initiating cancer stem cells (CSC) in solid tumors has come into highlights.

Materials and methods: A complex panel assay at cellular and molecular levels has been performed on primary and metastatic cancerous tissue biopsies and peripheral blood of patients with malignant melanomas (n = 153) (Ethical permission: ETU TUEB 16462-02/2010).

Results: Cell cultures grew out of the great majority of the starter metastatic tissue specimen and cancer initiating cells could be sorted (BD FACSAria Sorter) by colocalized unique siallated glycosphingolipids and anti CD20 binding capacity. Characteristic growth pattern, spheroid forming, CSC markers like CD133, Nestin, ABCB5, CD20 and unique GD3 gangliosides were found. Patients’ sera and selected patients’ Epstein Barr Virus transformed cell supernatants in bulk or after limiting dilution cloning were tested for cancer binding by ELISA and immunoffluorescence. Our novel tumor infiltrating B cell antibody phage display technique and DNA sequence cluster analysis (Vector NTI Advance 11, Bioedit 7.0, ClustalX2.0.11) resulted in some antibody fragments, belonging to representative tumor binding antibody variable region subgroups, with defined sialilated glycosphingolipid specificity.

Conclusion: Our strategy enables the detection and characterization of cancer stem cells in metastatic melanomas, with potential diagnostic importance. The novel peripheral blood and tumor infiltrating B cell antibody profile analysis proved to be a useful asset to reveal anti tumor humoral immune responsiveness and harness it by antibody engineering technique for further diagnostic and therapeutic usage.

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O2
Activation of the ErbB3-AKT axis promotes melanoma cell survival and proliferation in response to RAF/MEK inhibition

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Background: Therapy of advanced melanoma has been improved with the advent of BRAF inhibitors. However, a limitation to such treatment is the occurrence of resistance. Several mechanisms have been implied in the development of resistance, which in most cases lead to downstream MEK reactivation. In order to overcome resistance MEK inhibitors are being clinically developed with promising results. However, also in this case resistance inevitably occurs. It is commonly believed that the establishment of resistance is facilitated by adaptive changes that take place in cancer cells shortly after exposure to kinase inhibitors. Our laboratory is interested in the identification of these early adaptive changes with the intent to discover additional targets for therapeutic intervention.

Methods: Four melanoma cell lines were tested: LOX IMVI and M14 (BRAF V600E), MST-L (BRAF V600R) and WM266 (BRAF V600D). RTK arrays (R&D) were carried out with protein extracts from untreated of BRAFi and MEKi treated cells. Western blot analysis was performed on total protein extracts using anti-ErbB3, anti-AKT and anti-ERK 1/2 antibodies. The growth inhibitory effects of multiple combinations of BRAF and MEK inhibitors and/or anti-ErbB3 mAbs were evaluated by colony formation.
assays. Mouse xenograft studies were carried out with M14 cells injected s.c. at the dose of 5 × 10⁶ cells. Drug treatments began when tumors reached a mean volume of 100 mm³ and tumor growth was measured by caliper.

**Results:** We show that ErbB3 is the main RTK rapidly hyperphosphorylated in response to BRAF or MEK inhibition in melanoma cell lines harboring a variety of V600 BRAF mutations. This results in a strong activation of phospho-AKT. ErbB3 activation, which is caused by increased autocrine production of neuregulin, can be fully abrogated by two distinct anti-ErbB3 monoclonal antibodies, A3 and A4. Most importantly these two mAbs individually or in combination strongly enhance the ability of different BRAF/MEK inhibitors to silence the oncogenic MAPK and AKT pathways. This results in potentiation of growth inhibition and of apoptosis. Preliminary xenograft studies confirm that administration of BRAF inhibitors together with anti-ErbB3 mAbs exerts a more profound inhibition of tumor growth than single treatments which is accompanied by a stronger downregulation of oncogenic signaling.

**Conclusions:** Feedback activation of ErbB3/AKT phosphorylation is a fast and common response of melanoma cells to BRAF and/or MEK inhibitors. Our results suggest that combinatorial treatment of melanoma patients with BRAF/MEK inhibitors together anti-ErbB3 antibodies should be further explored as a potentially helpful clinical approach.

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**O3 Assessment of germline and somatic alterations in main candidate genes among patients with multiple primary melanoma**

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**Background:** A series of patients with multiple primary melanoma (MPM) has been studied for assessing frequency and distribution of alterations in candidate genes involved in susceptibility (CDKN2A and p16CDKN2A) and pathogenesis (BRAF, cKIT, CyclinD1) of such a disease.

**Methods:** Two hundred and twenty-seven genomic DNA samples from paired synchronous and/or asynchronous tumour tissues of 106 MPM patients (92 cases with two, 13 with three, and 1 with four primary melanomas) were screened for somatic mutations in BRAF gene and GNAS-based amplifications in cKIT and CyclinD1 genes. By 87/106 MPM patients from our series, the germline blood was available and genome DNA analyses were performed for mutations in p16CDKN2A and p14CDKN2A genes. All mutation analyses we performed by direct automated DNA sequencing. Family history for melanoma was defined according to standardized criteria.

**Results:** At somatic level, BRAF mutations were identified in 108/227 (48%) primary melanoma tissues, whereas amplification of cKIT and CyclinD1 genes was observed in 28/227 (12%) and 32/227 (14%) analyzed tissue samples, respectively. Considering all types of genetic events, paired samples provided a poor consistent distribution of somatic alterations in same patients 55/106 (52%) discordant cases). Among them, 35/106 (33%) patients presented discordant MPM lesions according to the BRAF mutation status. Among the 87 MPM patients whose germline DNA was available, 8 (9%) of them showed different CDKN2A germline mutations: 7 in p16CDKN2A and 1 in p14CDKN2A. Assessment of family history for melanoma revealed that 13/87 (15%) patients presented at least one additional family member affected; a total of ≥ 3 melanomas in family was observed in 20/87 (23%) cases of our series. The CDKN2A germline mutations were found significantly more frequent in patients with familial history of melanoma (7/13; 54%) compared with patients without (1/74; 1.4%) (P<0.001); analogously, CDKN2A mutations were observed in 1/67 (1.5%) and 7/20 (35%) patients with 2 and 3 or more melanomas in family, respectively (P<0.001).

**Conclusions:** The low consistency in mutation patterns at somatic level among MPM lesions from the same patients provide further evidence that melanomagenesis is heterogeneous and molecularly different cell types may participate to the development of multiple melanomas. Our findings on germline DNA indicate that occurrence of at least 3 melanomas (in patients or families) or familial recurrence of melanoma may represent strong indicators to address patients to CDKN2A mutation screening.

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**O4 Regulation of melanoma initiating cells by Hedgehog signaling and SOX2**

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**Background:** Recent reports suggest that within the heterogeneous population that constitutes a melanoma, certain cell types exhibit molecular and functional features similar to stem cells. These melanoma-initiating cells (MICs) have the ability of unlimited self-renewal, multilineage differentiation and the potential to initiate and maintain tumor growth [1]. Furthermore, MICs are believed to confer chemoresistance to conventional chemotherapy agents and newly developed molecularly-targeted drugs [2,3]. Therefore, defining the molecular and biochemical pathways that support MICs is of critical importance for the development of more efficient targeted therapies. We have previously shown that the HEDGEHOGL HLH signaling is required for melanoma growth [4] and for survival and expansion of MICs [5]. Here we investigate the mechanism by which inhibition of the HH signaling leads to a decrease of MIC stemness, addressing the role of the transcription factor SOX2.

**Materials and methods:** MICs were enriched by Fluorescence Activated Cell Sorting using the metabolic marker Aldehyde Dehydrogenase (AlDH+) and by establishing melanoma spheres in non-adherent culture conditions from 20 human melanomas. Expression of SOX2 and of HH pathway components was determined by real time PCR and Western blot analysis. Modulation of the HH signaling was performed by stable expression of lentiviral vectors encoding short-interference RNAs specific for SMO or GLI1 (to inhibit HH), and PTCH1 (to activate HH). Functional analysis of SOX2 expression was performed by stable overexpression and silencing using lentiviral vectors.

**Results:** We find that the HH signaling regulates the expression of SOX2 and the downstream effectors of the HH signaling, the transcription factors GLI1 and GLI2, bind to SOX2 promoter in melanoma cells. Functionally, we show that SOX2 is required for HH-induced melanoma cell growth and MIC self-renewal. We present evidence that SOX2 is highly expressed in a melanogenerally lethal blood was available and genome DNA analyses were performed for mutations in p16CDKN2A and p14CDKN2A genes. All mutation analyses were performed by direct automated DNA sequencing. Family history for melanoma was defined according to standardized criteria.

**Results:** At somatic level, BRAF mutations were identified in 108/227 (48%) primary melanoma tissues, whereas amplification of cKIT and CyclinD1 genes was observed in 28/227 (12%) and 32/227 (14%) analyzed tissue samples, respectively. Considering all types of genetic events, paired samples provided a poor consistent distribution of somatic alterations in same patients 55/106 (52%) discordant cases). Among them, 35/106 (33%) patients presented discordant MPM lesions according to the BRAF mutation status. Among the 87 MPM patients whose germline DNA was available, 8 (9%) of them showed different CDKN2A germline mutations: 7 in p16CDKN2A and 1 in p14CDKN2A. Assessment of family history for melanoma revealed that 13/87 (15%) patients presented at least one additional family member affected; a total of ≥ 3 melanomas in family was observed in 20/87 (23%) cases of our series. The CDKN2A germline mutations were found significantly more frequent in patients with familial history of melanoma (7/13; 54%) compared with patients without (1/74; 1.4%) (P<0.001); analogously, CDKN2A mutations were observed in 1/67 (1.5%) and 7/20 (35%) patients with 2 and 3 or more melanomas in family, respectively (P<0.001).

**Conclusions:** The low consistency in mutation patterns at somatic level among MPM lesions from the same patients provide further evidence that melanomagenesis is heterogeneous and molecularly different cell types may participate to the development of multiple melanomas. Our findings on germline DNA indicate that occurrence of at least 3 melanomas (in patients or families) or familial recurrence of melanoma may represent strong indicators to address patients to CDKN2A mutation screening.

**O5** Combination therapy with ipilimumab and electrochemotherapy: preliminary efficacy results and correlation with immunological parameters

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**Background:** Ipilimumab is the first agent approved for the treatment of advanced melanoma that showed a survival benefit in randomized phase 3 trials. Despite the survival benefit, due to its mechanism of action it is associated with a slow onset and low rate of responses and, in many cases, responses occur after other therapies, like chemotherapy, targeted therapy and radiotherapy. Electrochemotherapy (ECT) has been shown to be effective and well tolerated for local control of metastatic melanoma with superficial lesions. The current challenge is to improve ipilimumab efficacy by combination/sequence with other therapies. We performed a pilot study of combination with ipilimumab and ECT in order to verify the possible increase of response rate. Furthermore, due to the lack of predictive markers, we evaluated the possible predictive role of circulating T-regulatory cells (T-Reg) variations in peripheral blood mononuclear cells (PBMC) of treated patients.

**Methods:** We collected data from 15 patients (pts) with advanced melanoma (9 stage IIIC and 6 stage IV M1c) treated with ipilimumab at 3 mg/kg every 3 weeks for 4 cycles (day 1) and ECT with bleomycin at 15 mg/m2 (day 2) on superficial lesions. Blood draws were collected on day 0, 1 and 2, then on day 15 and 30 from ECT, at each cycle of ipilimumab and at every tumor evaluation (every 12 weeks). PBMC were thawed and labeled with anti-CD4-Pe-Cy5, CD25-PE and anti-FoxP3-AlexaFlour488 for T-Reg.

**Results:** 10/15 (67%) pts showed local objective responses (4 CR and 6 PR). 7/10 (70%) pts showed local response (6 PR and 1 CR) after the second ipilimumab dose and 3/10 showed response (3 CR) at week 12. Two pts with PR (28%) out of the group of 7 showed response on distant lesions at week 24 (absopal effect). All pts are still alive with a median follow up of 12.4 months (range 6-18).

We found in all responders a decrease of T-Reg of 0.10% (range 0.50-2.6%) per cycle and no variation of CD4+ and CD25+ lymphocytes. Furthermore 5/15 pts not responders had a delayed stable disease (local and distant) at week 16, with no variations of T-Reg.

**Conclusion:** Our preliminary results show that a combination approach with ipilimumab and ECT may increase responses to ipilimumab. T-Reg decrease in PBMC could be associated with early response to treatment. Further studies about this combination are warranted.

**O6** Efficacy of radiotherapy in patients on progression after treatment with ipilimumab 3 mg/kg

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**Background:** Ipilimumab, a fully human monoclonal antibody (IgG1) that promote antitumor immunity by blocking CTLA4, was the first agent which showed a long-term survival benefit, about the 20% of patients, for the treatment of metastatic melanoma. The combination of ipilimumab with other therapies might improve its efficacy.

The term “absopal effect” refers to a regression of metastatic lesions distant from the primary site of radiotherapy (RT). This new phenomenon represent the systemic response observed in patients who received ipilimumab.

Here we reported the outcomes from patients treated in the ipilimumab Italian expanded access program (EAP) who received RT after ipilimumab progression.

**Patients and methods:** Patients with advanced melanoma after ipilimumab progression were selected for analysis. Patients, who failed ipilimumab therapy and for whom no other therapeutic options were available, were eligible for radiotherapy.

**Results:** 21 out of 95 patients treated with ipilimumab in the Italian EAP were eligible for the analysis. The median age was of 58 years (range 21-77); The progression free survival (PFS) from ipilimumab treatment was 4 months (range 3-6), while the time from the end of treatment with ipilimumab and RT was of 5 months (range 4-8).

RT was performed on brain in 13 (62%) patients; 8 were treated with whole-brain RT and 5 patients with stereotactic RT. Other RT treatment included bone, metastatic distant lymph nodes, sub-cutaneous metastasis, spinal cord metastatis. The median doses of radiation was of 30 Gy (range 30-50). A local response to RT was detected in 13 patients (62%) while 8 patients (38%) did not show any local regression. The abscopal response has been detected in 11/21 (52%) patients: in details, we observed 9 abscopal partial response (42,8%), 2 abscopal stable disease (9,6%), and 10 progression (47,6%). The median of occurrence of the abscopal response was of 1 month (range 1-4). The median overall survival (OS) for all the 21 patients was of 13 months (range 6-26). The median OS for patients with and without abscopal responses was respectively of 22.4 months (range 2,5-50,3) and 8 months (range 7,6-9,0). 11 (84,6%) out of 13 patients with local response showed an abscopal effect.

**Conclusions:** RT could be a new treatment option after ipilimumab treatment for potentiate its effect. Local response to RT might be predictive for the abscopal response and outcome. Further studies are warranted in this field to better understand and define the role of RT in combination or sequencing with ipilimumab treatment.

**O7** Updated results and correlative FDG-PET analysis of a phase IIb study of vemurafenib and cobimetinib (MEK inhibitor [GDC-0973]), in advanced BRAF**V600**- mutated melanoma (BRIM7)

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**Background:** BRIM7 is a phase 1b study evaluating the safety and efficacy of combined BRAF and MEK inhibition with vemurafenib + cobimetinib. The utility of FDG-PET as an early predictor of clinical benefit was also evaluated in this study.

**Materials and methods:** Eligible patients (pts) had advanced BRAF**V600**- mutated melanoma and ECOG PS 0-1 and were either naive to BRAF inhibitor (BRAF-naïve) or had disease progression on vemurafenib (vem-progressor). Pts in the dose-escalation portion received vemurafenib 720 or 960 mg BID continuously and cobimetinib 60, 80, or 100 mg QD 14 days (d) on/14 d off (14/14); 21 d on/7 d off (21/7); or continuously (28/0). Two dose levels were expanded: vemurafenib (720 mg and 960 mg BID) continuously and cobimetinib 60, 80, or 100 mg QD 14 days (d) on/14 d off (14/14); 21 d on/7 d off (21/7); or continuously (28/0). Two dose levels were expanded: vemurafenib (720 mg and 960 mg BID) and on Day 15 of Cycles 1 and 2. Correlation between tumor glucose metabolism changes, baseline tumor burden and target lesion responses were evaluated.

**Results:** 128 pts were treated with vemurafenib + cobimetinib as of 21 June 2013; male 60%, median age 55 y (19-88), stage M1c 76% and BRAF-naive 49%. Median duration of follow-up in vem-progressor and BRAF-naive pts was 3 and 10 months, respectively. Most adverse events (AE) were mild to
moderate in severity and vem-pergessor pts reported fewer AEs compared to BRAFi-naive pts. Most common AEs in BRAFi-naive pts (n=63) were non-acneiform rash (89%), diarrhea (81%), photosensitivity/sunburn (70%), fatigue (67%) and liver test abnormality (59%). Most frequent grade ≥3 AEs in BRAFi-naive pts were liver test abnormality (19%), non-acneiform rash (13%), arthralgia (11%) and fatigue (10%). BRAFi-naive pts attained 85% confirmed response rate (RR), including 10% complete responses; median PFS was not reached at 10 months follow-up. Vem-pergessor pts attained 15% confirmed RR, stable disease of 43%, and median PFS of 2.8 months. Preliminary FDG-PET analysis showed that the magnitude of reduction in tumor glucose uptake correlated with maximal tumor reduction but the degree of correlation varied across time and in BRAFi-naive and Vem-pergessor pts.

Conclusions: Vemurafenib + cobimetinib can be safely administered at the respective single-agent MTDs of vemurafenib (960 mg BID) and cobimetinib (60 mg 21/7). Preliminary efficacy of the combination is encouraging in BRAFi-naive patients. FDG-PET is a potentially useful marker of early biologic response to the combination.

O8 Peripheral and tumor immune correlates in patients with advanced melanoma treated with nivolumab (anti-PD-1, BMS-936558, ONO-4538) monotherapy or in combination with ipilimumab

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Background: The fully human monoclonal antibodies nivolumab (Nivo) and ipilimumab (Ipi) block the interaction between the immune checkpoint receptors programmed death-1 (PD-1) and cytotoxic T lymphocyte antigen-4 (CTLA-4), respectively, and their cognate ligands, restoring antitumor immune response. Phase 1 studies of Nivo monotherapy (CA209-003; NCT00730639) or Nivo+Ipi combination therapy (CA209-004; NCT01024231) demonstrated durable clinical activity and objective response rates (ORRs) of 31% to 40%, respectively, in patients with advanced melanoma (MEL). Evaluation of PD-L1 expression using immunohistochemistry (IHC) suggested a correlation between pretreatment tumor PD-L1 expression and clinical response to Nivo monotherapy [1]. Identification of predictive markers of response would be valuable to guide effective use of Nivo and Ipi.

Materials and methods: MEL patients received Nivo monotherapy (n=107) or Nivo+Ipi combination therapy (n=86; concurrent regimen: n=53; sequenced regimen: n=33). Tumor and tumor infiltrating lymphocyte (TIL) surface programmed and death ligand 1 (PD-L1) expression in formalin-fixed, paraffin-embedded (FFPE) tumor tissue was evaluated by IHC with an automated assay (Dako) using the 28-Ab 8-B tumor PD-L1 positivity (PD-L1+) was defined as ≥5% cell membrane staining of any intensity; any expression on TILs was considered positive. Absolute lymphocyte counts (ALC) were measured in serial peripheral blood samples and lymphocyte subsets were evaluated using flow cytometry.

Results: Tumor PD-L1-positive expression was observed in 45% and 37% of samples from the 003 and 004 studies, respectively. In 003, inclusion of any immune cell staining increased PD-L1 positivity to 92%. A numerically higher ORR was observed in MEL patients with PD-L1+ tumors with Nivo monotherapy or with sequential but not concurrent combination therapy. Neither study demonstrated an obvious change in ALC; however, phenotypic changes in T-cell subsets, including increases in the percentage of CD4 and CD8 expressing HLA-DR, ICOS and/or Ki67, were seen with combination therapy. In both studies, responses were observed irrespective of tumor PD-L1 or ALC status. In an exploratory analysis low pretreatment myeloid derived suppressor cells (MDSC) correlated with higher ORR with combination therapy (P<0.05).

Conclusions: PD-L1 positivity is associated with tumor response with Nivo monotherapy; however, some responses were observed independent of PD-L1 or ALC status. No correlation between response and PD-L1 or ALC status was seen with combination therapy. MDSC levels may correlate with response to combination therapy. Future phase 3 randomized studies will explore these markers and other phenotypic changes in immune cell populations that might predict activity of Nivo in patients with MEL and other advanced cancers.

Reference

09 Ipilimumab treatment results in an early increase in the frequency of circulating granulocytic myeloid derived suppressor cells as well as their arginase1 production

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Background: Ipilimumab (Yervoy) is a fully human antibody that blocks CTLA-4 and has proven to extend overall survival in patients with unresectable stage III or stage IV melanoma [1]. Immune related adverse effects (IAEs) are frequent and can be severe but are reversible with early diagnosis and can be managed with corticosteroid therapy [2]. Most of the recently published immune monitoring studies focus mainly on the effect that ipilimumab has on T cell populations [3]. To date, little information is available on the possible impact that ipilimumab treatment may have on MDSC populations and their suppressive mechanisms [4]. In order to evaluate these effects, we conducted an in-depth immune monitoring study centered on peripheral blood MDSC populations as well as T cells in advanced melanoma patients undergoing treatment with ipilimumab.

Materials and methods: Fourteen patients with advanced stage melanoma received ipilimumab treatment at 3 mg/kg or 10 mg/kg doses and six of which were part of an ongoing double blind randomized trial (Bristol-Myers Squibb trial CA184-169). Blood samples were collected from each patient before treatment (baseline) and at the time of the second and fourth ipilimumab doses (3 and 9 weeks after the first dose). Peripheral blood mononuclear cells were isolated by density gradient centrifugation and stained for multicolored flow cytometric analysis. The staining protocol included five-color panels to analyze T cells (relative frequencies, activation, memory and Tregs) and MDSCs.

Results: Absolute lymphocyte counts showed an increasing trend during the course of treatment without significant differences. Analysis of circulating Treg (CD4+ CD25+ CD127− FoxP3+) frequencies revealed an initial increase, significantly decreasing after the second ipilimumab dose (week 9). The endpoint mean frequency of Tregs was lower than the baseline. Changes in MDSC populations with granulocytic and monocytic phenotype (Lin− HLA-DR−/Lo CD15+ CD33− CD11b+ and Lin− HLA-DR−/Lo CD14+, respectively) were monitored and the results showed that, after the first dose, the granulocytic MDSC population significantly decreased, remaining low at week 9. This decrease was accompanied by a significant increase in the population of ARG1+ myeloid cells.

Conclusion: The results obtained in this study provide a first look at the early responses of peripheral blood myeloid cell populations to ipilimumab treatment. The mechanisms by which these in trans effects are taking place should be further explored as well as their possible relations to clinical benefit.

References


O10 Harnessing host innate immunity may combat acquired resistance to BRAFi

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Background: Natural Killer (NK) cells are innate immunity lymphocytes that spontaneously recognise and kill melanoma cells [1]. Dacarbazine synergises with host immunity by inducing stress signals (NGK2D-L) that alert NK cells [2]. This provides an example of how cancer therapies and host innate immunity may be combined to improve management of melanoma patients. BRAFi inhibitors (BRAFi) are radically changing clinical practice in melanoma treatment because they offer unprecedented survival gains. However, melanoma patients nearly always relapse with drug-resistant disease after 6-12 months into the treatment. While evidence is emerging that BRAFi may modulate host immunity, the effect of BRAFi on the cross talk between NK cells and melanoma cells is unknown.

Material and methods: BRAFi-mutant melanoma cell lines 1520, SK-MEL-37 and Colo38 were cultured for 4 weeks in the presence of 5μM vemurafenib. BRAFi-resistant variants were sequenced to confirm the presence of the BRAF V600E mutation and exclude outgrowth of BRAFi-resistant clones. NK cells were isolated from the blood of healthy donors and activated with IL-2. Melanoma cell killing by NK cells was quantified before and after BRAFi treatment by FACS (CD107 degranulation assay) and/or by chromium release assays. MAPK signalling in melanoma cells was quantified by FACS (phospho-ERK). Expression of HLA class I, TRAIL, PD-1 as well as ligands for NKGD2, NCRs and DNAM-1 on melanoma cells was assessed by FACS. Receptor expression was assessed by FACS on blood NK cells from healthy donors or by confocal microscopy in lymph node biopsies of metastatic melanoma patients.

Results: Melanoma cells that acquired BRAFi resistance through increased MAPK signalling developed also resistance towards NK cell killing. Expression of HLA class I, PD-1 and TRAIL was often elevated in drug-resistant melanoma cells compared to parental melanoma cells, whereas the expression of other receptor ligands did not significantly change. PD-1 was found on a subset of NK cells from some melanoma patients and on IL-2 activated NK cells from some healthy donors.

Conclusion: These data suggest that the PD-1/PD-L1 pathway is an attractive target to restore NK cell activity towards melanoma cells in some patients, thus harnessing host innate immunity to combat acquired BRAFi resistance.

References


O11 Regulated intratumoral expression of IL-12 as a basis for combination therapy in melanoma

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Background: Major obstacles for the development of immunotherapeutics are the ability of tumors to escape the immune system coupled with toxicity associated with systemic administration. To overcome these challenges, we have developed an adenoviral vector, Ad-RTS-IL-12 (AD), administered intratumorally under control of the RheoSwitch Therapeutic System gene expression platform. Expression of IL-12 mRNA and IL-12 protein is tightly regulated by the oral administration of a small molecule activator ligand, veledimex (AL).

Materials and methods: In preclinical studies the antitumor activity of Ad-RTS-mIL-12 in combination with AL was studied in the mouse B16F0 melanoma model. Melanoma (B16F0) cells were inoculated subcutaneously into the right and left flanks of 6-8 week old female C57BL/6 mice. When tumors reached 70-100 mm3, animals received a single intratumoral (IT) injection of Ad-RTS-mIL-12 1 x 1010 viral particles into the right flank plus AL at a dose of ~200 mg/m2 in the chow 24 h before the duration of the study. Tumor growth (TG), tumor cytokines and infiltrating T cells were studied. In a human Phase I, 3+3 dose escalation study, subjects with nonresectable stage III/IV melanoma were injected Ad-RTS-hil-12 IT with a constant dose of 1 x 1012 viral particles on the first day of each 21-day cycle, and escalating oral doses of AL (5, 20, 100, and 160 mg) on Days 1-7 of each cycle. In the expanded Phase II portion 8 subjects have been treated with 160 mg.

Results: In mice, increase in local expression of IL-12 with increasing AL dose resulted in decrease in TG in both the treated and untreated tumors coupled with an increase in tumor infiltrating lymphocytes (TILs) as well as demonstration of systemic immunity. In human subjects, increase in tumor IL-12 mRNA expression and increases TILs (CD8+ CD45RO+) were observed following treatment of Ad-RTS-IL-12 + AL (100 or 160 mg). Clinical activity without significant toxicity correlated with the highest serum levels of IL-12 which induced interferon-gamma (IFN-γ). Based on these results, preclinical combination studies with signal transduction agents and immunotherapeutics are ongoing and will be presented.

Conclusions: Development of IL-12 intratumorally via an adenovector technology using RheoSwitch® technology enables finely-controlled expression of IL-12, which is well tolerated and results in an increase in TILs concomitant with a reduction in TG. These findings form the basis for ongoing combination studies in melanoma.

O12 Biomarker analysis of on-treatment and at progression biopsies from BRIM7 - a phase 1B trial of combined vemurafenib and cobimetinib treatment in BRAF V600 mutated melanoma

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Background: Combined BRAF and MEK inhibition may delay the onset of resistance compared with BRAF inhibition alone. The safety and tolerability
of the BRAF and MEK inhibitors, vemurafenib and cobimetinib were evaluated in a phase 1B trial, BRIM7 (NCT01271803). Modulation of signaling pathways, transcriptional outputs and T-cell dynamics were assessed in tumor samples obtained from BRIM7.

Materials and methods: Tumor tissues were collected at baseline prior to treatment, on-treatment at cycle 1 day 14 (C1D14) and at disease progression (PD). Modulation of the MAPK and PI3K pathways, cell proliferation, and CD8+ T-cells in tumor biopsies were assessed by immunohistochemistry. Changes in transcription profiles upon treatment were measured by qRT-PCR using the Fluidigm/Nanostaging platforms.

Results: 133 tumor samples have been collected representing 82 unique patients. There are six paired biopsies (baseline and C1D14) from the same patients, and seven evaluable PD samples. In the six paired biopsies (baseline and C1D14), robust inhibition of the MAPK pathway was observed in tumors following vemurafenib + cobimetinib treatment as measured by reduction of pERK levels compared to baseline (mean H-score inhibition 88 +/- 12%). This was observed in samples from BRAF inhibitor-naive patients (n=2) and also from patients who had progressed while on vemurafenib (n=4). The inhibition of pERK correlated with the reduction of the proliferation marker Ki67 in C1D14 tumor samples (77 +/- 10%), while inhibition of the PI3K pathway marker pS6 showed greater variability (68 +/- 28%). At PD, varying levels of MAPK pathway activity and moderate-to-high expression of Ki67 were seen in tumors. Ongoing analyses of combination treatment effects on the MAPK pathway, immune regulatory gene transcription and CD8+ T-cell status will be presented.


Reference:

POSTER PRESENTATIONS

P1
Long-term response after electrochemotherapy in patients with relapsed or refractory cutaneous melanoma
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Background: Treatment of early and multiple cutaneous unrespectable recurrences is a major therapeutic problem with around 80% of patients relapsing within 5 years [1]. For lesions refractory to elective treatments, electrochemotherapy (ECT) involving electropermeation combined with antineoplastic drug treatment appears to be a new potential option [2]. This study was undertaken to analyze the short- and long-term responses of lesions treated with ECT with intralesional injection of bleomycin in melanoma patients with in-transit disease or distant cutaneous metastases.

Materials and methods: Between January 2007 and September 2012, 60 patients with relapsed and refractory cutaneous melanoma metastases or in-transit disease underwent 100 courses of ECT with intralesional injection of bleomycin. Response to treatment was evaluated three months after ECT. A long-lasting response was defined as no cutaneous or in-transit relapse after a minimum of six months.

Results: Three months after ECT, a complete response was observed in 29 patients (48.4%), a partial response in 23 patients (38.3%) and no change or progressive disease in 8 patients (13.3%). The objective response rate of all treated lesions was 86.6%. Thirteen patients (44.3% of complete responders) experienced a long-lasting response to ECT and were disease-free after a mean duration of follow-up of 27.5 months.

Conclusions: The favorable outcome obtained in the present study demonstrates that ECT is a reliable, and effective procedure that provides long-term benefit in terms of curative and palliative treatment for unrespectable cutaneous lesions without adversely impacting the quality of life of patients [3-7].

References: P1

**P2**

A novel approach to target metastases of melanoma cells in an organ-selective manner.

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**Background:** Cancer is a leading cause of death globally and the majority of patients will die from the formation of metastases rather than from their primary tumor as most metastases are resistant to conventional therapies. Based on numerous clinical observations it is clear that different cancers have a propensity to metastasize to specific organs. The central hypothesis for our current study is that an organs vasculature is intimately involved in mediating the interaction between specific cancer cells and the target organ for which they will metastasize to. In this respect we have focused on how melanoma cancer cells metastasize to the liver and lungs, two major sites of metastatic disease within the body, in order to provide insight for the development of new treatments for metastastic cancer.

**Materials and methods:** Herein we used an unbiased combinatorial phage in vivo biopanning strategy to isolate a library of peptide-displaying-phage that home to the liver and lungs of animals treated with a pro-inflammatory stimulus, lipopolysaccharide (LPS). Using a functional screen we isolated a single phage that prevented the recruitment of leukocytes into the liver and lungs of animals following LPS treatment. We investigated if the lung/liver inhibitory phage were also able to inhibit the formation of lung metastasis using a human melanoma metastasis xenograft model.

**Results:** Using intravital microscopy, we identified a peptide-displaying-phage, and its corresponding displayed-peptide, that has the ability to inhibit adhesion and recruitment of neutrophils in the liver sinusoids in response to LPS. Excitingly, we also found that animals injected (i.v.) with human melanoma cells (70W) in the presence of the phage, or its corresponding displayed-peptide, showed reduced metastatic lung burden. As the isolated peptide was capable of inhibiting both leukocyte recruitment and melanoma lung metastasis, we asked if leukocytes played a functional role in the establishment of melanoma metastasis. We found that in animals that received LysG6 (Anti Gr-1) antibody to deplete circulating leukocytes there was a dramatic increase in 70W melanoma metastasis to the lungs and that this increase in metastatic state was still blocked by the addition of the peptide.

**Conclusion:** These results suggest two major findings, first that circulating leukocytes could have a protective role in preventing melanoma metastasis to the lungs and second that the peptide identified in this study may work by blocking molecules that are required for recruitment and/or extravasation of the cancer cells, a process that is shared by leukocytes.

**P3**

UV exposure and melanoma prognostic factors: results from Clinical National Melanoma Registry (CNMR)

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**Background:** Previous studies have reported an association between sun exposure and improved cutaneous melanoma (CM) survival. We analysed the association of UV exposure with prognostic factors and outcome in the National Clinical Melanoma Registry.

**Methods:** Clinical and socio-demographic features were collected melanoma patients at diagnosis, together with information on sunbed exposure and sunny holidays. Analyses were carried out to investigate the associations between UV exposure and melanoma prognostic factors.

**Results:** From December 2010, we retrieved information from 3299 melanoma patients from 39 IMI (Intergroupo Melanoma Italiano) sites geographically representative. 40% of the patients are over 60 years old, 52% are men, 49% live in the north of Italy and 56% are at high educational level (at least high school). 8% of the patients have melanoma familiarity and 55% have fair phenotype ( Fitzpatrick skin type I or II). 2% have metastases and 11% lymph-node involvement. 8% have very thick melanoma (Breslow>4 mm) and 21% ulcerated melanoma.

**Conclusions:** Holidays in the sun five years before CM diagnosis, among non-metastatic patients, were significantly associated with lower Breslow thickness (p=0.003), after multiple adjustment including socio-demographic status, degree of doctor making the diagnosis and residence. Sunbed exposure is not significantly associated with Breslow thickness.

**References**


**P4**

Psychological features in lower risk melanoma: an analysis on 204 patients

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**Introduction & objectives:** To describe psychological status in melanoma patients in a high risk distress moment: the follow up consultation. Secondly to know if there are any difference in distress and in other psychological variables between melanoma patients at stage Ib-II vs O-Ia
AJCC (American Joint Committee on Cancer) and to correlate these with clinical variables.

**Material & methods:** We recruited 204 continuous patients in 0-I-II melanoma stages during the follow-up visit from March to June 2011. They were submitted to a psychological interview and questionnaires (Distress Thermometer, Hospital Anxiety Depression Scale, Montgomery-Asberg Depression Rating Scale, SF-36, Brief cope). Patients were divided into two groups based on the stage of disease to achieve our second aim (0-Ia vs Ib-Ib AJCC).

**Results:** The prevalence of distress, anxious and depressive symptoms was detected in 8-44% of the sample. These range is formed by: distress (44.1%), anxious symptoms (25%) and depression symptoms (8.8%) measured both with HADS, while we used the Montgomery-Asberg Depression Rating Scale to evaluated depression. Globally, high values at the SF-36 test were demonstrated, reporting a good quality of life in lower stages AJCC melanoma patients.

The Brief Cope measured styles of coping that is a psychological construct that express the way to face problems and stressful events in our life. Denial coping style resulted significantly correlated with distress, anxious and depressive symptoms and worse quality of life. On the other hand, a bad quality of life seems to influence negatively the psychological status of patients. Of them, who used active coping was more able to face the pathology and reported better psychological arrangement in their life.

Comparing the two groups (0-Ia vs Ib-Ib AJCC) about levels of distress, anxious and depressive symptom, coping styles and quality of life we found no statistically significant differences regarding psychological distress, measured with the Distress Thermometer and there were no statistically significant differences for anxious or depressive symptoms, measured with HADS. Quality of life, measured with SF-36, revealed no significant differences between the two groups. There were no statistically significant differences between the two groups with regard to coping styles in the majority of subscales of the Brief Cope questionnaire.

**Conclusions:** This work shows the presence of psychological distress also in first stages melanoma patients population, suggesting the importance of psychological screening also in low risk melanoma patients. Moreover the findings corroborates that subjective factors, as the way of facing the pathology, are more associated with the adjustment of melanoma survivors than objective medical ones.

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**P6**

**In vivo targeting of cutaneous melanoma using an MSH-engineered human protein cage bearing fluorophore and MRI tracers**

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**Background:** Nanoparticle (NP)-based materials are very promising agents for enhancing cancer diagnosis and treatment. Once functionalized for selective targeting of tumor expressed molecules, they can specifically deliver drugs and diagnostic molecules inside tumor cells.

**Materials and methods:** In the present work, we evaluated the in vivo melanoma-targeting ability of a nanovesicle (HFT-MSH-PEG) based on human protein ferritin (HfF), functionalized with both melanoma-targeting melanoma stimulating hormone (α-MSH) and stabilizing poly(ethylene glycol) (PEG) molecules. We used two independent and complementary techniques, such as whole-specimen confocal microscopy and magnetic resonance imaging, to detect the in vivo localization of NP constructs endowed with suitable tracers (i.e., fluorophores or magnetic metals).

**Results:** Targeted HFT-MSH-PEG NPs were shown to accumulate consistently at the level of primary melanoma and with high selectivity with respect to other organs. Melanoma localization of untargeted HFT-MSH NPs, lacking the α-MSH moiety, was less pronounced and disappeared after a few days. Further, HFT-MSH PEG NPs accumulated to a significantly lower extent and with a different distribution in a diverse type of tumor (TS/A adenocarcinoma), which does not express α-MSH receptors. Finally, in a spontaneous lung metastasis model, HFT-MSH-PEG NPs localized at the metastasis level as well.

**Conclusions:** These results point at HFT-MSH-PEG NPs as suitable carriers for selective in vivo delivery of diagnostic or therapeutical agents to cutaneous melanoma.
Background: Metastatic melanoma represents the most deadly form of skin cancer. The poor response to chemotherapy and the brief response to the anti-BRAF vemurafenib in selected population of patients, make the identification of new therapeutic approaches an urgent need. Our goal is the evaluation of the efficacy of barasertib, an aurora B kinase inhibitor impairing cytokinesis, in both mutated and non-mutated melanoma cell lines.

Materials and methods: Panel of melanoma cells: BRAFV600E mutated cells (MBA72 and Hmel1), the same cell line in which the resistance to vemurafenib was induced by chronic exposure to it (MBA72R and Hmel1R) and BRAF wt (HBL and LND1). Cells were characterized for vemurafenib and barasertib effectiveness on cell growth by MTT assay after 3 and 6 days of continuous exposure. Cell cycle was determined by flow cytometry and migration was evaluated by wound-healing assay.

Results: Cells with BRAFV600E mutation are sensitive to vemurafenib conversely, those with BRAF wt and the resistant ones showed an IC50 of at least 10 folds higher. 3days-barasertib exposure strongly reduced cell growth (30-60% at 30 and 300nM, respectively) in all cell lines; when the drug was given together with vemurafenib, no gain in effectiveness was evident. Prolonged exposure to barasertib (6 days) showed a progressive increase of effectiveness particularly in cells BRAF wt. The analysis of cell death mechanisms involved in determining the effectiveness of barasertib and vemurafenib showed that the first drug induced both apoptosis and necrosis conversely, the latter mainly apoptosis. Cell cycle analysis demonstrated that barasertib induced an increase in cell size and in polyploidy, suggesting also the mitotic catastrophe as a further cell death mechanism. Moreover, the anti-mitotic behaviour of this agent has been evaluated in function of drug concentration and time exposure. Preliminary results showed a strong reduction of cell migration after drug exposure. 

Conclusions: The sensitivity of melanoma cells to barasertib is irreversible to BRAF mutational status; however, cells BRAF wt show an increased nuclear modification. In conclusion, our results suggest barasertib as a novel therapeutic approach in melanoma treatment irrespective of BRAFV600E mutation.

Overall survival of patients with chemotherapy-naive advanced melanoma treated with ipilimumab 3 mg/kg in clinical trials

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Background: Dacarbazine (DTIC) is currently the only agent approved throughout Europe for patients with previously untreated, advanced melanoma that is not restricted by tumour genotype (e.g. BRAF status). Based on recent trials in patients with previously untreated, advanced melanoma, median overall survival (OS) and a 1-year OS rate of approximately 9 months and 36% represent conservative, historical benchmarks for DTIC monotherapy [1–5]. Data from clinical trials and expanded access programmes show that ipilimumab treatment consistently provides a proportion of patients with long-term clinical benefit, irrespective of whether they are pretreated or treatment-naïve. In Europe, ipilimumab is indicated for use in adults patients who have received prior therapy. To support the use of ipilimumab in the previously untreated setting, data were analysed from ipilimumab clinical trials.

Materials and methods: Data were pooled from chemotherapy-naive patients who received ipilimumab 3 mg/kg as monotherapy in one of four randomised clinical trials: MDX010-20 (NCT00094653), a Phase 3 trial of ipilimumab alone or in combination with gp100 vaccine versus the vaccine alone; MDX010-08 (NCT00505102), a phase 2 multi-dose study of ipilimumab with or without dacarbazine; CA184-004 (NCT00263165), a prospective phase 2 biomarker study, and CA184-022 (NCT00289640), a phase 2, dose-ranging study. Details on these four trials have previously been published [6–9]. Patients received ipilimumab 3 mg/kg x 4 doses with the option for retreatment (MDX010-20) or maintenance therapy (CA184-004/022).

Results: Of the patients who received ipilimumab 3 mg/kg within one of the four clinical trials described above, 78% had not received prior chemotherapy and were eligible for analysis. Of these, 27% had elevated lactate dehydrogenase levels at baseline and 53% had received prior immunotherapy for advanced melanoma. With a median follow-up of 11.6 months (range: 0.03–69.7), median overall survival was 13.47 months (95% confidence intervals [CI]: 11.2–19.6) and the one- and two-year survival rates were 54.1% (95% CI: 42.5–65.6) and 31.6% (95% CI: 20.7–42.9), respectively. Survival outcomes compared favourably to historical observations with dacarbazine in phase 3 trials of patients with previously untreated, advanced melanoma.

Conclusions: Therapeutic options for treatment-naive patients remain limited. OS data from chemotherapy-naive patients are necessary.
Background: Metabolic reprogramming is commonly found in cancer but it is poorly understood in melanoma. Recent works [1,2] provided new insights concerning molecular mechanisms involved in mitochondrial biogenesis of melanoma. This work aims to find possible correlations between pathways involved in the onset and progression of the disease in order to provide supporting information in this field. In particular we studied the behavior of the mitochondrial master regulator gene PGC1alpha in novel sporadic melanoma cell lines and its relations with BRAF mutational status.

Materials and methods: We studied new cell lines extracted from sporadic metastatic melanomas (hmel1, M3, Mba72) and primary melanomas (hmel9, hmel11), genotyped for genes involved in melanoma development compared to control melanoma cell lines (HBL, LND1) wt for MC1R and BRAF genes. Hmel1, hmel9 and hmel11 have already been described in Zanna et al., 2011 [3] and Zanna et al., 2013 [4]. We evaluated PGC1α levels and some of its mitochondrial target genes and the mitochondrial respiratory capacity, the amount of ROS, and the lactate level. We related these data to BRAF mutational statuses and analyzed MITF and cAMP levels.

Results: The HBL and LND1 cell lines, wt for BRAF, highly express PGC1α while hmel1, hmel9, hmel11, Mba72, M3, presenting BRAF mutations at the V600 residue, show a downregulation of this gene. MITF expression levels were more abundant in HBL and LND1 cell lines with respect to the other cell lines harbouring BRAF mutations. There is a direct correspondence between PGC1α alpha and MITF levels: higher levels of PGC1α are associated with an enhanced MITF quantity. The analysis of cAMP levels in our melanoma cell lines showed a similar trend, being higher in wt BRAF cell lines compared to the other cell lines.

Conclusions: Our data confirm the key role of BRAF mutations, MITF and cAMP levels in melanoma biology, suggesting a very important association between PGC1alpha and MITF levels: higher levels of PGC1alpha are associated with an enhanced MITF quantity. The analysis of cAMP levels in our melanoma cell lines showed a similar trend, being higher in wt BRAF cell lines compared to the other cell lines.

References:

P10
Enrichment of KIR+CD57+ highly cytotoxic NK cells in sentinel lymph nodes of melanoma patients
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Materials and methods: We have collected 190 FFPE metastatic samples from melanoma patients treated with Ipilimumab, using the last lesion excised before the treatment: lymph node involvement or distant metastatic sites. We are characterizing the immunoscore with the expression of CD3, CD8, CD20, Foxp3, by immunohistochemistry and evaluated correlation with clinical outcome. Serial sections (3-4 micron in thickness) have been stained with each marker and counterstained with H/E. Moreover, one section has been stained with a multiplex of all markers including tumor marker for melanoma (S100). All slides will be digitized on a Leica SCN-400 system, and the results will be quantified by assessing the density of different cellular immune population at the center and invasive margin of the tumor using a digital images analysis application (Definiens Architect XD). The markers expression will be subsequently matched with the most important clinical information about all the patients.

Results: We have considered the clinical benefit from treatment with Ipilimumab as the parameter for evaluating the effectiveness of immunoscore. The treatment has been considered as effective if a patient met a partial or complete response, or had stable disease for more than 6 months from the start of therapy, or with an overall survival > 1 year. The evaluation of prognostic and predictive power of the immunoscore, in patients with metastatic melanoma, treated with ipilimumab, are nearing completion and definition.

Conclusion: We believe that the immunoscore is key as prognostic and predictive markers for immunotherapies in metastatic melanoma.
Conclusion: The hMENA splicing program is an early prognostic marker of NSCLC patients and may represent a surrogate marker of a permissive or not tumor microenvironment for lymphocyte recruitment.

References

P13

Natural immunomodulator preimplantation factor PIF affected cancer growth in malignant melanomas
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Background and objectives: Malignant and trophoblastic cells share common features in terms of migration and invasion, while they represent striking differences also (1). Our results on immune mechanisms in pregnancy failure (2) and use of immunotherapeutics (3) fostered the present new approach: Pregnancy-derived compounds for potential growth controlling effect in metastatic melanomas were studied.

Methods: Preimplantation factor (PIF), a novel peptide secreted by viable embryos was selected (4), as its immune regulatory effects were advantageous (5). Based on our tumorimmunological project (Ethical permission ETT TUKEB 1642-02/2010), minor tissue samples from surgically removed lymphodes of patients with metastatic melanomas were processed. Primary cultures were set up in special conditions with or without immunomodulator PIF. In a parallel system, we transfused human HT199 melanoma cells into immunodeficient NOD.Cg-PkdcrdcsIi2tg1m1Wj/J-Sz (NSG) mice and followed tumor burden in PIF-treated groups.

Results: We found that PIF treatment delayed tumor outgrowth in time. A tendency of lower tumor volumes was seen both after administration of immunomodulator peptide and use of additional physical (static

References
magnetic field) treatment. A synergistic effect could be achieved by this combination treatment strategy. Retained or primed expression of glycolipid based tumorassociated antigens on PIF treated melanoma cells could be defined by immunofluorescence FACS and confocal laser microscopy.

Conclusions: Our results suggest an indirect mechanism of PIF on tumor growth. Retained or enhanced tumor antigen expression is advantageous, as cancerous cells become predisposed to be recognized, while induced activation of antigen presenting cells is beneficial for elimination. Our complementary strategy resulted as effective and provides a new potential modality for cancer control.


References

Cite abstracts in this supplement using the relevant abstract number, e.g.: Kotlan et al.: Natural immunomodulator preimplantation factor PIF affected cancer growth in malignant melanomas. Journal of Translational Medicine 2014, 12(Suppl 1):P13